Molecular Events During Melanoma Transendothelial Migration

_In Vitro_

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Biochemistry
University of Toronto

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Abstract

Extravasation of tumour cells is integral to metastasis. However little information is available which describes the transendothelial migration of tumour cells. To examine the extravasation process of tumour cells, we developed an in vitro model of transendothelial migration which involves the co-culture of human melanoma cells on a monolayer of human endothelial cells cultured on Matrigel. Actin dynamics during the process were studied using laser scanning confocal microscopy. The results revealed that dramatic changes in the organization of the actin cytoskeleton take place, along with dynamic changes in the cell shape of both melanoma and endothelial cells, during melanoma transendothelial migration. Furthermore, the cytokine TNFα was found to increase the ability of poorly metastatic cells to undergo extravasation, suggesting that adhesion and diapedesis are distinct steps in the process.

In contrast to the transendothelial migration of leukocytes, which involves PECAM-1/CD31, CD31 was redistributed away from endothelial cell-cell contacts juxtaposed to the melanoma cells and the protein was absent from heterotypic contacts between the two cell types, suggesting that the attachment of melanoma cells can signal the dissolution of endothelial CD31-mediated adhesion complexes. These results indicate, for the first time, that tumour cell extravasation is different from that of leukocytes.
We also determined that the distribution of VE-cadherin during melanoma extravasation was similar to that observed for CD31. In contrast, we have evidence that classical cadherins are present in heterotypic contacts between melanoma cells and endothelial cells during the process, and suggest that N-cadherin might be one of these cadherins.

Finally, we investigated the role played by the αβ3 integrin, since levels of this integrin are known to increase with the progression of disease. We observed αβ3 integrin in melanoma membrane blebs and in heterotypic contacts between melanoma cells and endothelial cells. The transmigration of melanoma cells was inhibited both by antibodies directed against αβ3 and by a cyclic RGD peptide, suggesting that αβ3 is involved in the migration of melanoma cells through the endothelial junction. Our results also suggest that melanoma αβ3 interacts with the cell adhesion molecule L1 on the endothelial surface.

These results not only define the sequence of events involved, but also bring new insights into the mechanism of tumour cell extravasation. Our in vitro model system should provide a useful paradigm for further investigation of the molecular and cellular events which take place during tumour cell transendothelial migration.
Acknowledgments

First of all, I wish to thank my Supervisory Committee, Drs. Fred Keeley and Avrum Gotlieb for their wisdom, advice and helpful questioning over the last five years. I also would like to acknowledge my supervisor, Dr. Chi-Hung Siu, for providing me with the opportunity to complete my Ph.D. and to pursue my interest in this project. I also want to thank him for the knowledge I gained during my Doctoral training.

I feel that in any science research laboratory one of the most important factors contributing to an individual's successful research are those that also work in the laboratory. These are the people who supply both the encouragement and criticism to keep the work on track and create the atmosphere in which the research is done. One of the things that I will miss most from my Ph.D. studies are my lab mates. These are all very special people with whom I have made many memories and they have all contributed to my success and enjoyment during the last five years. Although too many to mention, I wish I could take them all with me as I continue my career. I hope that when we meet in future we will share some laughs and reminisce over our time spent together.

Any accomplishment of mine is also shared with my parents, Joe and Renate Voura. Completing my Doctoral studies was a long awaited event for the three of us and I could never thank them enough for their tireless support and love. Their faith in me has been the one constant in my life which has been the predominant force to get me to this point.

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<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
</tr>
<tr>
<td>ARM</td>
<td>Armadillo (protein)</td>
</tr>
<tr>
<td>arm</td>
<td>armadillo (gene)</td>
</tr>
<tr>
<td>BBE</td>
<td>Bovine Brain Extract</td>
</tr>
<tr>
<td>BODIPY FL</td>
<td>dipyrrometheneboron difluoride</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell Adhesion Molecule</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster Designation</td>
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<tr>
<td>CEA</td>
<td>Carcinoembryonic Antigen</td>
</tr>
<tr>
<td>cx-43</td>
<td>connexin 43</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in Colon Carcinoma</td>
</tr>
<tr>
<td>Dil</td>
<td>1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate</td>
</tr>
<tr>
<td>DPB</td>
<td>dense Peripheral Band</td>
</tr>
<tr>
<td>EBM</td>
<td>Endothelial Basal Medium</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial Cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGM</td>
<td>Endothelial Growth Medium</td>
</tr>
<tr>
<td>EGM-2MV</td>
<td>Endothelial Growth Medium 2 Microvascular</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Epithelial cadherin</td>
</tr>
<tr>
<td>eIF</td>
<td>eukaryotic Initiation Factor</td>
</tr>
<tr>
<td>EMMPRIN</td>
<td>Extracellular matrix metalloproteinase inducer</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal -Regulated Kinase</td>
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<td>E-selectin</td>
<td>Endothelial selectin</td>
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<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
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<tr>
<td>GST</td>
<td>Glutathione-S-Transferase</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein</td>
</tr>
<tr>
<td>GSK</td>
<td>Glycogen Synthase Kinase (3b)</td>
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<tr>
<td>HBSS</td>
<td>Hank's Buffered Saline Solution</td>
</tr>
<tr>
<td>hEGF</td>
<td>Human Epidermal Growth Factor</td>
</tr>
<tr>
<td>12(S)-HETE</td>
<td>12(S)-Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HEV</td>
<td>High Endothelial Venules</td>
</tr>
<tr>
<td>HMVEC</td>
<td>Human Microvascular Endothelial Cell</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cell</td>
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<tr>
<td>IAP</td>
<td>Integrin Associated Protein</td>
</tr>
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<td>ICAM-1</td>
<td>Intercellular Cell Adhesion Molecule 1</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin Linked Kinase</td>
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<tr>
<td>INFγ</td>
<td>Interferon γ</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
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<tr>
<td>LFA-1</td>
<td>Leukocyte Function Antigen-1</td>
</tr>
<tr>
<td>LEF</td>
<td>Lymphoid Enhancer binding Factor</td>
</tr>
<tr>
<td>LSCM</td>
<td>Laser Scanning Confocal Microscopy</td>
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<td>L-selectin</td>
<td>Leukocyte selectin</td>
</tr>
<tr>
<td>Lu-ECAM-1</td>
<td>Lung Endothelial Cell Adhesion Molecule-1</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td>Mac-1</td>
<td>Macrophage-1</td>
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<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<td>Mel-CAM</td>
<td>Melanoma-Cell Adhesion Molecule</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MIDAS</td>
<td>Metal Ion Dependent Adhesion Site</td>
</tr>
<tr>
<td>MKK7</td>
<td>MAP Kinase Kinase 7</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane Type-Matrix Metalloproteinase</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>Neuronal cadherin</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor -κB</td>
</tr>
<tr>
<td>pAb</td>
<td>polyclonal Antibody</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
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<td>P-cadherin</td>
<td>Placental cadherin</td>
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<td>Platelet selectin</td>
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<tr>
<td>RPMI 1640</td>
<td>Roswell Park Memorial Institute 1640</td>
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<tr>
<td>SAP kinase</td>
<td>Stress Activated Protein kinase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SLe&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Sialyl Lewis a</td>
</tr>
<tr>
<td>SLe&lt;sup&gt;x&lt;/sup&gt;</td>
<td>Sialyl Lewis x</td>
</tr>
<tr>
<td>TCF</td>
<td>T-Cell-(specific) Factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinase</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor α</td>
</tr>
<tr>
<td>TR</td>
<td>Texas Red</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular Cell Adhesion Molecule-1</td>
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<td>VE-cadherin</td>
<td>Vascular Endothelial cadherin</td>
</tr>
<tr>
<td>VGP</td>
<td>Vertical Growth Phase</td>
</tr>
<tr>
<td>VLA</td>
<td>Very Late Antigen</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula Occludins</td>
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CHAPTER ONE

Introduction
I. Extravasation of Leukocytes

Research conducted over the past twenty years has provided a great deal of information on how hematopoietic cells undergo transendothelial migration. This research has been driven primarily by a desire to understand and control the process of inflammation. It has been determined that blood cell transendothelial migration involves a hierarchical cascade of adhesion steps, each one stimulating the next step in the process. The initial cues come from a plethora of cytokines which stimulate endothelial cells to express molecules that are recognizable by blood cells as extravasation stimuli. The entire process of hematopoietic cell transendothelial migration, in fact, involves a partnership of adhesion, de-adhesion and extracellular stimuli. Obviously, these effectors initiate intracellular signals which are ultimately responsible for producing the correct protein expression for the next stage of the process. Despite many years of research the exact sequence of cytokines and the intracellular signals involved in blood cell extravasation is still not well characterized.

Recent studies have concentrated upon the sequence of adhesion molecules involved and this has resulted in a better understanding of the roles of the various extracellular stimuli and the resulting intracellular signals (Carlos and Harlan, 1994; Dunon et al., 1996). There are several classes of adhesion molecules involved in the process of extravasation. These include the selectins, integrins and immunoglobulin (Ig)-like molecules (Figure I.1). Most work has concentrated on these three superfamilies of cell adhesion molecules and very little is known about the role of cadherins during the transendothelial migration of hematopoietic cells, although interendothelial cadherins do pose a barrier to leukocyte extravasation.
Figure I.1. Structural Features of Cell Adhesion Molecules
(Modified from Sherman et al. (1994) and Humphries et al. (1996))
(A) Adhesion Molecules Involved in Leukocyte Transendothelial Migration

(1) Selectins

There are three known selectins: E (endothelial)-selectin, P (platelet)-selectin and L (leukocyte)-selectin. L-selectin is expressed only by leukocytes, while E- and P-selectins are expressed by endothelial cells. P-selectin, as its name indicates, is also expressed on platelets (Carlos and Harlan, 1994; Joseph-Silverstein and Silverstein, 1998; Sass, 1998). The selectins are characterized by the presence of an amino terminal lectin-like Ca\(^{2+}\)-dependent binding domain, an epidermal growth factor-like region and a variable number of complement-regulatory factor-like repeats. These extracellular domains are connected by a transmembrane domain to a short cytoplasmic domain. Given the lectin-like binding properties possessed by the selectins, it is not surprising that selectin ligands consist of carbohydrate moieties. These sugar chains seem to require sialic acid and fucose linkages and, in some instances, these ligands are sulfated. One major ligand, referred to as Sialyl Lewis X (Sle\(^{X}\)), is recognized by all three selectin molecules. Other ligands include sulfated polysaccharides and lipids, as well as phosphorylated monosaccharides and polysaccharides (Albelda et al., 1994).

(2) Integrins

Integrins are heterodimeric adhesion molecules containing non-covalently linked α and β subunits (Albelda et al., 1994). There are a number of α and β subunits which can combine to form a variety of integrins (Carlos and Harlan, 1994). Currently, twenty-two different integrin heterodimers are known (Hemler, 1998). Both α and β chains are transmembrane proteins containing a cytoplasmic domain. The α subunit has a region containing 7 tandem repeats which show homology with EF-hand structures. In some α
subunits, a region, referred to as an I domain, is found. All seven integrins containing an I domain are expressed by leukocytes (Stewart et al., 1995; Humphries, 1996). The I domain has been shown to possess homology to the A domain found in cartilage matrix protein and von Willebrand factor (Carlos and Harlan, 1994; Stewart et al., 1995; Humphries, 1996). Some α chains also have an extracellular cleavage site near the transmembrane domain. In such instances, the two α chain fragments are linked by a disulfide bond. The β chains have four cysteine-rich tandem repeats and an amino-terminal conserved sequence involved in heterodimer formation. They also express an I-like motif as found in the α subunits (Humphries, 1996). Both α and β chains are required for ligand recognition and both subunits have been shown to contain a motif referred to as the metal ion-dependent adhesion site (MIDAS). Divalent cations are required for integrin mediated adhesion (Gumbiner, 1996). Integrins are best known for their interactions with extracellular matrix (ECM), but various Ig superfamily members and soluble proteins, such as fibrinogen, are also known ligands (Joseph-Silverstein and Silverstein, 1998).

β₁ integrins are often referred to as very late antigens (VLA) and are frequently associated with adhesion to ECM (Carlos and Harlan, 1994). The VLA-4 (α₄β₁) integrin is one of the most common integrins of the hematopoietic system. This integrin will adhere to certain Ig-like molecules in addition to ECM molecules, such as fibronectin. Of all the leukocytes, only neutrophils do not express VLA-4. Other VLA integrins important for blood cell transendothelial migration include VLA-2 (α₂β₁) and VLA-5 (α₅β₁) which interact with collagen and fibronectin, respectively (Albelda, 1991).

Endothelial cells do not express β₂ integrins (Carlos and Harlan, 1994). The β₂ integrins involved in leukocyte extravasation, Mac-1 (α₅β₂) and LFA-1 (Leukocyte Function Antigen 1) (α₅β₂), therefore, are exclusively found on leukocytes (Stewart et al., 1995).
(3) Cadherins

The classical cadherins, E-(endothelial), P-(placental) and N-(neuronal) cadherins, are referred to as type I cadherins (Huber et al., 1996; Munro and Blaschuk, 1996; Wheelock et al., 1996; Barth et al., 1997). These transmembrane cell adhesion molecules are found in adherens junctions between cells and interact via homophilic binding. The only known exception being an interaction between E-cadherin and the lymphocyte α₅β₇ integrin (Higgins et al., 1998). The classical cadherins contain a highly conserved region within their cytoplasmic tail. The extracellular region usually contains Ca²⁺-binding pockets between five domains of approximately 110 amino acids. These domains are organized into anti-parallel β-strands (Overduin et al., 1995; Munro and Blaschuk, 1996; Joseph-Silverstein and Silverstein, 1998). It was proposed that, two cadherin monomers on the cell surface associate to form a “strand dimer” structure (Overduin et al., 1995; Shapiro et al., 1995). Strand dimers on opposing cells could then associate in an anti-parallel fashion to form cadherin adhesion complexes. These interdigitations would form cadherin “zippers” of adhesion between cells (Cowin and Burke, 1996; Gumbiner, 1996). Recently, it was discovered that anti-parallel cadherin interactions and clustering of these molecules depend on cadherin association with catenins and congregation of the cytoplasmic tails, while dimer formation does not (Chitaev and Troyanovsky, 1998; Katz et al., 1998). Dimer formation, rather, requires stabilization by Ca²⁺ ions (Nagar et al., 1996).

(4) Immunoglobulin Superfamily

This group of molecules contains proteins involved in cell adhesion (C2-type) or in antigen recognition (C1-type) (Carlos and Harlan, 1994). Members of the C2-type Ig-like proteins, are involved in leukocyte extravasation and compose a diverse group of proteins
capable of Ca\(^{2+}\)-independent homophilic interactions (Brown, 1997; Joseph-Silverstein and Silverstein, 1998). Most of these proteins contain a transmembrane domain and a cytoplasmic region. All the proteins possess a variable number of Ig-like domains consisting of a ternary structure of two \(\beta\)-sheets, which are usually stabilized by a disulfide bond found between two conserved cysteines (Carlos and Harlan, 1994). The major Ig superfamily members involved in leukocyte extravasation include vascular cell adhesion molecule-1 (VCAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1) or CD31 (cluster designation 31), intercellular adhesion molecule-1 (ICAM-1) and the mucosal addressin (MAdCAM-1) (Carlos and Harlan, 1994). Many members of the Ig family of cell adhesion molecules are also capable of heterophilic interactions with integrins and other Ig superfamily members. During extravasation, for example, endothelial VCAM-1 adheres to \(\alpha_i\beta_1\) expressed by leukocytes while ICAM-1 interacts with LFA-1 (\(\alpha_i\beta_2\)) and CD31 binds to integrin \(\alpha_i\beta_3\) (Fogler et al., 1996).

(B) Adhesion Complexes in the Interendothelial Contact Region

Signals contained in basement membrane complexes have been suggested to create many different endothelial types therefore the expression of adhesion molecules on the surface of different endothelia is variable (Gumbiner, 1996). On the other hand, adhesive structures expressed in endothelial cell-cell contacts remain quite homogeneous in composition, although their complexity might vary depending on the location of the vessel (Figure 1.2) (Kuzu et al., 1993; Dejana et al., 1995; Rajotte et al., 1998).

Endothelial monolayers express tight junctions, although a continuous barrier is not usually provided by these structures. In fact, these contacts have been shown to be highly dynamic (Gumbiner, 1996; Lampugnani and Dejana, 1997). Tight junctions are largely composed of occludin which associates with zonula occludins (ZO)-1 and -2, cingulin and the GTP-binding protein, rab 13. Gap junctions are also expressed between endothelial
Figure 1.2. Junctional Complexes Between Endothelial Cells
cells. The connexins found in endothelial gap junctions include connexins 43, 40 and 37. Gap junctions have been shown to facilitate communication between endothelial cells and between these cells and other cell types, such as muscle cells and macrophage (Dejana et al., 1995; Lampugnani and Dejana, 1997).

Adherens junctions connect cadherins with the actin cytoskeleton. Vascular endothelial-cadherin (VE-cadherin) is found only in endothelial junctions (Heimark et al., 1990; Lampugnani et al., 1992; Caveda et al. 1996). Other classical cadherins expressed by endothelial cells include E-, N- and P-cadherins (Albelda, 1991; Dejana et al., 1995). N-cadherin, however, is not expressed in adherens junctions. This cadherin is present over the entire surface of the endothelium (Navarro et al., 1998). Cadherins in adherens junctions are linked to the actin cytoskeleton via a complex of α, β- and γ-catenins (Dejana et al., 1995; Munro and Blaschuk, 1996; Lampugnani and Dejana, 1997). The endothelial actin cytoskeleton is expressed as a dense peripheral band of actin filaments in confluent monolayers in vitro (Ettenson and Gotlieb, 1993). This peripheral actin bundle is thought to be important for cell-cell contact. Central microfilament bundles, suggestive of cell-substratum adhesion, can also be observed in endothelial cells. Integrins found in endothelial cell-cell contacts include, α1β1, α1β2 and α1β3 (Albelda, 1991; Lampugnani et al., 1991). Antibodies to β1 were found to increase endothelial permeability and disrupt the integrity of the endothelial monolayer.

PECAM-1/CD31 is also found in endothelial cell-cell contacts as well as on most leukocytes, and was originally isolated in a screen for endothelial-specific molecules (Simmons et al., 1990; Muller, 1995; Newman, 1997;). Leukocyte transendothelial migration experiments using function blocking antibodies revealed the requirement of CD31 for most leukocytes to undergo diapedesis. In the presence of anti-CD31 antibodies, blood cells were trapped at the tight adhesion stage and could not traverse the endothelium. At most, leukocytes were able to insert a small pseudopod between endothelial cells, suggesting that anti-CD31 reagents could be used to control inflammation (Muller et al.,
1993; Muller, 1995; Muller, 1995; Liao et al., 1997; Newman, 1997). These results were reinforced by observations made using in vivo models of inflammation (Bogen et al., 1994). In vitro, the reagents were effective in the absence of Ca\(^{2+}\) and also when added to either the leukocytes or the endothelium, suggesting homophilic interactions were involved. Heterophilic interactions, however, were suggested to mediate migration following diapedesis (Liao et al., 1995).

(C) Sequence of Events during Leukocyte Transendothelial Migration

The sequence of adhesion molecules involved in leukocyte extravasation constitutes what is referred to as the "adhesion cascade" (Albelda et al., 1994; Carlos and Harlan, 1994; Joseph-Silverstein and Silverstein, 1998). Blood cells travelling in the direction of flow randomly contact the endothelium of the vessels (Figure I.3). From the sporadic contacts, leukocytes determine if the endothelium is expressing the molecules required for transmigration. Whether these molecules are required for inflammation and disease, or for recirculation of lymphocytes to the lymphatics, the stages of transendothelial migration are similar.

(1) Leukocyte Rolling

First, leukocytes begin to roll on the endothelium. This rolling is largely supported by selectins, as \(\beta_2\) integrins, for example, can only initiate such adhesion under low shear stress (Albelda et al., 1994; Carlos and Harlan, 1994; Dunon et al., 1996; Liu et al., 1998). Interestingly, VLA-4 can also mediate rolling (Stewart et al., 1995; Gumbiner, 1996). Anti L-selectin antibodies however, prevent rolling of both monocytes and neutrophils. L-selectin has been shown to be localized on the tips of microvilli and also to interact with the cytoskeleton during rolling (Butcher and Picker, 1996; Dunon et al., 1996; Joseph-
Silverstein and Silverstein, 1998). Both L- and P-selectins are involved in leukocyte rolling (Luscinskas et al., 1996). E-selectin, on the other hand, is only expressed by endothelial cells after several hours of cytokine stimulation (Brown, 1997).

Molecules stimulating the expression of E-selectin, VCAM-1 and ICAM-1 on endothelium during inflammation include interleukin-1 (IL-1), tumour necrosis factor α (TNFα) and lipopolysaccharide (LPS). Such stimulation and regulation of adhesion molecules is crucial to ensure the effectiveness of the adhesion cascade. Transcription of VCAM-1, for example, is increased during inflammation, while P-selectin, which is constitutively produced, is mobilized from cytoplasmic granules to the cell surface of the endothelium (Albelda et al., 1994). Differing induction kinetics of adhesion molecules by individual factors may contribute to the differential recruitment of leukocytes during the course of inflammation (Carlos and Harlan, 1994). In addition, some factors exert effects on certain cell adhesion molecules whereas others do not. Furthermore, several cytokines may work together to augment or block the expression of cell adhesion molecules.

Selectins are unique in their ability to support adhesion in the presence of high shear stresses and gene knock-out studies have indicated that they are vital to the maintenance of normal host physiology during infection (Brown, 1997). This feature stems from the rapid on-rate and affinity of selectin-mediated adhesion. Rolling is manifested in the reciprocation the rapid off-rate of selectin adhesion (Gumbiner, 1996).

Abnormalities in fucose metabolism may result in leukocyte adhesion deficiency type II. This abnormality highlights the importance of leukocyte rolling and selectin-mediated adhesion. Inflammatory cells in these patients have difficulty rolling on endothelium under normal physiological shear stress. Since this defect prevents the subsequent steps required for leukocyte extravasation, individuals with the disease have a compromised immune response (Albelda et al., 1994; Carlos and Harlan, 1994).
(2) Stable Adhesion of Leukocytes to the Endothelium

While rolling, L-selectin is rapidly removed from the leukocyte surface by proteolytic cleavage and this activity is thought to cause intracellular signals within the leukocyte, resulting in a more stable adherence to, and spreading on, the endothelium (Albelda et al., 1994; Carlos and Harlan, 1994; Dunon et al., 1996). These signals activate leukocyte integrins and Ig-like cell adhesion molecules required for firm adhesion, during which blood cells adopt a flattened morphology from the previously spherical one used during rolling (Liu et al., 1998). Furthermore, leukocyte signalling induced upon binding cytokines, such as IL-8 and IL-5, leads to an increase in β₁ and β₂ integrin adhesion (Carlos and Harlan, 1994; Dunon et al., 1996). Such cytokines induce signalling by binding to G-protein coupled receptors, which in turn activate the GTP-binding proteins of the ras family (Butcher and Picker, 1996; Dunon et al., 1996; Brown, 1997). These soluble factors can be produced by endothelium, stromal cells, microorganisms or other leukocytes in the vicinity (Carlos and Harlan, 1994). Importantly, functional IL-8, as well as other cytokines and growth factors, has been found on the surface of the endothelium and in the ECM. The rapid activation of integrins, via a conformational change in the heterodimer, is vital for a quick leukocyte adhesive response after rolling on the endothelium (Albelda et al., 1994). This modulation is equally important for the resulting migration on, and diapedesis of leukocytes through, the endothelial lining (Carlos and Harlan, 1994; Liu et al., 1998). Integrin activation can occur within minutes of cell stimulation and can be reversed, upon removal of the stimulus, with equal efficiency (Gumbiner, 1996; Brown, 1997). Interaction of leukocytes and endothelium likely results in mutual signalling events, producing more of the adhesion and cytokine stimuli necessary for extravasation. For example, binding of T-cells to endothelial CD31, leads to an increased adhesion by T-cell β₁ integrins (Carlos and Harlan, 1994).
Leukocyte adhesion deficiency type I is a syndrome which highlights the importance of $\beta_2$ integrins to firm adhesion (Albelda et al., 1994; Carlos and Harlan, 1994). Patients with this disease are lacking in these integrins and hence, monocytes and neutrophils from these patients fail to extravasate during inflammation. These cells, in fact, just continue to roll. Other leukocytes such as lymphocytes, eosinophils and plasma cells, however, can still traverse the endothelium, suggesting that different adhesion cascades function for the various types of blood cells. Furthermore, adhesion interactions during inflammatory transendothelial migration can differ from those involved in lymphocyte recirculation. In the latter, special high endothelial venules (HEV) display specialized cell adhesion molecules referred to as mucosal addressins since subsets of circulating lymphocytes, expressing complementary receptors, are selectively bound by these molecules (Carlos and Harlan, 1994; Picker, 1994). These proteins are required for initial loose and subsequent firm adhesion to the endothelium during lymphocyte homing.

Lymphocyte recirculation is a highly regulated event controlled by HEVs. HEVs are cuboidal endothelial cells with high walls. Mature and naive lymphocytes recirculate differently as governed by the particular vascular addressins expressed by these specialized endothelia (Picker, 1994; Butcher and Picker, 1996). Lymphocyte L-selectin binding to endothelial CD34 and MAdCAM-1 can mediate rolling during recirculation, whereas, interactions between $\alpha_4\beta_1$ and MAdCAM-1 can facilitate both rolling and firm adhesion of lymphocytes on HEV (Carlos and Harlan, 1994; Butcher and Picker, 1996). Adhesion between $\alpha_4\beta_1$ and VCAM-1, as well as CD44 and hyaluronate, is important for similar interactions. The ligands for E- and P-selectin have also been suggested to complement L-selectin mediated rolling on SLe$^+$ as observed for non-recirculation types of extravasation (Picker, 1994; Butcher and Picker, 1996).
Transmigration of Leukocytes

Following firm adhesion, leukocytes usually migrate to endothelial cell-cell junctions, a process requiring both integrin and Ig-like molecules (Carlos and Harlan, 1994). Leukocytes then squeeze through these junctions and enter the ECM in a process referred to as diapedesis. Often the adhesion of leukocytes to endothelium only results in diapedesis if a chemotactic gradient is present (Albelda et al., 1994). The $\alpha_\beta_3$ integrin is used by monocytes during transmigration (Weerasinghe et al., 1998). Furthermore, studies on leukocyte adhesion deficiency type I have suggested that $\beta_2$ integrins, such as MAC-1 ($\alpha_{4}\beta_2$) and LFA-1 ($\alpha_L\beta_2$), are also required for transmigration (Butcher and Picker, 1996; Brown, 1997). Other experiments have suggested that transmigration may also involve the VLA-5 ($\alpha_5\beta_1$) and VLA-6 ($\alpha_6\beta_1$) integrins. Stimulation of endothelial monolayers leads to the induction of VCAM-1 and E-selectin, suggesting that these molecules are also involved in transendothelial migration, and do not just play a part in rolling and adhesion (Carlos and Harlan, 1994). In many experiments, however, it is difficult to determine whether the identified cell adhesion molecules are involved in migration, diapedesis, or both.

Diapedesis, or the penetration of leukocytes through endothelium is the most poorly understood stage of extravasation (Brown, 1997). Recently, a series of in vivo and in vitro experiments have confirmed that PECAM-1/CD31, is involved in the diapedesis of most blood cells (Albelda et al., 1994; Carlos and Harlan, 1994). This molecule is expressed on blood cells and in endothelial cell-cell contacts. The CD31-mediated adhesive mechanism between leukocytes and endothelial cells involves homophilic CD31 interactions and heterophilic interactions between CD31 and the $\alpha_\beta_3$ integrin (Dunon et al., 1996). CD31 concentrated in endothelial cell-cell contacts has been suggested to provide a haptotactic gradient to guide leukocytes through the endothelium. In addition, CD31 mediated adhesion, the resulting intracellular signalling and the consequent stimulation of integrin-
mediated adhesion have been suggested to facilitate migration of leukocytes through the ECM following transmigration (Muller and Weigl, 1992; Carlos and Harlan, 1994).

Antibody blocking experiments have suggested that the integrin associated protein (IAP) may also be involved in diapedesis (Brown, 1997). This protein is physically associated with the $\alpha_\text{L}\beta_3$ integrin, further implicating this integrin in the process. IAP associates with the extracellular region of the $\beta_3$ subunit in both $\alpha_\text{L}\beta_3$ and $\alpha_\text{IIb}\beta_3$ (Hemler, 1998; Keely et al., 1998). IAP is a receptor for thrombospondin and acts to enhance $\alpha_\text{L}\beta_3$ mediated binding to vitronectin in the presence of thrombospondin (Gao et al., 1996). Recent experiments using monocytes have indicated that LFA-1 ($\alpha_\text{L}\beta_3$) is also required during diapedesis (Sandig et al., 1997). Studies on the transmigration of neutrophils have indicated that endothelial adherens junctions become dissociated during the process and that migration frequently takes place at tricellular corners between endothelial cells (Del Maschio et al., 1996; Allport et al., 1997; Burns et al., 1997). Diapedesis of leukocytes occurs rapidly, with no obvious disruption of the endothelium (Pawlowski et al., 1988; Muller and Weigl, 1992). Following diapedesis, blood cells use integrins to traverse the ECM and migrate toward the extravascular stimulus (Brown, 1997). This process is, most likely, facilitated by proteolytic digestion of the ECM (Dunon et al., 1996).

