Role of the MAPK in the Monocyte/Macrophage Procoagulant Response: Implications for Fulminant Viral Hepatitis

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Abstract:

Viral fulminant hepatic failure (FHF) is one of the most devastating diseases in clinical medicine, with progression from complete health to death occurring in a matter of days or weeks. Liver destruction is characterized by the dense infiltration of mononuclear cells (monocytes, macrophages and lymphocytes) with subsequent activation of the inflammatory coagulation cascade. We have studied the activation of the coagulation cascade by cells of the monocyte/macrophage lineage under conditions that model the integrin-dependent migration of the cells across the vascular endothelium and into the liver parenchyma, and the reaction of the cells to a viral pathogen known to induce FHF in mice. Tissue factor (TF), one of the chief inducers of coagulation, is expressed on monocytic cells in response to surface integrin crosslinking and following transendothelial migration. Flg-2/fibroleukin, a potent prothrombinase, is expressed on macrophages after infection with Murine Hepatitis Virus Strain-3 (MHV-3). Recent work has suggested that Mitogen Activated Protein Kinases (MAPK) are involved in integrin signaling and the monocyte response to infectious stimuli. We postulated that the ERK and p38 MAPK play critical roles in monocyte migration and the reaction of macrophages to MHV-3. We demonstrated activation of the ERK and p38 MAPK in response to integrin crosslinking, monocyte adhesion to activated endothelial beds, and infection of macrophages with MHV-3. Taking advantage of novel, selective inhibitors of the ERK MAPK pathway and the p38 MAPK (PD98059 and SB203580, respectively) we found that both MAPK modules are essential for the induction of TF in the adhesion dependent response to monocyte transendothelial migration. The p38 MAPK was necessary for fgl-2 expression in response to MHV-3; ERK was necessary for fgl-2 activity. These studies suggest that critical aspects of the monocyte/macrophage response which drives liver destruction in viral FHF are under the control of the ERK and p38 MAPK. Thus, the selective inhibition of these modules may be of use in attenuating the inflammatory damage of viral FHF.
List of Major Abbreviations:

Ab: Antibody
AMP: Adenosine Mono-Phosphate
AP-1: Activating Protein 1
APC: Activated Protein C
ATIII: Antithrombin III
ATF: Activating Transcription Factor
ATP: Adenosine Tri-Phosphate
CPLA2: Cytoplasmic Phospholipase A2
CSAID: Cytokine Suppressive Anti-Inflammatory Drug
APC: Activated Protein C
EGF: Epidermal Growth Factor
EMSA: Electrophoretic Mobility Shift Assay
ERK: Extracellular Signal-Regulated Kinase
FAK: Focal Adhesion Kinase
FCS: Fetal Calf Serum
FG: Fibrinogen
FGF-2: Fibrinogen-like protein-2 (Fibroleukin)
FN: Fibronectin
FHF: Fulminant Hepatic Failure
FRNK: FAK-Related Non-Kinase
FVH: Fulminant Viral Hepatitis
GAP: GTPase Activating Protein
GDI: Guanine nucleotide Dissociation Inhibitor
GDS: Guanine nucleotide Dissociation Stimulator
GPCR: G-protein coupled receptor
GRB: Growth Factor Receptor-Binding Protein
HBV: Hepatitis B Virus
HBsAg: Hepatitis B Virus Surface Antigen
HbsAb: Hepatitis B Virus Surface Antigen Antibody
HCV: Hepatitis C Virus
HIV: Human Immunodeficiency Virus
HOG-1: Hypoerosmolarity Glycerol Activated Kinase
HUVEC: Human umbilical vein endothelial cell
IAP: integrin-associated protein
ICAM: Intercellular Adhesion Molecule
IG: Immunoglobulin
IL: Interleukin
ILK: integrin-linked kinase
INFL: Interferon
JNK: c-Jun N-Terminal Kinase (or SAPK)
LFA: Leukocyte Function Antigen
LPS: Lipopolysaccharide
mAb: Monoclonal antibody
MAPK: Mitogen Activated Protein Kinase
MBP: Myelin Basic Protein
MEF: Myocyte Enhancer Factor
MEKK, MAPKKK: MAP Kinase Kinase Kinase
MHV-3: Murine Hepatitis Virus Strain 3
MHVR: Murine Hepatitis Virus Receptor
MKK, MEK, MAPKK: MAP Kinase Kinase
MKP: MAPK Phosphatase
MOF: Multiple Organ Failure
MRNA: Messenger Ribonucleic Acid
NAC: N-Acetyl Cysteine
NFκB: Nuclear Factor κB
PAb: Polyclonal Antibody
PAEC: Porcine Aortic Endothelial Cell
PAI: Plasminogen Activator Inhibitor
PAK: p21 Activated Kinase
PBM: Peripheral Blood Monocyte
PC: Protein C
PCA: Procoagulant Activity
PDGF: Platelet-Derived Growth Factor
PDTC: Pyrroldine Dithio Carbamate
PFU: Plaque Forming Units
PG: Prostaglandin
PKC: Protein Kinase C
PL: Poly-L-Lysine
PMA: Phorbol Myristic Acid
PP: Protein Phosphatase
PS: Protein S
PTK: Protein Tyrosine Kinase
PTP: Protein tyrosine phosphatase
RK: Reactivating kinase (p38 MAP kinase)
RKK: Reactivating Kinase Kinase
ROCK: P160 rho-associated coiled-coil containing protein kinase
RSK: Ribosomal S-Protein Kinase
SAPK: Stress Activated Protein Kinase (or JNK)
STAT: Signal Transducing Activator of Transcription
SH2: Src-homology domain 2 (phosphotyrosine)
SH3: Src-homology domain 3 (proline rich)
SHP: Src-homology-2-domain-containing protein tyrosine phosphatase
SIRS: Systemic Inflammatory Response Syndrome
SOS: Son of sevenless (adapter protein)
SPRK: SH3 domain-containing Proline Rich Kinase
TEM: Transendothelial Migration
TM: Thrombomodulin
TM4: Four transmembrane domain protein family
TNF: Tumour Necrosis Factor
TF: Tissue Factor
UV: Ultraviolet
VCAM: Vascular Cell Adhesion Molecule
VLA-4: Very Late Antigen 4
Section I. Monocytes and Macrophages: Key Inflammatory Cells

Cells of the monocyte/macrophage lineage play central roles in the response to infectious stimuli. They are critical both in the inflammatory response to the infectious organism and to its clearance. This dual role is well illustrated by the fact that disorders of monocyte function are associated with recurrent bacterial infections (Holland 1998), while monocyte/macrophage activation contributes to the systemic activation of inflammatory cascades which is often fatal in severe inflammatory states (Volk 1996).

The bloodborne monocyte first migrates to sites of extravascular infection and then responds to local infectious stimuli. Although monocytes and macrophages have a number of important functions, including phagocytosis and cytokine release, one crucial response is the activation of the coagulation cascade by increased expression of surface procoagulant molecules, particularly tissue factor (TF) and fibrinogen-like protein 2 (fgl-2). These molecules activate the coagulation cascade in a manner which can be measured as procoagulant activity (PCA). PCA is experimentally defined as the ability of washed cell lysates (or whole cells) to stimulate fibrin deposition when calcium is added to the cell preparation in the presence of all necessary clotting factors. The activation of the coagulation cascade by monocyte/macrophage PCA modulates and contributes to the developing inflammatory response and tissue damage.

One excellent example which integrates these roles of the monocyte is viral fulminant hepatic failure (FHF). This severe illness is characterized by the parenchymal infiltration of monocytes and macrophages, which contribute to tissue destruction via the induction of the coagulation cascade. Both the migration phase of monocyte infiltration and the reaction to the viral pathogen are likely to contribute to the activation of coagulation. At present there is no good medical therapy for viral FHF, which even with liver transplantation has a roughly 50% mortality (Pappas 1996). However, the ideal treatment for viral FHF would be medical, since even severely damaged livers have the capacity to regenerate if the patient survives the acute illness.

We therefore undertook a study of the mechanisms underlying the monocyte/macrophage procoagulant response induced by monocyte migration and by the reaction to a viral pathogen which causes viral FHF. Since earlier work has implicated tyrosine phosphorylation signaling as an early and powerful transducer of extracellular adhesive and viral stimuli, we focused on the role of downstream components of tyrosine phosphorylation cascades: the mitogen-activated protein kinases (MAPK). The MAPK have
clearly been implicated in monocyte/macrophage responses to inflammatory stimuli, although their role in monocyte adhesion or the monocyte response to viral stimuli is not known.

This thesis deals with the above issues in a sequential manner. A brief discussion of the mechanics of monocyte migration is followed by a detailing of the role of monocyte/macrophage procoagulant activity in local and systemic inflammatory responses, particularly in the liver. The regulation of expression of the two chief monocyte/macrophage procoagulants, TF and fgl-2, is discussed in some detail, as is the pathogenesis of viral FHF. The roles and regulation of the MAPK cascades are overviewed, focusing on monocyte migration and response to viral stimuli. Finally, the results of studies investigating the role of the MAPK in adhesion, migration and virally-induced procoagulant activity are presented and discussed.
Section II. Monocyte Migration: Mechanisms of leukocyte-endothelial cell interactions:

Summary: One of the earliest steps in the pathogenesis of extravascular inflammation, including viral FHF, is the infiltration of mononuclear cells into local tissues. This section briefly describes the mechanisms employed in the emigration of bloodborne monocytes, and suggests that intracellular signaling is an important part of the process.

Monocyte Migration:

The active migration of inflammatory cells carried passively by bloodstream currents into inflamed tissues is a multistep process characterized by surface molecule interactions, cytoskeletal changes and induction of second messenger pathways. Floating leukocytes are first slowed by rolling across an endothelial surface, then firmly adhere to the endothelial cell and transmigrate into the extravascular space. (Bevilacqua 1992, Hogge 1995) Three families of surface adhesion molecules are thought to be integral to this process. The selectins are lectin-like molecules expressed on endothelial cells (E-selectin, P-selectin) and leukocytes (L-selectin), and are either constitutively expressed (L-selectin), or are rapidly up-regulated by cell stimulation (E- and P-selectin). The glycocalyx to which the selectins bind is inducible by inflammatory stimuli. (Spertini 1992) Selectin binding to membrane glycoproteins produces cell rolling, and has also been shown to activate leukocyte second messenger pathways (reviewed in Waddell 1995, Lorant 1993) Firm leukocyte-endothelial adhesion and transmigration is mediated by the integrins, transmembrane αβ heterodimers which also interact with proteins of the extracellular matrix (ECM). 8 β subunits have been identified, each of which interacts with specific groups of 16 α subunits. Leukocyte adhesion depends on three important integrin subfamilies. All of the β2 integrins - α4β2 (LFA-1), αmβ2 (MAC-1), and αXβ2 (p150,95) - are expressed by neutrophils and monocytes, while lymphocytes express only LFA-1. The β1 integrin α4β1 (VLA-4) is found on lymphocytes and monocytes, but not on neutrophils. Lymphocyte adhesion is also a function of the α4β1 integrin. Leukocyte integrins bind to members of the Ig superfamily expressed on the endothelial surface. ICAM-1 and ICAM-2 bind the β2 integrins, while VCAM-1 binds both VLA-4 and α4β2, the latter also binding MadCAM-1. (Altieri 1993) Both ICAM and VCAM are upregulated in response to inflammatory signals. Thus, focal sites of infection or inflammation in extravascular tissues will recruit leukocytes via focal changes in vascular cell adhesion molecules.

The process of monocyte transmigration is currently understood in terms of the particular adhesion
molecules involved. The initial and necessary “firm adhesion” to activated endothelium is contributed to, both in vitro and in vivo, by β2-integrin/ICAM and by VLA-4/VCAM interactions, with the relative contribution of each pathway varying depending on the tissue type and inciting inflammatory event in vivo (Issekutz J Exp Med 1995, Issekutz JJ 1995), and on the type and length of endothelial activation, as well as the chemotactic factor used, in vitro (Takahashi 1994, Chuluyan 1995, Chuluyan 1993). In contrast, the PECAM-1 adhesion molecule, which mediates homotypic adhesion between endothelial cells and between endothelial cells and leukocytes, appears to be universally important for the monocyte transmigration step per se (Liao 1997, Muller 1993). All of these steps are dynamic, and involve crucial changes in integrin affinity states (“inside-out” signaling). Constitutively held in low-affinity states, the integrins must be “activated” to a higher affinity state for adhesion to occur, but must then revert to a lower affinity state for movement to occur. A dramatic example of this requirement is the fact that the TS2/16 mAb against the VLA-4 integrin on monocytes “locks” the integrin in a higher affinity state, and so enhances adhesion while blocking migration (Dominguez-Jimenez 1996, Sanchez-Mateos 1993). By contrast, transendothelial migration will not proceed in the absence of CAM and integrin activation: increasing ICAM or VCAM expression by transfection is not sufficient to increase transmigration in the absence of an integrin- and CAM-activating chemotactic factor (Chuluyan JJ 1995, Zocchi 1996). Equally, engagement of one surface adhesion molecule can dramatically affect cell adhesion mediated by other surface adhesion molecules (Simon 1995). This adhesion is a function of integrin affinity, integrin clustering and cytoskeletal re-arrangements (Peter 1995). Thus, the “outside-in” signal mediated by integrin engagement must lead to changes in both integrin affinity and cytoskeleton arrangement for migration to proceed, arguing for a regulatory potential and suggesting that cell migration may be best understood from a signaling perspective.
Section III. Monocyte/ Macrophage Reaction: Monocyte/Macrophage Activation of the Coagulation Cascade in Local and Systemic Inflammation:

Summary: Activation of the coagulation cascade has traditionally been synonymous with the need for hemostasis at sites of bleeding. However, over the past several decades, it has been increasingly recognized that initiation of coagulation is an integral and consistent element of the local and systemic response to inflammatory stimuli (see Figure 1). Cells of the monocyte/macrophage lineage are key players in the inflammatory activation of the coagulation cascade. In this section, the proinflammatory effects of coagulation at sites of local tissue injury as well as the systemic effects of the activation of this pathway will be addressed. Activation of the coagulation cascade can be seen as both an inducer of local organ damage and a contributor to systemic inflammation.

The contribution of fibrin deposition to the development of local inflammation:

Fibrin deposition is one of the hallmarks of the local response to extravascular inflammatory stimuli, both infectious and noninfectious. Fibrinous exudates are characteristic of bacterial peritonitis, chronic wound infections, ARDS, and delayed type hypersensitivity reactions (Bone 1992, Brisseau 1993), and fibrin deposition is also seen in the liver sinusoids and parenchyma of viral FHF (see Section IV).

Several studies support the concept that the process of fibrin deposition is an important contributor to the full development of the inflammatory response to infectious stimuli. This notion is perhaps best exemplified by the demonstration that anticoagulation is able to lessen inflammation in several animal models. For example, the induration of DTH response is markedly attenuated by systemic anticoagulants (e.g. heparin or warfarin) or local anticoagulants (high-affinity heparin) (Colvin 1975, Edwards 1978, Kakakios 1990). The precise mechanisms whereby coagulation contributes to the full expression of inflammation is an area of active study. During the process of activation of the coagulation cascade, several of the proteolytically active coagulation components as well as their byproducts have been shown to be proinflammatory. Both thrombin and Factor Xa are able to induce inflammation when injected into the subcutaneous tissues of experimental animals (Cirino 1996). Thrombin has direct chemoattractant activity for neutrophils (Bar-Shavit 1985) and monocytes (Bar-Shavit 1983) and has also been shown to induce mast cell degranulation with the release of bioamines causing increased vascular permeability (Stukova 1996). Similarly, Factor Xa causes mast cell degranulation, possibly through interactions with
Figure 1: Activation of the coagulation cascade in inflammatory states

Note the central role of Tissue Factor (TF), and the multiple points of intersection of the coagulation and inflammatory cascades. Coagulation acts very much to focus the inflammatory response in space.
its receptors on these cells (Cirino 1997). In the in vivo setting, specific inhibition of thrombin or Factor Xa interaction with their respective receptors has been shown to attenuate the magnitude of the local inflammatory response (Cirino 1996, Tapparelli 1993). Further, fibrinopeptide B, fibrin/fibrinogen degradation products as well as thrombin are known to have chemoattractant properties directing neutrophil migration into the inflammatory focus (Ferrara 1989, Senior 1986, Stecher 1972).

**Systemic activation of the coagulation cascade during inflammatory states:**

Derangement of the coagulation system is a frequent occurrence in patients with systemic inflammatory responses. This is seen both during the systemic propagation of inflammation, arising from diverse causes including sepsis and viral FHF. The abnormalities consist of increased procoagulant activity, reduced anticoagulant activity and impaired fibrinolysis. The net effect of these processes is the development of the clinical syndrome of disseminated intravascular coagulation (DIC). In DIC, overexuberant systemic clotting leads to bleeding complications due to depletion of coagulation proteins and platelets, while the concomitant development of microvascular thrombosis leads to end organ injury, partly on an ischemic basis (Senior 1986, Bick 1992, Bick 1988, ten Cate 1993, Taylor 1994, Shibayama 1987, Schlag 1985). Using stringent diagnostic criteria, the incidence of DIC in patients with septic shock may exceed 70% (Fourrier 1992). Furthermore, the coagulation defects of DIC are increasingly common as patients advance from SIRS to septic shock (Rangel-Frausto 1995), and correlate well with APACHE II scores (Voss 1990).

Systemic activation of the coagulation system likely also accompanies viral FHF. Viral FHF is characterized by cytokinemia and is often complicated by endotoxemia. Detailed studies in human volunteers and septic patients have documented the specific abnormalities of the coagulation system which occur following both endotoxemia and cytokinemia. In human volunteers injected with small doses of TNFα or LPS there is gradual activation of coagulation as evidenced by increases in thrombin-antithrombin (TAT) complexes, prothrombin activation fragments and fibrinopeptide A (Suffredini 1989, van Deventer 1990, van der Poll 1990, van der Poll 1991). This process begins by 2 hours, peaks at 4 to 5 hours, and persists for 6-12 hours. There is also an early increase in plasma fibrinolytic activity (1-2 hours) due to the presence of plasminogen activators which lead to plasmin generation. However, this activity is rapidly neutralized by increased levels of the plasminogen activator inhibitor-1 protein (PAI-1). These findings are consistent with the conclusion that systemic inflammatory states cause an imbalance of the procoagulant and anticoagulant systems, resulting in a sustained hypercoagulable state.
Interestingly, the coagulant and anticoagulant arms of the response appear to be differentially regulated. While TNF infusion induces the procoagulant arm of the response in both primates and human subjects (van der Poll 1990, van der Poll 1991, van der Poll 1994a), anti-TNF treatment of primates infused with endotoxin or E.coli does not prevent the procoagulant response (van der Poll 1994b). However, antibody to IL-6 effectively did so (van der Poll 1994c). By contrast, TNF infusion has been shown to induce the fibrinolytic response in humans via increased secretion of plasminogen activators (van der Poll 1991), and inhibition of TNF with monoclonal antibodies effectively prevented the activation of fibrinolysis in endotoxin-treated chimpanzees (van der Poll 1994b). Anti-IL-6 Therapy was without effect (Levi 1997, van der Poll 1994c).

**Mechanisms contributing to an inflammatory procoagulant state**

**A. Activation and regulation of the coagulation cascade systemically:**

**a. contact activation (intrinsic pathway):**

The initiation of coagulation in response to infection was traditionally thought to be mediated through the contact activation (or the intrinsic pathway) system. This concept was based on studies showing that high doses of endotoxin as well as bacterial proteinases were able to cause *in vitro* activation of Factor XII, one of the proximal proteins of this pathway (Kalter 1985). However, the notion was brought into question when studies of sublethal endotoxemia in man failed to induce activation of the contact system despite marked activation of coagulation (van Deventer 1990, van der Poll 1990). Moreover, while lethal bacteremia does induce activation of the contact system in baboons, (Piley 1992) blockade of Factor XII failed to prevent the development of DIC but did attenuate hypotension (Pixley 1993). Present dogma thus suggests that activation of Factor XII by LPS or bacterial proteinases (Kaminishi 1994) contributes to the kallikrein-kinin axis and to activation of the classical Cl-esterase dependent complement cascade (Bone 1992, Pixley 1993, Fourrier 1995) and may therefore contribute to the magnitude of the septic hypotensive response in man and animals (Hack 1993).

**b. Tissue Factor:**

Two lines of evidence suggest that expression of the cell-associated procoagulant molecule, tis-
Sue factor (TF) is crucial to the initiation of the coagulation cascade during inflammatory states. First, TF expression is tightly regulated and is absent from the bloodstream in non-inflammatory conditions. TF is expressed constitutively only by cells outside the bloodstream and therefore acts as an "extravascular envelope." triggering coagulation whenever the vascular endothelial integrity is breached (Drake 1989). Within the bloodstream only monocytes and the endothelium can be induced to express TF, and have been demonstrated to do so both *in vitro* and *in vivo* in response to *E. Coli*, LPS and various cytokine mediators of the host response to infection. (Taylor 1994, Drake 1993, Mackman 1993, Osterud 1983) Cells of the monocyte/macrophage lineage are especially important to the procoagulant state generated by inflammatory or infectious stress (Osterud 1983). For example, in meningococcal meningitis the fulminant bleeding and simultaneous thrombotic complication of *purpura fulminans* occurs concurrently with increased TF activity on circulating monocytes, and the degree of increase is associated with worsening clinical outcomes (Osterud 1983). Second, various strategies directed towards the neutralization of TF or the TF/VIIa complex have demonstrated efficacy in preventing organ damage associated with endotoxemia or bacteremia in experimental models. These include anti-tissue factor antibody (Taylor 1991, Levi 1994), anti-Factor VIIa (Biemond 1995), as well as administration of Tissue Factor Pathway Inhibitor (TFPI) (Creasey 1993). Considered together, these data suggest a central role for activation of TF-mediated coagulation in the full expression of the septic inflammatory response.

The mechanisms regulating TF expression have been examined in both monocytes/macrophages as well as endothelial cells. As noted earlier, TF upregulation in states of systemic inflammation appears to be predominantly on monocytes/macrophages (Osterud 1995). This contention is based in part on the lack of clear data demonstrating endothelial TF upregulation in inflammatory states *in vivo*. Relatively little endothelial TF is seen following septic insults. Experimentally, lethal *E. coli* infusion in baboons results in endothelial TF upregulation only in the spleen, despite marked activation of the coagulation cascade and increased circulating monocyte procoagulant activity (Drake 1993). Mice exposed to LPS demonstrate marked upregulation of TF mRNA in kidney and lung tissues (Mackman 1993); however, it is unclear whether this increase is endothelial in origin or arises from the high numbers of resident macrophages in these organs. Since increased monocyte/macrophage TF has been described in response to similar insults both in primates and man (Osterud 1983, Taylor 1994), it appears that cells of the monocyte/macrophage lineage are crucial in the induction of TF by systemic inflammatory stimuli *in vivo*.

Virtually every microbial species tested as well as their surface components have been shown to
induce monocyte/macrophage TF activity (Brisseau 1993). In addition, a multitude of cytokines and inflammatory mediators shown to play a role in local and systemic inflammatory responses - including TNF-α, IL-1, IL-2, C5a, IL-6, platelet activating factor - have been demonstrated in vitro to either upregulate or prime for TF expression. and have also been demonstrated in primate models to activate the coagulation cascade and contribute to septic mortality (van der Poll 1994c, Jansen 1995, Baars 1992).

c. Fgl-2/fibroleukin:

Another major procoagulant, less well-studied than TF, is the Fibrinogen-Like Protein-2 (fgl-2), or fibroleukin. This glycoprotein, expressed on the surface of macrophages, monocytes, and endothelial cells, acts as a direct prothrombinase both in human and murine cells (Levy 1999). Studies from our group have demonstrated a key role for fgl-2/fibroleukin in the pathogenesis of viral FHF as modeled by murine hepatitis virus strain 3 (MHV-3) infection in susceptible mouse strains (Li 1992). The regulation and function of fgl-2 is discussed in detail in Section IV.

d. Anticoagulation mechanisms:

In general, activation of the coagulation cascade induces anticoagulant mechanisms which function to limit progression of the coagulation cascade. However, during sepsis, impairment of the anticoagulant mechanisms leads to further promotion of the procoagulant state. Antithrombin III (ATIII) is a member of the serine protease inhibitor (serpin) family, which covalently binds to and inactivates all of the serine protease coagulation factors, including TF/VIIa (Jesty 1996). ATIII-coagulation factor complexes are rapidly cleared from the circulation by the reticuloendothelial system (Bick 1992). Protein C is a component of the Protein C/Protein S/C4bBP inhibitory axis. The endothelial surface protein thrombomodulin binds Xa, changing its specificity and leading to the activation of protein C (McVey 1994). The effect of Activate Protein C (APC) is amplified by complexing with Protein S. The APC/Protein S complex efficiently degrades Factors Va and VIIIa, thereby inhibiting coagulation, and APC also promotes fibrinolysis (Sakata 1986) Both ATIII and Protein C are rapidly consumed in the septic inflammatory state, and both are decreased in viral FHF by the combined effects of decreased synthesis and increased consumption (Fourrier 1992, Meesters 1996). Additionally, the downregulation of thrombomodulin due to both local and systemic release of cytokines such as TNF and IL-1 may result in impaired activation of the anticoagulant APC/protein S complex (Nawroth 1986). Finally, as mentioned above, several hours after the initiation of sepsis, increased generation of the antifibrinolytic PAI-1 serves to
abort the brisk fibrinolytic response seen in the first hours after endotoxemia.

B. Local activation of the coagulation cascade: The liver

Sinusoidal thrombosis in the liver during sepsis or viral FHF is a good example of how cellular interactions with the coagulation system results in microvascular changes. In sepsis, the endothelial-lined sinusoids are engorged with neutrophils, fibrin and red cells (Shibayama 1987, Schlag 1985). The human viral FHF liver is characterized by sinusoidal and parenchymal fibrin deposition, while MHV-3 infection of susceptible mice causes sinusoidal thrombosis with accumulation of neutrophils and cells of the monocyte/macrophage lineage.

Local fibrin deposition in liver inflammation is likely due both to monocyte/macrophage tissue factor and to upregulation of the fgl2 prothrombinase. The fgl-2 response is dealt with in detail in Section IV, while the role of TF is outlined below.

Several factors influence local monocyte/macrophage TF procoagulant expression in the inflamed liver. In migrating into the inflamed liver, circulating monocytes bind to and migrate across the inflamed vascular bed. Integrin-mediated interactions have been demonstrated to increase monocyctic cell TF (Fan 1994); thus, both the initial contact with the endothelial bed and the subsequent interaction with the parenchymal extracellular matrix could increase TF. In addition, circulating and local endotoxin and inflammatory cytokines can cause upregulation of TF on the surface of resident Kupffer cells, leading to local fibrin deposition (see Figure 1). Both systemic mediators and those released locally by Kupffer cells might further accentuate the local procoagulant response. As discussed earlier, in vitro LPS, TNF and IL-1 stimulate monocyte/macrophage TF expression and inhibit endothelial thrombomodulin expression (Nawroth 1986, Moore 1987). Moreover, in the local inflammatory response a variety of endothelial and Kupffer surface products are expressed, such as platelet activating factor, L- and P-selectin, and the Mac-1 integrin, which either synergistically increase the monocyte/macrophage procoagulant tissue factor response (Kucey 1991), are sufficient to induce it (Lo 1995, Celi 1994), or act to amplify procoagulant activity (Altieri 1988). The consequent initiation of local coagulation simultaneously promotes neutrophil and monocyte infiltration, priming and activation via generation of thrombin and fibrinogen/fibrin degradation products (Bar-Shavit 1983, Bar-Shavit 1985, Ferrara 1989, Stecher 1972, Senior 1986). The tethering of inflammatory cells within a developing clot may serve both to localize and promote the inflammatory response by a variety of adhesion-dependent processes, such as those mediated by the $\beta_2$-
class integrins (reviewed in Altieri 1993). Complement activation results from local generation of active coagulation factor XIIa, cellular damage, direct induction by the alternative route, or from local LPS or bacterial proteinases. The resulting C5a and C5b-9 attack complexes increase tissue factor expression (Caroson 1990), and C5b also leads to the generation of prothrombinase-containing vesicles from platelets and endothelial cell membranes (Sims 1988, Wiedment 1986, Hamilton 1990). This sequence not only increases the generation of thrombin, but disseminates circulating, coagulation-inducing particles (Taylor 1994). Finally, products of activated cells such as neutrophils, platelets, and mast cells are known to exert proinflammatory effects on both endothelial cells and macrophages further amplifying the local alterations. Considered together, these data demonstrate that both systemic and local factors involved in the inflammatory response contribute to microvascular thrombosis and fibrin deposition, in part through interactions with the coagulation system.
Section IV: Signaling Pathways of Tissue Factor Expression on Monocytes and Macrophages

Summary: The expression of surface tissue factor by cells of the monocyte/macrophage lineage is a major factor in the development and progression of local and systemic inflammatory reactions. A wide variety of extracellular stimuli act to upregulate tissue factor (TF), the principal inducer of the coagulation cascade in vivo. Since both the coagulation cascade and TF have multiple roles, the expression of TF has consequences both for the inflammatory milieu and for the monocyte. TF expression is controlled primarily at the transcriptional level, particularly by activation of AP-1 and NF-κB transcription factors. Control is mediated both at the level of NF-κB and AP-1 nuclear translocation and DNA binding and by altering their transactivation potential; however, recent data suggests that while these factors are necessary, they are not sufficient for TF expression. Similarly, a number of intracellular signaling cascades have been studied and may affect TF upregulation both at the transcriptional and post-transcriptional level. In particular, the roles of tyrosine protein kinases, protein kinase C and the downstream Mitogen Activated Protein Kinases (MAPK) suggest that signal integration occurs at many stages. In this review, the roles of monocyte TF are briefly overviewed and the signaling pathways regulating TF gene expression and functional activity are examined in detail.

Tissue Factor: Structure and Function:

TF is a 47 kD integral glycoprotein composed of a large, 219 amino acid extracellular domain, a 23 residue transmembrane domain and a short 21 residue cytoplasmic domain (Ruf 1994). TF avidly binds coagulation factor VIIa, and the complex has markedly enhanced proteolytic activity, rapidly converting the zymogen precursors of the serine proteases Factors IX and X to their active forms. All surface TF is active (Drake 1989). However, the ability of TF to lead to induce Factor Xa-containing prothrom-
binase complexes, thrombin activation and cleavage of fibrinogen to fibrin (a functional activity measured as procoagulant activity, PCA) is enhanced by the phospholipid bilayer of the cell membrane (Drake 1989) as well as by the ability of the cell surface to trap Factor X and Xa. In theory, accessory monocyte proteins such as the Mac-1 integrin (CD11b/CD18), which binds Factor X, and the recently described EPR-1 receptor for Xa, may contribute to PCA (Rozdzinski 1995, Mesri 1998, Plescia 1996, Altieri 1990, Bouchard 1997). The fact that antibodies against TF abolish the great majority of monocyte/macrophage PCA in response to various inflammatory stimuli is consistent with a primary role for TF in the induction of coagulation at the monocyte surface (McGilvray 1997, Ternisien 1993, Lyberg 1982, Lo 1995, Neumann 1996, Schwager 1994). Further, although these accessory molecules may enhance the effects of TF expression, the modulation of TF-dependent monocyte/macrophage PCA by interventions which affect cellular signaling has only been reported in association with decreased TF mRNA or protein: where TF-dependent PCA has been described, its regulation is a function of TF protein expression.

The induction of coagulation has many consequences for local and systemic inflammation (reviewed in McGilvray 1997). At a local level, activated coagulation enmeshes both leukocytes and bacteria in a fibrinous matrix, and many coagulation products, such as thrombin and the fibrinopeptides, are chemotactic and/or directly stimulatory to leukocytes (Bar-Shavit, Cirino 1996, Cirino 1997, McGilvray 1998). Systemically, TF expression contributes to the syndrome of disseminated intravascular coagulation (DIC) and to cytokine generation and mortality in experimental models of endotoxemia (Taylor 1994, McGilvray 1999). From this point of view the monocyte is of central importance. In the bloodstream, only endothelial cells and monocytes are capable of inducible TF expression. However, following endotoxin infusion in primates, TF expression is seen only in splenic vascular beds (Drake 1993). By contrast, blood borne monocytes consistently express high levels of PCA after similar insults (Taylor 1991, Taylor 1987). In humans, increased TF has been described in circulating monocytes in a variety of
inflammatory circumstances, including clinical sepsis (Vickers 1998), elevated plasma endotoxin (Saliola 1998), meningococcal meningitis (Osterud 1982), active coronary heart disease (Leatham 1995), and following intraabdominal surgery (Blakowski 1986). The importance of monocyte TF to the outcome of systemic inflammation is suggested by the fact that alterations in biochemical parameters of coagulation are highly prognostic of mortality in critically ill patients (reviewed in McGilvray 1997)

In addition to its role in the induction of coagulation, TF has other roles in monocyte signaling and trafficking. TF has homology with the interferon α/β and γ receptors (Bazan 1990); it is perhaps not surprising that the binding of VIIa to TF results in intracellular signaling. In particular, Factor VIIa binding to TF has been demonstrated to increase or decrease several mRNA species in fibroblastic WI-38 cells (Pendurthi 1997), induce Ca²⁺ transients in both human umbilical vein endothelial cells induced to express TF and bladder carcinoma J82 cells, which constitutively express TF (Rottingen 1995), and cause phosphorylation of the ERK1/ERK2 MAPK proteins in baby hamster kidney cells transfected with TF (Poulsen 1998). Although it is possible that the generation of Factor Xa, which is downstream of the TF/VIIa association may also trigger Ca²⁺ signaling (Camerer 1996), Poulsen et al demonstrated that MAPK signaling was independent of Factor Xa generation (Poulsen 1998). No work has yet been done in monocytes; however, it has been suggested that such signaling may help to explain the inhibition of cytokine release (IL-6, IL-8) that follows anti-TF pretreatment in primate endotoxemia models (Taylor 1994 NH). In addition to a signaling role, the association of TF expression with metastatic phenotypes in melanoma cells suggested that TF may play a role in cell trafficking (Mueller 1992). A more recent, elegant study by Randolph et al outlined a role for TF in the retrograde, basal-to-apical transmigration of monocytes across endothelial cells (Randolph 1998). Thus, the expression of TF by cells of the monocyte/macrophage lineage is likely to have many consequences for cell function and activity beyond induction of coagulation; understanding how TF comes to be expressed by the monocyte may therefore shed light on a number of important pathogenic processes.
**Induction of Monocyte/Macrophage Tissue Factor:**

A review of the factors which contribute to monocyte TF expression suggests the primary role of the monocyte in localizing and upregulating the inflammatory response, and offers insights into contributing signaling cascades. Although many inflammatory cytokines, such as IL-1, TNFα, and IL-8, complement factors (C5a), bacterial endotoxin and the bacteria themselves can increase monocyte PCA and TF expression both in vitro and in vivo (Neumann 1997, McGilvray 1998), many of the stimuli for TF expression are involved in the mechanics of monocyte migration and cell-cell crosstalk. Monocyte surface adhesion molecules are crucial for cell adhesion to and transmigration across endothelial surfaces, and also mediate interactions with the extracellular matrix and with other inflammatory cells. The β1 integrin VLA-4 (CD49d/CD29), β2 integrins Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18), and the P-selectin ligand CD15 all contribute to these processes. These adhesion molecules have also been implicated in monocyte TF expression: crosslinking of surface VLA-4, Mac-1, LFA-1, or CD15 induces or primes for TF expression (McGilvray 1997, McGilvray 1998, Fan 1991, Fan 1995, Lo 1995). Similarly, the adhesion of monocytes to activated endothelial surfaces induces TF in a manner that is at least partially due to the β2 integrins (Collins 1995) and CD15 (Lo 1995), while P-selectin can directly induce TF (Celi 1991) and P-selectin-expressing platelets enhance LPS-induced monocyte PCA and TF in a P-selectin-dependent fashion (Amirkhosravi 1996). In the developing inflammatory reaction, lymphocyte TH1 and TH2 subsets proliferate, and the balance can affect monocyte TF expression. Early works found that activated T-cells can induce monocyte TF (Edwards 1980); more recently it has been demonstrated that TH1 adhesive contact with monocytes synergizes with Th1 soluble factor production to induce TF, while TH2 subsets have no effect (Del Prete 1995). Furthermore, monocyte adhesion to artificial surfaces is sufficient to induce TF (Brozna 1988, Barstad 1998). These considerations suggest that in addition to the effects of cytokines and bacterial products, adhesive interactions and cell-cell crosstalk are critical for
monocyte activation and the generation of tissue factor.

Much work has been done in defining the intracellular signals that follow monocyte/macrophage exposure to cytokines or LPS, and a growing body of work has examined the response to adhesive interactions. Certain broad generalizations can be drawn. First, tyrosine phosphorylation signaling cascades are induced by virtually all of the stimuli that induce TF. For example, both cytokines and LPS have been demonstrated to increase tyrosine phosphorylation of intracellular proteins in macrophages (Sweet 1996, Lee 1996). Moreover, tyrosine phosphorylation is a virtually universal response to cell adhesion and to engagement of surface adhesion molecules (Miyamoto 1995, Aplin 1998). In human monocytic THP-1 cells, crosslinking of β1 integrins results in the accumulation of tyrosine phosphoproteins (Lin 1995). Within the broad response of tyrosine phosphorylation, recruitment of active MAPK proteins has been demonstrated to play critical roles in monocyte/macrophage activation following a wide number of stressful stimuli (reviewed in Lee 1996). In particular, MAPK pathways are induced in THP-1 monocytic cells or human monocytes following exposure to cytokines and LPS (Sweet 1996), after crosslinking of surface β1 and β2 integrins, or following adhesion to fibronectin matrices (Section VIII). Second, nuclear translocation and DNA binding of transcription factor NF-κB is a prompt response to these stimuli. TNFα and LPS rapidly induce NF-κB activation in monocytic cells (reviewed in Wulczyn 1996). Similarly, crosslinking of the β1 integrin VLA-4 on human THP-1 cells, or adhesion of these cells to the VLA-4 ligand, fibronectin, results in NF-κB activation (Fan 1995), as does the adhesion of monocytes to the β2 integrin ligand, fibrinogen (Sitrin 1998). There are many reported associations between tyrosine phosphorylation signaling, MAPK recruitment and activation of NF-κB, as well as with activation of other important nuclear factors such as AP-1 (see below). Thus, tyrosine phosphorylation, MAPK activation, and NF-κB could reasonably be predicted to be important in the expression of monocyte/macrophage TF following a number of stimuli.
**Regulation of TF Gene Expression in Monocytes: NF-κB and AP-1**

Tissue factor gene expression is controlled primarily at the level of transcription (Brand 1991, Gregory 1989). Its control has been studied principally in human THP-1 monocytic cells in response to LPS; the response of human monocytes to LPS is similar (Brand 1991). Resting blood monocytes express no tissue factor, but gene and surface expression are rapidly induced following stimulation with LPS (Drake 1989). The TF gene promoter region contains two sites for AP-1 transcription factor complexes, a single NF-κB complex site, five Sp1 sites, and three Egr-1 sites which overlap with the proximal three Sp1 binding regions (reviewed in Mackman 1997). There is constitutive binding of Sp1 and AP-1 complexes, which may contribute to basal expression of TF in transformed THP-1 cells (Oeth 1997) TF upregulation may be partially dependent on Egr-1, an inducible nuclear phosphoprotein, since LPS will weakly induce a truncated TF gene reporter construct which does not contain the AP-1 or NF-κB sites (Mackman 1991). However, the bulk of inducible TF mRNA is due to the synergistic effects of AP-1 and NF-κB heterodimers assembled on the TF promoter (Mackman 1997).

The TF NF-κB site is unusual in that a cytosine residue at position 1 confers selective binding of NF-κB complexes composed of the c-Rel/p65 (RelA), and precludes binding of the prototypical p50/p65 NF-κB complex (Mackman 1997). In THP-1 cells, LPS leads to the rapid phosphorylation and degradation of the IκBα protein which is otherwise tightly associated with the c-Rel/p65 complex, preventing its translocation to the nucleus (Mackman 1997, Hall 1999, O’Connell 1998). Degradation of IκBα in this system is primarily a function of the IκBα Kinase (IKK)-2, a serine kinase which can homo- or heterodimerize with IKK-1 to form the IKK complex (O’Connell 1998, Zandi 1997). IKK2 phosphorylates IκBα, leading to its rapid degradation, presumably by ubiquitin-dependent pathways (O’Connell 1998, Wulczyn 1996). Support for the central importance of this sequence in the LPS-induced upregulation of TF is found in work demonstrating that inhibition of IκBα degradation with salicylates or protease in-
hibitors prevents LPS-induced TF upregulation in THP-1 cells (Oeth 1995, Mackman 1994). While the great majority of studies have been performed in response to LPS, the increase in THP-1 TF following ligation of surface VLA-4 integrin is accompanied by increased TF NF-κB binding (Fan 1995). However, while NF-κB appears to be necessary for TF expression it is not sufficient for it: retinoic acid abolishes LPS-induced TF without affecting NF-κB binding or transactivation (Oeth 1998).

As for NF-κB, AP-1 plays a crucial role in the induction of the TF gene, but is not sufficient for its expression. Like NF-κB, AP-1 complexes are formed by the combination of two subunits. Homo- and hetero-dimers of Jun family proteins bind DNA, while Fos subunit members must first heterodimerize with Jun proteins (Whitmarsh 1996). Unlike NF-κB, there are high levels of constitutive AP-1 binding in unstimulated THP-1 cells (Oeth 1997, Fan 1995). The specific composition of AP-1 necessary for TF gene expression in stimulated THP-1 cells is controversial, with cFos/cJun, cFos/JunD and JunD/Fra-2 complexes described (Oeth 1997, Groupp 1996, Hall 1999). It is also not clear whether LPS stimulation leads to increased binding of AP-1 (Oeth 1997), a change in the binding of individual complexes (Groupp 1997), or an altered ability of constitutively bound AP-1 to induce transcription (Hall 1998). Whatever the case, deletion of either AP-1 site in the TF promoter results in decreased LPS induction (Mackman 1991, Oeth 1997). As for NF-κB, retinoic acid inhibits LPS induction without affecting either AP-1 binding or transactivation (Oeth 1998). These data suggest that regulatory pathways in addition to AP-1 and NF-κB are important to the induction of TF.

**Upstream Signaling Pathways in the Regulation of Monocyte TF:**

Just as large number of stimuli can induce monocyte/macrophage TF, so have many intracellular second messenger systems been studied in the context of TF expression. Early work noted that TF expression was dependent on changes in intracellular Ca^{2+}, required arachidonic acid metabolites, and
could be inhibited by increased generation of cAMP (reviewed in Lyberg 1984). More recently the roles of protein kinases have been elucidated, in particular protein kinase C, protein tyrosine kinases, and the MAPK family of serine/threonine kinases. The role of the cellular redox state has also been investigated. Components of these signaling routes interact with or are independent from the NF-κB and AP-1 pathways, and can disrupt TF gene expression or affect TF protein expression in a post-transcriptional manner. It is the integration of these diverse signals that determines the monocyte TF response.

A. Protein Kinase C:

Protein kinase C (PkC) is a serine/threonine kinase which integrates a myriad of diverse transduction signals and in turn regulates a wide variety of cellular functions (Newton 1995). Not surprisingly, PkC can have a number of roles in the regulation of monocyte TF, with occasionally contradictory results. For example, while pretreatment of human monocytes with PkC inhibitors has been reported to inhibit LPS-induced TF expression (Ternisien 1993, Car 1990), further activation of PkC once TF is expressed results in the rapid disappearance of TF activity and protein (Brozna 1988). This effect is in turn reversed by PkC inhibition (Brozna 1994). The data are contradictory even if only those studies in which PkC inhibition is initiated prior to LPS are considered. Pharmacologic inhibition of PkC with staurosporine, H7 or calphostin C has been variously reported to markedly inhibit TF-dependent PCA, TF protein, and TF mRNA expression following LPS treatment of human blood monocytes and bovine pulmonary alveolar macrophages (Ternisien 1993, Car 1990, van der Logt 1988). By contrast, we recently reported that inhibition of PkC with bisindolylmaleimide or staurosporine either did not affect or only partially reversed, respectively, LPS-induced PCA in murine macrophages (Dackiw 1997). The differing results may reflect cell type-specific responses, or be secondary to nonspecific pharmacologic effects. For example, staurosporine is known to directly affect tyrosine kinases (Hidaka 1992). Accepting that PkC likely does play a role in monocytic TF regulation, it is unclear what the mechanism for that effect
might be. While in LPS-stimulated human monocytes staurosporine inhibited TF transcription and not mRNA degradation (Ternisien 1993), suggesting an effect on NF-κB-dependent TF transcription, in other work staurosporine did not inhibit LPS-induced consensus NF-κB binding in human macrophages (Geng 1993). PkC has been shown to be upstream of macrophage pathways, such as the ERK MAPK, which may modulate TF expression in a fashion at least partially independent of NF-κB and AP-1 (Prokcyk 1999). Taken together these data illustrate the capacity of PkC to influence TF expression in monocytes, but suggest that its particular function will be dependent on cell type and stimulus.

