DEDICATION

To my children Iskra and Istok
Extracellular Matrix Affects Mesangial Cell Phenotype
Effect Of Collagen Type I and Heparin on Cell Proliferation and Contractility

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ABSTRACT

Accumulation of extracellular matrix (ECM) in renal disease is accompanied by a switch in mesangial cell (MC) phenotypes. From quiescent they become proliferative, from contractile they become hypotonic, and accumulate proteoglycans (PG) and collagen type I (collagen) among other ECM components. Heparin, a glycosaminoglycan related to heparan sulfate PGs, and collagen both suppress MC proliferation. We tested the hypothesis that heparin and collagen regulate MC phenotypes by affecting intracellular kinase cascades. Compared with growth on plastic, Matrigel, or mesangial matrix, collagen decreased cell number, total DNA per culture, mitogenic response, and c-fos induction. Intact collagen was required for these effects. Soluble collagen fragments were ineffective. Collagen decreased activation of mitogen-activated protein kinase (MAPK) by phorbol ester (TPA) or serum, or of Ca²⁺/calmodulin-dependent kinase (CaMK)-II by ionomycin. Loss of PKC activity upon trypsinization was restored in cells attached to plastic but not in cells on collagen. Collagen inhibited global phosphorylation of tyrosine in contrast to cells on plastic. Compared with plastic, cells grown on collagen were more contractile, showed a higher Ca²⁺ signal, and faster endothelin-1-dependent (ET-1) myosin light-chain (MLC) phosphorylation. Heparin suppressed the serum-dependent mitogenic response and inhibited the serum-stimulated entry of extracellular Ca²⁺ similarly to verapamil. Serum- and TPA-dependent or Ca²⁺-dependent c-fos induction in MCs appear to be suppressed by the ability of heparin to suppress MAPK or CaMK-II activation, respectively. MAPK activation elicited by serum or TPA was suppressed by heparin. Intracellular Ca²⁺ chelation prevented ionomycin-dependent c-fos induction.
induction. KT-5926 and KN-93 inhibited c-fos induction by ionomycin but not by serum or TPA. Heparin suppressed the development of autonomous CaMK-II activity but did not inhibit total CaMK-II activity or activation of CaMK-II in vitro. Heparin did not affect MLC phosphorylation or MC contractility. Thus, while collagen increased ET-1-dependent MC contraction, heparin was without effect. These results demonstrate that both heparin and collagen suppress MC mitogenic responsiveness by affecting c-fos induction and activation of MAPK and CaMK-II, and do so without compromising MC contractility.
ACKNOWLEDGMENTS

I am most indebted my supervisor, Dr. D.M. Templeton, for accepting me into his laboratory in order to complete these studies, for the provision of a fertile research environment in which to study and for his patience, guidance and encouragement throughout this period. I greatly appreciate the time and effort he spent in preparing this thesis. I also want to thank Dr. J. Parkes for providing helpful suggestion and criticism both during the completion and during writing of this thesis.

I would like to thank the other members of my advisory committee, Dr. C.I. Whiteside and Dr. P. Harper, for their helpful comments and advice during these years. I also want to thank all my colleagues from the lab for their advice and assistance.

I am also greatful for the financial assistance provided by the University of Toronto Open Doctoral Fellowship and Dr. Templeton's grant, which permitted me to pursue this study.

Finally I must thank my family for their love, support and understanding which have helped me throughout the years. I want to express my deepest appreciation to my parents, especially to my mom, who has always placed a premium upon education. Her beliefs and encouragement provided a firm basis of support which is and always will be inspirational. Particularly, I want to express my deepest appreciation to my wife, Jasmila, who was always there with unfalling support, constant encouragement and understanding, through the good and bad times. This has truly been a joint degree in which we both have participated.
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AGE</td>
<td>advanced glycation endopproducts</td>
</tr>
<tr>
<td>ANF</td>
<td>atrial natriuretic factor</td>
</tr>
<tr>
<td>ANG-II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>AT-III</td>
<td>antithrombin</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis-(2-aminophenoxy)ethane-N,N',N'-tetra-acetic acid</td>
</tr>
<tr>
<td>BGG</td>
<td>bovine-y-globulin</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>[Ca^{2+}]_i</td>
<td>intracellular calcium concentration</td>
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<tr>
<td>CaMK-II</td>
<td>Ca^{2+}/calmodulin-dependent kinase -II</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>CRE-binding protein</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulfate</td>
</tr>
<tr>
<td>CSA</td>
<td>cross-sectional area</td>
</tr>
<tr>
<td>CSF-1</td>
<td>colony stimulating factor 1</td>
</tr>
<tr>
<td>CSPG</td>
<td>chondroitin sulfate PG</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DDR</td>
<td>discoidin domain receptor</td>
</tr>
<tr>
<td>DexS</td>
<td>dextran sulfate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified essential medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DS</td>
<td>dermatan sulfate</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>Erk</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FFC</td>
<td>fibril-forming collagens</td>
</tr>
<tr>
<td>aFGF</td>
<td>acidic fibroblast growth factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>FN</td>
<td>fibronectin</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>Gal</td>
<td>galactose</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GBM</td>
<td>glomerular basement membrane</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GHRH</td>
<td>GH releasing hormone</td>
</tr>
<tr>
<td>GlcN</td>
<td>glucosamine</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>GlcUA</td>
<td>Glucuronic acid</td>
</tr>
<tr>
<td>GRIPS</td>
<td>glypican-related integral membrane PGs</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronic acid</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
</tbody>
</table>
HC-II heparin cofactor II
HEPES N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HMC human mesangial cells
HRP horseradish peroxidase
HS heparan sulfate
HSPG heparan sulfate proteoglycan
5-HT 5-hydroxytryptamine hydrochloride (serotonin)
ICAM-1 intracellular adhesion molecule 1
IdoUA L-iduronic acid
IGF insulin-like growth factor
IL-1 interleukin-1
ILK integrin-linked kinase
IP3 inositol 1,4,5-triphosphate
Kf glomerular ultrafiltration coefficient
KS keratan sulfate
LDH lactate dehydrogenase
LDL low-density lipoprotein
LM laminin
L-NMMA L-N-nomonomethyl arginine
LPS lipopolysaccharide
MAPK mitogen activated protein kinase
MC mesangial cells
MEK MAPK//Erk kinase
MEKK MEK kinase
MKHH modified Krebs-Henseleit/HEPES
MKSS modified Krebs-Henseleit-HEPES buffer
MLB myelin basic protein
MLC  myosin light chain
MLCK  MLC kinase
MMP  matrix metalloproteases
MOPS  3-[N-morpholino]propanesulfonic acid
MTT  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N-CAM  neuronal cell adhesion molecule
NO  nitric oxide
PAF  platelet activating factor
PAI-1  plasminogen activator inhibitor-1
PCNA  proliferating cell nuclear antigen
PDGF  platelet-derived growth factor
PGs  proteoglycans
PG (E,F,1)  prostaglandins E, F, 1
PI  phosphoinositol
PKA  protein kinase A
PKC  protein kinase C
PLC  phospholipase C
PMSF  phenylmethylsulfonyl fluoride
PBS  phosphate-buffered saline
RER  rough endoplasmic reticulum
RIPA  radiolmmunoprecipitation assay buffer
RMC  rat mesangial cells
SHR  spontaneously hypertensive rat
SLIPS  syndecan-like integral membrane PGs
SMA  smooth muscle actin
SMC  smooth muscle cells
SRE  serum response element
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>SSC</td>
<td>saline sodium citrate</td>
</tr>
<tr>
<td>SSPE</td>
<td>saline sodium phosphate EDTA buffer</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
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<tr>
<td>TGF-α</td>
<td>transforming growth factor alpha</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloprotease</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>t-PA</td>
<td>tissue-type plasminogen activator</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoyl phorbol-13-acetate</td>
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<tr>
<td>TRE</td>
<td>TPA response element</td>
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<td>Tris</td>
<td>tris(hydroxymethyl) aminomethane</td>
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<td>thrombospondin</td>
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<tr>
<td>TxA2</td>
<td>thromboxane A2</td>
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<tr>
<td>u-PA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>VER</td>
<td>verapamil</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular SMC</td>
</tr>
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<td>Xyl</td>
<td>xylose</td>
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1. INTRODUCTION

The intrinsic mesangial cell (MC) is an active participant in a variety of renal diseases. In chronic glomerular disease, MC proliferation and excessive matrix production and deposition cause progressive obliteration of the glomerular tuft capillaries with loss of filtration surface (1, 2). Fig. 1 outlines processes during the development of glomerulonephritis. In normal glomerulus MCs possess several phenotypic features. They are quiescent with low expression of PDGF chains, PDGF receptors, cyclins, cyclin-dependent kinases (CDKs), smooth muscle α actin, and increased expression of CDK inhibitor p27. They show low PCNA staining suggestive of a low proliferative rate (3-7). MCs are contractile and express contractile proteins such as actin and myosin in addition to receptors for vasoactive agents such as endothelin-1 (ET-1), and angiotensin-II (8-13). In addition, MCs maintain the mesangium with a balanced amount of extracellular matrix (ECM) proteins such as collagens (type IV and V), proteoglycans, fibronectin and laminin (14, 15).

After onset of glomerular injury either of immunologic, metabolic or mechanical nature MCs change phenotypically. They become proliferative, characterized by an increased expression of smooth muscle α actin, PDGF chains, PDGF receptors, cyclins, CDKs, and a decreased expression of p27 leading to increased PCNA staining (4-6). They deposit an increased amount of ECM proteins including collagen type I synthesized de novo, due to an increased expression of TGF-β and of protease inhibitors such as tissue inhibitor of metalloproteinase (TIMP) and plasminogen activator inhibitor-1 (PAI-1) (4, 5). Hypercellularity and accumulated ECM cause the glomerulus to swell, and there is an increase in the intraglomerular pressure and a decrease in glomerular filtration rate (GFR) culminating in glomerulosclerosis (16, 17). The decreased GFR suggests a lower contractile response of MC due to a lower expression of vasoconstrictor receptors, increased intraglomerular tensions, and increased amount of stress fibers, focal adhesions, or cGMP and cAMP (18-22).
Fig. 1 - Development of glomerular injury

GLOMERULAR INJURY
IMMUINOLOGIC METABOLIC MECHANICAL

QUIESCENT
CONTRACTILE
BALANCED SECRETION

HYPERCELLULARITY
ECM

SPLENOGENESIS
PDGF R
PDGF-B
CYCLINS
CDK2
PCNA
α-SMA
p21
p27

PROLIFERATIVE

HYPERSECRETORY

HYPOCONTRACTILE

GLOMERULOSCLEROSIS

TGF-β
COL-1, IV, V
PROTEOGLYCAN
FIBRONECTIN
TIMP
PAI-1

cAMP
cGMP
NO
VINCULIN
STRESS FIBERS
ET-1-R
AVP-R
This process of glomerular sclerosis is defined histologically at an early stage by deposition of hyaline material; with later progression towards MC hyperplasia, ECM expansion, capillary collapse, and adhesion to Bowman's capsule. ECM expansion in focal glomerulosclerosis is the result of a disturbed balance between synthesis and catabolism, in favor of synthesis. The accumulated ECM may influence MC phenotype by affecting MC proliferation, contraction and protein synthesis (23, 24). This thesis examines the role of two molecules (collagen type I and heparin/heparan sulfate) involved in ECM structure and cell-matrix communication, on proliferation and contractility of MC. The introduction discusses the ECM, with emphasis on collagen and heparin-like PGs, reviews the rather extensive literature on the antiproliferative effects of heparin on MCs and smooth muscle cells (SMC), and concludes with a discussion of characteristic phenotypic features of MC.

1.1. EXTRACELLULAR MATRIX

The origins of the ECM are intrinsic to the appearance and development of multicellular organisms (25, 26). ECM is generally believed to have evolved as a response to the mechanical forces acting on organisms consisting of more than a single cell. Contemporary models of protozoan aggregation suggest that single protozoans associated into colonies held together by extracellular material. It is also possible that multinucleated syncytia became partitioned into cellular and extracellular domains. Such extracellular material consists of embedded fibrous polymers in a mixture of nonfibrous components (25, 26). The matrix prevents tissue and organs from disintegrating under mechanical stress, but it also prevents cells from moving freely. In simple systems where the cells adhere but are not subjected to much mechanical stress, a ground substance develops as a link between cells (27). This link is the extracellular matrix in its simplest form (27). When assemblies of cells become more extensive, tensile forces become important and collagen fibers develop to hold the organism together, as in the sponges (26). Continuously changing tensile forces lead to the development of the highly branched polymer, a rubber-like protein, elastin (e.g., in major
vessels). Compressive forces, at least in higher organisms such as vertebrates, are opposed by the development of osteoid (skeleton), which is mineralized collagen (28). Continuously changing compressive forces lead to formation of cartilage - a collagen meshwork having the interstices filled with variety of proteoglycans and glycoproteins (29).

This view of the mechanical role of the matrix is now being supplemented by growing awareness of its importance in governing the behavior of cells (30-35). In most cases circulating nutrients and signaling molecules reach cells by first diffusing through a basement membrane and then through additional layers of extracellular matrix. Similarly cell products must pass through the same barriers in reverse order. These barriers govern the size and types of molecules that can pass. The matrix determines the shape, orientation, differentiation, and metabolic behavior of the cell. It is now appreciated that cells grown on plastic in vitro may behave in a different fashion from cells grown on natural extracellular matrices or matrix components.

ECM also functions in anchoring the cells. Anchorage is required for the growth of normal mesenchymal cells, implying a role for ECM receptors in regulation of the cell cycle. Using fibronectin as a substrate (36), it was shown that anchorage is required late in G1 for cells to enter into the cell cycle. If anchorage is not provided, the cells will not enter S phase even though growth factors are present.

In animals the major fibrous elements are the collagens, which are closely associated with linear, highly anionic polysaccharides, the proteoglycans. In plants the major fibrous element is the linear polysaccharide, cellulose, which is embedded in an amorphous matrix of considerable complexity. The relative proportion of the fibrous and nonfibrous components partially dictates the physical properties of a specific ECM, as does the diameter and weave of the fibers and the state of aggregation of the nonfibrous macromolecules. The major components of ECM are collagens, proteoglycans, fibronectin, laminin, elastin, entactin/ nidogen, tenascin and hyaluronan.
1.1.1 The Collagens

Collagen occurs in all but the simplest of organisms (37). In the more advanced phyla, collagen is prevalent in disparate tissues and organs, suggesting that collagen molecules originate as products of a number of cell types.

1.1.1.1. Nomenclature

Presently, at least 18 gene products have been identified as subunits of at least 19 collagen molecules (38-41). The distinct collagens are called "types" and are numbered with Roman numerals. The same numerals are added to the chains in brackets, following the Arabic number of the chain (42). The polypeptide chains are termed α-chains. The chains with different primary structure are numbered with Arabic numerals, e.g. α1, α2. They may be identical or different within the same molecule. Several stoichiometries can be observed within a single collagen type. For example, the composition of the most common type I collagen is [α1(I)2α2(I)]. It consists of two identical α1(I) chains and one α2(I). Different collagen molecules are individually indicated as type I, type II etc. Type II collagen is a homotrimer of three identical chains known as α1(II) chains which form molecules with composition [α1(II)]3. The numbering of collagen types and chains is arbitrary and reflects mostly the historical sequence of their biochemical characterization (43).

1.1.1.2. Structure

The principal features which affect helix formation are a high content of glycine and imino acid residues and the sequence of repeating residues Gly-X-Y, where X and Y can be any amino acid, often proline and hydroxyproline (44). The sequence Gly-Pro-Hyp makes up about 10% of type I collagen. A high content of Gly-Pro-Hyp triplets form the most stable region of the triple-helix. The left-handed helical structure of an individual chain is not stable when the chains are separated. The presence of glycine as every third residue is an absolute requirement for the triple-helix formation. Three glycine residues alternately from three
chains form a shallow center of the superhelical structure. The side chains of residues of amino acids other than glycine are directed outwards. Three left-handed helical chains form a supercoil with a pitch of approximately 8.6 nm. The distance between amino acids within each chain is 0.291 nm, and the relative twist is 109. Thus the distance between each third glycine residue is 0.87 nm (44-47).

All forms of collagens are made up of three left-handed polypeptide chains. These chains are coiled in a right-handed direction around a common axis to form the rope-like molecule (48). The major part of the collagen molecule has a triple-helical structure. Nonhelical domains are present at both ends of the chains, and in some types of collagen the helical part is interrupted by nonhelical domains. The presence of a bigger portion of the molecule with a triple-helical conformation is a basic feature for classification of the protein as collagen. Proteins containing only a small portion of triple-helical conformation are said to contain collagen sequences (49).

The primary structure of collagens was investigated by elucidating the sequence of residues of smaller peptides after fragmentation of the peptide chains. Cleavage with cyanogen bromide (CNBr) has proved to be particularly useful. CNBr splits polypeptides specifically at methionine residues (50). This results in a clear mixture of relatively few fragments. The unique sets of CNBr peptides serves as a definitive means of identifying each type of chain (51) and were the basis for the discovery of type II and III collagens (52).

1.1.1.3. Classification

Collagens have been classified by types that define distinct sets of polypeptide chains that can form homo- and heterotrimeric assemblies (43). Nineteen collagen types have been defined so far (38). These include fibril-forming collagens, collagens forming sheets, short chain collagens, fibril-associated collagens with interrupted triple helices (FACITs), collagens forming beaded filaments, collagens forming anchoring fibrils, multiplexins, and other
collagens. Domains and structures of the representative collagens are shown in Fig. 2.

1.1.1.3.1. Fibril-forming collagens (FFC)

This family of collagens is the most investigated. The molecular structure and amino acid sequence of the four chains of types I-III collagens are well established. The chains are products of different genes, but similarities in their structure have been shown (53-56). Collagen type I has studied most widely and is used in this thesis as representative of collagen matrix.

Each of the FFCs is synthesized, assembled, and secreted as a complex molecule with six distinct major structural and functional domains as indicated in Fig. 2. The genes of these collagens are derived from a single ancestral gene (57). The high conservation of the gene structure, including the C-propeptide region, can be traced from mammals to evolutionary distant species such as sponges (58) and sea urchins (59). The basic molecular plan for collagen type I and other collagens is illustrated in Fig. 2.

The reason for molecular diversity in the fibrillar collagen molecules is still very much a matter of speculation. The highly variable structure of the N-propeptide region suggests that the major biological reason for the diversity of these molecules resides in the amino-terminal domain (60). The higher O-glycosylation of hydroxylysyl residues in type II collagen compared to type I collagen may be the basis for the larger volume occupied by collagen molecules in cartilage fibrils (61). The higher degree of posttranslational modifications of collagen type II may be due, at least in part, to a slower folding of the triple helix. Lysyl hydroxylase and glycosyl transferases, like prolyl hydroxylase, are active only on nontriple helical chains, and the level of hydroxylation and glycosylation is thus a function of the rate of helix formation (62).
Fig. 2 - Domain structure of collagens

Schematic representation of collagen types indicated by Roman numerals. Triple-helical (collagenous) domains are indicated by hatched boxes (\(\overline{\overline{\text{---}}}\)). Lines (\(\overline{\ldots}\)) represent non-collagenous domains. The long collagenous domain of collagen type IV which is interrupted at several sites by noncollagenous sequences is presented as (\(\overline{\overline{\overline{\overline{\text{Z}}}}}\)). N and C indicate amino and carboxy terminal respectively, while other signs are explained in the text. The structures are based on data from (38, 39).
Fig. 2

- Fibril-forming collagens
  - TYPE I
  - TYPE V
- Collagens forming sheets
  - TYPE IV
- Collagens forming beaded filaments
  - TYPE VI
- Collagens forming anchoring fibrils
  - TYPE VIII
- Short chain collagens
  - TYPE IX
- FACITs
  - TYPE XII
- Multiplexins
  - TYPE XIV
- Other collagens
  - TYPE XV
  - TYPE XVII
1.1.3.2. Collagen forming sheets; collagen type IV

Type IV collagen is involved in the formation of sheets or protein membranes that surround tissues and organisms, such as basement membranes (BM). Basement membranes are extracellular structures found between cells and the connective tissue stroma. They are thin (40-60 nm), sheetlike structures, found in every organ of the body, and with few exceptions most cells are surrounded by them. Owing to the presence of type IV collagen the BM has significant strength and provides structural support for tissues. It is also responsible for cell attachment, probably due to an interaction of type IV collagen and laminin with specific binding proteins of the cell membrane. Selective filtration through the barrier of the BM is a third function (63).

Like the interstitial procollagens, type IV collagen contains both globular and triple helical domains, though the size and orientation of these domains differ from interstitial collagens. Type IV collagen appears not to be processed at all after secretion from the cell (64). The major type IV collagen consists of two constituent chains forming the \([\alpha 1(IV)]2\alpha 2(IV)\) heterotrimer. Clear evidence for the presence of three additional chains in some BM has been obtained through partial characterization of two chains bearing the epitopes responsible for Goodpasture syndrome (65). The type IV molecule contains cysteiny1 residues that form disulfide bonds within the triple helix. The type IV triple helix is imperfect, containing several regions in which the sequence Gly-X-Y is disrupted, allowing this domain to be susceptible to a large number of proteolytic enzymes (64). These disruptions also increase the flexibility of the triple helical domain at these sites. Both chains have a similar pattern of helical and nonhelical domains.

1.1.3.3. Other collagens

In this group belong molecules that fit the generic description of collagens, but are structurally and functionally distinct from fibril and basement membrane-forming collagens.
(43). These collagens (e.g., types XII and XIV) have a non-triple-helical domain as a major part of the protein, which could be functionally significant. They are present in smaller quantities in tissues and play the role of connecting elements between major structures and other tissue components. Their major characteristics are listed in Table 1 and Fig 2.

1.1.2. Proteoglycans

Proteoglycans (PGs) are the second major component of ECM but also found on all cell surfaces and in intracellular stores. They are versatile molecules responsible for imparting resilience to tissues (88). They also serve as repositories of growth factors and are implicated in modulating developmental processes. PGs are ubiquitous molecules present in all tissues and organs. Sponges are the most ancestral of the known metazoan animals and they are rich in PGs, some of which have been implicated in sponge cell aggregation (89).

1.1.2.1. Structure

A PG is a protein substituted with at least one glycosaminoglycan (GAG) chain. With the exception of hyaluronic acid, every GAG found in ECM is linked covalently to a protein. The number of GAG chains on a core protein may vary from 1 to 100, and their length may vary. The same core protein may be substituted with more than one type of GAG chain or, at times, with none at all (90).

GAGs are polyanionic chains of variable length constructed from disaccharide repeating units that contain a hexosamine residue (91). In mammalian tissues there are four major classes of GAGs: Hyaluronic acid (HA), chondroitin sulfate (CS), keratan sulfate (KS), and the heparan sulfate (HS)/heparin class (Fig. 3). The CS and HS/heparin GAGs are attached to protein by a characteristic oligosaccharide linkage region that contains glucuronic acid (GlcUA), galactose (Gal), and xylose (Xyl) in its structure (92).