Therefore, the overall scheme of blood cell extravasation, whether it be for inflammation, immune surveillance, or lymphocyte recirculation, can be broken down into several well recognized stages: contact and rolling, stable adhesion and spreading, diapedesis and migration through the ECM. Although the particular adhesion molecules involved might vary depending on the reason for extravasation, timing and the particular leukocyte involved, each instance does involve a cascade of adhesion molecules and a set of extracellular stimuli.
Figure 1.3. Stages and Molecules Involved in Leukocyte Transendothelial Migration

Modified from Carlos et al. (1994)
II. Extravasation of Tumour Cells

The understanding of tumour cell extravasation, when compared to that of leukocyte transendothelial migration, is still in its infancy. Many cell adhesion molecules expressed by tumour cells are inevitably involved in the migration of tumour cells through the endothelial cells. Comprehension of the role of these molecules during tumour cell extravasation should reveal novel targets for therapeutic intervention during metastasis. These adhesion molecules might be directly involved in adhesion between tumour and endothelial cells. Other molecules may stimulate intracellular signalling to enhance the expression of other cell adhesion molecules or even cytokines and proteolytic enzymes (Shioda et al., 1997). Cell adhesion might also be targeted following extravasation to prevent migration through ECM and, in that way, inhibit the establishment of secondary tumours. The molecules required for diapedesis of tumour cells may display striking similarities between different tumour cell types, as this has been the observation for many leukocytes during transendothelial migration.

(A) Cell Adhesion Molecules and Tumour Cell Extravasation

(1) Selectins

As described above, leukocyte extravasation requires selectin-mediated interactions to permit rolling prior to adhesion and diapedesis. It is likely, therefore, that some tumour cells utilize selectins in a similar fashion. Many carbohydrate ligands are unique to tumour cells (Sass, 1998). In fact, a variation of the SLe^a antigen, SLe^b, is not usually found on leukocytes, but is expressed on tumour cells and will interact with all of the selectin molecules (Carlos and Harlan, 1994). E-selectin has been shown by some investigators to mediate cancer cell adhesion to activated endothelium and to redirect the location of breast
and colon cancer cell metastasis (Tozeren et al., 1995; Sass, 1998). Antibody staining experiments revealed that levels of E- and P-selectin were increased in breast carcinoma while P-selectin down-regulation was found to decrease tumour growth and spreading (Kim et al., 1998; Sass, 1998). However, not all tumour cells require selectins or engage in rolling during extravasation.

(2) Integrins

Much work has been done on the expression and function of various integrins in many forms of cancer. Integrins play a role in all stages of metastasis and are, therefore, likely important during extravasation. Decreases in the level of certain integrins may be indicative of increased cell motility required when tumour cells migrate through the matrix. Other integrins may be increased because they aid in migration. These changes in adhesion may cause altered intracellular signalling, thus influencing many different aspects of cellular physiology. Down-regulation of fibronectin-mediated adherence by the $\alpha_2\beta_1$ integrin is generally associated with tumourigenesis, metastasis and increased cellular proliferation (Varner and Cheresh, 1996; Keely et al., 1998). Similarly, down regulation of $\alpha_2\beta_1$ adhesion to laminin induces a transformed phenotype, while increased levels of $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins are associated with increased tumour progression. $\alpha_6\beta_1$ has been shown to facilitate migration on laminin and extravasation of murine melanoma cells in vivo (Hangan et al., 1997). The role of the $\alpha_6\beta_4$ integrin is not clear as it has been shown to be upregulated in some tumours and down-regulated in others (Keely et al., 1998). The $\alpha_4\beta_1$ integrin has consistently been shown to be required for melanoma cell extravasation through cytokine-stimulated endothelium (Lafrenie et al., 1994; Okahara et al., 1994; Garofalo et al., 1995). $\alpha_4\beta_1$ has been suggested to bind to VCAM-1 in the process. The $\alpha_\gamma\beta_3$ and $\alpha_\delta\beta_3$ integrins are involved in the adhesion of colon cancer cells to fibronectin, while similar interactions involve $\alpha_\lambda\beta_3$ in human melanoma cells (Kemperman et al.,
In general, a decreased integrin expression pattern is associated with tumour progression, although a few integrins are upregulated. The particular integrins upregulated seems to depend largely on the specific type of malignancy being considered (Sanders et al., 1998).

In contrast to other integrins, the role of the $\alpha_\beta_3$ integrin in cancer progression has been quite consistent. In situ detection of $\alpha_\beta_3$ and the $\beta_3$ integrin subunit, as well as the analysis of $\alpha_\beta_3$ protein levels, indicate amounts of this integrin increase with progressive stages of melanoma metastasis (Albelda et al., 1990; Lafrenie et al., 1994; Merimsky et al., 1994; Varner and Cheresh, 1996; Natali et al., 1997). In fact, $\beta_3$ is not even expressed in normal skin melanocytes (Weterman et al., 1994). $\alpha_\beta_3$ can mediate adhesion and migration on a variety of ECM proteins including vitronectin, fibrinogen, fibronectin, laminin, collagen and osteopontin. This integrin is also important for tumour angiogenesis, and antagonists of the integrin compromise this process in tumours (Varner and Cheresh, 1996; Sanders et al., 1998). In contrast, such inhibitors have no effect on existing blood vessels (Keely et al., 1998). Data supporting the existence of different $\alpha_\beta_3$-mediated haptotactic and chemotactic signalling pathways in melanoma cells has recently been published (Aznavoorian et al., 1996). $\alpha_\beta_3$ is also implicated in melanoma cell interaction with platelets, which might enhance the invasive behaviour of these tumour cells (Merimsky et al., 1994). In addition to melanoma, $\alpha_\beta_3$ is important in other forms of cancer (Lafrenie et al., 1994; Yun et al., 1996).

(3) Cadherins

Studies on neutrophils have revealed that VE-cadherin leaves endothelial cell-cell contacts during extravasation (Del Maschio et al., 1996). This is accompanied by a dissociation of plakoglobin and $\alpha$- and $\beta$-catenin. These effects can be prevented by inhibiting neutrophil adhesion using antibodies against the $\beta_2$ integrin subunit. In contrast,
VE-cadherin is redistributed together with α-, β- and γ-catenin during tumour cell transendothelial migration (Lewalle et al., 1997). The dissolution of VE-cadherin is accompanied by a redistribution of vinculin during tumour cell extravasation. The redistribution of VE-cadherin during neutrophil and tumour cell transendothelial migration, however, does not preclude cadherin involvement in cell-cell interactions during the process for either leukocytes or tumour cells since other cadherins might be involved.

It has been suggested that cadherins can form heterophilic interactions with other cadherins (Munro and Blaschuk, 1996). This sort of interaction may play an important role when cells of different tissues interact, as they would when tumour cells or leukocytes undergo transendothelial migration. Furthermore, the possibility that the same cadherin molecules on two different cell types interact during such processes is an obvious consideration.

Many tumour cells show a reduced P- and E-cadherin expression (Munro and Blaschuk, 1996; Seline et al., 1996). However, it is reduced E-cadherin levels that seems to correlate with malignancy in most tumour types (Ben-Ze'ev, 1997). Conversely, melanoma cells have been shown to express many novel cadherins not found in normal melanocytes (Matsuyoshi et al., 1997). Such ectopic cadherin expression, consistant with malignancy, strongly suggests cadherin involvement in the metastatic phenotype and a possible importance to the process of extravasation.

(4) Immunoglobulin-Like Molecules

The carcinoembryonic antigen (CEA) is widely used as a tumour marker. This Ig cell adhesion molecule can mediate homophilic interactions and can promote colon cancer cell adherence to collagen. In addition, soluble CEA can be detected in the serum of cancer patients (Tang and Honn, 1994). Another Ig-like molecule used as an indicator of malignancy is the tumour suppressor, deleted in colon carcinoma (DCC). Decreased levels
of DCC correlate with increased malignancy (Tang and Honn, 1994). Conversely, increased ICAM-1 levels are commonly associated with a poor prognosis. In addition, the cell adhesion molecule Mel-CAM (Melanoma-Cell Adhesion Molecule) or MUC-18 is upregulated with progressive stages of melanoma (Shih et al., 1994; Shih et al., 1997; Meier et al., 1998). Adhesive interactions involving Mel-CAM are hypothesized to be involved in tumour cell extravasation (Meier et al., 1998). However, the adhesive ligand for Mel-CAM has not yet been identified.

(i) PECAM-1/CD31

CD31, or PECAM-1, is a member of the Ig-like superfamily of adhesion molecules presenting six Ig domains, a transmembrane spanning sequence and a cytoplasmic tail (Newman et al., 1990; Simmons et al., 1990). The complex cytoplasmic tail is encoded by eight different exons which can be alternatively spliced or modified to yield CD31 proteins with changed adhesive capacities (Baldwin et al., 1994; DeLisser et al., 1994; Kirschbaum et al., 1994; Sun et al., 1996; Famiglietti et al., 1997). Furthermore, processing of CD31 leads to the synthesis of soluble forms of the molecule (Goldberger et al., 1994). Ectopic expression has also been reported on a number of tumour cell types (Simmons et al., 1990; Tang et al., 1993). CD31 is capable of both homophilic and heterophilic adhesion (Albelda et al., 1991; Muller et al., 1992; Newton et al., 1997). The nature of the heterophilic partners involved in CD31-mediated adhesion are still in debate, although both sulfated glycosaminoglycans and the α₃β₁ integrin have been implicated (DeLisser et al., 1993; Piali et al., 1995; Buckley et al., 1996; Sun et al., 1996; Sun et al., 1996; Sun et al., 1998). Importantly, ligation of CD31 has been shown to stimulate β₁, β₂ and β₃ integrin adhesion which are most likely required for migration through ECM (Tanaka et al., 1992; Piali et al., 1993; Leavesley et al., 1994; Berman and Muller, 1995; Berman et al., 1996; Newman, 1997; Varon et al., 1998).
Since CD31 is expressed by both tumour cells and leukocytes, and is absolutely required for diapedesis during inflammation, it may have an equally important function during the extravasation of tumour cells. Furthermore, the association of CD31 phosphorylation with the cytoskeletal interactions of the molecule, suggest that CD31 adhesion may regulate motility and cell shape. No reports examining either an adhesive or signalling role for CD31 during tumour diapedesis have been published, although CD31 has been shown to mediate tumour cell attachment to endothelial monolayers (Tang et al., 1993).

(ii) L1

The highly conserved L1 protein has six Ig-like domains and five fibronectin type III-like repeats in its extracellular domain, a transmembrane sequence and a cytoplasmic domain (Moos et al., 1988; Hlavin and Lemmon, 1991; Hortsch, 1996; Burden-Gulley et al., 1997; Kamiguchi and Lemmon, 1997). Of the two alternatively spliced isoforms, the one expressed in non-neuronal cells is missing a short four amino acid sequence from the cytoplasmic domain and a stretch of amino acids from the N-terminal extracellular region. L1 is expressed primarily in the nervous system and L1 mutations correlate with various neurological disorders. L1 has been implicated in neurite out-growth, myelination, neuronal migration and axonal fasciculation (Lindner et al., 1983). Homophilic binding of the L1 molecule is mediated by sequences in the second Ig-like domain (Miura et al., 1992; Zhao and Siu, 1995; Zhao et al., 1998). On the other hand, heterophilic binding partners include chondroitin sulfate proteoglycans (Milev et al., 1994), laminin (Hall et al., 1997), TAG-1/axonin-1 (Kuhn et al., 1991) and the integrins α3β1, α3β1, α,β1 and α,β3 (Ruppert et al., 1995; Felding-Habermann et al., 1997). Integrin interactions with L1 are mediated by a RGD sequence in the L1 Ig-like domain six (Ruppert et al., 1995; Felding-Habermann et al., 1997; Montgomery et al., 1996; Yip et al., 1998). L1-dependent
adhesion is enhanced by cis interactions with neural cell adhesion molecule-1 and nectadrin (Kadmon et al., 1990; Horstkorte et al., 1993; Kadmon et al., 1995). The phosphorylation of the L1 cytoplasmic tail may have a role in L1 mediated signalling. Furthermore, L1 can be connected to the cytoskeleton, both by ankyrin as well as directly, through a short membrane proximal region. These interactions might also be required for L1-mediated signal transduction (Hortsch, 1996; Burden-Gulley et al., 1997; Kamiguchi and Lemmon, 1997; Hortsch et al., 1998). Interactions between L1 and the cytoskeleton are necessary for neuronal extension.

L1 is also found on leukocytes, epithelial cells and various cancer cells (Kowitz et al., 1992; Kujat et al., 1995; Hortsch, 1996). For instance, L1 is expressed on human lung cancer and melanoma cells in culture and expression on malignant gliomas is important for invasion (Linnemann et al., 1989; Izumoto et al., 1996; Katayama et al., 1997). L1 is also expressed by murine intestinal cells (Thor et al., 1987). Leukocyte L1 levels decrease following cellular activation (Hortsch, 1996). L1 expressed by leukocytes has been shown to adhere to endothelial cell α,β, integrin, suggesting yet another important interaction involved in leukocyte extravasation (Ebeling et al., 1996; Pancook et al., 1997).

Interactions between L1 and the α,β, integrin have been suggested to be important during cancer progression (Montgomery et al., 1996; Duczmal et al., 1997). Melanoma cells adhere to L1-coated substrate and this interaction can be inhibited by a function blocking antibody recognizing the α,β, heterodimer. These melanoma cells were shown to secrete L1 in a soluble form, suggesting L1 can be deposited in the ECM to provide an adhesive substrate for melanoma α,β, integrin. Indeed, L1 was detected in situ associated with ECM laminin (Montgomery et al., 1996).
(5) Lu-ECAM-1

Lu-ECAM-1 (lung endothelial cell adhesion molecule-1) is a cell adhesion molecule only expressed on endothelial cells of the lung and has been suggested to form a chloride channel (Zhu et al., 1991; Elble et al., 1997). There is a correlation with the expression of Lu-ECAM-1 and the location of melanoma cell metastasis (Zhu and Pauli, 1993). Lu-ECAM-1 has been shown to be required for adhesion of melanoma cells to lung endothelium under conditions of flow in vivo (Zhu et al., 1991; Goetz et al., 1996). Lung-specific adhesion of melanoma cells can be inhibited by antibodies and soluble recombinant protein (Zhu et al., 1992).

(6) CD44

CD44 is a transmembrane cell adhesion molecule which is a member of the hyaladherins, a family of proteins which recognize hyaluronic acid (Sherman et al., 1994). This adhesion molecule can also interact with other ECM proteins, such as collagen and fibronectin. The extracellular amino terminal region of the protein is responsible for CD44 interaction with hyaluronic acid. Furthermore, glycosylation and a properly formed loop structure are necessary for CD44 mediated adhesion. CD44 is capable of cytoskeletally mediated dimerization (Dunon et al., 1996). Many different isoforms of CD44 result from alternative splicing and post-translational modification of the protein. CD44s is the standard form of the cell adhesion molecule. Other characterized isoforms include CD44H, the hematopoietic version, and CD44v which occurs primarily on epithelial tissues and is also found on tumour cells.

CD44 has been shown to have a role in leukocyte transmigration and lymphocyte activation (Brown, 1997). Resting lymphocytes express high levels of CD44s and CD44-specific antibodies can effectively inhibit an immune response (Sherman et al., 1994).
Furthermore, endothelial cells expressing CD44H were shown to use the molecule to bind melanoma cell hyaluronic acid suggesting that CD44 can be used by tumour cells to adhere to endothelium prior to extravasation (Price et al., 1996; Sherman et al., 1994). In addition, introduction of CD44 variants into non-metastasizing tumour cells increased the metastatic ability of these cells (Sherman et al., 1994). Others have shown that CD44 is required for the spreading and invasion of cancer cells. CD44 is expressed at the leading edges of murine carcinoma cells and levels of CD44 decrease as the degree of cellular spreading increases (Ladeda et al., 1998). Furthermore, CD44 on melanoma cells is required for migration on type IV collagen and for invasion of the basement membrane (Knutson et al., 1996). These findings implicate CD44 as a major player in tumour cell invasion of the ECM following transendothelial migration.

(B) Association of Adhesion Receptors with the Cytoskeleton

Cellular contacts with ECM or with other cells requires links between adhesion molecules and the actin cytoskeleton (Gumbiner, 1996). These complexes provide points of contact to generate the traction forces required for cell migration. In fact, disruption of the cytoskeleton has been shown to reduce the transendothelial migration of monocytes in vitro (Kielbassa et al., 1998). Such contacts, therefore, are likely important for tumour cell extravasation. Adhesive forces must be generated at the leading edges of a migrating cell and then broken at the tail end to facilitate this migration (Gumbiner, 1996).

The ECM is linked to the actin cytoskeleton via clusters of integrins in focal adhesions. These structures provide the adhesive interactions that are required for cell migration. Cytoplasmic proteins including protein kinases, talin, α-actinin, vinculin, paxillin and tensin, complex integrins to the actin cytoskeleton in these focal adhesion sites (Huttenlocher et al., 1995; Craig and Johnson, 1996; Dedhar and Hannigan, 1996; Gumbiner, 1996). Vinculin provides an essential link between the cytoskeleton and
integrins during cell spreading (Ezzell et al., 1997). Focal adhesions are likely transient and difficult to visualize in highly motile cells, such as extravasating leukocytes and tumour cells (Gumbiner, 1996).

Dynamic interactions between the cell and the substrate are necessary for cell migration (Huttenlocher et al., 1995). Forward motion of the cell requires protrusion of the leading edge through the polymerization of actin. Dynamic lamellipodial and filopodial structures pull the cell and initiate forward contacts. These structures have dense arrays of actin filaments (Mitchision and Cramer, 1996). A long bundle of actin filaments pointed in the direction of motion is found in filopodial structures. In contrast, lamellipodia contain a meshwork of two sets of actin fibers oriented at 45 degree angles to each other. Therefore, a high concentration of monomeric actin molecules is required at the leading edge of cellular extensions during locomotion. The forward polymerization of actin is balanced by depolymerization at the back of the cell (Mitchision and Cramer, 1996; Welch et al., 1997). Adhesion to the ECM via adhesion molecules stabilize these cellular extensions. The strength of the resulting adhesions must be stronger at the front of the cell than at the back, in order to accommodate the release and retraction of the rear of the cell (Huttenlocher et al., 1995). This asymmetry of adhesive strength permits forward motion. Quick formation and disassembly of these adhesive structures is also a prerequisite for cellular motility.

Junctional clustering of cadherins also requires association with the cytoskeleton (Cowin and Burke, 1996). Cadherins are linked to the cytoskeleton through α- and β-catenin and, occasionally, α-actinin (Munro and Blaschuk, 1996; Wheelock et al., 1996; Barth et al., 1997). α-catenin is absolutely required for linking cadherins to the actin cytoskeleton (Gumbiner, 1996; Barth et al., 1997). Studies completed using chimeric molecules have indicated that β-catenin is dispensable if α-catenin is linked directly to the cadherin cytoplasmic tail. Plakoglobin, a protein also found in desmosomal structures, can substitute for β-catenin in cadherin linkages to the cytoskeleton (Cowin and Burke, 1996;
Wheelock et al., 1996; Barth et al., 1997). Each cadherin monomer in a single strand dimer can associate with either β-catenin or plakoglobin and there appears to be no requirement for each monomer to complex with the same protein. Since α-catenin is incapable of interacting with tyrosine phosphorylated β-catenin, the latter has been suggested to be a regulatory molecule in the process of adherens junction formation. Thus, phosphorylation of β-catenin prevents cadherin interaction with the cytoskeleton and reduces cellular adhesion.

Clearly, interactions of the cytoskeleton with adhesion molecules in focal contacts and in adhesive structures containing cadherins involve complex intracellular events. The assembly and disassembly of these structures are highly regulated and subject to dynamic changes. This organization is fundamental to cell migration and therefore to extravasation.

(C) Effects of Cytokines on Cell Adhesion Molecules

Cytokines elicit many different effects that are usually required to mediate a successful immune response. The overexpression of cytokines is often associated with the progression of disease (Tracey and Cerami, 1993). Vascular endothelial cells can be induced by cytokines to express altered levels of various cell adhesion molecules. It has been shown, for example, that levels of ICAM-1 can be increased following TNFα, IL-1, and interferonγ (INFγ) stimulation (Cartwright et al., 1995). E-selectin and VCAM-1, on the other hand, are induced by TNFα and IL-1 but not IFNγ. TNFα can also stimulate endothelial αβ3 integrin expression (Varner and Cheresh, 1996). The time required to observe the changed expression of cell adhesion molecules varies between molecules and inducers. The response of endothelial cells to cytokine stimulation may play a major role during tumour cell extravasation. Cytokine stimulation has also been shown to have profound effects on cell adhesion and cell motility by intracellular signalling, as serum factors have been shown to affect Rho, Rac and Cdc42-mediated cytoskeletal organization.
(see below) (Gumbiner, 1996). Tumour cells can express a variety of cytokines which can mediate such changes in endothelial cells. On the other hand, it has been suggested that cytokine production by endothelial cells can increase levels of integrins expressed by human cancer cells (Boccaletti et al., 1996; Narita et al., 1996). Significantly, cytokine-stimulated endothelial cells have been shown to increase melanoma metastasis in vivo (Scherbarth and Orr, 1997).

TNFα can be found attached to cell membranes but also in a soluble form. Thus, this cytokine is capable of both paracrine and juxtacrine signalling mechanisms (Fagotto and Gumbiner, 1996). Macrophage and endothelial cells are both capable of TNFα production along with cancer cells, lymphocytes, glial cells and adipocytes (Tracey and Cerami, 1993; Fagotto and Gumbiner, 1996). There are two TNFα receptors and these are largely co-expressed in most tissues. Ligand binding to these receptors results in the activation of separate signalling pathways through non-identical cytoplasmic domains. Interestingly, INFγ is known to upregulate the expression of both TNFα receptors (Tracey and Cerami, 1993).

TNFα has been shown to activate CD44 and increase levels of E-selectin and VCAM-1 on fetal astrocytes (Hurwitz et al., 1992; Maiti et al., 1998). TNFα also increases the expression of VCAM-1 and E-selectin on endothelial cells (Iademarco et al., 1995; Bradley and Pober, 1996). This upregulation was shown to occur through the sphingosine kinase pathway involving the stimulation of extracellular signal regulated kinase (ERK) and nuclear factor-κB (NF-κB) (Xia et al., 1998). By other mechanisms, this cytokine will induce the phosphorylation of eukaryotic initiation factor-4E (eIF-4E) and Jun by c-Jun N-terminal kinase (JNK) (Marino et al., 1996; Moriguchi et al., 1997; Xia et al., 1998).

In addition to inducing CAM expression, TNFα can also stimulate the production of other cytokines and their receptors (Kozawa et al., 1997; Lugli et al., 1997). These other cytokines can complement or inhibit the effects of TNFα (Tracey and Cerami, 1993).
Therefore, it is often difficult to assign the function and effects of different cytokines \textit{in vivo}. Given these facts and the plethora of cytokines and growth factors which can influence extravasation, it is likely that cytokines influence cancer progression and metastasis.

(D) Signalling via Adhesion Receptors

(i) Signalling via Integrins

Integrins can affect signalling in two ways (Keely \textit{et al.}, 1998). ECM adhesion by integrins can directly stimulate signal transducers. On the other hand, integrins can modulate signals initiated by growth factors. The assembly of focal adhesions is regulated by intracellular signalling and binding of ECM proteins (Gumbiner, 1996). Integrin-mediated adhesion and actin stress fiber assembly in melanoma cells was shown to require the activity of phosphatidylinositol 3-kinase (Metzner \textit{et al.}, 1996). Furthermore, ligand binding and integrin clustering are required for focal adhesion assembly (Galbraith and Sheetz, 1998). The actin cytoskeleton is then recruited to the site. FAK and phosphorylated forms of tension and paxillin seem to be required for this process. Tyrosine phosphorylation of FAK occurs upon ligand binding and aggregation of $\beta_1$ and $\beta_3$ integrins (Dedhar and Hannigan, 1996). Paxillin and tensin are consequently phosphorylated by FAK or some other FAK stimulated kinase. These phosphorylation events occur prior to actin association with focal adhesions. Integrins have also been shown to activate mitogen activated protein kinase (MAPK) by a pathway independent of FAK, suggesting integrins can transduce different sets of signals (Lin \textit{et al.}, 1997). Furthermore, aggregation of integrins has been shown to cause the upregulation of signalling molecules besides FAK, such as ERK and JNK (Galbraith and Sheetz, 1998).
ECM adhesion by integrins also causes increases in cellular pH, cyclin synthesis and formation of phosphatidylinositol 4,5-bisphosphate which can initiate a variety of signalling pathways (Craig and Johnson, 1996; Varner and Cheresh, 1996; Joseph-Silverstein and Silverstein, 1998). These pathways quite possibly stimulate signals involving the GTPases Rho, Rac and Cdc42 which are ultimately responsible for stress fiber formation during cell migration, the development of lamellipodia and the extension of filopodia, respectively (Dedhar and Hannigan, 1996; Keely et al., 1998). Changed expression of these GTPases most likely produce alterations in the cytoskeleton observed in tumour cells. These alterations might also stem from differences in levels of upstream or downstream effectors of these proteins. The exchange of GDP for GTP on these proteins, for example, is accomplished by guanine nucleotide exchange factors while the reverse is catalysed by GTPase activating proteins. In fact, lymphocyte invasion was promoted by the over-expression of Tiam-1, a Rac nucleotide exchange factor (Keely et al., 1998). These and other proteins which control the GTP bound state of Rho, Rac and Cdc42 are currently under intense investigation.

β3-endonexin associates with the integrin β3 subunit, while the serine-threonine kinase, integrin linked kinase (ILK), has been shown to associate with β1, and β3 integrin subunits (Dedhar and Hannigan, 1996; Boudreau and Bissell, 1998; Hemler, 1998). ILK overexpression is associated with a down-regulation of E-cadherin (Wu et al., 1998). Since ILK has been shown to regulate β-catenin signalling and LEF-1 transcription factor expression, this kinase might provide a link between integrins and the regulation of cadherins by signalling through the Wnt pathway (see below) (Hemler, 1998; Novak et al., 1998). Integrins might also influence growth factor signalling, as integrin clustering has been shown to recruit growth factor receptors to focal adhesions. α,β1, and β3 integrins have, for example, been found associated with active forms of the receptors for platelet-derived growth factor and insulin (Boudreau and Bissell, 1998).
(ii) Signalling via Cadherins

It has been shown that the establishment of adherens junctions, much like the formation of focal contacts, requires Rho and Rac-mediated signalling (Braga et al., 1997). In addition, phosphorylation of catenins can lead to a down-regulation of cadherin adhesion (Barth et al., 1997). Decreases in cell adhesion and migration following the phosphorylation of β-catenin and plakoglobin might be due to the activation of the hepatocyte growth factor and epidermal growth factor receptor tyrosine kinases.

It has been suggested that cadherins and integrins work together to affect cell adhesion and migration (Boudreau and Bissell, 1998). During integrin-mediated migration of neural crest cells on fibronectin, for example, N-cadherin levels were reduced in intercellular contacts. Furthermore, the absence of integrin mediated adhesion by antibody or peptide inhibition, resulted in an increase of N-cadherin-mediated cell clustering (Monier-Gavelle and Duband, 1997). In addition, N-cadherin-mediated intercellular contact between α5-expressing myoblasts resulted in decreased cellular motility. Inhibition of the α5-mediated adhesion prevented intercellular N-cadherin interactions, thus restoring myoblast cell migration (Huttenlocher et al., 1998). In light of these findings, it is not difficult to envisage that cadherins and integrins co-operate during transendothelial migration.

β-catenin and plakoglobin display homology to the protein produced by the Drosophila segment polarity gene armadillo (arm) (Cowin and Burke, 1996; Wheelock et al., 1996; Peifer, 1997; Barth et al., 1997). The ARM protein is involved in a signalling pathway stimulated by Wingless in Drosophila. In Xenopus a similar pathway signals through β-catenin following initiation by Wnt. Cadherin-mediated cell junction formation and transduction of the Wnt signal have been shown to be mediated by both β-catenin and ARM (Gumbiner, 1996; Barth et al., 1997). Cadherins are not directly involved in β-catenin signal transduction, but have an influence on the cytosolic levels of unbound β-
catenin. High cadherin levels can reduce β-catenin-mediated signalling through Wnt (Cowin and Burke, 1996; Gumbiner, 1996; Barth et al., 1997). Interestingly, recent work has suggested that α-catenin can also influence the Wnt pathway (Sehgal et al., 1997). β-catenin-mediated responses include changes in cell shape, adhesion and proliferation. Thus, cell adhesion and signalling may cooperate through β-catenin and plakoglobin. Increased cell adhesion might decrease signal transduction and vice versa. By these means, perhaps cadherins act as suppressers of cell invasiveness and metastasis. Decreased E-cadherin expression, for example, leads to increased malignant behaviour of cells (Gumbiner, 1996; Barth et al., 1997).

When excess levels of β-catenin and plakoglobin exist in the cytoplasm, these proteins can be found in the nucleus even though neither protein contains a nuclear localization signal (Rubinfeld et al., 1997; Kim et al., 1998). Complexes of β-catenin and T-cell factor (TCF) or lymphoid enhancer-binding factor-1 (LEF-1) are potent transcriptional activators in experimental models (Barth et al., 1997; Gumbiner, 1997; Korinek et al., 1997; Peifer, 1997; Hsu et al., 1998). Interestingly, the E-cadherin gene has a LEF-1 consensus sequence in its promoter (Ben-Ze'ev and Geiger, 1998). Certain sites of phosphorylation on β-catenin and plakoglobin target these proteins for degradation. Glycogen synthase kinase (GSK) has been suggested to mediate such phosphorylation (Ben-Ze'ev and Geiger, 1998). GSK is down-regulated upon Wnt stimulation (Cowin and Burke, 1996). Decreased GSK expression is associated with increased levels of β-catenin and plakoglobin. Adenomatous polyposis coli (APC) is another protein which will bind and down-regulate levels of both β-catenin and plakoglobin (Cowin and Burke, 1996; Peifer, 1997).