B. Protein Tyrosine Phosphorylation:

Nearly every cellular process is regulated, at least to some degree, by protein phosphorylation (Hunter 1995). Protein tyrosine phosphorylation is a broad response of monocytes/macrophages to inflammatory and adhesive stimuli (Lee 1996, Lin 1995). Using murine macrophages we have shown that LPS-induced TF mRNA and PCA is abolished by pretreatment with the tyrosine kinase inhibitors genistein and herbimycin A (Dackiw 1997). Consistent with this work, it has previously been demonstrated that genistein and herbimycin A inhibit LPS-induced consensus NF-κB binding in human macrophages (Geng 1993). Elements of the tyrosine phosphorylation response are both stimulatory and inhibitory for TF expression and activity following LPS. For example, the tyrosine phosphatase inhibitor vanadate caused a striking increase in tyrosine phosphorylation following LPS in murine macrophages, which did not affect the upregulation of TF mRNA but abolished PCA (Dackiw 1997). In human monocytes, cyclosporine A, which inhibits the tyrosine phosphatase calcineurin, has been shown to decrease LPS-stimulated PCA and TF mRNA in concert with reduced NF-κB binding (Holschermann 1996). These studies outline the importance of tyrosine phosphorylation to monocytic TF expression, but suggest that individual pathways will have specific and possibly competing roles.
C. Mitogen Activated Protein Kinases:

The MAPK family of serine/threonine kinases are downstream effectors of tyrosine phosphorylation pathways that have been demonstrated to play crucial roles in monocyte/macrophage functions (Lee 1996, Sweet 1996, DeFranco 1998). LPS stimulation of monocyctic cells leads to the tyrosine phosphorylation and activation of members of all three MAPK families: extracellular signal-regulated kinase (ERK) (Weinstein 1992), the stress activated protein kinase (SAPK) or c-jun N-terminal kinase (JNK) (Hall 1999, Hambleton 1996), and the p38/CSB/RK (Han 1994). It has been demonstrated that macrophages exposed to LPS require both ERK and p38 MAPK activity for the induction of inflammatory cytokines (Carter 1999, DeFranco 1998). Similarly, we have found that inhibition of either the ERK or the p38 MAPK pathway in murine macrophages stimulated with LPS results in inhibition of TF mRNA expression (I.D. McGilvray, unpublished observations). These results suggest that while both the ERK and p38 MAPK pathways are necessary for TF expression in monocytes, neither alone is sufficient to induce TF.

The ability of the MAPK proteins to regulate TF expression may flow, at least in part, through their effects on NF-κB and AP-1. There is considerable evidence that MAPK family members can influence either the binding or the transactivation potential of NF-κB and AP-1. Hall et al found that AP-1 phosphorylation increased following LPS stimulation in a manner that correlated with increased SAPK activation (Hall 1999). Whether AP-1 phosphorylation contributes to its ability to induce transcription is controversial (Whitmarsh 1996, Mackman 1997). However, there is clear evidence that the MAP kinases can regulate expression of the individual AP-1 components upregulated in response to LPS, including c-Fos and c-Jun (Whitmarsh 1996, Dokter 1993). Thus, MAPK-regulation of AP-1 may modulate both the immediate and the more delayed monocyte TF response.

MAPK pathways also influence NF-κB, in a cell and stimulus-specific manner. Control of NF-κB
is effected both at the level of its translocation from the cytosol to nucleus ("activation") and it ability to induce gene transcription once bound to promoter regions ("transactivation"). As noted earlier, translocation to the nucleus is often a function of degradation of the IκBα protein by the IKK complex. The recent finding that proteins which are upstream regulators of MAPK pathways (MAP kinase kinase kinase proteins: MEKK-1, MEKK-2, MEKK-3 and NIK) also regulate the IKK complex suggests that MAP kinase and NF-κB activation may flow in parallel (Nemoto 1998, Zhao 1999). Evidence to this effect includes the fact that NF-κB activation is not affected by either ERK or p38 MAP kinase inhibition in LPS-stimulated murine macrophage RAW264.7 cells (Hwang 1997), nor by p38 inhibition in mouse astrocyte cultures treated with IL-1α and TNFα (Da Silva 1997). Similarly, TNFα- and hydrogen peroxide-stimulated p38 MAP kinase activation in human embryonic kidney cells is independent of NF-κB activation (Wesselborg 1997). On the other hand, ERK1 is upstream of NF-κB activation following the binding of HIV to its lymphocyte receptor, CD4 (Briant 1998), and NF-κB transactivation is dependent on p38 MAP kinase, but not ERK, in RAW264.7 cells stimulated by either mycoplasma fermentas membrane associated lipoprotein-1 or LPS (Garcia 1998). The latter finding may suggest that NF-κB transactivation can be regulated independently of its activation; in support of this hypothesis, a recent study found that both ERK and p38 MAP kinase inhibition attenuated NF-κB transactivation, but not nuclear translocation and DNA binding, in mouse fibrosarcoma L929 cells exposed to TNFα (Vanden Berghe 1998). Further insight into the multiple roles of the MAPK in NF-κB signaling is provided by an intriguing study which found that the inhibition of IκBα degradation by salicylates, noted earlier, may in fact be mediated via the p38 MAP kinase (Schwenger 1998). The finding that tyrosine phosphorylation of IκBα can lead to NF-κB activation without proteolytic degradation of NF-κB (Imbert 1996), the fact that there are likely to be IKK-independent routes of IκBα degradation (Li 1998), and the recent description of regulatory proteins contained within the IKK complex (Mercurio 1999) suggests that there are many aspects of NF-κB control yet to be described. The multiple roles of the MAP kinases in this complex system suggest that they may play a crucial role in the integration of signals leading to monocytic TF expression.
D. Cellular redox state:

Many cellular stresses, including LPS, affect the redox state of monocytes and macrophages (Sweet 1996). Although most monocyte/macrophage regulation of TF is at the level of transcription, TF protein can be decreased in a posttranscriptional fashion by antioxidants. We have reported that pretreatment of human monocytes and murine macrophages with N-acetyl cysteine (NAC) or pyrrolidine dithiocarbamate (PDTC) markedly reduces LPS-induced PCA, without affecting the upregulation of TF mRNA in murine macrophages (Brisseau 1995). Intriguingly, NAC also abolished plasmalleular expression of TF protein, and by Western blot analysis abolished the induction of mature, 47kD TF protein. The non-glycosylated, immature 32kD form was upregulated even in the presence of NAC. These results suggest that cellular redox states can affect the processing or production of TF protein in a posttranscriptional manner. The mechanism of this effect is unclear. Redox states have been demonstrated to modulate NF-xB, AP-1 and MAP kinase activation (reviewed in Wulczyn 1996 and Chakraborti 1998); however, these pathways affect TF expression principally at the level of gene transcription. One possibility is that TF turnover is affected by cellular redox status. Reactive oxygen species were generated in a study which linked PKC activation with TF turnover; however, the authors used antioxidants (catalase, superoxide dismutase) to specifically exclude the possibility that their effect was dependent on intracellular oxidation (Brozna 1988). The effects of antioxidants on TF expression may be due to inhibition of glycosylation coupled with altered protein stability. NAC appeared to inhibit glycosylation of the immature form of TF. This glycosylation does not appear to affect TF activity, but may be necessary for optimal cell surface expression of TF (Bona 1987, Paborsky 1990). The cellular redox state can also influence protein stability within the endoplasmic reticulum (Young 1993). Whatever the underlying mechanism, the antioxidant studies illustrate another level of control of TF protein expression.
Conclusions for Section IV:

As befits a cellular product with important consequences for both the inflammatory milieu and cell function, control of the expression of monocyte/macrophage TF is tightly controlled by a series of integrating signaling pathways. Expression is regulated principally at the level of transcription. In response to inflammatory mediators, such as LPS, or to adhesive, contact-dependent stimuli monocytes and macrophages respond with the coordinated upregulation of intracellular signaling pathways, including NF-κB, AP-1, protein kinase C, tyrosine phosphorylation and MAP kinase pathways. Individual components of these pathways are necessary but not sufficient for the expression of TF. Studies have also suggested another level of control, in that TF turnover or protein processing can be influenced in a post-transcriptional manner. The response of the cell to a given stimulus represents the integration of these diverse signaling pathways and processes. In particular, the role of the MAP kinases in adhesion or integrin-related monocyte TF expression remains to be defined.

From a therapeutic viewpoint, the study of these signaling routes may suggest novel approaches to the therapy of inflammatory and coagulation disorders. In support of this contention, the systemic coagulation disorder that accompanies acute promyelocytic leukemia responds dramatically to treatment with all-trans retinoic acid (ATRA, Frankel 1994), just as ATRA inhibits monocyte TF expression (Oeth 1998). The observation that salicylates, protease inhibitors, and cyclosporine A inhibit monocyte TF production (Oeth 1995, Mackman 1995, Holschermann 1996) may ultimately be of benefit in the treatment of pathologic upregulation of monocyte TF, such as follows adhesion to prosthetic devices and clinical inflammatory states. Further investigation into the control of monocyte TF expression is therefore essential.
Section V: Insights into the Molecular Pathogenesis of Viral Fulminant Hepatic Failure: Roles and Regulation of fgl-2/fibroleukin

Summary:

Viral fulminant hepatic failure (FHF) is the most common cause of acute liver failure in North America. Although our understanding of the mechanisms underlying the massive liver destruction characteristic of FHF has improved, liver transplantation is often the only viable therapy. Ideally treatment of FHF would restore liver function or at least delay liver damage, either as a bridging measure prior to transplantation or potentially to allow regeneration to occur and obviate the need for transplantation. In this review the current understanding of the immunopathogenesis of viral fulminant hepatic failure will be briefly outlined. In viral FHF, the “quiet” removal of hepatocytes by apoptosis is accompanied by an intense inflammatory process which drives activation of the coagulation cascade. Recent data from our group has suggested that activation of coagulation may play a central role in the pathogenesis of viral FHF clinically. In particular, the human analogue of the murine macrophage prothrombinase fibrinogen-like protein-2 (fgl-2)/fibroleukin, identified as a key component of the murine hepatitis virus strain-3 model of FHF, may be important to the human disease. Future studies into the regulation and function of this interesting protein may suggest novel therapeutic approaches for viral FHF.

Introduction: The Clinical Background

Fulminant hepatic failure complicates only 1% of cases of acute viral hepatitis, but when it does occur the consequences are severe (Pappas 1995). Despite liver transplantation, overall mortality rates are roughly 50% (Pappas 1995, Williams 1996). The poor outcome reflects both the severity of the insult to the liver and the multiple organ failure (MOF) which accompanies the systemic cytokines, circulating endotoxin and bacteremia which are associated with FHF (Williams 1996, Muto 1988, Wyke 1982, Wilkinson 1974, de la Mata 1990). The medical management of FHF is made more difficult by the fact that by the time of presentation much of the liver damage has already occurred, viral replication may
by the time of presentation much of the liver damage has already occurred, viral replication may have ceased, and MOF may have set in (Williams 1996). Nevertheless, the ideal treatment of FHF would be medical, since if given time the tremendous regenerating potential of the liver can replace even the most damaged organ (Chenard-Neu 1996).

A wide range of medical therapies for FHF have been attempted with generally disappointing results. Hepatoprotective prostaglandin E may have some benefit if started early in the course of toxic, drug-induced FHF but despite early, encouraging results has not provided consistent evidence of its usefulness in viral FHF (Sinclair 1989, Sinclair 1991, Sheiner 1992, Peltekian 1996). Antioxidant therapy with N-acetyl cysteine has been reported to improve the hemodynamic and oxygen transport abnormalities of FHF, but has only a small effect on survival (Harrison 1991). Anti-viral interferon therapy has been suggested to be of benefit in two small, uncontrolled trials (Levin 1989, Yoshiha 1995), while another found no utility to the treatment (Sanchez-Tapias 1987). Reversal of the coagulation defect of FHF with fresh frozen plasma with or without heparin has been attempted, with anecdotal report of benefit (Rake 1970); a follow up study examining tylenol-induced FHF found no survival benefit (Gazzard 1974). FHF is associated with a dramatic reduction in a key regulator of coagulation, antithrombin III; however, antithrombin III replacement in 13 patients with FHF (3 viral, 10 toxic) did not improve survival — but also did not affect the coagulation defect — when compared to 12 control FHF patients (Langley 1993). The more invasive measures of charcoal hemoperfusion, artificial and bioartificial liver support devices and extracorporeal porcine liver perfusion have either not been demonstrated to improve survival or provide marked clinical benefits, or are at best very temporary in nature ((O’Grady 1988, Williams 1996, Catrall 1994, Chari 1994). In the future these types of therapies may prove very useful; in the meantime, a careful review of the immunopathogenesis of viral FHF is warranted in an effort to suggest alternative medical therapies.
Immunopathogenesis of Viral FHF: A Pro-Inflammatory State:

Current understanding of the immunopathogenesis of viral FHF suggests that profound liver damage is mediated by the inflammatory infiltration and activation of mononuclear lymphocytes and macrophages. F.V. Chisari has proposed an elegant sequence for the development of viral FHF, based on a murine model of FHF in which cytotoxic T cells (CTL) specific for the hepatitis B surface antigen are injected into transgenic mice expressing the antigen. The CTL induce hepatocyte apoptosis and incite the infiltration and activation of large numbers of non-antigen-specific lymphocytes. A local inflammatory focus develops with recruitment of macrophages. These leukocytes are activated by the interferon-γ (IFNγ) secreted by lymphocytes, and prompt a delayed-hypersensitivity-type reaction resulting in massive liver necrosis (Ando 1993, Chisari 1995).

The fact that viral FHF is caused by a large number of viruses in addition to Hepatitis B (Pappas 1995) makes it likely that the underlying mechanisms of liver damage are quite variable. Nevertheless, the classic pathology of viral FHF is strikingly similar to the HbsAg transgenic model. In the most damaged areas there is only a connective tissue framework denuded of cells and collapsed on itself. Although the massive tissue destruction in these areas leaves little evidence behind of the underlying pathogenic mechanisms, where parenchyma does remain there is generally inflammatory infiltration of lymphocytes and macrophages. Kupffer cell hyperplasia and the presence of acidophilic (Councilman) bodies presumed to reflect hepatocyte apoptosis (Popper 1948, Lau 1998). Clinical and experimental evidence support the general model of lymphocyte and macrophage activation in a local inflammatory setting, with the resultant cytokine secretion, lymphocyte-mediated cytotoxicity and activation of the coagulation cascade resulting in massive liver damage. Hepatic destruction is the result both of hepatocyte apoptosis (programmed cell death) and necroinflammatory necrosis.
A. Hepatocyte apoptosis:

Hepatocyte apoptosis has recently been recognized as a potential major mechanism of hepatocyte death in FHF. Hepatocyte apoptosis can arise in four ways. First, CTL-mediated cytotoxicity results from the injection of apoptosis-inducing proteins into target cells (perforin/granzyme pathway) or by the TcR-limited interaction of lymphocyte Fas ligand with Fas (CD95) hepatocyte surface protein (Kagi 1994, Vignaux 1995, Feldman 1998). Second, hepatocytes may be induced to undergo apoptosis in response to local secretion of cytokines, such as TNFα and IFNγ (Shingawa 1991, Morita 1995). Third, hepatocyte apoptosis may follow intracellular viral replication. Although hepatitis B is not considered to be cytopathic, and may actually inhibit hepatocyte apoptosis (Gottlob 1998), other viruses, including hepatitis A and HSV, may induce apoptosis as a result of direct cytopathic effects (Brack 1998, Irie 1998). Finally, hepatocytes may express Fas ligand themselves, and prompt “auto-killing” of surrounding cells (Strand 1998, Galle 1995).

Hepatocyte apoptosis may be a general effector of liver damage. Large scale hepatocyte apoptosis has been implicated in a number of experimental models. Hepatocyte apoptosis is a feature of T-cell driven FHF models (Tiegs 1997), of the viral FHF that follows HSV infection in silica-treated mice (Irie 1998), and in Propionibacterium acnes-sensitized, endotoxin treated mice (Kondo 1997). Hepatocyte apoptosis has the potential to destroy the liver, even in the absence of significant inflammatory infiltrates: the intraperitoneal injection of pro-apoptotic antibody specific to surface Fas antigen results in rapid liver destruction in mice (Ogasawara 1993). Apoptosis can also contribute to the development of a destructive liver response in inflammatory conditions. In the transgenic HbsAg murine model, the initial CTL-mediated apoptosis of hepatocytes is a necessary step in the progression to massive inflammatory liver necrosis. Inhibiting apoptosis with soluble Fas protein inhibits apoptosis and liver destruction but does not affect the early infiltration of inflammatory cells. Thus, although apoptosis is considered to remove
cells in a “quiet” fashion (Fadok 1998), in the case of viral FHF it may precipitate inflammatory damage. It is tempting to speculate that the marked distortion of liver architecture that results from massive hepatocyte apoptosis (Ogasawara 1993) may influence the dynamics of the inflammatory response. An inflammatory response generally requires spatial localization for amplification: the distorted, apoptotic liver architecture may confine and sequester leukocytes in such a manner that the inflammatory response is enhanced.

The actual contribution of hepatocyte apoptosis to clinical viral FHF remains to be determined. Although the formation of Councilman bodies in viral FHF is presumed to reflect hepatocyte apoptosis, there is conflicting data regarding the prevalence of apoptosis as determined by biochemical or molecular means. Fas ligand mRNA expression in hepatitis B virus acute liver failure is found primarily in areas of lymphocyte infiltration (Galle 1995), suggesting a clinical link between hepatocyte death and lymphocyte-mediated apoptosis. However, small studies have reported either large numbers or no significant apoptotic hepatocytes in patients with viral FHF (Ryo 1995, Afford 1995). Increased circulating Fas ligand has been described in patients with apparent viral FHF (Shiota 1998); however, the significance of this finding is unclear, since soluble Fas ligand loses its liver toxicity and can actually protect against membrane-bound Fas ligand-mediated damage (Schneider 1998, Tanaka 1997). It must be pointed out that apoptosis is also a mechanism used by leukocytes to limit the inflammatory response (Brown 1999): as such, the finding of mononuclear cell apoptosis in inflammatory liver disease suggests that leukocyte apoptosis may act to down-regulate the liver inflammatory response (Afford 1995, Nuti 1998). For this reason, efforts to inhibit “apoptosis” in clinical inflammatory liver disease should be approached with caution.
B. Evidence for a pro-inflammatory state:

Although apoptosis may remove hepatocytes in a non-inflammatory fashion, the viral FHF liver is markedly pro-inflammatory. Evidence for the local pro-inflammatory milieu of viral FHF is found in the local and systemic release of cytokines, upregulation of vascular adhesion molecules, inflammatory profile of infiltrating lymphocytes, and monocyte/macrophage infiltration and activation.

1. Cytokine release:

Viral FHF is accompanied by the local and systemic increases in cytokine levels. These may initially be induced by viral replication in hepatocytes (Larapezzi 1996, Inagi 1996), and are perpetuated and amplified by the infiltration and activation of mononuclear leukocytes (Andus 1997, Chisari 1995). Systemic increases in IL-1, TNFα, and IL-6 have been described in patients with viral FHF or acute hepatitis (Muto 1988, Sun 1992, de la Mata 1990). To some extent this increase may reflect the endotoxemia or bacteremia that often accompanies FHF, leading to the general syndrome of MOF (de la Mata 1990, Williams 1996). However, three lines of reasoning suggest that a substantial fraction of the systemic release of cytokines seen in viral FHF actually originates in the liver. First, all cells normally resident in the liver have the capacity to produce cytokines (Andus 1996), and strong TNFα staining has been reported in tissue macrophages and infiltrating mononuclear cells in acute viral hepatitis (Spengler 1996), while hepatocytes appear to secrete TNFα in chronic viral hepatitis (Gonzalez-Amaro 1994). Second, as a rule the organ most affected in MOF is the organ which secretes the most cytokines (Douzinas 1997). Finally, both the endotoxin and bacteremia of FHF are presumed to originate from the gastrointestinal system. The liver is the first organ on which these bioactive agents impact, and Kupffer cells clearly can be activated by both types of stimuli (reviewed in Winwood 1993). Experimentally there is good evidence that the local production of cytokines such as TNFα and IFNγ in T-cell driven models is essential to the development of a destructive liver response; equally, the local secretion is accompanied by an increase in

2. **Upregulation of Vascular adhesion molecules:**

   The inflammatory mononuclear infiltration of viral FHF is supported by local changes in vascular adhesion molecules. Increased adhesion molecule expression follows exposure of sinusoidal lining cells to cytokines, complement and lipid-derived mediators (Jaeschke 1996). Although to date there are no studies of adhesion molecule expression in viral FHF, in acute hepatitis the inflamed liver has upregulated expression of ELAM-1 (E-selectin) and ICAM-1 on sinusoidal lining cells (Volpes 1992, Volpes 1990). ICAM-1 has been demonstrated to be important for the migration of leukocytes, particularly neutrophils, into liver tissue (Jaeschke 1996). ICAM-1 is also increased on hepatocytes during acute hepatitis, in response both to viral replication and to local cytokines (Hu 1992, Schroder 1995, Satoh 1994). Interestingly, hepatocyte adhesion molecule expression may modulate the interaction of mononuclear cells with hepatocytes and so affect cytotoxicity: anti-ICAM-1 antibodies blocked lymphocyte cytotoxicity in concanavalin A-induced liver damage (Watanabe 1996). Thus, local upregulation of adhesion molecules can affect both the extent and the effect of the inflammatory infiltration.

3. **Inflammatory profile of infiltrating lymphocytes:**

   Although the immune response to hepatitis virus may be initiated by virus-specific CTL, the bulk of infiltrating lymphocytes are not specific to the pathogen (Abrignani 1997). Since migration is not antigen-specific but instead determined by adhesion molecule and chemokine expression, most of the T cells
that are recruited into an inflamed or infected site will not be specific for the inciting pathogen (Abrignani 1997, Springer 1994). These “bystander” lymphocytes are nonetheless activated by the cytokine milieu of hepatitis. While it is generally thought that individual cytokines act as growth, co-stimulation or differentiation factors, combinations of cytokines (such as IL-2/IL-4/IL-7 + IL-6 + TNFα) can activate both naïve and memory resting T cells in an antigen-nonspecific fashion (Abrignani 1997, Unutmaz 1994, Unutmaz 1995). These considerations help to explain why many more cells secrete cytokines than are pathogen-specific, and fit very well in the general schema proposed by Chisari, in which HbsAg-specific CTL only induce massive liver damage when CTL infiltration is followed by large numbers of autologous T cells with unrelated specificity (Ando 1993, Chisari 1995). This concept has been studied in the clinical context of chronic hepatitis C, where the majority of liver-infiltrating T cells display an activated phenotype, and when isolated from liver biopsies synthesize the Th1 proinflammatory IFNγ cytokine (Ballardini 1995, Nuti 1998). The cells responding to any of the HCV proteins are at a frequency of 1/100 or lower (Minutello 1993). The close parallel of these findings with the HbsAg transgenic mouse model of FHF makes it likely that similar mechanisms are at play in viral FHF. Nevertheless, the Th1/Th2 response should be examined critically in clinical viral FHF, since it is possible that a predominantly Th2 response, with less production of antiviral IFNγ, might predispose to FHF (Chung 1994, Pope 1996).

4. Macrophage activation:

Macrophage activation plays a critical role in the massive liver destruction that characterizes viral FHF. Infiltrating macrophages and Kupffer cell hyperplasia is a classic feature of viral FHF (Popper 1948); in the transgenic HbsAg mouse model, the delayed-hypersensitivity reaction initiated by macrophages is responsible for the bulk of liver necrosis (Ando 1993, Chisari 1995). Macrophages have a multitude of mechanisms which can lead to tissue damage, including cytokine generation, the production of
reactive oxygen species, and lysosomal protein release (reviewed in Winwood 1990). In the clinical context tissue macrophage activation is evidenced in acute hepatitis by positive immunostaining for TNFα (Spengler 1996). The general ability of tissue macrophages to induce liver injury by free radical release is supported by experimental data demonstrating Kupffer cell free radical production in conjunction with increased oxidative DNA damage in murine chronic active hepatitis (Hagen 1994), macrophage-mediated oxidative liver damage in D-galactosamine-sensitized mice (Shiratori 1988), and TNF-dependent macrophage superoxide release following ischemia-reperfusion (Shibuya 1997). In addition to these mechanisms, macrophage-induced activation of the coagulation cascade is likely central to the damage of viral FHF, as discussed below.

**Activation of Coagulation in viral FHF:**

Local pro-inflammatory effects make it inevitable that coagulation will be activated during the pathogenesis of FHF. Generally thought of in terms of hemostasis only, the coagulation cascade is at least as important as an inducer and modulator of inflammatory responses (reviewed in McGilvray 1998). There are tight interactions between inflammatory cascades and coagulation: not only is coagulation activated by many bioactive substances, including endotoxin, cytokines, bacterial products, and viral infections, but there is excellent experimental evidence in animal models detailing the often critical role of coagulation cascade activation in the outcome of septic and inflammatory insults (reviewed in McGilvray 1999). Activated coagulation results in the deposition of fibrin and the generation of active coagulation factors with direct inflammatory effects, such as Factor IIa - thrombin, Factor Xa, and the fibrinopeptides (McGilvray 1998). Fibrin deposition contributes to microvascular thrombosis, but also affects leukocyte accumulation and activity (McGilvray 1998). The effect of activated coagulation in the inflamed liver is likely increased by local upregulation of coagulation factor receptors: for example, it has been recently reported that thrombin receptor expression on monocellular cells is dramatically increased in clinical vi-
ral FHF (Marra 1998). Thus, in the pro-inflammatory milieu of viral FHF activation of coagulation may be important both to the genesis and to the outcome of the destructive liver response.

Cells of the monocyte / macrophage lineage are central to activation of coagulation in inflammatory states (Osterud 1996). The ability of the macrophage to induce activation of the coagulation cascade, measured as procoagulant activity (PCA), can in principle be mediated by four separate routes (see Figure 2). Macrophage de novo synthesis of tissue factor (TF), a cell surface glycoprotein which binds Factor VIIa and activates the so-called extrinsic coagulation pathway, is one of the principle mechanisms for activating coagulation \textit{in vivo} (Osterud 1996, McGilvray 1998). Expression of the potent prothrombinase, fibrinogen-like protein-2 (fgl-2), has been demonstrated in the Murine Hepatitis Virus Strain-3 model of FHF, and more recently by our laboratory in man (Levy 1999). In addition, macrophage release of proteolytic enzymes such as cathepsin may activate factor X bound to surface CD11b (Mesri 1998, Plescia 1996); this form of activation is more likely to enhance coagulation activation than act as a primary inducer of it (reviewed in McGilvray 1999). Finally, a direct Factor X activator has been described in rabbit hepatic macrophages in response to endotoxin (Maier 1981), but has not been described in human cells.

The local pro-inflammatory environment of viral FHF encourages activation of the coagulation cascade. Local activation of the coagulation cascade will follow macrophage and endothelial upregulation of Tissue Factor (TF) and fgl-2 prothrombinase, as well as the release of active lysosomal enzymes. Although TF has not been directly studied in viral FHF, the infiltration of macrophages in a pro-inflammatory milieu make it almost certain to be increased. The close approximation of macrophages with inflammatory T cells enhances the expression of TF initiated by cytokines (Edwards, Del Prete, McGilvray 1998). Increased vascular adhesion molecule expression leads to the local recruitment of bloodborne monocytes: the adhesive interactions necessary for such emigration have been demonstrated
Figure 2: Monocyte/macrophage-dependent activation of the coagulation cascade.
to be potent stimuli for the upregulation of monocyte TF (Collins 1995, Lo 1995, McGilvray 1997, McGilvray 1999). The liver may be particularly susceptible to the procoagulant effects of TF, since expression of the TF pathway inhibitor (TFPI) on hepatic sinusoidal cells appears to be relatively low (Arie 1995, Yamanobe 1997). Moreover, hepatic macrophages have the capacity for large-scale expression of TF: TF is strongly upregulated in response to ischemia-reperfusion injury, endotoxin, and hemorrhage (Kobayashi 1998, Arai 1998, Yamashita 1997). The release of proteolytic enzymes such as cathepsin is a general feature of activated macrophages and can also promote coagulation activation (Mesri, Plescia). Although likely involved, both TF and cathepsin-mediated mechanisms of coagulation activation remain to be confirmed in viral FHF.

A recent study from our group provides good evidence for local activation of the coagulation cascade. The livers from six consecutive patients with viral FHF (4 NANB, 2 HBV) who required transplantation were examined. Necrosis ranged from 60-90%, but in all six cases there was dramatic evidence of fibrin deposition in the areas where recognizable parenchyma remained. Vascular sinusoidal fibrin deposits were seen in areas of acute focal, confluent or bridging necrosis but were infrequent in the more advanced areas of panlobular necrosis in which only the consequences of the hepatocellular necrotizing process remained (Figure 3). Interestingly, fgl-2 prothrombinase mRNA was found in macrophages in the areas of acute necrosis seen in the 4 cases where the surviving parenchyma constituted more than 10% of the liver, in a striking colocalization with areas of fibrin deposition. These results suggest that fibrin deposition and fgl-2 expression may be involved in the development of the acute inflammatory lesions which ultimately degenerate into diffuse necroinflammatory necrosis.

Our findings are at first glance at odds with the classic description of the pathology of viral FHF, in which fibrin thrombi are relatively rare (Popper 1948). While some groups have described fibrin deposition as a classic feature of viral FHF (Mori 1984), most have found relatively infrequent, small fibrin
deposits in viral FHF, in contrast to larger and more frequent fibrin deposition in toxic, drug-induced FHF (Popper 1948, Rake 1971, Oka 1979). Some of the difference in results is technical: our group employed an immunologic assay for fibrin deposition which is more sensitive than the standard stains for fibrin (Dindzans 1986). However, the fact that fibrin deposition is mostly localized to acute, developing hepatic lesions suggests that it may be a transient finding which is eliminated along with the bulk of the cellular parenchyma during the massive necrosis which follows. This concept is supported by clinical parameters of coagulation and fibrinolysis activation in viral hepatitis and FHF, in which there appears to be generation of activated coagulation factors in parallel with increased secondary fibrinolysis (Rake 1970, Kernkes-Matthes 1991, Takahashi 1990, Takahashi 1989, Langley 1990). Thus, deposited fibrin would be expected to be promptly removed, either by secondary fibrinolysis or by progressive necrosis. As noted earlier, any deposition of fibrin is significant for what it implies about the developing inflammatory response. Moreover, as discussed below there is good reason to believe that significant parenchymal damage can occur in a coagulation-dependent fashion without overt fibrin deposition.

The finding that the human analogue of fgl-2 is upregulated in clinical FHF is very suggestive of the relation between coagulation activation and liver damage in viral FHF. Accumulated evidence in the Murine Hepatitis Virus Strain 3 model of viral FHF suggests that activation of coagulation via the fgl-2 prothrombinase is a major mechanism of liver damage.
**Figure 3**: Activation of coagulation in fulminant viral hepatitis

Liver biopsy specimen from an intensive care patient suffering from fulminant viral hepatitis, stained for fibrin deposition. Of note is the marked association between areas of damaged hepatocytes and strong sinusoidal staining for fibrin (in brown).
The Immunologic Response to Murine Hepatitis Virus Strain-3: Promoting the Expression of Macrophage Procoagulant Activity and fgl-2:

The Murine Hepatitis Virus Strain-3 (MHV-3) model has offered many insights into the pathogenesis of viral FHF. Although the transgenic HBsAg model is an elegant means of dissecting pathogenic mechanisms, it differs markedly from the clinical scenario in that there is no replicating viral pathogen. Injection of MHV-3 into susceptible mice results in FHF characterized by the presence of sinusoidal thrombosis and associated hepatocellular necrosis (Levy 1983, Levy 1989, Li 1992). These findings occur concomitant with the virally-induced expression of the fgl-2 prothrombinase protein in the tissue macrophages and sinusoidal lining cells of the liver (Ding 1997). The fgl-2 prothrombinase activates the coagulation cascade, an ability expressed as procoagulant activity (PCA) (Parr 1995, Fung 1991). Virus-induced PCA is central to the pathological changes observed in MHV-3 FHF. The hepatocellular necrosis which follows MVH-3 infection occurs in regions of sinusoidal fibrin deposition, concomitant with expression of fgl-2 in the sinusoidal lining cells and macrophages (Ding 1997). Other organs simultaneously infected with MHV-3 do not express fgl-2 and remain uninjured. Pretreatment of mice with monoclonal antibody directed against MHV-3 induced PCA prevents sinusoidal fibrin deposition, hepatocellular necrosis, and mortality in infected mice (Li 1992). Thus, there is good reason to conclude that the lethal hepatitis induced by MHV-3 is dependent upon fgl-2 upregulation.

As in clinical viral FHF, the immune macrophage and lymphocyte responses are critical to liver injury. The development of FHF following infection with MHV-3 is initiated by viral-induced upregulation of the fgl-2 gene, focal deposits of sinusoidal fibrin, and leukocyte infiltration with a predominance of macrophages and neutrophils (Yuwaraj 1996). There is good evidence that the macrophage PCA response contributes to disease. MHV-3 infection of macrophages results in the large expression of fgl-2 mRNA, fgl-2 surface protein, and PCA (Fung 1991). The degree of hepatocellular necrosis that follows
MHV-3 infection correlates well with the induction of PCA (Abecassis 1987, Li 1992, Pope 1995). Macrophages from susceptible mouse strains (Balb/cj) infected with MHV-3 develop a marked increase in PCA, in contrast to those recovered from resistant (A/J) mice (Chung 1994, Fingerote 1996). Resistant A/J mouse strains will develop both FHF and macrophage PCA following treatment with corticosteroids, which stabilize fgl-2 mRNA (Fingerote 1996). Furthermore, treatment with PGE2 abrogates viral induction of macrophage PCA both in vitro and in vivo, and prevents the development of FHF (Abecassis 1987, Chung 1991). Considered together these data suggest that macrophage PCA serves both as a marker of disease and contributes to the pathogenesis of FHF by virtue of the ability to sequester in the infected liver.

As noted above, macrophage PCA can result from increased fgl-2 expression or from tissue factor synthesis. However, the induction of PCA by MHV-3 is entirely dependent upon fgl-2 surface protein expression. The PCA which follows MHV-3 infection is dependent upon coagulation factor II but independent of factors VII and X, indicating a prothrombinase activity rather than tissue factor or factor X activator (Pope 1995). Monoclonal antibodies against the MHV-3 induced prothrombinase, which do not cross-react with tissue factor, completely abolish PCA following infection of macrophages both in vitro and in vivo (Li 1992, Fung 1991). Furthermore, MHV-3 infection of macrophages leads to fgl-2 mRNA and protein expression, and transient fgl2 protein expression in RAW 264.7 cells induces PCA and prothrombin cleavage activity (Parr 1995, Fung 1991, Pope 1995). These studies indicate that MHV-3 infection of murine macrophages results in the expression of fgl-2 prothrombinase, which in turn is responsible for virally induced PCA.

The lymphocyte response which accompanies the development of FHF in susceptible mice following infection with MHV-3 promotes macrophage PCA. CD4+ Th cells control the development of immune effector mechanisms through the secretion of cytokines: the synthesis of IL-2, IFNγ by Th1 cells
promote protective cellular immunity and viral clearance, while the IL-4, IL-10 and IL-13 secreted by Th2 cells promote humoral, B-cell-mediated immunity. In MHV-3 infection, susceptible mice develop a predominantly Th2 response, with less synthesis of the anti-viral IFNγ (Pope 1996). In addition, Th cells isolated from infected, susceptible mice will instruct uninfected macrophages to express PCA (Chung 1991). Pretreatment with a Th1 clone protects from MHV-3 induced FHF and death (Chung 1994). Similarly, T lymphocytes from mice resistant to FHF following infection with MHV-3 develop a protective ability to suppress PCA in macrophages, while susceptible mice do not (Chung 1991). Interestingly, viral pathogenicity correlates with replication of MHV-3 in T and B lymphocytes, with lysis of these cells in susceptible mice and lymphoid organ atrophy (Lamontagne 1989, Lamontagne 1991). Taken together these results suggest that MHV-3 infection of mice which go on to develop FHF results in a state of relative immunodeficiency, an inability to control viral replication and a Th cell response which encourages macrophage PCA.

In summary, the immunologic response of mice which develop FHF in response to MHV-3 is characterized by macrophage activation and expression of fgl-2 prothrombinase-dependent PCA in the context of a permissive Th2 reaction. By contrast, mice which do not develop FHF do not express macrophage fgl-2 and develop a predominant Th1 response. Interestingly, MHV-3 infection of macrophages from both susceptible and resistant mouse strains induces similar degrees of cytokine release (TNFα, IL-1) (Pope 1995): it is the expression of the fgl-2 prothrombinase which is critical for the development of FHF. These findings offer an intriguing analogy to the clinical setting, where the great majority of patients who develop acute hepatitis do not go on to develop FHF: the local Th1/Th2 balance of the immune response to a viral infection may determine both the ability to control viral replication and the aggressiveness of the immune coagulation response. The fgl-2 protein may serve actually serve two roles in determining whether or not liver infection progresses to FHF. In addition to triggering activation of the coagulation cascade, the fgl-2 protein may directly affect lymphocyte function and so change the local
Th1/Th2 balance (discussed below). In this context it is interesting to note that patients with viral FHF who go on to die have a greater increase in markers of activated coagulation than patients who survive (Langley 1991).

It is possible that viral FHF, like hyperacute allograft and xenogeneic rejection, represents a fundamental disorder of innate immunity. All three clinical entities involve pathologic, intense activation of the coagulation cascade with resultant destruction of liver parenchyma. The fgl-2 prothrombinase appears to play a role in clinical viral FHF; preliminary data in our laboratory has suggested that it may also play a role in the hyperacute rejection of xenogeneic livers. Future studies may define this intriguing possibility further.

Evidence for the role of fibrin deposition in other experimental models of FHF:

Although not directly analogous to viral immunopathogenesis, a number of experimental models have demonstrated that activation of the coagulation cascade with subsequent fibrin deposition can contribute to hepatic damage. Sinusoidal and parenchymal fibrin deposition has been observed in rat livers following administration of dimethylnitrosamine, endotoxin alone, or of endotoxin after sensitization with heat-killed Corynebacterium parvum (Fujiwara 1995, Hirata 1989, Shibayama 1987). Hepatic necrosis in the dimethylnitrosamine model was attenuated by treatment with antithrombin III concentrate (Fujiwara 1988), and in the endotoxin model by treatment with heparin (Shibayama 1987). Injection of portal vein thrombin results in sinusoidal thrombosis and focal areas of parenchymal necrosis (Shibayama 1987). However, fibrin deposition does not have to be marked to contribute to liver damage. As assessed by routine hematoxylin/eosin staining, hepatic sinusoidal fibrin deposition is small and relatively uncommon in rats exposed to carbon tetrachloride (CCl₄) (Rake 1973, Fujiwara 1995). Nevertheless, after the administration of CCl₄ radiolabelled fibrinogen accumulates more in the liver than in any
other organ, including the spleen, kidney, heart and lungs (Rake 1973). Heparin treatment delays the development of hepatic necrosis following CCl₄ (Rake 1973). These studies suggest that local activation of coagulation can contribute to hepatic damage even in the absence of gross evidence of fibrin deposition; rapid fibrinolysis may account for the relative absence of fibrin deposits. More recent immunohistochemical studies have demonstrated that CCl₄ intoxication leads to parenchymal fibrin deposition in pericentral necrotic areas (Neubauer 1995). Taken together these studies demonstrate that activation of the coagulation cascade by a number of disparate insults has the potential to damage the liver. Thus, there is good reason to suppose that activation of coagulation can damage the liver during viral infection, as evidenced by the MHV-3 model.

**The human fgl-2 analogue:**

We recently demonstrated expression of a human fgl-2 gene homologue in liver macrophages of patients suffering from FHF (Levy 1999). The human analogue for murine fgl-2 was first described in T-lymphocytes, and has been suggested to function as a secreted extracellular matrix protein with regulatory effects on T cell activation (Ruegg 1995, Koyama 1987, Marazzi 1998). Our group recently reported that the human fgl-2 nucleotide sequence predicts a 439 amino acid protein, having 70% overall homology with the murine fgl-2 (Levy 1999). In particular, a 225 amino acid sequence at the carboxyl end corresponds to the well-conserved “fibrinogen-related domain” (Parr 1995). Human fgl-2 has homology with other fibrinogen-related domain proteins with recognized immunomodulatory effects, such as tenascin and cytotaxin (Ruegg 1990, Xu 1990). However, it should be remembered that protein function is determined as much by its cellular location as by its structure. This concept holds true for coagulation cascade proteins. For example, tissue factor is biologically active only when expressed on the macrophage cell surface (McGilvray 1999 review). Similarly, although lymphocytes may secrete fgl-2 protein, in the murine MHV-3 model fgl-2-dependent procoagulant activity is restricted to mouse macrophages. Macro-
phages when stimulated appropriately express large amounts of fgl-2 on their surfaces (Chung 1991, Fung 1991, McGilvray 1998). In the human case, fgl-2 expressed on the surface of activated macrophages is likely to have a direct prothrombinase activity. Transient transfection of CHO cells with a full length cDNA of the human fgl-2 coding region results in the de novo expression of cellular procoagulant activity and the ability to directly cleave prothrombin to thrombin, concurrent with cell surface expression of the fgl-2 protein (Levy 1999). These results suggest that when the human fgl-2 protein is expressed on the cell surface it acts like its murine macrophage surface protein counterpart, and functions as a prothrombinase with the ability to activate the coagulation cascade.

**Regulation of murine fgl-2: Replication and Intracellular Signaling**

Human macrophage and/or endothelial expression of the fgl-2 prothrombinase may be critical to the development of clinical viral FHF. The MHV-3 model is an attractive method for defining the regulation of fgl-2, and for testing agents which might be useful clinically. For example, the observation that prostaglandin E (PgE) inhibited MHV-3 induced macrophage procoagulant activity and liver necrosis led directly to the testing of PgE in clinical FHF (Abecassis 1987, Peltekian 1996). As noted earlier, PgE ultimately did not provide consistent benefit in viral FHF, but was demonstrated to be useful in early toxic FHF, and in orthotopic liver transplantation complicated by primary graft non-function or recurrent hepatitis B infection (Sheiner 1992, Flowers 1994, Greig 1989). The inability of PgE to consistently improve viral FHF reflects the general difficulty of treating this overwhelming condition. Nevertheless, the high mortality of viral FHF and the shortage of organs available for liver transplantation make it critical that new medical therapies be developed and tested. Insights into the regulation of murine macrophage fgl-2 may suggest novel therapeutic approaches.

The tremendous immune response elicited by the causative virus can result in its virtual elimina-
tion by the time of clinical presentation (Williams 1996). Nevertheless, it is impossible to separate patients who present with viral FHF into those with residual virus and those who have eliminated it. Thus, the ideal therapy of viral FHF would inhibit both viral replication and the aspects of the immune response which result in liver damage. The MHV-3 model illustrates the essential nature of both viral replication and immune response to FHF pathogenesis. Replication is essential for the full response to the virus: UV-irradiated, non-replicating virus induces neither macrophage procoagulant activity nor FHF (G Levy, unpublished data). However, replication alone is not sufficient for disease, since MHV-3 replicates both in resistant and in susceptible strains of mice (Pope 1995). It is possible clinical therapy of viral FHF with prostaglandin E may have failed in part because the prostaglandin inhibits only the inflammatory axis, without attenuation of viral replication (Abecassis 1987).

Data from the MHV-3 model suggests that it may not be necessary to fully inhibit replication, but that partial inhibition may be enough to alter the immune response and in particular the expression of macrophage fgl-2. Ribavarin, a synthetic guanosine analog, decreased the 10 hour replication of MHV-3 in murine macrophages by a single log viral titer, but abrogated the induction of fgl-2 and inhibited liver necrosis (Ning 1998, Sidwell 1977). The method of inhibiting viral replication appears to be important: Oragen, a 2',5'-oligoadenylate analogue, inhibited 12 hour MHV-3 replication by roughly 60%, but failed to inhibit either macrophage PCA or liver necrosis (Fingerote 1995). Recent data from our group has suggested that proteins involved in MHV-3 replication are essential for the stimulation of macrophage fgl-2 (Ning 1999). Specifically, the viral nucleocapsid protein acts as a nuclear transcription factor to promote fgl-2 expression, possibly in concert with the liver-specific LF-A1 transcription factor. Ribavarin and oragen may have affected these factors differently. Thus, MHV-3 induced fgl-2 and liver necrosis may be inhibited if replication is attenuated appropriately.

Defining the intracellular signaling pathways which are necessary for fgl-2 expression may sug~
gest novel means of inhibiting the liver inflammation which follows viral infection. Inhibition of virally-induced macrophage procoagulant activity by agents which increase intracellular cAMP (PGE2, forskolin, isobutylmethylxanthine), inhibit GTP-binding proteins (pertussis toxin), or inhibit protein kinase C (staurosporine, H7), indirectly suggests that these signaling pathways are involved in the macrophage response to MHV-3 (Abecassis 1987, Chung 1994). More recently we have demonstrated induction of tyrosine phosphorylation signaling pathways in macrophages exposed to MHV-3 (Dackiw 1995). MHV-3 infection of murine macrophages consistently upregulates tyrosine phosphoproteins which migrate in the 38-44 kDa range; however, the roles of the p38 MAPK, p42 ERK2 and p44 ERK1 in MHV-3 signaling remain to be defined.

Whether these results can be translated into clinical interventions remains to be seen. Preliminary evidence from our laboratory has demonstrated decreased, but not wholly abolished, hepatic fgl-2 in mice pretreated with tyrosine kinase inhibitors and infected with MHV-3; however, viral replication \textit{in vivo} was increased and liver necrosis was not inhibited. These results are in keeping with previous work examining the effect of abolishing MHV-3 procoagulant activity with specific monoclonal antibodies (Li 1992). Inhibition of FHF was observed primarily at the higher doses of antibody. In other words, partial inhibition of fgl-2 was not sufficient to lessen liver damage. These data point out the difficulty of treating multifactorial viral FHF with a single intervention, but do suggest that the course of FHF can be modulated by efficient inhibition of fgl-2. When considered together with the replication data discussed above, these studies argue that both viral replication and the inflammatory response to it may need to be addressed to effect a clinical benefit.
Other roles of fgl-2:

The fgl-2 protein likely functions in several capacities in addition to its ability to induce coagulation. As mentioned early, the fgl-2 secreted by lymphocytes has been suggested to act as an extracellular protein with regulatory activity on T lymphocytes (Marazzi 1998). Soluble fgl-2 (also known as fibro-leukin, or FIBLP) is secreted primarily by memory T lymphocytes, and secretion is maintained by IFNγ (Marazzi 1998). Preliminary work by our group has suggested that soluble fgl-2 inhibits lymphocyte proliferation and mixed lymphocyte reactions (C. Chan and G Levy, unpublished observations). In theory this immunosuppressant activity may impair Th1 responses and viral clearance, and thereby contribute to the progression from acute viral hepatitis to viral FHF.

Coagulation activation by fgl-2 may also function in pathologic conditions other than viral FHF. Macrophage fgl-2 is known to be upregulated by stimuli other than MHV-3, including herpes simplex virus (personal communication, Dr. G. Levy) and IFNγ (Lafuse 1995). Cytokine induction of fgl-2 appears to be a selective function of IFNγ, since fgl-2 is not induced by other cytokines, including IL-2, IL-4, IL-10 and TNFα (Lafuse 1995). Surface fgl-2 upregulation on endothelial cells has been implicated in the induction of fetal loss by cytokines (TNFα and IFNγ) in CBA x DBA/2 mice (Clark 1998). It should be stressed that activation of coagulation is an inflammatory response: thus, fgl2 upregulation may contribute to a number of inflammatory processes.