HA consists of repeating disaccharide units composed of GlcUA and N-
<table>
<thead>
<tr>
<th>TYPE</th>
<th>MOLECULE</th>
<th>REPRESENTATIVE TISSUES</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI</td>
<td>α1(VI)α2(VI)α3(VI)</td>
<td>most ECM, vessels, skin, intervertebral disc</td>
<td>interface between cells and ECM (43, 66)</td>
</tr>
<tr>
<td>VII</td>
<td>α1(VII)</td>
<td>?</td>
<td>dermoepithelial junction (keratinocytes)</td>
</tr>
<tr>
<td>XIII</td>
<td>α1(XIII)</td>
<td>?</td>
<td>cartilage, intestine, skin, striated muscle/Endothelial cells (69)</td>
</tr>
<tr>
<td>XVII</td>
<td>α1(XVII)</td>
<td>?</td>
<td>hemidesmosome of stratified squamous epithelia</td>
</tr>
</tbody>
</table>

Short collagens:

| VIII | α1(VIII)α2(VIII) | ? | Descemet's membrane/endothelial cells | structure/proliferation/angiogenesis (72, 73) |
| X    | [α1(X)]3 | growth plate/chondrocytes | structural role (74, 75) |

FACITs:

| IX    | α1(IX)α2(IX)α3(IX) | Hyaline cartilage/vitreous humour | maintenance of cartilage after birth (66, 76) |
| XII   | [α1(XII)]3 | Embryonic tendon & skin/periodontal ligament | bridging ECM molecules (78-80) |
| XIV   | [α1(XIV)]3 | Fetal skin and tendon | bridging ECM molecules (80, 81) |
| XVI   | ? | contains 10 triple-helical domains which show homology to α1(IX) (82) |
| XIX   | ? | complete structure not known but similar to α1(IX) (83, 84) |

Multiplexins (collagens with multiple triple-helical domains):

| XV    | α1(XV) | ? | vascularized organs | perivascular matrix assembly (85-87) |
| XVIII | α1(XVIII) | ? | vascularized organs | perivascular matrix assembly (85-87) |
Fig. 3 - The basic repeating disaccharide units.

The five major classes of mammalian glycosaminoglycans: Hyaluronic Acid (HA), Heparan sulfate (HS), Chondroitin sulfate (CS), Keratan sulfate (KS), and Dermatan sulfate (DS). Arrows point at the positions where the unit could be alternatively sulfated, acetylated, or left unchanged.
acetylglucosamine (GlcNAc), linked with $\beta(1,3)$ glycosidic bond and disaccharide linkage is $\beta(1,4)$ glycosidic bond (Fig. 3). HA units are not sulfated.

CS consists of repeating disaccharide units composed of GlcUA and GalNAc linked with $\beta (1,3)$ glycosidic bond (Fig. 3). The sulfation occurs at C4 and/or C6 of the GalNAc residue. CS occurs at various sizes but almost always less than 100 kDa (93, 94).

DS consists of repeating disaccharide units composed of L-iduronic acid (IdoUA) and GalNAc linked with an $\alpha (1,3)$glycosidic bond (Fig. 3). The sulfation varies and takes place at C2 of IdoUA and C4/C6 of GalNAc, respectively (95-97).

The disaccharide unit repeats in KS are composed of Gal and GlcNAc (same structure as in lactose) linked with $\beta (1,4)$ glycosidic bond (Fig. 3). The sulfation occurs on the C6 position on both sugars in KS.

HS/heparin

HS is composed of repeating disaccharide units containing GlcUA or IdoUA and glucosamine (GlcN) linked with a $\beta(1,4)$ and $\alpha(1,4)$ alternating glycosidic bonds (Fig. 3). The amino groups of the GlcN units could be sulfated, acetylated or free amino groups. About 50% of the $N$-acetyl groups are normally converted to $N$-sulfates in HS. O-sulfate substituents are located at C6 of GlcN and occasionally at C3 and C2 of GlcN and GlcUA (98, 99). HS chains, usually below 50 kDa, occur bound to a large number of different core proteins, and are synthesized by virtually all cells investigated (100).

Heparin is isolated from animal tissues (pig intestinal mucosa; bovine lung) and is used in the clinic as an antithrombotic drug. In the intact tissue it is confined to mast cells, where it is stored in cytoplasmic granules. There is variation in the content and type of HS within tissues, and within the same tissues between species (101). HS on the other hand, has ubiquitous distribution on cell surfaces and in ECM. It is generally less sulfated than heparin.
and has a more varied structure. Heparin contains repeating disaccharide units of IdoUA and GlcN linked with an α(1,4)glycosidic bond. Like HS, heparin is synthesized by epimerization of GlcUA to IdoUA, but like HS amino groups of GlcN units are either sulfated, acetylated or free amino groups (Fig. 3 & 4).

Heparin is a glycosaminoglycan. Its chains are often found attached to selected serine residues of core proteins (102). It is highly polyanionic, carrying four potential anions per disaccharide: one carboxyl, one N-sulfate, and two O-sulfates. In therapy it is primarily used as an anticoagulant and its anticoagulant action depends on an interaction with antithrombin III (AT-III) thereby blocking the action of thrombin (103). A pentasaccharide sequence, (Fig. 4) encompasses the binding region for antithrombin (104). It consists of the trisulfated disaccharide sequence IV and V with regular regions of the polysaccharide (105). Besides this unit the sequence contains a central disaccharide of GlcA (II)-linked to a 3-O sulfated GlcN-6SO3 residue (III), and 2-N sulfated GlcN-6SO3 (I) to which is attached nonsulfated IdoA as the sixth residue (not shown). The IdoUA and GlcN are linked with an α(1,4) glycosidic bond. The molecular weight of heparin varies from 5 to 50 kDa (106). More than 70% of the N-acetyl groups are converted to N-sulfate in heparin. O-sulfation occurs at both C2 of IdoUA and C6 of GlcN. The O-sulfation at C2 of IdoUA is thought to prevent the back epimerization of IdoUA to GlcUA which would be greatly favored at equilibrium (107). In contrast, HS has about 50% of its GlcN units N-sulfated (108), resulting in lower IdoUA content and a lower overall degree of O-sulfation (109).

1.1.2.2. Classification of PGs by location

PGs show not only great diversity among different members of the family, but also exhibit considerable diversity within particular groups. Different types of PGs are found at different locations. According to their location with regard to the cell of origin they are classified as PGs stored in secretory granules, constitutively secreted into ECM, or associated with the plasma membrane (110-114).
Fig. 4 - Heparin - Proposed oligosaccharide sequence for AT-III specific binding.
1.1.2.2.1. The PGs stored in secretory granules

The heparin PG in the storage granule interacts via the GAG chains with cationic proteases, carboxypeptidases, and histamines which the cell stores for release when participating in a host defense reaction (107). Besides PGs containing heparin intracellular storage granules also could contain an oversulfated form of CS (107). The core protein is 17-19 kDa and is remarkably resistant to proteolysis. It contains an extended sequence of alternating Ser and Gly units after which it was named Serglycin (115-117). Serglycins contain different GAGs in different cells (107, 118, 119). They may occur as hybrid PGs, in which both CS and heparin chains are attached to the same core protein (120).

1.1.2.2.2. The PGs secreted into the ECM

Various PGs in the ECM can be classified into three groups: (i) large interstitial PGs, (ii) small interstitial PGs, and (iii) basement membrane PGs.

(i) The large interstitial PGs provide the basis for the ability of cartilage to withstand a compressive load with minimal deformation. They do so by eliciting a swelling pressure on the inextensible collagen fibrillar network after being compressed. The function of these large PGs in blood vessels seems to be to resist compression generated by pulsatile forces in the cardiovascular system. Members of this group include aggrecan and versican. Aggrecan is a CS/KSPG from cartilage (121) which has a core protein containing three globular domains (122). Through these domains, it interacts with HA and other matrix components, to provide the basis for forming aggregates in the ECM and to participate in the organization of the ECM (123, 124).

Versican has a core protein of about 260 kDa (125), and homology to two globular domains of aggrecan. It also interacts with HA. The core protein contains two epidermal growth factor (EGF)-like repeats and has 15 Ser-Gly and 16 Gly-Ser sequences for CS/DS attachment, 20 Asp-Xaa-Ser (Thr) sequences for N-linked oligosaccharides, and more than
(ii) The family of small interstitial PGs is made up of decorin, biglycan, lumican, and fibromodulin (107, 126). In all cases the core proteins but not the GAG chains are essential for binding to collagens (127, 128). Lumican and fibromodulin are preferentially located on the collagen surface near the a and c bands in cornea, while decorin is found near the d and e bands (129, 130). Both KS and CS/DS keep collagen fibrils apart and help maintain the right degree of swelling, and so are essential to corneal transparency (131).

Decorin and biglycan have a Mr value of 90-140 and 150-240 kDa, respectively. They both contain 30-40 kDa CS or DS chains and have about 45 kDa core proteins rich in Leu and Asp/Asn (29, 126, 132). Ser-Gly sequences for glycosylation of decorin and biglycan are located near the NH₂ termini (133, 134). Decorin and biglycan are expressed in different regions of tissues during development (135, 136) and their expression is differentially regulated by transforming growth factor-beta (TGF-β) (137) and retinoic acid (138), indicating differences in function (107). Both decorin and biglycan can bind to TGF-β via their protein cores and neutralize its growth-promoting activity (139, 140), suggesting roles in cellular events associated with both development and disease.

Lumican and fibromodulin have a similar core protein with 50% identity at the amino acid level but contain KS attached through N-linked oligosaccharides instead of CS/DS (126, 141). The core proteins of each contain about ten conserved Leu-rich motifs, but they contain no Ser-Gly sequences (133, 134).

(iii) Perlecan is the prototype BM HSPG, although BM contains both HSPGs and CSPGs. Originally perlecan was identified in the BM-producing murine Engelbreth-Holm-Swarm (EHS) tumor (142). Later it was found present in almost all BMs of the body and in the ECM produced by at least some mesenchymal cells (143). This HSPG contains a large core protein (ca. 400 kDa) with 3-4 60 kDa HS chains (144-146). It can interact with itself
and with other BM macromolecules such as collagen type IV (147) and laminin (148). It facilitates attachment of cells to their underlying BM (149), anchors acetylcholinesterase in the neuromuscular junction (150), and binds protease inhibitors (151) and growth factors (152).

Smaller HSFGs have been also isolated (153, 154). Our lab has reported that cultured glomeruli synthesize two small BM HSFGs with Mr values of 250 and 130 kDa. Both of them have a 30 kDa protein core with GAG chains of 25-45 kDa, and may be processed forms of perlecan (155).

Two CSPGs were also found in BM, one in Schwann cell BMs (156), and another in Reichert’s membrane (157).

1.1.2.2.3. The PGs associated with the plasma membrane

The plasma membrane associated PGs are classified into three groups:

- the Syndecan-Like Integral membrane PGs (SLIPS) which are intercalated into the membrane via a hydrophobic trans-membrane domain of the protein (158, 159)

- the Glypican-Related Integral membrane PGs (GRIPS) which are covalently linked to a phosphatidylinositol (PI) anchor in the membrane (159-161), and

- other cell surface PGs including peripheral PGs which are non-covalently associated with plasma membrane proteins (149, 162, 163).

1.1.2.2.3.1. SLIPS

SLIPS are a HSPG gene family (158, 164). The members of this family are: syndecan-1 (syndecan) (165), syndecan-2 (fibroglycan) (166), syndecan-3 (N-syndecan) (167, 168) and syndecan-4 (ryuocan/amphiglycan) (169-171). Most cell types generate more than one member of the SLIPS (158, 159, 164, 169, 172). Syndecan-1 and -2 are expressed
predominantly in epithelial cells and in tissues rich in endothelial cells, respectively. Syndecan-4 is found in most tissues while syndecan-3 is primarily in neural tissues. SLIPS have core proteins of 20-40 kDa, and their HS chains are attached to the N-terminal extracellular domains (167).

The potential glycosylation signal sequences for HS chains, the trans membrane and cytoplasmic domains of SLIPS are highly conserved (159). A protease-susceptible site adjacent to the trans-membrane domain is conserved indicating that this domain may function to position GAG chains at the cell surface (158, 159). A potential function of the cytoplasmic domain is interaction with the actin-containing cytoskeleton (173). Also, the length and sequence between the trans-membrane domain and first tyrosine are conserved. This tyrosine is surrounded by polar (Ser, Thr, Glu, Asn) or positively charged amino acids (Arg, Lys, His). These properties may reflect functions involving interactions with cytoskeletal proteins and/or signal transduction.

1.1.2.2.3.2. GRIPS

The members of the GRIPS family are glypican (174), cerebroglycan (175), OCI-S/glypican 3 (176-178), and K-glypican (179). Many different cell types synthesize glypican (159), while K-glypican is highly expressed in the mouse kidney and developing brain (179). Cerebroglycan and OCI-5 are expressed mainly in the nervous system and colon, respectively (175, 176). The core proteins of about 60 kDa have HS chains attached close to the cell surface in the C-terminal part. The N-terminal region also may contain one (glypican) or three (cerebroglycan) additional HS chains. Glypican remained highly conserved during evolution, with a 90% sequence identity between the rat, mouse and human forms (159), suggesting a specific role of this core protein (180). Glypican 3 is mutated in patients with the Simpson-Golabi-Behmel overgrowth syndrome (181). OCI-5 and glypican 3 are implicated in regulating growth. OCI-5 binds bFGF (182) whereas for glypican 3 the evidence is still controversial whether it binds to IGF-2 (178, 181, 182).
1.1.2.3. Other cell surface PGs

Two other membrane-intercalated PGs have been characterized, but they are not homologous to either SLIPS or GRIPS. These are betaglycan, the TGF-β type III receptor (183), and CD44 (184). Both of them are called 'part-time' PGs because betaglycan does not always carry GAG chains and only a small fraction of CD44 has GAG chains.

Peripheral PGs have also been described. The association of these PGs to the membrane is the weakest in the family. There are two groups of peripheral PGs, based on the type of association to the membrane. One is PGs which are non-covalently associated with plasma membrane proteins via GAG chains (162, 163, 185). The binding of these PGs to the membrane depends on presence of GAG receptors, termed as hyaladherins (163), which include 14 kDa protein in B-16 cells (162), CD44, or 85 kDa HA receptor of baby hamster kidney cells (186). The other group is PGs that are bound to the membrane via core protein. A 38 kDa protein, on the surface of hepatocytes, binds to the core protein of HSFGs of basement membrane (149).

1.1.2.3. Functions and interactions of PGs

Most of the functional properties of PGs depend on interactions between the GAG chains and proteins. Binding of heparin/HS sequences to proteins is ionic, thus involves positively charged, usually clustered, amino acid residues. The interactions may be nonspecific or specific and determined by (i) specific amino acid sequences with positive charges, or (ii) both specific oligosaccharide sequences with a defined sulfation pattern and basic amino acid sequences.

Two consensus sequences for GAG binding have been deduced from the heparin-binding proteins. One is Yaa-Baa-Baa-Yaa-Baa-Yaa, where Baa is a basic and Yaa a hydrophobic amino acid. This sequence is found in thrombin, heparin binding growth factors, and several cell adhesion molecules including fibronectin, and vitronectin (187, 188). Another
The consensus sequence is Yaa-Baa-Baa-Yaa-Yaa-Baa-Yaa. This occurs in heparin cofactor II, protein C inhibitor and some molecules containing the first consensus sequence (187, 189). These sequences are in an α-helical regions, and all basic amino acids are arrayed on the same face of the helix, to create close-approach, minimum-energy interactions between themselves and the sulfate and carboxylate residues in heparin (190).

Heparin has the highest negative charge of any known biological macromolecule (191). It thus interacts with a variety of proteins such as enzymes, enzyme inhibitors, extracellular-matrix proteins and cytokines (120). The protein binding activity and specificity of HS require moderate-to-high levels of sulfation. Many proteins may bind to the common sequence, \([\text{IdoUA(2-OSO}_3^-\text{-GlcNSO}_3^-\text{(6-OSO}_3^-\text{)]}_n\], which is predominant in heparin but less abundant in HS (192).

1.1.2.3.1. Enzymes/enzyme inhibitors

The enzymes that heparin/HS bind are numerous, such as mast-cell proteinases (193, 194), lipoprotein lipase (195), coagulation enzymes, elastase, and extracellular superoxide dismutase (100). HSPGs may immobilize active enzymes at an appropriate distance from the cell surface (120) and facilitate binding of low-density and very-low-density lipoproteins to lipoprotein lipase and subsequent internalization of the lipoproteins by the LDL receptor (196). Binding of heparin/HS to the plasma Ser protease inhibitors (serpins) intensifies their inhibition of protease activities (197).

The best known example is the interaction between AT-III and heparin (198, 199). The minimal size of heparin required for maximal AT-III binding is a pentasaccharide (200, 201, 202) (Fig. 4). The critical structural requirements for binding to AT-III are: 1) the 6-O-sulfate group of GlcN (unit I), 2) the 3-O-sulfate group on the central GlcN (unit III), 3) the N-sulfate group on units III and V, and 4) the carboxylate moiety of IdoUA on unit IV. The 3-O-sulfation on unit III is crucial for the binding to AT-III although both O-sulfation and N-
sulfation on units IV and V increase the anti-Xa activity (202). The primary binding site is the trisaccharide of I-II-III and units IV and V form a secondary one (201). The pentasaccharide-AT-III complex only inhibits free factor Xa but not thrombin. Heparin oligosaccharides of less than 5,400 Da are without cofactor activity for thrombin (203), indicating that heparin chain length is also an important determinant of thrombin inhibition. Whereas antithrombin binds to a specific pentasaccharide sequence, a much longer saccharide (>18 monosaccharide units) is required to induce thrombin inhibition (199), suggesting the requirement for a ternary complex in which both antithrombin and thrombin bind to the GAG chain (204).

In addition to AT-III (199), the serpins include HC-II (199, 205, 206), plasminogen activator inhibitor-1 (199, 207, 208), protein C inhibitor (199, 209, 210), and protease nexin 1 (199, 211). A major difference between AT-III and HC-II is their specificity for GAGs; HC-II binds either heparin or DS to inhibit thrombin in vitro, whereas AT-III does not interact with DS. DS potentiates HC-II inhibition of leukocyte cathepsin G while heparin does not (212), suggesting that the interaction of HC-II with heparin is less specific than with DS (213).

1.1.2.3.2. Adhesion molecules

PGs can interact with one another or with cell surface receptors via specific recognition sites in the protein and/or oligosaccharide sequences. One of these sites is the tripeptide sequence Arg-Gly-Asp (RGD) in the protein (214), which binds to the integrin receptor family to transmit information between the ECM and the cytoskeletal network of the cell membrane (215). One of the consequences of interaction between integrins (216) and ECM components (217) is the formation of focal adhesions that regulate attachment and polymerization of actin (218, 219). HSPGs also bind to ECM proteins such as collagens, fibronectin, laminin, thrombospondin, tenascin, vitronectin, and others (100, 158, 220-222). Through these interactions, cell-surface HSPGs can regulate cell adhesion. In addition to
interactions involving GAGs, the adhesive function of PGs can be mediated through the protein cores such as in perlecan (220, 223, 224).

PGs also interact with two neural adhesion molecules, N-CAM and myelin-associated glycoprotein, members of the immunoglobulin superfamily. Heparin/HS binds to a heparin binding domain located on N-CAM (225, 226).

1.1.2.3.3. Growth factors

Two major classes of heparin-binding polypeptides, which have related structures and functions, are known as acidic FGF (aFGF) and basic FGF (bFGF). The FGF family of growth factors binds heparin with relatively high affinity and interacts with HSPGs in the ECM or on the cell surface. Different FGFs require growth factor-specific saccharide structures (227), indicating the possibility that under physiological circumstance there is a specific mechanism tailoring HS to affect selected growth factors. Binding sites in heparin for bFGF (228-231) and aFGF (232) are composed of 5-8 monosaccharide units. The induction of a mitogenic response requires the presence of a GAG chain containing both IdoUA 2-O-sulfate and GlcN 6-O-sulfate groups (227) while the interaction with bFGF was shown to involve only 2-O-sulfate groups, indicating that GlcN 6-O-sulfate was required for GAG/bFGF receptor interaction. FGF receptor contains a heparin-binding region in addition to the FGF-binding site. Peptides representing this site interfere with bFGF binding to the receptor (233).

The length of polysaccharide sequence is also a determinant for eliciting a biological response to FGFs (227, 234-236). There is a sharp demarcation of promoting of mitogenic activity of bFGF, in Swiss 3T3 cells, between heparin decasaccharide (inactive) and dodecasaccharide (equivalent to full-length heparin) (227). According to a proposed model two regions on the dodecasaccharide are required for eliciting the activities of bFGF, one binding the growth factor and the other interacting with receptor. Alternatively, the longer
sequence might be required to bind two bFGF molecules adjacent to each other, thus
facilitating dimerization of the FGF receptor in the plasma membrane (235).

Studies on the interactions of HS with bFGF showed that the binding sequence in HS
contains a repeating core sequence of \([\text{IdoUA}(2-\text{OSO}_3)-\text{GlcNSO}_3]_5\) within a
tetradecasaccharide, although the actual size of the combining site may comprise only 5
residues in this sequence (237). Both 2-\text{O}-sulfate and N-sulfate are absolutely necessary for
high-affinity interaction. Although 6-\text{O}-sulfation dramatically increases the binding to a
pentasaccharide region composed of such trisulfated repeats (228, 229), 6-\text{O}-sulfate groups
do not appear to be essential to binding (194, 230, 237).

1.1.3. Other ECM molecules

1.1.3.1. Fibronectin

Fibronectin is multifunctional extracellular matrix and plasma glycoprotein that plays a
central role in cell adhesion (238). It is a dimer of 250 000 kDa subunits that are disulfide-
bonded at their C termini (239). The subunits are flexible and can be fully extended to form a
V-shaped molecule (240, 241), or folded into a compact globular molecule (242).
Fibronectin binds fibrin, heparin, collagen, integrins, and other molecules. It contains an
RGD sequence that is essential to the adhesive activity which is recognized by the \(\alpha_5\beta_1\),
integrin receptor (243). Among factors that regulate synthesis of fibronectin such as
hormones, growth factors, and derivatives of cAMP (238) TGF-\(\beta\) is the most potent (244).

1.1.3.2. Laminin

Laminin is a member of a closely related family of structural glycoproteins that are
abundant in BMs (245) where it plays both structural and cell-informational roles. It is
composed of three \(\text{A}, \text{B}1\) and \(\text{B}2\) disulfide-linked subunits (246) that combine to form an
asymmetrical four-armed structure. The multidomain nature of laminin is ideally suited to
mediate the interactions of a variety of BM components, and thus plays a key role in creating and maintaining the complex three-dimensional structure necessary for correct functioning of the BMs. Laminin exhibit a variety of biological activities, including promotion of attachment, growth and differentiation of a number of cell types (245, 247, 248).

1.1.3.3. Entactin/Nidogen

Nidogen, a degradation product of entactin, is an atypical and ubiquitous basement membrane protein with strong affinity for laminin. It has a dumbbell structure when visualized by rotary shadowing electron microscopy (249). Because of its broader interaction repertoire (250) nidogen is recognized to be a key mediator in basement membrane assembly (251). It binds to laminin (252), collagen type IV (250, 251), HSPG (253, 254), cellular receptors (255-257), Ca2+ (258), Co2+ (250), and Zn2+ (254).

1.1.3.4. Thrombospondin

Thrombospondin (TSP) is an adhesive glycoprotein. It is disulfide-bonded trimer of three identical chains with monomer molecular mass of about 140 kDa. Four related thrombospondin genes have been described, and their products are named TSPs 1-4 (259-261). Thrombospondin binds to other ECM components including collagens, fibronectin, fibrinogen, and laminin, and to proteases such as plasminogen and tissue plasminogen activator (262). Thrombospondins are present in the α granules of platelets and in fibroblasts, SMC, myoblasts, epithelial cells, monocytes and macrophages, endothelial cells, osteoblasts and chondrocytes, keratinocytes and melanocytes, and many neoplastic cells (263). In general, thrombospondin expression is the highest in tissues where cells are actively undergoing proliferation and migration and is restricted in adult tissues. Major recognition sequence in TSP, for many of the integrins are GRDA or GRDGI (264). In vitro studies suggest a functional role for thrombospondins in both cell migration and neurite outgrowth (265) and in inhibition of angiogenesis (266-268).
1.1.3.5. Tenascin

Tenascins are a complex set of ECM proteins that interact with cells and participate in the building up of the ECM scaffold (269). At present three members of the tenascin family have been described, tenascin-C, tenascin-R, and tenascin-X (270). In contrast to cells plated on fibronectin, cells plated on intact tenascin either do not attach at all, or if they attach, do not spread (271-274). That might be important to counteract other adhesive proteins to enable cells to detach from ECM and to migrate during morphogenic movements.