Expression of APC acts as a tumour suppresser in certain forms of colon cancer and melanoma (Gumbiner, 1997; Korinek et al., 1997; Rubinfeld et al., 1997). Mutant APC is often associated with increased β-catenin levels and the onset of these malignancies (Barth et al., 1997; Morin et al., 1997; Peifer, 1997; Rubinfeld et al., 1997). Gene
activation resulting from β-catenin mediated signaling may inhibit apoptosis and stimulate cell division (Ahmed et al., 1998; Ben-Ze'ev and Geiger, 1998). Perhaps wild-type APC overexpressed in such cells can decrease β-catenin levels in the cytoplasm and nucleus as well as decrease the amount of β-catenin complexed with transcription factors (Korinek et al., 1997). Also, mutant β-catenins might have lost their APC binding regions or amino-terminal sites required for GSK phosphorylation, thus preventing their degradation in colon cancer and melanoma (Ben-Ze'ev and Geiger, 1998; Morin et al., 1997; Rubinfeld et al., 1997). Interestingly, melanoma cells can present mutated forms of β-catenin in association with HLA molecules which may be recognized by T-cells (Robbins et al., 1996).

(E) Cell-Cell Communication via Gap Junctions

Recent studies on gap junction formation during extravasation have yielded provocative insights into the requirement of gap junctions and the resulting communication during transendothelial migration. The main endothelial connexin proteins include connexins 43, 40 and 37 (Dejana et al., 1995). Melanoma cells will form connexin 43 mediated gap junctions with endothelial monolayers in an adhesion-dependent fashion (El-Sabban and Pauli, 1994). The transfection of connexin cDNA increased the invasive properties of cultured tumour cells (Graeber and Hulser, 1998). Moreover, higher levels of connexin 43 were observed with greater metastatic ability of melanoma cells and this correlated with increased dye transfer from tumour cells to vascular endothelial cells in vitro (El-Sabban and Pauli, 1991). These contacts have been suggested to mediate the transfer of metabolites between the interacting cells and thereby facilitate transendothelial migration and metastasis. One metabolite suggested to mediate these effects is 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), a lipoxygenase metabolite of arachidonic acid. Lipoxygenase metabolites have been shown to be involved in the expression of endothelial
adhesion molecules and to stimulate extravasation of monocytes in a PKC-dependent
mechanism (Sultana et al., 1996).

Tumour cells express 12(S)-HETE and this expression correlates with the degree of
experimental metastasis (Chen et al., 1994; Tang and Honn, 1994). 12(S)-HETE is also
known to influence the arrangement of the tumour cell cytoskeleton (Timar et al., 1993).
Melanoma cells were induced by 12(S)-HETE to spread on fibronectin and this involved
the phosphorylation of FAK (Tang et al., 1995). In addition, tumour cell production of
12(S)-HETE was increased upon contact with the endothelium (Honn et al., 1994).

12(S)-HETE can also cause a rearrangement of the cytoskeleton and the retraction
of microvessel endothelial cells. This retraction is accompanied by a redistribution of
CD31, \(\alpha_\beta_1\) and \(\alpha_\beta_3\), away from cell-cell contacts to the non-contact cell surfaces (Tang et al., 1993; Tang et al., 1993; Honn et al., 1994; Tang and Honn, 1994). The resulting
increase in the surface accumulation of \(\alpha_\beta_3\) has been implicated in the adhesion of tumour
cells to the endothelium (Tang et al., 1993).

Interestingly, using cultured human keratinocytes it was found that interaction of
integrin \(\alpha_\beta_1\) with laminin 5 increased the assembly and function of connexin 43 containing
gap junctions (Lampe et al., 1998). These effects were a result of Rho mediated signalling.
These data suggest that adhesion to ECM molecules can differentially regulate the nature of
intercellular contact and the formation of gap junctions. Such findings, together with those
described above, suggest that gap junctions and the resulting intercellular communication
might influence tumour cell transendothelial migration.

(F) Role of Proteolytic Enzymes in Tumour Cell Extravasation

Proteolytic enzymes are thought to be important for cancer metastasis on two
fronts. First of all, for invasion of tumour cells through the ECM as they leave the host
tissue and migrate to blood vessels prior to intravasation. Secondly, enzymatic digestion of
ECM is required following extravasation to permit tumour cell invasion into a secondary site. Furthermore, angiogenesis is necessary for tumour development. This is another step of malignancy requiring proteolytic digestion of the ECM to permit the growth and invasion of newly formed blood vessels, which supply nutrients and oxygen to the expanding tumour cell population.

The main enzymes involved have been classified into several categories including plasmin and plasminogen activators, cysteine proteinases and metalloproteinases, such as the matrix metalloproteinases (MMPs) (Basbaum and Werb, 1996; Johnsen et al., 1998). These soluble enzymes are concentrated to the pericellular region, or found directly attached to the plasma membrane of invading cells (Basbaum and Werb, 1996). Soluble MMP-2, for example, is localized to the cell membrane through a complex of tissue inhibitor of metalloproteinases-2 (TIMP-2) and membrane-type MMP-1 (MT-MMP-1) (Strongin et al., 1995; Basbaum and Werb, 1996). Alternatively, MMP-2 has the unusual feature of being coupled to the leading edge of migrating cells via the α₁β₃ integrin (Basbaum and Werb, 1996; Brooks et al., 1996). On the other hand, plasmin is secreted by the liver as the inactive proenzyme plasminogen (Johnsen et al., 1998). Plasminogen is activated to plasmin when bound to its cell surface receptor by urokinase-type plasminogen activator. The consequent plasmin activity is localized to the cell surface as the activator must also bind to a receptor on the plasma membrane to be activated. Receptors, therefore, concentrate plasmin and plasminogen activator to the cell membrane (Kramer et al., 1994; Basbaum and Werb, 1996; Johnsen et al., 1998). Plasmin has been found at the invasive front of melanoma lesions and is activated by sulfated glycosaminoglycans (de Vries et al., 1996; Brunner et al., 1998). Interestingly, the urokinase plasminogen activator receptor can also bind ECM vitronectin and thereby influence the adhesive function of β₁ integrins (Basbaum and Werb, 1996; Chapman, 1997). Specific inhibitors target plasminogen activators, as TIMPs block MMP function, in an effort to control the proteolysis of these enzymes (Johnsen et al., 1998). When concentrated on the cell surface, proteolytic
enzymes can still function even in the presence of high concentrations of soluble inhibitors (Werb, 1997).

Proteolytic digestion of the ECM has been recognized to be fundamental to the progression of cancer for many years. In fact, many MMP genes were originally cloned from tumour cell lines (Basbaum and Werb, 1996). It is known that cancer involves aberrant regulation of proteinase functions. However, malignant tumours do not display common patterns of gene expression to permit universal management of malfunctioning proteolysis (Johnsen et al., 1998). Furthermore, stromal cells are the source of most of the involved proteinases (Basbaum and Werb, 1996; Werb, 1997). Tumour cells, therefore, must stimulate the expression of these proteins to meet their needs during invasion. It has been suggested that tumour cells even regulate protease secretion from endothelial cells during extravasation (Cottam et al., 1996). For instance, extracellular matrix metalloproteinase inducer (EMMPRIN) is an Ig superfamily member, which is shed from tumour cell vesicles and induces the expression of stromal cell MMPs (Basbaum and Werb, 1996).

Metalloproteinases have been suggested to have no effect on extravasation, but have been shown to be required for tumour invasion and growth at secondary sites (Koop et al., 1994). Blocking protease function can decrease tumour growth (Werb, 1997; Noel et al., 1998). Ectopic expression of TIMPs has been shown to control tumourigenesis and metastasis (Khokha, 1994; Martin et al., 1996; Werb, 1997; Kruger et al., 1998). Furthermore, proteolytic cleavage of ECM can expose cryptic sites that may cause cells to function differently than if interacting with the full-length protein (Werb, 1997). MMP-2, for example, was recently shown to degrade laminin-5 and thereby produce a proteolytic fragment capable of inducing tumour cell migration (Giannelli et al., 1997). In addition, MMP-2 can process type I collagen to permit α3β1 integrin-mediated adhesion in preference to adhesion via α2β1 to intact collagen (Werb, 1997).
III. Models for the Study of Tumour Cell - Endothelial Cell Interactions

Models, both in vivo and in vitro, provide controlled environments in which to examine the process of extravasation. Different model systems permit the analysis of different stages of the process and together, the various data obtained can be pieced together to formulate a better picture of the process. No single model system can provide all the necessary information. In vitro models do not duplicate all of the factors influencing the process in the natural physiological environment. In contrast, in vivo systems are too complex to dissect out the role of each of the important players.

The first models of transendothelial migration were developed for leukocytes. The initial studies simply examined the ability of various leukocytes to adhere to surfaces coated with different ECM molecules, in an effort to determine the adhesive interactions involved. These in vitro studies were then advanced to use endothelial cells grown on coverslips and filters, supported by various ECM formulations (Carlos and Harlan, 1994). Most of these studies focused upon adhesion to, and migration on, the surface of the endothelial cells. The introduction of cytokines permitted an analysis of diapedesis, as most leukocytes did not spontaneously penetrate the endothelium. Pretreatment of endothelial monolayers with IL-1 and TNFα promoted leukocyte transmigration (Carlos and Harlan, 1994). Antibody and peptide blocking experiments and the utilization of cells transformed to over- or under-express adhesion proteins, yielded data on the involvement of adhesion molecules in these processes. Studies conducted in vivo often made use of soluble recombinant CAMs injected into mice to compete for natural ligands in the process of leukocyte extravasation (Albelda et al., 1994). Antibodies and peptides have also been used. These studies depend on the observation and analysis of organ tissue sections for the appropriate hematopoietic cell infiltration. Often the mice used in these experiments are
transgenic or knock-outs for the particular molecule of interest. Similar methods are now being applied to studies of tumour cell extravasation.

(A) Melanoma Cells as Model Tumour Cells

Melanoma cell lines have been used in many in vitro and in vivo studies on the various stages of extravasation and metastasis. There are many reasons which make these particular tumour cells desirable in model systems. First of all, melanoma has been studied extensively and the progression of this cancer has been categorized into well defined stages (Weterman et al., 1994). Secondly, many highly studied human cell lines have been isolated from each of these stages (Herlyn et al., 1985; Herlyn et al., 1990). Furthermore, melanoma cells do not roll on endothelium, even when exposed to shear forces (Goetz et al., 1996). This fact is particularly important for an in vitro model since other cancers, such as colon and breast malignancies, behave differently under flow conditions (Giavazzi et al., 1993). This feature can also make comparisons between in vivo and in vitro results more feasible. Finally, much work has been done examining the cytokine requirements of these lines (Weterman et al., 1994). This again, is important with regards to culture systems, such that a base line of molecular expression can be established for each particular set of experimental conditions (Rodeck et al., 1987; Kath et al., 1991; Lu et al., 1992; Kobayashi et al., 1994; Rak et al., 1994).

(B) Models of Tumour Metastasis

To date, most work examining tumour cell extravasation has been accomplished using several in vitro models. Most in vivo scenarios utilize the same techniques as the inflammation models described above. Other semi-in vivo models have utilized the chicken chorioallantoic membrane (Chambers et al., 1982).
Early in vitro studies examining the process using tumour cells and cultured endothelial monolayers, helped outline the stages involved in the process and suggested unique features of tumour cell extravasation (Kramer and Nicolson, 1979; Nicolson, 1982; Ohigashi et al., 1989). Other studies utilized alternative monolayers to determine how tumour cells interact with stromal cells and mesothelium during invasion (Roos et al., 1981; Akedo et al., 1986; Horai et al., 1992). The invasion of malignant cells into an artificial blood vessel wall has also been examined (Jones et al., 1981). The vessel wall was simulated using cultures combining endothelial cells with smooth muscle cells. The muscle cells produced an ECM and the digestion of this structure by the tumour cells was monitored. Tumour cells multiplied in the region between the two cell types. Another early model made use of plasma clot cultures (Nicosia et al., 1986). In this case, aggregates of endothelial cells were cultured in a rotary shaker. Such clusters of endothelial cells permitted the formation of vascular channels and allowed an examination of the process of invasion by tumour cells into these structures.

More recent advances have largely focused upon changes in the endothelial cells mediated by extravasating tumour cells. It was discovered that endothelial cell Ca\(^{2+}\) levels increase and that these cells can be stimulated to undergo apoptosis upon exposure to extravasating tumour cells (Kebers et al., 1998; Lewalle et al., 1998). Despite these novel advances in the field of tumour cell extravasation, little is known about the complexities of these processes at the molecular level.

(C) Cancer Therapy Based On Tumour Membrane Components

Knowledge of specific molecules involved in the process of metastasis should permit the development of therapeutic strategies to control the spread of cancer cells. Some work has begun using identified melanoma associated antigens in antibody therapies. To accomplish this, extensive work is underway to identify progression markers with
heightened expression at specific stages in the development of this cancer (Weterman et al., 1994). The goal of these studies is to induce a host immune response to the tumour cells (Merimsky et al., 1994). Often, these monoclonal antibodies are derived against entire melanoma cells or are selective for specific glycoproteins or glycolipid molecules. Several attempts have also been made to conjugate plant toxins to these antibodies in order to poison the tumour (Merimsky et al., 1994).

Scientists are also striving to develop cancer vaccines as another means to stimulate an immune response in cancer patients. Many of these have been developed in an effort to treat melanoma and some have reached clinical trials. (Bystryn, 1998). The rationale for such therapies stems from the fact that melanoma cells differ from normal melanocytes and these variations may be sufficient to stimulate and mount an immune response to melanoma cells if the antigens are correctly presented. The ultimate desire is to use a single antigen or a limited number of purified antigens to stimulate the immune system. The problem is, however, that melanoma, like other malignancies, is antigenetically heterogeneous. In any case, murine studies have shown that melanoma development is effectively prevented if the mice are vaccinated prior to lethal injections of melanoma cells (Bystryn, 1998). These melanoma vaccines consist of several different antigen preparations and they can be non-purified, irradiated whole cells or differentially treated extracts of cells from a number of different donors, purified antigens and partially purified antigens (Soiffer et al., 1998). The limited results available from clinical trials have been positive. Some vaccines have lead to improved life-spans and others improved the quality of life of patients during treatment due to decreased side effects (Bystryn, 1998).

The more we understand about the process of metastasis, the more targets will become available to these novel cancer therapies. Given the growing success of anti-inflammatory techniques, excellent cancer treatments targeting adhesion molecules are a real possibility. An understanding of the process of tumour cell and endothelial cell interactions will aid in the search for unique molecules that are critical to successful cancer treatment.
IV. Objectives of the Thesis

The overall purpose of the studies conducted and contained in this thesis was to dissect the complexities of the process of tumour cell extravasation. This was achieved by paying particular attention to the involvement of the cytoskeleton and adhesion molecules at specific stages of diapedesis. The main objectives were: (1) to develop an in vitro system with which to outline the stages of tumour cell transendothelial migration using the laser scanning confocal microscope, (2) to analyze the actin cytoskeletal dynamics in both the tumour cells and the endothelium during the process, (3) to evaluate the role of CD31 during tumour cell diapedesis, and (4) to examine the involvement of L1 and the $\alpha_v\beta_3$ integrin during extravasation.

A description and characterization of the in vitro system is contained in chapter two. The outline of the stages involved during tumour cell transendothelial migration and the consequent reorganization of the actin cytoskeleton are included in this chapter. Changes in the actin cytoskeleton were observed at different stages of melanoma cell transendothelial migration, suggesting a highly dynamic, interactive process which involved active participation from both melanoma and endothelial cells.

Also in chapter two, an examination of the involvement of TNF$\alpha$ during melanoma transendothelial migration was conducted. The rationale behind this investigation stemmed from the fact that cytokines, such as TNF$\alpha$, are upregulated during disease states, including cancer. Since TNF$\alpha$ can increase adhesion molecule expression on both melanoma and endothelial cells, not to mention the ability to regulate the expression of other cytokines and signalling factors, it was important to determine if these features can directly influence transendothelial migration. Significantly, the results show that tumour cells which are not known for their ability to form secondary tumours in mice, can be induced to undergo transendothelial migration upon exposure to TNF$\alpha$. 
Since the cell adhesion molecule CD31 is required for diapedesis of most leukocytes, it presented a likely candidate for an adhesion molecule important for tumour cell diapedesis. Chapter three presents the significant finding that the binding of melanoma cells to endothelium leads to the redistribution of CD31 from endothelial cell contacts. Furthermore, in contrast to leukocytes, melanoma cells do not require CD31-mediated adhesion during extravasation. These discoveries pointed to a major difference between tumour cell transmigration and the extravasation of leukocytes.

Chapter four examines the role of L1 and $\alpha_\beta_3$ integrin during melanoma cell transendothelial migration. Previous publications have shown that $\alpha_\beta_3$ integrin on melanoma cells supports adhesion to L1-coated surfaces. In addition, L1 is secreted by malignant melanoma cell lines and associated with laminin in solid tumours. We, therefore, hypothesized that an interaction between L1 and $\alpha_\beta_3$ would be involved in tumour cell extravasation. Both L1 and $\alpha_\beta_3$ were observed in heterotypic contacts between melanoma and endothelial cells as well as in the contact regions between tumour cells and the Matrigel matrix. Antibody inhibition studies suggest that these cell adhesion molecules play a significant role during transmigration.

An appendix is also included which outlines an initial study examining the role of cadherins during tumour transendothelial migration. Significantly, antibody inhibition experiments indicated that N-cadherin is required for early stage adhesion of melanoma cells to endothelium during extravasation.

In summary, these studies have highlighted the important roles of several cell adhesion molecules on melanoma cells and endothelial cells during extravasation. Fundamental differences between tumour cell and leukocyte transendothelial migration also became evident. Furthermore, this thesis has shown that cytokines can have a direct influence on diapedesis and identified key players involved in the cascade of adhesive interactions required for the process. Future work will ultimately identify other adhesion
receptors and factors involved in tumour cell transendothelial migration, thus leading to a better understanding of cancer metastasis.
CHAPTER TWO

Cell Shape Changes and Cytoskeleton
Reorganization during Transendothelial
Migration
of Human Melanoma Cells

Portions of this chapter have been published as
E.B. Voura, M. Sandig, V.I. Kalnins and C.-H Siu

Dr. Martin Sandig contributed to the actin analysis.
I. INTRODUCTION

Cancer metastasis is a complex process which involves a variety of interactions between tumour cells and host tissues (Nicolson, 1988; Stetler-Stevenson et al., 1993). Metastasizing cells must first separate from the primary tumour and enter the circulatory system. Those cells that survive the hostile environment of the blood stream will eventually arrest in the capillary beds of specific organs. Here tumour cells will adhere to the surface of the endothelium and extravasate by migrating between the endothelial cells (ECs) of the capillaries. Successful extravasation is accompanied by cell proliferation, invasion of the surrounding tissue and the development of metastases. The endothelium provides a natural barrier to metastasis formation. A better understanding of the mechanisms involved in transendothelial migration of cancer cells, therefore, is important for the development of effective cancer therapies.

To date, the majority of work examining adhesive interactions in transendothelial migration has focused on leukocytes (Albelda et al., 1994; Carlos and Harlan, 1994; Picker, 1994; Stewart et al., 1995; Dunon et al., 1996; Voura et al., 1997). Work on the transendothelial migration of cancer cells has primarily involved the use of metastatic cell lines in in vivo models and examinations of in situ morphological characteristics (Albelda et al., 1990; Lauri et al., 1990; Kuzu et al., 1993; Renard et al., 1994; Garofalo et al., 1995). However, there have been few studies describing the passage of tumour cells through the endothelial lining. We have, therefore, developed an in vitro system that allows detailed examination of the transendothelial migration of tumour cells in a controlled environment.

In our model system, melanoma cells are seeded on a confluent layer of endothelial cells and their migration through the endothelium is monitored by microscopy. Melanoma cells have been chosen for this study since melanoma is one of the best characterized human malignancies. It has been classified into five clinically identifiable stages (Herlyn et al., 1990) with lesions ranging from some that are considered benign to those which are
highly metastatic. Consequently, melanoma provides an excellent starting point for studies of tumour progression. The WM series of cell lines used in this study provides a wide range of well characterized and commonly used human melanoma cells isolated from different stages of metastatic progression (Rodeck et al., 1987; Herlyn et al., 1985; Herlyn et al., 1990; Kath et al., 1991; Lu et al., 1992; Kobayashi et al., 1994). It is interesting that, in contrast to leukocytes and other tumour cells, melanoma cells simply adhere to, and do not roll on, endothelium under conditions of flow in vitro (Giavazzi et al., 1993; Albelda et al., 1994; Carlos and Harlan, 1994; Picker, 1994; Stewart et al., 1995; Goetz et al., 1996).

In this chapter, we have used laser scanning confocal microscopy (LSCM) to investigate the interactions between ECs and transmigrating melanoma cells. It is known that the cell-cell and cell-substratum adherens type junctions of ECs are associated with a dynamic network of cytoplasmic microfilaments and that these filaments are required for cell migration. We have, therefore, focused our studies on the changes of the F-actin cytoskeleton in both cell types, as the melanoma cells adhere to and migrate through the endothelial monolayer. The results obtained depict dynamic changes in the cell shape and the actin cytoskeleton of both cell types and provide evidence for the active participation of ECs in the transmigration of melanoma cells. The effects of tumour necrosis factor (TNF)α on several melanoma cell lines were also investigated. TNFα promoted the transendothelial migration of the non-metastatic cell line, WM35. This observation thus defines cell attachment and migration through the monolayer as two distinct steps, and suggests that cytokines, such as TNFα, can enhance the metastatic potential of tumour cells.
II. EXPERIMENTAL PROCEDURES

Cells and culture conditions

Human umbilical vein endothelial cells (HUVEC) and human lung microvascular endothelial cells (HMVEC) were purchased from Clonetics (San Diego, CA). HUVEC were cultured in endothelial growth medium (EGM), which contained 500 mL of EC basal medium, supplemented with 2 mL of 3 mg/mL bovine brain extract, 0.5 mL of 10 μg/mL human epidermal growth factor, 10 mL fetal bovine serum (FBS) (at a final concentration of 2%), 0.5 mL of 1 mg/mL hydrocortisone, 0.5 mL of 50 mg/mL gentamycin sulfate and amphotericin-B, all of which were purchased from Clonetics. HMVEC were cultured in EGM-2MV medium (Clonetics, San Diego, CA). 5 mL of 1000 U/mL penicillin and streptomycin (Gibco/BRL, Gaithersburg, MD) were also added to 500 mL of the medium.

The human melanoma cell lines, WM9, WM35, WM239 and WM115, were provided by Dr. Meenhard Herlyn of the Wistar Institute, Philadelphia, PA. All melanoma cell lines were cultured in RPMI (Roswell Park Memorial Institute)-1640 medium (Ontario Cancer Institute Media Kitchen, Toronto, ON), supplemented with penicillin and streptomycin at 2 U/mL and 10% FBS. All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂/air.

Antibodies

The LM609, function blocking, monoclonal antibody for α,β₃, was obtained from Drs. David Cheresh and Anthony Montgomery of the Scripps Research Institute (La Jolla, CA). Connexin 43, β₁, β₃, and L1 monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). The α, polyclonal antibody was purchased from Chemicon International (Temecula, CA).
Transendothelial migration assay

Round glass coverslips (12 mm in diameter and 1 mm thick) were coated with Matrigel purchased from Becton/Dickinson (Bedford, MA) at 1:8 dilution. Matrigel was diluted in ice-cold H₂O and 200 μL was applied to cold coverslips placed in 24-well plates. Exactly 100 μL of Matrigel was subsequently removed and the remaining amount was air dried overnight in a laminar flow hood at room temperature and then rehydrated in Hank's buffered saline solution (HBSS) (Ontario Cancer Institute Media Kitchen, Toronto, ON). Following rehydration of the Matrigel, the coverslips were transferred to 35-mm dishes. The Matrigel formed a thin layer of extracellular matrix material which optimized EC attachment and permitted subsequent examination by LSCM. ECs (1.5 x 10⁵ cells taken between passages 2 to 8) were added to the Matrigel-coated coverslips in a 200 μL drop of EGM or EGM-2MV and allowed to settle for 3 to 4 hours. Unless noted otherwise, transmigration assays were routinely done with HUVEC. Coverslips were then transferred carefully to 24-well plates and incubated in 0.5 mL EGM or EGM-2MV with TNFα (10 ng/mL) (Gibco/BRL, Gaithersburg, MD). Phase microscopy showed that confluent EC monolayers were achieved after 12 hours of culture and they rarely showed any gaps under these conditions. Melanoma cells were then added to the monolayers.

Melanoma cells were removed from culture dishes using 4 mM EDTA in HBSS and subsequently labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR). The 2.5 mg/mL DiI stock solution was diluted 1:200 with HBSS and added to the cells for 10 minutes at 37°C. The DiI-labelled cells were washed three times in 2 mL HBSS and the final pellet was resuspended in EGM at a concentration of 1.76 x 10⁶ cells/mL. DiI-labelled melanoma cells (8.8 x 10⁴ cells in 50 μL) were then added to the EC monolayer and incubated for different time intervals prior to fixation and staining. TNFα was included in co-cultures whenever TNFα-stimulated EC
monolayers were used. Unless noted otherwise, WM239 cells were routinely used in transmigration assays.

Specific TNFα stimulation of WM35 cells was accomplished via a preincubation of the WM35 cells in a rotating suspension at 37°C with 10 ng/mL TNFα for 30 minutes before washing in HBSS and seeding on an EC monolayer that had not been stimulated by TNFα. Experiments using cycloheximide inhibition of TNFα effects were carried out by incubating the WM35 melanoma for a further 30-minute period in 10 μg/mL cycloheximide in the rotating suspension at 37°C prior to a subsequent incubation with TNFα as described above. Inhibition using the LM609 antibody in these experiments was accomplished during the TNFα incubation step. The antibody was simply added to the suspension solution to yield a concentration of 40 μg/mL.

**Fixation, extraction and staining**

Cells were fixed using 3.5% (w/v) paraformaldehyde at 22°C for 5 minutes and washed three times (3 minutes each) in PBS, pH 7.4. Cells were then extracted for 5 minutes in a cytoskeleton stabilizing buffer, pH 6.9, containing 0.1 M 1,4-piperazinebis(ethanesulfonic acid) (Aldrich, Milwaukee, WI.), 1 mM EGTA, 4% (w/v) polyethylene glycol 8000 and 0.1% Triton-X 100 (Opas and Kalnins, 1985). The extraction was followed by another series of washes and a 5-minute blocking step in 1% (w/v) BSA. Cells were then labelled with a 1:10 dilution of dipyrrrometheneboron difluoride (BODIPY FL)-conjugated phallacidin (Molecular Probes, Eugene, OR.) in the blocking solution for 45 minutes at 22°C. Coverslips were then washed three times (3 minutes each) in PBS and mounted. Strips cut from plastic coverslips (stacked two high) were used as spacers to avoid squashing the cells when they were mounted on microscope slides. The mounting medium was composed of 80% glycerol in PBS and contained 2.5% (w/v) of the antioxidant 1,4-diazabicyclo-[2,2,2]-octane (Sigma, St. Louis, MO) as an antibleaching agent. On
occasion, 0.2% (w/v) \( p \)-phenylenediamine (Sigma, St Louis, MO) was added to the mounting medium as a further antioxidant. The preparations were sealed with nail enamel and stored in the dark at 4°C.

**Western blotting**

WM239 cell lysates were prepared from cultures grown in 100 mm dishes upon overnight exposure to HMVEC growth medium (EGM-2MV as described above). WM35 lysates were similarly prepared following 3 or 5 hours culture in EGM-2MV with or without 10 ng/mL TNF\( \alpha \) as indicated. 5 \( \mu \)L of the prepared cell lysates, each having started with approximately two million cells, were separated on 7.5% acrylamide gels by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Proteins were transferred to nitrocellulose at 100 volts for 1 hour. Membranes were blocked for 30 minutes at room temperature with 5% (w/v) skim milk powder in PBS plus 0.5% (v/v) Tween 20. The blots were incubated with primary antibodies overnight at 4°C at a dilution of 1:1000 (v/v) for the L1, \( \beta_1 \), \( \alpha \), and \( \beta_3 \) antibodies and 1:250 (v/v) for the connexin 43 antibody. The overnight incubation was followed by a 1-hour incubation at room temperature. The membranes were washed as suggested for ECL detection (Amersham Life Science, Buckinghamshire UK) and then incubated with 1:1000 (v/v) goat anti-mouse horse radish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA) for 2 hours at room temperature. The membranes were washed again and incubated with the ECL detection reagent as recommended (Amersham Life Science, Buckinghamshire, UK) and exposed to BioMax X-ray film (Kodak, Rochester, NY). All the antibody dilutions were carried out in the blocking solution, while all washes were done in PBS containing 0.5% Tween 20.
Fluorescence microscopy

LSCM X/Y images were obtained using either a Nikon Optiphot microscope, equipped with a 60x objective and the MRC 600 confocal imaging system (Bio-Rad Laboratories, Richmond, CA), or a Zeiss Axiovert 135 inverted microscope equipped with a 63x Neofluor objective and the LSCM 410 confocal attachment. Serial optical sections were routinely taken at 1 μm thickness in a basal to apical direction. Quantitative analysis of transmigration of melanoma cells was carried out using a Wild Leitz Orthoplan microscope equipped with epifluorescence optics.