The above considerations suggest a general model in which surface fgl-2 protein leads to coagulation activation and drives inflammatory processes such as viral FHF, while secreted fgl-2 acts as a relative immunosuppressant which may alter the Th1/Th2 balance. In the future it will be of interest to define whether fgl-2 affects other aspects of viral FHF immunopathogenesis, such as hepatocyte apoptosis.
Conclusions for Section V:

Fulminant viral hepatitis is a overwhelming condition which can result either in death or in liver transplantation. To date attempts at altering the course of viral FHF with medical therapies have failed; nevertheless, the relative birth of organs available for transplantation and the potential for native liver regeneration make the search for a medical intervention critical. The viral FHF liver presents a markedly pro-inflammatory milieu in which parenchymal destruction is mediated by a combination of lymphocytic and macrophage-dependent processes. Clinical and experimental data suggest that activation of the coagulation cascade is a key factor in liver necrosis. In particular, recent data from our group has suggested that macrophage expression of a potent prothrombinase, fgl-2, may play a role in the pathogenesis of the clinical disease. On the basis of recent data from the MHV-3 model of FHF we propose that medical therapy of FHF should aggressively target both viral replication and fgl-2-dependent activation of coagulation. Studies into the regulation of fgl-2 expression may offer new prospects for the treatment of a condition which to date has largely defied modern medical practices.
Section VI: Roles and Regulation of the MAP kinases in Monocytes and Macrophages

Summary: The MAP kinases are powerful transducers of extracellular signals which play key roles in many cellular responses. They may therefore represent a potential target both for the study and the therapy of monocyte/macrophage-driven diseases, such as fulminant viral hepatitis.

In this section the activation, regulation and function of the three MAPK modules are described in some detail. More specifically, discussion is made of their potential activation during monocyte integrin stimulation or migration, and during the monocyte response to viral infection and inflammatory stimuli.

A. MAP Kinase Modules:

1. MAPK Families:

The MAP kinases are divided into three families: the ERK, SAPK, and p38 MAPK. These ubiquitous Serine/Threonine kinases are differentially activated by a wide range of extracellular stimuli, including growth factors, mitogens, cytokines, and environmental stress (ultraviolet light, heat shock). Similarly, they have been implicated in a wide range of cellular functions, such as cell growth, oncogenic transformation, cell differentiation, apoptosis and inflammatory cytokine expression. As a rule they are described in terms of a "module," composed of the Ser/Thr MAP kinase (MAPK), a dual specificity Thr/Tyr MAP kinase kinase (MAPKK) which activate the MAPK, and a Ser/Thr MAP kinase kinase kinase (MAPKKK) which activates the MAPKK.

The ERK Family:

The extracellular signal regulated kinase (ERK) family currently comprises 5 members: p44 ERK1, p42 ERK2, p55 ERK3, p63 ERK4, and ERK5 (big map kinase, BMK) (Widmann 1999). All members are activated by dual Threonine/Tyrosine phosphorylation on a TEY motif located, as for the other MAPK, within the T loop adjacent to the catalytic cleft (Cobb 1995). Once activated, ERK family members phosphorylate substrate proteins on serine or threonine, within a proline-directed motif. Pro-Leu-Ser/Thr-Pro is the most stringent consensus sequence for ERK1 and ERK2 recognition (Casillas 1993).
Although classified as ERK proteins, there is actually more divergence between the ERK1/2 proteins and the ERK3/4/5 proteins than for any other MAPK (Widmann 1999). The ERK1 and ERK2 subfamily is the most extensively studied, and leads to activation of several transcription factors and other serine/threonine kinases contributing to cellular proliferation, differentiation, cell cycle regulation, and cell survival. The other ERK proteins are less well characterized: ERK3 is localized to the nucleus and is activated by PKC isoforms. ERK4 is activated in response to nerve growth factor and EGF in a Ras-dependent fashion, and ERK5 is activated by oxidative stress, hyperosmolarity and serum and can activate the MEF2C transcription factor (reviewed in Widmann 1999).

As a general rule, the ERK MAPK are induced by mitogenic stimuli, such as phorbol esters and growth factors, while the SAPK and p38 MAPK are induced by cellular stress. This concept is well-illustrated in neutrophils, in which endotoxin (LPS), a “stressful” stimulus, induces the p38 but not ERK MAPK (Nahas 1996). However, in the macrophage/monocyte, many “stressful” stimuli, including cytokines and LPS, induce ERK, SAPK and p38 MAPK in parallel (Weinstein 1992, Han 1994, Lee 1995). A similar broad activation of the MAPK families is seen in mast cells after ligation of the FceR1 receptor for IgE on mast cells (Widmann 1999). Thus, the MAPK response to a given stimulus is cell-specific; moreover, a single receptor can activate all three MAPK families.

The SAPK Family:

The prototypical SAPK/JNK (stress activated protein kinase, c-Jun N-terminal kinase) was originally identified as a 54kDa MAP-2 kinase activated in rat liver following the injection of cycloheximide (Kyriakis 1990). The SAPK family now comprises 12 isoforms encoded by at least three genes (α, β, and γ) and synthesized as alternative mRNA splicing variants (Kyriakis 1994, Kyriakis 1996, Derijard 1994, Sluss 1994). All SAPK contain the motif TPY at sites of regulatory phosphorylation in subdomain VIII, and are activated by dual Threonine/Tyrosine phosphorylation on this motif (Kyriakis 1994, Derijard 1994). SAPK activation has been described in response to a number of stressful stimuli. SAPK activity is induced in situ by ultraviolet light, heat shock, osmotic shock, anisomycin, cycloheximide and IL-2, while SAPK activation has also been described in vivo following reperfusion of ischemic kidneys (Kyriakis 1994, Coso 1995, Minden 1995, Pombo 1994). As a rule, the SAPK have been associated with apoptosis, while the ERK MAPK are associated with cellular differentiation (reviewed in Widmann 1999).
The p38 MAPK:

The p38 MAPK was first purified as a macrophage protein that became tyrosine phosphorylated in response to LPS (Han 1995), and was suggested to play an essential role in the subsequent release of cytokines (Lee 1994). An isoform of p38, Mxi2 (Max interactor), has been identified and is an RNA splice variant of p38 (Zervos 1995). Related p38 MAPK family members include p38δ, p38γ, and p38δ (Jiang 1996, Goedert 1997, Goedert 1997b, Jiang 1997). As for the SAPK, the p38 MAPK is preferentially activated by cellular stresses. Like other MAPK, p38 is activated by dual Tyr/Thr phosphorylation in subdomain VIII of the catalytic domain. As for the yeast Hog1 (hyperosmolarity glycerol) protein, the mammalian p38 MAPK is activated by dual phosphorylation on a Thr-Gly-Tyr (TGY) motif (Han 1994). The early association of the p38 MAPK with inflammatory settings and cytokine release prompted the suggestion that p38 might be most closely aligned with acute inflammatory responses (Lee 1994).

2. MAPKK/ MEK:

Immediately upstream of the MAPK family members are the MAP kinase kinases (MAPKK, MEK, MKK). These dual specificity Threonine/Tyrosine kinases activate the MAPK. The specificity of each MKK varies, so that in vitro some MKK may activate members of two or all three MAPK families. However, for the purpose of this thesis they are discussed within the context of the MAPK family for which they are most selective. An overview of the activation of the MAPK is presented in Figure 4.

ERK:

MEK 1 (MKK1) and MEK2 (MKK2) are highly homologous dual threonine/tyrosine kinases which phosphorylate ERK1 and ERK2 at their Thr-Glu-Tyr activation motif. All known signaling pathways are believed to use MEK1/2 to phosphorylate and activate ERK1/2 (Cobb 1995). Both very selective for the ERK proteins. MEK1 and MEK2 differ in their cellular location and activation patterns. MEK1 contains a nuclear export sequence which excludes it from the nucleus (Fuduka 1997). Inactive MEK1 may act to sequester ERK1/2 in the cytoplasm. Upon ERK1/2 activation, the ERK proteins dissociate from MEK1 and, if the signal is prolonged, translocate to the nucleus (Marshall 1994). “Functionally sequestered” active ERK1/2 remain in the nucleus until dephosphorylated by nuclear phosphatases, whereupon they return to the cytoplasm and re-associate with MEK1 (Widmann 1999).
Figure 4: Overview of MAPK cascade activation

GCK: germinal center kinase: constitutively active
Receptor tyrosine kinases, nonreceptor tyrosine kinases and G-protein coupled receptors (GPCRs) can lead to the simultaneous activation of both MEK1 and MEK2 (Marais 1996, Whitmarsh 1996). However, MEK-1 activation tends to be more prevalent than activation of MEK2. For example, Raf-1 can activate both MEK-1 and MEK-2 (Marais 1996). However, addition of recombinant RasGTP, the low molecular weight GTPase upstream of Raf-1, to cell lysates results in a complex composed of Ras, Raf and MEK-1, but not MEK2. Similarly, cells transfected with active forms of Ras are induced to activate MEK1 more than MEK2, as are NIH3T3 cells treated with serum or HeLa cells treated with EGF (Marias 1996, Whitmarsh 1996, Lange-Carter 1994, Jelinek 1994). These findings provide a good rationale for the pharmacologic inhibition of the ERK pathway with selective MEK1 inhibitors such as PD98059.

**SAPK:**

The dual Thr/Tyr phosphorylation at the SAPK TPY activation motif is mediated primarily by MKK4 (SEK1) and the recently cloned MKK7 (Tourrier 1997). MKK4 is a potent and specific activator of SAPK which does not activate ERK (Derijard 1995, Sanchez 1994), but which can phosphorylate and activate p38 in vitro and when over-expressed in situ (Derijard 1995). Although MKK4 remains the most extensively studied of the MAPKK upstream of SAPK, it is likely that others remain to be defined. For example, extracts of 3Y1 fibroblasts exposed to hyperosmolarity contain at least four chromatographically distinguishable SAPK-activating factors, only one of which co-purifies with MKK1 immunoreactivity (Moriguchi 1995). This data suggests that activation of the SAPK pathway may be the end-product of multiple upstream pathways. The number of extracellular stimuli and cell surface receptors that lead to SAPK activation is extensive (Fanger 1997). Moreover, a large and growing number of MAPKKK proteins have been described as activators of MKK4 (Widmann 1999). Although these facts suggest that SAPK activation may a relatively nonspecific byproduct of many extracellular stimuli, specificity of the SAPK signal may be conferred, at least in part, by the ability of proteins such as MEKK1 to act as “scaffolds,” shielding and facilitating the SAPK response to individual stimuli (see below).
p38:

Activation of the p38 MAPK flows through at least three dual specificity kinases: MKK3, MKK6, and the IL-1β activated RKK (Derijard 1995, Raingeaud 1996, Freshney 1994, Rouse 1994). MKK3 and MKK6 share homology with the yeast Pbs2p protein; the relation of RKK to MKK3/6 remains to be elucidated (Kyriakis 1996). The MAPKKK upstream of MKK3 and MKK6 are described below, but remain relatively poorly defined.

3. MAPKKK/ MEKK:

Discussed earlier in the context of NF-κB regulation, the MAPKKK/MEKK are a heterogeneous collection of proteins which activate M KK. As for the M KK, the MEKK proteins exhibit relative in vitro and in situ selectivity for individual MAPK modules.

ERK:

A number of MAPKKK have been described as activators of the MEK1 and MEK2 MAPK. As noted in Figure 3, both MEKK2 and MEKK3 are upstream of MEK1/2. In cotransfection studies, MEKK2 activates SAPK in slight preference to ERK, while MEKK3 is more selective for ERK activation; neither activate p38 MAPK (Blank 1996). Interestingly, while MEKK2 can phosphorylate both MEK1 and SEK1 in vitro, MEKK3 cannot: it may act on other, as yet unidentified substrates (Kyriakis 1996, Blank 1996) MEKK1 may also lie upstream of the ERK proteins: kinase inactive MEKK1 inhibits receptor stimulation of ERK1/2 and binds Ras-GTP (Fanger 1997, Russell 1995). MEKK1 also binds Cdc42/Rac1, and may lie upstream of the SAPK pathway (see Figure 3, also Fanger 1997). In other words, several MAPKKK may potentially lie upstream of individual MAPK.

The most extensively studied MAPKKK upstream of MEK1/2 is Raf-1, a component of the classical Ras-Raf pathway of ERK MAPK activation. Raf-1 activation is complex. GTP-bound Ras binds to the N-terminal regulatory domain of Raf-1 (Koide 1993) and prompts its translocation to the cell membrane (Koide 1993, Marais 1996). The association of Ras with Raf-1 is not sufficient to activate Raf-1: a second stimulus is needed (Zhang 1993, Vojtek 1994). This second signal may involve tyrosine phosphorylation of Raf-1 by membrane bound c-Src; however, mutation of the sites of tyrosine phosphorylation (Tyr 340, Tyr 341) does not eliminate Ras-dependent Raf-1 activation (Marais 1996). Alternatively, phosphorylation of serine residues by protein kinase C on RasGTP-bound Raf-1 may lead
phosphorylation of serine residues by protein kinase C on RasGTP-bound Raf-1 may lead to Raf-1 activation (Marais 1998). Oligodimerization of membrane-associated Raf-1 proteins, possibly by the scaffold-like 14-3-3 proteins, may be necessary for activation (Freed 1994, Fu 1994). Raf-1 activity will also reflect inhibitory phosphorylation, as by ERK1/2 and protein kinase A (Marais 1996, Mischak 1996, Vossler 1997), and stimulation by MEK-1 (Zimmermann 1997). In other words, Raf-1 activation is regulated at many levels, and illustrates the potential for integratory regulatory inputs.

JNK:

Activation of MKK4 may flow through a large number of upstream MAPKKK. These include MEKK1-4 (Widmann 1999), Ask1 (Wang 1996), Tak1 (Yamaguchi 1995), Mst 1(Hirai 1997), and Tpl-2 (Salmeron 1996). Within a given cell type it is possible that individual MAPKKK proteins transduce relatively specific stimuli. For example, TAK1 (transforming growth factor-β-activated kinase –1) is activated by TGFβ and can phosphorylate MKK4 in vitro (Yamaguchi 1995). Equally, the adapter protein NCK, a target for several growth factor receptors, binds NIK, which in turn associates with the N-terminus of MEKK1 (reviewed in Ferrell 1998). Thus, NCK may link MEKK1 to the growth factor response.

In addition to the MAPKKK noted above, mixed lineage kinases (MLK) can phosphorylate and activate MKK4. MLK are Ser/Thr kinases whose catalytic domains contain segments similar to tyrosine kinases (Kyriakis 1996). The SH3 domain-containing proline-rich kinase (SPRK), the MLK known variously as dual lineage kinase (DLK), ZPK or MUK, and MLK-3 can activate both cotransfected SAPK and SEK1 (Kyriakis 1996, Teramoto 1996). Similarly, immunoprecipitated SPRK can phosphorylate and activate SEK1 directly in vitro (Rana 1996).

p38:

A number of MAPKKK proteins have been demonstrated to phosphorylate and activate the MAPKK MKK3 and MKK6 upstream of p38. Several of these can also activate the SAPK pathway, such as Tak1 (Yamaguchi 1995), Ask1 (Wang 1996), and the MLKs SPRK and MLK3 (Teramoto 1996). MEKK1 transfection can lead to p38 MAPK activation, but only when MEKK1 expression far exceeds that required for maximal SAPK activation (Kyriakis 1996). MLK3 associates directly with the low molecular weight GTP binding proteins Rac1 and Cdc42, and so may directly link these GTPases with activation of the p38 MAPK (Teramoto 1996); for the most part, however, the pathways upstream of the
vation of the p38 MAPK (Teramoto 1996); for the most part, however, the pathways upstream of the p38-activating MAPKKK remain undefined (Widmann 1999).

4. Low molecular weight GTP binding (GTPase) Proteins:

The low molecular weight GTP-binding proteins (GTPases) are molecular switches which control a diverse array of biological processes, including the MAPK modules. Their activity is regulated by GTP binding: when bound to GTP, they transduce signals to effector proteins; when bound to GDP, they are inactive (Vojtek 1995). Control of the cycling between active and inactive states is maintained by positive and negative modulators that directly associate with the GTPases. Positive modulators include guanine nucleotide dissociation stimulators (GDSs), which catalyze the dissociation of GDP, leading to the binding of GTP. In contrast, GTPase-activating proteins (GAPs) stimulate the GTP hydrolytic activity which converts bound GTP to GDP and promotes an inactive state. There are at least 50 low molecular weight GTPases, divided into five classes (Ras, Rab, Arf, Ran, Rho) based on sequence homology. The five classes are presented in Table 1, along with a brief description of their major cellular functions.
### TABLE 1: Low molecular weight GTP binding proteins:

<table>
<thead>
<tr>
<th>GTPase Family</th>
<th>Members</th>
<th>Cellular Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ras</td>
<td>H-Ras, K-Ras, N-Ras, R-Ras, TC21, Rap1A/Rap1B, Rap2A/2B</td>
<td>cell growth and differentiation</td>
</tr>
<tr>
<td>Rab</td>
<td></td>
<td>monitor and direct movements of vesicles</td>
</tr>
<tr>
<td>Arf</td>
<td></td>
<td>as for Rab</td>
</tr>
<tr>
<td>Ran</td>
<td></td>
<td>nuclear protein import</td>
</tr>
</tbody>
</table>
In addition to (or as part of) the general cellular functions described in Table 1, the low molecular weight GTPases link the cell surface with the MAPK cascades. However, the association of these multifunctional proteins with the MAPK can reflect general roles in the construction of an intracellular signal - which may or may be dependent on cytoskeletal changes - more than a specific association of a particular GTPase with a particular MAPK module. For example, the Ras family is generally thought to be upstream of the ERK MAPK module. Ras-mediated recruitment of the Raf MAPKKK can be essential for Raf activation (Cobb 1995, Schlessinger 1994). However, both Ras- and Raf-independent activation of ERK has been described, while the Rho GTPase family can also participate in ERK activation in the context of integrin signaling, possibly by virtue of its ability to influence the actin cytoskeleton (Hotchin 1995, Renshaw 1996, and see Section 5).

Rac1 and Cdc42 can, in a manner that involves tyrosine kinases, lie upstream of the JNK/SAPK and p38 MAPK modules (Coso 1996, Minden 1995, Zhang 1995). The connections linking Rac/Cdc42 and JNK/p38 are largely obscure. Much interest has focused on the role of the p21 activated kinases (PAKs). PAK1, PAK2, β-PAK/PAK3, γ-PAK are 60-70kDa proteins whose C-terminal catalytic domain is 60-70% identical with the yeast MAPKKK Ste20p (Kyriakis 1996). The PAK N-terminal domains contain a Rac/Cdc42 binding segment, and the PAKs are activated directly upon interaction with the GTP-bound GTPase (Kyriakis 1996). Although tempting candidates to link Rac/Cdc42 with JNK/p38, PAK1 co-expression with activated Rac1 or Cdc42 actually inhibits JNK signaling (Teramoto 1996). Furthermore, PAK overexpression per se is not sufficient to activate either JNK or p38 (Zhang 1995, Bgrodia 1995, Polverino 1995). Recent work has suggested that in COS cells stimulated by EGF, Rac/Cdc42 may influence activity of the JNK MAPK module by associating directly with the MEKK1 and MEKK4 MAPKK (Fanger 1997). In this interesting study, PAK activation appeared to be independent of JNK. Other possible Rac/Cdc42 effectors are the mixed lineage kinases (MLKs). Some MLKs contain a Rac1/Cdc42 binding domain (Burbeio 1995), and several have been described to have the ability to activate the JNK/SAPK and p38 MAPK modules as MAPKKK (see discussion above). In particular, MLK3 has been noted to function as a PAK-independent, Rac1/Cdc42-dependent inducer of JNK and p38 (Teramoto 1996).

The activities of the various low molecular weight GTPases can be inter-dependent. Ras activity can control Rac and Rho-dependent nuclear events, and even Rac/Cdc42-dependent activation of JNK. Constitutively active Ras stimulates stress fiber formation by activating Rac and Rho (Ridley 1992,
Experiments with dominant negative mutants, toxin and GAPs indicate that Rac1 and RhoA are also required for morphological transformation by Ras (Qiu 1995, Pendergrast 1995), while Cdc42 can activate Rac and Rho (Nobes 1995). Linking the various GTPases may be phospholipid second messenger systems (Vojtek 1995). In mammalian cells, Rac1 can be activated by specific phosphoinositides that are produced by phosphoinositide 3-kinase (Hawkins 1995). Phosphoinositide 3-kinase, in turn, can be activated directly by Ras (Rodriguez-Viciana 1994). Similarly, Rho is activated by arachidonic acid metabolites produced when Rac1 is activated (Peppelenbosch 1995). Thus, the Ras-mediated activation of JNK/SAPK may represent a paracrine effect: Ras to PI3K to PI to Rac1 (see Figure 4). Again, these studies point out that the association between the low molecular weight GTPases and individual MAPK modules may reflect the general construction of an intracellular signal. They also demonstrate the potential for upstream crosstalk between signaling cascades: MAPK module specificity is likely to be controlled downstream of the low molecular weight GTPases.

B. MAPK Regulation:

1. Turning the modules on:

MAPK module activation is a broad response to a number of extracellular stimuli. Activation follows stimulation of receptor tyrosine kinases, G-protein coupled receptors, receptor-associated tyrosine kinases, and surface receptor clustering. MAPK activation may also be independent of cell surface events. A vast number of stimuli can activate the MAPK through a number of mechanisms: thus, it may not be surprising that MAPK activation is often non-linear, with overlapping and complementary upstream proximal pathways. For the purpose of this thesis, MAPK activation is reviewed from the cell surface in.

a. receptor tyrosine kinases:

The transduction of an intracellular signal following ligand binding of a receptor tyrosine kinase is one of the best studied routes of MAPK activation. The receptors for many growth factors, such as EGF and PDGF, are transmembrane proteins which comprise a cytosolic tyrosine kinase domain. After ligand binding, individual receptor tyrosine kinases (RTKs) are induced to dimerize or oligodimerize, resulting in activation of the kinase function. RTKs become autophosphorylated on tyrosine residues and
bind specific adapter proteins via Src-homology-2 (SH2) or PTB domains. Classically, the Grb2 (growth factor receptor bound protein-2) adapter recruits guanine nucleotide exchange factors with proline rich SH3 binding domain sites to the membrane (e.g. mSOS), in proximity with the isoprenylated small GTP binding proteins they activate. These exchange factors promote the association of the Ras GTPase with GTP. GTP-bound, active Ras then binds the protein kinase Raf-1, thereby targeting Raf to the cell surface where its activity is increased. The precise mechanism of Raf activation is unclear, but may involve oligodimerization, mediated in part by 14-3-3 proteins (Freed 1996, Fu 1994). MKK1/2 become phosphorylated and activated, and ERK activation ensues (Schlessinger 1994). RTKs can also activate MAPK in manners which differ from the classic, Ras-dependent route. For example, in rat fibroblasts RTKs can activate ERK1/2 in a Ca2+-dependent, but protein kinase C and Ras-independent fashion (Burgering 1993). Equally, not all growth factor receptors are RTKs. The receptor for growth hormone (GH) does not contain intrinsic tyrosine kinase activity, but GH does promote the association of its receptor with the JAK2 tyrosine kinase. JAK2 activation ensues, with the development of an intracellular signal (VanderKuur 1995). Thus, the transduction of extracellular signals to intracellular MAPK activation may begin with the proximal activation of a tyrosine kinase and the subsequent recruitment of adapter proteins and MAPKKK.

b. G protein coupled receptors (GPCRs):

Proteins coupled to heterotrimeric GTP-binding proteins (G proteins) represent another well-studied mechanism linking extracellular signals to the MAPK modules. G protein coupled receptors (GPCRs) are integral membrane proteins which act as receptors for a wide range of inflammatory stimuli, including chemotactic peptides, thrombin, substance P, endothelin, prostaglandin F2a, and lysophosphatidic acid, as well as for stimuli involved in other processes, such as neurotransmitters, hormones, phospholipids, photons, odorants and growth factors (Gutkind 1998). In keeping with their diverse stimuli, GPCRs can induce a wide range of cellular actions, including chemotaxis, exocytosis, mitogenesis, oncogenesis, and cellular proliferation. Some of these functions are likely to be mediated via MAPK activation, since activation of one or more of the MAPK modules is commonly associated with GPRC signaling (Gutkind 1998).

1 A discussion of the selectivity of this protein recruitment is beyond the scope of this thesis. However, it should be noted that although SH2 and PTB domains bind tyrosine phosphorylated residues, individual SH2 and PTB domains are specific for particular target sequences. SH2 domain specificity is conferred by the amino acids just to the C-terminus side of the tyrosine residue, while PTB specificity is a function of the N-terminus residues.
GPRC intracellular signaling follows the dissociation of cell membrane G protein subunits. G proteins are composed of G\(\alpha\) subunits (16 members, 4 families: \(\alpha_\alpha\), \(\alpha_n\), \(\alpha_q\), and \(\alpha_{12}\)) and G\(\beta\gamma\) complexes (11 G\(\gamma\), 5G\(\beta\)). The best known family of GPRCs, with over 1000 members, have the common structural motif of seven membrane-spanning regions (Gutkind 1998). Ligand binding induces a conformational shift in the structure of these GPRC, resulting in the exchange of GTP for GDP on the associated G\(\alpha\) subunit. This exchange results in the dissociation of the G\(\alpha\) subunit from the G\(\beta\gamma\) complex. While active, GTP-bound G\(\alpha\) subunits initiate a number of intracellular signals (e.g. G\(\alpha_i\) modulates ion channel activity, inhibits cAMP production, regulates phospholipases and phosphodiesterases, vs G\(\alpha_q\); promotes PIP2 catalyzed reaction of phosphoinositol to diacylglycerol and IP3), they do not appear to regulate the MAPK. By contrast, the G\(\beta\gamma\) subunits have been demonstrated to be upstream of the ERK and JNK/SAPK pathways in mammalian cells (reviewed in Gutkind 1998, Luttrell 1997). The signal is ended with the hydrolysis of GTP to GDP, and the reassociation of G\(\alpha\) and G\(\beta\gamma\) subunits.

As for other mechanisms of MAPK activation, the route from G\(\beta\gamma\) release to MAPK activation in mammalian cells can involve a number of inter-related upstream proteins. In the case of the ERK MAPK, both Ras-dependent and Ras-independent linkages have been described. Activation of GPRCs in a number of cellular systems leads to the rapid phosphorylation of the Shc adapter protein on tyrosine residues, the formation of Shc-GRB2 complexes, and mSOS-Ras-Raf-dependent activation of the ERK MAPK module (van Biesen 1995, Chen 1996). Src family tyrosine kinases, often bound to the cell membrane, or the distantly related Pyk2 kinase, are likely to link the active, membrane-associated G\(\beta\gamma\) to Shc tyrosine phosphorylation (Luttrell 1996, Wan 1996). Alternatively, G\(\beta\gamma\) complexes may activate growth factor RTKs and thereby recruit adapter proteins and MAPKKK to the cell membrane (Linseman 1995, Daub 1996), activate Ras-GRF, a distinct Ras guanine nucleotide exchange factor expressed in neuronal cells (Mattingly 1996), or stimulate a novel phosphatidylinositol 3-kinase (PI3K\(\gamma\)) which can activate Src-like kinases (Hawes 1996, Stoyanov 1995, Lopez-Ilașca 1997). While these mechanisms of ERK activation are likely Ras-dependent, G\(\beta\gamma\) activation of protein kinase C (PKC) may stimulate the ERK MAPK module a Ras-independent manner (Thomas 1992, Hawes 1995). The role of protein kinase C is a good illustration of how activation of ERK is likely to be cell and stimulus-specific: GPRC-mediated activation of ERK has been demonstrated to be fully PKC-dependent (Hawes 1995), fully PKC-independent (Charlesworth 1997), or partially PKC-dependent (Crespo 1994).

JNK/SAPK and p38 MAPK activation have also been described following GPRC stimulation. In NIH3T3 cells, GPRC stimulation, but not PDGF, leads to JNK activation (Coso 1995), while the
chemoattractant f-Met-Leu-Phe (FMLP) stimulates a robust increase in both JNK and p38 activity in neutrophils and HL-60 cells (Huang 1998, Rane 1997). Relatively less is known about the link between GPRC and these MAPK module than for the ERK module, although the small GTPases Rac and CDC42 are thought to be involved (Coso 1996). Although the p21 activated kinases (PAKs) have been suggested to link Rac/CDC42 to SAPK/p38, in neutrophils stimulated with FMLP, PAK activation is not sufficient for either JNK or p38 activation (Huang 1998). Whether the GPRC can directly stimulate intracellular activation of the SAPK and p38 MAPK modules, without a G-protein intermediary, remains to be confirmed (Huang 1998).

c. receptors associated with intracellular kinases: cytokines, LPS and HIV-1

Cell surface receptors may also regulate MAPK module via a direct association with intracellular tyrosine kinases. One excellent example of this concept is found in the JAK/STAT signaling pathway induced by cytokines. A Janus-activated tyrosine kinase (JAK), physically associated with the β1 or β2 cytokine receptor subunit, is activated after individual cytokine receptor subunits are induced to dimerize by ligand binding (Carpenter 1999). The subsequent phosphorylation of the cytoplasmic domain of the cytokine receptor recruits JAK substrates, such as the STAT transcription factors and the SHP-2 phosphatase, which become activated following phosphorylation by JAK (Stahl 1995, Carpenter 1999). Tyrosine phosphorylated SHP-2 can act as an adapter molecule, recruiting GRB2 and mSos, and hence Ras and the ERK MAPK module. As discussed below, the association of the Src family tyrosine kinase Lck with CD4 may be important in the Raf1-mediated stimulation of the ERK MAPK in response to human immunodeficiency virus-1 (HIV-1). CD14, the surface receptor for LPS, has also been suggested to be associated with Src family members, including Hck, Lyn and Fgr (Stephanova 1993, Beaty 1994). Activation of these kinases could link CD14 signaling to the MAPK modules. However, it is not clear whether the association between CD14 and the Src family members is true physical contact or simply the result of the co-localization of these proteins in detergent-resistant membrane subdomains (caveolae) (DeFranco 1998). Moreover, macrophages from mice deficient in all of the three major Src-family members expressed in macrophages (Hck, Fgr, and Lyn), have normal or elevated responses to LPS (Meng 1997). Thus, the Src kinases may not be necessary for CD14-induced signaling, even if associated with the receptor. Receptor association with kinases may also negatively regulate the MAPK. For example, β1 and β3 integrins are associated with the Ser/Thr Integrin-Linked Kinase (ILK), which is activated following integrin clustering during cellular adhesion; overexpression of this kinase downregulates ERK activity (Hannigan 1996). Taken together these data suggest that receptor associations with intracellular
kinases can modulate MAPK activity at a very proximal level in the signaling cascade.

d. construction of a supramolecular signaling complex: integrins

Implicit in the above discussion is the ability of cell surface molecules to generate an intracellular signal by recruiting adapter and intermediary proteins to a specific point in space, where MAPK module activation can proceed efficiently. One excellent illustration of this concept is the construction of a supramolecular complex of signaling proteins upon the intracellular scaffolding provided by the clustered cytosolic domains of integrins. Elegant studies by Miyamoto et al have demonstrated that in fibroblastic cells integrin clustering and binding of extracellular ligand results in the prompt accumulation of cytoplasmic cytoskeletal and signaling proteins in the region of integrin clustering (Miyamoto 1995, Miyamoto 1995b). Included in these proteins are many of the components necessary for activation of the ERK and SAPK MAPK modules, including GRB2, mSOS, Ras, Raf, MEKK, M KK1/2, ERK1/2 and JNK1, and ERK and JNK are activated with distinct time courses (Miyamoto 1995b). In these studies, the generation of a full response required cytoskeletal integrity, tyrosine phosphorylation, and both ligand occupancy and receptor clustering. In this context the role of the Rho family of small GTPases in MAPK activation may be to assist in the cytoskeletal-dependent construction of the signaling complex (Hotchin 1995), while tyrosine kinases are crucial for recruiting proteins to the area via SH2-mediated binding. Similar mechanisms are likely involved in other cell types: for example, in THP-1 monocytic cells VLA-4 (α4/β1) – dependent tyrosine phosphorylation requires integrin clustering, and is inhibited both by cytochalasin D and herbimycin (Lin 1995).

Although provocative, the spatial co-localization of integrin cytoplasmic units with signaling molecules does not fully explain the induction of MAPK modules by integrins. Regulatory molecules such as the Focal Adhesion Kinase (pp125FAK) are rapidly induced to associate with integrin cytoplasmic domains after integrin clustering and ligand binding. FAK contains SH2 binding sites for GRB2 and Src family kinases, both of which could act as proximal inducers of the MAPK pathways (Yamada 1997). However, both FAK-dependent and FAK-independent activation of the ERK MAPK module has been described in fibroblastic cells (see below). Several recent, intriguing studies described integrin-mediated concentration of growth factor receptors at adhesion/signaling complexes (Plopper 1995, Miyamoto 1997). The concentration and phosphorylation on tyrosine residues of these receptors required both integrin occupancy and clustering (Miyamoto 1997). Integrin-mediated MAPK activation may therefore follow recruitment of growth factor-receptor pathways, as described above, but this intracellular signal is
initiated by a spatially-localized signaling complex. These studies demonstrate the complexity and often overlapping nature of the upstream pathways involved in MAPK module activation, and point out that MAPK activation follows a spatially-organized response. These considerations help to explain the generation of MAPK specificity, as described below.

e. MAPK activation which is independent of surface receptors: ceramide, free radicals, and DNA damage

Activation of the MAPK is not necessarily dependent on cell surface receptors. For example, phorbol ester activation of the ERK MAPK is mediated by direct protein kinase C activation following the internalization of the compound (Thomas 1992, Hawes 1995). In addition, the identification of ceramide, free radicals and DNA damage as inducers of the p38/SAPK pathways suggests that certain cellular stresses do not need to interface with the cell surface to produce MAPK activation. One of the earliest events triggered by inflammatory cytokines (TNFa, IL-1) and environmental stresses (UV radiation, heat shock, H2O2), is the hydrolysis of membrane sphingomyelin to generate ceramide. Interestingly, ceramide when added exogenously or generated by the treatment of cells with sphingomyelinases, selectively activates the SAPK in situ, while dominant negative inhibitory mutants of SEK1 selectively inhibit ceramide-stimulated apoptosis (reviewed in Kyriakis 1996). A membrane bound, ceramide-activated protein kinase has been identified that may couple TNFa to the p38/SAPK protein kinase cascades (Joseph 1993). Similarly, there is accumulating evidence that DNA damage and free radical production may link radiation-induced damage to activation of the SAPK/p38. For example, UV-C radiation-induced activation of SAPK can be inhibited by the antioxidant N-acetyl cysteine in a manner that involves the inhibition of Ras and tyrosine kinases (Devary 1992, Adler 1993), while ionizing radiation-induced DNA damage activates the SAPK in a fashion that requires c-Abl and SEK1 (Kharbanda 1995, Kharbanda 1995).
2. Conferring specificity to the MAPK module signal:

The integration of various extracellular stimuli into a specific cellular response in mammalian cells may be mediated, at least in part, by MAPK module specificity. This concept derives from observations made in yeast. In *S. cerevisiae*, six major MAPK have been described, five of which have been assigned specific biologic functions. The MAPK Fus3p is specific for mating, Kss1p for filamentation, Hog1p for high osmolyte responses, Mpk1p for low osmolyte responses/cell wall remodeling, and Smk1p for sporulation (Madhani 1998, Sprague 1998). As in the mammalian situation described above, the MAPKK and MAPKK upstream of these MAPK have variable specificity, so that the MAPKKK Ste11 is common to the Fus3p, Kss1p, and Hog1p pathways, while the MAPKK Ste7 activates both Fus3p and Kss1p (Sprague 1998). Nevertheless, a mating peptide pheromone stimulus activates only Fus3p, and high osmolarity only Hog1p. The particular MAPK activated then transduces a specific cellular effect. Interestingly, there does not appear to be any crosstalk between the individual yeast MAPK pathways (Sprague 1998).

Mammalian MAPK differ from the yeast in several important ways. First, many different inputs can induce the same MAPK and yet have strikingly different effects on the cell. For example, the chemotactic peptide FMLP induces ERK, JNK and p38 MAPK activation in human monocytes, but does not lead to tissue factor expression, despite the fact that endotoxin, which activates the same pathways, does induce tissue factor (see Section 3). Secondly, there does appear to be crosstalk between individual MAPK pathways in mammalian cells. Mast cells stimulated by IgE crosslinking activate both ERK and p38 MAPK, but selective pharmacologic inhibition of p38 with SB203580 upregulates ERK activity (Zhang 1997). Although the mammalian situation may be more complex, the recent description of scaffolding proteins which can facilitate and shield MAPK module activation, and potentially act in a manner specific to a given extracellular stimulus, suggests that mammalian MAPK modules may also have the capacity to drive specific responses (Sprague 1998).

Mammalian MAPK specificity may be conferred by several mechanisms. These may explain how a non-linear signaling system can produce a specific cellular response, by virtue of a sensitive, switch-controlled and self-regulated combinatorial control of gene regulation.
a. scaffolding proteins:

MAPK module scaffold proteins create multienzyme complexes which lead to efficient and protected activation of an individual MAPK cascade. Two MAPK scaffold proteins have been described in *S. cerevisiae* and there are several possible such proteins in mammalian cells. In yeast, Ste5p and Pbs2p appear to facilitate MAPK activation in response to specific physiological stimuli, and insulate the bound MAP module against activation by irrelevant stimuli (Whitmarsh 1998). Ste5p coordinates activation of the Fus3p mating MAPK. It binds the MAPKKK Ste11p, the MAPKK Ste7p and the MAPK Fus3p. The Kss1p filamentation MAPK can also bind Ste5p *in vitro*; however, Fus3p possesses an activity in addition to its Ser/Thr MAPK function which limits the access of Kss10 to the Ste5p scaffold (Madhani 1997). Pbs2p potentiates activation of Hog1p in response to high osmolarity by binding the MAPKKK Ste11p, functioning as a MAPKK itself, and binding Hog1p (Posas 1997). It is important to note that the same MAPKKK is used in both responses: it is the scaffolding protein which determines a specific activation of individual MAPK modules.

In mammalian cells there are several candidate proteins which may act in a fashion similar to the yeast scaffolds. The N-terminus of MEKK1 associates with NIK (a Ste20-like protein kinase), which binds the adapter protein NCK, a target for several growth factor receptors. The C-terminus binds MKK4 (and Ras). Although the C-terminus does not appear to be essential, the interaction of NIK, NCK and MEKK1 potentiates the activation of JNK and so may link growth factor receptors to this pathway (Su 1997, Xu 1997). Indirect evidence for the selectivity of MEKK1 and the JNK pathway is found in the fact that MEKK1 overexpression fails to activate the p38 MAPK unless expressed at levels far beyond that required for maximal JNK/SAPK activation (Kyriakis 1996, Yan 1994). MEKK1 is a potent activator of MKK4, and MKK4 *in vitro* does activate p38 directly: the fact that it does not when MEKK1 is overexpressed suggests that the MEKK1 scaffold effect is directing activation of the JNK/SAPK pathway only. It is not known whether other MEKKs perform a similar function. JIP-1 (JNK-interacting protein-1) may also facilitate activation of the JNK/SAPK pathway. Originally described as an inhibitor of JNK, JIP-1 binds the MAPKKK MLK, the MAPKK MKK7, and JNK and so may direct the efficient activation of JNK (Dickens 1997). MP1 binds MKK1 and ERK1, but not ERK2 or MKK2 (Schaeffer 1998). Interestingly, adapter proteins may also perform a scaffold function. GRB10 contains a SH2 domain which binds to receptor tyrosine kinases, and in a phosphotyrosine-independent fashion also binds Raf-1 and MKK1. Like MP1, GRB10 may represent an ERK scaffold (Nantel 1998). Cytoskeletal protein may potentially perform similar functions. Actin binding protein 280 (ABP280) binds MKK4 and is re-
quired for maximal TNF-α-induced activation of JNK in melanoma cells (Marti 1997), while kinesin motor protein KIF3 colocalizes with activate JNK along microtubules (Nagata 1998). These potential scaffold proteins raise the possibility that specific extracellular signals will be channeled to specific activation of individual MAPK modules.

b. subcellular MAPK localization:

The question of how the subcellular location of an activated MAPK can affect subsequent cellular function deserves further study. As noted by Ferrell, "protein kinases shuttle to the plasma membrane to interact with upstream regulators and to the nucleus to interact with downstream targets; signaling molecules dock at autophosphorylated receptor tyrosine kinases, and scaffolds of different sorts organize and coordinate the actions of kinases, phosphatases, and entire signaling cascades" (Ferrell 1998). The cytoplasmic targets of the ERK and p38 MAPK suggest that their recruitment to specific cytosolic locations may have immediate effects in that area. For example, the co-localization of activated ERK in areas of integrin clustering or receptor binding of chemotactic factors might affect cytoskeletal function by virtue of its actions on microtubule associated proteins (MAPs) and regulatory proteins such as dynamin (see below). This localization may contribute to the role of ERK in alveolar rat macrophage spreading in response to oxidant stress (Ogura 1998), or to its role in neutrophil chemotaxis in response to FMLP, IL-8 and C5a (Kuroki 1997).

Translocation of MAPK to the nucleus may be part of a "switch-like" response, in which only strong signals are turned "on," while weaker ones are filtered out (Ferrell 1998). Roughly 30-50% of a cell's ERK1/2 accumulates in the nucleus in response to strong ERK-activating stimuli (Chen 1992); the longer the duration of the ERK-activating signal, the more ERK translocates to the nucleus (Marshall 1995). The nucleus contains many phosphatases that efficiently de-activate ERK and will suppress weaker signals. In the smaller volume of the nucleus ERK becomes much more concentrated than when in the cytoplasm, so a strong ERK-activating signal will saturate the nuclear phosphatases. As a result there is accumulation of large amounts of local, nuclear ERK – the ERK signal is turned "on" (Ferrell 1998). This mechanism may help to explain the fact that endotoxin stimulates monocytic tissue factor, while FMLP does not: in monocytes, the LPS-induced activation of ERK and p38 is much more prolonged than for FMLP (Weinstein 1994).
c. MAPK as specificity factors:

MAPK activation may act as its own specificity factor by limiting module activation. In *S. cerevisiae*, the high osmolarity Hog1p MAPK limits its own activation, and so inhibits crosstalk (O’Rourke 1998). As noted earlier, the mating Fus3p MAPK likely has a biochemical activity distinct from its catalytic activity which prevents interaction of Kss1p and other MAPK with the scaffolding protein Ste5p (Madhani 1997). Mammalian ERK1/2 may also act as a specificity factor. ERK1/2 can phosphorylate the EGF receptor, the Ras guanine nucleotide exchange factor mSos, the Raf-1 MAPKKK, and even the MEK-1 MAPKK (Widmann 1999). In all cases, the phosphorylation of these proteins is thought to reduce their activity, and so may represent a feedback inhibition circuit.

d. combinatorial control of gene activation:

The concept of combinatorial gene activation may help to explain how different stimuli can lead to similar patterns of MAPK activation and yet produce unique – and still MAPK-dependent – cellular responses. In yeast Fus3p and Kss1p share the downstream effector Ste12p, and yet have specific effects on cellular function. Ste12p appears to function in a combinatorial regulatory strategy: for mating (Fus3p), Ste12p functions either as a homodimer or a heterodimer with Mcm1p. For filamentation (Kss1p), Ste12p acts as a heterodimer with Tec1p. These combinations bind to promoter regions for mating or filamentation-specific genes (Madhani 1997, Johnson 1995, Madhani 1998). How Fus3p and Kss1p lead to homo/heterodimerization of Ste12p is unknown, and although attractive the relevance of this concept to mammalian systems remains to be defined.

3. Affecting cellular function:

**ERK Module Targets:**

**Cytoplasmic Targets:**

Cytoplasmic targets of the ERK1/2 proteins may act to amplify the ERK signal and can affect many aspects of cell function. Several cytoplasmic proteins have been identified as targets of the ERK1/2 proteins: S6 kinase p90^RSK^, cytosolic phospholipase A2 (cPLA2), and the juxtamembrane region of the EGF receptor (Lin 1993, Whitmarsh 1996, Widmann 1999). Phosphorylation of p90^RSK^ by ERK1/2 contributes
to the activation of the kinase (Nguyen 1993). p90RSK has multiple actions: once activated, it translocates to the nucleus and phosphorylates c-Fos (Widmann 1999). It also phosphorylates glycogen synthase kinase 3 (GSK3), inhibiting its activity; interestingly, GSK3 negatively regulates c-Jun (Eldar-Finkelman 1995). Transient inhibition of GSK3 activity following p90RSK phosphorylation may represent one way that the ERK1/2 proteins can rapidly activate c-Jun, and so, possibly, AP-1 (Eldar-Finkelman 1995).

Phosphorylation of cPLA2 at serine-505 has been suggested to activate the enzyme, leading to the release of arachidonic acid and to the subsequent generation of potent eicosanoids (Gordon 1996). Several microtubule-associated proteins (MAP) are also substrates for ERK1/2, including MAP-1, MAP-2, MAP-4 and Tau (Widmann 1999). The ERK proteins can be physically associated with microtubules, and can phosphorylate regulatory proteins such as dynamin (Morishima-Kawashima 1996, Earnest 1996, Cole 1995). Thus, ERK1/2 targets in the cytoplasm can influence both nuclear events (via AP-1) and alter cellular responses (via eicosanoid production, and potentially through effects on the cytoskeleton).

Nuclear Targets:

As noted earlier, prolonged stimulation of the ERK proteins leads to their translocation to the nucleus and to the local generation of a strong ERK signal (Marshall 1994, Ferrell 1998). Many transcription factors are thought to be phosphorylated and activated by ERK1/2, including Elk1, Ets1, Sap1a, c-Myc, Tal, and signal transducer and activator of transcription (STAT) proteins. By contrast, phosphorylation of Myb inhibits its activity (Widmann 1999). The interactions of the MAPK pathways with NF-κB and AP-1, described in Section IV, may also influence nuclear responses.