1.1.3.6. Elastin

The molecule of elastin is chemically inert, extremely hydrophobic and, in the fully cross-linked from, is insoluble under most conditions (275) which makes it one of the most stable proteins in the body (276, 277). The biological distribution of elastic fibers in the mammalian system is in those tissues that are subjected to continual physical deformation, tension, and high pressure differentials (39, 278).

1.1.4. ECM regulation

Tissue injury elicits an inflammatory reaction in which substances released from dead and dying cells activate monocyte/macrophage-type cells that may be acutely extravasated from circulation or be normally resident in the organ, as in the case of Kupffer cells in the liver (279) and pulmonary macrophages in the lungs (280). Notable amongst the many cytokines and inflammatory mediators that are released from activated macrophages are TNF-α and interleukins 1 and 6 (IL-1 and IL-6), which recruit further inflammatory cells into the local environment, acidic and basic PDGs, EGF, and possibly the two most important fibrogenic cytokines, PDGF and TGF-β (281, 282). Other cells contribute to this cytokine pool, including platelets, lymphocytes and fibroblasts, and, in the case of liver fibrosis, modified lipocytes or Ito cells (283, 284).
Deposition and degradation of the ECM play an important role in mesangial remodeling and provide mechanisms for maintaining normal amounts of matrix within the mesangium. TGF-β is a major factor that contributes to matrix protein synthesis in experimental glomerulonephritis (285-288). The increase in matrix secretion induced by TGF-β is also accompanied by increased protease mRNA expression. Inhibition of TGF-β by neutralizing antibody (287), decorin (289), or low protein diet (290) prevented matrix expansion in the Thy-1 model of glomerulonephritis. Various cytokines also have been implicated as stimuli for matrix protein synthesis, including IL-1 (291, 292), IL-6 (293), and PDGF (286). Studies using various inhibitors also implicated angiotensin II (ANG II) (294), endothelin-1 (295), and platelet-activating factor (296), in the matrix expansion that accompanies experimental glomerular disease. Based on in vivo studies or on studies with cultivated cells, inflammatory mediators including complement (297, 298) also have been recognized as possible stimulators of matrix protein synthesis in glomerular cells. Proteolytic enzymes are involved in the process of ECM turnover (299). Of all the enzymes involved, most attention has focused on two main groups of enzymes — the plasmin/plasminogen activator system of serine proteases and the matrix metalloproteases (MMPs). There is in vivo evidence that many of these enzymes interact to form activation cascades — particularly in the case of plasmin and plasminogen activators, which have been shown not only to activate one another, but also are implicated in the activation of several of the MMPs. MMPs can collectively catabolize all the major constituents of the ECM and as such play a major role in the remodeling events that occur in normal morphogenesis and wound healing.

1.1.5. Cell matrix communication

The interaction of cells with the ECM is of fundamental importance for many biological processes such as cell growth and differentiation, and embryogenesis. Conditions such as wound healing and tumor metastases are also influenced by cell-ECM interactions. Cells respond to ECM through cell surface adhesion receptors that have primarily a structural or
mechanical role. The integrins are family of cell surface receptors that mediate attachment to
the ECM. Specific classes of integrins also mediate important cell-cell adhesive interactions.
Integrin-mediated adhesive interactions are intimately involved in the regulation of many
cellular functions, including embryonic development, tumor cell growth and metastasis,
leukocyte homing and activation, bone resorption, clot retraction and response of cells to
mechanical stress (300-303). They promote reorganization of the cytoskeleton and regulate
adhesive strength, cell shape and motility (304). For example, leukocytes lacking the integrin
β2 family cannot extravasate, and platelets lacking integrin αIIβ3 [glycoprotein (GP)IIb-IIIa
complex] cannot bind fibrinogen or aggregate. Adhesion receptors often work cooperatively
with growth factor receptors to transmit and integrate cellular responses to specific signals
from the ECM (304). In addition, integrins function in cell surface-mediated ECM assembly
(305). Integrins can regulate intracellular second messengers by which ECM can control cell
functions. The occupation of integrin elicits a variety of intracellular signaling events. These
include expression of genes, tyrosine phosphorylation, the stimulation of PKC activity (306),
Na+/H+ antiporter activity (307), and phosphoinositide (PI) hydrolysis (308), in addition to
the elevation of intracellular free Ca2+ levels (309) and GTP-bound p21Ras levels (310).

1.1.5.1. Structure of integrins

All integrins are heterodimers of noncovalently associated α and β subunits (Fig. 5).
Single subunit can associate with more than one partner, providing a secondary level of
diversity within family. At present 16 different α and 8 different β subunits are identified,
that can form 22 different integrins (Fig. 5a) (311). Many integrins bind to more than one
ligand (Fig. 5a), and individual subunits can exist in multiple forms due to alternative splicing
of mRNA and/or as a result of post-translational modifications such as glycosylation,
phosphorylation and proteolytic cleavage (311, 312).
Fig. 5 - Subunit pairing and ligand specificity (a), and schematic diagram of integrin structure (b).

a) FN=fibronectin, VN=vitronectin, FG=fibrinogen, LM=laminin, COL=collagen(s), TSP=thrombospondin, vWF=von Willebrand factor, VCAM=vascular cell adhesion molecule, ICAM=Intercellular adhesion molecule, C3bi=complement factor C3bi, FX=factor X.

b) Prototypical model of integrin α and β subunit receptors with major disulfide bonds (S-S).
1.1.5.2. Integrin expression in smooth muscle and mesangial cells

Integrin expression in SMCs and MCs is well documented. SMC express $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins which are involved in cytokine-mediated fibronectin synthesis (313, 314). The compiled distribution patterns of $\alpha_1$, $\alpha_3$ and $\alpha_6$ subunits of integrins allow discrimination between tumors of smooth ($\alpha_1/\alpha_3/\alpha_6$-positive) and striated muscle ($\alpha_1/\alpha_3/\alpha_6$-negative) origin (315). SMC from heart express variants of $\alpha_3$ integrin subunits. "A" variant can be phosphorylated in contrast to "B" (316). SMC also use $\beta_3$ and $\alpha_6\beta_3$ integrins for sensing mechanical strain (317), and they express the activation epitope of $\beta_1$ integrin in injured arteries (318). The usual repertoire of integrins in SMC consists of $\alpha_8$ (319), $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$ and $\alpha_6\beta_5$, where $\alpha_5\beta_1$ (320), $\alpha_6\beta_3$ (320-323), and $\alpha_6\beta_5$ (323) are essential for SMC migration in vitro (320) and neointimal formation (324). In addition, $\alpha_6\beta_5$ integrin provides a logical link to mechanotransduction and myogenic phenomena through its role in mediating cellular attachment to the ECM and in cellular signaling involving Ca$^{2+}$ (325). Cultured SMC can recognize the native and unfolded triple helical structure of type I collagen by interacting with the collagen fibril-binding receptor ($\alpha_1\beta_1$ integrin) (326, 327) and collagen peptide-binding receptors ($\alpha_2\beta_1$ (326, 327) and $\alpha_5\beta_1$ integrins (326)). $\alpha_5\beta_1$ is a critical collagen receptor involved in matrix remodeling after injury (327). SMC in the contractile state adhere to native type I collagen through the $\alpha_5\beta_1$ and $\alpha_5\beta_1$ integrins (328). In contrast, cell adhesion to heat-denatured type I collagen is mediated only by $\alpha_5\beta_1$ integrin in the contractile state and by $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins in the synthetic state (328).

In glomeruli, $\alpha_1$ subunit of integrin characterizes MC while $\alpha_2$ and $\alpha_3$ subunits show expression in endothelial cells and podocytes, respectively (329). Integrins are used also as markers for MC in vivo and in vitro such as $\beta_1$, $\alpha_1$, and $\alpha_5$ chains (330). $\beta_1$ and $\alpha_6\beta_3$ integrins are highly expressed in proliferating MC in IgA nephropathy, membranoproliferative glomerulonephritis type I and diffuse proliferative lupus nephritis.
Usually, the distribution of integrin receptors for fibronectin (α3β1) and vitronectin (αvβ3β3) (333) follows the distribution of their ligands (334). MCs express α1, α2, α3, α4, α5, α6, αv, β1 (335-337), and α8 (319). α1β1 (on collagen and laminin) and α2β1 (on type I collagen) (338, 339), α3β1 (on type I collagen, laminin and fibronectin) and α5β1 (on fibronectin) are concentrated into focal adhesions (335, 336), while α6β1 is not detectable in focal contacts (335, 336). Endogenous blocking of protein synthesis by cycloheximide does not affect either cell attachment, spreading, or formation of talin and integrin-containing focal contacts (335, 336). αvβ3, and αvβ5 are expressed in MC and they are involved in adhesion to von Willebrand factor (vWF), while αvβ1 and αvβ3 are involved in integrin-induced apoptosis in HMC (340), and phagocytosis of apoptotic neutrophils (341).

1.1.5.3. Signal transduction by integrins

Integrins not only adhere to their ligands, they also transduce signals from their ligands to the cell interior (342). In cells adhering to a substrate the occupied integrins cluster at the basal side of the cell in so-called focal adhesions, which contain many cytoplasmic proteins, such as structural proteins involved in the organization of the cytoskeleton (talin, α-actinin, vinculin (343)) and also various signaling proteins such as focal adhesion kinase (FAK) (344), PKCε (345), integrin-linked kinase (ILK) (346), and β3-endonexin (347) which can transduce information from the occupied integrin. In addition to outside-in signaling processes, integrins are also involved in inside-out signaling and by the latter mechanism the activation state of an integrin can be tightly regulated. ILK interaction with integrins may regulate both inside-out and outside-in signaling as overexpression of ILK in rat epithelial cells results in decreased cell adhesion to ECM substrates; cell attachment and spreading on fibronectin results in altered ILK activity; and overexpression of ILK in epithelial cells induces anchorage-dependent growth (346), stimulation of the cell cycle, and malignant transformation (348). Cell attachment and spreading can be promoted through integrin
interactions with the cell binding domain of fibronectin (349). However, normal anchorage-dependent fibroblasts require an additional signal to form focal adhesions, which occur after binding of a heparin binding domain of fibronectin or a peptide from this domain to a cell surface heparan sulfate proteoglycan (349, 350). These interactions may stimulate PKC activity, since PKC inhibitors prevent focal adhesion formation, and pharmacological activation of PKC can substitute for stimulation through heparin binding domains (349). Syndecan 4 is selectively concentrated in focal adhesions in numerous cell types (351). It may transduce the signal generated on binding of heparin moieties to cells. A unique region of its cytoplasmic domain (LGKKPIYKK) can potentiate PKCα activity in vitro, and PKC interacts with its core protein in vivo and in vitro, and synthetic peptides of the LGKKPIYKK sequence (352).

1.1.5.3.1 Outside-in signaling

Ligand occupation of integrins triggers the accumulation and association of several signal transduction molecules with integrin, and cytoskeletal proteins (353, 354). This process appears to require tyrosine kinase activity in addition to cytoskeletal organization. The accumulation of talin, α-actinin and vinculin required ligand occupancy and clustering, but not tyrosine kinase activity or cytoskeletal organization. All four are required for accumulation of F-actin and paxillin (354).

The cytoskeletal proteins α-actinin and talin bind directly to the cytoplasmic domain of the β1A integrin (355, 356), and can bridge integrins to actin either by direct binding, as both are actin-binding proteins, or indirectly via vinculin or tensin (357, 358). In addition to interacting with cytoskeletal proteins, the integrin β subunits interact with intracellular proteins that participate in the regulation of integrin function and in integrin mediated signal transduction. Three proteins that were demonstrated to bind cytoplasmic domains of β integrin are ILK (346), β3-endonexin (347), and FAK (359). FAK, which was originally identified as a 120-kDa substrate of v-src (360), was shown to bind in vitro to peptides
derived from the cytoplasmic domains of $\beta_1A$, $\beta_2$, and $\beta_3$ integrin subunits (359). Activated FAK increases its own tyrosine kinase activity toward exogeneous substrates (361). It has not been possible so far to demonstrate a direct association of FAK with integrins in vivo, as FAK cannot be coimmunoprecipitated with integrins (348). This suggests that the interaction is of low affinity or although present in focal adhesion plaques, FAK does not associate directly with integrins. However, the $\beta$ integrin cytoplasmic domains may be required for FAK localization to focal adhesion plaques, as deletion of the carboxy-terminal amino acids 791-799 of $\beta$ integrin results in the loss of localization of FAK to microbeads coated with anti-integrin antibodies (362). ILK can associate with $\beta_1A$, $\beta_2$ and $\beta_3$ integrin subunits (346), while $\beta_3$-endonexin binding is specific for $\beta_3$ integrin (347). In contrast to FAK, the serine/threonine kinase ILK can be readily coimmunoprecipitated with various $\beta$ integrins which include $\beta_1A$, $\beta_2$ and $\beta_3$ integrins (346) suggesting a relatively strong in vivo association of ILK with cytoplasmic domains of $\beta$ subunits.

Interaction of integrins with ECM is not sufficient to induce integrin clustering. Focal complex formation requires the activity of members of the Rho family of GTPases (363). Rho regulates actin cytoskeletal rearrangement and assembly of focal adhesion plaques in response to growth factors and ECM (364), and it is suggested that the Rho-mediated pathway specifically couples integrin signal transduction to focal adhesion plaque assembly, independently of other mitogenetic signals (346, 365). In addition the p190 Rho GTPase-activating (GAP) proteins may also regulate integrin clustering, as a novel isoform of p190, p190-B Rho-GAP, is found to be present in integrin clusters (366), and constitutive activation of p190 Rho-GAP disrupts integrin clustering and focal plaque formation (367).

As a part of outside-in signaling integrins regulate PKC activity (306, 368, 369). An additional activation of PKC is needed to drive the cytoskeletal and receptor clustering that accompany the formation of stress fibers and focal adhesions, (349, 370). In this regard syndecan-4 was shown to regulate distribution and activity of PKC by binding to the catalytic
domain of PKCa and potentiate its activation by phospholipid mediators (352). Syndecan-4 can also directly activate PKCa through the sequence LGKKPIYKK in the short cytoplasmic domain of this PG, in the absence of other mediators.

Integrins can regulate tyrosine phosphorylation of cellular proteins (371, 372, 373). Several members of the src family of nonreceptor tyrosine kinases localize to sites of cell-ECM and cell-cell contact (374). Neither tyrosine phosphorylation of the relevant proteins nor c-src association with the cytoskeleton is observed in platelets lacking integrin αIIbβ3 (375). Tyrosine-phosphorylated β4 integrin associates with tyrosine-phosphorylated Shc and the adapter protein Grb-2, thus possibly linking αβ4 integrin to the ras signaling pathway (376), which includes the mitogenic MAPK cascade (377). Integrin-dependent MAPK activation was affected by cytochalasin D treatment, while PDGF-dependent activation of MAPK is not (378), suggesting that ECM-stimulated MAPK activity may be related to shape-dependent cell cycle progression.

Cell-ECM interactions are also essential for survival. Loss of such interactions results in the induction of apoptosis in epithelial and endothelial cells (379, 380) and in the arrest of fibroblasts in the G1 phase of the cell cycle (381). Integrin engagement is required for the suppression of apoptosis (382) and also for the transit of cells through the G1 → S phase of the cell cycle (383, 384). The suppression of apoptosis may involve the induction of expression of Bcl-2 (385) upon engagement of certain integrins. Concomitantly, the expression of the cell cycle inhibitor p27Kip1 is reduced from a high level upon cell adhesion after plating cells from suspension (383, 384).

The ratio of expression of the cytoplasmic domains of integrins in cells can regulate whether the cells will proliferate or differentiate. For example, the α5 cytoplasmic domain promotes proliferation and inhibits differentiation in myoblasts, whereas the α6 cytoplasmic domain promotes differentiation and decreases proliferation (386).
The adhesion of cells to ECM, cell-cell interactions, or treatment with antibodies to integrins stimulates expression of specific genes such as fos (387-389) and myc (388) and protease genes (387, 390). The rapid onset of responses suggests that they are primary responses to adhesion rather than secondary consequences of changes in growth or differentiation. Other genes whose expression can be regulated by integrins are alkaline phosphatase in osteosarcoma cells (391), PDGF, c-jun, and EGFR2 in monocytes (389), and casein in mammary epithelial cells (392).

Integrins can also regulate the angiogenic effects of bFGF, TGF-α and vascular endothelial growth factor (VEGF). The bFGF-induced effect is dependent on αβ3 and TGF-α- and VEGF-induced effects are dependent on αβ5 integrin (393) as cytokine-dependent angiogenesis is blocked by neutralizing antibodies or synthetic peptides against integrins.

Cell adhesion affects [Ca\(^{2+}\)]. Calcium spikes in neutrophils adhering to plastic are blocked by anti-αβ2 antibodies (394). Adherent cells release Ca\(^{2+}\) from intracellular stores in response to PDGF, but suspended cells fail to do so (395), suggesting that some step in the pathway linking the PDGF receptor to calcium release must require adhesion. Adhesion-dependent Ca\(^{2+}\) spikes are needed for calcineurin activation, leading to dephosphorylation of a substrate and detachment in neutrophils (396), and for activation of FAK in a tumor cell line (397), although, some integrins activate FAK without increase in [Ca\(^{2+}\)] (368).

Adhesion to FN stimulates the Na-H antiporter and elevates pH\(_i\) in fibroblasts (398-400), endothelial cells (401), lymphocytes (402), hepatocytes and SMC (368).

Integrins control inositol lipid synthesis while soluble growth factors control lipid breakdown. Stimulation of both events is required for optimum release of second messengers. Baby hamster kidney cells show an increase in levels of intracellular water-soluble inositol phosphates after attachment to FN (403). Kidney epithelial cells grow much better on
complex ECM than on plastic and have substantially higher levels of diacylglycerol and inositol phosphates (404).

1.1.5.3.2 Inside-out signaling

Integrin-mediated cell adhesion can be regulated either by altering the repertoire of integrin expression on the cell surface or by modulating the affinity of the integrins for their ligands. Two regions are important in the regulation of the affinity of integrin. Both motifs, NPXY and KLLxxxxD, are in the cytoplasmic domain of $\beta_1$-$\beta_7$ integrins. Point mutation of these motifs abolishes integrin activation (405, 406). In $\alpha$ subunits, a highly conserved membrane-proximal motif, KXGFFKR, also regulates integrin activation, as a truncation of the $\alpha_{1b}$ cytoplasmic domain that eliminated this motif resulted in constitutively active receptor (407). Calreticulin, a calcium-binding protein, is implicated in stabilizing integrin in an active conformation. It binds readily to an active, but not to the inactive form of collagen receptor $\alpha_2\beta_1$, through the KXGFFKR motif (408). Expression of constitutively active related (R)-Ras induced cells in suspension to adhere to the ECM by converting integrins to a high affinity state (409).

1.2. BIOLOGY OF MESANGIAL CELLS

1.2.1. Glomerulus and mesangial cells

The functional unit of the kidney is the nephron. Each human kidney contains about $(0.8 - 1.2) \times 10^6$ nephrons, whereas the adult rat kidney has $(3 - 4) \times 10^4$ nephrons (410, 411). The essential components of the nephron are the glomerulus, the proximal tubule, the thin limbs, the distal tubule, and the collecting ducts and tubuli.

1.2.1.1. Glomerulus

The glomerulus is composed of a capillary network lined by a thin layer of endothelial cells, a central region of mesangial cells with surrounding mesangial matrix, the visceral
epithelial cells, and the associated basement membrane. The average diameter of a
glomerulus is approximately 200 μm in the human kidney and 120 μm in the rat kidney
(410). The glomerulus is responsible for the production of an ultrafiltrate of plasma. The
filtration barrier between the blood and urinary space is composed of a fenestrated
endothelium, the peripheral glomerular basement membrane, and the slit diaphragms between
the foot processes of the visceral epithelial cells. The mean area of the filtration surface per
glomerulus has been reported to be 0.136 mm² in the human kidney (412) and 0.184 mm² in
the rat kidney (413). The tuft of specialized capillaries is attached to the mesangium, and
both are enclosed in a pouch-like blind extension of the tubule. Within the glomerular tuft,
three cell types are found, all of which are in contact with the GBM: endothelial cells,
mesangial cells, and podocytes. Their numerical ratio in the rat is 3:2:1, respectively (414).
The capillaries, together with the mesangium, are covered by epithelial cells (podocytes),
thereby forming the visceral epithelium that at the vascular pole is reflected to become the
parietal epithelium of Bowman’s capsule (415). At the interface between the glomerular
capillaries and the mesangium on one side and the epithelial layer of Bowman’s capsule on
the other, the glomerular basement membrane (GBM) is developed. The space between both
layers of Bowman’s capsule represents the urinary space which, at the urinary pole, passes
over into the tubule lumen. The GBM represents a complexly folded sack inside which the
glomerular capillaries pursue a tortuous course around centrally located mesangial axes (416,
417).

1.2.1.2. Mesangial cells

The mesangium consists of MC and the mesangial matrix. Since its first description by
Zimmermann (418) the mesangium has generally been believed to form a supporting
framework which maintains the structural integrity of the glomerular tuft. It occupies the
axial region of a glomerular lobule. MC have an irregular shape, with a dense nucleus and
elongated cytoplasmic processes that can extend around the capillary lumen and introduce
themselves between GBM and the overlying endothelium. MCs have an indented nucleus and a relatively small amount of cytoplasm that contains mitochondria, small vesicles and lysosomes (416, 419, 420). The organelles involved in the synthesis and secretion of proteins, such as ribosomes, rough endoplasmic reticulum and stacked Golgi cisternae are relatively few and poorly developed. The mesangial cell in all likelihood represents a specialized pericyte and possesses many of the functional properties of SMCs (16). A prominent feature of these cells is the presence of numerous bundles of small intracellular fibrils and associated attachment bodies similar to those found in SMC. The similarity of MC to SMC is further emphasized by the presence of receptors for ANG II on their plasma membrane. Microfilaments are composed at least in part of actin, α-actinin, and myosin (421, 422). There is a concentration of microfilaments situated along the paramesangial region and within the mesangial cell processes adjacent to the glomerular capillaries (423). Microfilaments in the MC processes appear to connect with the GBM either directly or through interposition of myofibrils (423). This apparatus within the mesangium serves primarily to prevent capillary wall distention secondary to elevation of the intracapillary hydraulic pressure (422, 423).

The potential roles of the mesangium in glomerular function are: a) structural support to the glomerulus, and specifically the capillary loops; b) generation and turnover of ECM; c) target site for vasoactive agents such as i) vasoconstrictors including ANG II, endothelin, vasopressin, and norepinephrine, and ii) vasodilators, such asatriopeptin, nitric oxide, prostaglandins PGE2 and PGI2 and dopamine; d) target site for inflammatory mediators, growth factors, and cytokines with effects on, for example, local hemodynamics, cell proliferation and matrix turnover; e) site of production of vasoactive mediators and growth modifying agents, such as prostaglandins, thromboxane, lipoxygenase products, platelet activating factor (PAF), nitric oxide, etc.; f) site production of various growth factors and cytokines such as PDGF, IL-1, CSF-1, TGF-β; g) expression of chemokines and adhesion molecules; h) generation of plasminogen activator and inhibitors; i) handling of
macromolecules such as lipids, immune complexes and advanced glycation endoproducts (AGE) (15, 16, 424-428).