Quantification of transmigration of melanoma cells

All experiments were done in triplicate unless indicated otherwise. Three times fifteen random fields for a total of 45 fields were scored for each coverslip. Each set of 15 fields contained 100-250 melanoma cells. Triplicate experiments resulted in over 1000 cells being scored for any one time point. All cell counts were carried out on F-actin stained coverslips with the melanoma cells preloaded with DiI for identification. Melanoma cells were divided up into three major categories and scored as described in Figure II.1. The relative distribution of melanoma cells in these categories was also confirmed by LSCM. For comparison, PC12 cells were seeded on the EC monolayer. PC12 cells remained fairly spherical throughout the assay and only those that were clearly showing signs of spreading were scored as transmigrated cells.
III. RESULTS

Establishment of an in vitro model system for melanoma transendothelial migration

An in vitro system was established to investigate the migration of tumour cells through the endothelium. A confluent EC monolayer cultured on a coverslip coated with Matrigel was used as the model endothelium to study the transendothelial migration of melanoma cells (Figure 11.1). The Matrigel was set to provide sufficient thickness to allow penetration of transmigrating cells but thin enough for detailed microscopic examination. To mark the melanoma cells, they were labelled with DiI prior to seeding on EC monolayers. The cultures were fixed at different time intervals after seeding and stained for F-actin using BODIPY FL-conjugated phallacidin for fluorescence microscopy. In general, images of melanoma cells in contact with the endothelium could be divided into three broad categories (Figure II.1). The first category consists of round melanoma cells with "smooth" surfaces attached to the top of the EC monolayer. The second category included cells at various stages of passage through the EC monolayer. These cells were characterized by the presence of numerous prominent surface protrusions and some became partially intercalated between EC. In the third category were melanoma cells with a fibroblast-like shape. These melanoma cells were either enveloped by ECs or located underneath the monolayer. They had come into direct contact with the Matrigel and were spreading on it.

To determine the kinetics of melanoma cell transendothelial migration, WM239 cells were seeded on a monolayer of HUVEC. It took approximately 30 minutes for the melanoma cells to settle on the monolayer and become attached to the ECs. Co-cultures were therefore fixed at 0.5, 1, 3 and 5 hours and melanoma cells belonging to each of the three categories were scored (Figure II.2a). Melanoma cells appeared to initiate the transmigration process soon after their attachment to the EC monolayer, since the number
of round attached cells constituted less than 20% of the melanoma cell population after 30 minutes of co-culture. On the other hand, approximately 80% of the cells already showed signs of transmigration. However, less than 1% of melanoma cells were spreading on the Matrigel. By 1 hour, approximately 15% of the attached melanoma cells were spreading on the Matrigel. The number of spreading cells increased steadily during the next 4 hours. This increase was accompanied by a corresponding drop in the percentage of transmigrating cells. Five hours later, approximately 60% of the melanoma cells showed signs of cell spreading on the Matrigel. Approximately 90% of the melanoma cells completed migration through the endothelium after overnight culture. Cells in the first category attached to the monolayer fairly loosely. Approximately 50% of cells in this category could be removed by agitation and washing (data not shown), indicating that they were loosely attached to the monolayer. In contrast, the number of cells in the latter two categories was not significantly affected by the same treatment, probably due to their firm attachment to ECs and/or the Matrigel.

The rapid penetration of melanoma cells during the first hour of seeding raised the question whether gaps were present in the EC monolayer. Incomplete closure of the EC monolayer would allow melanoma cells to fall through the monolayer and adhere directly onto the Matrigel. As a negative control, we seeded the non-metastatic peochromocytoma PC12 cells on the EC monolayer. Less than 5% of PC12 cells showed signs of spreading on the Matrigel after 5 hours of co-culture (Figure II.2a), indicating that junctional complexes had been formed between ECs and that there were few gaps in the monolayer.

It is known that melanoma cells have a tendency to form metastases in the lung. Therefore, transmigration of WM239 cells was also examined using human lung microvascular endothelial cells (HMVEC) as the model monolayer. Similar results were obtained for monolayers reconstituted with HMVEC (Figure II.2b). However, there was an initial delay of approximately 30 minutes, and only 2% of melanoma cells were in category 3 at 1 hour, compared to 18% for cells seeded on HUVEC. The number of
Figure II.1. Schematic drawing depicting the transendothelial migration assay. Details of the assay system are described in Experimental Procedures. For quantitative analysis, melanoma cells were divided into three major categories: (1) initial attachment of melanoma cells to the endothelial cell monolayer, (2) intercalation of melanoma cell between endothelial cells and (3) spreading of melanoma cell on Matrigel underneath the endothelial cell monolayer.
Coat coverslip with Matrigel.

Add EC. Settle for 3 h.

Stimulate EC with TNFα.

Add melanoma cells.

(1) Cell attachment  (2) Transmigration  (3) Cell spreading on ECM
**Figure II.2.** Kinetics of transendothelial migration by melanoma cells. Dil-labelled melanoma cells were seeded on top of tumour necrosis factor α-stimulated endothelial monolayers and the cultures were examined at different time intervals after seeding. The melanoma cells were separated into the three categories as shown in Figure II.1: attached cells (*open bars*), intercalating cells (*striped bars*), and transmigrated cells (*solid bars*). **a** WM239 cells cultured on a monolayer of human umbilical vein endothelial cells. **b** WM239 cells cultured on a monolayer of human microvascular endothelial cells. The total number of cells counted in each data set was taken to be 100% and the relative percentages of cells in the three categories were calculated. As a negative control, Dil-labelled PC12 cells were co-cultured on top of human umbilical vein endothelial cells. All values for melanoma cells represent the mean of three experiments, while those for PC12 cells were based on two experiments.
WM239 cells in category 3 increased linearly between 1 and 5 hours, and the rate of transmigration across the HMVEC monolayer was similar to that obtained with the HUVEC monolayer (Figure II.2a). By 5 hours, approximately 48% of WM239 cells were spreading on the Matrigel.

**Morphology of ECs and melanoma cells on Matrigel**

Confocal microscopy was used to study the process of melanoma cell migration through the endothelium at higher resolution. First, we examined changes in the actin cytoskeleton of ECs and melanoma cells cultured separately on Matrigel. ECs in a confluent monolayer examined 12 hours after seeding displayed an abundance of peripheral actin microfilaments (Figure II.3a). Stress fibers were often observed in regions where several ECs met. The WM239 melanoma cells, on the other hand, appeared spherical and expressed cortical F-actin, when examined shortly after plating. There was a higher concentration of F-actin along the periphery of the spreading front (Figure II.3b). Spreading melanoma cells could be seen as early as 1 hour after plating. Eventually, the melanoma cells became flattened, acquired a highly irregular shape, with numerous stress fibers and a high concentration of F-actin near the ends of many of the processes (Figure II.3c).

**Appearance of large membrane blebs in heterotypic cell-cell contacts of attached melanoma cells**

Next, we examined co-cultures of WM239 melanoma cells on HUVEC monolayers. Melanoma cells attached on the EC monolayer were identified by DiI staining. Prior to transmigration, melanoma cells invariably had a spherical shape and relatively few membrane protrusions. Cortical F-actin staining was present, but the cells lacked stress fibers (Figure II.4a). Frequently, the melanoma cells were located directly on top of
contact regions between ECs where a high local concentration of F-actin was evident and microfilament bundles from the underlying ECs were seen to terminate (Figure II.4b). Interestingly, these melanoma cells rarely spread on top of the EC monolayer and most of the cells that remained on top of the monolayer were round even after 5 hours of co-culture.

Most melanoma cells attached on the endothelium began to send out lateral pseudopods from the basal areas within a short time, while the upper part of the cell still retained a rounded shape (Figure II.5a). Cortical F-actin staining was evident along the periphery of melanoma cells. Large membrane blebs appeared on the pseudopods (Figure II.5b) and on the ventral surface of the cell (Figure II.5c). Although occasional small blebs were found along the cell surface in other regions, clusters of blebs with a diameter greater than 1 μm were only found along the heterotypic contact regions in the basal part of the melanoma cells. Often prominent membrane blebs extended in several directions from the basal end of the melanoma cell (Figure II.5d). A high concentration of F-actin was present between adjacent blebs. Interestingly, actin bundles from the underlying ECs frequently appeared to terminate near the melanoma membrane blebs (Figure II.5c) or regions between them where a high concentration of F-actin was present (Figure II.5d), suggesting active interactions between the two types of cells in this region. Occasionally, lamellipodial structures filled with F-actin were also found extending from the melanoma cell, that appeared to make contacts with the endothelial surface (Figure II.5e).

Changes in cell shape and actin cytoskeleton during transendothelial migration

During transendothelial migration, melanoma cells underwent drastic shape changes. As a result, melanoma cells differing widely in shape were observed. The images could, however, be arranged in a plausible sequence that reflected different stages in the transmigration process as follows. Melanoma cells penetrating the EC monolayer showed
Figure II.3. Confocal images showing the actin cytoskeleton of endothelial cells and melanoma cells cultured on Matrigel. Cells were examined by laser scanning confocal microscopy. a A confluent monolayer of endothelial cells 12 hours after plating; b an attached melanoma cell 30 minutes after plating; c spreading melanoma cell 3 hours after plating. Bars: 10 μm

Figure II.4. Shape and actin cytoskeleton of adherent DiI-labelled melanoma cells. a An X/Y image (a two dimensional section, through the horizontal plane, parallel to the coverslip) showing a melanoma cell 2 hours after seeding on a monolayer of endothelial cells, as seen in 1 μm sections using confocal microscopy. This optical section was taken through the middle of the round melanoma cell. b An X/Y image showing the actin cytoskeleton of endothelial cells in the region just below the melanoma cell. This optical section was taken 6 μm below a, showing the upper part of the endothelial cell monolayer. The melanoma cell is located directly above this region where endothelial cells join and several stress fibers of the endothelial cells terminate (arrowheads). Bars: 10 μm
Figure II.5. Membrane protrusions along the basal lateral surfaces of melanoma cells attached to the endothelial cell monolayer. Cells were observed by confocal microscopy after staining for F-actin. a-c Three X/Y optical sections through the middle and the basal end of a melanoma cell. a An optical section taken approximately 4 μm from its uppermost surface; b an optical section 3 μm below a; and c a section 4 μm below a. Membrane blebs from the ventral surface of the melanoma cell (M) and microfilaments from the endothelial cell (E) underneath are evident. Bars: 5 μm. d, e Confocal images showing the association of F-actin with membrane blebs and lamellipodial structures extending from the ventral surface of melanoma cells. d A X/Y section showing a high concentration of F-actin in regions between membrane blebs (arrowheads) near the ventral surface of a melanoma cell (M). Endothelial cell microfilaments that end in the actin-rich junctions of membrane blebs are indicated by arrows. e An X/Y image showing prominent lamellipodial structures (arrowheads) extending from the two adjacent melanoma cells (M). Bars: 10 μm
cortical F-actin staining, similar to that seen at earlier stages, but were flatter and often displayed a spindle-shaped morphology. Figure II.6 shows a melanoma cell with the lower half of its body being surrounded by adjacent ECs (panel a) and a pseudopod from its ventral surface penetrating downward through the EC junction (panel b). At a more advanced stage of transmigration, the ECs retracted further allowing more complete intercalation of melanoma cells between the ECs (Figure II.6c, d). Interestingly, the melanoma cells and ECs were always in close apposition and gaps were not detected between these two cell types. Transmigrating melanoma cells continued to exhibit cortical F-actin staining in the cell periphery and retained a spindle-shaped morphology.

Active spreading of ECs over melanoma cells during reclosure of the endothelial monolayer

Endothelial processes arising from adjacent ECs were often seen on top of the intercalated melanoma cells (Figure II.6c). These processes contained prominent microfilament bundles that extended upwards over the dorsal surface of the melanoma cell and terminated at contact sites on the melanoma cell, suggesting that the EC processes were actively spreading on top of the melanoma cell.

Transendothelial migration was only completed when the gap in the EC monolayer was closed by the spreading of EC processes. At this stage, the microfilament bundles in these EC processes stretched completely across the apical surface of the underlying melanoma cell (Figure II.7a,b). Upon contact with the Matrigel, melanoma cells spread along their long axis (Figure II.7c). Panels d to f of Figure II.7 show a well-spread melanoma cell which had invaded the space between ECs and Matrigel and acquired a fibroblastic shape. Cortical F-actin was evident along the periphery of the melanoma cell in regions above the Matrigel (Figure II.7c and e). However, close to the Matrigel, prominent arrays of stress fibers were seen in the spreading melanoma cell (Figure II.7f).
Figure II.6. Confocal images of melanoma cells at different stages of transmigration. 

a, b Confocal images of an ellipsoidal DiI-labelled melanoma cell (M) (arrowheads) penetrating through the endothelium. 

a An X/Y section taken 3 μm from the top, showing the melanoma cell surrounded by endothelial cells (*). 

b An optical section taken 2 μm below a, showing a DiI-stained pseudopod penetrating between the endothelial cells. 

c, d Confocal images of a spindle-shaped melanoma cell (M) situated between endothelial cells (E). 

c An X/Y section showing endothelial microfilaments terminating on their heterotypic contact surfaces. 

d A section taken 1 μm below c, showing cortical F-actin staining in the melanoma cell. 

Bars: 10 μm
Figure 11.7. Confocal series of transmigrated melanoma cells. a-c The top three confocal sections taken at 1 μm intervals of a melanoma cell covered by endothelial processes. The uppermost section shows microfilament bundles in endothelial cell (E) processes stretching over an underlying melanoma cell (labelled M in c). d-f Confocal sections of 1 μm thickness of a melanoma cell spreading on Matrigel. d The uppermost optical section showing microfilament bundles in endothelial cell processes covering a melanoma cell. e A section taken 5 μm below d, showing that the melanoma cell (M) adopts a fibroblast-like shape. f A section taken 1 μm below e, showing stress fibers in the melanoma cell. Bar: 10 μm
Effects of TNFα on melanoma transendothelial migration

To determine whether melanoma cells obtained from various stages of tumour progression differed in their ability to undergo transendothelial migration, several melanoma cell lines were examined. The WM9 cell line, like the WM239 cell line described above, was established from a metastatic human lesion, while the WM115 and WM35 cell lines were isolated from a late vertical growth phase lesion and a premetastatic lesion, respectively (Herlyn et al., 1990; Herlyn et al., 1985; Rodeck et al., 1987). The rates of transmigration of cells from these lines were compared using the in vitro assay described above (see Figure II.1).

TNFα is known to stimulate the expression of cell adhesion molecules in ECs (Luscinskas et al., 1995) and promotes the transmigration of leukocytes (Muller et al., 1993). The ability of melanoma cells from these cell lines to migrate through TNFα-stimulated and unstimulated EC monolayers was investigated. When the means obtained with and without TNFα stimulation of ECs for cell lines WM9, WM115 and WM239 were compared, these three cell lines showed similar percentages of transmigrated cells and none of the differences was found to be statistically significant (Figure II.8a). In contrast, melanoma cells from the WM35 cell line failed to transmigrate efficiently in the absence of TNFα (Figure II.8b). Instead of migrating through the EC monolayer, many cells began to spread on top of it.

Since it was difficult to determine the different stages of transmigration accurately using the epifluorescence microscope for the WM35 cell line, the co-cultures with this cell line were examined and quantified by LSCM. The results obtained showed that approximately 80% of WM35 cells remained on top of the EC monolayer 5 hours after seeding (Figure II.8b). About 50% of the WM35 cells displayed a round morphology (Figure II.9a,d), that belonged to category 1. In contrast, less than 5% of WM239 cells were scored in category 1 by 5 hours (see Figure II.2a). Among the remaining WM35
cells, many round cells had long filopodial structures extending from their basal-lateral surfaces into the EC monolayer (Figure II.9c,e), while others became flattened and spread on top of the ECs (Figure II.9c,f). Both types of morphology were novel and they were not observed previously with WM239 cells (Figure II.9g to l). When seeded on top of EC monolayers in the presence of TNFα, WM35 cells transmigrated and spread on the Matrigel with an efficiency comparable to the other three melanoma cell lines (Figure II.8). By 5 hours, less than 10% of WM35 cells remained on top of the EC monolayer, and most cells were either intercalated between ECs or spreading on the Matrigel, with morphologies comparable to those observed with WM239 cells (Figure II.9g to l).

To determine whether TNFα exerted a direct effect on WM35 cells, these cells were pre-incubated with TNFα for 30 minutes before seeding on an EC monolayer that had not been stimulated with TNFα. The percentage of these TNFα-treated WM35 cells which completed transendothelial migration was indistinguishable from that of TNFα-treated cocultures (Figure II.8b). The result suggests that TNFα had a direct influence on the ability of WM35 cells to transmigrate through the EC monolayer.

Effects of TNFα on the synthesis of adhesion molecules in WM35 cells

Since the WM35 cells appeared to have difficulty penetrating the monolayer, we hypothesized that TNFα stimulated the production of proteins required for WM35 cell extravasation. To address this issue, WM35 cells were incubated with cycloheximide to inhibit protein synthesis prior to the addition of TNFα. Cycloheximide blocked the stimulatory effects of TNFα and reduced transmigration of WM35 cells to control levels (Figure II.10). Since α,β3 integrin levels increase with melanoma disease progression (Albelda et al., 1990; Sanders et al., 1998; Weterman et al., 1994), antibody inhibition experiments were carried out using mAb LM609, which is known to block α,β3 function.
**Figure II.8.** Effects of tumour necrosis factor α treatment on the transendothelial migration of melanoma cells. The transendothelial migration assay was carried out by seeding cells from different melanoma cell lines on top of either tumour necrosis factor (TNF)α-stimulated (*solid bars*) or unstimulated (*open bars*) HUVEC monolayers. Cells were scored at 5 hours as described in the legend to Figure II.2.  

a The percentage of cells in category 3 was scored for WM239, WM115, and WM9 cell lines. PC12 cells, which do not transmigrate, were included as the negative control.  

b WM35 cells were scored by confocal microscopy. In a separate experiment, WM35 cells were pretreated with TNFα for 30 minutes, washed three times, and then seeded on an endothelial monolayer that was not previously stimulated by tumour necrosis factor α (*striped bar*). Data represent the mean ± S.D.
Figure II.9. Maximum projections of optical section series of melanoma cells at different stages of transendothelial migration. Each projection was rotated to provide different top and side views and the relationship between the melanoma cell and its surrounding endothelial cells was analyzed. Two images from each rotation series were chosen to represent the appearance of these stages from the top (a-c, g-i) and from the side (d-f, j-l). The red colour indicates the DiI label used to tag the melanoma cell while the green colour shows the phallicidin staining of F-actin in both endothelial cells and melanoma cells. Different shades of yellow and orange represent the co-localization of red and green colours. a-f Images of WM35 cells cultured on top of an endothelial cell monolayer in the absence of tumour necrosis factor α. a, d Top and side views of a round WM35 cell adhered to the endothelial cell monolayer; b, e a round WM35 cell with long filopodia-like structures (arrowheads) extending from its basal lateral surface into the endothelial cell monolayer; and c, f a WM35 cell spreading on the endothelial cell monolayer. g-l Images of WM239 cells cultured on a human microvascular endothelial cell monolayer. g, j Top and side views of a round WM239 cell on endothelial cells; h, k a spindle-shaped WM239 cell, with an endothelial process spreading over part of the cell body (arrowhead); and i, l a WM239 cell (*) spreading on the Matrigel underneath the endothelial cell monolayer. Similar results were obtained with WM239 cells cultured on human umbilical vein endothelial cells in the presence or absence of tumour necrosis factor α (data not shown). Bar: 10 μm
**Figure II.10.** Inhibition of TNFα stimulation of WM35 transmigration by cycloheximide and anti-α,β, integrin mAb. The transendothelial migration assay was carried out using WM35 melanoma cells following incubations of suspension cultures with various combinations of TNFα (10 ng/mL), cycloheximide (10 µg/mL) and the anti-α,β, mAb, LM609 (40 µg/mL) as described in the Experimental Procedures. Cells were scored at 5 hours by confocal microscopy. Data represent the mean ± S.D.
(Montgomery et al., 1996). WM35 transmigration was inhibited by 60% in these experiments (Figure II.10).

To identify molecules that might be upregulated by TNFα, WM35 cell lysates contributing equal amounts of proteins, were separated by gel electrophoresis and subjected to Western blot analysis (Figure II.11). Since α5β3 is a marker of melanoma disease progression, the blots were probed with antibodies recognizing α5 and β3. Levels of the β3 integrin subunit remained constant and while the level of α5 showed an approximate two-fold reduction in the presence of TNFα. Since the immunoglobulin superfamily member, L1, has been reported to be expressed by melanoma cells and soluble L1 is found in melanoma cultured supernatant (Montgomery et al., 1996) we decided to determine if this protein was upregulated by TNFα. Contrary to our predictions, TNFα did not stimulate the expression of L1.

Other reports have suggested that connexin 43-mediated gap junction formation between melanoma and endothelial cells facilitates melanoma metastasis (El-Sabban and Pauli, 1994; El-Sabban and Pauli, 1991; Graeber and Hulser, 1998). Western blot analysis showed that increases in connexin 43 levels were stimulated by TNFα (Figure II.11). Finally, the β1 integrin subunit was examined, since various β1 integrins have been implicated in melanoma cell metastasis (Hangan et al., 1997; Lafrenie et al., 1994; Okahara et al., 1994). Western blotting analysis indicated that the monoclonal antibody recognized intact β1 at 140 kDa, together with a lower molecular weight band of 110 kDa. Since unglycosylated β1 has a molecular weight of 88 kDa, the 110 kDa band possibly represents a partially glycosylated form of the protein. Both of the bands recognized by the β1 monoclonal antibody were upregulated in WM35 cell lysates derived from cells exposed to TNFα. (Figure II.11). To determine whether the metastatic cell line WM239 had a higher level of connexin 43 and β1 expression, WM35 and WM239 cell lysates obtained from cells not exposed to TNFα were compared. Western blot analysis indicated connexin 43 expression levels, as well as amounts of both native and intermediate molecular weight β1,
were distinctly higher in the WM239 cell lysates (Figure II.12).
Figure II.11. Effects of TNFα on the expression of specific adhesion molecules. Cell lysates were prepared from approximately 2 x 10⁶ WM35 cells cultured in HMVEC media for 3 and 5 hours with or without 10 ng/mL TNFα. Proteins from approximately 1 x 10⁴ cells were separated by SDS-PAGE and transferred to nitrocellulose as described in Experimental Procedures. The protein blots were incubated with antibodies directed against different adhesion molecules and then processed for ECL detection.
Figure II.12. Western blots comparing WM239 and WM35 cell lysates following culture in HMVEC media in the absence of TNFα. Cell lysates were prepared using approximately 2 x 10⁶ similarly treated WM35 and WM239 melanoma cells cultured in HMVEC media for 3 (1), 5 (2) or 12 (3 and 4) hours. The lysates from approximately 1 x 10⁴ cells were separated by SDS-PAGE and transferred to nitrocellulose as described in Experimental Procedures. The protein blots were incubated with antibodies directed against connexin 43 and the β₁ integrin subunit and then subjected to ECL detection.
Figure II.13. Schematic drawings depicting different stages of the transendothelial migration of melanoma cells. 

a There are five morphological stages (I to V) of melanoma cell transmigration through the endothelium. 

b These stages are accompanied by distinct cell shape changes in the melanoma cells as viewed from the top. 

F-actin distribution in endothelial cells at the heterotypic contact sites and in melanoma cells are indicated by lines inside these cells.
IV. DISCUSSION

We have established an in vitro model system which enables the detailed examination of molecular and cellular interactions during the process of melanoma transendothelial migration. Sequential events of transendothelial migration by melanoma cells can be divided into five stages and are summarized schematically in Figure II.13. Due to the low resolution of conventional fluorescence microscopy, melanoma cells in stages II and III were scored collectively as category 2 cells and stages IV and V as category 3 in our kinetic studies (Figures II.1 and 2). Subsequent studies using LSCM have allowed a detailed analysis of the transmigration process. Our results show that melanoma cells undergo drastic changes in cell shape during transendothelial migration. These shape changes are accompanied by the reorganization of the actin cytoskeleton.

The onset of transmigration is marked by membrane blebbing, lamellipodia and pseudopod formation on the basal surfaces of melanoma cells. These protrusions also extended laterally from the basal end of the melanoma cells. Significantly, the formation of extensive membrane blebs of greater than 1 μm in diameter on melanoma cells is noted here for the first time. Formation of the membrane protrusions of this size are normally not seen, suggesting that they may be triggered by interactions between melanoma cells and the endothelium. These cell protrusions can potentially increase the contact area between melanoma cells and ECs considerably and may lead to stronger cell-cell adhesion. Cortical F-actin is present in these membrane protrusions and microfilament bundles of ECs often extend upwards and terminate where these melanoma protrusions come in contact with the EC. This actin organization suggests that active cell-cell interactions occur between ECs and melanoma cells. The membrane blebs may be the precursors of lamellipodial and pseudopodial extensions that are used later to probe and to penetrate the region between the EC. The penetration of pseudopods into the region between the ECs somehow leads to the retraction of EC, providing space so that the entire melanoma cell can move into the gap.
As they become intercalated between EC junctions, melanoma cells flatten, often become spindle-shaped and acquire an increased level of cortical F-actin.

The retraction of ECs does not lead to detachment of melanoma cells from the endothelium. Quite the contrary, the cell membranes of both cell types remain in close apposition during the entire transmigration process, suggesting that active heterotypic adhesive interactions are taking place. Although VE-cadherin disappears from areas of tumour cell transmigration, immunostaining using a pancadherin antibody suggests the presence of a classical cadherin in the heterotypic contacts (Sandig et al., 1997). Also, numerous EC microfilament bundles generally organize in parallel arrays and terminate at the melanoma-EC contact sites, indicating an active response of the ECs to the transmigrating tumour cell.

As melanoma cells come into contact with the underlying Matrigel, they spread on its surface. The spreading melanoma cells eventually adopt a fibroblastic morphology and become sandwiched between the EC monolayer and the underlying Matrigel. The numerous stress fibers present in transmigrated melanoma cells at this stage suggest stable adhesive interactions between the cell and the Matrigel.

To reseal the gap left by the transmigrating melanoma cell, adjacent ECs reorganize their actin cytoskeleton and send out lamellipodial structures containing prominent stress fibers that spread along the apical surface of the melanoma cells. The behavior of ECs during closure of the gap is similar to that of ECs during the closure of small wounds, which is also accomplished by a reorganization of the actin cytoskeleton and extension of lamellipodia with stress fibers in the direction of the wound (Ettenson and Gotlieb, 1993).

In many ways, the transendothelial migration of melanoma cells resembles that of leukocytes (Pawlowski et al., 1988; Muller, 1995). However, there are also major differences. First, the time taken for melanoma cells to complete transendothelial migration is much longer. Studies using leukocytes indicate that virtually all cells can fully transmigrate through the endothelium within a couple of hours (Muller et al., 1993; Liao et
When our assay system was applied to the study of leukocyte transmigration, approximately 50% of monocytes completed migration across the EC monolayer within 1 hour (Sandig et al., 1997). In contrast, only 15% of WM239 melanoma cells showed signs of spreading on the Matrigel at 1 hour. Previous in vitro studies using different tumour cell lines and endothelial monolayers also report similar extravasation times (Kramer and Nicolson, 1979; Nicolson, 1982; Ohigashi et al., 1989). A recent report examining melanoma cell transendothelial migration through the chicken chorioallantoic membrane indicates that 50% of injected melanoma cells leave the blood vessels within 2 to 6 hours (Koop et al., 1995), suggesting that the time for transendothelial migration of melanoma cells in situ is similar to what we observed in our culture system.

Another major difference is that leukocytes migrate between ECs with minimal disruption of the EC monolayer. This is quite unlike melanoma cells which cause a considerable retraction of surrounding ECs to accommodate the larger melanoma cell. Leukocytes are capable of squeezing through much narrower openings between the ECs (Pawlowski et al., 1988; Sandig et al., 1997), possibly because the cell body and nucleus of leukocytes are much more flexible.

The contact region between ECs constitutes a permeability barrier to different solutes and controls the passage of cells through the endothelium. Recent studies from several laboratories have led to a much better understanding of EC contact regions. Several types of cell-cell junctions are known to exist between EC. While tight junctions and gap junctions are not found in all endothelia, adherens junctions appear to be ubiquitous (Dejana et al., 1995). The endothelial adherens junctions contain a specific type of cadherin, the vascular-endothelial (VE)-cadherin, which is expressed only by ECs (Lampugnani et al., 1992; Ayalon et al., 1994). Another component concentrated primarily in the contact region between ECs is the platelet-endothelial cell adhesion molecule PECAM-1/CD31, a member of the immunoglobulin superfamily of cell adhesion molecules (Albelda et al., 1990; Newman et al., 1990; Simmons et al., 1990; Albelda et al., 1991;
Newman, 1997). A recent report shows that adhesion of polymorphonuclear leukocyte to endothelium induces the disappearance of VE-cadherin from all endothelial cell-cell contacts (Del Maschio et al., 1996). This effect can be inhibited by anti-β2 integrin antibodies, suggesting that it may be induced by contact-mediated signalling involving β2 integrins.