SAPK Module Targets:

SAPK activation has mostly been implicated as a necessary but not sufficient signal for the induction of apoptosis (reviewed in Widmann 1999). It may therefore not be surprising that all of the SAPK targets identified to date are transcription factors which can be involved in the control of pro-apoptotic genes. These factors include c-Jun, Elk-1, p53, DPC4, and NFAT4 (Widmann 1999). Their phosphorylation by the SAPK proteins influences both their activity and stability. For example, SAPK phosphorylation of c-Jun on serine-63 and serine-73 not only increases the formation of Jun/Jun homodimers and Jun/ATF2 heterodimers, but renders c-Jun more resistant to ubiquitin-dependent degradation (Musti 1997). Phosphorylation of ATF-2 and Elk1 increases their respective transcriptional activity; the latter is a point of convergence between the ERK and SAPK pathways (Zinck 1995). Phosphory-
lation of DPC4 (human mothers against decapentaplegic (Mad)-related transcriptional factor) is important in the induction of apoptosis by TGFβ (Zhang 1996). By contrast, phosphorylation of the anti-apoptotic NFAT4 (nuclear factor of activated T cells) inhibits its function by preventing translocation to the nucleus (Chow 1997, Oukka 1998). These data suggest that the SAPK play a permissive role in the coordinated apoptotic response by regulating nuclear transcriptional events.

p38 MAPK Module Targets:

The p38 MAPK has been implicated in many different biologic functions, including cytokine generation, apoptosis and cellular proliferation (Lee 1994, Brenner 1997, Crawley 1997). It has many cellular targets, both cytoplasmic and nuclear. Following stimulation of p38, MAPKAP (MAPK activated protein kinase) 2 and 3 are phosphorylated and activated, with the subsequent phosphorylation of small heat shock proteins (hsp25, hsp27) and of LSP-1, a poorly understood protein which is diffusely associated with the actin cytoskeleton (Huang 1997, Rouse 1994). While ERK can phosphorylate MAPKAP-2 in vitro, activation of the ERK pathway in situ is not associated with MAPKAP-2 activation (Freshney 1994, Stokoe 1992). On the other hand, several of the nuclear targets of p38 MAPK do suggest an integration between the ERK, SAPK and the p38 pathways. For example, Elk1, a target of ERK1/2, is phosphorylated by p38 MAPK at several sites in its COOH-terminal activation domain (Rajneaud 1996). The transcription factor Max is phosphorylated by p38 MAPK, whereupon it heterodimerizes with c-Myc, an ERK substrate (Zervos 1995). The p38 MAPK was recently demonstrated to phosphorylate and activate myocyte-enhancer factor 2c (MEF2C), a member of the MADS superfamily of transcription factors (Han 1997). Interestingly, endotoxin stimulation of monocytic cells (murine Raw264.7, human THP-1) increased transactivational activity of MEF2C and resulted in increased c-Jun transcription. These studies may link the ability of p38 MAPK to increase c-Jun with the ability of the SAPK to phosphorylate and stabilize c-Jun, describing a feedback loop that could alter the response of the cell to prolonged or recurrent stressful stimuli. In theory the ERK5-mediated phosphorylation of MEF2C could accomplish the same result (Widmann 1999). In addition to these integratory reactions, p38 MAPK phosphorylation of ATF2 (at Thr69 and 71) and CHOP (GADD153, at Ser78 and 81) increases the transcriptional activity of these factors (Widmann 1999). Posttranscriptional stability of mRNA may also be influenced by p38 MAPK activity (Lee 1996, Sirenko 1997). These studies suggest that p38 MAPK activation can contribute to a number of nuclear events, and potentially – via mediators such as LSP-1 – to cytoskeletonally-dependent cellular functions.
4. Turning the MAPK off:

a. Dual Specificity Phosphatases: The MAP Kinase Phosphatases:

The MAPK are inactivated by dephosphorylation of the Thr(P)-X-Tyr(P) activation motifs, an event mediated largely by dual specificity phosphatases (DSPs). These proteins, capable of hydrolyzing both phosphotyrosine and phosphoserine/threonine, are the largest family of phosphatases regulating the MAPKs in mammalian systems (Lewis 1999, Keyse 1999). They include MKP-1, MKP-2, MKP-3, MKP-4, PAC1, VHR, B23 (hVH3), and M3/6 (Widmann 1999). All share a common homology with the prototypical VH1 DSP of vaccinia virus (Lewis 1999). From the point of view of substrate specificity, MKP-1, MKP-3 and MKP-4 are more selective for ERK1/2 dephosphorylation than for other MAPK. M3/6 is very specific for p38 MAPK and SAPK; the other phosphatases do not appear to differentiate between individual MAPK. MKP-3 and MKP-4 are exclusively cytoplasmic and so may be selectively important for control of cytoplasmic ERK1/2 (Widmann 1999, Lewis 1999). MKP-1 is located only in the nucleus of both quiescent and stimulated cells (Brondello 1995), as are PAC-1, MKP-2, and B23 (hVH3). By contrast, microinjected M3/6 varies in localization depending on the cell type, being nuclear in Swiss 3T3 cells, but cytoplasmic in MDCK and PC12 cells (Theodosiou 1996). Expression of the MKP is generally under the control of the MAPK themselves, though the role of individual MAPK differs on a cell to cell basis. For example, upregulation of MKP-1 in U937 monocytic cells is dependent on ERK2, but not p38α or SAPK1 (Bokemeyer 1996). By contrast, MKP-1 expression in fibroblastic NIH3T3 cells is regulated by ERK, not SAPK. Although much work remain to be done in this area, having the expression of the signal-ending MKP under differential control by the MAPK themselves represents another level of control, and potentially of specificity, for the regulation of the MAPK signal.

b. Protein Phosphatases:

Active, dually phosphorylated mammalian MAPK can also be inactivated by Ser/Thr protein phosphatases (PPs) and protein tyrosine phosphatases (PTPs). Although both Ser/Thr and Tyr phosphorylation are required for MAPK activation, dephosphorylation of either residue results in complete loss of activity (Keyse 1998). Of the protein phosphatases, PP1 and PP2A have been demonstrated in vitro to inactivate ERK1/2, as well as MKK1/2, by hydrolysis of phosphoserine/phosphothreonine residues within their activation lips (Lewis 1999). Similarly, inactivation of PP1 and PP2A with okadaic acid can activate the ERK MAPK module (reviewed in Lewis 1999). Rather less is known about the regulation of
the MAPK by PTPs in mammalian cells. A single ERK-inactivating PTP has been isolated from *Xenopus* eggs (Sarcevic 1993). Recent data suggests that other PTPs may act on proteins upstream of the MAPK. PTP1B-mediated dephosphorylation of p130Cas may be important in the regulation of adhesion-induced, but not growth factor-induced, ERK MAPK activity (Liu 1998). To add yet another layer of complexity to the already complicated regulation of the MAPK, the relative roles of PPs, DSPs and PTPs in MAPK regulation is likely to be cell specific. For example, in rat PC12 neuronal cells, 3T3-L1 fibroblastic cells, and porcine aortic endothelial cells, PP2A is thought to be the chief regulator of ERK and MKK1 (Alessi 1995), rather than the DSPs.

5. Monocyte Migration: Integrin-dependent ERK MAPK Signaling

The process of monocyte transendothelial migration to a site of extravascular inflammation, such as the liver parenchyma of viral FHF, involves sequential engagement and release of surface adhesion molecules. The signaling cascades induced by these adhesive interactions involve tyrosine phosphorylation cascades, and likely include activation of MAPK modules. The subsequent development of a monocyte/macrophage procoagulant response may involve these same pathways.

Integrin-mediated adhesion and signaling are intimately involved in the regulation of many cellular functions, including leukocyte homing and activation, hemostasis, cell growth and development (Clark 1995). Early work with the monocyte demonstrated that ligation of β1 integrins augmented phagocytosis of particles via β2 receptors, while prolonged treatment of cells with anti-β2 antibody induced the respiratory burst, upregulated Ia expression and reduced protein synthesis (Wright 1983). β2 engagement has also been shown to increase monocyte-associated IL-1 (Couturier 1990), augment the expression of TF and TNFα in response to LPS (Fan 1991, Fan 1993), and variably increase intracellular calcium (Fan 1991, Altieri 1992). Similarly, engagement of the β1 integrin very late antigen 4 (VLA-4, CD49d/CD29) consistently induces gene expression (Lin 1994, Shaw 1990, Lin 1995, Yurochko 1992, Fan 1995, Sporn 1990, Haskill 1991). VLA-4 engagement by antibody, or adhesion of monocytes to the physiologic VLA-4 ligand, fibronectin, induces upregulation of immediate early genes encoding IL-1β (Lin 1994), IL-8 and TNF; (Sporn 1990) and transcription factors IκB (Lin 1994), c-jun and c-fos (Shaw 1990), as well as promoting the nuclear translocation of NF-κB (Lin 1995, Rosales 1996). Clustering of VLA-4 by monoclonal antibody in both human peripheral blood monocytes and monocytic THP-1 cells promotes TF expression; antibody ligation of β2 integrins potentiates the increase in TF following exposure to LPS (Fan 1995, Fan 1991). Similarly, monocyte adherence to activated endothelial cells, a process mediated by
both $\beta_1$ and $\beta_2$ integrins, (Lo 1991, Collins 1995) induces monocyte TF-specific procoagulant activity. Considered together, these studies implicate VLA-4 as an important signal transduction molecule in monocyte adhesive interactions.

The induction of tyrosine phosphorylation is an early consequence of integrin engagement (Clark 1995). An elegant study examining the fibronectin integrin $\alpha_5\beta_1$ (VLA-5) in fibroblasts demonstrated that integrin clustering leads to the rapid formation of a focal supramolecular complex of cytoskeletal proteins and signaling molecules intrinsic to several second messenger pathways, including pp125$^{FAK}$, other tyrosine kinases (c-src, c-Yes, C-Fyn, C-Csk), G-proteins and adapter molecules (ras, Grb2, SOS), stress kinases involved in the SAPK and MAPKinase cascades (ERK 1 and 2, MEK1, MEKK, JNK 1), and proteins involved in $Ca^{2+}$ signaling (PI3K, PLC-γ) (Miyamoto 1995b). Inhibition of tyrosine kinases with herbimycin or genistein dramatically disrupted the assembly of this complex, with only a few cytoskeletal molecules - including talin and tensin - and pp125$^{FAK}$ concentrating after $\alpha_5\beta_1$ clustering. This study clearly demonstrates the importance of tyrosine phosphorylation to the integrin signal, and suggests that the process of integrin clustering induces an intracellular signal dependent upon both cytoskeletal integrity and tyrosine kinase activity.

The intracellular signals leading to VLA-4-mediated induction of immediate-early inflammatory genes in monocytes involve tyrosine phosphorylation (Lin 1994, Lin 1995). The potential involvement of the ERK MAPK module in this response is suggested by several studies. ERK MAPK phosphorylation has been demonstrated following VLA-4 crosslinking in lymphocytic cells and after $\beta_1$-integrin-mediated adhesion of fibroblastic NIH3T3 to fibronectin (Sato 1995, Morino 1995). Clustering of $\beta_1$ integrins on fibroblasts induces activation of ERK1 (Miyamoto 1995). Thus, activation of the ERK pathway may be a broad consequence of $\beta_1$ integrin engagement. As noted earlier, the fact that TF expression in response to LPS is dependent on ERK suggest that integrin-mediated procoagulant responses may also require ERK activity.
a. Integrin-Mediated ERK Activation: Rho, Ras and Syk?

Integrin-mediated ERK signaling is an excellent example of the complexity and non-linear regulation of a MAPK module. ERK activation flows from a number of upstream GTPases, including Ras and Rho, and may involve a proximal tyrosine kinase, pp76 Syk. This complexity argues that from a practical standpoint the best means of associating ERK MAPK activity with a given outcome is by the selective inhibition of ERK itself, or of kinases immediately upstream of ERK, such as MEK-1.

i. Ras. The mechanisms upstream of the ERK MAPK module may be cell specific. The classic ERK activation pathway, described in response to peptide mitogens, involves the recruitment to the ligand-bound receptor of guanine nucleotide exchange proteins such as Sos by adapter proteins (Grb2, Shc), and the subsequent activation of the Ras small molecular weight GTPase (Seger 1995). As a result, the Ser/Thr kinase Raf MAPKKK translocates to the cell membrane, becomes activated and phosphorylates MEK, leading to the phosphorylation and activation of ERK. Until recently it was assumed that integrin-mediated ERK activation flowed through the same pathway. Ras and Raf molecules are induced to co-cluster with integrins following α5β1 clustering on fibroblastic 3T3 cells, and this physical co-localization precedes the activation of ERK (Miyamoto 1995). Again in fibroblastic cells, Clark and Hynes used dominant negative Ras transfectants to demonstrate that Ras is necessary for integrin-mediated ERK and cPLA2 activation, but not cytoskeletal organization (Clark 1996) Equally, MEK and Raf are activated upon adhesion of fibroblastic cells to fibronectin (Chen 1996). An attractive, unifying hypothesis for integrin-mediated ERK activation suggested that the Grb2/Sos complex could be recruited to sites of integrin clustering by tyrosine phosphorylated focal adhesion kinase (pp125FAK), in a SH2-dependent fashion (Schlaepfer 1994).

However, recent work has defined alternative and possibly cell-specific pathways of ERK activation in response to integrin engagement. For example, a Ras- and pp125FAK-independent activation of ERK following β1 integrin clustering in fibroblastic NIH3T3 cells was recently reported (Lin 1997, Chen 1996). In monocytic THP-1 cells, Raf-1 tyrosine phosphorylation does not follow β1 integrin crosslinking (Lin 1995), although from the considerations above it is likely that ERK is activated, as it is in lymphocytic cells (Sato 1995). Moreover, human primary monocytes do not appear to express pp125FAK (Lin 1995), although they do possess FAK-analogues such as the Pyk kinase. There is a good chance that as yet unidentified MEK activators exist (Reuter 1995), in much the same way that multiple activators have been identified but not yet characterized for the stress-activated protein kinase pathway (Moriguchi...
Taken together, these studies suggest that while the Ras GTPases may play some role in β1-integrin-mediated activation of the ERK/MAP Kinase pathway, other upstream modulators of the ERK pathway exist and may be cell-type specific.

**ii. Rho** The Rho family of GTP binding proteins may be involved in β1-integrin signaling. The p21 Rho family consists of the Rho, Rac and Cdc42Hs GTPases. The Rac1 and Cdc42Hs members are well-recognized to be upstream, tyrosine-kinase dependent activators of the SAPK/JNK and p38/RK signaling pathways (Coso 1995, Minden 1995, Zhang 1995). RhoA regulates the formation of focal adhesions and actin stress fibers, which has been well-documented in Swiss 3T3 cells (Ridley 1992). The fact that integrin-dependent adhesion of these cells onto fibronectin-coated dishes induces the rapid formation of stress fibers, similar to that seen with injection of activated Rho (Nobes 1995), suggests that RhoA may be involved in integrin signaling. This contention is supported by the fact that both Rho and adhesion to fibronectin activate phosphatidylinositol-4-phosphate 5-kinase (Chong 1994), and selective RhoA inhibition with C3 exoenzyme blocks cellular responses in a manner similar to the loss of integrin mediated cell adhesion (Chong 1994, Kumagai 1995, Paterson 1990).

More direct evidence for the relation of Rho to integrin-mediated ERK signaling comes from two recent studies. The first used the fact that Rho is inactivated in serum-starved cells. Using NIH3T3 cells, the authors demonstrated that ERK activation following adhesion to fibronectin was dependent on Rho activity, while growth-factor induced ERK activation was not (Hotchin 1995). An elegant study by Renshaw et al noted that fibronectin-induced activation of ERK in NIH3T3 cells was blocked by dominant negative Rho transfection or C3 exoenzyme, and increased by activated Rho transfects or Lbc, a Rho-specific guanine nucleotide exchange protein (Renshaw 1996). It is unclear precisely how Rho contributes to ERK activation, although, as the authors point out, Rho activity may be essential for the actin cytoskeletal changes that help construct the integrin signaling complex. It is also possible that Rho inhibition, acting through the actin cytoskeleton, may more selectively disrupt the translocation of Src family kinases to sites of integrin clustering (Fincham 1996), and so abrogate the ERK signal. Whether the same processes would hold true in monocytic cells, which do not form true focal adhesions, is unclear. However, Rho activity clearly has some role in the maintenance of monocytic cytoskeletal integrity in THP-1 cells (Aepfelbacher 1996) and contributes to FcRI-mediated monocytic phagocytosis (Hackam 1997), suggesting that the cytoskeletonally-dependent integrin signaling events may also be affected.
**iii. Syk**  Another intriguing possibility which may link integrin engagement to ERK activation is the Syk nonreceptor tyrosine kinase. This kinase is expressed in several hematopoietic cells, including monocytes, macrophages and platelets (Lin 1995). Although much attention has been focused on the role of Syk in monocytic Fc receptor-mediated signaling and phagocytosis (Durden 1995, Schreiber 1997), Syk has also been demonstrated to be tyrosine phosphorylated and activated in response to VLA-4 crosslinking on THP-1 cells (Lin 1995). Syk is involved in the induction of ERK activity by muscarinic ACh receptors, which are linked to heterotrimeric G-proteins (Wan 1996). The relation of Syk to integrin-mediated cell signaling and activation of the ERK pathway is unknown, though Syk would appear to be ideally placed in the integration of cytoskeletonally-dependent processes, such as adhesion and phagocytosis, and the generation of an intracellular signal.

In sum, activation of the ERK MAPK module after integrin engagement may reflect the coordinate action of a number of upstream GTPases and tyrosine kinases. In a sense this concept is hardly surprising, given the multiple effects of integrin signaling. It implies also that the ERK MAPK module may act to integrate diverse signals: this integratory capability may place the MAPK module at the heart of cell activation.

**b. Integrin-mediated Activation of the p38 MAP Kinase:**

In contrast to the ERK pathway, the parallel p38/reactivating kinase pathway has not been extensively studied in the context of integrin stimulation. However, there is good reason to suppose that it might be involved: the related SAPK proteins are often induced by the same stimuli as p38, and have been demonstrated to be activated upon β1 integrin engagement (Miyamoto 1995). In addition, differentiated THP-1 cells recruit Cdc42HS, a Rho-family GTPase presumed to be upstream of p38, to their cell membranes when they undergo spreading (Aepfelbacher 1994). Moreover, p38 activity appears to be essential for many of the acute responses to inflammatory stimuli. First described in monocytes (Han 1994), specific p38 inhibition leads to blockade of the monocytic expression of TNFα and TF in response to LPS (Cuenda 1995), and inhibits IL-6 synthesis in response to TNFα (Beyaert 1996). Just as ERK activation influences both early responses and more long term processes, both the SAPK and p38 pathways have been implicated in programmed cell death (Yan 1994, Johnson 1996, Devary 1992). This function might help to explain the recognized association of β1-class integrin engagement with epithelial apoptosis (Boudreau 1995). To date the closest association of p38 with integrin function is a recent re-
port implicating the p38 MAPK pathway in collagen-induced platelet aggregation: selective p38 MAPK inhibition with SB203580 disrupted collagen-induced platelet aggregation with only minimal inhibition of thromboxane generation (Saklatvala 1996). Considered together, these studies suggest that surface integrins might contribute to inflammatory cell functional outcomes in part via activation of p38.

6. Monocyte/macrophage reaction to viral stimuli: Viral induction of the MAPK:

Once the bloodborne monocyte has migrated into the acutely inflamed liver parenchyma of viral FHF, it will be exposed to a number of factors capable of inducing the MAPK. Cytokines, complement, and endotoxin translocated from the gastrointestinal tract can all induce the MAPK (see above). In addition, viral infection of monocytes may itself lead to MAPK module activation. Hepatitis B, which accounts for roughly half of viral FHF cases, does not replicate in monocytes or macrophages. However, many of the other viruses capable of causing human FHF do replicate in cells of the monocyte/macrophage lineage. Importantly, Murine Hepatitis Virus Strain 3 (MHV-3) causes liver damage in large part via infection of macrophages (see Section IV). Viral infection can lead to MAPK activation via a number of routes, and have important consequences for cell differentiation, apoptosis and oncogenesis. However, the role of the MAPK in virally-induced inflammatory events remains to be defined.

a. Virally-induced ERK, SAPK, and p38 activation:

Activation of all three MAPK modules has been described following viral infection of mammalian cells. ERK is activated after expression of the Hepatitis B HBx protein (Benn 1996), JNK/SAPK is stimulated both by HBx and the murine leukemia virus (Renshaw 1996, Henkler 1998), while p38 MAPK activation has been noted following infection of primary human T cells with human immunodeficiency virus-1 (HIV-1) (Cohen 1997). The MAPK response to viral infection is likely to be cell and virus-specific. For example, binding of simian virus 40 (SV40) to growth arrested cells induced neither Raf nor ERK activation (Dagorian 1996).

Virally-induced activation of the MAPK has been suggested to play a role in oncogenesis, differentiation, apoptosis and to viral replication events. However, there are relatively few demonstrations of the roles of individual members of the MAPK in these functions. Viral induction of transcription factors may be under the control of the MAPK. The hepatitis B virus HBx protein induction of AP-1 is dependent on ERK in quiescent cells, but independent of it in replicating cells (Benn 1996, Henkler 1998). HBx
also appears to lead to NF-κB nuclear translocation in a manner that is partially dependent on Ras/Raf activation (Benn 1996). HBx-mediated activation of the SAPK MAPK module has been suggested to play a role in the deregulation of cell cycle checkpoints (Benn 1995). Activation of the p38 MAPK by HIV-1 may be critical to replication of the virus in human T cells (Cohen 1997). Taken together these studies suggest that virally-induced MAPK activation may play a role in a number of cell functions.

The role of the MAPK in virally-induced inflammatory events remains to be elucidated. To date the only association of virally-induced tyrosine phosphorylation signaling with an inflammatory cytokine is the finding that the Newcastle disease virus induces TNFα production in astrocytoma cells in a tyrosine kinase-dependent fashion (Fisher 1994). Intriguingly, tyrosine kinases may be critical to the ANTI-viral IgG response: mice lacking the Src family tyrosine kinase Lck do not clear lymphocytic choriomeningitis virus or vaccinia virus (Molina 1993). Thus, tyrosine kinases may be important both to the virally-mediated induction of an inflammatory response, and to its ultimate resolution by viral clearance.

b. Viral mechanisms leading to MAPK activation:

Viral infection of mammalian cells can lead to MAPK activation in a number of fashions. The initial binding of the virus to a surface receptor can trigger intracellular events which culminate in MAPK activation. For example, both HIV-1 and recombinant HIV-1 surface glycoprotein gp120 bind to CD4 on T cells. CD4 is associated with the Lck tyrosine kinase, which becomes activated after HIV-1 or gp120 binding. Lck dissociates from CD4, and activates the Raf-1 MAPKKK in manner independent of Ras (Popik 1996, Kanner 1995). Alternatively, virally-encoded MAPKKK, or virally-encoded tyrosine kinases which activate MAPKKK, may lead to MAPK activation. The classic example of this process is the induction of the Src tyrosine kinase by the Rous sarcoma virus, while murine leukemia virus encodes v-abl, a cytoplasmic tyrosine kinase that can associate with PI3K, Shc, and GRB2 and activates ERK2 in both a Ras and Rac-dependent manner (Renshaw 1996). The Moloney murine leukemia virus encodes Tpl-2, a Ser/Thr MAPKK which activates both MKK1 and MKK4; similarly, there are descriptions of viral oncogenic induction of the Raf and Mos MAPKK (Salmeron 1996). Viruses may also encode proteins which activate GTPases upstream of the MAPKKK. Cytosolic hepatitis B virus HBx protein activates Ras-GTP complex formation and hence the Ras/Raf/ERK cascade (Doria 1995, Benn 1994). Finally, viral induction of proteins which influence phosphatase activity may influence MAPK activation. The SV40 small tumor antigen (small t) specifically interacts with protein phosphatase 2A (PP2A) in situ, inhibiting its ability to de-activate MEK and ERK (Frost 1994).
c. MHV-3 and the MAPK:

It is not known whether MHV-3 infection of murine macrophages leads to MAPK activation. However, as noted earlier in Section IV, previous work from our laboratory has established that MHV-3 infection of murine peritoneal exudative macrophages (PEM) leads to accumulation of tyrosine phosphoproteins. Inhibition of tyrosine kinases with genistein or herbimycin abrogated both MHV-3 induced procoagulant activity (PCA) and fgl-2/fibroleukin prothrombinase mRNA (Dackiw 1995). The consistent upregulation of tyrosine phosphorylated proteins which migrate in the 40-44kDa range suggests that the ERK1/2 and p38 MAPK may be involved in the response to MHV-3.

C. Roles of the MAPK in monocytes/macrophages:

Summary: In the context of the developing inflammatory response of viral FHF, bloodborne monocytes first migrate to the site extravascular inflammation, and then respond to the local inflammatory milieu of the liver parenchyma. Both the migration and response stages are likely to involve activation of the MAPK modules, with important consequences for cell activation and the generation of inflammatory mediators. In particular, the generation of a local procoagulant response may require MAPK activation.

1. A Role for ERK and p38 in Integrin-Mediated Cellular Activation, Transmigration and Transmigration-Induced Activation?

Given that tyrosine phosphorylation is a universal consequence of integrin engagement and that ERK and p38 may be activated by both integrins and inflammatory stimuli, there is good reason to suppose that they should contribute to integrin-dependent cellular activation, monocyte transendothelial migration and transmigration-dependent activation.

Recently, Lin et al found that crosslinking of the VLA-4 integrin on human THP- monocyctic cells led to accumulation of tyrosine phosphoproteins and upregulation of IL-1β mRNA (Lin 1995). Inhibition of tyrosine kinases with herbimycin abrogated the induction of this inflammatory cytokine. The same group has found that monocyte adhesion to plastic tissue culture wells induces a number of inflammatory genes, which are sensitive to selective pharmacological inhibition of ERK and p38 (Yurokco 1992, Sirenko 1997). Studies from our group have found that crosslinking both of surface VLA-4 β1 in-
tegrin and CD11b/CD18 β2 integrin induces procoagulant activity and tyrosine phosphorylation in rat monocytes (Dackiw 1996). Inhibition of tyrosine kinases eliminated induction of procoagulant activity. Although the MAPK were not considered in these studies, examination of the data presented reveals a striking increase in tyrosine phosphorylated proteins in the 40-44 kDa range, suggesting that the ERK and p38 MAPK might be involved. Integrin-mediated monocyte activation may flow, at least in part, through the ERK and p38 MAPK.

MAPK activation may also help to explain the ability of integrin engagement to act as a co-stimulatory signal when combined with inflammatory mediators such as TNFa or LPS (Fan 1991, Liles 1995), or with engagement of other surface molecules, such as the TcR in lymphocytes (Sato 1995, Renner 1995, Dietsch 1994). Even more intriguingly, ERK activation, or activation of similar tyrosine phosphorylation routes, may play some role in the dynamic regulation of integrin avidity and signaling. For example, overexpression of active Ras, which leads to continuous ERK activation, results in decreased β1 integrin chimeric affinity (Hughes 1997). Similarly, PMA treatment of HL-60 granulocytic cells, which strongly activates ERK, also decreases phosphotyrosine accumulation following CD18 crosslinking (Hellberg 1995).

There is good evidence in a variety of cell types for the requirement of tyrosine phosphorylation in chemotaxis (migration stimulated by a soluble factor), and many chemotactic factors (including FMLP, MCP, RANTES and C5a) induce tyrosine phosphorylation as part of their signal. In monocytes, broad inhibition of tyrosine kinases with genistein has been demonstrated to inhibit monocyte-chemotactic protein (MCP)-1, MCP-2 and MCP-3-induced migration (Sozzani 1994). The question of which tyrosine phosphorylation signaling pathways are important to monocyte transendothelial migration is more difficult, given the complicated, dynamic interplay of monocytes, endothelial cells and chemotactic factors. Some measure of this difficulty is seen in a recent study which found differing cytoskeletal and pharmacological responses even in the related processes of chemotaxis and haptotaxis (migration in response to a substratum-bound factor), both mediated by the same integrin (Aznavoorian 1996). Moreover, it is difficult to predict cell responses during transmigration from results of antibody or adhesion studies, which may mirror but do not reproduce aspects of the process of transmigration. Integrin engagement by antibody compared to engagement by adhesion generates differing levels of cell activation in monocytes and neutrophils (Walzog 1994), as well as differing patterns of cytoskeletal re-organization and protein phosphorylation (Lin 1995, Petruzzelli 1996). As a further complication, a recent study suggests that some aspects of migration may not be dependent on tyrosine phosphorylation, since a novel
tumor autocrine motility factor (AMF) induced the redistribution of tyrosine phosphorylated proteins in focal adhesions but did not change the general level of cellular tyrosine phosphorylation (Silletti 1996). In other words, the only way to elucidate the role of individual components of a tyrosine phosphorylation cascade in monocyte transmigration is to directly study them during the process of transmigration.

More specifically, the MAPK may be involved in leukocyte motility and cytoskeletal responses. As noted earlier, the ERK and p38 MAPK have cytosolic, cytoskeletonally associated targets; the JNK/SAPK MAPK does not (Section V3). Intriguingly, a recent study found that TNFα inhibited macrophage chemotaxis in a manner downstream of Cdc42, the GTPase upstream of the p38 MAP kinase cascade (Peppelenbusch 1999). In contrast, the spreading of alveolar rat macrophage NR8383 cells in response to oxidant stress is partially (40-60%) inhibited by ERK inhibition with PD98059 (25-75 μM), transfection with dominant negative ERK mutants, p38 MAP kinase inhibition with SB203580 (10 μM), or transfection with dominant negative p38 mutants (Ogura 1998). In neutrophils, the ERK MAP kinase pathway has been reported both to be important for chemotaxis in response to FMLP, IL-8, C5a, and LTB4 (Kuroki 1997 BBRC), or to be independent of IL-8 induced chemotaxis (Knall 1997). Although present, p38 MAP kinase activity is not required for neutrophil chemotaxis in response to IL-8 (Knall 1997). Interestingly, in Rat1 fibroblasts, fibronectin-stimulated chemotaxis is inhibited by selective ERK inhibition, while directed migration in response to PDGF-BB is independent of ERK (Anand-Apte 1997). A recent study by Hansen et al found that the tyrosine phosphorylation cascades in response to PDGF have components which are inhibitory for chemotaxis (Hansen 1996), suggesting that downstream signaling pathways can either enhance or inhibit cell movement. Taken together these results suggest that the roles of the ERK and p38 MAP kinases in regulating cell movement are both cell and stimulus-specific. This work does suggest that the ERK and p38 MAPK have the potential to contribute to monocyte transmigration; however, their roles remain to be defined.

2. Roles of the MAPK in Monocyte/Macrophage Inflammatory Response:

In the inflammatory milieu of viral FHF liver parenchyma, cells of the monocyte/macrophage lineage will be exposed to a number of stimuli with the potential to activate the MAPK modules. These include direct interactions with the extracellular matrix, inflammatory cytokines, endotoxin translocated from the gastrointestinal tract, and direct contact with the inciting virus. Of the MAPK, the ERK and p38 modules have clearly been associated with monocyte/macrophage production of inflammatory mediators, while the SAPK MAPK may be more involved in cellular differentiation and programmed cell
The contribution of the ERK pathway to inflammatory outcomes is suggested by the fact that induction of ERK or its upstream activators is found upon stimulation of monocytes or monocytic cells with a variety of inflammatory stimuli, including LPS (Liu 1994), IFNγ (Liu 1994), TNFα (Belka 1995), IL-13 (Adunyah 1995), and engagement of the FcRI (Durden 1995). The ERK pathway may be acting in a physiologic sense to integrate diverse inflammatory stimuli (Crew 1993, Rosales 1995). Some evidence for this concept is found in the fact that VLA-4 crosslinking on T-cells leads to tyrosine phosphorylation of the ERK proteins and contributes to T-cell co-stimulation (Sato 1995), and that ERK inhibition by IL-10 may de-activate monocytes (Geng 1994). The potentially broad permissive function of the ERK pathway may explain its importance both to inflammatory outcomes and to its more classical association with cell growth and differentiation (Cowley 1994, Pang 1995). More recently the ERK pathway has been implicated in the induction of IL-1, IL-8, TNFα and PGE₂ in monocytes stimulated by endotoxin (Schelrle 1998), and in the Fc-γ-receptor-mediated increase in TNFα in murine macrophages (Rose 1997). In a very interesting paper, Rawadi et al described the differential roles of the ERK, JNK and p38 MAPK in the induction of inflammatory cytokines by RAW264.7 cells in response to Mycoplasma fermentans-derived membrane lipoproteins (LAMPf) (Rawadi 1998). In response to LAMPf, RAW264.7 cells secrete IL-6, IL1β and TNFα. Selective ERK inhibition with PD98059 inhibited IL-1β and TNFα production, but not IL-6, while selective p38 inhibition with SB203580 abrogated all three. Transfection with a dominant negative JNK mutant inhibited only IL-6 production. Thus, the MAPK modules may differentially affect aspects of the monocyte/macrophage inflammatory response.

There is relatively little direct data linking the JNK/SAPK MAPK pathway with the immediate monocyte/macrophage inflammatory response. As noted earlier, the JNK/SAPK MAPK module is activated in response to a wide variety of cellular stresses, including inflammatory cytokines and endotoxin (Widmann 1999). Endotoxin stimulates JNK activation in murine macrophages, RAW264.7 cells, human THP-1 monocytic cells and murine bone-marrow derived monocytes (Hambleton 1996, Kraatz 1999). Exposure to TNFα leads to JNK activity in both mouse macrophages and monocyte-derived human dendritic cells (Sato 1999, Chan 1998). Macrophages treated with IL-16 activate only SAPK and p38
MAPK, with no induction of ERK activity (Krautwald 1998). Thus, there is good evidence that an infectious stimulus will lead to monocyte/macrophage SAPK activity.

The role of SAPK activity in the subsequent generation of inflammatory mediators is less well-known. In lymphocytes, correlative data has suggested a role for the SAPK MAPK in cytokine production. PMA-induced activation of ERK in lymphocytes is insufficient to induce IL-2 production, which is only triggered following the addition of Ca2+ ionophores and SAPK activation (Su 1994). Moreover, lymphocyte SAPK and IL-2 production, but not ERK, are inhibited by cyclosporin A. Similar associative evidence suggests that SAPK may promote inflammatory gene induction, possibly by increasing the transactivation potential of the AP-1 transcription factor. For example, TNFα activates SAPK, ERK and p38 and leads to increased CD83, CD86, HL-DR and T cell stimulatory capacity in human dendritic cells (Sato 1999). Pretreatment of the cells with IL-10 inhibits the TNFα cellular effect and ERK, SAPK and p38 activation. Similarly, endotoxin-induced tissue factor expression in THP-1 monocytic cells is accompanied by increased AP-1 transactivational activity, which occurs in parallel with C-Jun phosphorylation and increased SAPK activity (Hall 1999). On the other hand, other groups have found that SAPK-mediated phosphorylation of AP-1 in monocytic cells does not increase its transactivational activity (Behre 1999). Using dominant negative SAPK transfects, Rawadi et al found that SAPK was important only for IL-6 production in RAW264.7 cells exposed to LAMPf, and not for TNFα or IL-1β (Rawadi 1998). The fact that p38 MAPK activity, induced by similar stresses, is important for production of all three cytokines suggests that SAPK function may not be primarily oriented towards the acute generation of inflammatory products. Instead, SAPK activation may be more important for cellular differentiation, as has been suggested for U937 and HL-60 cells stimulated by phorbol esters (Kaneki 1999).

p38 MAPK:

In contrast to the JNK/SAPK MAPK, the p38 MAPK module has clearly been associated with the production of inflammatory mediators in cells of the monocyte/macrophage lineage exposed to infectious stimuli. The cytokine-suppressive anti-inflammatory drugs (CSAIDS) were recognized to inhibit p38 MAPK shortly after its original description. This led to the development of p38-selective compounds, such as SB203580 and the identification of the p38 MAPK module as being of central importance in the monocyte/macrophage generation of TNFα and IL-1β in response to endotoxin (Han 1994, Lee 1994). By contrast, CSAID-mediated p38 inhibition has a relatively limited effect on T and B cell responses involving the production of cytokines such as IL-2 and IL-4 (Lee 1996, Lee 1993). As noted in
Section III, our group has also found that p38 inhibition with SB203580 abolishes the endotoxin-induced increase in tissue factor in THP-1 cells, murine macrophages and human monocytes. These studies and those discussed above suggest that the p38 MAPK may play a central role for the monocyte/macrophage response to inflammatory stimuli. Interestingly, p38 inhibition may also represent a useful tool for the modulation of inflammatory responses in vivo: SB203580 was recently noted to reduce mortality in a mouse model of endotoxemia (Badger 1996).
Section VII: Hypotheses

In light of the preceding discussion, we developed a series of hypotheses to better explain aspects of the pathogenesis of viral FHF. This monocyte/macrophage-dependent disease, driven by activation of the coagulation cascade, was approached in a sequential fashion. We began by investigating how the mechanics of monocyte migration leads to a procoagulant response, with particular emphasis on the MAPK. Of the three MAPK modules, the ERK and p38 families appear to be the most directly involved with monocyte/macrophage responses to inflammatory stimuli. For this reason, and due to the availability of pharmacologic agents which selectively target each pathway (PD98059 for the ERK module, SB203580 for p38), we chose to focus on the roles of the ERK and p38 MAPK. Once in the inflamed liver parenchyma, monocytes and macrophages are exposed to a number of inflammatory stimuli, not least of which is direct activation by the inciting viral pathogen. We therefore next chose to study the role of the ERK and p38 MAPK in MHV-3-induced procoagulant activity.

**Hypothesis 1.** Integrin clustering on the surface of human monocytic cells results in ERK and p38 MAPK activation, which are necessary for the production of tissue-factor specific procoagulant activity (PCA). Both \( \beta_1 \) integrins (VLA4, CD49d/CD29) and \( \beta_2 \) integrins (mac-1, CD11b/CD18, and LFA-1, CD11a/CD18) will recruit the MAPK and induce PCA.

**Hypothesis 2:** The process of transendothelial migration activates monocyte MAPK modules, specifically ERK and p38, with subsequent activation of the cell and expression of tissue factor.

**Hypothesis 3:** Macrophages exposed to Murine Hepatitis Virus Strain-3 will respond by activating ERK and p38 MAPK. The subsequent generation of a fgl-2/fibroleukin procoagulant response will depend upon these MAPK.

The studies which follow address these hypotheses in sequence. Together they provide novel insights into the role of the monocyte/macrophage MAPK activity in the pathogenesis of viral FHF and the monocyte/macrophage procoagulant response.
Section VIII: Data Chapters and Discussions:

Chapter 1. VLA-4 Integrin Crosslinking on Human Monocytic THP-1 Cells Induces Tissue Factor Expression by a Mechanism Involving the ERK MAP Kinase

Summary: Adhesion molecules such as VLA-4 are important not only for monocyte adhesion to extracellular matrix proteins, but also for subsequent cell activation. Monocyte adherence to fibronectin or engagement of VLA-4 has been demonstrated to stimulate production of potent inflammatory mediators such as TNF-α, interleukin-1, and the procoagulant tissue factor (TF) protein. However, the intracellular signaling cascades leading to gene expression have not been elucidated. Using the human monocytic THP-1 cell line, VLA-4 crosslinking by monoclonal antibodies directed against its α4 and β1 subunits produced a time-dependent increase in tyrosine phosphorylation of a broad range of cellular proteins. Using Western blot analysis directed against the phosphorylated form of the ERK MAP Kinase proteins, as well as immunoprecipitation and in vitro kinase assays, we found that VLA-4 crosslinking increased ERK1/ERK2 tyrosine phosphorylation and activity. In conjunction, integrin crosslinking also increased NF-κB nuclear translocation and 4-hour expression of tissue factor. Inhibition of tyrosine kinase activity with genistein (10μg/ml) as well as selective MAP Kinase inhibition with the MEK-1 inhibitor PD9059 abolished the VLA-4 dependent ERK tyrosine phosphorylation, inhibited NF κB nuclear binding and abrogated TF expression induced by both VLA-4 crosslinking and adhesion to fibronectin in THP-1 cells and human peripheral blood monocytes. These studies point to the involvement of the MAP Kinase pathway in the activation of monocytic cells during transmigration to inflammatory sites.

INTRODUCTION:

The integrin family of surface adhesion molecules plays a key role in leukocyte recruitment to areas of extravascular inflammation. These heterodimeric integral membrane proteins are important not only for the adhesion to and transmigration across endothelial barriers, but also for adhesive interactions with extracellular matrix proteins (Clark 1995, Osborn 1990, Hogg 1995). While initially considered important only for their adhesive properties, recent studies have suggested that integrin engagement can initiate signal transduction pathways contributing to cellular activation (Rosales 1995, Graham 1993, Gresham 1991, Garnotel 1995, Ng-Sikorski 1991).

Monocyte recruitment to extravascular sites is an important component of the host response to a variety of stimuli including bacterial infection, tumor deposits and atherosclerotic plaques. In this loca-
tion, the surface expression as well as release of a number of macrophage products serve to coordinate the local inflammatory response. Fibrin deposition induced by macrophage tissue factor (TF) expression is known to contribute significantly to the development of this response. Within the vascular space, adherence of monocytes to the endothelium stimulates expression of TF on monocytes, a process which likely contributes to local microvascular thrombosis (Brisseau 1993, Lo 1995). Further, at extravascular sites, products of both the coagulation and fibrinolytic cascades contribute to the generation of the inflammatory response through their interaction with infiltrating cells. Indeed, strategies aimed at reducing fibrin deposition or precluding TF expression have been shown to mitigate the full expression of both the local and systemic inflammatory response depending on the model system studied (Brisseau 1993, Ruf 1994, Taylor 1991, Levi 1994, Rotstein 1988, McRitchie 1991).

Recent work has defined a role for integrin engagement in the induction of monocyte TF expression as well as other immediate early genes such as IL-1β (Lin 1994), IL-8 and TNF, (Garnotel 1995) and transcription factors IkB (Ruf 1994), c-jun and c-fos; (Shaw 1990). While monocytes are endowed with a variety of surface integrins, engagement of very late antigen 4 (VLA-4) appears to consistently induce gene expression (Lin 1994, Shaw 1990, Lin 1995, Yurochko 1992, Fan 1995, Sporn 1990, Haskill 1991, Fan 1993). For example, ligation of VLA-4 by monoclonal antibody in both human PBM and in the monocytic THP-1 cell line promotes TF expression, whereas engagement of β2 integrins has little effect (Fan 1995). The intracellular signaling mechanisms leading to the VLA-4-mediated induction of TF as well as other inflammatory genes appears to involve the induction of tyrosine phosphorylation (Lin 1994, Lin 1995, Sato 1995). Recent studies by Lin and colleagues have implicated a possible signaling role for Syk tyrosine kinase in this process (Lin 1995). In human monocytic THP-1 cells, VLA-4 engagement caused prominent tyrosine phosphorylation as well as activation of Syk tyrosine kinase, an effect which occurred in concert with the induction of the interleukin-1β gene.

Integrin engagement through interaction with extracellular matrix proteins has been shown to contribute to the regulation of cellular growth and differentiation and to modulate tumor behaviour (Clark 1995). In fibroblasts, this process involves tyrosine phosphorylation and activation of MAP kinase through a cascade involving Raf-1 and MEK (Clark 1995, Mornio 1995, Crew 1993, Chen 1994). While this cascade is known to be activated in macrophages in response to various proinflammatory stimuli (Beyaert 1996, Cowley 1994, Geng 1994, Liu 1994, Adunyah 1995, Belka 1995, Liu 1994, Durden 1995), its contribution to the β1 integrin-induced activation of inflammatory cell gene expression is unknown. In the present studies, engagement of VLA-4 on the surface of THP-1 monocytic cells and on human monocytes, both through integrin crosslinking and attachment to a fibronectin substratum, was shown to induce tyrosine phosphorylation and activation of the ERK1/ERK2 MAP kinases. This effect
occurred in parallel with nuclear translocation of NF-κB and stimulation of TF on the surface of these cells. Further, we took advantage of the existence of a novel selective inhibitor of MEK1 to define the contribution of this pathway to integrin-induced TF expression. This agent, PD98059, caused a dose-dependent inhibition of tyrosine phosphorylation and activation of MAP kinase in response to VLA-4 engagement and concomitantly prevented NF-κB translocation and TF induction. Considered together, these studies support a contribution of the MAP kinase pathway to integrin-induced gene expression in cells of monocyte/macrophage lineage.

Experimental Procedures

Buffers and Reagents:
Genistein was purchased from Calbiochem and prepared in DMSO at 10mg/ml. The selective MEK-1 inhibitor PD98059 was the kind gift of Dr. R. Saltiel, and was prepared in DMSO. Escherichia coli O111:B4 lipopolysaccharide (LPS) was purchased from Gibco, as were endotoxin free RPMI and HBSS media. Fetal calf serum (FCS) was from Hyclone. The following antibodies were used in the integrin engagement studies: mouse IgG1 anti-CD49d (mAb HP2.1 [Immunotech], mAb 44H6 [Serotec]), CD29 (mAb K20, mAb Lia1.2 [Immunotech]), goat F(ab')2 anti-mouse IgG [Immunotech], mouse IgG1 anti-CD45 mAb 1214 [PDI Bioscience], negative mouse IgG1 [Serotec]. Inhibitory anti-tissue factor antibody (mAb 4509) was obtained from American Diagnostica.

Cell Preparation:
Human monocytic THP-1 cells (ATCC) were propagated in RPMI/10%FCS/ penicillin/ streptomycin at 37°C/5% CO2. Human peripheral blood monocytes (PBM) were isolated from the blood of normal healthy donors by centrifugation over a Ficoll-Hypaque gradient at 400xg for 20 min. The mononuclear layer was aspirated, washed twice and resuspended in RPMI/2%FCS/L-Glutamine. This cell population contained 25-35% monocytes as assessed by Wright's stain and CD14 expression (flow cytometry with FITC-conjugated anti-CD14 Ab [Becton-Dickinson]), with >96% viability by trypan blue exclusion and propidium iodide uptake.