1.2.2. Proliferation of mesangial cells

Glomerular MC in normal adult kidney show essentially no replication (429). Only 2% of rat glomerular cells are renewed each day, and this is accounted for mostly by endothelial cells (3). In both clinical and experimental renal disease, MC proliferation is a part of the mesangial pathology that follows a variety of initial triggers and culminates in glomerular obliteration (17, 429). Following 5/6 nephrectomy, MC in the remnant kidney begin to proliferate after a characteristic series of changes including expression of α-SMC actin, PDGF B chain, and the PDGF receptor (4). Proliferation of MC represents a condition in which the equilibrium between proliferation, quiescence and apoptosis is not balanced (429, 430). In such a state MC readily proliferate in response to growth factors such as PDGF, bFGF and insulin-like growth factor (IGF-I) (431). In addition, all growth factors mitogenic for MC also stimulate fibroblasts and VSMC to proliferate, indicating a common mode of action (431). The proliferation of MC as a major feature of glomerular injury was first documented by incorporation of [3H]thymidine in the Habu snake venom model of mesangio proliferative nephritis (432). Although this study showed that mesangial hypercellularity resulted from cell proliferation within the glomerulus and not from the localization of migrating mononuclear cells, it does not exclude infiltrating monocytes and macrophages which may proliferate within the glomerulus. Other studies were undertaken in the anti-Thy-1 model and confirmed a proliferative phenotype of MC, by immunostaining tissue sections with monoclonal antibodies to the proliferating cell nuclear antigen (PCNA) (433), the common leukocyte antigen (CD45) (435), or monocyte-macrophages (434). More than 85% of the PCNA-positive cells were shown to exclude the leukocyte markers (433, 434), strongly suggesting that proliferation was of the intrinsic MC. In addition numerous glomerular diseases, including IgA nephropathy, lupus nephritis, and variants of idiopathic
focal glomerulosclerosis are characterized by an increase of cells in mesangial areas (435, 436).

Although the mechanism of MC proliferation is still unclear, accumulating evidence suggests that platelets play important roles in the mediation in vivo of MC proliferation. Platelets contain many growth factors and vasoactive substances that mediate MC proliferation including PDGF, bFGF, TGF-α, TGF-β, EGF, IGF-1 and -2, serotonin, and adenine nucleotides (437-442). These factors can be released during platelet activation. Activated platelets or platelet antigens were found in glomeruli in many experimental and human proliferative glomerulonephritides (4, 443-447). In diseases such as mesangioproliferative and membranoproliferative glomerulonephritis, platelet activation can be demonstrated by decreased platelet survival, the presence of platelet-released products in plasma, and circulating platelets with reduced granule contents (448-450). Intraglomerular platelets accumulate before development of hypercellularity or proliferation in several experimental diseases (445, 451). Furthermore, anti-platelet agents (452, 453), and platelet-depleting antibodies, reduced MC proliferation in experimental models of mesangial proliferative nephritis (433, 454).

In vivo evidence suggests that MCs produce and respond to growth factors and cytokines to participate in the destructive and restorative processes that follow the initiation of glomerular injury (16). PDGF is the most important among several growth factors studied (455-457) because it is responsible for much of the mitogenicity of serum (458). It is a known mitogen for MC proliferation, and can be released by a variety of cells present in the glomerulus during inflammation (456, 459). In addition, an up-regulated synthesis of glomerular PDGF B-chain was found in several disease models (4, 434, 460-463). Mesangial localization of PDGF mostly in cells which express α-SMC actin, observed by in situ hybridization (461), and by immunostaining (434), suggests that most of the PDGF is produced by the activated MC (434, 461). Direct evidence that PDGF mediates MC
proliferation in glomerulonephritis comes from studies in which rats with anti-Thy-1 nephritis were administered a polyclonal neutralizing antibody to PDGF. The antibody reduced cell proliferation by 60% (464). Furthermore, in vivo transfection of the PDGF-B gene into the kidney increased MC proliferation and induced glomerulosclerosis (286). Another growth factor that influences glomerular mesangial expansion is bFGF. MC release bFGF, as suggested by the decrease in bFGF in glomeruli after anti-Thy-1 Ab-induced MC lysis (465). During repair, bFGF produced by glomeruli increased the proliferation of glomerular cells, but the response was less than observed after PDGF infusion (466). GH, GHRH and IGF-1 are important in the pathogenesis of human lesions (467). Lines of animals transgenic for these peptides exhibit abnormal growth, and the increase in size is particularly marked for GH and GHRH (468, 469). Mice transgenic for GH and GHRH show an increase in the size of the glomeruli and a severe progressive mesangial sclerosis which affects all the glomeruli and leads to end-stage glomerular disease (470). On the other hand, GH deficiency has been shown to result in resistance to glomerulosclerosis (471). Intact glomeruli and cultured MC along with endothelial and epithelial cells have cell surface IGF-1 receptors. When IGF-1 binds to its receptor, a mitogenic response is elicited followed by de novo synthesis of IGF-1 (472, 473). In addition, IGF-1 can mediate GH-dependent effects on growth and development (474, 475).

Interleukins also stimulate MC to proliferate in vivo. Mice transgenic for IL-6 exhibit a proliferative glomerulonephritis (476). The urine from patients with proliferative glomerulonephritis, including a large proportion of patients with IgA disease, contains high amounts of IL-6 activity (477). In contrast, urine from patients with nonproliferative forms of glomerular disease contain amounts of IL-6 comparable to those found in healthy subjects (477). A granulocyte inhibitory protein that regulates IL-6 production was reported in chronic renal disease, and IL-8 increases transcription and expression of IL-6 and IL-8 in human mesangial cells (478). Increased urinary IL-6 is associated with the development of mesangial cell proliferation and glomerular sclerosis in patients with IgA nephropathy as well.
as in mice transgenic for IL-6 (479, 480). Intrагlomerular expression of the cytokines, IL-1 and TNF-α, has also been demonstrated in both human and experimental glomerulonephritis (481, 482).

Besides factors that trigger MC proliferation, there is growing number of agents that suppress it. They include cAMP, ANF, TGF-β, PGI2, PGE2, TxA2, and NO. However, heparin is the only agent that has been shown to inhibit MC proliferation both in vivo and in vitro (106).

1.2.3. Contractility of mesangial cells

Although the geometrical arrangement of the mesangial contractile apparatus suits it to the role of decreasing ultrafiltration surface area, it was suggested that the shortening of MC processes connecting opposing mesangial angles would only bring the angles closer together, compressing the mesangial capillary interface, but leaving the peripheral capillary wall area unaltered (483). Furthermore, the contraction of MC and of preparations of whole glomeruli in culture is not opposed by intracapillary hydrostatic pressure as it is in vivo. These considerations and the absence of measurable glomerular tuft dimensional changes in response to vasoactive agents in vivo (484), led Kriz and colleagues to suggest that the mechanical action of MC contraction is essentially static in nature, developing tension that serves to counteract expansive forces on the tuft without including significant changes in capillary dimensions (423). They suggest that MC, in the case of the acute alteration of glomerular ultrafiltration coefficient (Kf) – the product of filtration surface area and hydraulic permeability – do not change filtration surface area (483). In addition the isoforms of actin in MC are subject to change, which may alter the contractile potential of the cells. Reports are somewhat conflicting. For instance rat MC in culture express the smooth muscle isoform of α-actin whereas those under normal conditions in vivo do not (485). However, recently it was shown by in situ hybridization that α-smooth muscle actin was expressed not only in vitro, but also in vivo in MC from normal kidneys of one-week-old to adult mice, as opposed
to new-born mice (13).

Despite questions regarding dynamic contraction in vivo, based on their SMC-like characteristics and their syncytial continuity with the juxtaglomerular apparatus in addition to their central position, a contribution of MC to regulation of glomerular filtration rate (GFR) has long been suspected. As early as 1924 Richards and Schmidt (486) observed similarity in changes between glomeruli and capillary loops, after maneuvers such as nerve stimulation, or administration of caffeine, epinephrine, or pituitary extract to perfused glomeruli. It is frequently found in glomeruli, where branching of afferent vessels is distinguishable, that the greater portion of entering blood passes in steady flow into only one of three available channels (16). This pattern could change. Such changes in the number of glomerular capillaries being perfused would result in a change of glomerular surface area available for filtration (16). Micropuncture experiments showed that a number of vasoactive agents can change glomerular $K_f$ (16, 487, 488). It seems that vasoactive agents can decrease the effective intraglomerular filtration area in large part by reducing blood flow to certain capillary loops, thus excluding them from contributing to filtration. It appears that MCs, located at the glomerular hilus, where they totally surround the capillaries at their branching points are best able to regulate the number of perfused glomerular capillaries (16). Recently, Inkyo-Hayasaka (489) reported that mesangial loops surround about 15% of the capillary branches in a glomerulus, indicating that mesangial loops have a significant influence on the distribution of blood flow within the glomerulus. The peripheral MC would also be important in providing a force counter to the intracapillary pressure, thereby holding secure the invagination of the basement membrane (16). As noted by Latta et al. (416) failure of this function would explain the occurrence of capillary aneurysms occasionally seen in glomerular disease. That this may be accomplished by contraction of MC is supported by reports that many of the vasoactive agents including ANG II, AVP, norepinephrine, thromboxane, leukotrienes, PDGF, and ET-1 capable of reducing the $K_f$ in vivo will also contract isolated glomeruli and cultured MC in vitro (9, 16, 19, 488, 490, 491). Furthermore, receptors for
ANG II, AVP, PDGF, and ET-1 were demonstrated on MCs (9, 11, 16, 492-494). Local modulation of glomerular filtration may depend on a delicate balance between the actions of circulating vasoactive agents on MCs and of locally generated autacoids such as prostaglandins (488).

1.2.4. Mesangial extracellular matrix

MCs are surrounded by porous ECM. A large number of common ECM structural proteins have been demonstrated to be present within the mesangial matrix, including several components of elastic and nonelastic microfibrils such as collagens type III, IV, V and VI (495-501), fibrillin (502), microfibril-associated glycoprotein (503), microfibrillar proteins 78 and 340 (504), fibronectin (497, 505-507), laminin (497, 506, 508), enactin (509), thrombospondin (510), and IG 10 Antigen (511). The matrix also contains several types of proteoglycans including HS-PGs (512), and CS-PGs (513) such as biglycan and decorin (514).

The basic ultrastructural organization of the matrix is a network of microfibrils (483). The mesangial matrix shows up as basement membrane-like material, however more fibrillar in character than a basement membrane proper (417). In specimens prepared by a technique that avoids osmium tetroxide and uses tannic acid for staining, the mesangial matrix is seen to contain a dense network of microfibrils (421, 515). Within the mesangium, the three-dimensional network of microfibrils establishes a tough, functionally continuous medium anchoring the mesangial cells to the GBM (421).

The network form of the ECM in the mesangium subserves the transmission of mechanical forces in all directions. This counters expansion of the axial mesangium, just as spanning mesangial cell processes anchor the pericapillary GBM against expansion at the mesangial angles. Bundles of microfibrils may be considered to be microtendons transmitting contractile mesangial forces to specific sites of the GBM. Microfibrils are coated
with fibronectin (516). This fibronectin could serve to connect the microfibrils, as well as to anchor cells to the matrix (517). Superfibronectin, a highly crosslinked form of fibronectin (518), may form another kind of fibrous nexus, further contributing to the mechanical strength of the mesangial matrix. The high content of fibronectin in the mesangial matrix (505) may be the result of chronic high levels of mechanical stress to which the mesangium is exposed (519). The abundant, well-hydrated proteoglycans in the matrix probably afford a certain degree of resistance to compression to the axial mesangium as well (520).

The mechanical coupling of ECM to the signaling apparatus of mesangial cells is established by proteins including α-actinin, vinculin, talin, syndecan 4, and membrane-spanning integrins (351, 483). The contact of individual microfibrils to the cell surface is often seen to be tangential, an arrangement that would provide for an extensive interface of surface fibronectin on microfibrils and cell membrane-bound integrins. Microfibrils appear to penetrate into the lamina densa of the GBM, thus anchoring directly to the structure that can be expected to possess the greatest tensile strength in this system. How these fibrils are attached to the components of the GBM is at present unknown.

ECM proteins are synthesized and degraded by MCs. The balanced and small amount of ECM is produced in healthy kidney, whereas in glomerulonephritis the production of ECM is enormously increased thereby contributing to the development of glomerulosclerosis (514, 519, 521). ECM synthesis by MC (519) may increase with elevated mesangial cell/matrix strain, which will tend to decrease the magnitude of the stress on individual cells, especially together with mesangial proliferation. MC production of neutral protease and other ECM-degrading enzymes (522) suggests that MC may be constantly remodeling matrix structures including the perimesangial GBM. It is possible that this remodeling results in minimizing mechanical stress on the matrix, MC, and GBM, suggesting adaptive and structure-stabilizing functions of the mesangial matrix over the long term.
1.2.5. Mesangial cells in culture

Cultures of homogeneous populations of renal cells allow identification of features of the cell’s phenotype that are not easily accessible in vivo, partly because of the multiplicity of cell types in the glomerulus (523). Homogeneous populations of smooth muscle-like MC in culture can be obtained from many species, including man, rat, mouse, and pig (424). Cultures are derived from decapsulated glomeruli isolated from renal cortex by differential sieving (524-528). In an early method whole glomeruli were plated on plastic flasks and MC were allowed to grow out gradually in medium containing fetal bovine serum (FBS) after an initial and transient outgrowth of epithelial cells (424, 529-531). Several methods have been developed since to increase the purity of MC cultures, including cell cloning, enzymatic digestion of glomeruli, and use of selective cytotoxic agents (532-534). Two different types of MC have been described: the intrinsic smooth muscle-like MC and a bone marrow-derived phagocyte (529, 535, 536). Because of their ability to contract in vitro and to synthesize ECM proteins, the smooth muscle-like MC in culture are considered myofibroblasts (5, 523). The macrophage-like cells are not present. Most rat glomerular MC cultures are carried out in medium supplemented with 10 - 20% serum (523, 537). Relatively high concentrations of serum are required to establish and maintain mesangial cells in vitro (431), in order to provide growth factors such as PDGF for the cells to proliferate (431). A consequence of such conditions is that the cells in culture represent activated cells rather than the quiescent cell observed in the normal glomerulus. In order to determine the stage of MC differentiation the expression of SM α-actin, α1(IV), and α2(I) collagen mRNAs could be used. For instance, it was shown that the levels of α-SMA mRNA paralleled those of α1(IV) collagen, both features of SMC, and they were inversely correlated with the MC doubling time (13). On the other hand, the levels of α2(I) collagen, a characteristic of a fibroblast phenotype, were proportional to the cell doubling time (13). It was the lowest in the cells with high levels of SM α-actin, but increased in cells with a shortened doubling time and lower levels of SM α-actin (13). The same authors also showed that the observed change in phenotype was due to
an actual change in the phenotype rather than on overgrowth of a minor population. Namely, the observed changes in phenotype did not appear until after the cells had been passaged over 25 times, and they were reversible by changing the methylation state of the CCGC motifs (13). Because multiple passages may favor the emergence of a cell subpopulation with peculiar characteristics or undergoing dedifferentiation, only low-passage MC should be studied (538).

Cultured MC in plastic Petri dishes appear large and stellate with many irregular cytoplasmic projections as seen by light microscopy (523). In long-term culture, they can build multilayers and hillocks, the formation of which is increased by heparin (539-541). At the ultrastructural level, they show an elongated nucleus, a well-developed RER and Golgi apparatus, and many bundles of microfilaments associated with attachment bodies, as in SMC. At variance with smooth muscle-like MCs, mesangial bone marrow-derived macrophages rapidly adhere to plastic but cannot be subcultured (523).

Resting MC in culture show an elaborate array of microfilament bundles of stress fibers which stain for F-actin and myosin (542). Furthermore, the MC show scattered streaks of vinculin localized in areas of focal contact with the culture substrate. Stress fibers and vinculin-containing substrate attachment plaques have been shown to arise during development of tight cell-to-substrate adhesion (19). They are believed to play a structural role in anchoring the cytoplasmic matrix to the substratum and in distribution of forces applied on cells to attachment sites on the substratum or basement membrane (543). Stress fibers develop in cells that are exposed to high fluid shear (543). Harris et al. (544) suggested that stress fibers in living cells are in a state of isometric contraction under conditions of normal growth. Willingham et al. (545) showed that stress fiber development was the result of tight cell-to-substrate adhesion, and that the development of stress fibers was greater on plastic than on poorly adhesive substrata. Kreisberg (543) suggested that the development of tight cell-to-substrate adhesion in mesangial cells may explain why the cells...
no longer isotonically contract to AVP. Therefore, development of stress fibers inversely correlates with the ability of cells to contract isotonically and to move (19).

Besides culturing on plastic, MC can be cultured on dishes coated with various ECM proteins, such as laminin, fibronectin, and types I, IV, and V collagen (523). During such culture they acquire different morphologies, indicating that ECM can induce changes in the cell phenotype (23). For instance, MC grow in sprouts and bundles on fibronectin, whereas the degree of intracellular organization is lower on laminin and type IV collagen. In three-dimensional culture carried out in native type I collagen gels, MC rapidly organize into multicellular aggregates with numerous pseudopodia and regions of cell membrane fusion (23) supporting the notion that the mesangial cell phenotype in three-dimensional culture is closely related to the in vivo phenotype (546). In basement membrane-type gel matrix, MC usually have a lower proliferative rate (547, 548), except in fibronectin gel, which promotes both cell elongation and proliferation. Laminin impedes MC migration, and collagen type IV and heparin-like PGs inhibit cell elongation, migration, and proliferation (547-549).

1.2.5.1 MC receptors and growth factors

MC in vitro are both target and source of hormones, cytokines, and autacoids. Numerous receptors for these biomolecules were observed on MC membranes (424, 473, 490, 550, 551). In general these agents can be subdivided into (i) those that appear to have a direct mitogenic effect, such as PDGF (550, 552, 553), bFGF (465, 554), EGF (553, 555) and IGF-1 (473, 556, 557); (ii) those that are likely to act indirectly to induce the synthesis of a direct mitogen like PDGF, such as IL-1 (558, 559), IL-6 (477, 560), IL-8 (561, 562) and TGF-β (563, 564); and (iii) an intermediate group that may have both direct and indirect mitogenic actions, such as endothelin (424, 490, 565) and angiotensin II (431, 566). The direct mitogens have in common an action through receptors with intrinsic tyrosine kinase activity, whereas those with indirect action do not.
Different second messenger systems activated by growth factors have been linked to the proliferative response of cultured MCs. An important role is postulated for phosphorylation events such as tyrosine phosphorylation after binding the agents to their respective tyrosine kinase receptors (567) or after activation of other cellular tyrosine kinases (568), and serine/threonine phosphorylation after activation of PKCs (569). Intracellular events following mitogenic stimulation of cultured MC were also studied by evaluating changes at the level of early genes. There are a number of genes that may couple early second messenger signals to long-term changes in gene expression. Cellular genes that are induced rapidly after cell stimulation without the requirement for de novo protein synthesis are referred to as immediate early response genes. They represent the initial genetic response of cells to growth factors. Several of these genes encode transcriptional regulatory proteins that modulate the expression of other genes, ultimately resulting in a specific biological response. The group of genes that are both cellular immediate early genes and transcription factors includes the c-fos family (570, 571), the jun family (572-574), a member of the steroid hormone receptor superfamily (575), and the early growth response gene family (576-578). Transcription of the c-fos proto-oncogene is rapidly and transiently induced in cells exposed to a remarkably wide range of external stimuli, including growth and differentiation factors, neurotransmitters, drugs, and mechanical stimuli (579). These diverse agents act on cells through a series of divergent signal transduction pathways, whose activation triggers c-fos transcription. These pathways include those involving PKC, MAPK, Ca2+, and cAMP.

1.2.5.2. Vasoactive agents and eicosanoids

In addition to many studies on the effect of ANG-II and AVP (9, 19, 543, 580-582), a constitutive expression of pre-proendothelin-1 transcripts and ET-1 secretion were demonstrated in cultured MC (583, 584). ET-1 production is stimulated by vasoactive agents and growth factors, including PDGF, ANG-II (585), AVP (586), TGF-β (583), thrombin (583, 587), and thromboxane analogs (583). ET binds to two types of receptors. The low
ET-B receptor binds all three isoforms ET-1, ET-2 and ET-3, and is a G protein-coupled receptor that induces a biphasic increase in cytosolic Ca\textsuperscript{2+} with a rapid spike followed by a lower but sustained plateau (491, 588). In contrast, the high-affinity ET-A receptor binds specifically ET-1 and ET-2 but not ET-3 (11). Binding of the agonist to the receptor activates PLC to cleave phosphatidylinositol biphosphate with formation of two second messengers, inositol triphosphate (IP\textsubscript{3}) and diacylglycerol (DAG). The transient IP\textsubscript{3}-dependent Ca\textsuperscript{2+} release activates calmodulin which in turn activates a number of Ca-dependent enzymes (589), whereas DAG activates PKC leading to phosphorylation of specific proteins which together cause the cell to contract. An increase in intracellular cAMP induced by forskolin or the \beta-adrenergic agonist isoproterenol inhibits serum-stimulated ET-1 production (584). Production of vasodilators such as PGE\textsubscript{2} and PGI\textsubscript{2} would function in a negative feedback manner to attenuate the action of the vasoconstrictor peptides (558, 559).

ET-1 enhances tyrosine phosphorylation of at least 5 distinct target proteins, probably by an indirect mechanism because the endothelin receptor is not known to possess intrinsic tyrosine kinase activity. The vasoactive peptides, in addition to their effect on MC tone, exert multiple biological effects including the induction of oncogenes c-fos and c-jun, alterations of the synthesis of extracellular and intracellular proteins, and cell growth (565, 590, 591).

1.2.5.3. Extracellular matrix

MC in culture also secrete ECM proteins. As visualized by immunofluorescence the major constituents of ECM are the collagen types I and III in addition to types IV and V. The glycoproteins such as laminin and fibronectin and HSPGs such as perlecan and CS/DSPGs such as versican, decorin and biglycan are also synthesized by MC in culture (424, 540, 592-596). Type I collagen represents about 95% of total collagen synthesis but only 10% is incorporated into the cell matrix (394). Half of type I collagen is present in trimers \([\alpha_1(1)\text{II}]_3\), a form often associated with wound healing, which suggests that cultured MC express phenotypic characteristics reminiscent of those observed in glomerulonephritis. Cultured MC
also synthesize thrombospondin (597) and vitronectin, which can be incorporated into pericellular matrix or secreted in the cultured medium (540). Deposition of ECM is increased by serum and cell confluency (593). The production of ECM by cultured MC is regulated through matrix degrading enzymes and their inhibitors. A neutral protease activity has been characterized in the conditioned culture medium of rat MC, in addition to a small amount of casein and PG-degrading activity and tissue inhibitor of metalloprotease-1 (TIMP-1) (598).

1.2.5.4. Other characteristics of cultured MC

The effect of immune complexes and the terminal attack complex on MC in vitro indicated that these cells behave like true inflammatory cells. When stimulated with C5b-9 at concentrations which do not induce cell lysis, they respond with increased production of several proinflammatory molecules, reactive oxygen products, and enhanced production of ECM (599-602). Fc-mediated endocytosis of immune complexes associated with production of reactive oxygen species has also been described in MC (603) in addition to morphologic aspects of the phagocytic properties of MC (604). Uptake of tracers such as ferritin (417), colloidal gold (605), globin (606), aggregated proteins (607), and immune complexes has been described (16). It was suggested that phagocytosed material may be cleared from the mesangium by cell-to-cell transport to the extraglomerular mesangial region at the vascular pole of the glomerular tuft (607, 608). The endocytosis of immune complexes by cultured MC was associated with stimulation of PGE2 and platelet activating factor (PAF) and was dependent on Fc receptor activity.