Attachment of melanoma cells results in the dissolution of VE-cadherin complexes in the region of EC contacts (Sandig et al., 1997). In contrast to the disappearance of VE-cadherin from all endothelial junctions during transendothelial migration of polymorphonuclear leukocytes, the dissolution of VE-cadherin during melanoma cell transmigration is a highly localized event and it occurs only in regions directly beneath the attached melanoma cells. Other regions of EC contact in the vicinity are not affected.

Studies from several laboratories suggest that melanoma cells adhere to EC via a RGD-dependent mechanism, which may involve the vascular cell adhesion molecule-1 (VCAM-1) on the EC and the α4β1 integrin on the melanoma cells (Lauri et al., 1990; Giavazzi et al., 1993; Offner et al., 1993; Garofalo et al., 1995: Lafrenie et al., 1994: Sanders et al., 1998). Resting ECs express a basal level of VCAM-1. TNFα induces not only an elevated level of VCAM-1, but also its redistribution to regions between EC (Carlos and Harlan, 1994; Cartwright et al., 1995; Iademarco et al., 1995; Luscinskas et al., 1995; Bradley and Pober, 1996). In addition, TNFα has been reported to induce the production of various cytokines, which in turn can stimulate the transendothelial migration of leukocytes (Muller et al., 1993). Interestingly, our results show that TNFα does not seem to have any major effects on the transmigration of melanoma cells from cell lines established from metastatic melanoma. Since it has been reported that tumour progression can lead to a loss of sensitivity to many paracrine growth factors (Lu et al., 1992; Rak et al., 1994), melanoma cells from these cell lines are likely no longer responsive to the effects of TNFα.

Significantly, most melanoma cells from the non-metastatic WM35 cell line remained attached to the unstimulated EC monolayer in our assay system, but successfully
migrated through the endothelial layer only in the presence of TNFα. This observation, therefore, defines cell attachment and cell penetration as two distinct steps in the transendothelial migration process and suggests that TNFα may enhance the metastatic potential of otherwise non-metastatic melanoma cells.

Since TNFα has been shown to induce the expression of E-selectin, VCAM-1 and ICAM-1 in human fetal astrocytes (Hurwitz et al., 1992), it may likewise trigger the WM35 melanoma cells to express an elevated level of cell adhesion molecules, thus enabling them to mimic the invasive behavior that is characteristic of melanoma cell lines derived from later stages of metastatic progression. We have found that only a short pre-incubation of WM35 melanoma cells with TNFα is sufficient to enhance the ability of these cells to undergo transendothelial migration up to the level of the metastatic lines and that cycloheximide can inhibit the TNFα effect. These results suggest that TNFα does, indeed, stimulate the synthesis of proteins which facilitate transmigration of this cell line. Since unstimulated WM35 cells appear to be arrested at a point just prior to extravasation, the newly synthesized proteins may be required for this process. Our data indicate that protein levels of both the gap junctional protein connexin 43 and the β1 integrin subunit are increased by incubation with TNFα.

It is likely that β1 integrins, such as VLA-4, are not only required for tumour cell adhesion to endothelium (Lauri et al., 1990; Giavazzi et al., 1993; Offner et al., 1993; Lafrenie et al., 1994; Garofalo et al., 1995; Sanders et al., 1998), but also play a role in extravasation. Indeed, melanoma VLA-4 and endothelial VCAM-1 have been shown to be required in TNFα enhanced experimental metastasis in vivo (Okahara et al., 1994). Additionally, VLA-6 has been suggested to play a role in melanoma extravasation (Hangan et al., 1997) through interactions with endothelial laminin. Therefore, β1 integrins may be involved in extravasation as well as subsequent invasion through the basement membrane.

Formation of gap junctions via connexin 43 has been suggested to be required for melanoma metastasis (El-Sabban and Pauli, 1991; El-Sabban and Pauli, 1994; Graeber and
Hulser, 1998). These junctions are thought to facilitate the passage of biological mediators such as lipoxygenase metabolites from melanoma to endothelial cells to facilitate metastasis (El-Sabban and Pauli, 1991; Tang et al., 1993; Tang et al., 1993; Chen et al., 1994; El-Sabban and Pauli, 1994; Tang and Honn, 1994). These mediators could elicit specific responses in endothelial cells and facilitate the migration of the tumour cells. Such events are also reported to occur during monocyte transmigration (Sultana et al., 1996). Interestingly, lipoxygenase metabolites have been shown to induce rearrangements of the endothelial actin cytoskeleton probably via the activation of protein kinase C (Tang et al., 1993; Tang et al., 1993; Sanders et al., 1998).

TNFα has been shown to influence a variety of cellular functions although the mechanisms are only now beginning to be defined (Tracey and Cerami, 1993; Marino et al., 1996; Lugli et al., 1997; Moriguchi et al., 1997). Since TNFα has been shown to upregulate MKK7 (MAP Kinase Kinase 7), which in turn phosphorylates SAP (Stress Activated Protein) kinase and JNK (c-Jun N-terminal kinase) (Hunter, 1997; Moriguchi et al., 1997), it is conceivable that the transcriptional activators Jun and Fos could be stimulated to upregulate the expression of the β1 subunit and connexin 43. Importantly, TNFα has recently been shown to induce adhesion molecule expression through the sphingosine kinase pathway in endothelial cells (Xia et al., 1998). Alternatively, TNFα may act indirectly to upregulate β1 and connexin 43 in the WM35 cell line by stimulating the production of other cytokines which could act through an autocrine mechanism inducing transcription of the molecules by a multitude of possible pathways.

We have made the significant finding that cancer cells may arrest at a point just prior to diapedesis and our results suggest that the adhesion molecules may be involved in this intrinsic process. In order to produce metastases, perhaps primary tumour cells need to acquire an ability not only to gain access to the blood stream, but also to exit the vessels through the process of diapedesis. In light of these findings, it will be of importance to determine the molecular events triggered by TNFα in WM35 melanoma cells. Our in vitro
model system should provide a useful paradigm for more detailed investigation of the molecular and cellular events during transendothelial migration of tumour cells.
CHAPTER THREE

Redistribution of Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1)/CD31

During the Transendothelial Migration of Melanoma Cells in vitro

This chapter has been prepared for submission.

Figures II.2 and II.11 were completed in collaboration with Ning Chen, a graduate student in the laboratory.
1. INTRODUCTION

Transendothelial migration is a critical step during metastasis as tumour cells must traverse the endothelium twice, once during intravasation, and then again during extravasation. Knowledge of the molecules involved in these processes is crucial to gain a better understanding of metastasis. To date, only molecules involved in initial adhesion and rolling of tumour cells on the endothelium, or those involved in migration on extracellular matrix (ECM), have been identified (Lauri et al., 1990; Giavazzi et al., 1993; Offner et al., 1993; Lafenie et al., 1994; Garofalo et al., 1995; Aznavoorian et al., 1996; Goetz et al., 1996; Knutson et al., 1996). In contrast, the in vitro assay system which we have developed, permits the examination of invasiveness and morphological changes during tumour cell extravasation, as well as the molecular and cellular interactions required during the process. This information provides us with previously unavailable information on tumour cell extravasation.

Previously, using our in vitro system, and a confocal microscopic analysis of the actin cytoskeleton, we demonstrated that melanoma transendothelial migration is a step-wise process involving intimate contacts between both melanoma and endothelial cells. We also discovered that endothelial cells are active participants in the process (chapter 2). Our data also show that TNFα can enhance the migration of poorly metastatic melanoma cells, which may be due to increased β1 integrin levels, induced by TNFα, in these cells.

Members of the immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs) have been implicated in the final stages of leukocyte transendothelial migration. These latter stages include spreading on the endothelium, diapedesis and migration in the ECM (Albelda et al., 1994; Carlos and Harlan, 1994; Picker, 1994; Stewart et al., 1995). Here we used our assay system to examine the role of CD31 or PECAM-1, one of these Ig superfamily members, during the process of tumour cell extravasation.
CD31 is a transmembrane glycoprotein composed of six extracellular Ig-like domains and a cytoplasmic tail (Newman et al., 1990; Kirschbaum et al., 1994; Newman, 1997). CD31 is present in cell-cell contacts between endothelial cells and on the surface of most white blood cells (Simmons et al., 1990; Albelda, 1991; Albelda et al., 1991; Metzelaar et al., 1991; Bogen et al., 1992; Lampugnani et al., 1992; Ayalon et al., 1994; Dejana et al., 1995; Fawcett et al., 1995; Zocchi et al., 1996; Newman, 1997). The CD31 molecule functions in both Ca$^{2+}$-independent homophilic and Ca$^{2+}$-dependent heterophilic interactions (Albelda et al., 1991; Muller et al., 1992; DeLisser et al., 1994; Muller, 1995; Sun et al., 1996; Farniglietti et al., 1997; Newman, 1997). CD31 antibodies and recombinant CD31 have been shown to block transmigration of monocytes and neutrophils in vitro and inflammation in vivo (Muller et al., 1993; Bogen et al., 1994; Liao et al., 1995; Muller, 1995; Zocchi et al., 1996; Liao et al., 1997; Newman, 1997; Rattan et al., 1997). Although CD31 has been suggested to be a potential target for anti-inflammatory therapy (Muller, 1995), the exact role of CD31 in inflammation has not been fully defined (Muller et al., 1993).

Since CD31-mediated adhesive complexes are concentrated in endothelial cell-cell contacts, CD31 presents a natural barrier to the transendothelial migration of tumour cells. The involvement of CD31-mediated heterotypic interactions during leukocyte transendothelial migration suggests that it may play an equally significant role during tumour cell extravasation. Surprisingly, in contrast to data collected using hematopoietic cells (Muller et al., 1993; Bogen et al., 1994; Liao et al., 1995; Muller, 1995; Zocchi et al., 1996; Liao et al., 1997; Newman, 1997; Rattan et al., 1997), blocking antibodies against CD31 failed to prevent the transmigration of melanoma cells. When laser scanning confocal microscopy (LSCM) was used to inspect the distribution of CD31 during the process, dissolution of junctional endothelial CD31 was observed prior to melanoma diapedesis and CD31 was not present in heterotypic contacts between melanoma cells and endothelial cells. Furthermore, melanoma cells were able to transmigrate through
monolayers of CD31-negative endothelial cells, suggesting that CD31 is not required during melanoma diapedesis.
II. EXPERIMENTAL PROCEDURES

Cells and culture conditions

Human umbilical vein endothelial cells (HUVEC) and human microvascular endothelial cells (HMVEC) were cultured as outlined in chapter 2. The CD31-negative HUVEC cell line, ECV 304 (Hughes, 1996; Takahashi et al., 1990), was obtained from Dr. Robert Kerbel (Cancer Biology Research, Sunnybrook Health Science Centre, Toronto, ON) and was maintained in the identical media as the normal HUVEC cells. Human melanoma cell lines WM35, WM239 and WM115 were also cultured as described in chapter 2. The A375 human melanoma line was provided by Dr. Peter Newman (The Blood Research Institute, The Blood Center of Southeastern Wisconsin, Milwaukee, WI) and cultured in Dulbecco's H12 media prepared by the Ontario Cancer Institute Media Kitchen (Toronto, ON) supplemented with 5 mL 2M (20 mM) glutamine (Sigma, St. Louis, MO) and 5 mL 1000 U/mL penicillin and streptomycin (10 U/mL) and 10% FBS, both of which were purchased from Gibco/BRL. All cells were maintained in an humidified 37°C atmosphere containing 5% CO2/air.

Transendothelial migration assay

Details of the assay system were described in chapter 2. In these experiments, 1 x 10^5 to 1.5 x 10^5 HUVEC, HMVEC (passage 3-9) or ECV 304 were added to the Matrigel-coated coverslips in a 200 μL drop of the appropriate endothelial cell media. These were allowed to settle for three to four hours to permit monolayer formation. Coverslips were then carefully transferred to 24 well plates and left overnight in endothelial media with 10 ng/mL TNFα (Gibco/BRL, Gaithersburg, MD). Melanoma cells were added to the monolayers 12 hours later.
Melanoma cells were removed from culture plastic using 4 mM EDTA in HBSS and subsequently labeled with DiI as described in chapter 2. The cells were washed and resuspended at $1.76 \times 10^6$ cells/mL of HUVEC media. DiI-labelled melanoma cells ($8.8 \times 10^4$ cells in 50 µL) were added to the EC monolayer and incubated for different time intervals prior to fixation and staining. In blocking experiments, 25 µL containing an average of $6 \times 10^4$ melanoma cells were added to the endothelial monolayer. Monoclonal antibody (mAb) P2B1 (ascites) directed against CD31 was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (Ashman et al., 1991) and was diluted 1:12.5 and preincubated with the HUVEC monolayer for 30 minutes prior to the addition of WM239 melanoma cells in blocking experiments. Similarly, blocking experiments were also carried out using 100 µg/mL anti-CD31 mAbs 1.1, 1.2 and 1.3. These three antibodies were all obtained from Dr. Peter Newman (The Blood Research Institute, The Blood Center of Southeastern Wisconsin, Milwaukee, WI).

Fixation, extraction and staining

F-actin labelling was carried out as described in chapter 2. CD31 staining using the P2B1 mAb was done following sample fixation in 100% methanol precooled to -20°C. Fixation was followed by three washes of three minutes each and a five minute blocking step using 1% BSA in PBS. Coverslips were incubated with P2B1 (diluted 1:100 in blocking solution) for 45 minutes at room temperature. Secondary biotinylated goat anti-mouse polyclonal antibody was diluted 1:500 in PBS (Gibco/BRL, Gaithersburg, MD) and incubated on coverslips for 45 minutes at room temperature. After washing, coverslips were incubated with a 1:500 dilution of FITC-conjugated streptavidin (Gibco/BRL, Gaithersburg, MD) for 45 minutes at room temperature. The coverslips were then washed and mounted as described above.
Immunofluorescence using CD31 mAb 1.1 and the mAb for connexin 43 (cx-43) (Transduction Laboratories, Lexington, KY) was carried out using coverslips fixed with 3.5% (w/v) paraformaldehyde. Extraction, washing and blocking were carried out as for actin staining described in chapter 2. After a 45-minute incubation with a 1:100 dilution of the primary antibody at room temperature, the coverslips were washed and then incubated with Texas Red-conjugated (Molecular Probes, Eugene, OR) or FITC-conjugated (Sigma, St. Louis, MO) goat anti-mouse secondary antibodies at a dilution of 1:300 in blocking solution. Coverslips were then washed and mounted for fluorescence microscopy.

In those instances where cells were labelled with red cell tracker (Molecular Probes, Eugene, OR), a 10 mM stock solution of the dye was prepared and this was diluted 1:10000 (1 μM) in melanoma cell culture media and the cells were incubated with the dye for 1 hour. Subsequently, the media containing the dye solution was removed and fresh media was applied. The melanoma cells were used for transmigration experiments after overnight culture.

**Western blotting**

HMVEC, HUVEC, WM239, WM115 and ECV 304 cell lysates were prepared from cultures grown in 100-mm dishes in their respective culture media. Equivalent amounts of protein (4 μg) were separated on 7.5% acrylamide gels by SDS-PAGE. For co-culture lysates, HMVEC were cultured identically with the same starting cell numbers in 100-mm dishes to confluence and then 1 x 10^6 WM239 melanoma were added per dish and incubated for the required times. Cell lysates were prepared and SDS-PAGE was carried out using equivalent amounts of lysates - 15 μL for each cell type, total of 30 μL when two lysates were combined. Proteins were transferred to nitrocellulose at 100 volts for 1 hour. Membranes were blocked for 30 minutes at room temperature with 5% (w/v) skim milk powder in PBS plus 0.5% (v/v) Tween 20. Primary CD31 polyclonal antibody (pAb)
(Transduction Laboratories, Lexington, KY) was incubated overnight at 4°C at a dilution of 1:250 (v/v), followed by a 1 hour incubation at room temperature. The membranes were washed as suggested for ECL detection (Amersham Life Science, Buckinghamshire UK) and then incubated with 1:1000 (v/v) goat anti-rabbit horse radish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules CA) for 2 hours at room temperature. The membranes were then washed and incubated with the ECL detection reagent as recommended (Amersham Life Science, Buckinghamshire UK) and exposed to BioMax X-ray film (Kodak, Rochester NY). All the antibody dilutions were completed in the 5% skim milk blocking solution, while all washes were done in PBS containing 0.5% Tween 20.

Fluorescence microscopy

LSCM images were obtained using either a MRC 600 confocal imaging system (Bio-Rad Laboratories, Richmond, CA) on an Optiphot microscope (Nikon), equipped with a 60x objective, or a Zeiss Axiovert 135 inverted microscope equipped with a 63x Neofluor objective complete with an LSM 410 confocal attachment. Cell counting was carried out using a Wild Leitz Orthoplan microscope equipped with a 25x objective.

Quantification of transmigration of melanoma cells

All scoring was completed as outlined in chapter 2.
III. RESULTS

Effects of CD31 antibodies on the transendothelial migration of human melanoma cells

Several monoclonal antibodies are known to block CD31-mediated cell-cell adhesion and impair leukocyte transendothelial migration (Figure III.1a) (Liao et al., 1995; Newman, 1997). To assess the role of CD31 in tumour cell transendothelial migration we examined the ability of these monoclonal antibodies (Figure III.1b) to block the transendothelial migration of melanoma cells. The in vitro transendothelial migration assay was carried out using WM239 melanoma cells and HMVEC monolayers. The percentage of spreading cells was scored at 3 and 5 hours. The results show that neither monoclonal antibody 1.2, nor 1.3, known to block CD31 heterophilic and homophilic interactions respectively, had any detectable inhibitory effect on the transendothelial migration of WM239 melanoma cells. Even the combination of both blocking antibodies was not able to inhibit the transendothelial migration of the melanoma cells. Similar rates of transmigration were observed in the presence of the non-blocking antibody, 1.1, and in the absence of any antibody. The P2B1 monoclonal antibody against CD31 also failed to inhibit WM239 transendothelial migration (data not shown). An analysis of the total number of melanoma cells for a fixed area in these specimens did not reveal any apparent difference (Figure III.1c), indicating that the CD31 blocking antibodies did not affect the adhesion of the melanoma cells to the endothelial monolayers.

Efficient extravasation in the absence of CD31

To further investigate whether CD31 is required for the transendothelial migration of melanoma cells, co-culture experiments were carried out using WM239 and A375
melanoma cells. While immunoblot analysis confirmed that WM239 cells did not express CD31 (Figure III.2), the lack of CD31 expression on the A375 cell line was determined by both flow cytometric analysis (P. Newman, unpublished) and Western blotting (Figure III.2).

In addition to using HMVEC monolayers, transmigration of melanoma cells through monolayers of ECV 304 cells was examined. ECV 304 is an endothelial cell line which does not express CD31 (Hughes, 1996) (Figure III.2). DiI-labelled WM239 (Figure III.3a to d) and A375 (Figure III.3e to h) cells were stained for F-actin to examine their transmigration through ECV 304 monolayers. Both lines of melanoma cells were able to fully transmigrate through the CD31-negative ECV 304 cells and these monolayers were able to reseal the remaining gaps above the melanoma cells as efficiently as the normal HUVEC monolayers.

When time course assays were performed using CD31-positive HMVEC monolayers (Figure III.2), no significant difference in the migratory ability of WM239 and A375 cells was observed (Figure III.4a). Identical assays were carried out using the CD31-negative ECV 304 cell line (Figure 4b). Both melanoma cell lines were able to fully extravasate through ECV 304 monolayers, however the A375 melanoma cells appeared delayed at the one hour timepoint. Since there was no apparent difference in the rate of transmigration between the two melanoma cell lines after this time point, the altered migration rate of the A375 cells through the ECV 304 monolayers was likely a result of differences in the initial adhesive interactions between these two cell types. To examine if cell attachment was affected, the number of melanoma cells bound to the ECV 304 monolayers was compared to those attached to HMVEC. These results did not display any striking differences (Figure III.4c).
**Figure III.1.** Effects of CD31 antibodies on the transendothelial migration of melanoma cells. *In vitro* transendothelial migration was performed in the presence of various anti-CD31 mAbs. The antibody specificities are summarized in table form (a). The percentage of melanoma cells spreading on the Matrigel was estimated at 3 and 5 hours of co-culture (b). The three hour blocking data and the five hour mAb 1.1 blocking data were collected from two experiments while the rest of the 5 hour blocking data was averaged from four experiments. In c the total number of attached cells scored for both 3 hour experiments was averaged, while the total number of attached cells was averaged for two of the four experiments carried out at 5 hours. Given that a total of 45 fields were scored for each experiment, the area scored represents an estimated 0.6 percent of the total area of the 24 well plates to which the melanoma cells were added during each experiment. Assuming homogeneous mixing, no effect due to the negative meniscus found in the wells, exactly the same starting number of cells for each experiment and 100% attachment, the theoretical number of cells which would be found in this area can be estimated at 528 cells for both the 3 hour and 5 hour experiments. Data represent the mean ± S.D..
a

<table>
<thead>
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<th>ANTIBODY</th>
<th>SPECIFICITY</th>
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<tr>
<td>PECAM 1.1</td>
<td>mouse anti-human PECAM-1 Ig domain 5 (non-blocking control)</td>
</tr>
<tr>
<td>PECAM 1.2</td>
<td>mouse anti-human PECAM-1 Ig domain 6 (blocks heterophilic binding)</td>
</tr>
<tr>
<td>PECAM 1.3</td>
<td>mouse anti-human PECAM-1 Ig domain 1 (blocks homophilic binding)</td>
</tr>
</tbody>
</table>

b

![Bar graph showing % spreading cells](image)

- 3 hours
- 5 hours

Antibody used for blocking:
- No mAb
- 1.2
- 1.3
- 1.2+1.3
- 1.1

<table>
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<th>1.3</th>
<th>1.2+1.3</th>
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<td>422</td>
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</table>
Figure III.2. CD31 Western blot of total cell lysates. Western blotting was completed as outlined in Material and Methods. In a, CD31 protein expression was detected in individual lysates of; HMVEC (1), ECV304 (2), A375 (3), WM35 (4), WM115 (5) and WM239 (6). Mature CD31 at 130 kDa is observed only in the HMVEC lysates. The blot shown in b was reprobed with an antibody recognizing actin to ensure equal loading.
Figure III.3. Confocal images of transmigrated WM239 and A375 melanoma cells following extravasation through ECV 304 monolayers. Fully transmigrated single red DiI labelled WM239 melanoma (a-d) and A375 melanoma (e-f) cells are seen in a series of confocal sections covered by ECV 304 monolayers. Melanoma cells were labelled and added to confluent ECV 304 and allowed to transmigrate for 5 hours prior to fixation and actin staining using BODIPY-FL phallacidin as seen in green. The transmigration procedure and staining were carried out as outlined in Experimental Procedures. The series were taken in 1 μm intervals and are shown from top to bottom. The first images shown from each series (a and e) were taken 9 μm above the bottom sections shown in d and h. The next sections (b and f) were taken 3 μm and 6 μm (c and g) below a and e. The final section shown in each series, d and h, was taken 1 μm from the bottom. Bar: 10 μm
Figure III.4. WM239 and A375 melanoma migrate through HMVEC monolayers with similar kinetics. Timecourse experiments of WM239 and A375 passing through HMVEC monolayers (a) and ECV 304 monolayers (b) were carried out as outlined in Experimental Procedures. Data for a and b were collected from two experiments for each timepoint. Shown in c are the total number of attached cells scored for one experiment on HMVEC and an average of both experiments for each timepoint on ECV 304. Data represent the mean ± S.D..
**Dissolution of CD31 endothelial junctions juxtaposed to melanoma cells**

Since CD31 antibodies did not inhibit melanoma transendothelial migration, the fate of CD31 during melanoma extravasation was examined by confocal microscopy. Beneath attached and early migrating WM239 melanoma cells, the EC junctional staining of CD31 became diffuse while remaining intact (Figures III.5 and III.6- arrows) in neighbouring junctions not in direct contact with melanoma cells. No CD31 appeared to be enriched in early melanoma-HUVEC contacts (Figures III.5 and III.6). On occasion, however, some residual CD31 was observed between endothelial cells and melanoma cells (Figure III.6 arrowheads). The CD31 staining pattern in EC junctions was regularly observed in oblique angles or in folds of membrane between these cells. In these instances the endothelial junctions appeared slanted and CD31 had a meshwork-like distribution between the apposing membranes (Figure III.5c). This morphology is particularly evident in confocal sections taken near the bottom of the monolayer, close to the Matrigel, beneath round melanoma cells (Figure III.5e). This staining pattern suggested that the adhesion complexes made up of CD31, begin to dissociate upon contact with the melanoma cell.

At a slightly later stage of melanoma cell transendothelial migration through the endothelial monolayers (Figure III.7), it was apparent that CD31-rich endothelial contacts were abruptly interrupted by the presence of the migrating melanoma cell (Figure III.7e-arrowheads) and CD31 was absent from the heterotypic contacts between the melanoma cells and the adjacent endothelial cells. However, endothelial cell-cell contacts away from the melanoma cell, maintained a normal CD31 distribution pattern (Figure III.7). A comparable CD31 distribution was observed when WM239 melanoma cells migrated through HMVEC monolayers (not shown).
**Figure III.5.** Dissolution of CD31 complexes under a spherical melanoma cell. The *in vitro* transendothelial migration assay was carried out by incubating WM239 melanoma cells on a HUVEC monolayer. The coverslip was fixed with 100% methanol and stained for CD31 using the mAb P2B1. A series of LSCM images showing a Dil-labelled melanoma cell sitting on top of the endothelial monolayer was taken at 1 μm intervals. Three images, from top to bottom, separated by 1 μm, are shown with CD31 staining on the left (a to e) and the Dil staining on the right (b to f). Images in e and e indicate CD31 became diffuse beneath the melanoma cell at this early stage of migration and no longer clearly defined the endothelial contacts. *Bar:* 10 μm
Figure III.6. Residual CD31 observed in heterotypic contacts between melanoma and endothelial cells. A LSCM series of two Dil labelled WM239 melanoma cells penetrating a HUVEC monolayer. The specimen was fixed with 100% methanol and stained with P2B1 mAb to identify CD31 as outlined in the Experimental Procedures. The stack of 13 single μm sections was merged to achieve the appearance of a regular fluorescence image. The left panel (a) shows CD31 staining while the right panel (b) shows the location of two Dil-labelled melanoma cells. The arrows point to CD31 in endothelial contacts while the arrowheads indicate the location of CD31 in contact regions between endothelial cells and melanoma cells. Bar: 10 μm
**Figure III.7.** CD31 is absent from EC-melanoma contacts during transendothelial migration. A series of confocal images was taken at 1 μm intervals through a DiI labelled WM239 melanoma cell which became intercalated in endothelial monolayer. Four images of the series are shown, from top to bottom, with CD31 staining on the left (a to h) and the DiI staining on the right (b to h). The location of the melanoma cell is indicated by the DiI label shown in b through h. CD31-rich endothelial contacts are apparent in a through g. These contacts abruptly lost their CD31 staining when contact with the melanoma cell was made (*arrow heads*). All sections (a to g) were separated by 1 μm. *Bar: 10 μm*
Reappearance of CD31 to the leading edges of endothelial cells spreading over melanoma cells

As the endothelial cells began to close the gap over a transmigrated melanoma cell (Figure III.8) CD31 remained in endothelial cell-cell contacts not involved in WM239 extravasation (Figure III.8c and g-arrows). Diffuse patches of CD31 were found in the lamellipodia-like structures spreading over the melanoma cells (Figure III.8c). CD31 was also observed around the gap in the endothelial monolayer (Figure III.8a) occupied by the apical region of the melanoma cell (Figure III.8b, d and f-arrowheads). The morphology of the CD31 patches above transmigrated melanoma cells was similar to that observed in the initial stages when attached melanoma cells began to penetrate the monolayer. Endothelial cell-cell contacts reformed above fully extravasated melanoma cells (Figure III.9). Finally, the patterns of CD31 in the late stages of WM239 migration through HMVEC monolayers was comparable to that observed using HUVEC monolayers (Figure III.10).

Redistribution of CD31 during melanoma transendothelial migration

To determine whether CD31 is degraded during melanoma transendothelial migration Western blot analysis was carried out. As described above, Western blot analysis of total cell lysates indicated that the WM239 melanoma cells did not express CD31 (Figure III.2). Lysates derived from the CD31-negative ECV 304 cell line, used as a negative control, corroborated these results. Since WM239 cells do not express CD31, it was possible to examine changes in the amount of CD31 in the endothelial cells by Western blot analysis of lysates obtained from co-cultured WM239 melanoma and HMVEC cells (Figure III.11). These blots showed minimal changes in the CD31 protein levels during melanoma cell extravasation. Also, smaller degradative products were not detected on these blots. These
results suggest that CD31 is redistributed away from endothelial cell-cell contacts and not down-regulated, during melanoma cell transendothelial migration.