Cell Activation:
For integrin engagement studies, THP-1 cells were suspended in RPMI/2%FCS/L-Gln at 5x10^6 cells/ml. Surface CD29, CD49d and CD45 antigens were ligated with monoclonal antibody for 25 min at 15 ug/ml and 4°C, washed twice in cold RPMI, and then crosslinked with 5 ug/ml goat anti-mouse F(ab')2 for 25 min at 4°C. The concentration of primary antibody was shown to be saturating by flow cytometry (data not shown). Cells were washed twice in cold RPMI, resuspended in RPMI/2% FCS/L-Gln and incubated at 37°C/5%CO2 for times ranging from 1 minute to 4 hours. Reactions were stopped by placing the cells
on ice. For cell adhesion studies, 6-well polystyrene culture plates were coated either with 1 mg/ml poly-L-lysine or 0.05 mg/ml fibronectin for 1 hour at 25°C. Poly-L-lysine was crosslinked under UV light for 2 hours; all wells were then washed twice in cold RPMI and blocked for 1 hour with RPMI/10% FCS at 37°C. THP-1 (1x10^6) or PBM (0.5 x 10^6) were layered onto the coated wells and allowed to settle for 25 min at 4°C, and then brought to 37°C for up to 4 hours before being placed on ice. In inhibition studies, THP-1 cells or human PBM were preincubated in the presence of 1 and 10 uM PD98059, or 10 ug/ml genistein for 45 min at 4°C.

**Measurement of procoagulant activity:**
At 4 hours, THP-1 cells (0.5 x 10^6) were sedimented at 1000xg for 3 min. The cell pellet was resuspended at 10^6 cells/ml RPMI, freeze-thawed at -70°C, and procoagulant activity (PCA) measured by single stage recalcification clotting assay (Brisseau 1995). PCA was expressed as mU/10^6 cells by comparison to rabbit brain thromboplastin. In a typical experiment, β1 integrin crosslinking shortened clotting times from 70 seconds to 56 seconds, representing an increase in PCA from 74 mU/10^6 cells to 376 mU/10^6 cells. In the cell adhesion studies, THP-1 or PBM were harvested using a rubber policeman over ice after a 4 hour incubation, washed and resuspended at 10^6 cells/ml RPMI for PCA assessment. PCA was attributed to tissue factor (TF) expression on the basis of a complete reversal of PCA with inhibitory anti-TF antibody (see RESULTS) and failure of FVII-deficient serum to clot (data not shown).

**Western Blot Analysis:**
Following integrin crosslinking, THP-1 cells were lysed in ice-cold cell lysis buffer containing 1% Triton X100, 150mM NaCl, 10mM Tris-HCl (pH 7.4), 2mM sodium orthovanadate, 10ug/ml leupeptin, 50mM NaF, 5mM EDTA, 1mM EGTA, and 1mM PMSF. Postnuclear supernatants were collected following centrifugation at 10000xg for 5 min and diluted with 2X Laemmli buffer/0.1M dithiothreitol (DTT). Following adhesion to poly-L-lysine or fibronectin substrata, PBM were lysed in the culture wells with ice cold lysis buffer and prepared in a similar fashion. Lysates prepared from 100 000 cells were separated on 12.5% SDS-PAGE and transferred to PVDF membrane (Mobilon). Blots were then probed with polyclonal rabbit (Transduction Labs) anti-phosphotyrosine antibody, rabbit anti-phosphoERK antibody (New England Biolabs), rabbit anti-ERK1 or ERK2 antibody (Santa Cruz Biotechnologies). Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Sigma), blots were developed using an ECL-based system (Amersham).

**Immunoprecipitations and Immune Complex Kinase Assays:**

**Phosphotyrosine and ERK Immunoprecipitations:**
Cells (3x10^6) were lysed as above and the postnuclear supernatant pre-cleared with Protein G-Sepharose
(Pharmacia Biotech Inc). Cellular proteins were immunocomplexed using Pc anti-phosphotyrosine antibody (Transduction) or anti-ERK-1 and ERK-2 antibody (SCB) for 1 hour at 4°C. Protein G-Sepharose was added and incubated at 4°C for 1 hour. The resulting immune complexes were washed 5 times with cold PBS/0.01% Tween 20, and then separated from beads by 2X Laemmli buffer/0.1M DTT and boiling at 100°C for 5 min. Beads were then sedimented by ultracentrifugation and the supernatant collected for Western blot analysis.

**ERK-2 Immune Complex Kinase Assays:**

ERK-2 immunocomplexes were washed with 5 changes of cold PBS/0.01% Tween 20, and then incubated for 30 min at 30°C with 20 ug of ultra-pure myelin basic protein (MBP, Upstate Biotech Inc) in kinase assay buffer composed of 0.4mM cold and 0.4mM P$_32$-γATP (NEN), 50mM Tris-HCL (pH 7.4) and 10mM MgCl$_2$. Reactions were stopped with the addition of 2X Laemmli buffer/0.1M DTT and boiling at 100°C for 5 min. Equal volumes were loaded and run on 10% SDS-PAGE. The radioactivity of the phosphorylated MBP band running at 20 kDa was quantified on a Molecular Dynamics SI Phospholmage.

**Preparation of Nuclear Extracts:**

Following cell activation by VLA-4 crosslinking, 5 million THP-1 cells were washed twice in cold HBSS and lysed in 10mM HEPES (pH 7.9), 1.5mM MgCl$_2$, 10mM Kcl, 0.5mM DTT, 0.5mM PMSF and 0.1% NP-40. Following centrifugation at 13000rpm (4°C) for 10 min, the nuclear pellet was resuspended in 15ul/10$^7$ cells extract buffer containing 20mM HEPES (pH 7.9), 25% glycerol, 420mM NaCl, 1.5 mM MgCl$_2$, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF, 0.5mM spermidine, 0.15mM spermine, and 5ug/ml each of leupeptin, pepstatin and aprotinin. Supernatants were collected after a 15 min centrifugation at 14000 rpm (4°C) and diluted with 75ul of buffer containing 20mM HEPES (pH 7.9), 20% glycerol, 0.2mM EDTA, 50mM Kcl, 0.5mM DTT, and 0.5mM PMSF and immediately frozen on dry ice. Protein concentrations were determined using the Bradford protein assay (Bio-Rad).

**Electrophoretic Mobility Shift Assay:**

5ug of nuclear extract protein were pre-incubated with the nonspecific DNA competitor poly(dI-dC) (5ug, Pharmacia) for 10 min at room temperature. $^{32}$P-radiolabelled probe containing 2 NF-κB sites derived from the human immunodeficiency virus-1 enhancer (HIV-ENH), or containing the TF-specific NF-κB site (49), was incubated for an additional 20 min at room temperature. DNA-protein complexes were resolved on a 5% non-denaturing polyacrylamide (60:1 crosslink)/Tris glycine gel and autoradiographs prepared by exposure at -70°C using a Kodak X-OMAT film. To demonstrate specificity of the protein-
DNA complex. 125 molar excess of unlabelled probe or mutated TF κB probe were added to the nuclear extract before adding the radiolabelled probe. The sequence of the plus strands of the oligonucleotides used was:

- HIV-ENH 5'-AGGGACTTTCCGCGTGTTCC-3'
- TF κB 5'-AATTCTTGGAGTTTCCTACGGG-3'
- TFm κB 5'-AATTCTTGCAGTTTAGTACGGG-3'

**Statistical Analysis:**

The data are represented as the mean and standard error of the indicated number of experiments. Where representative studies are shown, these are indicative of at least three equivalent studies. Statistical comparisons were made for continuous data using one-way ANOVA with post hoc Tukey. For comparisons of experiments involving two treatment groups only, the two-tailed Student’s T-test was employed.

**RESULTS**

*Induction of Tyrosine Phosphorylation by crosslinking VLA-4:*

Figure 1A demonstrates that crosslinking of either the β1 or α4 subunits of VLA-4 induces a time-dependent increase in phosphotyrosine accumulation in THP-1 cells. The increase occurred within 1 minute, reached a maximum at 5-10 min and persisted for ~30 min. While receptor ligation alone caused a small increase in phosphotyrosine residues, crosslinking markedly amplified this effect (Figure 1B). This effect was not due to the addition of the secondary antibody per se, since this did not induce phosphotyrosine accumulation (Figure 1C). We also compared the effect of crosslinking using antibodies directed against different epitopes of the α4β1 integrin. As shown in Figure 1D, crosslinking of α4β1 with the inhibitory antibody Lial 1.2 directed against the β1 subunit caused a similar pattern of phosphotyrosine accumulation compared to the non-inhibitory antibody K20, although the magnitude was somewhat less. HP2/1, an inhibitory antibody directed against the α4 subunit caused similar accumulation of phosphotyrosine residues, while use of 44H6, also directed against α4, failed to induce tyrosine phosphorylation despite surface binding equivalent to mAb HP2/1. (data not shown, as assessed by flow cytometry, Coulter EPICS MCL). Finally, the effect was not due to the process of crosslinking of surface antigen per se, since neither crosslinking with mAb 1214 (Figure 1C) nor 44H6 induced an increase in tyrosine phosphorylated proteins (Figure 1D). As for 44H6, mAb 1214 had surface binding characteristics equivalent to K20 and HP2/1 (data not shown, as assessed by flow cytometry).

Endotoxin is known to cause tyrosine phosphorylation in monocytic cells (Liu 1994, Liu 1994). The observed effect of crosslinking was unlikely to be due to endotoxin contamination of the system.
Figure 1: VLA-4 Ligation and Crosslinking Induces Tyrosine Phosphorylation

**A. Time course:** THP-1 surface VLA-4 was crosslinked using either mAb HP2/1 or K20 and the anti-mouse F(ab')² fragment. The cells were incubated for the times indicated, lysed and the lysates probed for phosphotyrosine residues by Western blot.

**B. Crosslinking vs ligation:** VLA-4 was engaged by various mAb either by crosslinking or simple ligation, and cell lysates probed for phosphotyrosine residues.

**C. Controls:** the phosphotyrosine accumulation induced by crosslinking the β1 (K20) or α4(HP2/1) VLA-4 subunits was compared to that induced by crosslinking CD45 (mAb 1214), or by incubating cells in the presence of goat anti-mouse F(ab')² or negative mouse IgG. Cells were lysed at 5 minutes in each case.

**D. Epitope specific PTK activation:** VLA-4 was engaged by crosslinking β1 (K20, Lia1.2) and α4 (HP2/1, 44H6) subunits. THP-1 cells were lysed 5 minutes after crosslinking and probed for phosphotyrosine accumulation.
Endotoxin can lead to tyrosine phosphorylation in monocytes via either the CD14 or the Toll-like receptor (Medvedev 2000). However, binding of LPS with polymyxin B did not reverse the stimulation of phosphotyrosine accumulation and heating of the primary antibody prior to incubation with the cells completely prevented induction of tyrosine phosphorylation. Further, as indicated above, neither primary nor secondary antibody alone mimicked the stimulatory effect.

**Tyrosine Phosphorylation and Activation of ERK1/ERK2 following VLA-4 Crosslinking:**
While crosslinking induced a pattern of tyrosine phosphorylation encompassing a broad range of proteins, crosslinking with K20 caused some degree of persistent phosphorylation in the 42-50 kDa range. To determine whether p44 ERK1/p42 ERK2 MAP kinases might be among the candidate substrate proteins, cell lysates were probed first for phosphotyrosine residues, and the blots stripped and reprobed for p42 ERK2 and p44 ERK1. Both ERK proteins comigrated with an area of VLA-4-induced persistent tyrosine phosphorylation (data not shown). Importantly, the absolute amount of ERK protein in the cell lysates did not change over time following integrin engagement.

In order to conclusively identify the ERK proteins as targets of the VLA-4-induced tyrosine phosphorylation, two approaches were used. First, immunoprecipitation studies were carried out at 5 min after cell stimulation. The upper panel of Figure 2A shows a blot of immunoprecipitated tyrosine phosphoproteins probed with anti-ERK2 Ab under each of the treatment conditions. Integrin crosslinking with K20 markedly enhances the amount of tyrosine phosphorylated ERK2 protein compared to control cells, while engagement alone causes a slight increase. A similar effect is observed when cell lysates are sedimented with either anti-ERK1 Ab or anti-ERK2 Ab and then probed with antiphosphotyrosine Ab (Figure 2A, middle and lower panel respectively). Similar results were obtained when cell surface integrins were crosslinked with mAb against the α4 subunit (data not shown). ERK phosphorylation induced by VLA-4 crosslinking was also evaluated by Western blot studies using an antibody specific to ERK phosphorylated on tyrosine residues. Crosslinking of VLA-4 by mAb against the β1 subunit induced a time-dependent increase in ERK tyrosine phosphorylation, particularly of the p42 ERK2 (Figure 2B). This effect occurred early, peaked at 30 min and persisted for at least 60 min. This was accompanied by a slight retardation in the electrophoretic mobility, consistent with phosphorylation of ERK2. Integrin ligation alone caused a somewhat delayed rise in phosphoERK accumulation, although crosslinking induced more phosphorylation at any given time point.

Having demonstrated tyrosine phosphorylation of ERK1/ERK2 proteins, studies were performed to evaluate their level of activation. Using MBP as a substrate, Figures 3A and 3B show that crosslinking of α4 and β1 subunits of VLA-4 respectively cause a time-dependent increase in immunoprecipitated
Figure 2: VLA-4 Induced ERK Tyrosine Phosphorylation

A. THP-1 cells were lysed 5 minutes after engagement of VLA-4 by mAb K20, following which ERK1, ERK2 or proteins phosphorylated on tyrosine residues were immunoprecipitated. Western Blotting was performed as indicated. Similar results were obtained with mAb HP2/1.

B. Following VLA-4 engagement THP-1 cells were lysed at the times indicated and prepared for Western Blotting with Ab specific to phosphorylated ERK. Note that while both ligation (binding) and VLA-4 crosslinking induce the same pattern of ERK phosphorylation, crosslinking induces considerably more at any given time. All studies were replicated a minimum of three times.
Figure 3: Activation of ERK2 by VLA-4 Engagement

The VLA-4 integrin on THP-1 cells was engaged by crosslinking the α4 (mAb HP2.1) or β1 (mAb K20) subunit, and the cells incubated at 37°C for the times indicated. For comparison, cells were treated with 1 μg/ml LPS for 30 min. Following cell lysis, ERK-2 protein was immunoprecipitated as described in Experimental Procedures, and the immunocomplexes allowed to phosphorylate ultra-pure MBP for 30 minutes at 30°C. Proteins were then separated on 15% SDS-PAGE. A. and B. Time course of ERK-2 activation by VLA-4 engagement; the upper panels are autoradiograms of the 20kD 32P-MBP band, and the lower panels represent the radioactivity of the corresponding bands as determined by phosphoimager. The data shown are representative of two independent experiments for each antibody.
ERK2 activity, the magnitude of which is similar to that seen with 1ug/ml LPS. Similar results were seen with immunoprecipitated ERK-1, although the increase in activity stimulated by VLA-4 engagement was less, approximately 2 to 3 fold (data not shown). As expected from the pattern of induction of phosphotyrosine accumulation, crosslinking mAb Lia1.2 markedly increased immunoprecipitated ERK2 activity, while crosslinking 44H6 or mAb 1214 had little to no effect (data not shown).

Adherence to fibronectin stimulates tyrosine phosphorylation of ERK

To determine whether adhesion to a physiological substratum might induce a similar activation of ERK, human cells were plated onto fibronectin or poly-L-lysine substrata. Figure 4A shows the pattern of accumulation of tyrosine phosphorylated proteins in response to adhesion to the indicated substratum. In particular, proteins with molecular masses corresponding to 42 kDa, 72 kDa, 85 kDa and 95 kDa were tyrosine phosphorylated in fibronectin-exposed cells but not in cells adhered to poly-L-lysine. This pattern generally corresponded to that observed for VLA-4 crosslinking by antibodies in suspended, although the pattern was less complex (compare to Figure 1). Tyrosine phosphorylation of ERK following adhesion was also evaluated by immunoprecipitation. Cell lysates were recovered at varying time points after adhesion to fibronectin or poly-L-lysine and ERK2 was immunoprecipitated and probed with antiphosphotyrosine antibody. As shown in Figure 4B, there was there was a time-dependent rise in phosphorylated ERK in fibronectin-exposed cells, but not in those adhered to poly-L-lysine. Total ERK protein after immunoprecipitation did not differ between substrata (data not shown).

PD98059 Inhibits Both ERK Activation and Tyrosine Phosphorylation:

Recent studies have reported the development of a synthetic inhibitor of the MAP kinase pathway. This compound, PD98059, was shown to specifically block the activation of MEK 1, the upstream activator of ERK1/ERK2, without an effect on other protein kinases including the stress activated protein kinases (SAPK) and p38 (Dudley 1995, Pang 1995). Having demonstrated that VLA-4 crosslinking was able to induce activation of ERK1/ERK2, we used this agent to discern the role of this pathway in stimulating gene expression in macrophages in response to integrin engagement. First, the effect of PD98059 on VLA-4-stimulated ERK activation was studied. PD98059 prevented ERK-2 activation following VLA-4 crosslinking in a dose-dependent manner, with complete inhibition at 10 μM (Figures 5A and 5B). Binding studies with the flow cytometer and FITC-labelled secondary Ab demonstrated that PD98059 did not influence overall binding of antibody to integrin (data not shown). Consistent with its known mechanism of action, PD98059 also inhibited the increase in phospho-ERK which occurs in response to VLA-4 crosslinking (Figure 5C). In addition, this agent prevented the slight mobility shift ob-
Figure 4: Induction of PTK Activity and ERK Phosphorylation by Matrix Adhesion

A. Human PBM were allowed to adhere either to poly-L-lysine or fibronectin. Cell lysates taken at the times indicated were probed with antiphosphotyrosine Ab.
B. Human PBM were allowed to adhere to FN or PL for the times indicated, when they were lysed and ERK-2 was immunoprecipitated. The resulting blot was probed with antiphosphotyrosine Ab. In parallel experiments it was determined that over the first 30 minutes, >70% of all cells of the human PBM/lymphocyte layer that bound to the fibronectin or poly-L-lysine matrices were monocytes by CD14 analysis on the flow cytometer.
Figure 5: Inhibition of MAP Kinase Activity and Tyrosine Phosphorylation by PD98059

A. Following preincubation with PD98059 or genistein, THP-1 cells were lysed 30 minutes after K20 engagement of VLA-4, and ERK2 was immunoprecipitated. Immunocomplexes were assayed for MBP phosphorylation activity as described; $^{32}$P-MBP autoradiograms are presented.

B. Immunoprecipitated p42 ERK2 activity 30 minutes after VLA-4 engagement by mAb HP2/1.

C. THP-1 cells were preincubated with PD98059, and VLA-4 engaged by K20 or HP2/1 crosslinking. Cells were lysed 20 minutes after crosslinking, and lysates probed for ERK phosphorylated on tyrosine residues.

D. THP-1 cells were preincubated with Genistein or PD98059, and VLA-4 engaged by K20 crosslinking. Cells were lysed 5 minutes after crosslinking and lysates probed for phosphotyrosine residues.
served with activation of ERK. The inhibitory effect of PD98059 occurred without altering cell viability, as assessed by propidium iodide uptake and also had no effect on total cellular levels of the predominant isoform ERK2 (data not shown). In a similar fashion, the tyrosine kinase inhibitor, genistein (10ug/ml) also decreased ERK2 activation (Figures 5A and 5B). Figure 5D demonstrates that genistein causes a global reduction in the level of tyrosine phosphoproteins following crosslinking of β1, while PD98059 has little effect on the overall pattern of phosphorylation. Considered together, these studies demonstrate that both tyrosine kinase inhibition as well as inhibition of MEK-1 activation are able to prevent ERK activation in response to VLA-4 crosslinking.

**PD98059 and genistein inhibit VLA-4-stimulated monocytic tissue factor expression**

Consistent with previous reports (Fan 1995), VLA-4 engagement by antibody crosslinking or adhesion to fibronectin caused marked increases in monocyte PCA (Figure 6A and 6B respectively). The ability to stimulate PCA correlated with the increase in phosphotyrosine accumulation in response to treatment. In Figure 6A, PCA was increased following crosslinking with K20, HP2/1, and Lia1.2, but not 44H6 or CD45 (compare to Figure 1). Further, the increase did not occur following engagement with primary Ab alone or secondary antibody alone. The induction of PCA was comparable to that observed following stimulation with LPS (1 μg/ml). The effect of adhesion to fibronectin was observed both in freshly plated human blood monocytes as well in THP-1 cells (Figure 6B). PCA was attributed to tissue factor induction on the basis of inhibitory studies with anti-TF Ab. In a typical experiment, control cells had clotting times of 67+/−5 seconds (112±58 PCA μU), while cells crosslinked with mAb K20 exhibited induction of PCA (56±1 secs, 393±50μU). The ability of crosslinked cells to shorten clotting time was completely eliminated by inhibitory anti-TF Ab (to 66±1 secs, 118±1 μU), but not by mouse anti-human IgA Ab (Jackson). Further, this effect was not due to LPS contamination, since heated mAb did not induce PCA and polymyxin B (50μg/ml) did not reverse the effect of crosslinking.

Figure 7 shows the effect of PD98059 and genistein on VLA-4-induced PCA. At concentrations of PD98059 which preclude ERK activation in response to integrin crosslinking (10 μM for HP 2/1 crosslinking and 1 μM for K20 crosslinking), this inhibitor prevented induction of PCA (Figure 7A). Similarly, genistein prevented the rise in PCA following crosslinking of VLA-4 (Figure 7A), at a concentration which caused near complete inhibition of MBP phosphorylation. Figure 7B demonstrates the ability of PD98059 to abrogate the rise in PCA which occurs in response to attachment to a fibronectin substrate in both THP-1 cells and human monocytes.

**VLA-4 Induced NF-κB Nuclear Translocation is Dependent on ERK Activation:**

Previous studies have reported that VLA-4 engagement dramatically increases NF-kB nuclear translo-
Figure 6: Tissue Factor Induction by Integrin Engagement and Matrix Adhesion

A. The VLA-4 integrin on THP-1 cells was engaged either with mAb alone (ligation), or with mAb crosslinked with F(ab')2 secondary Ab, and cells were harvested after 4 hours for PCA determination.

B. THP-1 cells or human PBM were allowed to adhere to poly-l-lysine or fibronectin matrices for 4 hours, and then harvested for PCA assessment. The cumulative results of three separate experiments are shown; data: mean +/- SEM, n 6-8/group.

*** p<0.001 vs control (ANOVA with Tukey)

** p<0.0001 vs control (Student T-Test)
Figure 7: PTK and MAP Kinase Inhibition Attenuate Adhesion-Dependent TF Production

Human PBM and THP-1 cells were allowed to bind to a poly-L-lysine or fibronectin matrix, or VLA-4 on THP-1 cells was engaged by mAb as described in Experimental Procedures. After 4 hours of stimulation, cells were harvested and assessed for PCA. Data represents the cumulative results of three separate experiments, and is presented as mean+/−SEM, n 6-8/group. Statistical analysis was performed by one-way ANOVA with Tukey post hoc.
cation and specific binding in THP-1 cells (Fan 1995). Since NF-κB binding to the promoter of the tissue factor gene is required for induction of gene transcription, we examined whether PD98059 might exert its effect through inhibition of NF-κB translocation. Figure 8 illustrates two representative studies. Crosslinking VLA-4 induces a dramatic increase in both HIV-ENH and TF-specific NF-κB translocation. Preincubation of the cells with 10μM PD98059 largely abolished the HIV-ENH shift, and consistently effected a partial inhibition of the TF κB shift.

DISCUSSION:

Macrophage-mediated fibrin deposition via the surface expression of the procoagulant molecule tissue factor contributes significantly to the pathogenesis of both intravascular and extravascular inflammation. Induction of tissue factor occurs in response to a variety of proinflammatory stimuli including tumor necrosis factor, C3a and various bacterial species and their surface components (Brisseau 1993, Ruf 1994). Recent studies including those reported here demonstrate that engagement of monocyte surface VLA-4 by specific antibody is able to induce tissue factor expression (Fan 1995). The present results clearly demonstrate that tyrosine phosphorylation and activation of ERKI/ERK2 MAP kinase are involved in integrin-induced signalling pathway leading to tissue factor expression. Several lines of evidence support this conclusion. First, integrin aggregation through crosslinking causes tyrosine phosphorylation of these proteins. This was definitively shown by immunoprecipitation studies as well as by experiments using an antibody directed against the phosphorylated form of ERK. Further, adhesion of both THP-1 and human monocytes to fibronectin caused phosphorylation of ERK. Second, VLA-4 crosslinking caused a time-dependent activation of ERKI/ERK2 MAP kinase as assessed by its ability to phosphorylate its substrate protein myelin basic protein. Finally, two strategies shown to prevent phosphorylation and activation of ERK precluded the induction of tissue factor, not only in response to crosslinking, but also following adhesion to fibronectin. These included the use of the tyrosine kinase inhibitor genistein as well as the novel specific inhibitor of the upstream activator of ERK, PD98059. Considered together, these findings invoke a role for the MEK-1/MAP kinase cascade in the integrin-induced activation of monocyte coagulation molecules.

The data presented here are consistent with those recently reported by Lin and colleagues showing that adhesion of THP-1 monocytes to fibronectin lead to the tyrosine phosphorylation of pp125FAK, paxillin, and the nonreceptor tyrosine kinase Syk (Lin 1995, Kornberg 1992). Syk phosphorylation was also associated with its activation. At least two pathways leading to the activation of the Ras signalling cascade may link these effects to the activation of MAP kinase demonstrated in this study. First, phosphorylation of FAK creates an SH2-binding site for Grb2, resulting in localization of Grb2/SOS com-
The VLA-4 integrin on THP-1 cells was crosslinked either with mAb K20 or HP2/1, and the cells incubated for 3 hours at 37°C. 5μg nuclear extracts were taken and electromobility shift analysis (EMSA) was performed using 32P labeled HIV-ENH probe (0.2ng), or TF κB. These studies are representative of results replicated in 4 independent experiments.
plexes and subsequent activation of Ras (Schlaepfer 1994). In addition, in cultured mast cells, activated Syk has been shown to cause tyrosine phosphorylation of Shc in response to engagement of the FcεRI with antigen (Jabril-Cuenod 1996). Consequent association with Grb2 may lead to Ras activation. The precise signalling pathways leading to MAP kinase activation following integrin engagement, however, require further definition. While the activation of MEK-1/MAP kinase following integrin engagement in monocytic cells suggests the involvement of the Ras as well as the downstream kinase Raf in the signalling pathway, two lines of evidence suggest that the pathway does not align precisely along the lines suggested for growth factor induced cell signalling. For example, a recent study using NIH 3T3 fibroblasts demonstrated activation of Raf-1, MEK-1, and MAP kinase following adhesion to fibronectin in a manner which was independent of Ras (Chen 1996). Second, the studies reported by Lin and colleagues demonstrated that neither phosphorylation nor activation of Raf-1 occurred following β1 engagement in monocytes (Lin 1995). Recent studies have suggested the existence of unidentified MEK activators that may not have been detected in these studies (Reuter 1995). Further studies are required to evaluate the upstream activation pathway leading to MAP kinase activation following β1 integrin engagement in cells of monocytic lineage.

Although integrin ligation by antibody without aggregation induces phosphorylation and activation of p42 ERK-2, crosslinking consistently induces the most phosphorylation, ERK activation and procoagulant response (Figures 1 and 3). In addition, clustering by either adhesion-inhibiting antibodies as well as non inhibitory antibodies induced comparable effects. These findings are consistent with previous reports demonstrating the need to aggregate integrin receptors in monocytes as well as in other cell types to achieve maximal tyrosine phosphorylation and gene expression (Lin 1994, Miyamoto 1995, Miyamoto 1995). Considered together, the data suggest that the clustering of integrins plays a central role in initiating the signalling cascade leading to inflammatory gene induction. However, while integrin aggregation is necessary, it does not appear to be sufficient. The antibody 44H6 bound to THP-1 cells with equivalent affinity, yet was unable to induce either tyrosine phosphorylation or tissue factor expression when crosslinked. Particular VLA-4 epitopes as defined by mAb clones have already been suggested to play selective roles in adhesive functions (Letarte 1993). For example, Sato et al demonstrated that mAb 8F2 - directed against the α4 VLA-4 subunit epitope "C" - induced little phosphotyrosine accumulation in Jurkat T-cells, while two other "C"-specific clones did so strongly (Sato 1995). From a physiological standpoint, the process of integrin aggregation using mAb is a rather artificial one. Monocyte adhesion to endothelial cells or extracellular matrix proteins in vivo would presumably result in both engagement of the integrin ligand as well as aggregation of integrin receptors. In this regard, adhesion of monocytes to fibronectin (Figure 6) as well as interaction with endothelial cells has been shown to induce monocytic tissue factor expression (Lo 1995, Wharram 1991). It should
tissue factor expression (Lo 1995, Wharram 1991). It should be noted that the increase in TF induced by THP-1 or human PBM adherence to culture plates coated with 50ug/ml fibronectin is only a moderate stimulus for TF expression when compared to that obtained with 1ug/ml LPS. However, the observed increase is consistent with the ability of fibronectin to induce phosphotyrosine accumulation and stimulation of immediate early genes, as reported by Lin et al (Lin 1995).

The promoter region of the tissue factor gene has a cis-acting integrin response element containing two AP-1 sites and a single kappa B-like sequence (Fan 1995, Moll 1995, Mackman 1991). Mutation of either site resulted in reduced integrin responsiveness (Fan 1995). In the present studies, induction of tissue factor expression following integrin crosslinking and TF κB nuclear translocation were fully or partially precluded, respectively, by treatment with the MEK-1 inhibitor PD98059. HIV-ENH binding was abrogated by PD98059. Taken together, These data suggest an association between MAP kinase activation and NF-κB translocation leading to gene activation. Interestingly, in response to LPS, macrophages similarly exhibit both ERK activation and NF-κB translocation to the nucleus. However, these two events do not appear to be causally related, although both contribute to gene activation (Fan 1995, DeFranco 1995). Similar dissociation has been demonstrated in response to cellular hypoxia (Koong 1994). By contrast, overexpression of ERK-1 in Jurkat cells caused a marked increase in the DNA-binding activity of NF-κB (Park 1993). Together, these data suggest possible interaction between these two events which may be cell and stimulus specific.

The present study is the first to describe a contributory role for the ERK pathway in the induction of adhesion-dependent inflammatory response in cells of monocyte/macrophage lineage. Since endothelial cell adhesion of monocytes via engagement of surface integrins is an early event in the mobilization of cells to sites of inflammation, it will be of interest to discern how inflammatory and antiinflammatory mediator molecules acting via this or other signalling cascades might interact with this activated pathway to modulate the inflammatory response.
Chapter 2: MAP-Kinase Dependent Induction of Monocytic Procoagulant Activity by β2-Integrins

Summary: β2-integrin adhesion molecules play crucial roles in monocyte transmigration and adherence to the inflamed extracellular matrix. While integrin engagement contributes to inflammatory cell activation, little is known about the precise signaling pathways that are important to integrin-dependent monocyte activation. We examined the role of tyrosine phosphorylation and ERK activity in β2-integrin signaling in monocytes. Crosslinking of the LFA-1 (CD11a/CD18) and MAC-1 (CD11b/CD18) integrins on the surface of THP-1 monocytic cells induced the accumulation of tyrosine phosphoproteins. As part of this signal both ERK-1 and ERK-2 are tyrosine phosphorylated. In vitro kinase assays documented an increase in ERK-2 activity following both LFA-1 and MAC-1 crosslinking. β2-integrin crosslinking also led to a marked increase in 4 hour, tissue factor-specific procoagulant activity (PCA) in THP-1 cells and purified human monocytes. Inhibition of tyrosine phosphorylation by genistein (10μg/ml), or selective ERK inhibition with PD98059 (10μM), was able to block the integrin-dependent induction of PCA in both THP-1 cells and human monocytes. Thus, β2 integrin signaling in monocyctic cells can flow through the tyrosine phosphorylation and activation of the ERK MAP Kinases, which is essential for the subsequent expression of tissue factor. These results suggest that the ERK proteins likely function to integrate various adhesion-dependent signals during the process of monocyte transmigration.

Introduction:

Monocyte adhesion to and transmigration across an endothelial surface is the initial step in the recruitment of blood-borne monocytes to sites of extravascular inflammation. The subsequent expression of Tissue Factor (TF), the principal in vivo regulator of the coagulation cascade, is a key component of the host response to a variety of proinflammatory stimuli (McGilvray 1998, Drake 1989).

The integrin family of surface adhesion proteins is crucial to monocyte migration to extravascular sites. These heterodimeric structures are composed of alpha and beta subunits, and often function in conjunction with one another. For example, there is both β2-class integrin (LFA-1: CD11a/CD18, MAC-1: CD11b/CD18) and β1-class integrin VLA-4 (CD49d/CD29) dependent migration of cells across endothelial layers in vitro (Chuluyan 1993, Chuluyan 1995). These in vitro studies have been supported in in vivo models examining the effects of class-specific adhesion blocking antibodies on monocyte recruitment to sites of joint or dermal inflammation (Issekutz 1995, Issekutz 1995).

In addition to serving crucial adhesive functions, integrin-mediated signaling events can contribute to inflammatory cell activation as well. Studies performed using neutrophils have suggested a significant
role for β2-class integrins in this regard. Antibody crosslinking of surface β2-integrin on neutrophils, or adhesion to a physiologic substrate, primes the cells for an enhanced respiratory burst (Liles 1995, Garnotel 1995) and induces azurophilic granule exocytosis (Walzog 1994). These effects occurred in a tyrosine phosphorylation-dependent fashion (Liles 1995, Walzog 1994), and several groups have noted that neutrophil β2 integrin clustering induces the tyrosine phosphorylation of cellular proteins (Zheng 1996, Walzog 1996, Graham 1994, Berton 1994). These studies suggest that β2-class integrins have the capacity to contribute to neutrophil activation via the induction of tyrosine phosphorylation.

The role and mechanism of β2-integrin signaling in monocytes is less clear. The fact that monocyte/endothelial co-cultures can induce monocytic TF (Lo 1995, Collins 1995), an adhesion-dependent effect which is decreased by blocking the β2-ligand, ICAM-1 (Collins 1995), suggests that β2-integrins might play a more directly activating role to monocytes. In addition, monocyte/endothelial co-cultures, or the culture of monocytes onto ICAM-1 coated plates, results in the production of MIP-1α (Lukacs 1994). Since recent work has suggested that one key aspect of integrin signaling is integrin clustering (Miyamoto 1995), it is possible that simple ligation of the surface integrin is not sufficient to generate enough of an intracellular response to mediate cell tyrosine phosphorylation or cellular activation. Consistent with this notion, recent work in our laboratory has demonstrated that for β1-class VLA-4 integrin signaling and activation of the monocyte, simple antibody ligation has some effect, but much more so if the integrins are clustered by crosslinking (McGilvray 1997).

We therefore postulated that β2 integrin crosslinking can contribute to monocyte activation and tissue factor expression via the induction of tyrosine phosphorylation. We found that crosslinking of LFA-1 and MAC-1 on the surface of THP-1 cells or human monocytes led to increased procoagulant activity (PCA) due to surface tissue factor expression, an event occurring concurrent with the tyrosine phosphorylation and activation of the Extracellular-signal Regulated Kinase (ERK) Mitogen Activated Protein Kinase (MAPK) proteins. Inhibition of tyrosine phosphorylation with genistein, or selective ERK inhibition with the novel MEK1 inhibitor, PD98059, abrogated β2-integrin dependent PCA. These studies point to a significant role for the ERK MAP Kinase pathway in integrin-mediated monocyte/macrophage activation.
Experimental Procedures

Buffers and Reagents:
Endotoxin-free HBSS and RPMI media were purchased from Life Technologies, Inc. Fetal calf serum (FCS) was from HyClone. Genistein (Calbiochem) and PD98059 (RBI) were prepared in DMSO. The following antibodies were used in the integrin engagement studies: mouse IgG1 anti-CD11a (mAb 25.3.1, Immunotech) and anti CD11b (mAb Bear 1, Immunotech), goat F(ab')2 anti-mouse IgG (Immunotech), mouse IgG1 anti-CD45 mAb 512 (PD1 Bioscience), and negative mouse IgG1 (Serotec). FITC-conjugated mAb against human tissue factor was obtained from American Diagnostica (Massachusetts, USA).

Cell Preparation:
Human monocytic THP-1 cells (ATCC) were propagated in RPMI/10%FCS/penicillin/ streptomycin at 37°C, 5% CO2. Human monocytes were isolated from the blood of healthy volunteers as previously described (Issekutz 1995). Briefly, the cell-rich fraction from 3% dextran separated whole blood was spun over a Ficoll-Hypaque gradient at 400 x g for 20 minutes. The buffy coat fraction was aspirated, washed twice in cold RPMI, and slowly brought to an osmolarity of 360mOsm by the addition of 9%NaCl. After centrifugation over a 40/55/58 Percoll gradient at 1900 rpm for 30 minutes, the monocyte-rich layer was collected and washed twice in cold RPMI. This procedure consistently yields a platelet-free population of purified monocytes (>96% neutral red granule positive, >80% CD14 positive by flow cytometry), which are not activated (minimal baseline procoagulant activity and TNF secretion) and have a >98% viability by trypan blue exclusion.

Cell Activation:
For integrin engagement studies, THP-1 cells or purified human monocytes were suspended in RPMI/2%FCS/L-Gln at 5x10^6 cells/ml. Surface CD11a, CD11b, and CD45 antigens were ligated with monoclonal antibody for 25 minutes at 10µg/ml and 4°C, washed twice in cold RPMI, and then crosslinked with 5µg/ml goat anti-mouse F(ab')2 for 25 minutes at 4°C. The concentration of primary antibody was shown to be saturating by flow cytometry (data not shown). Cells were washed twice in cold RPMI, resuspended in RPMI/2%FCS/L-Gln and incubated at 37°C, 5% CO2 for times ranging from 1 minute to 4 hours. For inhibition studies, cells were pretreated with or without genistein (10µg/ml) or the ERK1/ERK2 inhibitor PD98059 (10µM) for 45 minutes at 37°C, 5% CO2. Crosslinking was performed as before, and the concentration of the inhibitors was maintained for the entire duration of the experiment.

Measurement of Procoagulant Activity and Surface Tissue Factor Expression:
THP-1 and human monocytes were sedimented at 1000 x g for 3 min after 4 hours of stimulation. The
cells pellet was resuspended at 10^6 cells/ml RPMI, freeze-thawed at -70°C, and procoagulant activity (PCA) measured by single stage recalcification assay (Brisseau 1995). PCA was expressed as milli-units/10^6 cells by comparison to rabbit brain thromboplastin (Brisseau 1995), or in some experiments by comparison to lipitated recombinant human tissue factor (American Diagnostica). In the latter case, 50 ng/ml recombinant tissue factor was arbitrarily assigned a value of 100 000 mU. For assessment of surface tissue factor, 250 000 human monocytes were sedimented 4 hours after integrin crosslinking and resuspended in 50-100 μl cold RPMI/2%FCS/L-Gln. The cells were then stained with FITC-conjugated anti-TF monoclonal antibody (American Diagnostica) according to the manufacturer’s instructions, washed twice in cold RPMI, and read on a Coulter EPICS XL Cytofluorometer.

Western Blot Analysis, Immunoprecipitations and Immune Complex Kinase Assays:
Phosphotyrosine accumulation, ERK tyrosine phosphorylation and ERK-2 kinase assays were performed as previously described (McGilvray 1997). Briefly, at various times after integrin crosslinking, THP-I cells were lysed in ice-cold cell lysis buffer containing 1% Triton X100, 150mM NaCl, 10mM Tris-HCl (pH 7.4), 2mM sodium orthovanadate, 10μg/ml leupeptin, 50mM NaF, 5mM EDTA, 1mM EGTA, and 1mM PMSF. For Western blot studies, postnuclear supernatants were collected after centrifugation at 10 000 x g for 5 min and diluted with 2X Laemmli buffer, 0.1 M dithiothreitol (DTT) prior to boiling for 4 minutes. Lysates prepared from 100 000 cells were separated on 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Mobilion). Blots were then probed with polyclonal rabbit anti-phosphotyrosine (Transduction Laboratories) or anti-phospho ERK antibody (New England Bioloabs). Following incubation with the appropriate HRP-conjugated secondary antibody (Amersham), blots were developed using an ECL-based system (Amersham).

For immunoprecipitation, 3x10^6 THP-I cells were lysed as above and pre-cleared with Protein G-Sepharose (Pharmacia Biotech Inc.). Cellular proteins were immunocomplexed using polyclonal anti-phosphotyrosine (Transduction) or anti-ERK1 and -ERK2 antibody (Santa Cruz Biotechnologies), and then bound to Protein G-Sepharose. The resulting immune complexes were washed five times with cold phosphate-buffered saline/0.01% Tween 20. For immunoprecipitation studies, proteins were separated from beads by 2x Laemmli buffer, 0.1M DTT and boiling for 5 min. Beads were sedimented by ultracentrifugation and the supernatant collected for Western blot analysis. For kinase assay studies, immune complexes were incubated for 30 min at 30°C with 20μg of ultra-pure myelin basic protein (MBP, Upstate Biotechnology, Inc.) in kinase assay buffer containing 0.4mM cold and 0.4mM [γ-^32P]ATP (Dupont NEN), 50mM Tris-HCl (pH 7.4), and 10mM MgCl₂. Reactions were stopped with the addition of 2x
Laemmli buffer, 0.1 mM DTT and boiling for 5 min. Equal volumes were loaded and run on 10% SDS-PAGE. The radioactivity of the phosphorylated MBP band running at 20kDa was quantified on a Molecular Dynamics SI Phosphorimager.

Statistical Analysis:
The data are represented as the mean and standard error of the indicated number of experiments. Where representative studies are shown, these are indicative of at least three equivalent and independent studies. Statistical comparisons were made for continuous data using one-way ANOVA with post hoc Tukey. For comparisons of experiments involving two treatment groups only, the two-tailed Student’s t-test was employed.

Results:

\( \beta_2 \) Integrin Crosslinking Induces Phosphotyrosine Accumulation in THP-1 Cells

Crosslinking of either CD11a (mAb 25.3.1) or CD11b (mAb Bear1) on the surface of monocytic THP-1 cells led to a dramatic accumulation of phosphotyrosine residues in a time-dependent fashion, peaking around 10-15 minutes and fading by 20-30 minutes (Fig 1A and 1B). A broad range of cellular proteins are tyrosine phosphorylated in response to \( \beta_2 \)-integrin crosslinking, especially in the 42-48kDa, 54-56kDa, 72-80kDa and 90-110kDa molecular weight ranges. Clustering of surface integrins was required for the full response, as demonstrated by the relatively mild increases in tyrosine phosphorylation induced by integrin ligation alone (Fig 1C). In contrast to clustering of the \( \beta_2 \)-integrins, CD45 crosslinking induced only a very mild increase in tyrosine phosphoproteins, while crosslinking with a negative mouse IgG1 control and the secondary F(ab’)2 alone, induced no cellular tyrosine phosphorylation response (18). By flow cytometry all three antibodies against CD11a, CD11b and CD45 bound to a marked degree to THP-1 cells, demonstrating that the lack of effect of CD45 crosslinking was not due to an absence of binding (data not shown). Taken together, these results argue for an integrin-specific induction of tyrosine phosphorylation in THP-1 monocytic cells that is not due to Fc-receptor ligation or to a non-specific antibody effect.

CD11a and CD11b Crosslinking Induces ERK Protein Tyrosine Phosphorylation

Since part of the tyrosine phosphorylation signal induced by \( \beta_2 \)-integrin crosslinking involves an increase in tyrosine phosphorylated proteins in the 42-44kD range, we next investigated whether the integrin crosslinking signal targets the 44kDa ERK-1 or 42kDa ERK-2 protein. Both CD11a and CD11b crosslinking induce the tyrosine phosphorylation of ERK-1 within 5 minutes of crosslinking (a represen-
Figure 1: β₂-Integrin Crosslinking Induces Tyrosine Phosphorylation

THP-1 cells were lysed at various times after CD11a or CD11b ligation or crosslinking by specific mAb as described in Experimental Procedures. Cellular proteins were separated by SDS-PAGE and probed for tyrosine phosphorylated residues.

A. and B. Effect of integrin crosslinking on total cellular tyrosine phosphorylation. Note the dramatic, time-dependent accumulation of tyrosine phosphoproteins.

C. Direct comparison of the effects on cellular tyrosine phosphoproteins after 5 minutes of integrin ligation with mAb alone or crosslinking with mAb primary and F(ab')₂ secondary (II').
tative study using antibody against CD11a is shown in Fig 2A; similar results were obtained for CD11b. Equally, crosslinking of either CD11a or CD11b leads to increased ERK-2 tyrosine phosphorylation within ten minutes of crosslinking, while II\(^{a}\) F(ab\(^{+}\))\(_{2}\) fragments alone do not (Fig 2B). These results clearly indicate that both ERK-1 and ERK-2 are early targets of the \(\beta_{2}\)-integrin response. To study the time course of ERK protein tyrosine phosphorylation, Western blot analysis of stimulated THP-1 cells was performed using antibody specific to the tyrosine-phosphorylated ERK proteins. For both CD11a and CD11b, integrin crosslinking led to a time-dependent increase in ERK-1 and ERK-2 tyrosine phosphorylation persisting to 30 minutes and beyond (Fig 2C). Note that while simple integrin ligation with divalent antibody increases ERK-1 and ERK-2 tyrosine phosphorylation, clustering of the integrin by crosslinking consistently results in considerably more ERK tyrosine phosphorylation for each time point studied (Fig 2C).

**CD11a and CD11b Crosslinking Induces ERK Activation in THP-1 Cells**

Having identified the ERK proteins as targets of the \(\beta_{2}\)-integrin tyrosine phosphorylation response, we ensured that this phosphorylation led to increased ERK kinase activity using *in vitro* kinase assays. As shown in Fig 3, crosslinking of surface CD11a and surface CD11b consistently led to a marked increase in ERK-2 kinase activity (roughly four-fold by thirty minutes after crosslinking). This increase in kinase activity is more pronounced upon integrin crosslinking than integrin ligation, and is not generated either by secondary antibody alone or by CD45 crosslinking (data not shown). Taken together, these studies and those above suggest that ERK activation is a consistent and a relatively specific effect of integrin crosslinking on THP-1 cells.