PAF (1-Alkyl-2-acetyl-sn-glycero-3-phosphocholine), is produced by MC stimulated by the calcium ionophore A23187, LPS (609, 610), TNF-α or IL-1β, complement activation, and ANG II (611). PAF stimulates multiple pathways in MC, including increase in intracellular Ca2+ concentration mediated by phospholipase C activation and PGE2 generation by phospholipase A2 (611).
Cultured MC constitutively express cyclooxygenase and lipoxygenase enzymes and convert arachidonic acid into prostaglandins in response to various agents such as ANG II, AVP, ET-1, and thrombin (612-614). Cultured MC generate L-arginine-derived NO+ and NO2/NO3 on exposure to TNF-α or IL-1 (615). NO+ generation is associated with soluble guanylate cyclase activation and cGMP accumulation (616-618). Rat MC in culture also generate superoxide anion and H2O2 when stimulated with opsonized serum-treated zymosan, a protein-carbohydrate complex from yeast cell wall, whereas nonopsonized zymosan has no effect (619).

1.2.6. MC phenotypes

MCs possess different characteristics in vivo and in vitro, and they are also different in normal and diseased glomeruli. Some important differences also exist between MC from different species such as human and rat. For instance, the role of the renin-angiotensin system on the glomerulus in the rat is clearly demonstrated (424, 620-622), while a direct contractile effect of ANG II on human MC was never demonstrated (622). One way to clarify data obtained with MCs would be to reach a consensus on basic phenotypic features of MCs in standardized culture conditions. Such features can be grouped into three categories regarding: (i) cell proliferation, (ii) contractility, and (iii) synthesis and secretion of proteins such as ECM.

1.2.6.1. Phenotypic aspects of cell proliferation

The quiescent phenotype is mostly expressed in vivo, in normal healthy glomeruli where MC are positive for MC-specific OX-7 antibodies (6). MC associated with nodules after prolonged culture, also express a quiescent phenotype indicated by an absence of mitogenic activity, expression of smooth muscle α-actin, and production of collagens (7). Quiescent MC do not express smooth muscle α-actin, PDGF B chain, or PDGF receptor (4). They differentially express cyclin kinase inhibitors; p27Kip1 is highly expressed but levels for p21 (Cip1), Waf1, Sdi1, Cap20) are low (6). In addition, quiescent MC in vivo do not express
the proteins for cyclins D1, E or A (6), as found in vitro studies in other cell lines (623, 624). Very low or no staining for CDK2 and CDK2 kinase activity were observed in quiescent glomerular MC (6). PCNA staining is also very low or absent in quiescent MC in vivo (6). Quiescent cells also display down regulation of receptors for growth factors as found in three-dimensional cell culture (546).

MCs with a proliferative phenotype have the opposite characteristics to those of quiescent cells. These, are for instance, expression of smooth muscle α-actin, PDGF B chain and PDGF receptor, along with low expression of p27Kip1, and highly expressed p21. Proliferative MCs occur in diseases such as experimental glomerulonephritis induced by 5/6 nephrectomy or anti-Thy-1 antibodies (4) and also in MC cultures (431).

1.2.6.2. Phenotypic aspects of contractility

Contractile MC express ANG II receptors, and also smooth muscle α-actin which is found in vivo (13), and in vitro (5, 13, 424, 431). A prominent feature of these cells is the presence of numerous bundles of small intracellular fibrils and attachment bodies similar to those found in SMC. In addition the presence of both actin and myosin (8), tropomyosin, caldesmon, α-actinin and vinculin (12) in MC are well documented. The contractile phenotype of MC is emphasized by expression of receptors for contractile agents such as AVP and E-1 (9, 10, 11, 493). Another indication that MC are contractile is the phosphorylation of MLC (20, 625), after exposure to contractile stimuli.

The concept of hypocontractile MCs was introduced when lack of response to vasoactive agonists was observed (10, 21, 626, 627). Hypocontractile cells contain vinculin and develop stress fibers, which produce a tight cell-to-substrate adhesion (19). In addition hypocontractile MC exhibit reduction in the number of vasopressor receptors (22, 628), and produce increased amounts of cAMP in response to isoproterenol (18, 20), dopamine (629), PGE2 and PG12 (630). They also produce cGMP in response to ANF and nitroprusside.
(21), thereby attenuating agonist-dependent contraction of MC. There are also indications that hypocontractility of MC results from a lack of cytoskeletal response to Ca\(^{2+}\) (582, 631, 632).

### 1.2.6.3. Phenotypic aspects of protein synthesis and secretion

SMC were shown to switch from a contractile to a synthetic phenotype in atherosclerotic plaques and in cultures (633, 634) as determined by dramatic changes in cell morphology, with loss of myofilaments and the formation of an extensive RER and large Golgi complex, and by gaining the ability to synthesize DNA and divide in response to mitogens. MC in culture are biosynthetically very active and seem to acquire a synthetic phenotype as do SMC. Morphologically, MC in culture exhibit well-developed RER and Golgi apparatus in the so-called active state, as compared to quiescent cells of healthy glomeruli where organelles involved in the synthesis and secretion of proteins are few and poorly developed (416, 419, 420). In addition activated MC showed prominent upregulation of 5-10 different proteins after exposure to AVP and 5-HT (635), and enhanced DNA and RNA synthesis (5, 432, 636, 637). In contrast to SMC other cytoskeletal proteins associated with cell movement are also upregulated in experimental mesangiotrophic nephritis, including vimentin, desmin and moesin (5, 638). As a consequence of the synthetic phenotype MC become hypersecretory. A balanced and low amount of ECM proteins in the normal glomerulus (14, 483) is replaced by increased amounts of ECM proteins including now de novo synthesized interstitial collagens type I and III (298, 596, 639, 640). The increased expression of collagen type I along with other features such as smooth muscle \(\alpha\)-actin and increased mitogenic activity was used to characterize activated/dedifferentiated MCs such as those occurring in pathological states or in cell culture (5, 7, 24, 431). The increased matrix deposition is not only due to increased synthesis but also due to inhibition of matrix degrading-proteins by TIMP (641) or plasminogen activator inhibitor (642), which are upregulated in the hypersecretory phenotype.
1.3. HEPARIN AND MC PROLIFERATION

1.3.1. Effect of heparin on SMC

Similar to SMC, MC are sensitive to the antiproliferative effect of heparin (106). An extensive literature has dealt with the effect of heparin on SMC. The observation by Clowes and Karnovsky in 1977 (643), later confirmed by Guyton et al. (103), that intravenous administration of heparin in rats inhibited the proliferation of SMC normally seen following deendothelialization of blood vessels suggested that heparin might play an important role in the regulation of cell growth, particularly in conditions such as atherosclerosis in which the biosynthetic capacity for these molecules may be altered (644). This effect was observed with both anticoagulant and nonanticoagulant fractions of heparin. These results suggest that heparin inhibits both medial SMC proliferation and neointimal expansion which was supported by experiments of Clowes and Clowes (645) who observed that the intravenous administration of heparin to rats for 14 days significantly inhibits the increase in the DNA content of the common carotid artery caused by balloon catheter injury (645, 646). Reduction of neointimal thickening was also observed in the balloon-injured rabbit aorta after a continuous intravenous infusion of heparin (647). The antiproliferative action of heparin is accompanied by an increase in the SMC content of α-actin and its corresponding mRNA, although the levels of α-actin may also be influenced by culture conditions (648). Further direct evidence of an effect of heparin on arterial SMC proliferation in vivo was obtained by Majesky et al. (649) who measured the [3H]thymidine labeling index of SMC in the rat common carotid artery. Local release of heparin from a gel enclosed in a periadventitial cuff around the rat common carotid artery significantly inhibited balloon catheter-induced neointimal thickening in the vessel (650).

These in vivo studies were supported by the results of experiments with SMC in culture. Hoover and colleagues showed that heparin dose-dependently inhibits the serum-stimulated proliferation of rat aortic SMC in culture, with 50% inhibition of growth occurring at a
concentration of approximately 10 μg/ml (651). Heparin inhibits vascular SMC proliferation and migration in vitro (651-653). Heparin’s inhibitory action is probably in G1 of the cell cycle since it must be added before the cells enter the S phase to achieve maximum inhibition (646, 649, 654). Heparin interferes with various G1 events (655-657), and the expression of several oncogenes such as c-myb, c-fos and c-myc (658, 659). Heparin also alters the deposition of extracellular matrix proteins (660, 661) and suppresses the expression of matrix-degrading proteases (662, 663).

1.3.2. Effect of heparin on MC in vivo

After several reports about the beneficial effect of heparin on experimental glomerulonephritis (664, 665), Kincaid-Smith, in the late 1960s, proposed the therapeutic use of heparin in nephrology (666). However, based on the putative pathogenic role of the coagulative cascade in acute kidney rejection and in rapidly progressive glomerulonephritis, that idea was gradually abandoned. Subsequent investigations carried out in different models have provided strong evidence that heparin is effective in ameliorating the natural progress of glomerulonephritis including proliferation of MC. This resulted in the renewal of heparin administration with a novel rationale (667, 668). The administration of intact or partially degraded heparin, resulted in a dramatic decrease in the number of cells detected in the mesangial areas in habu snake-venom-induced nephrotic rats (669). Many other studies showed that heparin suppresses MC proliferation in rat models such as puromycin- and aminonucleoside-induced nephritis (432, 669, 670), anti-Thy-1 experimental mesangio proliferative glomerulonephritis (671), glomerulosclerosis in the growth hormone (GH)-transgenic mouse (672), and nephropathy in the streptozotocin (STZ)-induced diabetic rat (673-675). As a part of its antiproliferative effect, heparin also reduced mesangial bFGF expression and PDGF receptor up-regulation (671, 676).

In addition to its antiproliferative effects, heparin prevents imbalanced matrix protein metabolism and accumulation (677). Chronic administration (every 12 h) of heparin
(anticoagulant or nonanticoagulant) resulted in a decrease in antibody-induced mesangial cell proliferation, which, in turn, was associated with a decrease in the size and number of areas of focal mesangial matrix increase (678). Heparin treatment of diabetic rats does not affect the metabolic control of diabetes but modulates ECM synthesis (673, 674) and completely prevents the increased α1(IV) collagen deposition and expression (679). Heparin treatment markedly inhibited not only accumulation of collagen type IV but also of other ECM proteins, including laminin, type I collagen, fibronectin and entactin. In addition, GAG administration modified renal matrix composition by normalizing collagen gene expression and increasing glomerular [35S]-sulfate incorporation (676).

The renoprotective effect of heparin does not depend on its anticoagulant ability. The progression of glomerulosclerosis in subtotal nephrectomized rats is prevented by heparin, but not by other anticoagulant drugs such as coumadin (680). In a similar model N-desulfated/acetylated heparin, which is almost completely devoid of anticoagulant properties, was as effective as intact heparin in preventing proteinuria and reducing the severity of the glomerular lesions (681). A treatment with a non-anticoagulant fraction of heparin or with intact heparin at dosage levels which did not prolong the activated thromboplastin time resulted in significant functional and histological improvement in aminonucleoside-induced focal and segmental glomerulosclerosis in rats (670). In addition, in the STZ-diabetic rat, nephropathy is prevented by both modified heparins and a dermatan sulfate which are poor anticoagulants (673, 674).

The effect of heparin is not mediated either by hypertension. An antihypertensive therapy regimen did not reduce glomerular injury as effectively as heparin (682). Similarly heparin does not ameliorate the increased glomerular hydrostatic pressure in focal and segmental glomerulosclerosis and the effects of heparin could not be explained by a change in glomerular hemodynamics (670). In addition, Ichikawa et al. (683) found that the protective effect of heparin on glomeruli of remnant nephrons was independent of glomerular
hyperfusion, hypertension or hyperfiltration as measured by glomerular capillary hydraulic pressure, glomerular plasma flow rate and single nephron filtration rate. They suggested, instead, that heparin plays a role in protecting glomerular structure.

1.3.3. Effect of heparin on MC in vitro

The confirmation of heparin's antiproliferative effect came from in vitro experiments. In vitro heparin interferes with (i) cell growth, (ii) proto-oncogene expression, (iii) synthesis of specific proteins, (iv) extracellular matrix composition, (v) cell adhesion and migration, and (vi) binding of immune complexes and LDL to MC.

(i) Growth

Heparin profoundly inhibited the growth of cultured MC (684) by 70% (685, 686) in a dose dependent manner, with an ED50 = 5-10 μg/ml (685). Furthermore, Castelot et al. (685) suggested that glomerular endothelial cells may participate in part in mesangial cell growth regulation via a heparin-mediated mechanism, after they showed that glomerular endothelial cell-conditioned medium inhibited MC growth by 60-70%. The antiproliferative activity of heparin was reversible and specific for mesangial cells as the target cell in the glomerulus. In addition cultured glomerular epithelial cells were found to secrete both stimulators and inhibitors of mesangial-cell growth (687). Approximately 70% of the antiproliferative activity was destroyed by a highly purified heparinase; the other 30% was sensitive to trypsin. It appears that heparin and HS are much more effective than other glycosaminoglycans (636, 685). In keeping with this idea, CS produced no inhibition while heparin and HS caused dose-dependent inhibition of RMC growth (688). The low sulfated fraction of HS produced more inhibition than the high-sulfated fraction.

Heparin does not inhibit only serum-dependent MC proliferation. Poly-L-lysine-enhanced MC proliferation in culture was inhibited by heparin and HS (689). Also thrombin-dependent stimulation of DNA synthesis and growth of cultured human MCs (HMC) were
inhibited by heparin and antithrombin III, separately or together, and the magnitude of inhibition was equal (690). Experiments carried out in serum-free (antithrombin-free) medium revealed that heparin’s inhibitory effects are independent of antithrombin III. TSP, in a concentration dependent manner (5 to 20 µg/ml), caused an increase in thymidine uptake, first detectable at 28 hours and more prominent at 48 hours. This effect was inhibited by heparin and heparan sulfate (691).

In addition to high concentrations of heparin used in vivo and in vitro (> 5 µg/ml), much lower concentrations, similar to those found in physiological conditions, are effective. Bovine lung heparin (1 µg/ml) decreased by 45% the incorporation of [3H]-thymidine into DNA after release of serum-starved RMC from growth arrest (636). The same authors went further and showed that 1 - 0.05 µg/ml of heparin significantly suppressed [3H]-thymidine incorporation into DNA in a concentration-dependent fashion. Concentration of 1 µg/ml of heparin showed the highest inhibitory effect on MC proliferation which decreased and delayed the entry of cells into S/G2 (637). Wang et al. also demonstrated that heparan sulfate glycosaminoglycans derived from mesangial proteoglycans are potential negative autocrine growth regulators (636). Heparin acted prior to the G1/S interface. The endogenous HSPGs had a slight antimitogenic effect on the RMC, but heparan sulfate chains from both the medium and cell layer had a potent effect. On an equivalent mass basis, only the free glycosaminoglycan chains were more potent than heparin in this regard, decreasing thymidine incorporation by over 90% when present at 1 µg/ml. Studies in vivo demonstrated that the biosynthesis of potent antiproliferative HS in both EC and SMC occurs only after confluence is reached with less potent antiproliferative molecules being produced during the growth phase (692).

(ii) Proto-oncogenes

It was suggested, by an immunocytochemical investigation, that heparin could inhibit the expression of nuclear oncogenes c-fos and c-jun (686). Low concentrations of heparin and
heparan sulfate suppress the mitogenic response of mesangial cells to serum and inhibit c-fos mRNA induction through an effect of cell surface-bound GAG on a signaling pathway downstream of PKC (637). Added at the time of serum stimulation, heparin (1 μg/ml) causes a decrease in the subsequent expression of c-fos mRNA in RMC, and a similar effect is observed with heparan sulfate chains isolated from RMC-cultures themselves. In addition to inhibition of c-fos, heparin suppressed egr-I mRNA expression by 44% (693).

(iii) Protein expression and synthesis

Heparin also affects the expression of different proteins. For instance, immunocytochemical studies showed that the amounts of FN and LM in the cytoplasm of MC decreased after heparin treatment (686). The profound direct metabolic effects of heparin and non-anticoagulant N-desulfated acetylated heparin significantly reduced the FN content in the conditioned media of subconfluent, confluent, and supraconfluent RMCs in culture (694). Both heparins significantly increased the amount of cell-associated fibronectin in sparse and subconfluent MCs. Heparin and non-anticoagulant N-desulfated acetylated heparin were found to significantly decrease cell-associated collagen in subconfluent but not in confluent MCs. No effects were seen on newly synthesized collagen secreted into the culture medium. Neither heparin nor N-desulfated acetylated heparin affected total protein synthesis, studied by metabolic labeling with 35S-methionine. One particular intracellular protein (molecular weight 54 kD, pI 5.91) was consistently overexpressed in heparin treated cells. Both heparins affected an identical set of another 19 different intracellular MC proteins (over-/underexpression or shift to higher molecular weights).

Heparin also reduced ET-1-activated prostaglandin endoperoxide synthase (PGHS)-2 mRNA expression and protein formation probably through a protein tyrosine kinase-dependent pathway (695). AVP, PDGF, or phorbol myristate acetate (PMA), stimulated ET-1 secretion was inhibited by heparin (696). In addition, the inhibitory effect of heparin was completely abolished in PKC-depleted mesangial cells, by 24-hours pretreatment with a high
TPA concentration. Heparin also reversed protamine sulfate-dependent stimulation of synthesis of prostaglandins E2, F2α, 6-keto-PGF1α and thromboxane B2 (697) and dose-dependently poly-L-lysine-evoked PGE2 synthesis and [Ca2+]i increase (698). IL-1β and heparin selectively counter-regulate broad substrate-spectrum matrix metalloproteinase, transin (rat stromelysin-1), expression in mesangial cells (699). When mesangial cells were stimulated by IL-1β in the presence of heparin, transin (MMP) expression was markedly suppressed in a dose-dependent manner. The inhibitory effect of heparin was specific to transin, since induction of procollagen α1(IV), laminin B2 or TIMP-1 by IL-1β were not affected. Heparin also inhibits PKC-independent cell proliferation and t-PA synthesis, suggesting multiple intracellular sites of action for heparin, unrelated or distal to PKC activation (700).

(iv) ECM composition

Heparin promotes the expression of HS and increases that of CS (701). Moreover, heparin modifies the expression of decorin and biglycan, involved in adhesion, and fibrillogenesis, while not affecting perlecan. An increased matrix production observed as a shift in the balance of α1(IV)COLL, MMP-2 and TIMP-2 was found in high glucose, which was reversed by heparin supplementation (702). The new equilibrium was mostly due to the down-regulation of type IV collagen expression, rather than reduction of proteolysis.

(v) Cell adhesion and migration

Heparin inhibits PKC-dependent cell shape changes but not PKC-dependent t-PA or PAI-1 synthesis in HMC (700). It appears that heparin stimulates mesangial cell hillock formation, possibly by decreasing cell adhesion. Heparin caused a 50% inhibition of mesangial cell growth and stimulated hillock formation threefold to fourfold. Irradiated cells developed hillocks to the same extent as did nonirradiated cells, and the addition of heparin also increased hillock formation threefold to fourfold (539). Dextran sulfate and chondroitin
B sulfate had no effect on mesangial cell hillock formation. Heparin together with antithrombin III inhibited thrombin-dependent inactivation of MC single-chain urokinase plasminogen activator (scu-PA) and inhibition and reversal of cAMP-stimulated loss of adhesion and cell shape (703). In addition heparin inhibited cationic bovine gamma-globulin-induced morphological changes, and significantly reduced LDH release (704). The MC migratory response was specifically inhibited in a dose-dependent and reversible manner by heparin and heparin-like GAGs (705). Chondroitin sulfates and hyaluronic acid did not significantly inhibit MC migration. Glomerular basement membrane heparin-like GAGs may normally prevent MC extension into the pericapillary space. Accompanying inhibition of migration, elongation and proliferation were inhibited by heparin and heparin-like proteoglycans in MC seeded on fibronectin (706) suggesting to a potential regulatory role of heparin/heparan sulfates in all three processes.

(vi) Binding and uptake of immune complex and LDL

Along with attenuation of ANG II- and ET-1-dependent \[^{3}H\]thymidine incorporation, heparin was found to suppress MC uptake of IgG complexes in a concentration-related manner. ANG II- and ET-1- enhanced uptake of IgG complexes were significantly antagonized by coincubation of MC with heparin at a therapeutic concentration (1 U/ml). Heparin was also found to inhibit surface binding of IgG to MC in a concentration-related manner (55% reduction at 1 U/ml) (707).

Heparin can block the binding of immune complexes to the Fc receptor of cultured mesangial cells, but not to peritoneal macrophages (708). It was found almost to eliminate binding of radiolabelled immune complexes to cultured RMC. Pretreatment of the cells with heparin for 24 h followed by washing before the addition of immune complexes had the same effect. High-molecular-weight dextran sulphate was found to inhibit the subsequent binding of immune complexes to the mesangial cells, although the effect appeared to be smaller than with heparin. Low-molecular-weight dextran sulfate had no detectable effect. The success of
heparin pretreatment indicates that the effect is on the cells rather than on the complexes in suspension (708). They also suggested that heparin destabilizes the immune complex structure.

Heparin also inhibits the uptake of LDL by HMC (709), by inhibiting LDL-specific binding to MC (710, 711)

1.3.4. Mechanism of heparin's effects

Even though the effects of heparin on cell behavior are being studied extensively, the mechanism of its actions are still poorly understood. The literature suggests several factors conferring an antiproliferative effect.

Although there are some variations in sensitivity, cell donor age does not seem to be an important factor in determining susceptibility to heparin (653).

1.3.4.1. Do structure and anticoagulant activity contribute to heparin's antiproliferative effect?

It was suggested that the antiproliferative action of heparin is to accelerate the inactivation of thrombin at sites of platelet aggregation (643), but subsequent studies indicate this mechanism to be unlikely (651). The antiproliferative action of heparin is not related to, nor dependent upon its anticoagulant activity (103, 651, 712), nor is it via interference with platelet adhesion to endothelium (103). The anticoagulant and antiproliferative properties are mediated by separate regions of the heparin molecule. For instance, some low molecular weight heparin species which lack anticoagulant properties were shown to inhibit cell proliferation in vitro and in vivo (670, 681, 713). The antiproliferative actions require, at a minimum, 5 or 6 sugar residues which are O-sulfated (713, 714). Anticoagulant activity requires N-sulfated groups. Structure-function studies have demonstrated the importance of the 3-O-sulfate glucosamine group for heparin's antiproliferative effect (714). In contrast,
the presence of a 2-O-glucuronic acid is not essential for its antiproliferative activity (715). Chemical modifications affect heparin's biological activity. Nonantiproliferative fragments can be rendered antiproliferative by O-oversulfation and vice versa, suggesting that the degree of sulfation in heparin fragments is an important determinant of antiproliferative effect (715). O-desulfated and N-desulfated heparins lose their antiproliferative activity (716). However, when the positive charge generated by desulfation is neutralized by acetylation, the N-desulfated N-acetylated heparins partially retain their antiproliferative effect. Recent work indicates that 2-, 4- or 6-carbon N-acetylated-low molecular weight nonanticoagulant heparins also have potent antiproliferative actions (717).

1.3.4.2. Role of charge

Structurally unrelated polyanionic compounds, i.e., heparin, suramin, poly-L-aspartic acid, and poly-L-glutamic acid, strongly inhibited 10% fetal bovine serum-stimulated cell proliferation (718). On the other hand, two polycations, protamine sulfate and poly-L-lysine, were equally effective in inhibiting cell growth. The antiproliferative activity of each compound was neutralized by molecules with opposite net charge. These data indicate that both anionic and cationic macromolecules exert an antimitogenic effect on cultured HMCs. This inhibitory effect is dependent upon charge density rather than on the net charge of each compound, but charge alone is not sufficient to induce such an effect. Anionic heparin sodium inhibited cationic bovine γ-globulin (BGG)-induced morphological changes and, when coincubated with cationic BGG, significantly reduced LDH release (P < 0.0001) to levels equal to or less than those with the neutral BGG control (704). This heparin effect was lost if addition was delayed until 10 minutes after the addition of cationic BGG, indicating an irreversible effect within this time and suggesting that charge alone is not sufficient for polycations to induce LDH release.