An efficient redistribution of CD31 from endothelial cell-cell contacts most likely requires a quick means of communication between melanoma cells and the endothelium. Such a mechanism might be afforded by gap junctions. To determine if gap junctions are formed between melanoma and endothelial cells, we stained co-cultures of melanoma cells and endothelial cells with an anti-connexin 43 mAb. Indeed, connexin 43 (cx-43) staining was observed in melanoma-endothelial contacts between HMVEC and WM239 melanoma cells(Figure III.12a-d) as well as HMVEC and WM35 melanoma cells (Figure III.12e-h) in the presence of TNFα. Interestingly, we have preliminary data suggesting that WM35 melanoma form fewer gap junctions with HMVEC in the absence of TNFα (data not currently shown).
**Figure III.8.** CD31 returns to reforming endothelial contacts above melanoma cells late in extravasation. A DiI-labelled WM239 melanoma cell was examined as it was being covered by the HUVEC monolayer as observed following fixation with 100% methanol and staining for CD31 using the P2B1 mAb. A series of LSCM images was taken at 1 μm intervals. Five images of the series are shown, from top to bottom, with CD31 staining on the left (a to i) and the DiI staining in the melanoma cells on the right (b to j). In the top section (a), CD31 was just out of focus in the cell contacts which were more clearly seen in c through g as indicated by the arrows. In b, the DiI image corresponding to a, the presence of DiI staining from the very top of the melanoma cell is seen (arrowhead). The uncovered portion of the melanoma cell corresponded to a gap in the CD31 staining as indicated by the star (a). A patch of diffuse CD31 staining (c) was located above the melanoma cell, in the HUVEC, and extended around the gap (star) in the monolayer. This diffuse patch of CD31 was also seen in sections a, e and g. The location of the melanoma cell is indicated in d and f by the arrowheads. Sections a, c, d and g and their corresponding DiI images (b through h) were all separated by 1 μm. The final section shown in i is 3 μm below g. In j, the DiI image equivalent of i, the contours of the melanoma cell are evident. *Bar: 10 μm*
Figure III.9. A DiI labelled WM239 melanoma cell under a monolayer of endothelial cells. The coverslip was fixed using 100% methanol and CD31 was labelled using P2B1 mAb as outlined in Experimental Procedures. The sample was sectioned using the LSCM in 1 μm intervals giving a total of 13 images. Shown in a are the top sections showing fully closed endothelial cell-cell contacts enriched in CD31. In b, the bottom sections, the contours of the melanoma cell are indicated by DiI crystals. The sections were merged to simulate regular fluorescence microscopy. *Bar:* 10 μm
Figure III.10. Melanoma transendothelial migration through HMVEC monolayers has the same effect on CD31 as observed using HUVEC monolayers. Two late stage Dil labelled melanoma cells are shown with surrounding ECs. The sections were fixed with paraformaldehyde and CD31 was labelled using the CD31 1.1 mAb. Both cells were imaged using 1 μm LSCM sections. The Dil label in red shows the location of the melanoma cells while green is indicative of CD31 staining. The first cell is shown in a and b. In a endothelial cell-cell contacts, rich in CD31, can be seen around a melanoma cell. These contacts, which end at the location of the melanoma cell, are indicated by arrowheads. The image in b was taken 1 μm below a. Two images from the second LSCM series are seen in c and d. In c, the endothelial monolayer has fully reformed above a transmigrated WM239 melanoma cell, and CD31 is observable in the EC cell-cell contacts (arrows). 3 μm below c the melanoma cell is readily visible as shown in d. In d, The other part of the endothelial cell (E) outlined in c by the CD31 rich EC contacts can be seen. Bars: 10 μm
**Figure III.11.** CD31 Western blot of total cell lysates from co-cultured melanoma and endothelial cells. Western blotting was completed as outlined in Experimental Procedures. No change in CD31 expression is detectable even after 5 hours of co-culture. The lysates in include: WM239 melanoma (1), HMVEC (2), a combination of the first two lanes (3), 1 hour co-culture (4), 3 hour co-culture (5), 5 hour co-culture (6) and the Jurkat lymphoma positive control (7).
Figure III.12. Connexin 43 staining between melanoma and endothelial cells during extravasation. WM239 cells (a-d) and WM35 cells (e-g) were tagged with Dil (red) and added to HMVEC monolayers. These coverslips were fixed with paraformaldehyde and stained for connexin 43 (green) as outlined in Experimental Procedures. Four early stage cells are shown. The first cell can be seen in a and it is just touching the EC monolayer in b. These sections are separated by 6 μm. The second cell in c is a later stage cell than the one imaged in a and forms more complete contacts as shown 2 μm below in d. The early stage cell seen in d is comparable to the one in a and can be seen contacting the monolayer in e which is 5 μm below. The cell shown in f makes good gap junctions as is still apparent at the bottom of the cell, 2 μm below, shown g. In all the images, arrowheads indicate representative locations of connexin 43 staining between melanoma and endothelial cells, while arrows show the location of connexin staining in endothelial homotypic contacts. Bar: 10 μm
Figure III.13. Schematic drawing depicting the distribution of CD31 in the different stages (I to V) of the transendothelial migration of melanoma cells. A detailed description of these stages is outlined in chapter 2. The distribution of CD31 at each stage is indicated by the heavy black lines.
CD31 is present in endothelial cell-cell contacts and on the surface of most white blood cells (Simmons et al., 1990; Albelda, 1991; Albelda et al., 1991; Metzelaar et al., 1991; Ayalon et al., 1994; Dejana et al., 1995; Zocchi et al., 1996; Newman, 1997). CD31 antibodies and recombinant proteins have been shown to block hematopoietic cell diapedesis both in vitro and in vivo (Muller et al., 1993; Bogen et al., 1994; Ferrero et al., 1995; Liao et al., 1995; Muller, 1995; Zocchi et al., 1996). In addition, ectopic expression of CD31 has been suggested to occur on a variety of tumour cell types (Tang et al., 1993). This information prompted us to examine the role of CD31 in relation to melanoma cell diapedesis in more detail.

In light of the success of CD31 mAbs and peptides in blocking hematopoietic cell diapedesis, we attempted similar blocking studies using various CD31 mAbs in our transendothelial migration system. These studies were conducted using a panel of mAbs whose binding sites and inhibitory abilities during hematopoietic cell transendothelial migration were previously characterized (Liao et al., 1995; Newman, 1997). Surprisingly, WM239 melanoma cells displayed no decrease in their ability to migrate through HMVEC monolayers in the presence of these antibodies. Furthermore, in contrast to results reported previously using murine melanoma cells (Tang et al., 1993), there also appeared to be no difference in the ability of the melanoma cells to adhere to the endothelium, despite using higher antibody concentrations. This discrepancy could be due to a role for CD31 in the initial cell attachment process of murine melanoma cells.

Our data, therefore, indicate that CD31 is not required for the diapedesis of human melanoma cells through human endothelial monolayers. This is in direct contrast to data collected using hematopoietic cells (Muller et al., 1993; Bogen et al., 1994; Liao et al., 1995; Muller, 1995; Del Maschio et al., 1996; Luscinskas et al., 1996; Wakelin et al., 1996; Zocchi et al., 1996; Liao et al., 1997; Newman, 1997; Rattan et al., 1997),
providing clear evidence that the process of diapedesis for solid tumour cells is distinctly different from that of blood cells.

The fate of CD31 during diapedesis of the WM239 melanoma cells through the EC monolayers was examined using LSCM. The results reveal the dissolution of CD31 complexes in EC contacts adjacent to extravasating melanoma cells. Strikingly, CD31 was not apparent in heterotypic contacts between melanoma cells and endothelial cells. A summary of these staining results is shown in Figure III.13. Furthermore, time courses conducted using CD31 negative melanoma cells and endothelial cells did not demonstrate any inhibition of melanoma transendothelial migration.

VE-cadherin is also found in EC cell-cell contacts (Lampugnani et al., 1992) and we have previously found that VE-cadherin redistributes away from endothelial contacts juxtaposed to migrating melanoma cells (Sandig et al., 1997). Similarly, leukocyte transendothelial migration also results in the dissolution of VE-cadherin complexes from endothelial cell-cell contacts (Del Maschio et al., 1996; Allport et al., 1997) suggesting that some similarities do exist between leukocyte and tumour cell transendothelial migration.

Recently, Lewalle et al. (1997) also reported the redistribution of VE-cadherin during the transendothelial migration of breast cancer cells (Lewalle et al., 1997). Significantly, they found that various protease inhibitors did not impair the transendothelial migration of tumour cells. Consistent with their observations, we found that the level of CD31 on endothelial cells remained more or less constant during co-culture with melanoma cells and no degradative products were detected. Therefore, it is likely that the CD31 is redistributed, as in the case of VE-cadherin, via a dynamic process out of endothelial cell-cell contacts juxtaposed to transmigrating melanoma cells. On the other hand, our results do not rule out a low level (<10%) of CD31 degradation.

Our transendothelial migration assay makes use of TNFα stimulated endothelial cells. This cytokine has been suggested to influence endothelial CD31. Some investigators have presented evidence that TNFα reduces the amounts of CD31 mRNA and protein in T-
cells and endothelial cells in vitro (Zehnder et al., 1992; Stewart et al., 1996). This cytokine has also been shown to cause a dose-dependent phosphorylation and redistribution of CD31 from away from EC cell-cell contacts to the cell body, resulting in an increase in endothelial permeability (Romer et al., 1995; Ferrero et al., 1996). However, others have reported that, in short term organ cultures and cultured endothelial cells, TNFα stimulation did not produce changes in CD31 expression (Ioffreda et al., 1993; Bradley and Pober, 1996). Similarly, in our hands, no redistribution of endothelial CD31 was observed in cell staining experiments following TNFα stimulation in the absence of melanoma cells. Others have used TNFα stimulated EC monolayers to examine the transendothelial migration of leukocytes. These investigators, likewise, did not observe any significant effects on the distribution of CD31 (Del Maschio et al., 1996). It is likely, therefore, that the dissolution of CD31 observed in our experiments was due to a specific melanoma-mediated effect.

Results from our Western blotting experiments suggest that CD31 is redistributed away from endothelial junctions upon contact with extravasating melanoma cells. It is conceivable that melanoma cells elicit these responses in endothelial cells through some means of direct communication. This idea is corroborated by our previous findings that extravasating melanoma cells do not simply push away endothelial cells, but rather elicit active participation from endothelial cells during transmigration (see chapter 2). Several recent reports have suggested that tumour cell adhesion is followed by cx-43-mediated gap junction formation at sites of contact between tumour cells and endothelial cells (El-Sabban and Pauli, 1991; El-Sabban and Pauli, 1994; Honn et al., 1994; Tang and Honn, 1994). Indeed, we have also observed positive cx-43 staining between melanoma cells and the endothelium in co-cultures.

Gap junctions between tumour cells and endothelial cells have been hypothesized to permit the transfer of metabolites such as 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), a lipoxygenase metabolite of arachidonic acid, from tumour cells to endothelial
cells, and thus permit a locally confined retraction of endothelial cells (El-Sabban and Pauli, 1991; El-Sabban and Pauli, 1994). This molecule has been implicated in a protein kinase C dependent rearrangement of the actin cytoskeleton (Tang et al., 1993) and in the movement of CD31 from EC cell-cell borders, and α,β3 integrin from EC focal contacts, to the cell body (Tang et al., 1993; Tang et al., 1993; Tang and Honn, 1994; Tang and Honn, 1994). Tumour cell 12(S)-HETE has also been shown to elicit changes in tumour cells themselves. Among these changes are increases in matrix adhesion (Chen et al., 1994), cAMP levels (Tang et al., 1995), cytoskeletal rearrangements (Timar et al., 1993) and phosphorylation of focal adhesion kinase. Interestingly, lipoxygenase metabolites have been shown to support monocyte transendothelial migration (Sultana et al., 1996). These concepts allude to a process of tumour cell extravasation requiring physical interactions and responses between both endothelial cells and the transmigrating tumour cells.

We have now shown that melanoma cell transendothelial migration in vitro does not require CD31, thus providing evidence of an important difference between the transendothelial migration of tumour cells and leukocytes. These results suggest, for the first time, that tumour cell extravasation is not identical to the process that has been elucidated for leukocytes. Our findings also indicate that inhibitory therapies targeting tumour cells and not effecting normal inflammatory processes may be a distinct possibility.
CHAPTER FOUR

Transendothelial Migration of Melanoma Cells In Vitro: Involvement of the $\alpha_v\beta_3$ Integrin and L1

This chapter has been prepared for submission.
I. INTRODUCTION

In chapter 2 we described an *in vitro* system to study molecular interactions and morphological changes during tumour cell extravasation. We outlined the stages of transmigration and discovered that the cytokine, TNFα, can increase the migratory ability of poorly metastatic melanoma lines (Voura *et al*., 1998). Furthermore, we also found that, while CD31 and VE-cadherin are not required for melanoma extravasation, heterotypic interactions with other classical cadherins play a role in the process (Sandig *et al*., 1997). In this chapter, we used our assay system to examine the role of the $\alpha_5\beta_3$ integrin and L1 during melanoma transendothelial migration.

The $\alpha_5\beta_3$ integrin was first identified as the "vitronectin receptor", but will adhere to a host of other extracellular matrix (ECM) proteins. In addition, the $\alpha_5\beta_3$ integrin undergoes heterophilic binding with CD31 and L1 (Buckley *et al*., 1996; Montgomery *et al*., 1996). L1, like CD31, is a member of the Immunoglobulin (Ig)-superfamily of cell adhesion molecules (CAMs), and contains six Ig-like domains and five fibronectin type III-like repeats (Moos *et al*., 1988; Hlavin and Lemmon, 1991; Hortsch, 1996; Kamiguchi and Lemmon, 1997). Binding of L1 to $\alpha_5\beta_3$ and other integrins has been shown to occur through an RGD sequence found in Ig domain 6 (Ruppert *et al*., 1995; Montgomery *et al*., 1996; Felding-Habermann *et al*., 1997; Yip *et al*., 1998). In addition, L1 can undergo heterophilic interactions with laminin (Montgomery *et al*., 1996; Hall *et al*., 1997) and other Ig-like molecules (Hortsch, 1996). Furthermore, L1 can mediate cell-cell binding via homophilic interactions (Miura *et al*., 1992; Zhao and Siu, 1995; Zhao *et al*., 1998).

Although L1 is largely known for its role in neuronal development, the function of L1 on a variety of normal and transformed cell types is now being investigated in many laboratories (Thor *et al*., 1987; Linnemann *et al*., 1989; Hortsch, 1996; Izumoto *et al*., 1996; Pancook *et al*., 1997). L1 expressed by monocytes has been implicated in interactions with endothelial $\alpha_5\beta_3$ integrin, which in turn may be involved in leukocyte
extravasation (Ebeling et al., 1996; Pancock et al., 1997). Conversely, $\alpha_\beta_3$ expressed by T-cells and tumour cells has been suggested to bind to L1 (Ebeling et al., 1996; Duczmal et al., 1997). L1 is shed from melanoma cells and has been suggested to provide an adhesive matrix for these cells via cell bound $\alpha_\beta_3$ integrin (Montgomery et al., 1996). Melanoma cell $\alpha_\beta_3$ integrin can also mediate haptotaxis of melanoma cells on L1 coated surfaces. Likewise, soluble L1 has been shown to provide an adhesive matrix for glioma cells (Izumoto et al., 1996).

It is known that $\alpha_\beta_3$ integrin levels increase with melanoma disease progression and expression of the integrin correlates with metastatic ability (Albelda et al., 1990; Weterman et al., 1994; Natali et al., 1997) Furthermore, interactions between L1 and $\alpha_\beta_3$ are suggested to be important for leukocyte extravasation (Ebeling et al., 1996; Duczmal et al., 1997; Pancock et al., 1997). We have, therefore, used our in vitro model of melanoma cell transendothelial migration to determine if melanoma $\alpha_\beta_3$ integrin-mediated interactions with L1 are required for the process.
II. EXPERIMENTAL PROCEDURES

Cells and Culture Conditions

Human microvascular endothelial cells (HMVEC) and WM239 melanoma cells were cultured as outlined in chapter 2. The M21 melanoma cell lines (M21, M21-L, M21-L12 and M21-L4) were obtained from Dr. Anthony Mongomery from the Scripps Research Institute (La Jolla, CA) and were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640) prepared by the Ontario Cancer Institute Media Kitchen (Toronto, ON). Melanoma cell RPMI-1640 media was supplemented with 5 mL of 1000 U/mL penicillin and streptomycin (10 U/mL) and 10% FBS. All cells were maintained in an humidified 37°C atmosphere containing 5% CO₂/air.

Antibodies and Peptides

The LM609 monoclonal antibody (mAb) against αβ₃ was obtained from Dr. David Cheresh from the Scripps Research Institute (La Jolla, CA). The polyclonal antibodies (pAbs) recognizing L1 domains 1-3, domains 4-6 and fibronectin repeats were described previously (Zhao and Siu, 1995). These pAbs were generated against GST (glutathione-s-transferase) fusion proteins of the designated L1 domains. The L1 mAb was purchased from Transduction Laboratories (Lexington, KY). The mAb P2B1 (acites) against CD31 was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (Ashman et al., 1991). This antibody was used as a non-blocking control mAb as it was previously shown to have no inhibitory effects on melanoma transendothelial migration (see Chapter 3). Polyclonal antibodies raised against a Dictyostelium protein were used as the control pAb. The N-cadherin GC-4 mAb was obtained from Sigma (St. Louis, MO).
Finally, the bromodeoxyuridine mAb was purchased from Boehringer Mannheim (Indianapolis, IN).

The cyclic RGD peptide was purchased from Peptides International (Louisville, KY). Both the linear RAD (PSITWRADGRDLQEL) and linear RGD (PSITWRGDGRDLQEL) peptides were derived from L1 domain 6 RGD sequences and were obtained from Dr. Anthony Mongomery of the Scripps Research Institute (La Jolla, CA).

**Transendothelial Migration Assays**

We continued to use the assay system as described in chapter 2. 1 x 10^5 to 1.5 x 10^5 HMVEC (passage 3-9) were added to the Matrigel coated coverslips and an average of 6 x 10^4 DiI-labelled melanoma cells were added in a volume of in 25 µL for each experiment. For inhibition studies, antibodies and peptides were incubated with the HMVEC monolayers for 30 minutes prior to the addition of the melanoma cells and these reagents were kept in the system for the duration of the experiment. In other experiments, ECs or melanoma cells were preincubated with antibodies individually and then washed prior to co-culture. DiI-labelled WM239 melanoma cells were rotated in suspension in the presence of either the appropriate inhibitor or HBSS for 30 minutes. The melanoma cells were subsequently washed 3 times with HBSS, resuspended at the appropriate concentration and added to HMVEC monolayers. Preincubations of HMVEC monolayers with inhibitors were carried out for 30 minutes. The reagents were then diluted more than 2000x by 3 washes in HMVEC media. TNFα was added to the media at the usual concentration before the addition of Di-I labelled melanoma cells.
Cell Staining

To label F-actin, cells were fixed, stained and mounted as described in chapter 2.

To label α,β integrin, the LM609 mAb was applied to samples fixed with 100% methanol at -20°C. Samples were washed 3 times for 3 minutes each, prior to a five-minute blocking step. LM609 was diluted 1:100 in blocking solution and incubation was carried out for 45 minutes at room temperature. After washing, samples were incubated with FITC-conjugated goat anti-mouse secondary antibody for 45 minutes at room temperature. The coverslips were then washed and mounted as described previously.

To label cells with antibodies directed against bromodeoxyuridine and L1, cells were fixed with 3.5% (w/v) paraformaldehyde. Extraction, washing and blocking were carried out as for actin staining described above. Following a 45 minute incubation with a 1:100 dilution of the primary antibody at room temperature in blocking solution, the coverslips were washed and then incubated for another 45 minutes with Texas Red-conjugated or FITC-conjugated goat anti-mouse or Texas Red-conjugated or FITC-conjugated goat anti-rabbit secondary antibodies at a dilution of 1:300 in blocking solution. The coverslips were washed and mounted for microscopic observation.

To label cells with red cell tracker (Molecular Probes, Eugene, OR) melanoma cells were incubated with 1 μM dye (1:1000 dilution of a 10 mM stock solution) in the culture medium for 1 hour. The dye-containing medium was then replaced with fresh media and cells were incubated overnight before collection for co-culture assays.

Bromodeoxyuridine-tagged melanoma cells were also used. In this case, a 10 mM stock of bromodeoxyuridine was diluted 1000x in the melanoma media for overnight culture. Incorporation of bromodeoxyuridine into DNA was detected by mAb as described above with the addition of 1.5 μL DNAse I (50 μg/mL) and 1.5 μL of 0.5 M MgCl₂ to the primary antibody solution.
**Microscopes**

LSCM images were obtained using a Zeiss Axiovert 135 inverted microscope equipped with a 63x Neofluor objective together with an LSM 410 confocal attachment. Cell counting was accomplished observing epifluorescence using a Wild Leitz Orthoplan universal large-field microscope through a 25x objective.

**Quantification of transmigration of melanoma cells**

All experiments were carried out on separate coverslips. The cells were counted as described in chapter 2.
III. RESULTS

Distribution of $\alpha_\beta_3$ on HMVEC and WM239 melanoma cells

Both melanoma and endothelial cells are known to express the $\alpha_\beta_3$ integrin. To examine the distribution of $\alpha_\beta_3$ on both HMVEC and WM239 melanoma cells, the LM609 mAb directed against $\alpha_\beta_3$ was used for immunofluorescence experiments. The $\alpha_\beta_3$ integrin was found in endothelial cell-cell contacts (Figure IV.1a) and some staining of this integrin was also observed in the endothelial cytoplasm. WM239 melanoma cells expressed $\alpha_\beta_3$ primarily on the cell surface, with a higher concentration in the cell-cell contact regions (Figure IV.1b).

Localization of $\alpha_\beta_3$ integrin on melanoma and endothelial cells during transendothelial migration

To determine the distribution of $\alpha_\beta_3$ during extravasation, the LM609 mAb directed against $\alpha_\beta_3$ was used in immunofluorescence labelling studies on co-cultures of melanoma and HMVEC (Figures IV.2 and Figure IV.3). The $\alpha_\beta_3$ integrin was associated with the entire melanoma cell membrane prior to extravasation. On the basal lateral regions, the attached melanoma cells displayed characteristic membrane blebs which stained for $\alpha_\beta_3$. During transmigration, $\alpha_\beta_3$ staining was also observed in junctions between melanoma and endothelial cells and appeared on melanoma cell membrane protrusions (Figure IV.2). Spreading cells were found to express $\alpha_\beta_3$ in heterotypic junctions with the endothelial cells, and also expressed the integrin over the base of the cell, contacting the Matrigel basement membrane matrix (Figure IV.3). The $\alpha_\beta_3$ integrin, therefore was expressed in all heterotypic contacts between the two cell types during all stages of melanoma cell transendothelial migration, as well as in contacts between melanoma cells and the ECM.
These results confirmed that the $\alpha_\text{v}\beta_3$ integrin was appropriately distributed during melanoma transendothelial migration to mediate diapedesis and spreading.

**Effects of an anti-$\alpha_\text{v}\beta_3$ antibody on melanoma transendothelial migration**

Given that $\alpha_\text{v}\beta_3$ was found in the heterotypic contacts during melanoma cell transmigration, we next examined the effects of the function blocking mAb, LM609, on melanoma cell transendothelial migration. When the antibody was added to the co-cultures, melanoma transendothelial migration was inhibited by 40 to 50% (Figure IV.4a) at 5 hours. The presence of a non-blocking control mAb did not result in any detectable inhibition (not shown). The observed reduction in the number of spreading cells was not due to a decrease in melanoma cell attachment, as the numbers of attached cells was comparable with or without antibody at all time points tested (Figure IV.4b).

Since we determined that both melanoma and endothelial cells express $\alpha_\text{v}\beta_3$, it was of interest to determine on which cell type the antibody was eliciting its effects. To address this issue, either melanoma or endothelial cells were preincubated with the LM609 mAb and then washed prior to co-culture. Preincubation of the melanoma cells resulted in a 40% reduction in spreading cells at 5 hours (Figure IV.5a). In contrast, no significant inhibition of melanoma cell transendothelial migration was observed following endothelial cell preincubation with the antibody (Figure IV.5b). Significant inhibition of melanoma cell transendothelial migration was most apparent at 5 hours in these experiments, implying, therefore, that $\alpha_\text{v}\beta_3$ is likely involved in the later stages of melanoma transendothelial migration. We previously determined that most melanoma cells undergo diapedesis and spreading at this assay time (see chapter 2).
Figure IV.1. $\alpha,\beta_3$ distribution on HMVEC and melanoma cells. Cell staining was carried out using the LM609 mAb directed against $\alpha,\beta_3$ following methanol fixation. LSCM images of HMVEC (a) and WM239 melanoma (b) depict the cell-cell contact distribution of $\alpha,\beta_3$. Bars: 10 $\mu$m
Figure IV.2. Expression of \( \alpha_3\beta_3 \) integrin on the surfaces of WM239 melanoma cells and in heterotypic contacts with endothelial cells during transendothelial migration through HMVEC monolayers. Seen are LSCM images, sectioned in 1 \( \mu \)m intervals, of Dil-labelled melanoma cells fixed after 3 hours of co-culture with endothelial cells and stained using a mAb (LM609) against \( \alpha_3\beta_3 \). In a and b, a melanoma cell is seen attached to the surface of the endothelium. \( \alpha_3\beta_3 \) is apparent on the surface of the melanoma cell and on melanoma membrane blebs in contact with the endothelium (arrowheads in b). Section a is 4 \( \mu \)m above b. \( \alpha_3\beta_3 \) is expressed in heterotypic contacts between melanoma cells and endothelial cells during melanoma transendothelial migration (c-f). An early migrating melanoma cell is seen in c and d, which are 7 \( \mu \)m apart. A "spindle-shaped" cell is depicted in e and f which are separated by 4 \( \mu \)m. Part of the cell has reached the Matrigel and shows signs of spreading. Heterotypic, \( \alpha_3\beta_3 \) containing, contacts are pointed out by the arrows (d to f). Melanoma protrusions are frequently observed in these transmigrating cells (arrowheads). Bars: 10 \( \mu \)m
Figure IV.3. Expression of $\alpha_\beta_3$ integrin in heterotypic melanoma-endothelial contacts and on the basal surface of spreading WM239 melanoma cells during melanoma transendothelial migration through HMVEC monolayers. The LSCM images show two spreading Dil-labelled melanoma cells fixed after 3 hours of co-culture with endothelial cells and stained using a monoclonal antibody (LM609) directed against $\alpha_\beta_3$. $\alpha_\beta_3$ integrin is apparent in membrane contacts (arrows) between melanoma and endothelial cells, as well as the bottom of melanoma cells where they come in contact with the Matrigel as indicated by the arrowheads. The cells were sectioned in 1 $\mu$m intervals. The images of the first cell, shown in a and b, are 1 $\mu$m apart. The images shown in c and d represent the second spreading cell. These sections are separated by 2 $\mu$m. Bars: 10 $\mu$m
Figure IV.4. Transmigration of WM239 melanoma cells through HMVEC in the presence of an antibody directed against $\alpha_\beta_3$. WM239 melanoma cells were added to HMVEC monolayers and transmigration occurred in the presence of the LM609 mAb recognizing $\alpha_\beta_3$ (a). Coverslips were fixed at 1, 3 and 5 hours following addition of the melanoma cells. The antibody was added to the endothelial cells 30 minutes prior to the addition of the melanoma cells. The stage of transmigration was scored as outlined in Experimental Procedures. Shown are the percentage of spreading cells at each time point. Transmigration in the absence of antibody is shown by the open bars. The solid bars represent the percentage of spreading cells in the presence of 40 $\mu$g/mL LM609 while the striped bar indicates migration with 120 $\mu$g/mL of the antibody. An asterix indicates a statistically significant reduction in spreading cell numbers (p<0.001). Data represent the mean $\pm$ S.D.. The antibody did not affect cell attachment as shown by the number of melanoma cells attached to the endothelium (b). The attached cell data represent the total number of cells scored for 45 fields in representative coverslips at the times indicated. The concentration of LM609 used is indicated in parentheses with the hours of incubation.
a

![Bar chart showing percentage of spreading cells over time with and without antibody.]

b

<table>
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<th>Time (h)</th>
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<th>With antibody</th>
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<td>405</td>
</tr>
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<td>3 (40 µg/mL)</td>
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</tr>
<tr>
<td>5 (40 µg/mL)</td>
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<td>1003</td>
<td>1150</td>
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**Figure IV.5.** Transmigration of WM239 melanoma cells through HMVEC following a preincubation with an antibody recognizing α₃β₃. In a, WM239 melanoma cells were preincubated with the LM609 mAb (40 μg/mL) against α₃β₃ for 30 minutes at 37°C. Unbound antibodies were washed out of the melanoma cell suspension prior to adding the melanoma cells to the HMVEC monolayers. In b, the HMVEC monolayer was preincubated with the LM609 antibody (40 μg/mL) for 30 minutes at 37°C. The unbound antibodies were washed out of the system prior to the addition of the melanoma cells. Coverslips were fixed at 1, 3 and 5 hours of co-culture. The melanoma position in transmigration was scored as outlined in chapter 2. Shown are the percentage of spreading cells at each time point. Transmigration in the absence of antibody treatment is shown by the open bars. The solid bars represent the percentage of spreading cells after preincubation with LM609. The asterix indicates a statistically significant reduction in spreading cell numbers (p<0.001). Data represent the mean ± S.D.
a

% Spreading cells

Time (h)

b

% Spreading cells

Time (h)
Inhibition of melanoma transendothelial migration by RGD peptides

To further evaluate the role of $\alpha_\beta_3$ integrin in melanoma cell transendothelial migration, peptide inhibition studies were undertaken. The $\alpha_\beta_3$ integrin mediates adhesion, like many integrins, via a RGD sequence (Rouslahti and Obrink, 1996). Therefore, peptides containing the RGD sequence were tested for their inhibitory effects on melanoma transendothelial migration at various concentrations. The linear RGD peptide inhibited melanoma cell transmigration by 40%, while a linear RAD peptide was not able to inhibit melanoma extravasation. In comparison, the cyclic RGD peptide was most effective and achieved 70% inhibition at 20 $\mu$M (Figure IV.6a). The cyclic RGD peptide has previously been used as a specific $\alpha_\beta_3$ function blocking peptide (Brooks et al., 1996; Pfaff et al., 1994). The observation that the cyclic peptide was more potent than its linear counterpart, suggests that $\alpha_\beta_3$ is the major integrin involved during melanoma transendothelial migration. Time course studies using the cyclic RGD peptide (Figure IV.6b) indicated that, while inhibiting at all time points, the $\alpha_\beta_3$ peptide inhibitor, like the inhibiting antibody, had its greatest effect at 5 hours. The number of cells attached at all time points tested was scored and the results indicated that the cyclic RGD peptide did not adversely affect cell adhesion to the monolayer (Figure IV.6c).