**\(\beta_{2}\)-Integrin Crosslinking Stimulates THP-1 Procoagulant Activity**

Previous work in our laboratory and others has demonstrated that \(\beta_{1}\)-integrin clustering leads to the expression of tissue factor in monocytic cells (McGilvray 1997, Fan 1995). To study the effect of \(\beta_{2}\)-integrin clustering, 4-hour procoagulant activity (PCA) was measured after integrin crosslinking by monoclonal antibody. Clustering of either CD11a or CD11b led to a marked increase in THP-1 PCA (Figure 4). As for the induction of tyrosine phosphorylation, primary antibody alone was insufficient to generate an increase in PCA. Although monocyte PCA can also be stimulated by LPS or other contaminants, it is unlikely that these contributed to our results. Heat denaturation of the antibodies markedly attenuated PCA induction, while pre-treatment of the cells with 50\(\mu\)g/ml polymyxin B (to bind endotoxin) had no effect (data not shown). In addition, endotoxin-free eppendorfs and media were used throughout all experiments. Together, these data argue that \(\beta_{2}\)-integrin crosslinking is sufficient to in-
Figure 2: β₂-Integrin Crosslinking Leads to ERK Tyrosine Phosphorylation

A. ERK-1 Tyrosine Phosphorylation by CD11a

B. ERK-2 Tyrosine Phosphorylation by CD11a and CD11b

C. Time Course of ERK Tyrosine Phosphorylation

Figure 2: β₂-Integrin Crosslinking Leads to the Tyrosine Phosphorylation of the ERK Proteins

A. 5 minutes after integrin crosslinking by 25.3.1 mAb (anti-CD11a), THP-1 cells were lysed and the ERK-1 proteins immunoprecipitated. The purified proteins were assessed for tyrosine phosphorylation by Western blot. Similar results were obtained with the Bear1 clone (anti-CD11b).

B. 10 minutes after CD11b or CD11a integrin crosslinking THP-1 cells were lysed and ERK-2 immunoprecipitated. Western blot analysis was performed with anti-phosphoERK antibody. The blots were then stripped and probed for ERK-2 to demonstrate equal loading.

C. THP-1 cells were lysed at increasing times after 25.3.1 or Bear1 ligation or crosslinking of surface integrins, and cell lysates prepared for Western blot. Antibody specific to tyrosine phosphorylated ERK was used to probe the blots.
Figure 3: Induction of ERK2 Kinase Activity by CD11a and CD11b Crosslinking

CD11a and CD11b were crosslinked on the surface of THP-1 cells as before. 30 minutes later, cells were lysed and in vitro ERK-2 kinase assays performed as described in Experimental Procedures. The study shown is representative of results replicated on three separate occasions.

A. Autoradiogram of the 20kDa $^{32}\text{P}$-MBP band generated by purified ERK-2.

B. Quantification of the radioactivity of the autoradiogram in A.
Figure 4: Induction of Procoagulant Activity by β2-Integrin Crosslinking on THP-1 Cells

4 hours after CD11a and CD11b crosslinking THP-1 cells were washed and prepared for assessment of PCA as noted in the text. PCA mU were determined by comparison to a rabbit thromboplastin standard curve. Data=mean+/-SEM, n>4/group. *p<0.05 vs control (ANOVA with post hoc Tukey)
crease THP-1 PCA.

*Induction of PCA and Surface TF by β2 Integrin Crosslinking on Human Monocytes*

As for THP-1 cells, crosslinking of surface CD11b on purified human monocytes led to a large increase in 4-hour PCA (Figure 5A). Similar results were found for CD11a (Figure 7). We have previously reported the fact that the integrin-dependent induction of PCA is due solely to increases in tissue factor, since it can be abolished by treating the cells with inhibitory anti-TF antibody (McGilvray 1997). This finding is consistent with the results of other laboratories (Fan 1995). Further evidence for the fact that integrin-dependent PCA is due to increased TF is found in the fact that CD11b crosslinking also leads to a moderate increase in surface tissue factor expression by flow cytometry (Fig 5B). Taken together, these results suggest that β2-integrin signaling can contribute to the tissue factor response of human monocytes.

*Tyrosine Kinase and Selective ERK Inhibition Abrogates Integrin-Dependent PCA in THP-1 Cells and Human Monocytes*

To test the significance of the ERK pathway to the integrin-dependent induction of monocyte PCA, THP-1 cells and purified human monocytes were pretreated with either the nonspecific tyrosine kinase inhibitor Genistein, or the selective ERK pathway inhibitor, PD98059. PD98059 selectively inhibits the gateway tyrosine kinase MEK-1 immediately upstream of ERK1 and ERK2 (Dudley 1995, Cohen 1997). Using these inhibitors - at doses which we have previously found to be non-toxic and to completely inhibit VLA-4 induced ERK-2 activity (McGilvray 1997) - we found that both nonspecific tyrosine kinase inhibition as well as selective ERK inhibition were able to abrogate the induction of PCA by β2-integrin clustering. PD98059 was equally effective in THP-1 cells (Fig 6A) and human monocytes (Fig 7). Moreover, PD98059 did not affect the absolute amount of integrin clustering on the surface of THP-1 cells as assessed by flow cytometry (Fig 6B), arguing that its effect was pharmacologic rather than steric in nature. These studies point to the ERK pathway as being of central importance to the induction of tissue factor by β2-integrin clustering.

*Discussion:*

Our results are consistent with a growing body of evidence that suggests that integrin signaling events (so-called "outside-in" signaling) may be as crucial to inflammatory cell function as integrin-ligand binding is to cell adhesion and transmigration. In particular, the induction of ERK MAP Kinase activity by integrin crosslinking appears to be of central importance to integrin signaling in monocytes. We have found that crosslinking of the surface LFA-1 and MAC-1 integrins leads to a dramatic accumulation of
Human monocytes were purified from the blood of healthy volunteers as described in Experimental Procedures. CD11b was crosslinked and cells incubated at 37°C, 5%CO₂ for 4 hours. 

A. 4-hour PCA as determined by single stage recalcification assay. PCA mU were determined by comparison to rabbit thromboplastin. Data=mean+/-SEM, n=5/group. 

B. 4-hour surface TF expression assessed by flow cytometry with FITC-conjugated anti-TF mAb. Data=mean+/-SEM, n=6/group. 

* p<0.05 vs control (unpaired Students t test)
Figure 6: Tyrosine Phosphorylation and ERK Inhibition Abrogate β₂ Integrin Dependent Induction of THP-1 PCA

A. Effect of Genistein and PD98059 on THP-1 PCA

B. Effect of PD98059 on CD11a Crosslinking

Figure 6: Tyrosine Kinase and ERK Inhibition Abrogate the β₂-Integrin Induction of THP-1 PCA

A. THP-1 cells were incubated in the presence or absence of genistein or PD98059 for 45 minutes at 37°C, after which CD11a was crosslinked as before. Cell reactions were stopped 4-hours after crosslinking by placing the cells on ice, and PCA was determined as before. PCA mU were determined by comparison to rabbit brain thromboplastin. Data=mean+/−SEM, n>3/group. *p<0.01 vs control (ANOVA with post hoc Tukey)

B. THP-1 cells were pretreated in the presence or absence of PD98059, after which CD11a was crosslinked. A FITC-conjugated goat anti-mouse F(ab')₂ was used to crosslink the surface integrin, and mean cell fluorescence determined by flow cytometry. Data=mean+/−SEM, n=3/group. ***p<0.001 vs control (ANOVA). Entirely analogous results were obtained using the Bearl clone.
Figure 7: Selective ERK Inhibition Abrogates β2-Integrin Induction of Human Monocyte PCA

After pretreatment with or without PD98059, mAb 25.3.1 and Bear1 were used to crosslink CD11a and CD11b, respectively, on the surface of highly purified human monocytes. Following incubation for 4 hours at 37°C, 5% CO₂, cells were prepared for assessment of PCA as before. PCA mU were obtained by comparison to lipidated recombinant tissue factor, as described in Experimental Procedures. Data=mean+-SEM, n=4/grp, ***p< 0.001 vs control (ANOVA with post hoc Tukey)
tyrosine phosphoproteins within THP-1 cells, preceding the increased expression of procoagulant activity in these cells as well as in human monocytes. While much of the broad induction of tyrosine phosphorylation is gone by 30 minutes after integrin crosslinking, ERK tyrosine phosphorylation and kinase activity remain considerable at this time (Fig 2C, Fig 3). The prolonged activation of ERK in response to integrin crosslinking suggests a central role in the subsequent activation of the cell. Consistent with this notion is the finding that both tyrosine kinase and selective ERK inhibition abrogates the induction of TF-specific PCA in THP-1 cells and human monocytes. Considered together, these data suggest that the ERK pathway is crucial to β2-integrin signaling in monocytes.

When compared to the effect of integrin ligation by antibody alone, the finding that clustering of CD11a and CD11b by crosslinking markedly enhances the accumulation of tyrosine phosphoproteins, ERK tyrosine phosphorylation and activity, and functional output (TF) is consistent with work done in fibroblastic cells, and may explain conflicting data with respect to the role of the β2-integrin in monocyte activation. As noted previously, one of the key mechanisms involved in the induction of integrin signaling seems to be the physical clustering of the molecules on the surface of the cell (Miyamoto 1995). Support for this concept in the monocyte is found in work both from our laboratory and others. For example, Lin et al found that monovalent VLA-4 integrin ligation with Fab fragments was insufficient to generate the tyrosine phosphorylation seen with F(ab')2 fragments, whole antibody or Fab fragments crosslinked with secondary antibody (Lin 1994). These authors also noted increased IL-1β expression when whole antibody was crosslinked with secondary. In our work, divalent ligation of VLA-4 with whole antibody did generate tyrosine phosphorylation and ERK activation, but less so than when crosslinked with secondary antibody (McGilvray 1997). Moreover, the functional TF response was dependent on integrin crosslinking. Previous work has demonstrated that β2-integrin divalent antibody ligation does not appear to induce tyrosine phosphorylation (Lin 1994), TNFα or TF expression (Fan 1995, Fan 1993, Fan 1991), while nevertheless priming the monocyte for increased responsiveness to LPS and Th-derived cytokines (Fan 1991, Fan 1993). The absence of β2-integrin-dependent tyrosine phosphorylation and TF functional responses in these works may reflect the fact that the divalent ligation used by these labs was not sufficient to generate a marked intracellular response.

The functional response of the inflammatory cell to integrin engagement depends on both integrin- and adhesion-dependent signaling processes. Integrin crosslinking by antibody, though it generates a signal specific to the integrin clustered, reflects only a portion of the adhesive process in vivo. Similarly, the process of static adhesion to a physiologic substrata such as fibrinogen does not accurately model the stresses the monocyte undergoes during the initial steps of transmigration, which is a tightly controlled sequence of events involving changes in integrin affinity ("inside-out" signaling), cytoskeletal protein
shifts, and integrin signaling to the cell. Some measure of the importance of these considerations is found in the different pattern of cytoskeletal proteins phosphorylated by VLA-4-dependent cell adhesion to fibronectin - which leads to the tyrosine phosphorylation of paxillin, pp125 FAK and pp76 Syk - when contrasted to simple integrin ligation, when only Syk is tyrosine phosphorylated (Lin 1995). Similarly, homotypic cell adhesion is required for the LFA-1-dependent tyrosine phosphorylation of p130cas in JY B-lymphoblastoid cells (Petruzelli 1996). Moreover, neutrophil adhesion to CD18 mAb coated wells can induce a respiratory burst, while crosslinking of surface CD18 does not (Walzog 1994). On the other hand, the adhesion of human monocytes to fibronectin is a relatively weak stimulus for PCA when compared to VLA-4 crosslinking or stimulation by LPS (McGilvray 1997). Thus, the fact that adhesion to fibrinogen does not induce monocyte TF (Fan 1995), while crosslinking of the β2-integrins involved in the adhesion to fibrinogen does, likely reflects the overall response of the cell to the model of integrin engagement employed. However, the fact that monocyte adhesion to fibrinogen does enhance the TF response to LPS suggests at least a priming role for β2 integrins in the monocyte TF response (Fan 1991); the fact that adhesion to the β2-ligand ICAM-1 can increase MIP-1α expression de novo suggests that β2 integrin signaling has the capacity to be directly activating (Lukacs 1994). As mentioned previously, monocyte/endothelial cell co-cultures induce monocyte TF, a process at least partially accounted for by the β2 integrins (Lo 1995, Collins 1995). These studies and our own argue for the role of β2-integrins in the monocyte TF response.

Our results with crosslinking of the β2-integrins are comparable to those we have obtained with crosslinking of the β1-integrin VLA-4. From a physiologic point of view, it seems plausible that both β1-class and β2-class integrins, both of which are involved in the process of monocyte adhesion and transmigration, act coordinately to influence the cell. Antibody-induced crosslinking of either leads to a tyrosine kinase and ERK-dependent induction of monocytic TF. There is a growing body of evidence suggesting that ERK activation ensues from adhesion molecule engagement in a variety of cell types and settings (Miyamoto 1995, Sato 1995, Morino 1995, Waddell 1995). Our finding that ERK is also essential for monocyte activation following either β2- or β1-integrin crosslinking argues that the ERK proteins may perform an integratory role in inflammatory cell function. Specifically, diverse inflammatory stimuli such as TNFα, LPS, IFNγ (Geng 1994, Liu 1994, Liu 1994) and Fc-receptor crosslinking (Durden 1995) all induce ERK activity, thereby providing some coordination of adhesion and response to inflammatory stimuli during monocyte migration to inflammatory sites. From a clinical standpoint this concept could have important implications, since targeted inhibition of the ERK pathway may serve as a novel anti-inflammatory therapy.
Chapter 3: Activation of the p38 MAP kinase is necessary for the induction of monocytic Tissue Factor after crosslinking of the VLA-4 integrin: Evidence for MAPK crosstalk

Summary: Tissue factor (TF) expression on monocytic cells is a key feature of extravascular inflammatory responses. The VLA-4 (CD49d/CD29) integrin, crucial for monocyte transendothelial migration, can also generate poorly understood intracellular signals which lead to TF expression and its biologic activity, procoagulant activity (PCA). We postulated that VLA-4 crosslinking would phosphorylate and activate the p38 MAP kinase. Using western blot, immunoprecipitation and in vitro kinase assays we found that crosslinking of surface VLA-4 with monoclonal antibodies results in an early upregulation of p38 MAPK phosphorylation and activity in both purified human monocytes and THP-1 monocytic cells. Phosphorylation and activation peaked 5-10 minutes after crosslinking and then faded. Having recently demonstrated that VLA-4 dependent increases in PCA require ERK MAPK activation, we investigated the relation of p38 MAPK activation to ERK. Selective inhibition of p38 MAPK activity with SB203580 (1 μM) considerably increased phosphorylation and activation of ERK at all time points examined. Similarly, VLA-4-induced activation of p90RSK, a known substrate of ERK, was markedly increased by SB20380 pretreatment. Despite the augmentation of ERK MAPK and p90RSK activity, SB203580 abrogated the VLA-4 dependent increase in PCA. These studies demonstrate that monocytic VLA-4 signals through the p38 MAPK, and suggest that one function of the p38 MAPK is to restrict excessive activation of ERK and its substrates.
Introduction:

In the development of an extravascular inflammatory response, bloodborne monocytes are induced to migrate across the vascular endothelium, whereupon they bind to and interact with the proteins of the extracellular matrix. Very Late Antigen-4 (VLA-4, \(\alpha_4\beta_1\), CD49d/CD29), a surface integrin present in high amounts on the surface of the circulating monocyte, is known to mediate many of the adhesive interactions required for these processes (Issekutz 1995, Lin 1995). Signals developed by the integrin may also play a crucial role in monocyte activation. In particular, our group and others have found that VLA-4 engagement and crosslinking leads to expression of Tissue Factor (TF), a potent inducer of the coagulation cascade (McGilvray 1997, Fan 1995). TF expression in sites of extravascular inflammation contributes to diverse processes, including infectious abscesses, atherogenesis and the rejection of allografts (McGilvray 1998, Drake 1989, Candinas 1996).

The signals linking surface crosslinking of VLA-4 to the synthesis of TF are poorly understood. We recently reported that crosslinking of VLA-4 on human monocytic THP-1 cells leads to the phosphorylation and activation of the ERK MAP kinase (McGilvray 1997). The activation of ERK was required for TF expression. In cells of the monocyte/macrophage lineage, ERK activation in response to inflammatory stimuli such as LPS is often found in parallel with activation of the related p38 MAPK (Weinstein 1993, Han 1994). The p38 MAPK is known to be essential for the subsequent expression of cytokines (Lee 1996). Interestingly, in this context ERK and p38 may function in concert, with both signals being required for cytokine elaboration (Carter 1999, DeFranco 1998). It is not known whether VLA-4 signaling induces the p38 MAPK.

While there is much data linking activation of the ERK MAPK to integrin signaling, the association with the p38 MAPK is less clear. Recent reports have linked the p38 MAPK with the \(\beta_2\)-integrin dependent respiratory burst in endotoxin-treated neutrophils (Detmers 1998), and with \(\beta_1\)-integrin dependent elaboration of collagenase-3 by human skin fibroblasts (Ravanti 1999). In the latter work, the ERK and p38 MAPK had opposing functions, with ERK inhibiting and p38 inducing the collagenase. Thus, the roles of the ERK and p38 MAPK are likely cell- and stimulus-specific.

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2 Abbreviations used in this paper: ERK: extracellular signal-regulated kinase; mAb: monoclonal antibody; MAPK: mitogen activated protein kinase; PCA: procoagulant activity; RSK: ribosomal S6 kinase; TF: tissue factor; VLA-4: very late antigen-4
We hypothesized that VLA-4 crosslinking would lead to p38 MAPK activation, which in turn would be essential for the generation of a potent inflammatory mediator, tissue factor. Using purified human monocytes, we found that crosslinking VLA-4 led to the rapid and transient phosphorylation of p38 MAPK, peaking at 5-10 minutes and then fading. A similar kinetic was observed in THP-1 cells, with rapid kinase activation. Intriguingly, inhibition of the p38 MAPK with SB203580 (1μM) consistently increased ERK phosphorylation and activation, and increased the activation of p90RSK, a known substrate of ERK (Nguyen 1993). Despite the increase in ERK and p90RSK activity, VLA-4 dependent TF-specific activity (procoagulant activity, PCA) was abrogated by SB203580. These results suggest that in the monocyte both ERK and p38 MAPK are necessary but not sufficient for integrin-dependent cell activation.
Experimental procedures:

Buffers and Reagents:

PD98059 was purchased from Calbiochem and prepared in sterile DMSO at 10 mg/ml and 10 mM, respectively. SB203580 was the kind gift of Dr. J.C. Lee (Smith Kline Beecham, PA), and was made up in sterile DMSO to 20 mM. Piceatannol and the Src-selective PP-1 inhibitors were from Sigma, and were made up in sterile DMSO. Endotoxin-free RPMI 1640 was obtained from Gibco, endotoxin-free fetal calf serum from Hyclone, and L-glycine from Sigma. The following antibodies were used in the integrin engagement studies: mouse IgG1 anti-CD49d (mAb HP2.1 [Immunotech], mAb 44H6 [Serotec]), CD29 (mAb K20, mAb Lia1.2 [Immunotech]), goat F(ab')2 anti-mouse IgG [Immunotech], mouse IgG1 anti-CD45 mAb 1214 [PDI Bioscience]. Negative IgG1 control antibody was from Serotec.

Cell Preparation:

Human monocytic THP-1 cells (ATCC) were propagated in RPMI/10%FCS/penicillin/streptomycin at 37°C, 5% CO₂. Human peripheral blood monocytes (PBM) were isolated from the blood of normal healthy donors by centrifugation over a Ficoll-Hypaque gradient at 400xg for 20 min. The mononuclear layer was aspirated, washed twice and resuspended in RPMI/2%FCS/L-Glutamine. Monocytes were further purified using hypertonic conditions and centrifugation over a 40/50/55% Percoll (Pharmacia) gradient (McGilvray 1998, Chuluyan 1993). The resulting cell population has >96% viability (trypan blue exclusion), is not activated (minimal baseline TNFα secretion and PCA), is consistently >96% positive for neutral red granules, and contains >70% monocytes as defined CD14 positivity, and by flow cytometric forward and side scatter characteristics.

Cell Activation:

For integrin engagement studies, THP-1 cells or purified human monocytes were suspended in RPMI/2%FCS/L-Gln at 5x10⁶ cells/ml. Surface CD29, CD49d and CD45 antigens were ligated with monoclonal antibody for 25 min at 10 ug/ml and 4°C, washed twice in cold RPMI, and then crosslinked with 10 ug/ml goat anti-mouse F(ab')2 for 25 min at 4°C. Cells were washed twice in cold RPMI, resuspended in RPMI/2% FCS/L-Gln and incubated at 37°C, 5%CO₂ for times ranging from 1 minute to 4 hours. Reactions were stopped by placing the cells on ice. In inhibition studies, cells were preincubated with 1-20 μM SB203580, 10 μM PD98059, 10 μg/ml piceatannol, or 10 μM PP-1 for 45 min at 4°C.
**Western Blot Analysis:**
Following integrin crosslinking, THP-1 cells were lysed in ice-cold cell lysis buffer containing 1% Triton X100, 150mM NaCl, 10mM Tris-HCl (pH 7.4), 2mM sodium orthovanadate, 10ug/ml leupeptin, 50mM NaF, 5mM EDTA, 1mM EGTA, and 1mM PMSF. Postnuclear supernatants were collected following centrifugation at 10000xg for 5 min and diluted with 2X Laemmli buffer/0.1M dithiothreitol (DTT). Following adhesion to poly-L-lysine or fibronectin substrats, PBM were lysed in the culture wells with ice cold lysis buffer and prepared in a similar fashion. Lysates prepared from 100 000 cells were separated on 12.5% SDS-PAGE and transferred to PVDF membrane (Mobilon). Blots were then probed with polyclonal rabbit (Transduction Labs) anti-phosphotyrosine antibody, rabbit anti-phosphoERK antibody (New England Biolabs), rabbit anti-ERKI or ERK2 antibody (Santa Cruz Biotechnologies). Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Sigma), blots were developed using an ECL–based system (Amersham).

**Immunoprecipitations and Immune Complex Kinase Assays:**
ERK2 and p38 MAPK immunoprecipitations and kinase assays were performed as described previously (McGilvray 1997, McGilvray 1998). In brief, cells (3x10^6) were lysed as above and the postnuclear supernatant pre-cleared with Protein G-Sepharose (Pharmacia Biotech Inc). Cellular proteins were immunocomplexed using polyclonal anti-ERK-2 or anti-p38 MAPK antibody (Santa Cruz Biotechnologies) for 1 hour at 4°C. Protein G-Sepharose was added and incubated at 4°C for 1 hour. The resulting immune complexes were washed 5 times with cold PBS/0.01% Tween 20, and then separated from beads by 2X Laemmli buffer/0.1M DTT and boiling at 100°C for 5min. Beads were then sedimented by ultracentrifugation and the supernatant collected for Western blot analysis.

For ERK2 kinase assays, ERK2 immunocomplexes were washed with 5 changes of cold PBS/0.01% Tween 20, and then incubated for 30 min at 30°C with 20 μg of ultra-pure myelin basic protein (MBP, Upstate Biotech Inc) in kinase assay buffer composed of 0.4mM cold and 0.4mM P^32-γATP (NEN), 50mM Tris-HCL(pH 7.4) and 1 0mM MgCl2. Reactions were stopped with the addition of 2X Laemmli buffer/ 0.1M DTT and boiling at 100°C for 5 min. Equal volumes were loaded and run on 10% SDS-PAGE. Kinase assays using p38 MAPK immunocomplexes were performed in a similar fashion, substituting ATF-2 (Santa Cruz Biotechnologies) for MBP. The radioactivity of the phosphorylated MBP band running at 20 kDa, or the phosphorylated ATF-2 band at 60 kDa, were quantified on a Molecular Dynamics SI Phosphoimager.
**Measurement of procoagulant activity:**

Four hours after integrin crosslinking, cells were sedimented at 1000xg for 3 min. The cell pellet was resuspended at $10^6$ cells/ml RPMI, freeze-thawed at -70°C, and procoagulant activity (PCA) measured by single stage recalcification clotting assay (Brisseau 1995). PCA was expressed as mU/$10^6$ cells by comparison to rabbit brain thromboplastin. We have previously established that VLA-4-dependent increases in monocytic PCA are attributable to TF (McGilvray 1997).

**Statistical Analysis:**

The data are represented as the mean and standard error of the indicated number of experiments. Where representative studies are shown, these are indicative of at least three equivalent studies. Statistical comparisons were made for continuous data using one-way ANOVA with post hoc Tukey.
Results:

**VLA-4 crosslinking induces p38 MAP kinase phosphorylation in human monocytes:**

Having previously demonstrated that crosslinking of the VLA-4 integrin induces phosphotyrosine accumulation and ERK MAP kinase activation in human THP-1 cells (McGilvray 1997), we investigated the signaling that follows crosslinking of VLA-4 on purified human monocytes. As seen in Figure 1A, crosslinking of either the α4 subunit (CD49d) with monoclonal hp2.1 antibody, or crosslinking of the β1 subunit with monoclonal K20 or Lia1.2, induces strong tyrosine phosphorylation of cellular proteins. Crosslinking consistently induced far more tyrosine phosphorylation of cellular proteins than simple divalent ligation. As with THP-1 cells, neither the 44H6 anti-CD49d clone nor anti-CD45 antibody (mAb 1245, Figure 1B) induces tyrosine phosphorylation. Tyrosine phosphorylation peaked 10-20 minutes after crosslinking and then faded (Figure 1B). As part of this signal, the p38 MAP kinase is strongly phosphorylated, with an early peak 5-10 minutes after crosslinking (Figure 1C). To avoid nonspecific Fc-receptor mediated binding, Fc receptors were first blocked via incubation with human IgG Fc fragments. Since neither secondary antibody alone nor CD45 crosslinking induced tyrosine or p38 MAPK phosphorylation, these results suggest that the induction of tyrosine and p38 MAPK phosphorylation is a specific result of VLA-4 crosslinking on human monocytes.

**VLA-4 crosslinking induces p38 MAP kinase phosphorylation and activation in monocytic THP-1 cells:**

The induction of tissue factor in THP-1 monocytic cells is similar to that seen in human monocytes (Mackman 1995, McGilvray 1997). We therefore employed THP-1 cells to more fully define the signaling events that follow crosslinking of surface VLA-4, and began by confirming the integrin-dependent phosphorylation and activation of the p38 MAPK. Using THP-1 cells, we previously reported that crosslinking of the α4 subunit with the mAb hp2.1 clone, but not the 44H6 clone, induces accumulation of tyrosine phosphoproteins and phosphorylated ERK1 and ERK2 MAPK (McGilvray 1997). Consistent with these findings, crosslinking of CD49d with the mAb hp2.1 clone, but not the 44H6 clone, leads to an early accumulation of dually phosphorylated p38 MAPK in THP-1 monocytic cells (Figure 2A). Again, divalent ligation is less effective at inducing p38 MAPK phosphorylation than crosslinking. Crosslinking of the β1 integrin subunit with K20 also induces the prompt appearance of dually phosphorylated p38 MAPK as assessed by Western blot; by contrast, crosslinking of CD45 evokes only mod-
Figure 1: VLA-4 crosslinking induces tyrosine and p38 MAPK phosphorylation in purified human monocytes

Highly purified human monocytes were obtained as described in Experimental Procedures. After Fc blockade by incubation at 4°C with human IgG Fc fragments, surface CD49d (hp2.1, 44H6) CD29 (lia1.2, K20), or CD45 (mAb 1243) proteins were ligated or crosslinked and the cells brought to 37°C, 5% CO₂. Cells were lysed at the times indicated and prepared for Western blot analysis.

A. Comparison of the effect of divalent surface ligation vs crosslinking; t=10 minutes all groups.

B. Time course of induction of tyrosine phosphorylation after crosslinking of CD49d, as compared to crosslinking of surface CD45 (mAb 1243), or treatment of cells with II0 F(ab')2 fragments alone.

C. The blot in B., stripped and probed for phosphorylated p38 MAPK.

All studies presented are representative of results obtained on at least three independent experiments.
Figure 2: Crosslinking of VLA-4 on monocytic THP-1 cells induces the phosphorylation of the p38 MAPK.

CD49d (hp2.1, 44H6), or CD29 was ligated or crosslinked as described in experimental procedures, and cellular reactions were allowed to proceed for the times indicated before lysis of the THP-1 cells. p38 MAPK phosphorylation was assessed by Western blot in A. and B. (using an antibody specific to the dually phosphorylated p38 MAPK), or, in C., by staining for tyrosine phosphorylation after immunoprecipitating p38 MAPK protein.

A. Effect of ligating or crosslinking CD49d with the hp2.1 or 44H6 mAb clones on accumulation of dually phosphorylated p38 MAPK.

B. Effect of CD29 crosslinking on p38 MAPK phosphorylation, as compared with CD45 crosslinking or secondary F(ab')² fragments alone (II°).

C. Effect of CD49d or CD29 crosslinking on p38 MAPK tyrosine phosphorylation, as assessed by immunoprecipitation.

All studies shown are representative of results obtained on at least three separate occasions.
est p38 MAPK phosphorylation (Figure 2B). Confirmation of these Western blot studies was obtained by immunoprecipitating p38 MAPK after crosslinking of either CD49d or CD29 and staining for tyrosine phosphorylation. As seen in Figure 2C, both treatments lead to an early increase in p38 MAPK tyrosine phosphorylation which peaks 5-10 minutes after crosslinking. This time course is at marked odds with VLA-4 dependent increase in ERK tyrosine phosphorylation, which peaks 20-30 minutes after crosslinking of surface VLA-4, and persists through 60 minutes (McGilvray 1997). As predicted by the time course of phosphorylation, crosslinking of surface VLA-4 leads to a marked early increase in p38 MAPK activity by in vitro kinase assay (Figure 3). As seen in Figure 2C, both treatments lead to an early increase in p38 MAPK tyrosine phosphorylation which peaks 5-10 minutes after crosslinking. This time course is at marked odds with VLA-4 dependent increase in ERK tyrosine phosphorylation, which peaks 20-30 minutes after crosslinking of surface VLA-4, and persists through 60 minutes (McGilvray 1997). As predicted by the time course of phosphorylation, crosslinking of surface VLA-4 leads to a marked early increase in p38 MAPK activity by in vitro kinase assay (Figure 3). As seen in Figure 2C, both treatments lead to an early increase in p38 MAPK tyrosine phosphorylation which peaks 5-10 minutes after crosslinking. This time course is at marked odds with VLA-4 dependent increase in ERK tyrosine phosphorylation, which peaks 20-30 minutes after crosslinking of surface VLA-4, and persists through 60 minutes (McGilvray 1997). As predicted by the time course of phosphorylation, crosslinking of surface VLA-4 leads to a marked early increase in p38 MAPK activity by in vitro kinase assay (Figure 3). Note again that while divalent antibody ligation induces some p38 MAPK activity, crosslinking induces both a more intense and more persistent activation. Considered together with the data discussed above, these studies clearly identify the p38 MAPK as a target of VLA-4 signaling both in human monocytes and THP-1 monocytic cells.

Several lines of evidence suggest that these results are not due to nonspecific antibody effects. First, both α4 and β1 subunit crosslinking induce intense and early phosphorylation of the p38 MAPK. Second, the fact that the 44H6 clone, which induces neither tyrosine phosphorylation, ERK phosphorylation or functional cell activation (McGilvray 1997) also does not induce p38 MAPK phosphorylation suggests that it is the manner of crosslinking which is as important to generating an intracellular signal as the presence of antibody per se. Crosslinking of another prevalent surface antigen, CD45, induces far less p38 MAPK phosphorylation, and the secondary F(ab')2 fragments do not induce p38 MAPK phosphorylation, whether alone or in the presence of an irrelevant IgG1 antibody control (Figure 2B). Finally, blockade of Fc receptors by incubating the THP-1 cells with human IgG Fc fragments does not affect the induction of tyrosine phosphorylation by the antibody clones employed (data not shown). These results strongly suggest that the p38 MAPK phosphorylation we observe following crosslinking of surface VLA-4 on THP-1 cells is due neither to nonspecific antibody effects nor to an Fc-receptor-mediated process.

**Effect of SB203580 and tyrosine kinase inhibition on VLA-4 dependent phosphotyrosine accumulation and the ERK MAPK:**

Since crosstalk between the ERK and p38 MAPK pathways has been reported both by our group and others (McGilvray 1998, Zhang 1998), we investigated whether a similar mechanism might be at play in VLA-4 dependent signaling. Since pp76Syk has been suggested to be important in VLA-4 signaling in monocytic cells (Lin 1995), and Src family kinases are well-recognized upstream modulators of the MAPK, we chose to compare the effects of selective p38 MAPK inhibition with pp76Syk and Src inhibi-
Figure 3: VLA-4 crosslinking induces p38 MAPK activation in THP-1 cells

Surface VLA-4 was ligated or clustered on human THP-1 monocytic cells as described in Experimental Procedures. Cells were lysed at various times after stimulation and the p38 MAPK immunoprecipitated. An ATF-2 in vitro kinase assay is presented for the K20 mAb clone specific to CD29; equivalent results were obtained using the hp2.1 mAb clone specific to CD49d.
tion. Both inhibition of the Src family of tyrosine kinases with PP-1 (Hanke 1996) and of the Syk tyrosine kinase with piceatannol (Oliver 1994) inhibited the general accumulation of tyrosine phosphoproteins following VLA-4 crosslinking (Figure 4A). However, selective inhibition of the p38 MAPK with SB203580 (1 μM) had little effect on total tyrosine phosphorylation, but consistently increased the accumulation of active, dually phosphorylated ERK MAPK forms (Figure 4B), at all time points examined (Figure 4C). By contrast, piceatannol abolished and PP-1 partly inhibited ERK phosphorylation (Figure 4B). An index of the specificity of these compounds is found in the fact that neither inhibited the induction of ERK phosphorylation after phorbol myristate acid (PMA) treatment. Since previous works have suggested that phosphorylation of cPLA2 by p38 MAPK may downregulate ERK activity (Zhang 1997), we examined the effect of a selective cPLA2 inhibitor, AACOCF3, on VLA-4 dependent ERK phosphorylation. Like selective p38 MAPK inhibition, pretreatment of THP-1 cells with AACOCF3 (10 μM) did increase VLA-4 induced ERK phosphorylation; however, we were unable to demonstrate a consistent activation of cPLA2 following VLA-4 crosslinking (as assessed by electrophoretic mobility shift), and other cPLA2 inhibitors, such as MAFP, gave conflicting results (data not shown). By contrast the effects of SB203580 were very consistent. Confirming the Western blot data, in vitro kinase assays demonstrated that SB203580 increased VLA-4 dependent ERK activity, while both piceatannol and the MEK-1 selective PD98059 abrogated the increase (Figure 5). SB20380 alone had no effect either on ERK phosphorylation or on ERK activity (data not shown). Together these results suggest that the early activation of the p38 MAPK after crosslinking of VLA-4 acts to inhibit the subsequent activation of the ERK MAPK.

Effect of selective ERK and p38 MAPK inhibition on p90 RSK activity:

To investigate whether the apparent feedback inhibition of ERK phosphorylation and activity by the p38 MAPK might have physiologic significance, we examined the effect of p38 inhibition on the activation of a known ERK substrate, p90 ribosomal S6 kinase (p90RSK). In vitro kinase assays demonstrated that VLA-4 crosslinking results in a small increase in p90RSK activity which is abolished by pretreatment of the cells with PD98059 (Figure 6). However, pretreatment with a 1 μM dose of SB203580 greatly increased p90RSK activity after VLA-4 crosslinking, with no effect on activity of the kinase alone. In control studies we found that p90RSK did not co-precipitate with either the ERK or p38 MAPK proteins, suggesting that these data reflect RSK and not MAPK activity (data not shown). The synergistic augmentation of p90RSK activity observed in the presence of VLA-4 crosslinking and p38 MAPK inhibition suggests that integrin-activated p38 MAPK may function in part to restrict ERK-dependent proc-
Figure 4: Effect of tyrosine kinase, Src and p38 MAPK inhibition on VLA-4 dependent induction on tyrosine phosphorylation and ERK MAPK phosphorylation

THP-1 were pretreated with the pp76Syk tyrosine kinase inhibitor piceatannol (30μg/ml), the Src-selective PP-1 (10μM), or the p38 MAPK specific SB203580 (1μM) and then stimulated either by crosslinking of surface CD49d/CD29 or with PMA (15ng/ml). Cells were lysed at various times after stimulation and prepared for Western blot analysis.

A. Western blot of tyrosine phosphorylation after crosslinking of CD29 with the K20 mAb; t = 10 minutes for all groups.

B. Western blot of dually phosphorylated ERK after crosslinking of CD49d with hp2.1; t = 10 minutes for all groups.

C. Time course of ERK dual phosphorylation after CD49d crosslinking in the presence or absence of the selective p38 MAPK inhibitor SB203580.
Figure 5: Upregulation of VLA-4 dependent ERK activity by selective p38 MAPK inhibition.

THP-1 cells were pretreated in the presence or absence of the pp76Syk inhibitor piceatannol, the MEK-1 specific PD98059, or the p38 MAPK-selective SB203580. Following crosslinking of CD29 with the K20 mAb clone, THP-1 cells were incubated at 37°C, 5% CO₂ for 30 minutes prior to lysis. ERK2 was immunoprecipitated and MBP in vitro kinase assays performed as described in Experimental Procedures. The upper panel is an autoradiogram of the 18kDa ³²P-MBP band, while the lower panel represents phosphoimager data pooled from three independent ERK2 in vitro kinase experiments. Similar results were obtained with the anti-CD49d hp2.1clone.
Figure 6: Effect of p38 MAPK inhibition on integrin-dependent p90RSK activity

THP-1 cells were pretreated in the presence or absence of the indicated inhibitors. Cells were lysed 30 minutes after crosslinking of CD49d with the hp2.1 mAb clone and p90RSK immunoprecipitated. RSK activity was assessed by allowing the p90RSK immunocomplexes to phosphorylate myelin basic protein (MBP) for 30 minutes at 30°C. An autoradiogram typical of results obtained on three separate occasions is presented.
Effect of selective p38 MAPK inhibition on VLA-4 dependent procoagulant activity:

We have previously demonstrated that inhibition of the ERK MAPK blocks VLA-4 dependent increases in tissue-factor specific procoagulant activity in monocytic THP-1 cells (McGilvray 1997). Since the p38 MAPK is considered to be essential for monocyte/ macrophage elaboration of inflammatory cytokines (Lee 1996), we asked whether the same might be true of VLA-4 integrin dependent increases in procoagulant activity. Using purified human monocytes, we found that p38 MAPK inhibition with SB203580 was sufficient to block the increase in PCA following VLA-4 crosslinking (Figure 7A). Similar results were obtained using THP-1 cells (data not shown). In dose response studies, a 1 μM dose of SB203580 was sufficient to abrogate the induction of PCA (Figure 7B). Importantly, SB203580 did not affect the amount of antibody binding to the cell surface, as assessed by flow cytometry (data not shown). These results clearly implicate the p38 MAPK as essential for VLA-4 integrin-dependent increases in PCA.
Figure 7: Effect of selective p38 MAPK inhibition on VLA-4 dependent PCA

Purified human monocytes were obtained as described in experimental procedures, and pretreated in the presence or absence of SB203580 at the indicated doses. Surface VLA-4 was crosslinked and the cells incubated at 37°C, 5% CO₂ for 4 hours. The cells were then pelleted, resuspended at 10⁶/ml in fresh, calcium-free medium, and PCA determined after a single freeze-thaw cycle at -70°C.

A. Effect of SB203580 on the induction of PCA following crosslinking of CD49d (hp2.1 clone) or CD29 (K20 clone). n=3/group.

B. Dose response of SB20380 in the inhibition of PCA after crosslinking of CD29. n=3/group.

*** p<0.001 vs control (ANOVA with post hoc Tukey)
Discussion:

The studies presented in this paper clearly demonstrate that monocytic VLA-4 integrin signaling involves the p38 MAPK. Crosslinking of surface VLA-4 with monoclonal antibodies specific either to the $\alpha_4$ or $\beta_1$ subunit leads to the early phosphorylation and activation of the p38 MAPK, peaking 5-10 minutes after integrin clustering and then fading. Interestingly, this early activation of the p38 MAPK appears to inhibit the later, and more prolonged, activation of the ERK MAPK. Inhibition of p38 MAPK activity with SB203580 both hastens and enhances ERK phosphorylation and activity, and increases activity of the ERK substrate p90RSK. Nevertheless, pretreatment with SB230580 abolishes the induction of TF-dependent PCA following integrin crosslinking. We have previously shown that ERK activity is necessary for the VLA-4 dependent PCA (McGilvray 1997): these additional studies suggest that the ERK and p38 MAPK act in concert to induce TF.

These studies are the first direct identification of the p38 MAPK as a target of integrin signaling in monocytic cells, and suggest that the p38 MAPK has a crucial role in integrin-mediated inflammatory events. Our data are consistent with an interesting study which employed a pharmacologic p38 MAPK inhibitor (SK&F 86002) to suggest that the p38 MAPK is essential for increased IL-1$\beta$ mRNA after monocyte adhesion to plastic culture wells; however, these results are inferential, since the authors did not demonstrate increased p38 MAPK phosphorylation or activation (Sirenko 1997). In related work we have found that adhesion of human monocytes either to plastic or to wells coated with the VLA-4 ligand, fibronectin, increases p38 MAPK phosphorylation (data not shown). Other studies have implicated p38 MAPK activation in the respiratory burst induced by $\beta_2$-integrin dependent adhesion in endotoxin-treated neutrophils (Detmers 1998). In platelets, collagen-induced aggregation, but not thromboxane generation, is inhibited by SB203580 (Saklatvala 1996). These studies suggest that the p38 MAPK can function in integrin-related inflammatory cell functions. Our work specifically implicates the p38 MAPK with integrin-dependent induction of TF; similarly, inhibition of p38 MAPK abrogates the induction of TF following monocyte transendothelial migration (McGilvray 1999).

The intracellular mechanisms linking VLA-4 clustering on the cell surface to MAPK activation remain to be elucidated. However, the work presented in this paper illustrates several points. For example, clustering of the integrin appears to be essential for the full generation of an intracellular signal. Divalent ligation of either the $\alpha_4$ or $\beta_1$ VLA-4 subunit induces far less intracellular tyrosine phosphorylation, and less p38 MAPK phosphorylation and activity, than does integrin clustering by antibody.
crosslinking (Figures 1 and 2). We have previously reported a similar effect in VLA-4 dependent activation of the ERK MAPK (McGilvray 1997). These findings are consistent with the concept, elegantly described by Miyamoto et al in fibroblastic cells, that integrin clustering acts as a scaffolding upon which a large number of signaling molecules are recruited (Miyamoto 1995). It is not clear whether monocytic integrins recruit the individual low molecular weight GTP-binding proteins generally considered to be upstream of the MAPK modules. However, differentiated THP-1 cells do recruit Cdc42HS, a Rho-family GTPase presumed to be upstream of p38, to their cell membranes when they undergo spreading (Aepfelbacher 1994). The fact that both piceatannol, a selective pp76<sup>Syk</sup> inhibitor, and PP-1, which is specific for Src family kinases, diminished VLA-4 dependent MAPK and tyrosine phosphorylation (Figure 3), suggests that both pp76<sup>Syk</sup> and Src kinases are involved in the generation of a VLA-4 signal. This finding supports the work of Lin et al, who found increased pp76<sup>Syk</sup> phosphorylation and activity after divalent antibody ligation of VLA-4 on monocytic THP-1 cells (Lin 1995). Our studies support a general schema in which monocytic VLA-4 integrin supports the generation of an intracellular tyrosine phosphorylation signal in an manner dependent on integrin clustering, with involvement of the pp76<sup>Syk</sup> and Src-family tyrosine kinases.

Induction of ERK activity by the VLA-4 integrin on monocytic cells is regulated in part by the earlier activation of the p38 MAPK. Inhibition of p38 MAPK activity with SB203580 consistently increased ERK phosphorylation and activity after integrin crosslinking (Figure 4 and 5). We have previously reported a similar effect in murine macrophages infected with Murine Hepatitis Virus Strain-3 (McGilvray 1998), as have others in the signaling that follows aggregation of the high affinity IgE receptor (Fc-ε-RI) in a mast cell line (Zhang 1997). The mechanism underlying this crosstalk is unclear: although cPLA<sub>2</sub> has been suggested to act as an intermediate (Zhang 1997), we were unable to consistently mirror the effects of p38 MAPK inhibition with a number of selective cPLA<sub>2</sub> inhibitors (data not shown). Feedback modulation of ERK activity has been previously noted to proceed via the mSOS adapter protein (Cherniack 1994), ERK-dependent expression or modulation of phosphatases (Peraldi 1994, Widmann 1999), or inactivation of the MAP Kinase kinase kinase Raf-1 by a GTP-sensitive tyrosine phosphatase (Dent 1996). Further studies may help to clarify the precise mechanism of p38MAPK/ERK crosstalk in monocytic cells.

The significance of the ERK/p38 MAPK crosstalk may suggest either a certain MAPK specificity or a mechanism of regulating MAPK-dependent events. Unlike the situation in S. cervisiae yeast, individual mammalian MAPK members cannot be assigned individual biologic functions (reviewed in
Sprague 1998). Instead, the roles of particular MAPK proteins are determined on a cell- and stimulus-specific basis. The inhibition of ERK by an earlier activation of the p38 MAPK may act to focus the intracellular MAPK signal, and in so doing prevent activation of ancillary ERK functions. For instance, prolonged ERK activation will lead to a more pronounced accumulation of ERK in the nucleus (Marshall 1994), which may overwhelm intra-nuclear MAP kinase phosphatases and "switch on" a ERK-dependent genetic program (reviewed in Ferrell 1998). For example, in response to Fc-ε-RI aggregation on a mast cell line, ERK, but not p38, appears to be important for production of TNFα and release of arachidonic acid (Zhang 1997). Inhibition of the p38 MAPK with SB203580 enhanced these ERK-dependent functions.