The observation that nonheparin GAGs such as CS have little or no antiproliferative action suggests that more than simple nonspecific electrostatic interactions are involved (719).
It was also proposed (669) that exogenous heparin exerts its beneficial effect on the progression of glomerular disease by replacing lost endogenous HS, and consequently restoring the normal proliferation of glomerular cells. However heparin does not affect the normal proliferation of MC and other glomerular cells in STZ diabetic nephropathy (674). The notion that the protective effect of heparin is simply due to the restoration of electronegative glomerular charge by this polyanionic compound seems to be unrealistic (673).

1.3.4.3. Role of cell binding

The means by which heparin mediates its action could be either by direct binding to receptors on the cell, or by binding to other growth regulatory substances or ECM components, or both. Heparin decreases the incorporation of newly synthesized thrombospondin into the cell matrix in vitro, and thus interferes with thrombospondin's role in cell cycle progression (660). MC display a single class of heparin-inhibitable TSP binding sites ($B_{max} = (3.8 \pm 1.8) \times 10^6$/cell, $K_d = 80 \pm 29$ nM). Based on these observations, the existence of an autocrine positive feedback loop of MC proliferation involving TSP and growth factors, regulated by heparan sulfate is possible (691). There are also heparin binding sites on fibronectin, laminin, and type IV collagen (720-723) which could be involved in regulation of proliferation. Heparin, bound to these matrix molecules, could localize growth factors in the region of mesangial cells. This was shown to be the case for the action of bFGF on bovine endothelial cells and baby hamster kidney cells (724, 725). Once bFGF is fixed to heparin or HS, it is protected from proteolytic degradation (724-726). It may thus serve as a reservoir for growth factors.

Specific binding sites ($K_d = 10^{-9}$ M; 100,000 sites/cell) for heparin were demonstrated on normal SMC and RMC (637, 719), and quiescent cells have a greater number of these sites than growing cells, which may explain the greater sensitivity of the former to the action of heparin. SMC from spontaneously hypertensive rats have a reduced number of heparin
receptors but retain the same receptor affinity and rate of heparin internalization as normal Wistar-Kyoto rats (727). Additionally, in SMC from both strains of rat, pretreatment with heparin reduced EGF binding to a similar extent. Thus, the enhanced proliferation observed in spontaneously hypertensive rat (SHR) SMCs (728) is attributable to a lower number of heparin receptors and hence a reduced response to the antiproliferative action of heparin. A lower degree of sulfation in the heparan sulfate chains produced by SHR SMC may also contribute to the higher proliferation rates observed for these cells compared with cells from normal animals.

Heparin improves diabetes related glomerular sulfate incorporation (674), and reduces the abnormal expression of the α1(I)collagen gene and deposition of corresponding molecule (674, 679). It is not clear at the moment whether heparin downregulates overexpression of collagen by interfering with autocrine/paracrine loops (possibly the TGF-β one) that are known to be activated in the diabetic kidney and in other experimental models. However, although heparin was reported to have cytokine-unrelated cellular activities, recent observations support the possibility that heparin may also downregulate renal TGF-β1 gene overexpression observed in STZ diabetic rats (729). Furthermore the inhibitory effect of heparin on bovine SMC in culture was shown to be dependent upon the presence of TGF-β. TGF-β is bound to α2 macroglobulin in a biologically inactive form in serum (730). Based on the electrophoretic mobility of the TGF-β/α2-macroglobulin complex in the presence or absence of heparin, it has been postulated that heparin frees TGF-β from its binding site on α2-macroglobulin and forms a new stable complex in which TGF-β becomes biologically active and could therefore, regulate cell proliferation and matrix synthesis (731).

Heparin may work by decreasing the binding of growth factors, which leads to a decreased stimulus for growth. It decreases the binding of EGF by decreasing the number of receptors on the cell surface (655). Heparin also affects binding of PDGF. It binds to the growth factor and interferes with binding to its receptor (657).
1.3.4.4. Role of internalization of heparin

Heparin has been shown to bind to the cell membrane and it can be internalized (637, 719). The heparin internalization and metabolizing pathways in both EC and SMC were characterized and the process appears to be slower in the latter cell type (732). After endocytosis by aortic SMC, labeled heparin is cleaved into low molecular weight fragments which retain the capacity to inhibit proliferation in vitro (733). Although these cells internalize and degrade heparin, upregulation of heparin binding to the SMC surface is required for an antiproliferative response, indicating an extracellular action of heparin (733). Furthermore, the binding to the cell surface is decreased but uptake of heparin is not affected in cells selected for resistance to heparin by growth in heparin containing medium (733). In addition, the antiproliferative effect of 10 μg/ml of HS is reduced by 50% by as little as 1 μg/ml of dextran sulfate, while dextran sulfate has little effect on the uptake of HS (734). The prolonged course of uptake of 125I-heparin from low extracellular concentrations suggests that at least the surface-bound component of heparin’s effect at 15 min is not dependent on internalization (637). 125I-heparin binds specifically to a single class of high affinity sites on the cell surface. Labeled heparin is taken up by cells and degraded with a half-time around two hours (637, 733). In addition, in cells selected for resistance to heparin by growth in heparin-containing medium, uptake of heparin is not affected, while the binding of heparin to the cell surface is decreased (733).

1.3.4.5. Mechanism of heparin’s effect on cell cycle and oncogene regulation

The inhibitory action of heparin is also at the level of the control of the cell cycle. The addition of heparin to cultures of bovine SMC resulted in a significant decrease in the expression of a mid-G1 phase protooncogenes (658). Heparin had to be added at least 4 h before the beginning of the S phase to efficiently prevent progression to DNA synthesis (654). Furthermore, after 48 h exposure to heparin the SMC reverted to G0 phase, raising the possibility that prolonged heparin exposure caused cells to return to quiescence. Thus
heparin may exert its antiproliferative action at multiple points of the cell signal transduction pathways.

The principal action of heparin appears to be on gene expression, including the inhibition of thymidine and uridine uptake required for DNA and RNA synthesis (735). Protooncogenes are regulated by several signal transduction pathways elicited by external stimuli. Receptor activation by ligand binding causes a transient activation of several kinases including PKC, MAPK, CaMK, PKA, and CREB kinase, which in turn translocate to the nucleus and regulate oncogene transcription after phosphorylation of transcription factors.

Heparin has a negative effect on histone H3 mRNA expression (a specific S phase cell cycle marker) and also inhibits expression of transcriptional proteins such as c-myb and 2F1-mitochondrial adenosine diphosphate or ATP carrier protein. This inhibition prevents proliferation by interfering with the mid-to late G1 phase of growth and preventing entry into S phase in SMC (654, 658, 717) and fibroblasts (736).

The effect of heparin on c-fos induction is independent of interaction with cytokines or cytokine receptors because the magnitude of its proliferative effect is not diminished when heparin-binding substances are removed from serum by heparin-Sepharose (637). The growth inhibitory effect of heparin on rat cervical epithelial cells cannot be explained by the inactivation of mitogens present in serum (737). Heparin treatment of these cells does not result in a reduction in the binding of EGF to the cells. The effect of heparin is not due to interference with the binding of growth factors in serum or the uptake of phorbol esters by the cells, since the addition of heparin can be delayed by 2-4 h. Direct evidence for a role of heparin independent of growth factors is provided by experiments with 12-O-tetradecanoylphorbol-13-acetate (TPA). Under serum-free conditions direct activation of PKC with phorbol ester leads to an induction of c-fos that is inhibited by heparin (637).

Since heparin suppresses the transcription of two different classes of proteases, it might
affect a common regulatory element such as TRE, the binding site for trans-acting factor AP-1 (738), present in the genes of t-PA and collagenase. Heparin suppresses TPA-induced CAT expression from the collagenase promoter containing TRE (663). Even though the entry of heparin into nucleus was implicated in the inhibition of binding of AP-1 to the response elements in both SMC (663), and HeLa cells (739), and HS has been detected in nuclear preparations (740), the intranuclear mechanism of heparin and HS actions was ruled out by studies from Hasca1's group. They demonstrated the difficulty in isolating pure nuclei and pointed out to misleading conclusions by previous methods (740). They showed the possible presence of dermatan sulfate but not HS in the nuclei of rat ovarian granulosa cells (741).

Heparin may suppress the activity of the Jun and Fos proteins by affecting their phosphorylation. Dephosphorylation of c-Jun at specific sites increases AP-1 binding activity (742). Heparin was shown to inhibit IP4-phosphate kinase activity in vitro (743). It may suppress AP-1 binding by decreasing one or more of the AP-1 proteins. Heparin may decrease AP-1 binding by inducing an inhibitor. Inhibitor protein 1 (IP-1) inhibits binding of AP-1 to TRE and is deactivated by dephosphorylation (744).

1.3.4.6. Effect of heparin on signal transduction pathways

MAPK is involved in activation of transcription of c-myc, c-jun and c-fos (745, 746, 747). Activation of MAPK by serum or TPA is inhibited by heparin (748). Heparin does not act directly on PKC since it does not diminish the TPA-dependent enzyme activity (736). Thus the effect of heparin involves activation of the PKC pathway, but the block is distal to the activation of PKC. Furthermore, heparin inhibits PKC-dependent c-fos induction in the absence of serum by 65% (637). While heparin inhibited serum-dependent c-fos induction, serum, heparin or combination of serum and heparin had no effect on the levels of PKC activity observed in quiescent cells (749). Phorbol ester caused an increase in the proportion of histone H1-active PKC associated with the cell membrane fraction, from approximately
25% to 70% of total activity. Heparin affected neither the total activity of the kinase nor the proportion associated with the membrane. When PKC was inhibited with staurosporine, only very low levels of c-fos were induced by serum (637). However, caution should be exercised in interpreting the effect of heparin on PKC activity because of weak specificity of staurosporine. Recently it was reported that heparin blocks SMC proliferation through PKC-α isoenzyme (750). Heparin's antiproliferative effect on SMC in vivo and in vitro was lost after preincubation of cells with a 20-mer phosphorothioate PKC-α antisense oligonucleotide.

Heparin was shown to have multiple targets in signal transduction pathways. For instance, G-proteins were implicated as a part of heparin's action. The antiproliferative effect of heparin is attenuated by the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester and by the guanylate cyclase inhibitor methylene blue, suggesting that a nitric oxide-cGMP-dependent mechanism may account for the antiproliferative effect of heparin (707). Heparin is also an effective inhibitor of intracellular inositol-1,4,5-phosphate receptors, but sufficient heparin to achieve this effect does not enter the cell unless aided by permeabilization or microinjection (751, 752), strongly suggesting that heparin internalization is not sufficient for its effects on intact cells. Heparin interference in the formation of IP₃ and Ca²⁺ mobilization may account for the inhibition of Na⁺-H⁺ exchange observed in SMC (753). Furthermore, heparin binds with high affinity to the α₁-subunit of the L-type Ca²⁺ channel (754) and blocks uptake of ⁴⁵Ca²⁺ through this channel at an extracellular site (755).

Apart from the antiproliferative mechanisms already outlined, heparin (and dextran sulfate) also has a protective effect on vascular cells by scavenging oxygen-derived radicals from cellular metabolism (756, 757). In general, HS and DS are less potent scavengers than heparin and dextran sulfate (757). This beneficial effect is unlikely to be via a stimulation of superoxide dismutase (SOD) because both heparin and dextran sulfate are reported to inhibit extracellular human SOD-C activity (758).
1.3.4.7. Mechanism of effect heparin on cell migration

Proteases are involved in cell growth and migration (759, 760). The major plasminogen activators secreted by cells are t-PA and u-PA. Their role is to convert plasminogen into plasmin. Plasmin is a trypsin-like enzyme, which degrades a wide range of noncollagenous matrix proteins. t-PA and interstitial collagenase expression are increased in the G1 phase of the cell cycle, whereas u-PA expression is decreased. Heparin suppresses the expression of both t-PA and collagenase and has little effect on u-PA (662, 663). This effect is specific to heparin-like GAGs and does not depend on anticoagulant activity.
1.4. HYPOTHESIS AND OBJECTIVES

An expanded mesangium and decreased glomerular filtration rate are major characteristics of glomerulosclerosis (5, 17). Two factors that contribute to such expansion are hypercellularity due to hyperproliferation of MCs, and accumulation of ECM proteins due to overexpression of TGF-β and protease inhibitors (5). MCs in culture possess a similar proliferative phenotype. They are dedifferentiated into myofibroblasts with characteristics of both smooth muscle cells (production of α-actin) and fibroblasts (production of collagen type I) (5). Besides other components, accumulated ECM contains HS proteoglycans (513, 531) and collagen type I not present in a normal ECM (639, 761). Both components suppress MC proliferation in culture (593, 636, 685, 763), consistent with the idea that ECM is a key factor in maintaining the MC phenotype (763). MCs in culture also secrete a significant proportion of their proteoglycans into the medium (531), and there may act as autocrine factors to suppress cell proliferation (636). Heparin, a GAG structurally related to HS (764), may suppress progression of arteriosclerosis (643) and of glomerulonephritis (668) in part by suppressing proliferation of SMC (651) and MC (636, 637), respectively. The antiproliferative effect of heparin correlates with its binding to a high affinity site (637) and is not dependent on anticoagulant activity (103), internalization (733), or binding to growth factors or their receptors (637, 765, 766). We hypothesize that the ECM components collagen type I (part of pathological mesangial matrix) and HS (a heparin-related GAG), can potentially regulate MC phenotype by affecting intracellular signaling pathways via specific receptors.

To test our hypothesis experiments were performed to compare proliferation and contraction of MCs grown on collagen type I gel, or grown on plastic in growth medium supplemented with heparin, with MCs grown on plastic culture dishes in heparin-free medium. Growth on plastic served as a model for the proliferative MC phenotype. To study the effect on growth stimulation, cells were rendered quiescent and tested for mitogenic
response, c-fos induction, and activation of kinases. To study the effect on contraction, quiescent cells were tested for Ca\textsuperscript{2+} signaling, a change in cross-sectional area, and the level of myosin light chain phosphorylation.
2. MATERIALS AND METHODS

2.1. REAGENTS AND MATERIALS

Myelin basic protein (MBP) was generous gift from Dr. Mario Moscarello (Hospital for Sick Children, Toronto). [H]thymidine (6.7 Ci/m mole) was from ICN (Mississauga, Ont.). [α-32P]dCTP (3000 Ci/m mole) and [γ-32P]ATP (45 Ci/m mole) were from Du Pont Canada (Mississauga, Ont.). [32P] orthophosphate (carrier-free, HCl-free) was from Amersham (Oakville, Ont.). Aclar plastic coverslips were from Proplastic Inc. (Linden, NJ). Enlightning™ autoradiography enhancer was from New England Nuclear (Boston, MA). Vitrogen-100 was from Celtrix Laboratories (Santa Clara, CA). Non-muscle anti-myosin polyclonal antibody was from Biomedical Technologies, (Stoughton, MA). Polyclonal rabbit anti-Erk-2, and rabbit anti-mouse FAK antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal mouse anti-phosphotyrosine antibody PY20 was from Transduction Laboratories, (Lexington, KY). A rat c-fos cDNA was cloned by T. Curran (455) and obtained from T. Cruz (University of Toronto). A cDNA for mouse 18s rRNA was obtained from J. Koropatnick (University of Western Ontario). ECL-Western blotting system was from Amersham Canada (Oakville, Ont.). Chromatography column packings such as Sephacryl S-300 HR, and Sephadex G-25 and G-50 were from Pharmacia LKB (Uppsala, Sweden). Other reagents used in this study and materials and their suppliers are listed in Table 2.

2.2. RMC CULTURE

2.2.1. Establishment of RMC culture

RMC cultures were established in our lab from glomeruli of 100 g male Wistar rats (767). Glomeruli were isolated from the renal cortices by sieving through graded stainless steel sieves, following the procedure of Simonson and Dunn (768). Glomeruli were cultured in RPMI 1640 medium in a humidified CO₂ atmosphere, containing 20% calf serum, penicillin
Table 2. List of reagents and materials.

<table>
<thead>
<tr>
<th>Reagent/Component</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose (g)</td>
<td>Marigel (EHS tumor basement membrane)(c)</td>
</tr>
<tr>
<td>Acrylamide (sc)</td>
<td>2-Mercaptoethanol (b)</td>
</tr>
<tr>
<td>Adenosine 5'-triphosphate (ATP) (s)</td>
<td>ML-7 (f)</td>
</tr>
<tr>
<td>Albumin (bovine serum, BSA) (s)</td>
<td>3-[N-Morpholino]propanesulfonic acid (MOPS)(s)</td>
</tr>
<tr>
<td>Amphotoline (pH 4-6, pH 3.5-10) (p)</td>
<td>3-[4,5-Dimethylthiazol-2-yl]2,5-/</td>
</tr>
<tr>
<td>Autocamidine-2 (b)</td>
<td>diphenyltetrazolium bromide (MTT) (s)</td>
</tr>
<tr>
<td>Benzamidine-HCl (s)</td>
<td>N,N'-methylene-bis-acrylamide (r)</td>
</tr>
<tr>
<td>Bromophenol blue (f)</td>
<td>N-ethylmaleimide (s)</td>
</tr>
<tr>
<td>Calmodulin (f)</td>
<td>N-lauroylsarcosine (s)</td>
</tr>
<tr>
<td>CNBr (f)</td>
<td>Na3VO4 (s)</td>
</tr>
<tr>
<td>Collagenase A (m)</td>
<td>NaF (f)</td>
</tr>
<tr>
<td>Chondroitin sulfate A and C (s)</td>
<td>Nonidet P-40 (s)</td>
</tr>
<tr>
<td>Chondroitin sulfate C (shark cartilage)</td>
<td>NuSerum™ IV (c)</td>
</tr>
<tr>
<td>Dextran sulfate (average Mr, 500 kDa) (s)</td>
<td>Octyl glycoside (s)</td>
</tr>
<tr>
<td>Diethyl pyrocarbonate (DEPC) (s)</td>
<td>PDM9059 (s)</td>
</tr>
<tr>
<td>Dimethylthielenblue (DMMB) (b)</td>
<td>Penicillin-streptomycin (g)</td>
</tr>
<tr>
<td>DMEM (g)</td>
<td>Pepstatin A (s)</td>
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<tr>
<td>EDTA (s)</td>
<td>Phenylmethylsulfonfyl fluoride (PMSF) (s)</td>
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<tr>
<td>EGTA (s)</td>
<td>Pluronic F-127 (s)</td>
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<tr>
<td>Endothelin-1 (ET-1) (s)</td>
<td>Polyvinylpyrolidone (s)</td>
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<td>Ethidium bromide (s)</td>
<td>Protein A immobilized on Sepharose 4B (p)</td>
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<tr>
<td>FBS (g)</td>
<td>Random primer DNA labeling kit (m)</td>
</tr>
<tr>
<td>Ficoll (s)</td>
<td>RPMI 1640 (g)</td>
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<tr>
<td>Formaldehyde (b)</td>
<td>Salmon sperm DNA (s)</td>
</tr>
<tr>
<td>Formamidine (b)</td>
<td>SDS (i)</td>
</tr>
<tr>
<td>Fura 2-AM (s)</td>
<td>Sodium deoxycholate (f)</td>
</tr>
<tr>
<td>Glycerol (f)</td>
<td>Sodium pyrophosphate (s)</td>
</tr>
<tr>
<td>Heparin (s)</td>
<td>Sodium nitrite (b)</td>
</tr>
<tr>
<td>N-2-Hydroxyethylpiperazine-N-2/-ethanesulfonic acid (HEPES) (s)</td>
<td>12-O-Tetradecanoyl phorbol 13-acetate (TPA)</td>
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<tr>
<td>Hoechst 33258 dye (s)</td>
<td>Tricloroacetic acid (TCA) (g)</td>
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<tr>
<td>Hybond-N nylon membrane (a)</td>
<td>Trifluoroethane sulfonic acid (TFMS) (s)</td>
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<td>Hydrogen peroxide (g)</td>
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<td>Triton X-100 (s)</td>
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<tr>
<td>Chemical Name</td>
<td>Supplier</td>
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<td>------------------------------------</td>
</tr>
<tr>
<td>Hydroquinone (s)</td>
<td>Trizol® (g)</td>
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<tr>
<td>Ionomycin (s)</td>
<td>Tween 20 (s)</td>
</tr>
<tr>
<td>KN-93 (l)</td>
<td>Urea (ultra-pure) (l)</td>
</tr>
<tr>
<td>KT-5926 (l)</td>
<td>Verapamil-HCl (s)</td>
</tr>
<tr>
<td>L-N-monomethyl arginine (L-NMMA) (s)</td>
<td>Xylene cyanole FF (j)Leupeptin (s)</td>
</tr>
</tbody>
</table>

(a) Amersham Canada (Oakville, Ont.); (b) BDH (Toronto, Ont.); (bbi) Bachem Bioscience Inc., (c) Collaborative Biomedical Products (Bedford, MA); (f) Fisher Scientific (Fair Lawn, NJ); (g) Gibco/BRL (Burlington, Ont.); (h) Serva (Heidelberg); (i) ICN; (j) Calbiochem, (La Jolla, CA); (m) Boehringer Mannheim Canada; (p) Pharmacia LKB, (Uppsala, Sweden); (q) Mallinckrodt Canada Inc. (Point-Claire, Que); (r) Bio-Rud; (rd) R&D System (Minneapolis, MN); (s) Sigma Chemical Company (St.Louis, MO), (sc) Sangon Co., (Beaconsfield Que).
G (100 IU/ml), and streptomycin (100 μg/ml). Amphotericin B (0.25 μg/ml) was included for the first week. MCs were subcultured by trypsinization at 28 days and subsequently maintained by weekly passage in RPMI 1640 medium with penicillin, streptomycin, 10% serum, insulin (5 μg/ml), transferrin (5 μg/ml), and sodium selenite (5 ng/ml). The cells were characterized as mesangial cells by their morphology, function, and cytoskeletal antigens (636). Their identity was confirmed by their appearance, visible contractility in response to ANG II and ET-1, absence ouabain-sensitive 86Rb uptake, a profile of proteoglycan synthesis distinct from other glomerular cell types, and growth-suppressive responsive to heparin (653). Light microscopy revealed large, stellate or spindle shaped cells with many irregular cytoplasmic extensions. Immunocytochemical analysis showed positive staining for desmin and smooth muscle actin, characteristic of mesangial cells (769). RMC were used between passages 5 and 15. They were cultured in RPMI 1640 containing 10% FBS and passaged at confluence by trypsinization for 5 min with a solution of 0.025% trypsin-0.5 mM EDTA.

2.2.2. Storage of RMC

Sub-confluent RMC at passage 4-5 in 75-cm² plates were trypsinized and centrifuged at 200 x g for 3 min. The pellets were resuspended in 2 ml RPMI 1640 containing 20% (v/v) FBS and 10% (v/v) dimethylsulfoxide (DMSO) and then aliquoted into 1.8-ml Costar Biofreeze vials (Costar Plastics). After 1 h at -20°C and 1 h at -70°C, the vials were transferred to liquid nitrogen for long term storage. RMC were shown to be stable for at least 1 year when stored frozen in liquid nitrogen (768). To thaw frozen cells, a vial was transferred to a 37°C water bath and the contents were thawed rapidly (< 2 min) while mixing. The suspension was diluted with 10 ml RPMI 1640 containing 20% FBS and seeded into a 25 cm² flask. When cells reach confluence, they were passaged by trypsinization.
2.2.3. Growth on different substrata

2.2.3.1. Collagen type I

Collagen type I was purchased as Vitrogen-100 containing 2.5-3.0 mg/ml in 0.12 M HCl. Collagen coated dishes were prepared as described earlier (768). Briefly, after mixing 9 parts of Vitrogen solution and 1 part of 10 X RPMI 1640 on ice, the solution was adjusted to pH 7.4 with 1M NaOH. While still liquid, the collagen mixture was pipetted into plates (0.25 ml/well in 24-well plates, 0.5 ml/well in 12-well plates, 1.0 ml/well in 6-well plates, or 4 ml in 10 cm petri dishes), and allowed to polymerize by placing it in a humidified incubator for 60 - 120 min at 37°C. The polymerized collagen type I, prepared this way, is referred to as a collagen gel throughout this study because of its gel-like coat on the plate, after polymerization (768).