$\alpha_\beta_3$-negative melanoma cells are impaired in transendothelial migration

The key role played by $\alpha_\beta_3$ was further supported by our study of the transendothelial migration of M21 melanoma cell variants which did not express the $\alpha_\beta_3$ integrin. The parental $\alpha_\beta_3$-expressing cell line was sorted by FACS for stable cell lines which expressed altered amounts of $\alpha_\gamma$ (Cheresh and Spiro, 1987). One of these cell lines, M21-L, did not express $\alpha_\beta_3$ since it did not synthesize $\alpha_\gamma$. The $\alpha_\gamma$ integrin subunit was introduced into the M21-L cell line on an expression vector yielding the M21-L4 line (Felding-Haberman et
The M21-L4 cell line therefore expressed $\alpha_v\beta_3$. The M21-L12 line was derived by transfecting the M21-L cells with an empty expression vector. Consequently, the M21-L12 cells remained $\alpha_v\beta_3$-negative. Here we examined the ability of these different cell lines to undergo transendothelial migration through HMVEC monolayers (Figure IV.7). The $\alpha_v\beta_3$ negative variants of the parental M21 line (M21-L and M21-L12) were compromised in their ability to undergo transendothelial migration. However, the M21-L4 line, in which $\alpha_v\beta_3$ was reintroduced, was able to transmigrate with similar efficiency as the parental cell line.

**L1, like $\alpha_v\beta_3$, is expressed by both melanoma and endothelial cells**

Our results, thus far, have established a role for melanoma $\alpha_v\beta_3$ integrin during transendothelial migration. Next, we endeavoured to identify the $\alpha_v\beta_3$ ligand. Since we previously documented that interactions with CD31 were not required for melanoma transendothelial migration (see chapter 3), we turned our attention to the Ig-superfamily member, L1. L1-coated substrates have been shown to support melanoma cell attachment and spreading via interactions with the $\alpha_v\beta_3$ integrin (Montgomery et al., 1996). To begin this investigation, we examined the distribution of L1 on endothelial and melanoma cells (Figure IV.8). We found that both endothelial cells and melanoma cells expressed L1. However the level of L1 at the cell membrane was relatively low compared with the level of cytoplasmic staining. Both cell types showed punctate perinuclear staining, suggesting L1 was actively being synthesized.

**Effects of anti-L1 antibodies on melanoma transendothelial migration**

The RGD sequence in the sixth immunoglobulin-like domain of human L1 has been shown to interact with the $\alpha_v\beta_3$ integrin (Montgomery et al., 1996; Felding-Habermann et al.,
To determine whether L1 was involved in melanoma cell transmigration, we added a polyclonal antibody directed against L1 Ig-like domains 4, 5 and 6, to melanoma transendothelial migration experiments and examined the percentage of spreading cells at various time points (Figure IV.9a). While the Ig456 pAb did not exhibit significant inhibitory effects on melanoma cells during the first 3 hours of co-culture, it inhibited extravasation by approximately 30% at 5 hours. In contrast, the addition of a control pAb did not have any inhibitory effect (not shown). Again, the number of attached cells at each time point (Figure IV.9b) indicated that the Ig456 pAb had negligible effects on the attachment of melanoma cells to the endothelium. L1 is known to mediate homophilic interactions through a region in Ig-like domain 2 (Zhao and Siu, 1995; Zhao et al., 1998). Since L1 is expressed by both melanoma and endothelial cells, inhibition experiments were performed using a polyclonal antibody raised against L1 Ig-like domains 1, 2 and 3. We did not observe any detectable inhibitory effects when the Ig123 pAb was present during melanoma transendothelial migration, thus ruling out the involvement of homophilic L1 interactions during the process. Importantly, like the $\alpha_v\beta_3$ inhibition experiments, reduced spreading cell numbers were observed only at the 5 hour timepoint, suggesting that L1 and the $\alpha_v\beta_3$ integrin function together during extravasation.

These results caused us to speculate that L1 on endothelial cells, and not melanoma cells, was involved in tumour cell extravasation. To address this issue, either endothelial cells or melanoma cells were preincubated with the L1 Ig456 pAb before co-culture. Preincubation of the WM239 melanoma cells with the L1 Ig456 pAb did not inhibit transendothelial migration (Figure IV.10a). However, preincubating the HMVEC with the pAb did produce a small, but statistically significant, level of inhibition (Figure IV.10b). Again, these inhibitory effects were most apparent at 5 hours. The observed endothelial-specific inhibition was approximately 15% and this inhibition confirmed that L1 on the endothelial cells, and not the melanoma cells, has a role during melanoma transendothelial migration.
**Figure IV.6.** Peptide inhibition of WM239 melanoma transmigration through HMVEC. Shown are the peptide inhibition curves for various peptides at five hours of co-culture (a). WM239 melanoma cells were added to HMVEC monolayers and transmigration occurred in the presence of the cyclic RGD peptide (squares), the linear RGD peptide (diamonds) and the linear RAD control (circles). The data are expressed relative to controls with no peptide present. The concentration of the reagents was as indicated. In b, a timecourse for the transmigration of WM239 melanoma cells through HMVEC monolayers in the presence of the cyclic RGD peptide is shown. For this experiment, WM239 melanoma cells were added to HMVEC monolayers and transmigration occurred in the presence of 90 μM of the cyclic RGD peptide. The coverslips were fixed at 1, 3 and 5 hours as indicated. Transmigration in the absence of the peptide is indicated by the open bars and that with peptide present is represented by the solid bars. The melanoma position in transmigration was scored as outlined in chapter 2. An asterisk indicates a statistically significant reduction in spreading cell numbers (single asterisk corresponds to p<0.001 while a double asterisk indicates p<0.01). Data represent the mean ± S.D.. The cyclic RGD peptide did not affect cell attachment as shown by the number of melanoma cells attached to the endothelium (c). The attached cell data represent the total number of cells scored in representative coverslips at the times indicated.
Protein concentration (μM)

Time (h)

No peptide

With peptide

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<th>With peptide</th>
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<tr>
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<td>437</td>
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Figure IV.7. Transendothelial migration of the M21 series melanoma cells through HMVEC monolayers. The transmigration of the M21 series melanoma cells through HMVEC monolayers was monitored at 1, 3 and 5 hours: M21 parental, $\alpha_\text{v}\beta_3$-positive cell line (squares), the FACS-sorted, M21-L $\alpha_\text{v}\beta_3$-negative cells (triangles), the M21-L4 $\alpha_\text{v}$ transfected, $\alpha_\text{v}\beta_3$-positive melanoma (diamonds) and the mock-transfected, M21-L12 $\alpha_\text{v}\beta_3$-negative line (circles). Shown are the percentage of spreading cells at each time point. Data represent the mean $\pm$ S.D.
Figure IV.8. L1 distribution on endothelial and melanoma cells. LSCM images of HMVEC (a), and a melanoma cell (b) are shown. Cells were fixed with paraformaldehyde and staining was carried out as outlined in Experimental Procedures. The monoclonal L1 antibody was used in a, while the L1 polyclonal fibronectin repeat antibody was used in b. Both coverslips were paraformaldehyde fixed. In a, L1 can be seen in endothelial contacts and in cytoplasmic vesicles. In contrast, melanoma cells show prominent vesicular staining in the perinuclear region (b). Bars: 10 μm
**Figure IV.9.** Transmigration of WM239 melanoma cells through HMVEC in the presence of antibodies directed against L1. WM239 melanoma cells were added to HMVEC monolayers and transmigration occurred in the presence of L1 polyclonal antibodies (a). Coverslips were fixed at 1, 3 and 5 hours of co-culture. The stage of transmigration was scored as outlined in chapter 2. Shown are the percentage of spreading cells at each time point. Transmigration in the absence of antibody is shown by the open bars. The solid bars represent the percentage of spreading cells in the presence of 1:10 dilution of serum raised against L1 Ig-like domains 4 to 6, while the grey bar indicates the percentage of spreading cells in a 1:100 dilution of this pAb. The striped bars indicate the percentage of spreading cells in the presence of a 1:10 dilution of the serum directed against L1 Ig-like domains 1 to 3. The asterix indicates a statistically significant reduction in spreading cell numbers (p<0.001). The data represent the mean ± S.D.. The Ig456 antibody did not effect cell attachment as shown by the number of melanoma cells attached to the endothelium (b). The attached cell data represent the total number of melanoma cells scored in 45 fields from representative coverslips at the times indicated.
a

% Spreading cells

Time (h)

1 1 3 3 5 5 5

With antibody

No antibody

b

<table>
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<td>5</td>
<td>216</td>
<td>251</td>
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**Figure IV.10.** Transmigration of WM239 melanoma cells through HMVEC following a preincubation with the L1 Ig456 pAb. In a, WM239 melanoma cells were preincubated with the L1 Ig456 pAb (1:10 dilution) for 30 minutes at 37°C. Unbound antibodies were washed out of the melanoma cell suspension prior to adding the melanoma cells to the HMVEC monolayers. In b, the HMVEC monolayer was preincubated with the L1 Ig456 pAb (1:10 dilution) for 30 minutes at 37°C. The unbound antibodies were washed out of the system prior to the addition of the melanoma cells. Coverslips were fixed at 1, 3 and 5 hours of co-culture. The melanoma position in transmigration was scored as outlined in chapter 2. Shown are the percentage of spreading cells at each time point. Transmigration in the absence of antibody treatment is shown by the open bars. The solid bars represent the percentage of spreading cells after preincubation with L1 Ig456. The asterix indicates a statistically significant reduction in spreading cell numbers (p<0.001). Data represent the mean ± S.D.
a

% Spreading cells

Time (h)

b

% Spreading cells

Time (h)
\textit{L1 and \(\alpha_5\beta_3\) interactions during extravasation are distinct from those mediated by N-cadherin}

The above results suggest that L1 and \(\alpha_5\beta_3\) may function as an adhesive pair during melanoma extravasation. When the transendothelial migration assay was carried out in the presence of both anti-L1 and anti-\(\alpha_5\beta_3\) antibodies, no additive inhibitory effects were observed, suggesting that L1 and \(\alpha_5\beta_3\) may interact with each other during melanoma transendothelial migration (Figure IV.11). Previously, we determined that a mAb against N-cadherin (GC-4) was able to inhibit melanoma extravasation at 3 hours, but not at 5 hours (see appendix) (Sandig \textit{et al.}, 1997). We, therefore, tested the effect of GC-4 in combination with LM609 and Ig456 during melanoma extravasation (Figure IV.11). Additive inhibitory effects were achieved at 3 hours but not at 5 hours. These results indicated therefore, that N-cadherin-mediated cell adhesion is distinct from the L1-\(\alpha_5\beta_3\) adhesive system, and that these interactions may operate at different stages of melanoma cell transendothelial migration.

\textit{L1 expression in melanoma cells changes during transendothelial migration}

To examine the subcellular localization of L1 during transendothelial migration, co-cultures of melanoma and endothelial cells were stained with L1 antibodies (Figure IV.12). The immunofluorescence staining patterns revealed that the perinuclear distribution of L1-containing vesicles in the melanoma cells was reduced when cultured with the endothelial cells. Furthermore, melanoma L1 expression changed from the largely vesicular pattern, observed when the melanoma cells were cultured alone, to one reminiscent of the endothelial cells. In fact, were it not for the tagging of the melanoma cells, the location of the melanoma cells would not have been easily deciphered. L1 was associated with large membrane blebs that characterized the ventral surface of melanoma cells when contacting
the endothelium. In addition, L1 staining was observable in membrane protrusions during the extravasation process. Immunostaining using the L1 mAb reduced background staining and made L1-containing vesicles and endothelial cell contacts containing L1 readily observable (Figure IV.12 g and h). Furthermore, L1 was also detected at the base of melanoma cells as they spread on the Matrigel basement membrane matrix.

We also observed that melanoma cells, when cultured alone, shed L1. L1-containing vesicles were seen associated with the periphery of cells (Figure IV.13a). In addition, intensely labelled vesicles deposited on the coverslips were also observed (Figure 13 c-g). The Matrigel-associated vesicles suggested to us that L1 was collected into these structures and then shed into the medium.
Figure IV.11. Transmigration of WM239 melanoma cells through HMVEC in the presence of antibodies for α,β3, L1 and N-cadherin. Melanoma cells were added to endothelial monolayers and transmigration occurred in the presence of various combinations of the LM609 antibody for α,β3 (40 µg/mL) or the L1-Ig456 (1:10 dilution) antibody or the GC-4 antibody to N-cadherin (370 µg/mL). Inhibition by the GC-4 monoclonal antibody was previously published (see appendix I) (Sandig et al., 1997). Coverslips were fixed at 3 and 5 hours as indicated. The percentage of spreading cell was scored as outlined in chapter 2. Shown are the percentage of spreading cells relative to controls for at each time point. The open bars represent the percentage of spreading cells in the absence of any antibody. The percentage of spreading cells in the presence of only the LM609 mAb is indicated by the grey bars and only the L1 Ig456 pAb by the horizontal line bars. The percentage of spreading cells in the presence of both the α,β3 and L1 Ig-456 antibodies is shown by the solid bars, while the percentage of spreading cells in the presence of all three antibodies is indicated by the diagonal line bars. An asterix indicates a statistically significant reduction in spreading cell numbers (p<0.001). Data represent the mean ± S.D..
Figure IV.12. L1 staining of melanoma cells transmigrating though endothelial monolayers. LSCM images were taken at 1 μm intervals though WM239 cells at different stages of transmigration. Nuclei of melanoma cells labelled with bromodeoxyuridine were stained red (a to f). The cells were fixed at three hours and stained with a L1 pAb (green). An attached melanoma cell is shown in a and b. In a, the spherical melanoma cell can be observed expressing diffuse L1 staining on the surface and in the cytoplasm. b is 6 μm lower, at the point of contact between the melanoma cell and the endothelial monolayer. L1 staining is seen in association with melanoma membrane blebs in this contact region (arrowhead) at the bottom of the melanoma cell. Sections shown in c and d are representative of a melanoma cell during diapedesis. Diffuse L1 staining is present in both melanoma and endothelial cells as observed in c and 5 μm below in d. A bleb rich in L1 is seen in d (arrowhead). Sections through a spreading cell shown in e and f. The appearance of the spreading cell reveals the similarity of the L1 staining pattern between the melanoma cells and the endothelial cells (compare with Figure IV.8b). e and f are separated by 1 μm. LSCM images of a DiI labelled spreading melanoma cell (red) stained with an anti-L1 mAb (green) can be observed in g and h. The observed L1-containing vesicles are no longer localized to the perinuclear region. The basal region of the melanoma cell (h) is covered in L1. g and h are separated by 2 μm. Bars : 10 μm
Figure IV.13. Shedding of L1-containing vesicles by melanoma cells. Melanoma cells were fixed with paraformaldehyde and stained with the L1 pAb as outlined in Experimental Procedures. Confocal images showing L1-containing vesicles associated with cell membranes (a and b) and in the culture medium (c to g) are presented. An X-Z image along the line in a is shown in b and an X-Z section through the line shown in c is shown in d. L1 vesicles found in the medium are shown near a melanoma cell at low magnification in e while others are shown at high magnification in f and g. Bars: 10 μm
Figure IV.14. Summary of the observed distribution of $\alpha_\varepsilon\beta_3$ during melanoma transendothelial migration. Schematic drawing depicting the distribution of $\alpha_\varepsilon\beta_3$ at the different stages of the transendothelial migration of melanoma cells. The *heavy lines* indicate the abundance of the integrin on the surface of the melanoma cells and in the heterotypic contacts between melanoma cells and endothelial cells. A detailed description of these stages has been outlined previously (see chapter 2).
IV. DISCUSSION

Previously, we used our *in vitro* model of melanoma cell transendothelial migration to examine cell morphologies, actin dynamics and the involvement of CD31 and cadherins during the process (see chapters 2 and 3 and appendix) (Sandig *et al.*, 1997; Voura *et al.*, 1998). Here we have continued our earlier work by demonstrating that the $\alpha_\text{v} \beta_3$ integrin is an integral cell adhesion molecule involved in melanoma extravasation.

Immunofluorescence labelling studies indicated that $\alpha_\text{v} \beta_3$ integrin is expressed by endothelial cells and over the entire surface of melanoma cells. During transmigration, $\alpha_\text{v} \beta_3$ was detected in large melanoma cell membrane blebs contacting the endothelial cells. The surfaces of these membrane protrusions originating from melanoma cells were also observed to express $\alpha_\text{v} \beta_3$. Our previous studies of the actin cytoskeleton revealed that melanoma cells produce blebs during extravasation (see chapter 2). The $\alpha_\text{v} \beta_3$ integrin is the first adhesion molecule to be observed on these structures. The purpose of these membrane extensions, however, is currently unknown. One might speculate that they may be precursors of lamellipodia (Chen, 1981), or perhaps they serve as a means to release melanoma cell proteins into the microenvironment to facilitate extravasation (Basbaum and Werb, 1996; Ginestra *et al.*, 1997).

Our immunofluorescence results also indicate that the $\alpha_\text{v} \beta_3$ integrin is present in melanoma-endothelial heterotypic junctions, as well as on the basal membrane of spreading melanoma cells contacting the ECM, during melanoma extravasation (see Figure IV.14). In combination, our staining data suggest that, during the diapedesis and spreading stages of melanoma transendothelial migration, the $\alpha_\text{v} \beta_3$ integrin on melanoma cells is available for interaction with endothelial cell membrane proteins and may also adhere to ECM molecules in the Matrigel.

Our antibody inhibition experiments suggest that the $\alpha_\text{v} \beta_3$ integrin present on melanoma cells, rather than endothelial cells, is responsible for the $\alpha_\text{v} \beta_3$-mediated contacts.
during melanoma cell extravasation. Furthermore, transendothelial migration of melanoma cells was significantly delayed following specific incubation of the melanoma cells with the \( \alpha_\beta_3 \) mAb. Since the inhibitory effects were most pronounced at 5 hours, \( \alpha_\beta_3 \) is likely required during the later stages of transendothelial migration, consistent with the staining results discussed above.

Inhibition studies using the cyclic RGD peptide further highlighted the importance of the \( \alpha_\beta_3 \) integrin for melanoma transendothelial migration. Time course studies carried out in the presence of the cyclic peptide indicated that \( \alpha_\beta_3 \)-mediated adhesion was most pronounced at 5 hours, suggesting that, while \( \alpha_\beta_3 \)-mediated adhesion is likely involved in all stages of the process, it may have a crucial role during later stages of transendothelial migration. The degree of inhibition observed at 5 hours using the cyclic RGD peptide corroborated both our immunofluorescence experiments and our antibody inhibition results. The lesser effect of the blocking antibody, when compared to the inhibition produced by the cyclic peptide, was probably due to a reduction in antibody concentration caused by internalization and turnover of the bound antibody during the incubation period.

M21 melanoma cells deficient in \( \alpha_\beta_3 \) migrated poorly when compared to their \( \alpha_\beta_3 \)-positive counterparts. The decreased efficiency of transmigration displayed by \( \alpha_\beta_3 \)-negative cells, which were derived from the M21 \( \alpha_\beta_3 \)-positive parental line, correlated with their decreased tumorigenicity in nude mice (Felding-Haberman et al., 1992). These findings suggest that decreased tumorigenicity is possibly related to the efficiency of transmigration. Our previous findings using the poorly metastatic WM35 melanoma cell line (see chapter 2) are reminiscent of our M21 transendothelial migration results. The migratory ability of the WM35 cell line, like the \( \alpha_\beta_3 \)-negative M21 cell variants, was depressed compared to their more aggressive counterparts. TNF\( \alpha \) was found to increase the ability of the WM35 cells to extravasate, and this effect was inhibited by the \( \alpha_\beta_3 \) antibody, LM609. Since the \( \alpha_\) and \( \beta_3 \) integrin subunits were not upregulated by TNF\( \alpha \) in
the WM35 cells, the cytokine may function by activating the $\alpha_\beta_3$ integrin already expressed by these cells.

The Ig-like molecules, L1 and CD31, are both ligands for the $\alpha_\beta_3$ integrin (Buckley et al., 1996; Montgomery et al., 1996; Felding-Habermann et al., 1997). Since CD31 is redistributed away from endothelial junctions associated with migrating melanoma cells and is not found in heterotypic junctions between the two cell types (see chapter 3), we hypothesized that endothelial L1 might adhere to melanoma cell $\alpha_\beta_3$ during melanoma cell diapedesis. This suggestion was supported by the finding that melanoma cell $\alpha_\beta_3$ can adhere to L1-coated substrates via a RGD sequence in human L1 Ig-like domain 6 (Montgomery et al., 1996).

Our studies using cells precoated with rabbit antibodies directed against L1 Ig-domains 4, 5 and 6 suggest that endothelial cell surface L1, and not L1 expressed on melanoma cell membranes, is involved in melanoma cell diapedesis. Inhibition experiments were also conducted with a rabbit antiserum directed against L1 Ig-like domains 1, 2 and 3, to block L1 homophilic interactions mediated by sequences in L1 Ig-like domain 2 (Zhao and Siu, 1995; Zhao et al., 1998). The results suggest that homophilic interactions between melanoma cell and endothelial cell L1 are not involved during extravasation.

Inhibition experiments carried out in the presence of both the L1 Ig-456 and $\alpha_\beta_3$ antibodies did not produce additive inhibition, suggesting that the two proteins may influence melanoma extravasation by a common mechanism during the diapedesis and spreading stages of melanoma transendothelial migration. Unfortunately, technical difficulties precluded us from double-labelling L1 and $\alpha_\beta_3$ integrin during melanoma transendothelial migration. L1, however, was present in much the same location as $\alpha_\beta_3$, and therefore, might provide an adhesive ligand for the $\alpha_\beta_3$ integrin during diapedesis and spreading. Our data also indicate that adhesion mediated by L1 and the $\alpha_\beta_3$ integrin is separate in timing and function from that of N-cadherin. Previously, we documented that a
N-cadherin antibody can inhibit melanoma transendothelial migration at an earlier time point than that suggested by L1 and α₃β₃ antibodies (Sandig et al., 1997). These results suggest, therefore, that a cascade of protein-protein interactions is necessary during tumour cell extravasation.

Our results using the cyclic RGD peptide, α₃β₃-specific mAb and the α₃β₃-negative melanoma cell lines, indicate that α₃β₃-mediated adhesion is pivotal during melanoma transendothelial migration. However, there are other possible L1-integrin-mediated interactions. Notably, the α₅β₁ and α₅β₃ integrins, which are also expressed by melanoma cells, can also form heterophilic interactions with L1 and may provide supplementary forms of adhesion to L1 in parallel with that mediated by α₃β₃ (Weterman et al., 1994; Ruppert et al., 1995; Felding-Habermann et al., 1997).

A portion of cellular L1 is expressed in vesicular structures in endothelial and melanoma cells, and the perinuclear localization of these vesicles decreases in the melanoma cells when cultured with the endothelial cells. Previously, it was reported that melanoma cells can secrete L1 and that L1 associates with laminin in the extracellular matrix found in tumour sections (Montgomery et al., 1996). We also observed that melanoma cells can shed L1 containing vesicles and secrete L1 into the media. However, we did not detect any co-localization of vesicular L1 with the actin cytoskeleton (data not shown). It remains to be determined if shed and secreted L1 provides an additional adhesive substrate for melanoma α₃β₃. L1 released by the melanoma cells might adhere to the surfaces of the endothelial cells and interact with laminin in the Matrigel. In this way, melanoma cell L1, released into the media, might contribute to our observed melanoma cell α₃β₃-mediated interactions with the endothelium and the ECM during the diapedesis and spreading stages of melanoma extravasation.

Our previous results examining CD31 show a significant difference between the transendothelial migration of leukocytes and melanoma cells (see chapter 3). We found that CD31 is redistributed away from endothelial contacts and is not detectable in heterotypic
junctions during the extravasation of melanoma cells. In contrast, CD31 is an important component of leukocyte adhesion with the endothelium during diapedesis (Muller, 1995; Muller, 1995; Newman, 1997). Our findings caused us to question what adhesion molecules are expressed, instead of CD31, in the contacts formed between melanoma and endothelial cells. We have evidence that classical cadherins are expressed in these junctions (see appendix). However, our current findings indicate that melanoma cell α,β3 can interact with endothelial L1, and thereby contribute to these heterotypic contacts.

In conclusion, we have used our in vitro model of tumour cell extravasation to demonstrate that the α,β3 integrin is required during the diapedesis and spreading stages of melanoma transendothelial migration. Furthermore, we demonstrated that heterophilic adhesion between melanoma α,β3 and endothelial L1 is important during melanoma extravasation. We also determined that L1/α,β3-mediated adhesion provides a separate adhesive function from N-cadherin (Sandig et al., 1997) and that these interactions function at different points during extravasation (Voura et al., 1998). These results, therefore, begin to describe the sequence of adhesive events required during melanoma cell transendothelial migration, and have lead to a greater understanding of tumour metastasis and the progression of metastatic disease.
CHAPTER FIVE

Conclusions and Future Perspectives
Using an in vitro model of melanoma transendothelial migration, we have made considerable progress in understanding the process of tumour cell extravasation. Other systems were used to make initial observations of tumour cell interactions with endothelial cells (Kramer and Nicolson, 1979; Jones et al., 1981; Chambers et al., 1982; Nicolson, 1982; Nicosia et al., 1986; Ohigashi et al., 1989). These earlier studies involved the use of artificial blood vessels, the chorioallantoic membrane, plasma clot cultures and cultured endothelial cells (ECs). These models, while quantitating adhesion and providing a measure of tumour invasiveness, were unable to permit the careful documentation of cellular and molecular interactions during the process. In contrast, the assay system we have developed permits the examination of invasiveness and morphological changes during tumour cell extravasation in a non-disrupting and easily manipulatable system, and also a means to successfully examine molecular and cellular interactions during the process. Therefore, our assay system provides information about tumour cell transendothelial migration which was not previously available.

It must be remembered, however, that our results have been gathered using an in vitro system of tumour cell transendothelial migration, and are consequently limited by the chosen culture conditions. Any results, therefore, must ultimately be examined using an in vivo environment to determine if the interactions discovered are indeed physiological. Another limitation of our model system stems from the lack of flow conditions. Even though melanoma cells do not roll on endothelium (Giavazzi et al., 1993; Albelda et al., 1994; Carlos and Harlan, 1994; Picker, 1994; Stewart et al., 1995; Goetz et al., 1996), the shear force on the cell membrane produced by the blood stream possibly produces a response in both endothelial and tumour cells, which would be lacking in our system. Future modifications to our in vitro model system might include the introduction of the element of shear force. Perhaps shear forces would identify the strongest adhesive interactions because weak connections might not be able to form under these conditions. Furthermore, another component lacking in our strategy is the interaction between tumour
cells and blood cells, such as platelets, which have been suggested to increase the efficiency of tumour cell transmigration (Dardic et al., 1997). The role of the ECM components and the universality of the established molecular interactions must also be explored by experimenting with individual ECM constituents and different tumour types.

Nevertheless, our studies led to several novel and provocative conclusions. Briefly, we examined the dynamics of the actin cytoskeleton during melanoma cell transendothelial migration and thereby, suggested the stages involved in the process. Furthermore, we discovered that cytokines, such as TNFα, are able to augment the transendothelial migration of a non-metastatic cell line. This observation thus defined cell attachment and transmigration as two distinct steps of extravasation. Our studies also indicate that CD31-mediated adhesion is not required for melanoma diapedesis. While CD31 in endothelial junctions provides a substrate for the transmigration of leukocytes, CD31 complexes in the endothelial junctions are disassembled and redistributed during the passage of melanoma cells. These data provided us with evidence that tumour cell transmigration is distinct from that of leukocytes. Finally, antibody and peptide inhibitory studies highlighted the pivotal role of the α,β1 integrin in melanoma cell transendothelial migration, and suggested that L1 on the the endothelial membrane likely serves as a substrate for melanoma cell attachment and migration using this integrin.

We have just begun, however, to explore the distribution and function of various adhesion molecules in the process of tumour cell transendothelial migration. Further investigation will be necessary to determine what other adhesive proteins are required. Other investigations in our laboratory have indicated that classical cadherins, including N-cadherin, might be involved in the process (Sandig et al., 1997). Furthermore, our observation that TNFα upregulates the expression of the β1 integrin subunit suggests that an increase in the expression in β1 integrins might confer invasive capacity to non-invasive cells. Much work is thus required to elucidate the adhesive components involved in tumour
cell transendothelial migration and further research will also necessitate studies of the
signalling pathways initiated by these adhesive receptors.