In our studies, inhibition of either ERK or p38 MAPK resulted in the complete abrogation of integrin-dependent TF (Figure 7). These results are more in keeping with studies demonstrating that in cells of the monocyte/macrophage lineage, both ERK and p38 MAPK are required for the elaboration of immediate early inflammatory genes. For example, the induction of IL-1β mRNA following human monocyte adhesion to plastic culture wells is sensitive both to p38 MAPK inhibition (as noted above) and to ERK inhibition with PD98059 (Sirenko 1997). Similarly, monocytic cells or macrophages exposed to endotoxin require both ERK and p38 MAPK for the induction of inflammatory cytokines (DeFranco 1998, Carter 1999). The fact that p38 MAPK inhibition markedly increased both ERK2 and p90RSK activity (Figures 5 and 6) after crosslinking of VLA-4 is consistent with an increased potential for cellular differentiation events (Nguyen 1993). It is tempting to speculate that the differentiation of monocytes into dendritic cells following integrin-dependent transmigration might be dependent on this type of signaling response (Randolph 1998). However, THP-1 cells continue to divide after crosslinking of VLA-4 whether in the presence or absence of SB203580, while neither suspended nor adherent human monocytes develop gross morphologic differences in the first 3-4 days after VLA-4 crosslinking and p38 inhibition (data not shown). Further studies are required to clarify the role of ERK, p38, and other co-stimulatory adhesive events in monocyte differentiation.

In summary, we present clear data linking describing the role of the p38 MAPK in VLA-4 dependent induction of TF in human monocytes and THP-1 monocytic cells. Our work suggests that the parallel activation of the ERK and p38 MAPK by clustering of the VLA-4 integrin is essential for subsequent cell activation. The identification of ERK/p38 MAPK crosstalk in this setting may reflect a fine-tuning of acute inflammatory activation vs more long-term cellular differentiation events. Monocyte function after the integrin-dependent process of transendothelial migration by bloodborne monocytes into
sites of extravascular inflammation is likely to involve both the ERK and p38 MAPK signaling cascades. Thus, the p38 MAPK represents a potential target for the selective pharmacologic modulation of inflammatory disorders which involve TF upregulation on infiltrating monocytes/macrophages, including allograft rejection and viral fulminant hepatic failure.
Chapter 4: Monocyte transendothelial migration induces Tissue Factor: Role of the MAP kinases

Summary: The expression of tissue factor (TF) by monocytes which have transmigrated across the endothelium to sites of extravascular inflammation acts both to focus and amplify the inflammatory response. Since clustering of the integrins responsible for endothelial adhesion and transmigration induces tyrosine phosphorylation and activation of the Mitogen Activated Protein (MAP) Kinases, we postulated that transmigration leads to monocyte activation and TF production by virtue of these signaling routes. Monocytes were migrated across TNFα-primed ECV304 cells grown on fibronectin (FN)-coated Transwell chambers in response to FMLP (10⁻⁸M). Following transmigration monocytes developed a marked, time-dependent increase in surface tissue factor and procoagulant activity. TF expression was dependent on monocyte-endothelial adhesive interactions: TF was not induced by FMLP, migration across FN alone, or by soluble factors induced during migration, while monocyte-ECV304 adhesion during migration was sufficient to stimulate TF. Antibodies against CD29 (β₁ integrin), but not against CD18 (β₂ integrin) or CD31 (PECAM-1), inhibited TF expression. Monocyte adhesion to ECV304 cells induced tyrosine phosphorylation of cellular proteins, and specifically of the ERK and p38 MAP kinases. Tyrosine kinase inhibition with genistein (10 μg/ml) or piceatannol (30 μg/ml) blocked transmigration, while selective ERK inhibition with PD98059 (50 μM) or p38 inhibition with SB203580 (20 μM) did not. However, both ERK and p38 inhibition dose-dependently abolished TF expression. These studies suggest that an extravascular focus of infection or inflammation can simultaneously promote both intravascular thrombosis and extravascular fibrin deposition by inducing monocytes to bind and transmigrate across the endothelial barrier. The selective inhibition of the MAP Kinases may offer a novel therapeutic means of modulating this inflammatory sequence.
**Introduction:**

Monocyte adhesion to and transmigration across the vascular bed are the initial steps in the recruitment of blood-borne monocytes to sites of extravascular inflammation. The subsequent expression of Tissue Factor (TF)\(^3\), leading to increased procoagulant activity (PCA), is a key component of the host response to inflammatory stimuli (McGilvray 1998). By triggering fibrin deposition and an altered inflammatory response, monocyte/macrophage TF expression contributes both to acute infectious processes, such as intraabdominal abscesses, chronic inflammatory events such as atherogenesis (McGilvray 1998, Drake 1989, Taubman 1997), and rejection of allo- and xeno-grafts (Kopp 1998, Candinas 1996).

It is not known whether monocyte transendothelial migration is a sufficient stimulus for upregulation of TF. Evidence from our laboratory and others has demonstrated that broad stimulation of individual surface receptors responsible for monocyte adhesion to and migration across endothelial cells can lead to increased monocyte TF (McGilvray 1997, Fan 1995, McGilvray 1998), as does prolonged (> 30 minutes) adhesion to activated endothelial beds in non-migratory conditions (Lo 1995, Collins 1995). In contrast to the relatively lengthy adhesive interactions modeled in these studies, monocyte transendothelial migration occurs in a matter of minutes (Muller 1992). Leukocytes respond differently to engagement of the same adhesion receptor depending on how the receptor is stimulated, whether the stimulation is transient or more long-term, and on whether other adhesion receptors are concurrently stimulated (Lin 1995, Petruzelli 1996, Walzog 1994). Whether the transient adhesive interactions the monocyte experiences during transendothelial migration are enough to induce cell activation and expression of TF is an unanswered but nevertheless important question, given the often central role of the transmigrated monocyte and coagulation cascade activation in extravascular inflammatory responses (McGilvray 1998).

Blockade of leukocyte emigration has been suggested as a therapeutic maneuver to attenuate prevent inflammatory responses (Isssekutz 1995, Muller 1993). However, if the transient adhesive interactions incurred during monocyte transmigration are sufficient to stimulate TF, it follows that blockade of transmigration *per se* may prevent leukocyte sequestration at sites of inflammation but result in the accumulation of activated monocytes locally (adherent to the inflamed endothelium) and/or systemically. In this case it might be more effective to target therapy to the intracellular pathways responsible for cell activation. We recently demonstrated that the ERK Mitogen Activated Protein Kinase (MAPK) is essential for upregulation of TF activity (PCA) following crosslinking of surface \(\beta_1\) (VLA-4, CD49d/CD29) and \(\beta_2\)

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\(^3\) Abbreviations used: DMSO: dimethyl sulfoxide; ECV: endothelial cell venous; ERK: extracellular signal-regulated kinase; FMLP: f-met-leu-phe; HUVEC: human umbilical vein endothelial cell; LPS: endotoxin; mAb: monoclonal antibody; MAP: mitogen activated protein; MBP: myelin basic protein; PBM: peripheral blood mononuclear cells; PCA: procoagulant activity; TEM: transendothelial migration; TF: tissue factor
(mac-1, CD11b/CD18, and LFA-1, CD11a/CD18) integrins (McGilvray 1997, McGilvray 1998); we have also found that the p38 MAPK plays a necessary role as well (ID McGilvray and OD Rotstein, unpublished observations). The roles of these pathways in monocyte transmigration is unknown, while there is conflicting data regarding their roles in neutrophil migration (Kuroki 1997, Knall 1997).

We postulated that the process of monocyte transendothelial migration would stimulate TF expression on migrated monocytes in an adhesion-, ERK- and p38 MAPK-dependent fashion, and investigated whether adhesion during migration alone was sufficient for activation, or whether monocyte diapedesis between endothelial cells was also required. We found that monocyte migration across TNFα-primed ECV304 layers resulted in a marked upregulation of surface TF. Interestingly, β1 integrin-mediated adhesion was essential for cellular TF expression. Both the ERK and p38 MAPK were tyrosine phosphorylated during the process of adhesion to primed ECV304 cells. Selective pharmacologic inhibition of the ERK pathway with PD98059 and of p38 with SB203580 did not inhibit transmigration but did attenuate TF upregulation. These studies suggest that transient adhesive interactions, and in particular interactions with β1 integrins, are sufficient for monocyte TF upregulation: the selective inhibition of ERK or p38 MAPK may represent a novel therapeutic approach to the treatment of mononuclear-driven inflammatory processes.
Experimental Procedures:

Buffers and reagents:

RPMI and Medium 199 were purchased from Sigma, fetal calf serum (FCS) from Hyclone. The tyrosine kinase inhibitors genistein and piceatannol were obtained from Sigma and were prepared in DMSO at 10 and 50 mg/ml concentrations, respectively. The selective MEK-1 inhibitor PD98059 was purchased from RBI and was diluted in DMSO to a 10 mM concentration, while SB203580 was the kind gift of Dr. J.C. Lee (SmithKline Beecham Pharmaceuticals, PA, USA) and was prepared in DMSO at 20 mM. The following antibodies were used: rabbit pAb against the dually phosphorylated forms of the ERK and p38 MAP Kinases (New England Biolabs), phosphotyrosine (Transduction Labs), p44 ERK2 and p38 MAPK (Santa Cruz Biotechnologies); murine mAb against PECAM-1 (hec7, Endogen), CD18 (7E4, Immunotech) and CD29 (Lia 1.2, Immunotech). FITC-conjugated mAb against human tissue factor was obtained from American Diagnostica.

Cell isolation:

Peripheral blood mononuclear cells (PBM) were isolated from the blood of normal, healthy volunteers as described previously (McGilvray 1998, Chuluyan 1993). Briefly, erythrocytes were sedimented with 3% Dextran T500, and the buffy coat layer collected after centrifugation over Ficoll (Pharmacia). In some experiments monocytes were purified further using hypertonic saline and centrifugation over a Percoll gradient. The resulting homogeneous monocyte population is >96% positive for neutral red granules, >96% viable by trypan blue exclusion and is not activated (minimal baseline TNFα secretion and PCA, with upregulation of CD11b in response to endotoxin). All cells were washed extensively in RPMI and suspended at a concentration of 1 x 10^6 monocytes/ml RPMI/2% FCS/ L-Gln.

Transmigration studies:

Polystyrene transwell plates with 3.0 µm pores (Corning Costar) were coated with 50 µg/ml fibronectin (Boehringer Mannheim). 1 x 10^6 ECV304 cells (ATCC) were seeded onto the plates grown to confluence in Medium 199/10% FCS/ Pen/Strep. To ensure tight confluence, 1125 albumin permeability studies were routinely performed: less than 5% of albumin instilled in the upper chamber leaked into the lower transmigration chamber over 1 hour incubations at 37°C, 5% CO2 (data not shown). On the day of the experiment, the ECV304 cells were stimulated for 4 hours with low dose TNFα (20 µg/ml, Genzyme) to increase adhesion molecule expression. After TNFα priming, the ECV304 cells were washed with four changes of RPMI at room temperature. 1 x 10^6 PBM or purified monocytes were layered over the endothelial cells, and transmigration was initiated by the addition of 10^-8 M FMLP to the lower chamber. Migration was allowed to proceed for 60 minutes at 37°C, 5% CO2 in the presence or absence of PD98059.
(10-50 μM) or SB203580 (1-30 μM). In some experiments, PBM were first exposed to 10 μg/ml mAb specific to PECAM-1, CD29 or CD18 for 25 minutes at 4°C, then washed twice in fresh RPMI. Transmigrated monocytes were then aspirated from the lower chamber. In some experiments nonadherent PBM from the upper chamber were washed away with 3 changes of RPMI at room temperature, and adherent cells collected by vigorous pipetting. Cells were transferred to sterile 6 ml polypropylene culture tubes, incubated for an additional 4 hours at 37°C, 5% CO₂, then pelleted and prepared for flow cytometry or PCA determination.

Evaluation of surface TF and Procoagulant activity:
PBM were sedimented at 1000 x g for 3 min after 4 hours of incubation at 37°C, 5% CO₂. For determination of procoagulant activity (PCA), the cell pellet was resuspended at 10⁶ cells/ml RPMI, freeze-thawed at -70°C, and PCA measured by single stage recalcification assay (Brisseau 1995). PCA was expressed as milliunits/10⁶ cells by comparison to rabbit brain thromboplastin. For assessment of surface tissue factor, the cell pellet was resuspended in 100 μl of ice-cold RPMI/ 10% FCS/ L-Gln. The cells were then stained with FITC-conjugated anti-TF mAb (American Diagnostica) according to the manufacturer’s instructions, washed twice in cold RPMI, and read on a Coulter EPICS XL Cytofluorometer. In all cases, the cytometer output was gated to the monocytic cell population, as determined by distinct forward and side scatter characteristics. This population was confirmed to be monocytic cells on the basis of staining with anti-CD14 (Becton-Dickinson) and anti-HLA-DR mAb (data not shown).

Adhesion studies for signaling:
In determining the signaling cascades which follow monocyte-endothelial interactions, highly purified human monocytes were allowed to adhere to washed, TNFα-primed ECV304 cells grown to confluence on autoclaved glass coverslips. 500 000 monocytes in 500 μl RPMI/ 2% FCS/ L-Gln were layered onto the endothelial cells and incubated for 30 minutes at 37°C, 5% CO₂. Alternatively, monocytes were left in suspension in a similar medium for the same time period. At the end of this time, 1 ml of 4% paraformaldehyde/ PBS (pH 7.4) solution was added and left for 20 minutes at room temperature before quenching with 100 mM glycine and permeabilization with 0.1% Triton X 100/ PBS for 5 minutes at room temperature. Suspension cells were cytopspun onto a glass microscope slide and prepared in a fashion comparable to the monocyte/endothelial preparations. Cells were washed with 5 changes of cold PBS and blocked overnight with 5% normal goat serum/ PBS at 4°C. Cells were incubated with primary rabbit pAb directed against phosphotyrosine residues (Transduction labs), phosphorylated ERK or phosphorylated p38 MAPK (New England Biolabs) for 2 hours at room temperature, washed 5 times with PBS, and
incubated for 1 hour with 1:500 goat anti-rabbit secondary conjugated to Cy3 (Jackson Immunologicals). After five washes in PBS coverslips were mounted using SlowFade antifade reagents (Molecular Probes) and evaluated by confocal microscopy using a BioRad MRC 600 confocal microscope with Comos 7.0 software.

**Measurement of transmigration index:**

In order to measure monocyte transmigration, highly purified human monocytes were labeled with $^{51}$Cr and transmigration was performed as above for 60 minutes. At the end of this time migrated cells were harvested from the lower chamber. To ensure complete harvest of migrated cells, the lower chamber was washed with 1 ml of a solution containing 1% Triton X100 / PBS, and the washing added to the previously harvested cells. The radioactivity of this sample was assessed by gamma counter. A transmigration index was calculated as the radioactivity of the harvested cells divided by the radioactivity of a sample of $1 \times 10^6$ labeled monocytes in suspension. Results were checked by manual counting of transmigrated cells and found to be accurate.

**Statistical analysis:**

Continuous data is represented as the mean and standard error of the indicated number of experiments. Where representative studies are shown, these are indicative of at least three equivalent studies performed independently. Statistical comparisons were made using one-way ANOVA with post hoc Tukey.
Results:

**Monocyte transendothelial migration induces surface TF expression and procoagulant activity in an adhesion-dependent fashion:**

Monocyte transendothelial migration is a function both of expressed adhesion molecules and of the chemotactic stimulus (Issekutz 1995, Chuluyan 1995). Previous work has suggested that migration of a mixed peripheral blood mononuclear preparation across endothelial layers is selective for monocytic cells (Muller 1992). In a series of preliminary studies we examined which population of leukocytes is induced to cross a TNFα-primed surface of ECV304 cells. As seen in the left panel of Figure 1B, only monocytes—defined by distinct side and forward scatter characteristics, and with $>85\%$ positive staining for CD14—undergo transendothelial migration in response to FMLP at a $10^{-8}$M dose, when compared to cells in suspension (Figure 1A, left panel). ECV304 cells constitutively express both the $\beta_2$ integrin ligand, ICAM-1 and the $\beta_1$ integrin ligand, VCAM-1 (Mutin 1997, Ricard 1997), facts we confirmed by Western blotting (data not shown). ECV304 layers were primed with TNFα to further enhance expression of these proteins. Under these conditions, monocyte migration was eliminated by pretreatment with the combination of blocking $\beta_2$ and $\beta_1$ antibodies (7E4 and Lia1.2, respectively), partially inhibited by pretreatment with anti-$\beta_2$ antibody alone (23±9% inhibition, mean±SEM, n=7, by manual counting), and more fully inhibited by blocking anti-$\beta_1$ antibody (75±5% inhibition, n=7). Thus, as has been described in other models of monocyte transmigration, migration across TNFα-stimulated ECV304 cells in response to FMLP is characterized by monocyte purification, and involves both $\beta_1$ and $\beta_2$ integrins (Muller 1992, Meerschaert 1995, Chuluyan 1993, Chuluyan 1995 JLB, Takahashi 1994).

We went on to use the ECV304 model to study the effect of transmigration on cell activation. Monocytes harvested from the lower transmigration chamber and incubated for 4 hours at 37°C, 5% CO$_2$ demonstrated a large increase in the expression of surface TF as assessed by flow cytometry (Figure 1B, right panel). The increase in TF was biologically active, as evidenced by the large increase in procoagulant activity (PCA) from the migrated cells, and was comparable to that observed following stimulation of suspended cells with LPS (1 μg/ml) for four hours (Figure 2A). Importantly, the rise in PCA and surface TF can not be attributed to FMLP stimulation alone, since the chemoattractant increases neither measure (Fig 2A and Figure 2B, respectively). Transmigration caused a time-dependent increase in surface TF which peaks 4 hours after migration and thereafter gradually fades (Figure 2B). To determine whether transmigration-induced TF was a function of monocyte/endothelial interactions, monocyte/fibronectin interactions, or both, we studied migration across fibronectin alone or ECV grown on a fibronectin matrix. Cells which migrated across the endothelial cell layer expressed TF, while transmigration across fibronectin alone had little effect (Figure 2C). Since monocyte/endothelial adhesion has
Figure 1: Monocyte Transendothelial Migration Induces Surface Tissue Factor Expression

PBM from the whole blood of normal, healthy volunteers were prepared by Ficoll separation as described in Experimental Procedures. This population was characterised by flow cytometric analysis both before (A, left panel) and after (B, left panel) transendothelial migration across a confluent layer of TNFα-primed ECV304 cells, and surface TF was determined after 4 hours of suspension (A, right panel), or 4 hours after migration (B, right panel). Note that only cells identified as monocytes by size, granularity and CD-14 positivity (region “A” in the left panels) migrate across the endothelial layer in this model. Migrated cells have a dramatic increase in surface tissue factor when compared to cells held in suspension.
Figure 2: Monocyte Transendothelial Migration Induces Procoagulant Activity

A. Four hours after treatment of PBM in suspension with $10^{-8}$ M FMLP or 1 µg/ml LPS, or 4 hours after monocyte transendothelial migration, PCA was determined as described in experimental procedures.

B. PBM were migrated across a confluent, TNFα-primed ECV304 layer in response to $10^{-8}$ M FMLP, and were assessed for surface TF expression by flow cytometry at various times after transmigration. Alternatively, PBM were treated with FMLP ($10^{-8}$ M) or held in suspension for the times indicated. The data shown is representative of results obtained in two independent experiments.

C. PBM in suspension were treated with FMLP ($10^{-8}$ M), or were migrated across either a confluent ECV304 layer or a polycarbonate filter coated with fibronectin alone.

D. PBM were migrated as before, then harvested from the lower well of the transmigration chamber and sedimented by centrifugation. The pelleted cells were washed and resuspended in RPMI/2% FCS/L-Gln, while the supernatant from the original migration cohort was used to suspend fresh PBM. All groups were then incubated for 4 hours at 37°C, 5% CO₂, and surface TF determined by flow cytometry. In all experiments presented, data=mean±SEM, n ≥3/group. *p<0.05 vs control, **p<0.01 vs control, δp<0.001 vs suspension and FMLP groups (ANOVA with post hoc Tukey).
been demonstrated to result in increased secretion of biologically active factors, such as IL-8 (Takahashi 1996), we next evaluated whether the transmigration-induced TF was due to a secreted factor. PBM were migrated across the endothelial cell layer for 1 hour, at which point the lower well contents were harvested. Following rapid sedimentation, the supernatant from the harvested, migrated cells was used to stimulate fresh PBM. Migrated cells were resuspended in fresh medium, and all cells were then incubated for four hours. As shown in Figure 2D, monocytes which had undergone transendothelial migration expressed large amounts of surface TF, whether or not they were washed after migration, but suspended cells treated with the migration supernatant did not. These results are most consistent with a process of monocyte activation dependent on the physical interactions of these cells with the endothelial cell layer, and not with fibronectin alone or with migration-induced soluble factors.

**Endothelial adhesion during migration is sufficient for the induction of TF:**

To determine whether the initial adhesive interactions with the endothelial surface are sufficient to stimulate TF expression in monocytes, we compared monocytes which had undergone transendothelial migration to cells which had adhered to the endothelial surface either during migration or under static conditions. As seen in Figure 3A, both migration and adhesion in the presence or absence of FMLP stimulated similar amounts of surface monocyte TF expression. Equally, adherent monocytes harvested from the endothelial surface at the end of the 1 hour transmigration period expressed similar amounts of TF as cells which had migrated across the layer (Figure 6B). By contrast, non-adherent monocytes harvested from the top well of the Transwell chamber did not express TF (data not shown). Taken together these results demonstrate that monocyte endothelial adhesion, either alone or during migration and whether or not in the presence of a chemotactic factor, is a sufficient stimulus for TF expression.

**The roles of β₁ integrins and β₂ integrins in monocyte activation during transmigration:**

As noted above, monocyte migration across primed ECV304 cells is a function both of β₁ and β₂ integrins. To determine which family of adhesion molecules contributes to transmigration-dependent cell activation, we pretreated PBM with adhesion-blocking mAb against CD29 (β₁) or CD18 (β₂). Anti-PECAM-1 antibody was used as a negative control, since ECV304 cells do not express PECAM-1 (Muttin 1997). In a series of control experiments, we determined that none of the antibodies used stimulated TF expression, either in the presence or absence of FMLP (data not shown). The hec7 clone failed to affect the upregulation of monocyte TF following transmigration (Figure 4). By contrast, the anti-CD29 Lia1.2 clone decreased post-transmigration TF expression (Figure 4) and PCA (data not shown), while CD18 blockade did not. These results are not due to steric interference with binding of the anti-TF mAb,
Figure 3: Adhesion vs. Migration-Dependent Induction of TF on Monocytes

A. PBM in the presence or absence of $10^{-8}$ M FMLP were allowed to adhere to a TNFα-primed layer of ECV304 cells for 1 hour, after which non-adherent cells were washed away and adherent cells harvested by vigorous agitation. Alternatively, PBM were subjected to TEM for 1 hour as before, and the transmigrated monocytes harvested. Following a 4 hour incubation at 37°C, 5% CO₂, surface TF was determined by flow cytometry.

B. PBM were allowed to migrate for 1 hour in response to $10^{-8}$ M FMLP, at which point migrated cells were harvested from the lower chamber. Non-adherent cells were washed away from the upper chamber, and adherent cells harvested by vigorous agitation.

Data=mean SEM, n=3/group. Data presented are representative of results obtained in four independent experiments. *** p<0.001 vs control by ANOVA with post hoc Tukey.
Figure 4: Effect of CD18, CD19 and PECAM-1 Blockade on Migration-Induced Tissue Factor

PBM were pretreated with 10μg/ml adhesion blocking monoclonal antibodies against CD18 (7E4), CD29 (Lia 1.2) and PECAM-1 (hec7), washed and migrated across a confluent, TNFa-primed ECV304 layer as before. 4 hours after migration, surface TF was determined by flow cytometry. Data=mean±SEM, n≥3/group. *** p<0.001 vs control; δ p<0.001 vs migration (ANOVA with post hoc Tukey).
since LPS-induced increases in surface TF are not affected by monocyte pretreatment with Lia1.2 (data not shown). It is unlikely that the inhibition of surface TF by \( \beta_1 \) integrin blockade is due to an altered population of migrated monocytic cells, since the great majority (>80%) of cells which migrated and fell within the flow cytometric parameters established for TF analysis remained strongly positive for CD14. Interestingly, pretreatment of monocytes with Lia1.2 did not inhibit the induction of monocyte TF following static adhesion to primed ECV layers (data not shown). These studies implicate the monocyte \( \beta_1 \)-integrin family as being of particular importance for transmigration-induced monocyte TF.

**Phosphorylation of the ERK and p38 MAP Kinases monocyte adhesion to endothelial cells:**

Monocyte transendothelial migration reflects the coordinate action of both \( \beta_1 \) and \( \beta_2 \) class integrins. We have previously demonstrated that clustering of both \( \beta_1 \) and \( \beta_2 \) class integrins on the surface of monocytic cells leads to the tyrosine phosphorylation and activation of the ERK MAP Kinase (McGilvray 1997, 1998), and p38 MAP Kinase phosphorylation and activation follows \( \beta_1 \) integrin crosslinking (I.D. McGilvray, unpublished observations). To determine whether these pathways are induced during the physical interaction of monocyte and endothelial cell, highly purified human monocytes were allowed to adhere to primed endothelial cells in the absence of FMLP for 30 minutes. At this time the monocyte/endothelial preparations were fixed in 4% paraformaldehyde and assessed for phosphotyrosine, phospho-ERK and phospho-p38 accumulation by confocal microscopy. As shown in Figure 5, when compared to monocytes held in suspension adherent monocytes demonstrated marked increases in tyrosine phosphorylation and phosphorylation of both the ERK and p38 MAPK. These results are consistent with the signaling patterns induced in monocytic cells following integrin crosslinking, and implicate the ERK and p38 MAP Kinases in our model of monocyte transmigration.

**Role of the MAP Kinases in monocyte transmigration per se:**

To determine the physiologic significance of MAP Kinase activation during transmigration we first evaluated the effect of selective ERK and p38 MAPK inhibition on monocyte migration *per se*. Figure 6 documents the effect on migration of the tyrosine kinase inhibitors genistein (10\( \mu \)g/ml) and piceatannol (30\( \mu \)g/ml) when compared to selective ERK inhibition with PD98059 (50\( \mu \)M) and selective p38 MAPK inhibition with SB203580 (30\( \mu \)M). While pretreatment with both genistein and piceatannol significantly attenuated monocyte transmigration, PD98059 had little effect, and SB203580 did not inhibit migration. These doses of PD98059 and SB203580 inhibited ERK and p38 activation, respectively, as assessed by *in vitro* kinase assay following monocyte exposure to FMLP (data not shown); similarly, we have previously found these doses to be effective at selectively inhibiting the MAP kinases in primary
Figure 5. Induction of ERK and p38 MAP Kinase Phosphorylation by Adhesion to ECV304 cells.

Highly purified human monocytes were allowed to adhere to a confluent layer of TNFα-primed ECV304 cells for 30 minutes, at which time the cells were fixed in 4% paraformaldehyde/ PBS for 20 minutes and prepared for confocal microscopy as described in Experimental Procedures. Cells in suspension (left panel) are compared to adherent cells (right panel) for phosphotyrosine accumulation, phosphorylation of the ERK MAP Kinase and p38 MAP Kinase phosphorylation.
Figure 6. Effect of Tyrosine Kinase, ERK and p38 MAP Kinase Inhibition on Monocyte Transendothelial Migration

Purified human monocytes were labeled with $^{51}$Cr and pretreated with broad spectrum tyrosine kinase inhibitors (genistein, 10μg/ml, and piceatannol, 30μg/ml) or selective MAP Kinase inhibitors (PD98059, 50μM, and SB203580, 30μM) and migrated across a confluent ECV304 layer in response to a $10^{-8}$ M dose of FMLP. Cells in the lower well were harvested as described in Experimental Procedures, and migrated counts compared to total counts (for $10^6$ cells) to obtain a transmigration index. Data=mean± SEM, n≥3/group. **p<0.01, ***p<0.001 vs control (ANOVA with post hoc Tukey).
macrophages (McGilvray 1998). Genistein blocks monocyte migration across fibronectin alone as well as attenuating the baseline migration across endothelial layers in the absence of a chemotactic factor, while PD98059 has no effect in either setting (data not shown). None of the inhibitors used affected ECV304 permeability as assessed by $^{125}$I albumin leakage (data not shown); similarly, none were toxic to the monocyte as determined by $>95\%$ viability after 6 hour incubations (trypan blue exclusion, data not shown). These results suggest that while tyrosine phosphorylation cascades are crucial for monocyte migration mechanics, the ERK and p38 MAP Kinases are not necessary for monocyte transmigration.

**Role of the MAP Kinases in transmigration-dependent monocyte activation:**

To evaluate the role of the ERK and p38 MAP kinases in the expression of TF following monocyte transendothelial migration, PBM were induced to migrate across an endothelial barrier and into a lower chamber into which various doses of PD98059 and SB203580 had been instilled. As shown in Figure 7, both selective ERK and selective p38 MAPK inhibition caused a dose-dependent reduction in transmigration-dependent monocyte TF. Neither inhibitor affected monocyte viability as evidenced by $>95\%$ viability after 6 hour incubations (by trypan blue exclusion, data not shown). Thus, the ERK and p38 MAPK are essential for transmigration-induced monocyte activation.
Figure 7: Effect of Selective ERK and p38 Inhibition on Migration-dependent TF Expression

To evaluate the effect of selective MAP Kinase inhibition on monocyte activation following migration, PD98059 or SB203580 was instilled into the lower well of the transmigration chamber and PBM migrated in response to FMLP as before. Migrated cells were harvested and incubated for 4 hours at 37°C, 5% CO₂ for 4 hours, at which time surface TF expression was determined by flow cytometry. Data=mean±SEM, n≥6/group. ***p<0.001, *p<0.05 vs control (ANOVA with post hoc Tukey).
Discussion:

In these studies we present evidence that monocyte transendothelial migration stimulates the expression of biologically active surface tissue factor. Following transmigration, monocyte surface expression of TF increases in a time-dependent fashion and is accompanied by a large increase in PCA. Adhesive interactions between the monocyte and the endothelial cell are essential in this sequence, particularly those of the $\beta_1$ integrin family, and adhesion of the monocyte to the endothelial surface during transmigration is sufficient to stimulate monocyte TF expression. Monocyte adhesion to primed ECV304 cells leads to tyrosine phosphorylation and recruitment of active forms of the ERK and p38 MAP Kinases: these kinases, while not required for monocyte migration *per se*, are nevertheless crucial for TF expression. Thus, these studies define the inflammatory potential of the process of monocyte transendothelial migration, suggest that the monocyte which has adhered to a primed endothelial surface is committed to activation whether or not it goes on to migrate across the layer, and offer a signaling rationale for the activation.

Monocyte transmigration across a confluent, cytokine-primed layer of ECV304 cells led to a large and time-dependent increase in surface tissue factor and PCA. Our findings are most consistent with monocyte activation being a function of cellular adhesive interactions, since TF is not stimulated by migration across fibronectin alone, nor by supernatants from the migrated cells, nor from exposure to FMLP alone (Figure 2). These results are consistent with the ability of integrin-dependent signaling to stimulate TF expression (McGilvray 1997, McGilvray 1998, Fan 1994), and are the first clear demonstration of the fact that the transient adhesive interactions between monocytes and endothelial cells during transmigration are sufficient for monocyte activation. It is important to make a distinction between resting and activated endothelial cells in this regard. Previous studies have shown that the increase in monocyte TF that follows prolonged adhesion to endothelial layers occurs primarily with activated endothelial cells (Lo 1995, Collins 1995). A recent study suggested that TF was increased following transmigration across resting HUVEC grown on collagen matrices impregnated with FMLP (Randolph 1998). However, it was not clear if transmigration itself had stimulated TF, since the FMLP-impregnated matrices stimulated increased monocyte PCA to the same degree as monocyte/HUVEC co-culture. Thus, while adhesion-dependent events clearly have the potential to increase monocyte TF, it is the nature of these interactions which determines whether TF synthesis is actually induced.

Our results further suggest that $\beta_1$-integrins are crucial for the induction of TF by the process of
transendothelial migration. Blockade of β1 integrin-mediated adhesion abrogates the induction of TF following transmigration (Figure 4). This finding is consistent with previous work in our lab and others, detailing the ability of β1 integrin engagement to induce TF in monocytic THP-1 cells (Fan 1994, McGilvray 1997). However, TF upregulation has been demonstrated in response to stimulation of a number of other monocyte adhesion molecules, whether by anti-β2 integrin antibodies (McGilvray 1998), P-selectin (Celi 1994), or anti-Lewis S sialoglycan antibodies (Lo 1995). These results suggest that the actual contribution of a given adhesion molecule to TF stimulation on monocytes depends both on how they are recruited experimentally and on the presence or absence of additional co-stimulatory signals. Thus, while divalent antibody ligation of β2 integrins does not directly increase TF (Fan 1991, McGilvray 1998), it does prime for increased TF expression in response to TNFα or LPS (Fan 1991). In contrast, antibody crosslinking of β2 integrins increases the biologic consequence of TF expression, PCA, in human THP-1 cells and monocytes (McGilvray 1998), and engagement of β2 integrins during monocyte adhesion to vascular smooth muscle cells, CHO cells transfected with ICAM-1, or unstimulated, ICAM-1-expressing HUVEC has been reported to mediate the whole or a part of the subsequent increase in monocyte TF (Marx 1998, Collins 1995). Both soluble P-selectin and adhesion to CHO cells transfected to express P-selectin increase monocyte TF (Celi 1994); however, while crosslinking of the monocyte selectin ligand, CD15, does increase PCA, the increase in monocyte PCA following co-culture of monocytes with stimulated HUVEC expressing ICAM-1, VCAM-1 and E-selectin was only 30% attenuated by antibodies against E-selectin, but not by antibodies against ICAM-1, VCAM-1, or CD11/CD18 (Lo 1995). Taken together, these studies suggest that the contribution of individual adhesion molecules to adhesion-dependent monocyte activation varies considerably under different circumstances: for transendothelial migration, β1-family integrins appear to be crucial.

Adhesion to a primed endothelial layer during the process of transendothelial migration was sufficient to induce upregulation of TF (Figure 3). This finding may have implications for the design of clinical anti-migration strategies: it may be deleterious to block inflammatory cell infiltration if the upshot is the accumulation of activated monocytes in the vasculature. This caveat is particularly relevant in the setting of infectious insults, where the adhesion molecules used in transmigration vary with the particular infectious organism, and blockade of leukocyte transmigration can be deleterious (Garcia 1995, Sharar 1991).

Since multiple adhesion molecules have the potential, individually or collectively, to increase TF in the setting of migration and adhesion, it may be useful to study the process from a signaling perspec-
tive. Based on our previous studies, we postulated that the ERK and p38 MAP kinases might represent necessary signals for transmigration-dependent monocyte activation. We found that short-term (<30 minutes) adhesion of purified human monocytes to a cytokine-primed ECV304 layer leads to increased monocyte tyrosine phosphorylation, and in particular, to accumulation of the dually-phosphorylated, active ERK and p38 MAP kinase moieties (Figure 5). This finding is consistent with the fact that clustering of either β₁ or β₂ integrins leads to tyrosine phosphorylation and activation of the ERK MAP kinase (Lin 1995, McGilvray 1997, McGilvray 1998), and we have found that β₁ integrin clustering leads to p38 MAP kinase activation as well (ID McGilvray and OD Rotstein, unpublished observations). The induction of tyrosine phosphorylation signaling cascades following clustering and ligand engagement of surface adhesion molecules may be a universal leukocyte response: recent work has suggested a role for tyrosine phosphorylation in PECAM-1 signaling in monocytes (Elias 1998), while in neutrophils the crosslinking of L-selectin increased tyrosine phosphorylation and led to ERK MAP kinase activation (Waddell 1994). In addition, many chemotactic factors stimulate tyrosine phosphorylation in monocytic cells (Sozzani 1994, Han 1994). More specifically, we have found that 10⁻⁸ M FMLP stimulated tyrosine phosphorylation as well as ERK and p38 MAP kinase phosphorylation (by Western blot) and activation (by in vitro kinase assay, data not shown) in purified human monocytes. Thus, there is good reason to suppose that tyrosine phosphorylation and MAP kinase signaling are induced during leukocyte migration.

Tyrosine phosphorylation is recognized to be an important signal for monocyte migration and movement, although the roles of the ERK and p38 MAP kinases are less clear. For example, genistein inhibited MCP-1, MCP-2 and MCP-3 induced monocyte migration across a polycarbonate filter (Sozzani 1994). In our work, broad-spectrum tyrosine kinase inhibition with genistein and piceatannol markedly diminished monocyte transendothelial migration (Figure 6). Similarly, genistein blunted monocyte transmigration in the absence of FMLP and inhibited migration across a fibronectin-coated Transwell filter (data not shown). These data are consistent with a role for monocytic tyrosine phosphorylation in transmigration. On the other hand, selective ERK inhibition with PD98059 only blunted transendothelial migration, while selective p38 MAP kinase inhibition with SB203580 tended to increase migration (Figure 6); selective ERK inhibition also failed to attenuate monocyte migration in the absence of FMLP or across a fibronectin-coated filter (data not shown). Although monocytes and endothelial cells do communicate through adhesion (Lorenzon 1998), the fact that similar results were obtained in the presence or absence of an endothelial layer suggests that inhibition of monocyte tyrosine phosphorylation is sufficient to block transendothelial migration. Our data confirms the role of tyrosine phosphorylation signaling cascades in monocyte transmigration, but suggests that neither the ERK nor the p38 MAP kinases are
necessary for the process to occur.

The ERK and p38 MAP kinases are likely to play cell- and stimulus-roles in migratory events. Intriguingly, a recent study found that TNFα inhibited macrophage chemotaxis in a manner downstream of Cdc42, the GTPase upstream of the p38 MAP kinase cascade (Peppelenbusch 1999). In contrast, the spreading of alveolar rat macrophage NR8383 cells in response to oxidant stress is partially (40-60%) inhibited by ERK inhibition with PD98059 (25-75 μM), transfection with dominant negative ERK mutants, p38 MAP kinase inhibition with SB203580 (10 μM), or transfection with dominant negative p38 mutants (Ogura 1998). In neutrophils, the ERK MAP kinase pathway has been reported both to be important for chemotaxis in response to FMLP, IL-8, C5a, and LTB4 (Kuroki 1997), or to be independent of IL-8 induced chemotaxis (Knall 1997). Although present, p38 MAP kinase activity is not required for neutrophil chemotaxis in response to IL-8 (Knall 1997). Interestingly, in Rat1 fibroblasts, fibronectin-stimulated chemotaxis is inhibited by selective ERK inhibition, while directed migration in response to PDGF-BB is independent of ERK (Anand-Apte 1997). A recent study by Hansen et al found that the tyrosine phosphorylation cascades in response to PDGF have components which are inhibitory for chemotaxis (Hansen 1996), suggesting that downstream signaling pathways can either enhance or inhibit cell movement. Taken together these results suggest that the roles of the ERK and p38 MAP kinases in regulating cell movement are both cell- and stimulus-specific; our data suggests that neither is essential for monocyte transendothelial migration in response to FMLP.

Although not essential for monocyte transmigration, both the ERK and p38 MAP kinases are crucial for migration-induced cell activation. Both PD98059 and SB203580 abolished TF expression in a dose-dependent fashion after transmigration (Figure 7). These results are in keeping with our previous finding that the expression of TF following crosslinking of the β1 integrin VLA-4 on human monocytic THP-1 cells is dependent on ERK MAP kinase activation (McGilvray 1997), and suggest that the ERK and p38 MAP kinase family members coordinate cell activation. The fact that FMLP also induces ERK and p38 MAP kinase activation, but is not able to induce TF expression alone, suggests that the MAP kinase family members are necessary but not sufficient for monocyte activation. Together these results argue that MAP kinase activation is a necessary step in the progression to monocyte activation. Interestingly, IL-10 has been shown to inhibit the expression of TF when monocytes are exposed to cytokines (Ernoffson 1996), and may mediate its effects via inhibition of the MAP kinases (Geng 1994).

In summary, the work presented in this paper suggests that an extravascular inflammatory stimu-
lus has the potential to simultaneously promote extravascular fibrin deposition and intravascular thrombosis by modulating monocyte-endothelial cell interactions. We propose that the β1 family of integrins is of particular importance in migration-induced monocyte activation, that adhesion during migration is sufficient to stimulate TF expression, and that the MAP kinases are signaling intermediaries in the activation sequence. These results have important implications from the viewpoint of disease pathogenesis, and help to explain the intense accumulation of coagulation endproducts in sites of extravascular infection and inflammation. The recent finding that transendothelial migration may lead to monocyte differentiation into dendritic cells (Randolph 1998) is of particular interest in light of our findings, since the MAP kinases are known to be involved with cellular differentiation (Widmann 1999). The fact that the MAP kinases are also at a point in the tyrosine phosphorylation hierarchy which is involved in cellular activation may offer a novel means of influencing inflammatory cascades in vivo, by permitting monocyte migration to sites of infection but tempering their subsequent activation:
Chapter 5: Murine Hepatitis Virus Strain 3 induces the macrophage prothrombinase fgl-2 through p38 MAP Kinase activation

Summary: The clinical syndrome of acute liver failure produced by fulminant viral hepatitis can be reproduced in mice by infection with Murine Hepatitis Virus Strain 3 (MHV-3). Though it is clear that MHV-3-induced hepatitis depends upon macrophage activation and the expression of a specific prothrombinase, fgl-2, the signaling pathways involved in virally-stimulated cell activation are unclear. Since we had previously found that MHV-3 induces the tyrosine phosphorylation of cellular proteins, we investigated the roles of the Mitogen Activated Protein Kinase (MAPK) proteins. In a series of Western blot, immunoprecipitation and in vitro kinase assay studies we found that both the ERK and p38 MAPK proteins are tyrosine phosphorylated and activated following exposure of murine peritoneal exudative macrophages (PEM) to MHV-3. While p38 phosphorylation and activity are induced soon after MHV-3 exposure, peaking by 1-5 minutes, ERK phosphorylation and activity increase more gradually, peaking at 20-30 minutes and gradually fading thereafter. Interestingly, while selective p38 inhibition with SB203580 (1-20 μM) abolished the virally stimulated induction of fgl-2 mRNA, protein and functional activity, selective ERK inhibition with PD98059 (1-50 μM) limited fgl-2 functional activity but had little to no effect on fgl-2 mRNA or protein levels. Moreover, while inhibition of ERK had no effect on p38 activity, p38 inhibition consistently increased MHV-3 induced ERK activity. To ensure that these pathways were relevant in vivo, MHV-3 was injected intraperitoneally and PEM collected. Again, MHV-3 exposure led to increased p38 and ERK tyrosine phosphorylation. These data argue that MHV-3 induces tightly interconnected ERK and p38 MAPK cascades in the macrophage both in vitro and in vivo. Though the ERK and p38 MAPK proteins have discordant effects at the level of fgl-2 expression both converge at the level of its activity, suggesting that targeted MAPK inhibition may ultimately be useful in the modulation of viral hepatitis.

INTRODUCTION:

The mortality rate associated with fulminant hepatitis remains in excess of 25-45%, despite the use of liver transplantation as an acceptable form of therapy (Pappas 1995). Studies using a model of viral hepatitis induced by infection with Murine Hepatitis Virus Strain 3 (MHV-3)\(^4\) have provided sig-

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\(^4\) The abbreviations used are: Ab, antibody; ATF-2, activating transcription factor-2; DTT, dithiothreitol; ERK, extracellular signal-related kinase; FCS, fetal calf serum; IP, immunoprecipitation; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MBP, myelin basic protein; MEK, MAP kinase kinase; MHV-3, Murine Hepatitis Virus Strain-3; MOI, multiplicity of infection; pAb, polyclonal antibody; PAGE, polyacrylamide gel electrophoresis; PCA, procoagulant activity; PEM, peritoneal exudative macrophages; PFU, plaque forming units; PMSF, phenylmethylsul-
nificant insights into the mechanisms underlying the pathogenesis of this disease and have suggested novel approaches to therapy (Pappas 1995, Abecassis 1987). Fulminant hepatitis induced by this virus is characterized by the presence of sinusoidal thrombosis and associated hepatocellular necrosis (Levy 1983, Levy 1989, Li 1992). These findings occur concomitant with the expression of a virus-induced procoagulant molecule in the sinusoidal lining cells of the liver. This prothrombinase protein, encoded by the $fgl-2$ gene, has the ability to directly activate the coagulation cascade - an ability expressed as procoagulant activity, PCA, and measured by standard clotting assays (Fung 1991, Parr 1995). Accumulated evidence suggests that the virus-induced PCA plays a central role in the pathological changes observed in this disease. Following infection with MHV-3, hepatocellular necrosis is seen to occur in regions of sinusoidal fibrin deposition. where concomitant expression of the $fgl-2$ gene and its protein product is observed in the sinusoidal lining cells (Ding 1997). By contrast, other organs simultaneously infected with MHV-3 fail to express $fgl-2$ protein and remain uninjured (Ding 1997). Importantly, pretreatment with a neutralizing monoclonal antibody directed against MHV-3-induced PCA prevents sinusoidal fibrin deposition, hepatocellular necrosis and mortality in infected mice (Li 1992). Thus, there is good reason to conclude that $fgl-2$ upregulation is essential to the lethal hepatitis induced by MHV-3. These considerations become all the more relevant with the recent discovery of a human $fgl-2$ analogue (Ruegg 1995), which may contribute to inducible endothelial PCA (Sekiya 1994, Liu 1996).

MHV-3 infection of murine macrophages represents an excellent model to study the induction of $fgl-2$ for several reasons. First, the degree of hepatocellular necrosis following infection correlates well with the induction of macrophage PCA (Abecassis 1987, Li 1992, Pope 1995). Macrophages from susceptible mouse strains (Balb/cJ) infected with MHV-3 exhibit a marked increase in PCA, while those recovered from resistant mice (A/J) fail to do so (Chung 1994, Fingerote 1996). Moreover, the resistant A/J strains will develop both fulminant hepatitis and macrophage PCA following pretreatment with corticosteroids, which stabilize $fgl-2$ mRNA (Fingerote 1996). Second, administration of exogenous prostaglandin $E_2$ completely abrogates viral induction of macrophage PCA both in vitro and in vivo and prevents the development of fulminant hepatitis (Abecassis 1987, Chung 1991). Considered together, these findings suggest that macrophage PCA may serve both as a marker of disease and contribute to the pathogenesis of the process by virtue of the cells’ ability to sequester in the infected liver.