2.2.3.2. Solubilization of collagen type I with CNBr

Collagen was solubilized according to O'Driscoll et al. (770), with minor modifications. Aliquots (0.5 ml) of collagen solution (about 300 mg/ml) were freeze-dried, dissolved in 70% (v/v) deoxygenated formic acid, and mixed 1:1 (v/v) with CNBr (60 mg/ml in formic acid). After the mixture was deaerated by bubbling with nitrogen for 2 min, the tubes were capped, agitated for 60 min, and left to stand in the dark at room temperature for 3 h. At the end of this time, 1.5 ml of dH2O were added, and the solution was desalted by passing twice through a Sephadex G-25 column and freeze-dried. Gel filtration of the CNBr reaction mixture was carried out on a 0.9 X 110-cm column of Sephacryl S-300 HR, equilibrated and eluted with 2.0 M guanidine-HCl containing 0.05 M tris, pH 7.5, at 15-20 ml/h. Blue dextran and phenol red were used to mark the column void (volume between the particles, Vd) and total (volume within support particles, Vt) volumes, respectively. Samples were dissolved in 100 μl dH2O with 1 μl 2-mercaptoethanol prior to loading. The elution profile was monitored by absorbance at 280 nm and by sodium dodecyl sulfate-polyacrylamide gel
electrophoresis (SDS-PAGE). Fractions in the mass range from 10 to 60 kDa were desalted twice on Sephadex G-25, freeze dried, and used for further testing.

2.2.3.3. **Matrigel**

Matrigel basement membrane matrix is a solubilized basement membrane preparation extracted from the Engelberth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major component is laminin, followed by collagen type IV, HSPGs, and nidogen (771). It also contains TGF-β, FGF, tissue-type plasminogen activator, and other growth factors which occur naturally in the EHS tumor. Matrigel was thawed in ice-cold conditions, added to six-well plates (1 ml/well), and allowed to gel for 60 -120 min at 37°C in a humidified CO₂ atmosphere.

2.2.3.4. **Mesangial matrix**

Mesangial matrix-coated plates were prepared by the method of Crowley et al. (772). Cells were grown for 2 weeks in tissue culture flasks with media changed twice weekly. After removal of media, the cell layer was dissociated from the matrix by 15-min exposure to 2.5 mM NH₄OH and 0.1% Triton X-100, followed by washing with PBS. This leaves insoluble matrix behind in the tissue-culture flask (773).

Prior to cell passage all substrata were washed briefly 3 X with PBS and once with RPMI 1640.

2.2.4. **Determination of cell number**

Cells were counted using a particle counter (model 0013, Coulter Electronics, Hialeah, FL). RMC were released from plastic and mesangial matrix by trypsinization, from collagen type I by exposure to collagenase A (5 mg/ml) for 5-30 min or from Matrigel by exposing to Dispase for 30 min (50 caseinolytic units/ml; Collaborative Research, Bedford, MA).
2.2.5. DNA content

DNA content was measured by using a DNA-specific bisbenzimidazole dye (Hoechst 33258) at 1 µg/ml in phosphate-saline buffer (2.0 M NaCl in 0.05 M sodium phosphate, pH 7.4) (774). Cell homogenates were sonicated and diluted 1:10 in the dye solution. Fluorescence was measured in an Aminco Model SPF-500 fluorescence spectrophotometer with excitation at 350 nm and emission at 455 nm. A standard curve was constructed with thymus DNA.

2.2.6. Cell attachment assay

After releasing RMC from culture flask by trypsinization, cells were resuspended in Dulbecco's modified Eagle medium (DMEM) containing BSA (1 mg/ml) and plated in 12-well plates (4 X 10^5 cells per plate) that were either noncoated or coated with collagen type I or mesangial matrix. The cells were allowed to attach for 60 min, then unattached cells were removed, and attached cells were released by trypsin/EDTA or by collagenase. Cell attachment was measured both by direct counting of attached and unattached cells with a Coulter counter or by scintillation counting of radiolabeled cells as described by Grinnell and Feld (775). For the later procedure cells were prelabeled with [3H]thymidine for 36-48 h prior to reaching 90% confluence, whereupon they were grown for a further 24 h without label and plated onto plastic, collagen type I or mesangial matrix. Percent attachment was calculated as 100 X [attached(cpm)/attached(cpm) + unattached(cpm)].

2.2.7. Cell viability studies

Cell viability was assessed by the tetrazolium salt (MTT) method as well as Trypan blue dye exclusion. The MTT assay tests the metabolic ability of cells to reduce the tetrazolium salt by mitochondrial succinate dehydrogenase to a formazan dye which is soluble in DMSO. The conversion takes place only in living cells and is a marker of mitochondrial function. Trypan blue dye exclusion assay tests viability by examining membrane integrity. Trypan
blue exclusion is an indicator of plasma membrane integrity and was used as an independent assessment of viability.

2.2.7.1. **MTT assay**

This assay was modified from previously described method (776). Cells were cultured on noncoated or collagen type I-coated 24-well plastic plates in a concentration of $10^5$ cells/well and allowed to grow overnight in RPMI-1640 and 10% FBS. The following day medium was replaced with RPMI-1640 containing 0.4% FBS and starved for 48 h. Cells were then washed with RPMI-1640 and incubated with MTT in RPMI at a concentration of 1 mg/ml for 1 h at 37 °C. After washing the medium, cells were treated with 2 ml DMSO for 30 min and lysates were measured for optical density (OD) at a wavelength of 570 nm. The results were expressed as a percentage of OD of control cells.

2.2.7.2. **Trypan blue assay**

Cells were prepared in a similar fashion to those described above. The cells were detached from the substratum they were grown on, removed from the plate, stained with Trypan blue in a concentration of 1 mg/ml, and counted in a hemocytometer using a phase contrast microscope.

2.2.8. **Mitogenic assay**

To render RMC quiescent, cultures grown on plastic or mesangial matrix- or collagen type I-coated plates at approximately 60-70% confluence were washed with RPMI 1640 medium and placed in fresh medium containing 0.4% FBS for 48 h. A mitogenic response was induced for different times with 5% NuSerum, 100 nM ET-1, or 100 nM ionomycin and thymidine incorporation was measured by pulse-labeling cells in 12- or 24-well plates with 2 μCi of $[^3H]$thymidine in 1 ml of serum-free medium for 45 min. After the exposure to $[^3H]$thymidine cells grown on plastic or mesangial matrix were washed three times with 5%
TCA at 0°C, dissolved with 0.1 M NaOH, and counted for radioactivity according to Jaffer et al. (563). Cells cultured on collagen type I were treated with collagenase until detached and then scraped from the well, collected on GF/B Whatman glass microfiber filters (Whatman, Maidstone, UK), and washed three times with ice-cold 5% TCA and once with methanol. The filters were placed in scintillation vials for counting.

2.3. CONTRACTILITY MEASUREMENT

RMC were passaged on plastic, mesangial matrix, or collagen type I. After 24 h culture medium was replaced with Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution (HBSS), buffered 10 mM HEPES, pH 7.4, containing 10% FBS. Eighteen hours later, medium was changed to serum-free, Ca²⁺- and Mg²⁺-replete HBSS containing either 2.5 μM ANG II, 0.1 μM ET-1, 20 nM AVP, 2 mM L-NMMA, 10 μM KT-5926, or heparin (1 μg/ml). Plates were placed on the thermostated stage of a phase-contrast microscope, inside a humidified plexiglass incubation chamber built around the stage, with monitoring of temperature and atmospheric CO₂ content. Images from a charge-coupled device video camera (Sony, XC-77) mounted on the microscope, were captured with RosterOps Image Capture hardware at intervals after addition of agonist and stored on a Macintosh Quadra 700 computer for analysis using NIH Image 1.4 image analysis software. Changes in cell shape were quantitated in two ways: 1) by calculating the percentage of cells that showed a significant response and 2) by measuring the maximum change in cell size as a cell cross-sectional area (CSA) for each cell on the video image. A cell was scored positive for contraction if the decrease in CSA was >7%, as described by Simonson and Dunn (777).

2.4. CYTOSOLIC Ca²⁺ MEASUREMENT

Ca²⁺ measurements were performed by using fura 2 dye, whose fluorescence shifts wavelengths upon binding Ca²⁺. Therefore the ratio of dye fluorescence intensities at two excitation wavelengths is sufficient to calculate [Ca²⁺], independent of total dye
concentration, path length, or absolute sensitivity of the instrument (778). During course of experiments some cells were loaded with BAPTA, after exposure to fura 2/AM. According to report by Tymianski et al. (779), neither the intensity nor wavelength of the excitation maximum of fura 2 was affected by BAPTA, so fura 2 fluorescence can be used to monitor \([\text{Ca}^{2+}]_{\text{i}}\) in the presence of BAPTA without recalibration. Intracellular \(\text{Ca}^{2+}\) measurement was performed as previously described (780). RMC were grown to confluence on Aclar coverslips with or without mesangial matrix or collagen type I coating and loaded with fura 2/AM (2 \(\mu\)M) for 40 min at 37°C in DMEM with 1 mg/ml BSA and 1 \(\mu\)l/ml Pluronic F-127. The coverslips were mounted in a chamber containing 1.5 ml of MKHH with 0.8 mM MgSO4 and 0.1% BSA and placed in the sample compartment of a Nikon Diaphot-TMD inverted microscope at 37°C. Test agents were introduced with a rapid infusion set (21G X 3/4 in; Terumo, Tokyo, Japan). Fluorescence intensity (F) at 500 was measured by alternating excitation at 340 and 380 nm (SPEX DM3000 spectrofluorometer). For calibration, maximum fluorescence (\(F_{\text{max}}\)) was determined by the addition of 2.5 mM \(\text{Ca}^{2+}\) and 25 \(\mu\)M ionomycin at the end of each experiment. Auto-fluorescence (\(F_{\text{auto}}\)) was analyzed by adding 8 mM Mn\(^{2+}\) to quench fura 2 fluorescence and was subtracted from all signals. The unquenched fluorescence in the presence of EGTA (\(F_{\text{min}}\)) is a constant fraction of the corrected maximum signal (\(F_{\text{max}} - F_{\text{auto}}\)) when the dye is saturated with \(\text{Ca}^{2+}\) and was found to be 0.07 \(\times (F_{\text{max}} - F_{\text{auto}})\). Cytosolic \(\text{Ca}^{2+}\) concentration was calculated as \([\text{Ca}^{2+}]_{\text{i}} = K_d(F - F_{\text{min}})/(F_{\text{max}} - F)\), where \(F = F - F_{\text{auto}}\) and the dissociation constant \(K_d\) for fura 2 is 224 nM (778).

### 2.5. KINASE ASSAYS

#### 2.5.1 Myosin light chain phosphorylation assay

Subconfluent cultures of RMC grown on 100-mm Corning tissue culture dishes were preincubated for 3 h at 37°C in modified Krebs-Henseleit/HEPES solution [MKHH (in mM)
NaCl, 5 KCl, 1.5 CaCl₂, 1.2 MgSO₄, 5.5 D-glucose, 20 mM HEPES (pH 7.4)]
containing 100 μCi/ml of carrier-free, HCl-free [³²P]orthophosphate before the addition of agonist. Cells were treated with ET-1 (0.1 μM) for 2, 5, 10, 20, and 40 min in the [³²P]-
containing MKHH. In some experiments heparin (1 μg/ml), or 10 μM KT-5926 were
included to inhibit MLCK-dependent MLC phosphorylation. Control cells were kept in [³²P]-
containing MKHH without addition of ET-1. The extraction of myosin complex was
achieved either by pyrophosphate-PAGE or by immunoprecipitation.

Extraction of myosin complex by pyrophosphate-PAGE was done as described earlier
(781, 782) with minor changes. Following the removal of the incubation medium, the ice-
cold extraction buffer consisting of 100 mM sodium pyrophosphate (pH 8.8), 5 mM EGTA,
1 mM EDTA, 50 mM NaF, 3 mM dithiothreitol, 10% glycerol, 1 mM PMSF, 0.1 mM
leupeptin, 5 mM benzamidine, and 1 μg/ml of aprotinin was added, cells were scraped and
cell homogenates were centrifuged at 7000 x g for 15 min. KI (final 0.6 M) was added to the
collected supernatant from a 4 M stock, followed by 20 min incubation on ice, to prevent any
myosin from remaining at the top of the pyrophosphate-polyacrylamide gel (781). This
solution was made 20% in a saturated sucrose solution and samples were electrophoresed on
the pyrophosphate-polyacrylamide gel. Pyrophosphate-polyacrylamide mini slab gels
contained 20 mM sodium pyrophosphate which separates native myosin complex and inhibits
P-light chain phosphatases (pH 8.3), 10% glycerol, 5 mM ATP, 100 mM NaF, 5 mM
EDTA, 4% acrylamide and 0.2% bisacrylamide. The running buffer contained 25 mM Tris
(pH 8.3), 190 mM glycine, 5 mM ATP, 50 mM NaF and 10% glycerol. After loading
samples together with myosin standard from rabbit skeletal muscle, electrophoresis was
performed in a cold environment at 120 V for 2-4 h. To visualize protein bands gels were
stained with 0.1% Coomassie Blue (R-250) in destaining solution (50% methanol and 10%
acetic acid). Protein bands that comigrated with myosin standard bands were excised from
the gel and incubated for 2 x 15 min with 2 changes of 20-30 volumes of a solution
containing 42 mM Tris-HCl (pH 6.8) and 8 M urea. The gel slices were then minced and
incubated for 30 min in a minimal volume of the isoelectric focusing buffer containing 8 M urea, 2% Ampholine (1.6%, pH 4-6, 0.4% pH 3.5-10), 15 mM 2-mercaptoethanol, 5 mM dithiothreitol, 2% (w/v) Nonidet P-40, and 0.01% bromophenol blue.

Immunoprecipitation of labeled myosin was achieved with non-muscle anti-myosin polyclonal antibody following the procedure of Ludowyke et al. (783, 784). Medium was removed and without rinsing, the cells were scraped with two washes in 100 µl each of ice-cold immunoprecipitation buffer 25 mM Tris-HCl (pH 8.1), 1% (w/v) Nonidet P-40, 100 mM sodium pyrophosphate, 100 mM NaF, 250 mM NaCl, 10 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 10 µg/ml leupeptin, 10⁻³ mM aprotinin, and 10⁻³ M pepstatin. Samples were centrifuged for 12 min at 10,000 x g, 4°C, and the supernatant was incubated for 2 h at 4°C with 20 µl of antibody. The mixture was then incubated 100 µl of 50% slurry of protein A immobilized on Sepharose 4B for 1 h at 4°C. The beads were washed twice with 750 µl ice-cold immunoprecipitation buffer and once with 750 µl ice-cold water. Immunoadsorbed proteins were then solubilized in sample buffer (63 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.2% 2-mercaptoethanol, and 0.005% bromophenol blue) or solubilized for 1 h at room temperature in 100 µl of a buffer containing 8 M urea, 2% Ampholine (1.6%, pH 4-6, 0.4% pH 3.5-10), 15 mM 2-mercaptoethanol, 5 mM dithiothreitol, 2% (w/v) Nonidet P-40, and 0.01% bromophenol blue, and subjected to SDS-PAGE (785) or isoelectric focusing, respectively.

Isoelectric focusing on slab polyacrylamide gels with a pH gradient of 4-7 was used to separate the phosphorylated forms of MLC (782, 786). Minced samples, or solubilized immunoprecipitates, were loaded to the prefocused (20-30 min at 200 V) mini slab gels, consisting of 6.25% bisacrylamide in 8 M urea, 1% Triton X-100, and 2% Ampholine (1.6%, pH 4-6; 0.4% pH 3.5-10), and subjected to isoelectric focusing with 20 mM NaOH as the cathode solution and 80 mM H₃PO₄ as the anode, solution for 6-8 h at 500 V in the cold. The gels were fixed for 1 h in 15% TCA, soaked overnight in 50% methanol and 10% acetic
acid, silver stained and prepared for autoradiography. Carbonic anhydrase (pI = 5.95) and soya bean trypsin inhibitor (pI = 4.5) were used as standards on all isoelectric focusing gels.

In order to define bands of the isoelectric-focused myosin complex, the isoelectric focused gel was stained and cut according to pI as follows 4.0-4.5, 4.5-5.0, 5.0-5.5, and 5.5-6.0, and incubated in buffer containing Tris 100 mM, dithiothreitol 10 mM, β-mercaptoethanol 100 μM and glycine 200 mM four times for 30 min. The gel pieces were homogenized in 1 X sample buffer according to Laemmli (785), boiled 3-5 min and subjected to SDS-PAGE on 10% gel.

2.5.2. MAPK assay

Quiescent RMC on collagen type I or plastic in 10-cm Petri dishes were treated under varying conditions for times indicated in individual experiments and then washed with ice-cold phosphate-buffered saline and scraped into 800 μl of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 5 mM EGTA, 1 mM EDTA, protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin), and phosphatase inhibitors (1 mM Na3VO4 and 1 mM NaF). For the cells grown on collagen type I, prior to exposing to cell-lysis buffer collagen gel was treated with 3 mg/ml collagenase I in buffer containing 1 mM Na3VO4, 30 mM sodium pyrophosphate, and 50 mM NaF and centrifuged briefly for 5 min at 700 x g. Cells were sonicated for 5 s (Branson Model W-350 sonifier) and centrifuged at 100 000 x g for 15 min. Cytosol was assayed for protein by the method of Bradford (787), and aliquots containing ∼500 μg of protein were precleared by adding 1.0 μg of normal rabbit IgG with 20 μl of protein A-Sepharose and centrifuged for 5 min at 4°C in a microcentrifuge. The supernatant was incubated with 2 μg of anti-Erk-2 antibody for 3 h at 4°C, and immunoprecipitates were recovered by incubation with a 50% slurry of protein A-Sepharose for further 2 h. Portions of the immunoprecipitates were subjected to 12% SDS-PAGE (785) and transferred to polyvinylidene difluoride membranes for Western blotting with anti-Erk-2 antibody and
detection with an Amersham ECL detection system. MAPK activity was determined by phosphorylation of myelin basic protein (MBP) (788). Immunoprecipitates were mixed with 20 mM HEPES buffer, pH 7.4, containing 10 mM MgCl₂, 2 mM MnCl₂, 0.5 mM EGTA, 10 mM NaF, 0.5 mM Na₃VO₄, 1 mM diithothreitol, 0.5 μM ATP, 2 μM AMP-dependent protein kinase inhibitor, and 5 μCi of [γ-³²P]ATP and incubated at 30°C for 20 min. Reaction mixtures were mixed with 2 X sample buffer for electrophoresis according to Laemmli (785) and separated on 16% SDS-PAGE for silver staining and autoradiography.

2.5.3. PKC assay

RMC were plated in collagen type I-coated or noncoated 24-well plates, and after 16-24 h the FBS content of the medium was decreased to 0.4% for 48 h. The cells were then washed twice with RPMI 1640 medium, treated under various conditions for 15 min, washed twice again, and incubated for 10 min at 30°C in 100 μl reaction solution 137 mM NaCl, 5.4 mM KCl, 10 mM MgCl₂, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 25 mM β-glycerophosphate, 5.5 mM D-glucose, 5 mM EGTA, 1 mM CaCl₂, 20 mM HEPES (pH 7.2), 0.1 mM ATP, and 50 μg/ml digitonin containing 120 μg/ml peptide VRKRTLRL and 10 μCi/ml [γ-³²P]ATP as described (789). Each reaction was terminated by adding 50 μl of ice-cold 30% (w/v) TCA on ice, briefly centrifuged and supernatant spotted onto phosphocellulose circles (p81) prewashed sequentially with water, buffer, and 75 mM H₃PO₄. After standing for 15 min at room temperature, the circles were washed with gentle shaking 4-5 times (10 min each) in 75 mM H₃PO₄, and once in 2.75 mM sodium phosphate, pH 7.5, before liquid scintillation counting.

2.5.4. CaMK assay

RMC were seeded onto plastic or collagen type I-coated Petri dishes. After cells were treated with various agonists cells were washed twice with ice-cold PBS and lysed by several freeze-thaw cycles in buffer containing 50 mM HEPES, 50 mM sodium pyrophosphate, 1
mM EGTA, 25 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM dithiothreitol, 0.5% (v/v) Nonidet-P-40, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM PMSF. For the cells grown on collagen type I, prior to exposing to cell-lysis buffer collagen gel was dissolved by treatment with 3 mg/ml collagenase I in buffer containing 1 mM Na$_3$VO$_4$, 30 mM sodium pyrophosphate, and 50 mM NaF, and centrifuged briefly for 5 min at 700 x g. After brief sonication (3 x 5 s) and centrifugation (10 min at 17,000 x g), the protein content of the supernatant was determined by the method of Peterson (790). Fractions of the supernatant containing 5 µg protein were incubated with a 10 volumes of CaMK II assay buffer at 30°C for 3 min. The assay buffer for autonomous activity contained 50 mM HEPES, 10 mM MgCl$_2$, 0.1 mM ATP, 0.01 mM autocamtide-2 (KKALRRQETVDAL, (791)), 1 mM EGTA, and 5 µCi/ml [γ-^32P]ATP, at pH 7.5. For total CaMK II activity, EGTA was replaced with 3 mM CaCl$_2$ and 1 µM calmodulin. Reaction was stopped by addition of TCA (final 5%; w/v), and reaction mixture was spotted onto phosphocellulose paper circles. P81 circles were washed several times with 75 mM H$_3$PO$_4$, until negligible counts were found in the washing solution, and were placed into vials for counting of radioactivity.

2.6. NORTHERN HYBRIDIZATION ANALYSIS

Total RNA was isolated from synchronized cells in a single step by lysis in a monophasic solution of phenol and guanidine isothiocyanate using TRizol Reagent, as described by Chomczynski and Mackey (792). Equal amounts of RNA (ca. 10 µg) were denatured by the method of Gong (793), separated by electrophoresis on agarose-formaldehyde gels (1 % agarose, 2.2 M formaldehyde, 1 mM EDTA, 5 mM sodium acetate, 20 mM MOPS, pH 7.0), and transferred by overnight capillary blotting to Hybond-N nylon membrane. Membranes were prehybridized for 5 h at 42°C in prehybridization solution (10 % dextran sulfate, 5 x SSPE [3 M NaCl, 0.2 M Na$_2$PO$_4$+H$_2$O, 0.025 M EDTA], 50 % formamide, 5 x Denhardt’s solution, 250 µg/ml heat-denatured salmon sperm DNA, and 2 % SDS). Hybridization was carried out at 42°C for 18-20 h in prehybridization solution containing c-
fos cDNA and labeled with [α-32P]dCTP by the random primer method. After hybridization, blots were washed twice for 15 min at 50°C in 2 x standard saline citrate (SSC)/0.1 % SDS, followed by one 30 min wash in 0.1 x SSC/0.1 % SDS. Levels of mRNA were quantitated by densitometry of the Northern blot autoradiographs using a White/UV Transiluminator UVP, and normalized to 18S rRNA after probing with labeled cDNA to a rat 18S rRNA fragment.