For example, to achieve the endothelial shape changes that accompany melanoma
transendothelial migration, contacts with the migrating melanoma cell likely trigger
signalling events in the surrounding endothelial cells (see Figure V.1). Initial interactions
may lead to clustering of adhesion molecules and cytoskeletal elements beneath the EC
surface. Such structures have been reported to occur on apical, lateral, and basal surfaces
of cells (Katoh et al., 1995; Katoh et al., 1996). Integrin adhesion and clustering has been
shown to mediate the reorganization of the cytoskeleton causing changes in cell shape, cell
spreading and focal adhesion assembly (Joseph-Silverstein and Silverstein, 1998). The
formation of focal adhesions and stress fibers has been shown to be mediated by the GTP
binding protein Rho and is enhanced by Rho kinase (Amano et al., 1997; Burridge et al.,
1997; Tapon and Hall, 1997). It is conceivable that melanoma cells produce extracellular
matrix molecules to which EC integrins may bind (Albelda, 1991; Sanders et al., 1998) and
thereby permit focal contact formation or simple adhesive contacts to facilitate cell motility.
Alternatively, these actin bundles could bind to transient adherens junction-like structures
which result from cadherin interactions between the two cell types. However, if such
junctions occur, they would be unusual in that the actin from the EC is not reciprocated by
obvious actin filaments in the melanoma cells as would be expected for classical adherens
junction formation (Joseph-Silverstein and Silverstein, 1998). Since tumour cells have
been found to have defective cytoskeletal assembly, perhaps the melanoma cells may be
incapable of forming typical actin filaments in this situation (Sanders et al., 1998).

The stress fiber formation in endothelial cells, initiated from heterotypic EC-
melanoma cell contacts, exhibits a morphology suggestive of EC migration. Perhaps the
EC migrate over the surface of the melanoma cells much as they would over extracellular
matrix. Endothelial lamellipodial spreading during closure over fully migrated
melanoma cells and the consequent F-actin distribution is likely mediated by Rac (Burridge
**Figure V.1.** Schematic drawing depicting different stages of the transendothelial migration of melanoma cells and possible mechanisms for the observed changes in the actin cytoskeleton. All five steps outlined in chapter 2 have been combined into one stylized diagram. The left half of the diagram indicated by the *dashed line* is suggestive of the earlier stages of transmigration, while the right half is representative of the later stages. *Black boxes* represent connexin 43 and *thin lines* within the cells represent actin filaments. *Black circles* signify complexes of proteins known to link cadherins and integrins to the cytoskeleton. The individual components in these structures are not discussed here. See chapter 1 for a description of these linking molecules.
et al., 1997; Tapon and Hall, 1997) another GTP-binding protein in the same family as Rho. Major changes in melanoma F-actin morphology do not become apparent until the tumour cells contact the Matrigel. The resulting stress fiber formation is likely due to contacts with the extracellular matrix and caused by Rho-mediated signalling. Melanoma cells express a variety of integrins which can interact with extracellular matrix molecules found in Matrigel to mediate stress fiber formation (Merimsky et al., 1994; Weterman et al., 1994). Motility of melanoma cells under the endothelial monolayer is the result of the extension of lamellipodia and filopodia provided by further actin polymerization mediated by Rac and cdc42, respectively (Lauffenburger and Horwitz, 1996; Burridge et al., 1997; Tapon and Hall, 1997). Cdc 42 is yet another GTP-binding protein in the same family as Rho and Rac. The formation of adhesive complexes, the regulation of actin polymerization by Rho, Rac and cdc42 and the resulting mechanism of cell motility is currently under intense investigation (Huttenlocher et al., 1995; Lauffenburger and Horwitz, 1996; Galbraith and Sheetz, 1998).

Included with the CD31 results in chapter 2 was the observation that cx-43-mediated gap junctional formation occurs during melanoma transendothelial migration. These junctions possibly permit the transfer of metabolites, such as 12(S)-HETE, between tumour and endothelial cells (El-Sabban and Pauli, 1991; El-Sabban and Pauli, 1994). These metabolites might elicit specific effects in the endothelial cells juxtaposed to the melanoma cells, such as the dissolution of CD31 from EC junctions. It has been suggested that many effects of 12(S)-HETE are mediated in a protein kinase C dependent fashion. Interestingly, CD31 is phosphorylated on serine by protein kinase C and this has been shown to facilitate leukocyte transendothelial migration (Zehnder et al., 1992; Rattan et al., 1996; Rattan et al., 1997). There is increasing evidence for the tyrosine phosphorylation of CD31 and its role in various signalling pathways (Famiglietti et al., 1997; Jackson et al., 1997; Lu et al., 1997; Newman, 1997; Rattan et al., 1997; Sagawa et al., 1997; Varon et al., 1998). Tyrosine phosphorylation of CD31 has been shown to be mediated by src and
Figure V.2. Co-localization of CD31 with the actin cytoskeleton. WM239 melanoma cells were tagged with red cell tracker dye as outlined in Experimental Procedures in chapter 3 to give red nuclear staining. CD31 staining was carried out following paraformaldehyde fixation. The mAb 1.1 was used to detect CD31 and is observable as red surface staining between endothelial cells in these images. Actin was stained green as outlined in the methods section in chapter 2. LSCM was used to scan at 1 μm intervals through the co-cultures. A spreading melanoma cell transmigrating through a monolayer of HMVEC is shown. The arrowheads point to areas between the two cell types devoid of CD31, while the arrows indicate yellowish areas suggestive of the co-localization of CD31 and actin. Bar: 10 μm
**Figure V.3.** Schematic drawing depicting different stages of the transendothelial migration of melanoma cells and possible mechanisms for the observed changes in the distribution of CD31. All five steps outlined in chapter 2 have been combined into one stylized diagram. The left half of the diagram indicated by the *dashed line* is suggestive of the earlier stages of transmigration, while the right half is representative of the later stages. *Black circles* signify the complex connecting CD31 to the cytoskeleton. *Black boxes* represent connexin-43 gap junctions, while *hatched ovals* represent CD31. *Thin lines* in the cells represent actin. All mechanisms shown are outlined in the Discussion.
redistribution of CD31 to cell contacts (via recoupling to cytoskeleton? dephosphorylation?)

redistribution of CD31 to cell body (via uncoupling from cytoskeleton? phosphorylation)

lipooxygenase metabolites [12(S)-HETE]

other metabolites
csk (Lu et al., 1997; Cao et al., 1998). Importantly, SHP2 has been shown to be associated with these phosphorylated residues, linking CD31 to additional signalling pathways (Jackson et al., 1997; Jackson et al., 1997; Newman, 1997). The tyrosine phosphorylation of CD31, in contrast to serine or threonine phosphorylation, has been shown to be independent of changes in Ca$^{2+}$ concentrations and the activity of protein kinase C. (Osawa et al., 1997). CD31 phosphorylation has been suggested to alter CD31 cytoskeletal association and these changes may putatively be responsible for the movement of CD31 away from EC cell contacts (Newman et al., 1992; Romer et al., 1995; Ferrero et al., 1996). CD31 redistribution from EC cell-cell contacts has been associated with decreased CD31 interactions with the cytoskeleton (Romer et al., 1995). Others have observed increased serine phosphorylation of CD31 in correlation with the loss of CD31 from EC contacts and the destabilization of CD31-cytoskeletal connections (Ferrero et al., 1996). In light of these results, it is interesting that we observed CD31 co-localization with actin in EC cell-cell contacts which were not juxtaposed to transmigrating melanoma cells (Figure V.2). The possibility that signalling pathways triggered by 12(S)-HETE, or other metabolites, transferred through gap junctions between melanoma cells and the endothelium, leading to the phosphorylation and redistribution of CD31 in our system is worth further consideration (see Figure V.3).

Our results suggest that the ligation of L1 and the $\alpha_\text{IIb}\beta_3$ integrin is involved in the transmigration process of melanoma cells. L1 has been proposed to be linked to the actin cytoskeleton via ankyrin and spectrin during L1 homophilic interactions mediating neuronal growth cone extension (Miura et al., 1992; Hortsch, 1996; Burden-Gulley et al., 1997; Kamiguchi and Lemmon, 1997; Hortsch et al., 1998). Our immunostaining results, however, do not suggest co-localization of L1 and actin during melanoma transendothelial migration. Ongoing research by several groups has suggested that neuronal L1 Ca$^{2+}$-independent homophilic interactions activate fibroblast growth factor receptors which mediate the opening of Ca$^{2+}$ channels through the activation of phospholipase C $\gamma$.
stimulated second messengers during neurite outgrowth (Hortsch, 1996; Kamiguchi and Lemmon, 1997). Since L1 homophilic interactions result in changes which can mediate intracellular signalling, it is reasonable to consider that heterophilic interactions can also induce intracellular signals. However, little work has been published regarding L1-stimulated signalling resulting from heterophilic interactions. Since we have found that endothelial L1 interacts with melanoma cell αvβ3 during extravasation, it would be important to consider if L1-mediated signalling pathways are stimulated in these heterotypic contacts.

Our L1 Ig-456 pAb produced slightly more inhibition when present during melanoma transendothelial migration than when preincubated only with the endothelial cells. However, we also found that this pAb had no detectable effect when preincubated with the melanoma cells. In light of these findings, we suggest that our results corroborate previously published results (Montgomery et al., 1996) which indicate that L1 secreted from melanoma cells, contributes to the adhesion of melanoma αvβ3 integrin to L1. Such soluble L1 would not be blocked in our experiments which specifically treated the two cell types with pAb. Soluble L1 possibly binds to laminin in the Matrigel used in our system (Hortsch, 1996; Burden-Gulley et al., 1997; Hall et al., 1997; Kamiguchi and Lemmon, 1997). Importantly, other investigators have also observed the interaction of tumour cell αvβ3 with substrate bound L1 (Ebeling et al., 1996; Duczmal et al., 1997). Significantly, soluble L1 has been shown to stimulate neurite out-growth (Kamiguchi and Lemmon, 1997) indicating that the conformation of soluble L1 is suitable for adhesion and is capable of producing intracellular signals. Soluble L1 can also provide an adhesive substrate for glioma cells (Izumoto et al., 1996). In contrast to the results by Montgomery et al. (1996) using melanoma cells, at least a component of glioma cell adhesion to soluble L1 was mediated by a homophilic mechanism. In our system, soluble L1 is possibly released from melanoma cells via secretory granules or shedded vesicles. We observed that melanoma cells bleb during all stages of migration and our L1 staining showed that blebs do contain
Figure V.4. Schematic drawing depicting different stages of the transendothelial migration of melanoma cells and possible events involved in the observed changes in the distribution of L1 and \( \alpha_\text{v}\beta_3 \). All five steps outlined in chapter 2 have been combined into one stylized diagram. The left half of the diagram indicated by the *dashed line* is suggestive of the earlier stages of transmigration, while the right half is representative of the later stages. The *black circles* represent L1 containing vesicles. The blebs can contain soluble L1 as well, indicated by the *dark speckle pattern*. *Dashed arrows* represent L1 being deposited upon exposed surfaces and on the Matrigel at all stages of migration. The *heavy black lines* represent surfaces coated with \( \alpha_\text{v}\beta_3 \). The *dark squares* are representative of MMP-2 which may be linked to melanoma \( \alpha_\text{v}\beta_3 \). The *lined oval* signifies general integrin adhesion. *Open squares* indicate the location vinculin and/or focal contacts. All mechanisms shown are outlined in the Discussion.
some adhesion mechanism leading to increased L1 release by secretion or vesicle shedding

$\alpha_\beta_3$ mediated-adhesion may upregulate integrins

L1-mediated signals

integrin → FAK phosphotylation stimulation phosphatidylinositot 3 kinase → Rho/Rac/cdc42 → actin paxillin phosphorylation assembly
L1. Shed vesicles containing L1 were also evident. The morphology of L1 staining in the melanoma cells appeared to change when cultured with endothelial cells. Melanoma cells showed a reduction in L1 containing vesicles in the perinuclear region, suggesting a decrease in the cytoplasmic pool of L1. L1 secreted or released from broken vesicles, may deposit on the EC surface, increasing the local concentration of L1. L1 on the EC surface may thus provide a substrate for melanoma cells to cross the endothelial junctions. We can only speculate how and why the L1 expression of the melanoma cells changes when in contact with the endothelium. As we discussed in chapters 2 and 3, some sort of communication likely takes place between the melanoma cells and endothelial cells. One might consider that any L1 on the surface of the melanoma cells may provide the melanoma cells with an advantage, such that the endothelial cells may accommodate an invading cell, as if it was another endothelial cell. Such adhesion could provide the tumour cells facile access to the ECM. In fact, our results do suggest that the endothelial monolayer does readily accommodate extravasating melanoma cells (chapter 2) (see Figure V.4).

Our preliminary studies on the distribution of vinculin during melanoma transendothelial migration suggest vinculin-containing structures exist in melanoma cell junctions with ECs, as well as at the basal surface of melanoma cells spreading under the endothelial monolayer. Vinculin has been suggested to couple integrins to the cytoskeleton in focal contacts and thereby facilitate spreading and migration (Ezzell et al., 1997). Since we detected the α,β3 integrin on these surfaces, as well as vinculin, it is likely that the integrin is also found in focal contacts (Hemler, 1998). In addition, since antibody and peptide inhibition experiments show maximal effects at later time points of melanoma cell extravasation, it is likely that L1-α,β3-mediated contacts are important during cell spreading on the Matrigel. Since spreading melanoma cells may also secrete L1 and shed L1-containing vesicles, the deposition of L1 on the ECM in our system might also contribute to spreading and migration of the transmigrated melanoma cells via cell surface α,β3. Therefore, focal contact formation and interactions with L1 deposited on the Matrigel may
contribute to other $\alpha_i\beta_j$-mediated adhesion mechanisms which may be inhibited in our antibody and peptide blocking experiments.

Monocyte $\alpha_i\beta_3$ has been shown to aid in transmigration by increasing $\alpha_i\beta_2$ integrin-mediated adhesion of these cells to vascular ICAM-1 (Weerasinghe et al., 1998). Such additional adhesion could also be down-regulated by $\alpha_i\beta_3$ inhibitors, such as the cyclic RGD peptide, during melanoma extravasation in our experiments. Furthermore, $\alpha_i\beta_3$ has been shown to mediate chemotaxis and haptotaxis of melanoma cells in response to vitronectin (Aznavoorian et al., 1996; Yun et al., 1996). Notably, $\beta_1$ and $\beta_3$ integrin adhesion results in the phosphorylation of FAK resulting in cytoskeletal reorganization and focal adhesion assembly through members of the Ras superfamily of small GTPases (Rho, Rac, and cdc42) (Dedhar and Hannigan, 1996; Burridge et al., 1997). Other investigators have discovered phosphatidylinositol 3-kinase is required for signal transduction during the migration of melanoma cells (Metzner et al., 1996), thus suggesting a means for actin stress fiber formation in association with $\alpha_i\beta_3$-mediated adhesion. One might envisage that such intracellular signals may be produced during $\alpha_i\beta_3$-mediated melanoma haptotaxis on ECM molecules and laminin bound L1 in our experiments (see Figure V.4).

The $\alpha_i\beta_3$ integrin has recently been reported to localize MMP-2 to the surface of invasive cells and this interaction was shown to be inhibited by the $\alpha_i\beta_3$ function blocking antibody LM609 (Brooks et al., 1996). Metalloproteinases such as MMP-2 as well as other ECM degrading enzymes, including plasmin, have been shown to be important for cancer progression, and the localization of these enzymes to the cell surface is important for their function (de Vries et al., 1996; Chapman, 1997; Werb, 1997; Brunner et al., 1998). Membrane type matrix metalloproteinase 1 can also localize and activate MMP-2 on the cell surface of invasive cells. Therefore, inhibiting MMP-2 interaction with the $\alpha_i\beta_3$ integrin does not preclude the membrane association of the enzyme during melanoma cell extravasation (Strongin et al., 1995; Werb, 1997). However, it is possible that a further component of our observed $\alpha_i\beta_3$ inhibitor functions is provided by a reduced localization
of MMP-2 to the surface of the melanoma cells during transmigration. Such blocking would, most likely, result in reduced melanoma cell spreading on the Matrigel matrix. Interestingly, MMP-2 was recently shown to degrade laminin-5 and thereby produce a proteolytic fragment of laminin capable of inducing tumour cell migration (Giannelli et al., 1997).

Future studies will undoubtedly be required to determine how proteolytic enzymes and cytokines co-operate with adhesion molecules to mediate tumour cell transendothelial migration. It is also exciting to speculate how these factors mediate their responses. For instance, the source of the required cytokines is currently unknown. Further experiments will be required to determine if melanoma cells stimulate themselves to undergo diapedesis by an autocrine mechanism, if endothelial cytokines are required for the process, or if a cytokine from one cell type is required for the release of another factor from the other cell type and that this combination leads to transmigration. Another possibility that must be considered is the ECM itself. The Matrigel basement membrane has been suggested to contain cytokines and growth factors which might be important for tumour cell extravasation. Furthermore, proteolytic enzymes active during tumour cell transendothelial migration might make matrix cytokines more readily available. Initially, this work will require and in-depth study of what factors are produced by the cells and which ones are found in the Matrix. Such a preliminary characterization would be followed by experiments focused on how these proteins interact to produce cellular responses and what further cytokines are released as a consequence of the initial stimuli.

Likewise, studies of proteolytic enzymes must start with a characterization of which ones are involved and which cell type is the source of these proteins. Perhaps cytokines are also important in stimulating the production of these enzymes or, on the other hand, are responsible for down-regulating their inhibitors. Another aspect to be considered is the ability of proteolytic fragments of ECM components to stimulate migration, as opposed to
adhesion, and how this might be involved in the completion of diapedesis (Giannelli et al., 1997).

All this information will lead to a complex web of proteins and pathways involved in tumour cell transendothelial migration. Ultimately, we will have to consider how all these molecules interact and in what sequence. Furthermore, models of metastasis suffer from the added complexity of the shear heterogeneity of the many tumour types and the variety of locations for metastatic dissemination. The progress made over the last twenty years examining leukocyte transendothelial migration does however, provide some assurance that the mechanisms for tumour cell extravasation will be sorted out.

Insight into leukocyte extravasation was motivated by the desire to control inflammation. As a result, monoclonal antibody therapies are being implemented (Joseph-Silverstein and Silverstein, 1998). For example, graft failure in human lymphocyte antigen mismatched bone marrow transplants has been controlled using anti-LFA-1 monoclonal antibodies (Albelda et al., 1994). In an effort to avoid immunogenicity problems however, soluble adhesion molecules may also be considered in future. Unfortunately, however, the biological activity of such proteins is quite low. Peptide mimetics and anti-sense oligonucleotides, on the other hand, might provide alternative approaches (Carlos and Harlan, 1994). Recently, a peptide mimic has been implemented to inhibit osteoporosis (Engleman et al., 1997). Work using animal models has suggested that orally administered, small molecular weight compounds, might also control inflammation (Albelda et al., 1994). It is hoped that cancer metastasis will be targeted by similar therapies in future. Our work examining tumour cell extravasation, as well as prospective research in this field, will provide evidence of new protein targets for these future cancer treatments. These studies, as well as those examining; angiogenesis, apoptosis, signalling, gene regulation and others, will ultimately lead to a common understanding of cancer and to an eventual cure for the disease.
APPENDIX

Involvement of Cadherins During Transendothelial Migration of Human Melanoma Cells

Portions of this appendix have been published as

This Appendix was completed in collaboration with Dr. Martin Sandig.
I. INTRODUCTION

Transendothelial migration of tumour cells is mediated by various soluble factors and adhesive interactions which activate those interactions required for the subsequent step. These different adhesive stages are referred to as the "adhesion cascade" (Carlos and Harlan, 1994). In chapter 2, we described an in vitro system to study these adhesive interactions during the transendothelial migration of human melanoma cells.

The role of cadherins in cancer is under active investigation in many laboratories. Many transformed cells have been shown to express decreased levels of cadherins and this decreased surface expression has been associated with increased invasiveness and metastasis (Joseph-Silverstein and Silverstein, 1998). E-cadherin, in particular, is believed to be a potent suppresser of epithelial tumour cell invasion and metastasis (Gumbiner, 1996; Munro and Blaschuk, 1996; Yoshimura et al., 1996; Barth et al., 1997).

To determine the role of cadherins in tumour cell transendothelial migration, we used our in vitro system to examine the distribution of cadherins, including VE-cadherin, during the transendothelial migration of melanoma cells. Our results are summarized schematically in Figure A.1. VE-cadherin was of interest because it is an endothelial specific cadherin found in the cell-cell contacts of all endothelia examined (Heimark et al., 1990; Lampugnani et al., 1992; Caveda et al., 1996). We observed that VE-cadherin is concentrated in EC complexes, but is redistributed from those EC contact sites immediately juxtaposed to melanoma cells. VE-cadherin is absent from melanoma-EC heterotypic contacts, but reappears in EC homotypic junctions upon closure of the gap in the endothelial monolayer created by the melanoma cell. On the other hand, the pan-cadherin antibody, which recognizes the cytoplasmic domain of classical cadherins, stained the heterotypic contacts between EC and melanoma cells. The pan-cadherin results indicate that cadherins, other than VE-cadherin, are present in these junctions. Immunofluorescence labelling experiments using the anti-N-cadherin antibody, GC-4,
showed that high concentrations of N-cadherin were present in the leading edges of endothelial cells spreading over transmigrated WM239 melanoma cells. However, N-cadherin was not apparent in the heterotypic contacts between the two cell types (Sandig et al., 1997).

To assess the role of classical cadherins in melanoma cell transendothelial migration, inhibition experiments were carried out using HAV containing peptides and a function blocking mAb directed against N-cadherin. The results suggest that cadherins are involved in tumour cell transmigration and that N-cadherin may be one of the cadherins having a role in this process.
II. EXPERIMENTAL PROCEDURES

Cells and culture conditions

Human umbilical vein endothelial cells (HUVEC), human lung microvascular endothelial cells (HMVEC) and the human melanoma cell line, WM239, were all cultured as described in Experimental Procedures as found in chapter 2.

Antibodies and peptides

The GC-4, mAb against N-cadherin was purchased from Sigma Chemical (St Louis, MO). Monoclonal antibody (mAb) P2B1 against CD31 was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (Ashman et al., 1991). The HAV peptide was obtained from Dr. Orest Baschuk (McGill University, Montreal, PQ).

Transendothelial migration assay

The assay was prepared as previously described in chapter 2. For inhibition experiments, the endothelial monolayer was incubated with the antibody or peptide at the indicated concentration for 30 minutes prior to the addition of melanoma cells and was left in the culture system for the duration of the experiment.

Microscopy and quantification of transmigration of melanoma cells

The co-cultures were fixed, stained with BODIPY FL-conjugated phallacidin (Molecular Probes, Eugene, OR.) and scored as outlined in chapter 2.
III. RESULTS

Effects of anti-N-cadherin monoclonal antibody on the transendothelial migration of melanoma cells

To further assess the role of N-cadherin during melanoma transendothelial migration, the function blocking N-cadherin mAb, GC-4, was used to inhibit the transendothelial migration of the WM239 melanoma cell line through HUVEC monolayers (Figure A.2). Time course experiments showed that the presence of the antibody in the culture media caused a slow-down in the migration rate at earlier hours. This inhibition, however, was readily overcome by 5 hours of co-culture.

Effects of cadherin blocking reagents on the transmigration of melanoma through HMVEC monolayers

The ability of the N-cadherin antibody to inhibit the transendothelial migration of WM239 melanoma was also examined using HMVEC monolayers (Figure A.3). The P2B1 mAb to CD31 was previously shown by us to have no inhibitory effects on melanoma transendothelial migration (see chapter 3) and was used here as a non-blocking control mAb. The GC-4 mAb inhibited the migration of the melanoma cells through the HMVEC monolayer by 50% while the control antibody showed no inhibitory effects. Next, we tested the effect of an HAV-containing peptide which is known to inhibit cadherin function (Blaschuk et al., 1990). Melanoma cell transmigration was reduced by 40% in the presence of 1.5 mM of the HAV peptide (see Figure A.3).
Figure A.1. Cadherin distribution during melanoma transendothelial migration. Schematic drawing depicting the distribution of cadherins in the different stages (I to V) of the transendothelial migration of melanoma cells. The observed pan-cadherin staining pattern is represented by the purple colour, the N-cadherin distributions is shown in red while the expression of VE-cadherin is indicated by green.
Figure A.2. Effects of anti-N-cadherin mAb (GC-4) on the transendothelial migration of melanoma cells. GC-4 was added to the HUVEC monolayer cultured on the coverslip at 0.37 mg/mL before the seeding of DiI-labelled melanoma cells. Co-cultures were carried out for 1, 3 and 5 hours before fixation and staining for F-actin. The number of transmigrated cells which had spread on the Matrigel was scored for each sample. Solid bars represent cultures with no mAb present while grey bars indicate the presence of mAb. The asterix indicates statistically significant inhibition (p<0.001). The data represent the mean ± S.D..
Figure A.3. Inhibition of melanoma cell transendothelial migration by mAb GC-4 and HAV containing peptides at 3 hours. WM239 cell transmigration through HMVEC was carried out in the presence of the anti-N-cadherin mAb, GC-4 or control mAb (P2B1) (solid bars). Transendothelial migration of WM239 cells was also assayed in the presence of 0.8 mM or 1.5 mM of the HAV containing peptide (hatched bars). All data were normalized to the no antibody, or no peptide, control which was taken to be 1. An asterix indicates statistically significant inhibition (p<0.001). The antibody and peptide concentrations used are as indicated. All data indicate the mean ± S.D..
Inhibitors and controls

% Spreading cells relative to control

0.0 0.5 1.0 1.5

mAb control 1:10
GG-4 mAb 0.4mg/mL
1.5 mM KLVND control
1.5 mM LRAHAVDING
0.8 mM LRAHAVDING
IV. DISCUSSION

Using our in vitro model of melanoma transendothelial migration, we have observed that VE-cadherin is removed from endothelial cell-cell junctions immediately juxtaposed to melanoma cells (Sandig et al., 1997). However, other cadherins, as indicated by pan-cadherin antibody staining, are present in cell-cell contacts between endothelial cells and melanoma cells during transmigration. Also, N-cadherin is localized to the leading edges of EC processes spreading over transmigrated WM239 melanoma cells.

Antibody inhibition experiments reported here, indicate that N-cadherin may also be required at an earlier stage of melanoma transendothelial migration. Other investigators observed that N-cadherin is expressed on the cell surface of endothelial cells, but not in the cell-cell contacts, unless VE-cadherin is removed from these junctions. Therefore, it is likely that the loss of VE-cadherin from EC junctions promotes the localization of N-cadherin to this location (Navarro et al., 1998). Since, melanoma cells also express N-cadherin (Matsuyoshi et al., 1997), the melanoma cells and endothelial cells might interact by N-cadherin, prior to diapedesis, in our system. Such an interaction might explain the inhibition by the N-cadherin antibody, which we observed at early hours of melanoma cell transmigration. Correspondingly, it has been suggested that VE-cadherin is required for endothelial homotypic interactions while N-cadherin is responsible for the adhesion of endothelial cells to other N-cadherin expressing cell types (Navarro et al., 1998). However, in our system, blocking of this early adhesion event was only transient, since the rate of transmigration was reduced only in the first 3 hours. Therefore, it is likely that N-cadherin is only one of many adhesion mechanisms involved in the process at this stage of melanoma migration.

Even though we did observe N-cadherin in EC processes spreading over migrated melanoma cells, we did not detect a significant amount of inhibition by the mAb at 5 hours.
when many melanoma cells are at this stage of migration. The GC-4 effect may have dropped by 5 hours due to a gradual depletion of the antibody in the system. Furthermore, our scoring method only considered a spreading melanoma cell morphology to indicate the culmination of transmigration. These experiments therefore, did not assess whether gaps were present over a higher percentage of these spreading cells in the antibody experiments. Such an assessment might have suggested that endothelial cell spreading over the melanoma cells was inhibited at 5 hours. Therefore, the absence of inhibition at later hours in these experiments could simply reflect the fact that our scoring method was insensitive to the possible antibody effects at later hours.

Our pan-cadherin staining indicated that cadherins are present in the heterotypic contacts between melanoma cells and endothelial cells during transmigration. The inhibition obtained with the HAV peptide is consistent with the immunostaining results and together these data indicate that cadherins are integral to these junctions. The mechanism by which these cadherins could associate at these lateral junctions is still under investigation (Cowin and Burke, 1996; Katz et al., 1998). We are only now attempting to determine which cadherins are found in these contacts. Since heterophilic adhesion mechanisms between cadherins and other cell adhesion superfamily types is limited, we assume that these cadherin interactions are homophilic (Higgins et al., 1998). Also, we believe that the observed cadherin staining between the melanoma and endothelial cells is due to the presence of classical cadherins, since the pan-cadherin antibody recognizes a common region of the cadherin cytoplasmic domain found in classical cadherins. This, however, does not preclude the existence of non-classical cadherins in the heterotypic junctions.

Not all endothelial cell cadherin molecules have been identified. One study has suggested that a minimum of 5 cadherins are expressed by endothelial cells (Ayalon et al., 1994). Melanoma cells, on the other hand, express many cadherins which are not expressed by normal melanocytes, and many of these are believed to be novel (Matsuyoshi et al., 1997). It conceivable that the discovered novel melanoma cadherins are also
expressed by endothelial cells, since not all EC cadherins have been identified. Any of these novel cadherins might then interact during melanoma cell transendothelial migration. Interestingly, the expression of E- and P- cadherin in melanoma cells is less than observed in normal melanocytes (Seline et al., 1996). One might speculate that the reduced expression of these cadherins might promote melanoma cell migration.

Our experiments indicate that cadherins are part of the mechanism used by tumour cells to transmigrate through endothelial monolayers, and imply that N-cadherin is one of the participating cadherins in both early and late stages of the process. Future studies will attempt to identify which other cadherins are involved, if cadherins are specifically produced to form these interactions, as well as the nature of the cadherin-mediated adhesion within heterotypic melanoma-endothelial cell-cell contacts.
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