The cellular mechanisms underlying the induction of this protein in macrophages as well as in other cells are presently being defined. Detailed studies by Holmes and colleagues have characterized

*fonyl fluoride; WB, Western blot.*
the MHV receptor as a 110-120-kDa glycoprotein in the carcinoembryonic antigen family of glycoproteins (Dveksler 1993, Nedellec 1994). Expression of this receptor in hamster cell lines confers susceptibility to MHV infection (Nedellec 1994). Subsequent events in the signaling pathway(s) leading to expression of fgl-2 in MHV-infected macrophages have not been clearly elucidated. Our group previously demonstrated that incubation of macrophages with MHV-3 caused the rapid accumulation of tyrosine phosphoproteins over a range of molecular masses from 33 to 91 kD. In addition, the nonspecific tyrosine kinase inhibitors genistein, herblmycin and tyrphostin AG51 inhibited virus-induced PTA, both at the functional level and at the level of gene expression (Dackiw 1995). Together, these findings suggest that tyrosine kinase activation in response to MHV-3 stimulation is an important component of the signaling cascade leading to fgl-2 expression. In this regard, a clustering of tyrosine phosphorylation around the 38-44 kDa region was suggestive of activation of members of the MAP Kinase family, specifically p38/CSBP/Reactivating Kinase and Extracellular Signal-Related Kinase (ERK)-1 and ERK2. These proteins, which undergo dual phosphorylation on tyrosine and threonine residues during their activation, are known to be involved in the response of cells to a variety of infectious and inflammatory stimuli (McGilvray 1997, Liu 1994, Liu 1994, Geng 1994, Durden 1995, Han 1994, Han 1997). We therefore hypothesized a role for these proteins in MHV-induced macrophage fgl-2 expression. In the present studies, we demonstrate that MHV-3 induces the phosphorylation and activation of both ERK and p38. However, while both of these kinases are activated, the use of specific inhibitors clearly demonstrate that p38, but not ERK, is integral to the induction of fgl-2 mRNA and its protein product.

EXPERIMENTAL PROCEDURES:

Animals, Buffers and Reagents:

Pathogen-free female Swiss-Webster mice aged 6-7 weeks were obtained from Taconic, and were chow fed and allowed to acclimatize for 1 week prior to experiments. 3% thioglycollate (Gibco) was prepared as per the manufacturer’s instructions. Endotoxin-free RPMI and HBSS were purchased from Gibco, fetal calf serum (FCS) from Hyclone. The p38 selective inhibitor SB203580 was the kind gift of Dr. J.C. Lee (Smith Kline Beecham), and was prepared in DMSO to a 20 mM solution. The selective MEK-1 inhibitor PD98059 (Research Biochemicals International) was prepared in DMSO to a 10 mM stock solution.

Cell and MHV-3 Preparation:

Peritoneal exudative macrophages (PEM) were harvested in ice-cold HBSS 5-6 days after the ip injection of 2 cc sterile thioglycollate. The cells were washed twice in cold HBSS and resuspended in RPMI/2%FCS/L-gln at 1-10 x 10⁶ cells/ml. This procedure consistently yields a >96% macrophage cell
population by Wright’s stain, with >97% viability by Trypan blue exclusion (26). Cells were incubated for 60 minutes at 37°C, 5% CO₂ prior to experimentation. MHV-3 was obtained and purified as previously described (Dackiw 1995). Virus was grown to titers of 10-50 x 10⁶ PFU/ml RPMI on confluent 17CL cells with strictly aseptic technique. For studies using dead virus, MHV-3 preparations were irradiated under UV light for 20 minutes (UVG-11 ultraviolet lamp; Ultra-Violet Products Inc.).

**Cell Activation:**

Cells were incubated at 37°C in 5% CO₂ in the presence or absence of MHV-3 for times ranging from 1 minute to 6 hours. Unless otherwise indicated, a multiplicity of infection (MOI) of 5:1 was employed. In some studies, PEM were preincubated in the presence or absence of 1-50 μM PD98059 or 1-20 μM SB203580 for 45 minutes at 37°C, 5% CO₂. Control cells were exposed to vehicle, 0.1% DMSO, during the preincubation period. At the end of the incubation period, reactions were stopped by placing the cells on ice.

**Measurement of Procoagulant Activity (PCA):**

PEM were pelleted 6 hours after exposure to viral particles, and resuspended at 1x10⁶ cells/ml RPMI. Following a single freeze-thaw cycle at -70°C, PCA was measured by single stage recalcification clotting assay. PCA was expressed as mU/10⁶ cells by comparison to rabbit brain thromboplastin as previously described (Dackiw 1995, Brisseau 1995). Previous work has established that MHV-3-induced PCA is entirely dependent on the induction of the fgl-2 prothrombinase (Li 1992, Parr 1995, Fung 1991, Pope 1995); for details, see RESULTS.

**Western Blot Analysis:**

At various times after virion exposure, PEM were pelleted, and lysed in ice-cold cell lysis buffer. Whole cell lysates were prepared with 2X Laemmli/0.1 M dithiothreitol (DTT) buffer followed by immediate boiling at 100°C for 5 minutes. Cytosolic fractions were isolated with 1% Triton X100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 2 mM sodium orthovanadate, 10 μg/ml leupeptin, 50 mM NaF, 5 mM EDTA, 1 mM EGTA, and 1 mM PMSF. Postnuclear supernatants were collected following centrifugation at 10000 x g for 5 min and diluted with 2X Laemmli buffer/0.1 M dithiothreitol (DTT). Lysates prepared from 100 000 cells were separated on 12.5% SDS-PAGE and transferred to polyvinilidene difluoride membrane (Mobilon). Blots were then probed with polyclonal rabbit anti-phosphotyrosine (Transduction Laboratories), anti-phosphoERK or anti-phospho-p38 (New England Biolabs) antibody, or rabbit anti-fgl-2 antibody (Dr. G. Levy, University of Toronto). Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham), blots were developed using an ECL-based system (Amersham).

**Immunoprecipitations and Kinase Assays:**
ERK-2 and p38 Immunoprecipitations - Cells (3x10^6) were lysed as above and the postnuclear supernatants precleared with Protein G-Sepharose (Pharmacia Biotech Inc.). Cellular proteins were immunocomplexed using rabbit polyclonal anti-ERK-2 or anti-p38 antibody (Santa Cruz Biotechnologies) for 1 h at 4°C. Protein G-Sepharose was added and incubated at 4°C for 1 h. The resulting immune complexes were washed five times with cold phosphate-buffered saline/0.01% Tween 20, and then separated from beads by 2x Laemmli buffer, 0.1 M DTT and boiling at 100°C for 5 min. Beads were then sedimented by ultracentrifugation and the supernatant collected for Western blot analysis.

ERK-2 and p38 Kinase Assays - ERK2 or p38 immunocomplexes were washed with 5 changes of cold phosphate-buffered saline/0.01% Tween 20, and then incubated for 30 min at 30°C with 20 μg of ultrapure myelin basic protein (MBP, Upstate Biotechnology, Inc.) or 5 μg of recombinant Activating Transcription Factor-2 (ATF-2, Santa Cruz Biotechnologies), respectively, in kinase assay buffer composed of 0.4 mM cold and 0.4 mM [γ-32P]ATP (DuPont NEN), 50 mM Tris-HCl (pH 7.4), and 10 mM MgCl2. Reactions were stopped with the addition of 2x Laemmli buffer, 0.1 M DTT and boiling at 100°C for 5 min. Equal volumes were loaded and run on 10% SDS-PAGE. Autoradiograms developed by exposure of the dried gels to Kodak BIOMAX MR film.

RNA Extraction and Northern Blot Analysis:
Total RNA from 10 x 10^6 PEM was obtained using the guanidium-isothiocyanate method (Chomczynski 1987). RNA was denatured, electrophoresed through a 1.2% formaldehyde-agarose gel and transferred to nylon membrane. Hybridization was carried out using a 32P-labeled, random primed murine fgl-2 cDNA probe, after which the blots were stripped and probed for 18s RNA or GAPDH mRNA to ensure equal loading.

Immunofluorescence:
PEM were allowed to adhere to autoclaved glass coverslips for 1 hour at 37°C, 5% CO2, incubated in the presence or absence of 50 μM PD98059 and then infected with MHV-3 at a MOI of 1. Following a 6 hour incubation with the virus, the cells were fixed in fresh 4% paraformaldehyde (Sigma) for 20 minutes at room temperature, quenched for 10 minutes with 100mM glycine, and blocked overnight at 4°C with 5% normal goat serum (Sigma) in PBS (pH 7.4). Rabbit anti-fgl-2 primary antibody was diluted 1:100 in PBS/1% normal goat serum and incubated with fixed PEM for 2 hours at room temperature. Cells were washed with five changes of PBS, and then incubated with a 1:500 dilution of Cy3-conjugated goat anti-rabbit pAb (Jackson) for 1 hour at room temperature. After five washes in PBS coverslips were mounted using SlowFade antifade reagents (Molecular Probes) and evaluated by confocal microscopy using a Bio-Rad MRC 600 confocal microscope with Comos 7.0 software.

In vivo PEM Stimulation:
At day 5 following ip injection of thioglycollate, mice were infected with \(50 \times 10^6\) virions in 500 \(\mu\)l RPMI/2%FCS/L-Gln at 37°C by ip injection. Control animals were injected with 500 \(\mu\)l medium alone, pre-warmed to 37°C. At 10, 20 and 30 minutes after injection of the virus, mice were euthanized by cervical dislocation and PEM collected by peritoneal lavage using 10 cc of ice-cold HBSS. Cells were immediately placed on ice, sedimented and prepared for Western blot analysis as described above. Protein amounts were standardized by Bradford protein assay (Bio-Rad) prior to gel loading.

**Statistical Analysis:**

Continuous data is represented as the mean and standard error of the indicated number of experiments. Where representative studies are shown, these are indicative of at least three equivalent studies performed independently. Statistical comparisons were made using one-way ANOVA with post hoc Tukey.

**RESULTS:**

**Induction of ERK tyrosine phosphorylation and activity by MHV-3**

Two approaches were used to determine whether the ERK MAP Kinase was targeted by the response to MHV-3. Figure 1A shows the time course of the appearance of tyrosine-phosphorylated p44 ERK1 and p42 ERK2 in response to MHV-3 stimulation. MHV-3 induced a rise in phospho-ERK as early as five minutes, reaching a peak at 20-30 min and fading over the ensuing 30 min. As confirmation of this effect, ERK2 was immunoprecipitated and evaluated for tyrosine phosphorylated residues. As shown in Figure 1B, tyrosine phosphorylation of ERK2 peaked at 20-30 minutes after exposure to MHV-3, and persisted through 45 minutes. Having shown the tyrosine phosphorylation of ERK2, *in vitro* kinase assays were performed to evaluate its activation (Figure 1C). MHV induced ERK2 activation with a time course consistent with its pattern of tyrosine phosphorylation.

**Induction of p38 tyrosine phosphorylation and activity by MHV-3**

MHV-3 also induced rapid tyrosine phosphorylation of p38. Figures 2A and 2B illustrate the time course of phosphorylation of p38 using the phosphospecific anti-p38 antibody and immunoprecipitation, respectively. It should be noted that in our work and that of others, the p38 MAPK migrates at 42 kDa (Krump 1997). In contrast to the activation of the ERK pathway, MHV-3 induces a much faster and briefer tyrosine phosphorylation of p38. Phosphorylation was seen as early as 1 minute, generally peaking by 1-5 minutes, and fading over the next 10-20 minutes. The time course of p38 tyrosine phosphorylation was reflected directly in p38 activity, as revealed by *in vitro* kinase assay (Figure 2C). Considered together with the results in Figure 1, these studies demonstrate that MHV-3 induces both ERK and p38 activation, albeit with markedly different time courses.
Figure 1: Induction of ERK tyrosine phosphorylation and activity by MHV-3

Following activation with MHV-3, cytosolic PEM proteins were prepared for Western blot analysis or immunoprecipitation of the p42 ERK2 as described in Experimental Procedures. A. Western blot (WB) staining PEM lysates with pAb specific to the tyrosine phosphorylated form of ERK (New England Bioloabs). Note the bands of tyrosine phosphorylated ERK migrating at 42 and 44 kDa and corresponding to ERK2 and ERK1, respectively. B. Immunoprecipitated (IP) ERK2 was probed for tyrosine phosphorylated residues using polyclonal anti-phosphotyrosine pAb. To control for loading, the blot was stripped and probed for ERK2 protein, as shown in the lower panel. C. Immunoprecipitated ERK2 was allowed to phosphorylate ultra-pure MBP (UBI) for 30 minutes at 30°C, using 32p-ATP. A typical autoradiogram of the 20kDa band is shown.
Figure 2: Induction of p38 tyrosine phosphorylation and activity by MHV-3

PEM were activated by treatment with MHV-3, lysed at the indicated times and prepared for Western blot analysis or p38 MAPK immunoprecipitation.
A. PEM lysates were probed with an antibody specific to the tyrosine phosphorylated form of the p38 MAPK (New England Biolabs). Note the early induction of phosphorylated p38, in marked contrast to the time course of ERK tyrosine phosphorylation.
B. Immunoprecipitated p38 was probed with polyclonal anti-phosphotyrosine antibody. The lower panel is the same blot, stripped and probed for p38 protein to control for loading.
C. Immunoprecipitated p38 was allowed to phosphorylate purified ATF2 (SCB) for 30 minutes at 37°C, using 32P-ATP. A typical autoradiogram of the 60kDa band is shown.
Although endotoxin (LPS) has been shown to activate both ERK and p38 in cells of the monocyte/macrophage lineage (21, 24), it is very unlikely that LPS contamination contributed to our results for the following reasons. All media and culture materials were endotoxin-free, both by commercial testing and Limulus assay, and strict attention was paid to sterile technique. Moreover, endotoxin induces a very different pattern of ERK and p38 activation in PEM: a 1 μg/ml dose of E. coli 0111:B4 LPS leads to a more profound tyrosine phosphorylation of the ERK1 and ERK2 proteins that persists through 60 minutes and beyond, and induces p38 tyrosine phosphorylation that peaks at 20-30 minutes and persists to 60 minutes (data not shown). Furthermore, pretreatment of cells with 50 μg/ml of LPS-complexing polymyxin B greatly attenuated LPS-induced PEM activation as assessed by PCA, but had no effect on MHV-3-dependent PCA (data not shown). Finally, MHV-3 preparations induced fgl-2 expression, while LPS does not. Taken together, these results argue that our findings are not due to LPS contamination.

Inhibition of Virus-Induced ERK and p38 activation using PD98059 and SB203580:

Two compounds have recently been described which act as selective inhibitors of the ERK and p38 pathways. PD98059 selectively inhibits MEK-1, the tyrosine kinase immediately upstream of ERK (29, 30), while the bicyclic imidazole SB203580 directly inhibits p38 kinase activity (Cuenda 1995, Kumar 1997). Prior to evaluating the role of these MAP kinases in virus-induced fgl-2 expression, initial studies were performed to determine the effect of these inhibitors on activation of ERK2 and p38 following MHV-3 stimulation. Pretreatment of cells with PD98059 caused a dose-dependent decrease in ERK2 activity, with complete inhibition of ERK2 activity generally achieved at a 10-50 μM dose (Figure 3A), though most consistently at 50 μM. Confirming its selectivity, PD98059 failed to inhibit the MHV-3 induced p38 activation (Figure 3B, compare lane 4 to lane 6). Pretreatment of PEM with 10 μM SB203580 markedly attenuated the activation of p38 following treatment with MHV-3 (Figure 3B). As shown in Figure 3C, SB20380 did not cross-inhibit ERK2 activation and in fact, caused a consistent augmentation. Neither inhibitor caused cellular toxicity, as evidenced by >95% trypan blue exclusion after 6 and 24 hours of incubation.

Induction of fgl-2 by MHV-3: differential roles of p38 and ERK

Having defined the ability of the two agents to inhibit their respective kinase activities following MHV-3 stimulation, studies were performed to evaluate their effect on fgl-2 expression. Figure 4A is a representative Western blot examining the effect of PD98059 and SB203580 on fgl-2 protein in response to MHV-3. At concentrations shown to completely inhibit ERK2 kinase activation, PD98059 had little
Figure 3: Selective inhibition of the ERK and p38 pathways: PD98059 and SB203580

A. In a series of ERK2 in vitro kinase assays, preincubation of PEM with increasing doses of PD98059 abrogated the MHV-3 induction of ERK2 activity. A typical autoradiogram of the 20kDa $^{32}$P-MBP band is presented; all points are taken at 20 minutes after treatment with MHV-3.

B. PEM pre-incubated in the presence or absence of PD98059 or SB203580 were treated with MHV-3 as before, and in vitro kinase assays performed on immunoprecipitated p38 MAPK. Note that while a 10 µM dose of SB203580 greatly decreased p38 activity, a similar dose of PD98059 had no effect. A typical autoradiogram of the 60kDa $^{32}$P-ATF2 band is shown.

C. Following pretreatment with 50 µM PD98059 or 20 µM SB203580, PEM were exposed to MHV-3 for 20 minutes, after which ERK2 protein was immunoprecipitated and an in vitro kinase assay performed. Note that while ERK2 activity was effectively eliminated by PD98059, it was consistently increased by SB203580. Similar results were seen by in vitro ERK2 kinase assay (data not shown).
Figure 4: Abrogation of prothrombinase protein expression by p38 MAPK inhibition

PEM preincubated in the presence or absence of PD98059 or SB20380 were exposed to MHV-3 for 4 hours, lysed and prepared for Western blot analysis with polyclonal anti-fgl-2 Ab. Note the large induction of the fgl-2 prothrombinase that followed treatment with MHV-3. Although 50 and 100 μM doses of PD98059 only minimally blunted the increase in prothrombinase expression, SB 203580 (20 μM) effectively eliminated it.
inhibitory effect on MHV-induced fgl-2 protein levels. By contrast, SB203580 (20 μM) caused a marked attenuation of fgl-2 protein expression. Consistent with the effect on fgl-2 protein, PD98058 failed to reduce the MHV-stimulated increase in fgl-2 mRNA levels, while SB203580 caused almost complete inhibition (Figure 5 presents results obtained with a 20 μM dose of PD98059; doses up to 50 μM failed to inhibit fgl-2 mRNA). These data therefore suggest that while MHV-3 activates both the ERK and p38 pathways, it appears that only the latter is required for fgl-2 expression.

**Effect of p38 and ERK inhibition on MHV-induced fgl-2 activity:**

The effect of the specific MAP kinase inhibitors on functional PCA was evaluated. Previous work from our group has established that MHV-3-induced PCA is entirely dependent on the fgl-2 prothrombinase, as distinct from the other major macrophage procoagulant, tissue factor. In brief, MHV-3-induced PCA is dependent on Factor II but independent of Factors VII and X, indicative of prothrombinase activity rather than tissue factor or Factor X activator (Pope 1995). Monoclonal antibodies directed against the MHV-3-induced prothrombinase, which do not cross-react with tissue factor, completely abolish PCA following infection of macrophages with MHV-3 both in vitro and in vivo (Li 1992, Fung 1991). Finally, MHV-3 infection of macrophages clearly leads to fgl-2 mRNA and protein expression, and the transient expression of this protein in RAW 264.7 cells induces PCA and prothrombin cleavage activity (Parr 1995, Fung 1991, Pope 1995). Taken together these studies demonstrate that MHV-3 infection of murine macrophages stimulates the expression of the fgl-2 prothrombinase, which in turn is responsible for virally-induced PCA.

As shown in Figure 6, SB203580 caused a dose-dependent inhibition of the MHV-3-induced PCA at concentrations shown to reduce expression of fgl-2. Interestingly, PD98059 at 10 and 50 μM caused a 40% and 80% reduction, respectively, in PCA compared to MHV-3 alone. Since these concentrations were able to inhibit MHV-3-stimulated ERK2 activation, but had no effect on fgl-2 expression, these findings suggest either a posttranscriptional effect of ERK2 activation on functional fgl-2 activity, or an effect on an as yet unknown fgl-2 cofactor.

**Effect of ERK Inhibition on fgl-2 Protein Localization:**

Recent studies have suggested that the ERK MAP kinase proteins can be associated with cytoskeletal elements such as microtubules, and phosphorylate regulatory proteins such as dynamin, suggesting that they may play some role in intracellular transport (Morishima-Kawashima 1996, Earnest 1996, Cole 1995). We postulated that one mechanism through which ERK inhibition could be affecting fgl-2 activity
Figure 5: *fgl-2* mRNA expression is selectively abrogated by inhibition of p38 MAPK

Following pretreatment with PD98059 (20 μM) or SB203580 (20 μM), PEM were exposed to MHV-3 and incubated for 4 hours at 37°C/5% CO₂. The mRNA from 10 x 10⁶ cells was isolated, separated and probed for FGL-2 as described in Experimental Procedures. Note that while PD98059 had little to no effect on MHV-3 induced FGL-2 mRNA expression, the increase was blocked by selective p38 MAPK inhibition with SB203580. A typical Northern blot is shown, and is representative of results obtained in at least 4 independent experiments. Note that doses of PD98059 up to 50 μM had no effect on *fgl-2* mRNA expression (data not shown).
Figure 6: Effect of ERK and p38 MAP Kinase inhibition on fgf-2 activity:
6 hours after exposure to MHV-3, PEM were washed, re-suspended at 10^6 cells/ml RPMI, and freeze-thawed at -70°C. Fgf-2 activity, manifested as PCA, was determined by single stage recalcification assay. Both PD98059 and SB203580 were able to dose-dependently inhibit PCA. Data=mean±SEM, n≥3/group. Stats: ANOVA with post hoc Tukey, ***p<0.001, *p<0.05 vs control.
is through inhibition of its transport to its biologically-active location in the plasma membrane. To address this issue PEM were infected with MHV-3 and then assessed for fgl-2 localization using immunofluorescence. As demonstrated in Figure 7, PEM pretreatment with a 50 mM dose of the ERK-selective PD98059 had no gross effect either the amount or localization of the fgl-2 protein. When considered together with the studies outlined above, these results suggest that the ERK MAP kinase is not essential for either the expression or the intracellular transport of fgl-2.

**Effect of UV Irradiation on MHV-3 Induced PCA and p38 Activation:**

Having determined that p38 MAPK activation is essential for fgl-2 synthesis, we performed studies using UV-irradiated virus in an effort to dissect whether this activation is sufficient for macrophage prothrombinase expression. As shown in Figure 8A, UV irradiation eliminated the ability of MHV-3 to stimulate functional PCA. This data is consistent with the finding that UV-irradiated MHV-3 does not induce fgl-2 mRNA (Dr. G. Levy, unpublished observation). However, the induction of phosphorylated p38 MAPK was unchanged (Figure 8B). This data suggests that p38 MAPK activation is necessary but not sufficient for fgl-2 synthesis.

**In vivo p38 and ERK tyrosine phosphorylation in murine PEM by MHV-3:**

To determine whether MHV-3 was able to stimulate ERK and p38 phosphorylation in vivo, animals were inoculated ip with MHV-3 or medium vehicle at 5 days after PEM elicitation with thioglycollate. At various times following MHV-3 injection, cells were harvested from animals and subjected to Western blot analysis. Compared to cells recovered from mice injected with medium alone, cells from MHV-3-treated animals exhibited a significant increase in the tyrosine phosphorylated forms of p38 and ERK. (Figures 9A and 9B, respectively). The lower panel in each figure confirms equivalent loading of the indicated protein among lanes.

**DISCUSSION:**

The present data provide a detailed assessment of the cellular mechanisms by which a pathogenic virus, MHV-3, causes the activation of physiologic intracellular signaling cascades and leads to the production of a biologically active protein responsible for disease. Expression of the prothrombinase encoded by the fgl-2 gene is central to the pathogenesis of hepatitis caused by MHV-3 (Li 1992). A previous report from our group demonstrated that induction of tyrosine phosphorylation by MHV-3 was necessary for macrophage expression of this molecule (Dackiw 1995). In the present studies, components of the
Figure 7: Effect of ERK Inhibition on fgl-2 Prothrombinase Localization

PEM adherent to glass coverslips were infected with MHV-3 (MOI of 1), incubated for 6 hours at 37°C/5% CO₂, then fixed and prepared for immunofluorescence using polyclonal anti-fgl-2 antibody. After counterstaining with Cy.3-labelled goat anti-rabbit secondary antibody, cells were visualised with a Bio-Rad MRC 600 confocal microscope. The following conditions were tested:

a. control PEM - the open-faced arrows indicate cell positions;
b. PEM infected with MHV-3;
c. PEM pretreated with a 50μM dose of PD98059, then infected with MHV-3;
d. control PEM treated with a 50μM dose of PD98059.
Figure 8: UV Irradiation of MHV-3
MHV-3 was killed by 20 minutes of UV irradiation as described in Experimental Procedures.
A. Comparison of live and UV-irradiated virus in their ability to stimulate 6-hour PCA, using a MOI of 1:1. Points are taken in duplicate, and are representative of results obtained on three separate experiments. * p<0.05 vs control (ANOVA with post hoc Tukey)
B. Following incubation in the presence or absence of live or UV-irradiated MHV-3, PEM were lysed and prepared for Western blot analysis with anti-phospho-p38 MAPK antibody. Both live and irradiated virus were able to induce p38 phosphorylation.
Figure 9: Tyrosine phosphorylation of the p38 and ERK MAP Kinases in vivo

Five days after intraperitoneal injection of sterile 3% thioglycollate, 100 μl of RPMI/2% FCS or 50 x 10⁶ PFU MHV-3 in 100 μl RPMI/2% FCS was instilled ip. Mice were euthanized by cervical dislocation 10, 20 and 30 minutes after virus injection, and PEM rapidly harvested and prepared for Western blot analysis as described in Experimental Procedures.

A. Cell lysates probed with pAb specific to the phosphorylated form of p38. The same blot, stripped and probed for p38 protein, is shown in the lower panel to control for loading.

B. Cell lysates probed with pAb specific to phosphorylated ERK. Note the induction of both p42 ERK2 and p44 ERK1 protein. In the lower panel the same blot has been stripped and probed for ERK2 protein to control for loading.
signaling cascade leading to fgl-2 expression are further defined. Specifically, we demonstrate that MHV-3 is able to rapidly induce the tyrosine phosphorylation and activation of two members of the MAP Kinase family, p38 and ERK. Using specific inhibitors of both pathways, p38 activation is shown to be required for induction of fgl-2 gene expression and elaboration of its protein product. Despite being activated by MHV-3, ERK does not appear to be essential for fgl-2 gene induction, although it may participate in the post-translational modification of the protein, or alternatively in the action of a co-factor required for its biological activity. When considered in conjunction with the in vivo data demonstrating tyrosine phosphorylation of both these kinases in peritoneal macrophages following intraperitoneal injection of MHV-3, these findings strongly support the idea that these pathways are critical in the development of hepatitis following MHV-3 infection.

The signaling pathways upstream of the MHV-3-induced ERK and p38 activation remain to be determined. The rapid activation of ERK and p38 activity is consistent with the conclusion that viral replication per se is not required for the early signaling events which ultimately contribute to fgl-2 expression. This is further supported by the observation that UV-irradiated MHV-3 similarly induces p38 phosphorylation. The receptor for MHV-3 is a 110 kDa glycoprotein, which is a member of the murine carcinoembryonic antigen family (Dveksler 1993). Its short intracellular domain lacking tyrosine residues precludes its ability to function as a receptor tyrosine kinase or act as a binding site for SH2 domains of non-receptor tyrosine kinases. However, variants of the receptor with a long cytoplasmic tail containing tyrosine residues - derived by alternative mRNA splicing - have been reported to serve as MHV receptors (Dveksler 1993). Phosphorylation of one of these CEA-related glycoproteins may have participated directly in the signaling pathways (Culic 1992, Afar 1992). In this regard, members of the CEA-related glycoprotein family have been reported to associate with molecules which could feed into downstream MAP kinases. Tyrosine phosphorylated biliary glycoprotein can reversibly associate with the protein tyrosine phosphatase SHP-1 (Beauchemin 1997), while CD66a, a human homologue, can be tyrosine phosphorylated and associate with pp60c-src, leading to increased c-src activity in vitro (Skubitz 1993, Brummer 1995). Activated c-src is a classic inducer of the Ras and Raf proteins, both potentially upstream of ERK (Stokoe 1997, Erpel 1995), and it is clear that tyrosine phosphatase activity can regulate both the ERK and p38 MAP Kinases (Chu 1996, Sun 1993, Muda 1996, Cook 1997, Dent 1996). An analogous situation may be found in the HIV tyrosine phosphorylation response, in which gp120 binds to the T-cell CD4 antigen, leading to the release of the CD4-associated, src-family tyrosine kinase p56 Lck and the subsequent phosphorylation of the MAP kinase kinase kinase Raf-1 (Popik 1996). Consistent with the notion of the role of c-src in the MHV-3 ERK signal, we have found that PP-1, a src-family inhibitor
(Hanke 1996), inhibits MHV-3 dependent ERK tyrosine phosphorylation (data not shown). Alternatively, it is possible that the extracellular domains of MHV receptor lacking a cytoplasmic tail might associate with a transmembrane receptor capable of initiating an intracellular signal, as has been reported for the IL-6 receptor (Hirano 1998). It is interesting to speculate that the different responses to MHV-3 in susceptible and non-susceptible mice might be partially due to differences in receptor-mediated signaling, since Bgp variants derived from alternative mRNA splicing are expressed differently in susceptible and non-susceptible mouse strains (Nedellec 1994).

Inhibition of fgl-2 protein by selective p38 inhibition with SB203580 appears to be at the level of transcription. Although the decrease in mRNA levels may be due to decreased transcription rates, other groups have also described a role for p38 MAPK in the maintenance of mRNA transcript stability (Sirrenko 1997). This finding is consistent with the presence of AUUU-rich regions in the 3'-region of the fgl-2 mRNA transcript (Dr. G.A. Levy, unpublished observations). By contrast, ERK inhibition did not affect fgl-2 mRNA or protein expression but abolished its activity as reflected in the PCA clotting assay. Control studies testing the effect of PD98059 on PCA assay itself indicated that this inhibition was not related to a direct effect of the compound on clotting per se. As demonstrated in Figure 7, selective ERK inhibition did not appear to affect fgl-2 localization within macrophages. Rather, the inhibition appears to be either posttranslational or related to the synthesis of a necessary cofactor. In this regard, our findings are consistent with previous observations made during evaluation of the effect of Prostaglandin E2 on fgl-2 function. PgE2 inhibited fgl-2-dependent PCA and liver necrosis (Abecassis 1987), but had no effect on fgl-2 protein levels (Chung 1991). A recent study found that PgE2 reduced the induction of ERK activity by PDGF and EGF in rat mesangial cells (Li 1995), suggesting that the effect of PgE2 on MHV-3 stimulated ERK activity may, in part, be due to inhibition of the ERK pathway. Consistent with increased cyclic AMP leading to decreased ERK activation, adenosine simultaneously increased cyclic AMP and inhibited the tyrosine phosphorylation of ERK-2 in human cultured mast cells following Fc epsilon RI receptor crosslinking (Suzuki 1998). The role of the ERK MAP kinase in regulating fgl-2 activity deserves further investigation. The fact that ERK inhibition does not affect fgl-2 protein migration by PAGE argues against protein cleavage as a post-translational modification; however, fgl-2 phosphorylation - should it occur - could be under the influence of ERK. It is possible that a co-factor protein is necessary for fgl-2 function, in a manner analogous to the recently described Effector Cell Protease Receptor-1 protein and the classical Factor Xa/Va prothrombinase complex (Bouchard 1997). The expression or activity of such a protein could be directly influenced by ERK activity. In any case, our data suggest that the ERK and p38 MAPK pathways act in a coordinated fashion to regulate MHV-3-induced pro-
Further evidence for the close interaction of the ERK and p38 MAPK pathways comes from the fact that selective p38 inhibition with SB203580 consistently increased ERK tyrosine phosphorylation and activity in response to MHV-3. This finding suggests an inhibitory feedback of ERK by p38 MAPK activation. These data are consistent with the recent finding of a similar p38-mediated inhibition of ERK in mast cells stimulated by IgE aggregation (Zhang 1997). Although the mechanism for such crosstalk is unclear, feedback modulation of ERK activity has been previously described to proceed via the mSOS adapter protein (Cherniack 1994), ERK-dependent expression or modulation of phosphatases (Peraldi 1994, Widmann 1999), inactivation of the MAP Kinase kinase kinase Raf-1 by a GTP-sensitive tyrosine phosphatase (Dent 1996), and possibly cPLA₂-mediated signaling events (Zhang 1997). The functional significance of this crosstalk between MAPK pathways following MHV-3 stimulation is unclear. Further elucidation of the significance of ERK activation in response to MHV-3 may clarify this issue.

Our results have important implications both for viral hepatitis and for virally-induced inflammatory responses. Several viruses have been demonstrated to stimulate tyrosine phosphorylation and through this contribute to aspects of the acute inflammatory response as well as virus-induced cellular transformation. For example, both the increased TNFα expression by astrocytomas exposed to Newcastle Disease Virus and the increased c-myc and c-jun expression which follows Simian Virus 40 binding to growth arrested cells occur in a tyrosine kinase dependent fashion (Fisher 1994, Dangoria 1996). Hepatitis B has been shown to activate MAP Kinase cascades via the virus-specific HBx protein (Benn 1996, Doria 1995, Benn 1994), and Human Immunodeficiency Virus infection of lymphocytic cells leads to the prompt activation of both the p38 MAP kinase and the MAP kinase kinase Raf-1 (Popik 1996, Cohen 1997). Although MAP kinase cascades have been suggested to be important for virally-induced cell cycle events (Benn 1995), their role in virally-induced inflammatory events remains unclear. The finding that the p38 MAP kinase in particular is essential for the viral induction of the fgl-2 prothrombinase, an important inflammatory mediator in viral hepatitis, adds to a growing body of evidence suggesting that this kinase can play an integral role in the inflammatory response elicited by a number of stimuli (Han 1994, Han 1997, Lee 1996, Saklatvala 1996, Ridley 1997, Pouliot 1997).

The relative roles of the ERK and p38 MAP Kinase pathways in the pathogenesis of viral hepatitis in vivo remains to be determined. We have demonstrated that both ERK and p38 are tyrosine phosphory-
lated *in vivo* in PEM exposed to MHV-3 by intraperitoneal injection. Since it has been previously demonstrated that inhibition of *fgl*-2 PCA by specific monoclonal antibody greatly attenuates MHV-3 induced liver necrosis and mortality (Li 1992), the data suggest that selective inhibition of p38 MAPK activation, and possibly of ERK, might be of benefit *in vivo*. In favor of this notion is recent work using the Tyrphostin family of nonspecific tyrosine kinase inhibitors, resulting in improved survival and organ function in a lethal endotoxemia model in mice and an intra-abdominal sepsis model in dogs (Novodgrodsky 1994, Vanichkin 1996, Sevransky 1997). Preliminary work in our laboratory has suggested that pretreatment of mice with Tyrphostin AG126 markedly inhibits the increase in hepatic *fgl*-2 mRNA following infection with MHV-3. Future studies to define the effect of nonspecific and selective inhibition of tyrosine phosphorylation signaling routes in this murine model of fulminant viral hepatitis may ultimately suggest novel treatment strategies for the clinical disease.
Section IX: Conclusions:

The preceding studies outline the roles of the ERK and p38 MAPK in the monocyte response to adhesive and viral stimuli. Although the induction of monocyte/macrophage procoagulant activity has broad implications for many local and systemic inflammatory states, we have integrated our findings within the context of viral fulminant hepatic failure. This immunopathogenesis of this disease may be conceptualized in two stages: first, the migration of bloodborne monocytes to the inflamed liver parenchyma, and second, the activation of cells of the monocyte/macrophage lineage by virally-induced stimuli. One of the more important activating stimuli infiltrating leukocytes may encounter is direct infection by the inciting virus. The elaboration of procoagulant activity focuses and upregulates the inflammatory response, leading to local tissue damage. Dealing with these stages in sequence, our work can be summarized on the basis of our original hypotheses:

**Hypothesis 1.** Integrin clustering on the surface of human monocytic cells results in ERK and p38 MAPK activation, which are necessary for the production of tissue-factor specific procoagulant activity (PCA). Both β₁ integrins (VLA4, CD49d/CD29) and β₂ integrins (mac-1, CD11b/CD18, and LFA-1, CD11a/CD18) will recruit the MAPK and induce PCA.

Blood borne monocytes use a variety of surface molecules in the process of transendothelial migration and the subsequent interaction with extracellular matrix proteins. We have shown that antibody-induced crosslinking of both β₁ and β₂ integrins leads to strong phosphorylation and activation of the ERK MAPK, while crosslinking of the β₁ integrin VLA-4 also prompts the phosphorylation and activation of the p38 MAPK. Both of these MAPK modules are required for the subsequent expression of Tissue Factor (TF), a chief inducer of the coagulation cascade. Our studies also shed some light on the manner in which a surface integrin generates an intracellular signal in monocytic cells: clustering is essential, while the pp76^Syk^ and Src family tyrosine kinases are likely to be important upstream modulators of the MAPK. Further, we have identified crosstalk between the p38 and ERK MAPK. Although the significance of this crosstalk remains to be defined further, the consistent upregulation of the activity of ERK-dependent kinases – specifically, pp90^Rsk^ - suggests that this crosstalk may act to hold ERK-dependent cellular events in check, perhaps altering the balance between acute activating and more longterm differentiation programs. Taken together, these studies clearly identify the ERK and p38 MAPK as being of crucial importance in the induction of monocytic TF by integrins.
**Hypothesis 2:** The process of transendothelial migration activates monocyte MAPK modules, specifically ERK and p38, with subsequent activation of the cell and expression of tissue factor.

Based on the results outlined above, there is good reason to suppose that the ERK and p38 MAPK would play a role in the cellular activation induced by transendothelial migration. We developed an *in vitro* model of transendothelial migration which mimics the migration across an activated vascular bed. Using this model, we were able to demonstrate that the process of monocyte transmigration induces a marked upregulation of surface TF. TF upregulation is dependent upon early, integrin-mediated adhesive events. The act of monocyte adhesion to an activated endothelial layer induces the accumulation of dually phosphorylated, active ERK and p38 isoforms. Interestingly, selective pharmacologic inhibition of ERK and p38 had little effect on the act of transmigration *per se*, but both ERK and p38 were required for the elaboration of TF. These results suggest that the process of migrating to sites of extravascular inflammation can contribute to the induction of a procoagulant environment. Thus, part of the local activation of coagulation seen in viral FHF may require ERK- and p38-dependent signaling.

**Hypothesis 3:** Macrophages exposed to Murine Hepatitis Virus Strain-3 (MHV-3) will respond by activating ERK and p38 MAPK. The subsequent generation of a fgl-2/fibroleukin procoagulant response will depend upon these MAPK.

Once recruited to the liver, cells of the monocyte/macrophage lineage may become infected with the inciting viral pathogen. MHV-3 infection of murine macrophages induces a large increase in procoagulant activity based on the induction of the potent prothrombinase, fgl-2/fibroleukin. Recent studies have suggested that this molecule may have significance in clinical viral FHF. Exposure of MHV-3 to murine macrophages led to phosphorylation and activation of both the ERK and p38 MAPK. Interestingly, inhibition of p38 activity with SB203580 again consistently increased ERK phosphorylation and activity. Pharmacologic inhibition studies demonstrated that while the p38 MAPK was essential for fgl-2 mRNA and protein expression, the ERK MAPK was not required for either. However, ERK inhibition did abolish fgl-2-dependent PCA. Interestingly, infection of peritoneal macrophages by intraperitoneal inoculation of virus also led to ERK and p38 phosphorylation, suggesting that these pathways are relevant to the *in vivo* setting. These data suggest that differential activation of the ERK and p38 MAPK by viral infection can directly impact on the expression and activity of a monocyte/macrophage product which has immediate implications for the pathogenesis of viral FHF. Considered together, the work presented in these studies argues that the targeted inhibition of the ERK and p38 MAPK may be of some benefit in alleviating aspects of viral FHF.
Section X: Future Directions:

“All’s well that ends well.”
William Shakespeare (1564-1616)

We have defined a link between the ERK and p38 MAPK and monocyte/macrophage procoagulant activity in response to integrin signaling and MHV-3 infection. Many mechanistic details remain to be elucidated, as do the roles of these pathways in the *in vivo* pathogenesis of inflammatory disease.

The upstream mediators of integrin signaling in monocytic cells should be identified. As described in Section V, integrin “outside-in” signaling may lead to ERK MAPK activation via a number of pathways, including the Ras and Rho families of low molecular weight GTPases, and the pp76<sup>Syk</sup> tyrosine kinase. Transfection experiments with dominant negative Ras and Rho isoforms would help answer which of the GTPases is involved. As a preliminary to co-transfection experiments, we found that we could transiently transfec THP-1 cells with hemaglutinin-tagged ERK constructs. However, transfection rates were very low. As an adjunct to our pharmacologic data linking the pp76<sup>Syk</sup> tyrosine kinase with integrin signaling, we were able to selectively deplete Syk protein from primary human monocytes with antisense oligonucleotides. Syk-depleted cell populations did demonstrate less tyrosine phosphorylation signaling in response to VLA-4 integrin crosslinking; however, it was difficult to separate this inhibition from the general toxicity of the transfection process (which resulted in a roughly 80% cell viability by trypan blue exclusion). Nevertheless, the theory behind these types of experiments is entirely viable, and it may be that subtle improvements in transfection techniques will yield more definitive results in the future.

Recent data in neutrophils has suggested that the pp126<sup>FAK</sup> analogue, Pyk-2, may play an integratory role in linking integrin-mediated signals to cellular effects. For example, neutrophil adherence to fibrinogen (a process dependent on beta-2 integrins) in the presence of TNFα leads to Pyk2 activation (Yan 1999), and the TNFα-induced respiratory burst in adherent neutrophils is inhibited by wortmannin, which concurrently inhibits Pyk2 (Fuortes 1999). Pyk2 may help to link monocyte/macrophage integrin signaling to functional cellular events as well, though its role remains to be defined. Monocyte adhesion to fibronectin, which we have shown to stimulate strong ERK phosphorylation, stimulates only minimal Pyk2 activity (Li 1998). On the other hand, adhesion to plastic or exposure to LPS or colony stimulating factor-1 does lead to Pyk2 activation in monocytes (Li 1998, Hatch 1998). These data suggest that while Pyk2 activation is unlikely to explain the monocyte activation that follows adhesion to fibronectin or
crosslinking of the fibronectin receptor, the VLA-4 integrin, Pyk2 may play a role in other integrin-mediated events or in integrating integrin and other activating stimuli.

Ultimately it will be of interest to apply transfection techniques to test our findings. The bulk of our work uses selective pharmacologic inhibitors to define the importance of the ERK and p38 MAPK pathways in monocyte/macrophage PCA responses. Recognizing the inherent shortcomings of pharmacologic agents, we initiated a series of antisense and transfection studies to bolster our arguments. However, we found that high toxicity (with antisense technology) and low transfection rates (of THP-1 cells) effectively precluded our ability to employ these techniques. Although both PD98059 and SB203580 are among the most selective pharmacologic agents ever described, recent data has suggested that, at least in platelets, both agents have the capacity to inhibit cyclooxygenase enzymes (COX-1 and COX-2) (Borsch-Haubold 1998). It is very unlikely that COX inhibition influenced our results. For example, the production of PgE2 by monocytes/macrophages actually inhibits PCA, so that inhibition of COX would be expected to increase PCA. We and others have found that COX inhibition with indomethacin has no or little effect on the generation of tissue factor in response to LPS (Dackiw 1997, Oeth 1995). Moreover, in preliminary studies we found no inhibition of tissue factor after transendothelial migration in the presence of indomethacin (data not shown). Nevertheless, transfection and antisense technology will be useful to further explore the specificity of the ERK and p38 MAPK effect in monocytes/macrophages.

One fascinating question is the significance of the crosstalk we have observed between the p38 and ERK MAPK pathways. Inhibition of the p38 MAPK with SB203580 increased ERK phosphorylation and activity in response both to VLA-4 crosslinking and infection of macrophages with MHV-3. Although speculative, it is tempting to think that the differentiation of bloodborne monocytes into tissue-specific macrophages or dendritic cells may have something to do with this process. In this formulation, the ERK pathway could be seen as a primary differentiation program, with the p38 MAPK – which is the more consistently associated with monocyte/macrophage inflammatory gene upregulation – being of primary importance for the acute upregulation of immediate early inflammatory genes. An overbalance of inflammatory stresses might increase p38 MAPK activity and so decrease the potential for ERK-dependent cell differentiation. The transendothelial, integrin-dependent model of monocyte migration we have developed may help approach this question. In preliminary work we have found that the migration of monocytes across an activated endothelial bed results in a shift in cell surface expression of "differentiation" markers, including the HLA-DR antigen. The relative roles of the ERK and p38 MAPK in this process deserves further study.
Relatively little is known about the upstream mediators of MHV-3 signaling. As noted in Chapter 5, the inhibition of viral signaling with PP-1, a selective Src inhibitor, suggests that the Src kinases play an important role. Extending these observations, Dr. Alice Wei from our laboratory has defined activation of the Hck and Lyn Src kinases in response to MHV-3 infection. The fact that UV-irradiated, non-replicating virus can still induce intracellular signaling suggests that it is the binding of the virion to its cell surface receptor which triggers signaling. It will be of interest to define precisely how the Src kinases are activated by viral binding to the MHV-R, since its intracellular domain is small and does not contain an SH2 docking region.

Most importantly, there is the question of whether the selective inhibition of the ERK and p38 MAPK will attenuate aspects of the inflammatory damage characteristic of viral FHF. As noted in Section IV, and in Chapter 5 of Section VII, we have found that broad-spectrum tyrosine kinase inhibition with Tyrphostin AG124 administered to Balbc/j mice prior to infection with MHV-3 attenuates the upregulation of liver fgl-2 mRNA. However, liver damage was not inhibited, possibly due to increased viral replication. From a practical point of view, it may be necessary to simultaneously inhibit viral replication — with agents such as ribavarin — and attenuate aspects of the virally-induced inflammatory response, potentially with ERK and/or p38 MAPK inhibitors. The ability of tyrosine kinase and p38 MAPK inhibitors to improve indices of organ function and even mortality in models both of systemic and local inflammation suggests that these agents may in the future prove useful in clinical settings. Our work suggests that the role of these inhibitors in viral FHF deserves investigation.
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