2.7. IMMUNOBLOTTING ANALYSIS

After treatment with various agents, cells were released from Petri dishes, lysed in 800 μl of modified RIPA containing 50 mM Tris-HCl, pH 7.4, 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 5 mM EGTA, 1 mM EDTA, protease inhibitors 1 mM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin), and phosphatase inhibitors (1 mM Na3VO4 and 1 mM NaF), and subjected for total protein determination by the method of Peterson (790). Aliquots (about 1 ml) containing ca. 500 μg of protein were precleared by adding 1.0 μg of normal rabbit IgG with 20 μl of protein A-Sepharose and centrifuging for 5 min at 4°C in a microcentrifuge. The supernatant was incubated with 2 μg of anti-Erk-2 or anti-FAK antibodies for 3 h at 4°C, and immunoprecipitates were recovered by incubation with 50% slurry of protein A-Sepharose for further 2 h. Tonal cell lysates, dissolved in SDS-PAGE sample buffer, or portions of the immunoprecipitates dissolved in sample buffer were subjected to 12% SDS-PAGE (785) and transferred by electrophoretic transfer to polyvinylidene difluoride membranes in transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 15% methanol. After blocking with 5% BSA, 5% Carnation skim milk or both, in buffer containing 30 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2.6 mM KCl, and 0.05% Tween-20, the membranes were probed with anti-Erk-2, anti-FAK or antiphosphotyrosine antibodies in modified RIPA and signal was detected with an Amersham ECL detection system.
2.8. MISCELLANEOUS BIOCHEMICAL PROCEDURES

Total proteins were measured by the method of Peterson which is a modified Lowry method (750), or by the method of Bradford (787) after solubilization of the cell layer in lysis buffer.

2.9. STATISTICS

Differences between means were compared by Student’s t-test. The means and standard errors of the pooled data of the contraction studies were calculated and shown graphically.
3. RESULTS

3.1. EFFECT OF COLLAGEN TYPE I ON RMC

3.1.1. Effect on proliferation

In mesangioproliferative glomerulonephritis proliferating MC secrete increased amounts of ECM. To determine whether the presence of matrix components, including newly synthesized collagen type I, causes functional changes in MC they were cultured on plastic, collagen type I, Matrigel, and mesangial matrix. After growing for 72 h on all four matrices significantly less proliferation occurred on collagen type I than on other substrata, whereas mesangial matrix stimulated proliferation (Fig. 6). Consistent with cell number, total DNA per well was also lower on collagen type I and higher on mesangial matrix, compared with plastic. Cells grown on Matrigel showed proliferation similar to those on plastic. The DNA content per cell was constant (9.43 ± 0.29 pg/cell, mean ± SE) on all matrices.

3.1.2. Effect of collagen type I on RMC mitogenic response

Next the effect of different matrices on RMC mitogenic responsiveness was examined using [\(^3^H\)]thymidine incorporation as an indicator of DNA synthesis in cells. Cells grown on plastic showed maximal DNA synthesis between 16 and 18 h after stimulating quiescent cells with serum, indicating progression through S phase. DNA synthesis was markedly decreased and delayed on collagen type I, whereas peak thymidine incorporation was increased on mesangial matrix, as compared to plastic in each case and consistent with results of the proliferative studies (Fig. 7). The decrease in cell number and mitogenic response is not due to lower adhesion on collagen type I. Cells were labelled with [\(^3^H\)]thymidine for 48 h and passaged onto either collagen type I or plastic. Then they were allowed to attach for 1 h, and the adherent radioactivity was counted. In five experiments, cell adhesion studies indicated that 85 ± 9% of the plated counts adhered to plastic compared with 86 ± 11% of counts on collagen type I. In four independent experiments, attached cells were harvested.
RMC were passaged at a 1:4 split ratio into 12-well plastic plates and grown in complete medium with 10% FBS for 72 h. They were then detached as described in methods and counted in a Coulter particle counter (solid bars) or processed for total DNA measurements (open bars). Four pairs of the bars to left represent cells grown on plastic, Matrigel, mesangial matrix, or collagen type I. The four pairs of bars at right represent cells grown on plastic in medium supplemented with collagen type I (10 μg/ml), or CNBr-solubilized collagen type I (40 μg/ml), or CNBr reagent and CNBr-cleaved albumin (40 μg/ml). Values are mean ± SD of 6 experiments. *P < 0.001, significantly different from value on plastic, by unpaired Student's t-test.
Twelve-well plates were precoated with collagen type I (○) or mesangial matrix (■) or left uncoated. RMC were allowed to attach in RPMI 1640 with 10% FBS and grown to 60-70% confluence. Serum content of the medium was then lowered to 0.4% FBS, and cells were growth arrested for 48 h. After stimulation with 5% NuSerum, triplicate wells were exposed to [3H]thymidine (2 μCi/well) for 45 min. Cells grown on plastic were either supplemented with CNBr-solibilized collagen (40 μg/ml, □) or not (●). Other cells were stimulated with 0.1 μM ET-1 instead of NuSerum while growing on collagen type I (▲) or plastic (●). Cells were then processed as described in Methods and counted for radioactivity. Values are mean ± SD for triplicate wells and are from 1 of 3 representative experiments with plastic, collagen, or mesangial matrix substrata, and from 2 experiments with soluble collagen or ET-1.
and counted directly in a Coulter particle counter. Again, 79 ± 12% of cells adhered to plastic and 90 ± 3% to collagen. Nor is the inhibitory effect of collagen type I due to impaired viability of cells. Cell membrane integrity, examined by dye exclusion assay, was complemented by assessment of metabolic activity using tetrazolium salt. About 93% of cells grown on collagen type I were viable 48 h after starvation as measured by Trypan blue exclusion, compared to approximately 95% of cells starved on plastic (Table 3). In addition, in the MTT assay 95% and 93% of starved cells were viable on collagen type I and plastic, respectively, indicating that collagen type I did not affect RMC viability. Therefore, the decrease in cell number after 72 h is not due to initial lower adhesion nor to lower viability, but rather to decreased proliferation on collagen type I.

Cell attachment to intact collagen type I is required to suppress proliferation and mitogenesis. Dissolved collagen at concentrations up to 10 μg/ml did not affect proliferation nor mitogenic response (Fig. 6). Collagen type I began to precipitate at higher concentrations. Therefore, to determine if a higher concentration of collagen was needed for the antiproliferative effect, it was solubilized by CNBr cleavage, and tested to see if it could mimic the effect of growth on collagen type I matrix. Like soluble collagen at 10 μg/ml, CNBr cleaved collagen did not affect proliferation of RMC at concentrations up to 40 μg/ml [(4.3 ± 0.3, 4.6 ± 0.8, and 3.6 ± 0.4) X 10^5 cells/well and (4.1 ± 0.4, 3.9 ± 0.6, and 3.8 ± 0.3) μg DNA/well (n=6) on plastic only, or supplemented with dissolved collagen, or with CNBr cleaved collagen, respectively]. Nor did it affect mitogenic response. RMC incorporated (1.6 ± 0.2) X 10^4 cpm of [3H]thymidine 18 h after activation with serum on plastic, and (2.0 ± 0.2) X 10^4 cpm in medium supplemented with CNBr-cleaved collagen (n=2) (Fig. 6 and 7). Albumin was treated with CNBr in a similar manner, and the cleavage products were used to control for effects of contaminants possibly arising from the reaction mixture or purification scheme. As an additional control, CNBr dissolved in 70% formic acid was processed the same way as the cleavage products of collagen type I and albumin.

Neither proliferation [(4.4 ± 0.2 and 4.6 ± 0.3) X 10^5 cells/well and (4.0 ± 0.2 and 3.9 ±
Table 3 — Cell integrity on plastic and collagen type I.

Mesangial cells were passaged onto plastic or collagen type I-coated 24-well plates, allowed to reach 60-70% confluence and exposed to RPMI 1640 containing 0.4% FBS to render them quiescent. After 48 h cells were subjected to Trypan blue exclusion assay, MTT assay, or processed for determination of DNA content, all as described in Methods. The values are mean ± S.D. of measurements from 24 individual wells for each assay.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Plastic</th>
<th>Collagen type I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypan blue exclusion (% of cells)</td>
<td>95.7 ± 10.9</td>
<td>93.1 ± 13.1</td>
</tr>
<tr>
<td>MTT (O.D. 570 nm)</td>
<td>0.44 ± 0.005</td>
<td>0.43 ± 0.06</td>
</tr>
<tr>
<td>DNA content (μg/well)</td>
<td>1.22 ± 0.47</td>
<td>1.44 ± 0.23</td>
</tr>
</tbody>
</table>
0.3 μg DNA/well (n=6) nor mitogenic response ((1.9 ± 0.3 and 1.9 ± 0.4) X 10^4 cpm of [3H]thymidine at 18 h (n=4)) were affected by albumin or CNBr control.

3.1.3. Effect of collagen type I on c-fos induction in RMC

The cellular c-fos proto-oncogene is rapidly induced to high transcriptional activity upon treatment of quiescent cells with serum growth factors (579, 794-796). Because the growth of RMC on collagen type I suppressed their proliferation and mitogenesis we studied the effect of collagen type I on induction of c-fos expression. For that purpose we measured steady state mRNA levels of c-fos by Northern blotting. Because basal levels of transcript are nearly undetectable, it is assumed that the appearance of c-fos mRNA represents initiation of c-fos transcription. As shown previously in our lab (637), MC grown on plastic displayed a strong increase of c-fos steady state mRNA after release from quiescence with 5% NuSerum (Fig. 8). The time course of c-fos induction in RMC grown on collagen type I indicates that c-fos activation peaked modestly 30 min after activation and gradually returned to the basal level 4 h later (Fig. 8). However, the peak c-fos signal at 30 min in RMC grown on collagen type I is 5-6 times lower than that in cells grown on plastic, suggesting a strong suppression of c-fos induction, consistent with the previous results.

3.1.4. Effect of collagen type I on MAPK activity

As indicated earlier, growth on collagen type I suppresses RMC proliferation and c-fos induction. To further investigate the mechanism of collagen's action we chose to study the effect of collagen type I on MAPK activity because i) activated MAPK induces c-fos transcription (Fig. 45), ii) MAPK is readily activated by extracellular stimuli such as serum and phorbol esters (Fig. 45), and iii) collagen type I suppressed serum-dependent c-fos induction.

Fig. 9 and 10 show a time course of MAPK activity in RMC grown on collagen type I...
RMC were seeded onto collagen type I precoated or non-coated plastic petri dishes. Quiescent RMC were treated with 5% NuSerum and total RNA was extracted at the indicated time points. Control cells were not treated with NuSerum. The level of c-fos mRNA was determined by Northern blotting and autoradiography. The intensity of the signal representing hybridization of the c-fos cDNA was normalized to the intensity of hybridized 18S rRNA probe, determined by laser densitometry. The histogram shows the intensity of the ratio of the c-fos signal to the 18S signal in arbitrary units. The result is representative of 2 separate experiments.
Cells were seeded onto plastic 10 cm petri dishes. Quiescent RMC were treated with 5% NuSerum and cytosolic extracts were obtained by scraping cells in modified RIPA, as described in Methods, at the indicated times. Cytosolic extracts were then immunoprecipitated with anti-Erk-2 antibody, and portions of immunoprecipitates were used either for Western-blotting or for in vitro kinase assay. Upper autoradiogram: Western blot of the immunoprecipitate run on a denaturing PAGE shows a major band corresponding to Erk-2 (42 kDa). The controls are as follows: (RM) reaction mixture containing no cell extract, no protein-A-Sepharose, and no antibody; (PA) contains protein A-Sepharose and cytosolic extract of cells treated for 2 min, while (PAB) contains anti-Erk-2 antibody plus protein A-Sepharose. Lower autoradiogram: The immunoprecipitates were used to phosphorylate MBP with [γ-32P]ATP, and the reaction mixture was then subjected to SDS-PAGE. The prominent MBP band was visualized by autoradiography, and also demonstrated on silver-stained gels of the starting material (not shown). Some cells were pretreated (30 min) with PD98059, a specific MEK inhibitor, before stimulation (lanes 2/PD). The histogram was obtained by laser densitometry and shows the level of incorporation of 32P into MBP. The autoradiograms are representative of 2 similar experiments.
Fig. 10 - Effect of collagen type I on MAPK activity in serum-treated RMC.

Cells were seeded onto plastic or collagen type I-coated petri dishes. Quiescent RMC grown on plastic were processed as described in Fig. 9. Cells grown on collagen were first released from the substratum by a brief exposure to collagenase A at the indicated times, and cytosolic extracts were obtained as described in Methods. Cytosolic extracts were immunoprecipitated with anti-Erk-2 antibody, and portions of immunoprecipitates were used either for Western-blotting or for in vitro kinase assay, as described in Fig. 9. Upper autoradiogram: Western blot of the immunoprecipitate run on a denaturing PAGE showing a major band corresponding to Erk-2 (42 kDa). Lower autoradiogram: The immunoprecipitates were used to phosphorylate MBP with \([\gamma^{32}P]ATP\), and reaction mixtures were then subjected to PAGE, as described in Fig. 9. Similar to cells grown on plastic, some were pretreated with PD98059 prior to stimulation with serum (lane 2/PD). The histogram was obtained by laser densitometry from the autoradiograph showing MBP phosphorylation. The autoradiographs are representative of 2 similar experiments.
and on plastic after activation with serum. At time zero, cells grown on plastic had little or no MAPK activity. When cells were activated with serum, an increase in MAPK activity was observed starting at 1 min after addition of serum and peaking at 5 min. MAPK activity then remained more or less constant for 30 min, and started to decrease 60 min later. When cells were pretreated with PD98059, a specific MEK inhibitor, inhibition of phosphorylation of MBP was observed consistent with a MEK/MAPK-dependent process. The specificity of the assay was addressed by testing the effect of protein-A-Sepharose beads alone or with an anti-Erk-2 antibody. Furthermore, the immunoprecipitate, protein-A-Sepharose or anti-Erk-2 antibody were omitted from the reaction mixture and also tested for the amount of incorporated radioactivity. As expected none of these controls caused phosphorylation of MBP (Fig. 9).

Growth of cells on collagen type I strongly suppressed MAPK activity, as indicated in Fig 10. A modest peak of MAPK activity was still observed between 2 and 5 min. However, it was four times lower than in cells grown on plastic. As indicated in lane 2/PD (Fig. 10) PD89059 suppressed MAPK activation, and together with controls similar to those described for Fig. 9, this supports a MAPK-dependent incorporation of [γ-32P] into MBP.

It is well established that PKC activates MAPK (Fig. 45). Therefore we tested the effect of collagen type I on PKC-dependent MAPK activation. When cells were treated with TPA, almost identical results were obtained to those with cells treated with serum, that is a very modest induction of MAPK activity in cells grown on collagen type I, that again peaked between 2 and 5 min (Fig. 11). As expected, a four times stronger MAPK induction was observed in cells grown on plastic (Fig. 12). The maximum activity was between 2 and 5 min and started to decrease earlier than is the case when cells were treated with serum. Down regulation of PKC activity by a 24-h pretreatment of cells with TPA (shown in Fig 15) totally suppressed MAPK activity, consistent with a PKC-dependent process. Similar to Fig. 9, controls including reaction mixture without immunoprecipitates, or supplemented with
Fig. 11 – Effect of collagen type I on TPA-dependent MAPK activity.

RMC grown on plastic or collagen type I-coated petri dishes were rendered quiescent, and treated with 200 nM TPA for the indicated times. Cells were then processed as described in the legend to Fig. 10. Some cells on both surfaces were pretreated with TPA for 24 h prior to activation in order to down-regulate PKC activity (indicated by †). Controls, upper and lower autoradiograms, and the histogram are as described in legend to Fig. 10, and are representative of 2 separate experiments.
Fig. 12 – Time course of TPA-dependent MAPK activity in RMC on plastic.

RMC grown on plastic petri dishes were rendered quiescent and treated with 200 nM TPA for the indicated times. Cells were then processed as described in the legend to Fig. 9. Some cells on both surfaces were pretreated with TPA for 24 h prior to activation in order to down-regulate PKC activity (indicated by †). Upper and lower autoradiograms, histogram, and controls are as described in the legend to Fig. 9, and are representative of 2 separate experiments.
protein-A-Sepharose, and/or anti-Erk-2 antibody, showed no observable phosphorylation of MBP (Fig. 12).

These results indicate that growth of RMC on collagen type I suppresses serum-dependent MAPK activation and support our previous observations of collagen's suppressive effect on RMC proliferation, mitogenic responsiveness, and c-fos induction.

3.1.5. Effect of collagen type I on tyrosine phosphorylation

Tyrosine phosphorylation is implicated in stimulation of cellular growth and differentiation (797, 798), and is required for MAPK activation. After a ligand causes its receptor to autophosphorylate on tyrosine residues, phosphorylated tyrosine binds the SH2 domains of adapters such as Grb2. Attached guanine nucleotide exchange factors (e.g., mSos) with proline-rich SH3 domain-binding sites are thus brought to the membrane (799). Attached to the membrane in proximity to the isoprenylated small G proteins, exchange factors promote the association of Ras with GTP. The GTP-bound form of Ras binds the protein kinase Raf-1, thereby targeting it to the membrane where Raf protein kinase activity is increased. The MAPK cascade is then activated after Raf-1 activates MAPK kinase (MEK) (799). Because growth on collagen type I suppressed RMC proliferation, mitogenic response, c-fos induction, and MAPK activity, we asked whether growth on collagen type I would affect tyrosine phosphorylation in RMC. Cells were grown on collagen type I-coated dishes and released from quiescence with serum, and the level of tyrosine phosphorylation of total cell protein was compared with that of cells grown on plastic. A strong tyrosine phosphorylation of proteins running at > 200, 120, 70, and 40 kDa was observed upon activation with 5% NuSerum by probing Western blots with an antiphosphotyrosine antibody (Fig. 13A). In RMC grown on plastic, the degree of phosphorylation was highest between 2 and 5 min (Fig. 13A; lanes 2 and 5) and decreased thereafter (Fig. 13A; lanes 10 - 60). When cells were pretreated with genistein (50 μM), a tyrosine kinase inhibitor, the phosphorylation of proteins at > 200, 70, and 40 kDa was inhibited, indicating a tyrosine
Fig. 13 – Time course of serum-dependent tyrosine phosphorylation in RMC on plastic.

Quiescent RMC, grown on plastic, were treated with 5% NuSerum for the indicated times and total cell extracts were obtained as described in Methods. Some cells were pretreated with 50 μM genistein for 30 min (lanes 1G and 2G). The same amount of protein was loaded onto the gels and the level of tyrosine phosphorylation was detected by ECL and autoradiography, after probing Western blots with the HRP-conjugate anti-phosphotyrosine antibody. A) time course of tyrosine phosphorylation. Cells at t=0 were untreated. An increase in tyrosine phosphorylation of proteins at 200 kDa, 120 kDa, 80 kDa, and 42 kDa (indicated to the outer sides of two autoradiographs) was observed, as determined by positions of prestained molecular mass markers, indicated between two autoradiographs. B) depletion of FAK and Erk-2 proteins. A portion of 5-min-serum-treated total cell extract was exposed to either anti-FAK or anti-Erk-2 antibody, and protein A-Sepharose. Supernatants from FAK depletion (lane F) or Erk-2 depletion (lane E) were run on SDS-PAGE along with non-depleted sample (lane C), and subjected to Western blotting as described in A. Western blots are representative of 3 separate experiments.
kinase-dependent phosphorylation. Phosphorylation of the protein at 120 kDa was inhibited to a lesser extent. However, the interpretation of the effect of genistein is limited because of its broad specificity, and toxicity which might cause similar effect. Therefore, an use of lavendustin A, or comparison of the effect of genistein to an effect of diadzein, its inactive analog, could define better the inhibition of tyrosine phosphorylation (800).

Cells grown on collagen type I (Fig. 14) showed decreased and delayed tyrosine phosphorylation of proteins at 120 kDa, and 70 kDa (lanes 4-11). Tyrosine phosphorylation of a protein above 200 kDa, that was strong after activation of cells grown on plastic, was not present in cells grown on collagen type I. The tyrosine-phosphorylated protein bands almost disappeared 30 min after activation. Tyrosine-phosphorylated EGF receptor was included on the electrophoretic gel as a positive control (Fig. 14; lane 1). The bands at approximately 20 kDa and lower represent nonspecific signals since the tyrosine-phosphorylated EGF receptor preparation displayed a similar pattern (Fig. 14).

To address the nature of the phosphorylated bands, anti-FAK and anti-Erk-2 antibodies were used to deplete the corresponding proteins in the cell extract. As indicated in Fig. 13B, exposure of total extract from cells grown on plastic to the anti-FAK (lane F) or anti-Erk-2 antibodies (lane E) at 5 min after activation (lane I) caused depletion of tyrosine-phosphorylated proteins at 120 kDa and 42 kDa, respectively. Lane 2 in Fig. 14 is the control for sample buffer and possible contaminants during immunoprecipitation process. These results suggest that growth on collagen type I suppresses serum-dependent tyrosine phosphorylation, including that of FAK and MAPK which are involved in induction of early response genes. This supports our previous findings that growth on collagen type I suppresses RMC proliferation and mitogenic response.
Fig. 14 - Effect of collagen type I on serum-dependent tyrosine phosphorylation in RMC

Quiescent RMC grown either on plastic or collagen-coated petri dishes were treated with 5% NuSerum for the indicated times. Cells grown on collagen type I were detached as indicated in the legend to Fig. 10, exposed to the same extraction buffer as cells grown on plastic, and subjected to Western blotting as described in Fig. 13. A time course of tyrosine phosphorylation of RMC grown on collagen type I (lanes 0 - 60) is compared to the peak of tyrosine phosphorylation at 5 min on plastic (lane 5). Tyrosine-phosphorylated EGF receptor was included on the gel as a positive control (lane 1, EGFR) in addition to sample buffer with extraction solution. Prestained molecular mass markers are indicated to the right, and tyrosine phosphorylated proteins to the left. The figure is representative of 3 separate experiments.
3.1.6. Effect of collagen type I on PKC activity

Because TPA-dependent MAPK activation was strongly suppressed in RMC grown on collagen type I we measured PKC activity in cells growing on collagen type I and plastic. For that purpose we used an in situ PKC assay (789) where the PKC-specific substrate (VRKRTLRL) is exposed to cellular PKC after permeabilizing the cells with digitonin in the presence of [γ-32P]ATP. First, we determined PKC activity in cells grown on plastic. Some basal activity was observed when no substrate was present in the reaction mixture (Fig. 15, lane NS). When cells were activated with TPA a several-fold increase in PKC activity was observed, peaking broadly between 1 and 60 min and slowly decreasing after 120 min to about 70% of maximal activity (Fig. 15, lanes 0-120). The highest PKC activity was at 5 to 10 min after addition of TPA. The observed increase in substrate phosphorylation is due to PKC activity since it was inhibited by down regulation of the enzyme after exposure to TPA for 24 h (Fig. 15, lane 5T).

Next the effect of growth on collagen type I on PKC activity was studied. In a similar experiment, a modest increase in PKC activity was observed in cells on collagen type I, with a broad peak from 5 to 30 min decreasing slowly thereafter (Fig. 16). The peak of PKC activation on collagen type I was several times lower than that on plastic, indicating that growth of cells on collagen type I strongly suppressed PKC activity. Once again, TPA pretreatment abolished substrate phosphorylation indicating PKC-dependent processes on both surfaces (Fig. 16, lane 5T).

We wondered whether PKC activity was downregulated by the growth of RMC on collagen type I. Therefore, a time course of PKC activity in RMC was determined following attachment to collagen type I or plastic. Unattached cells had very low PKC activity averaging a phosphate incorporation of about 0.2 nmol/min/μg DNA (Fig. 17). PKC activity was abruptly increased 1 h after passage of cells onto plastic and sustained at a similar level (5-6 nmol/min/μg DNA) over 48 h. In contrast, in RMC attached to collagen type I, PKC
Fig. 15 - TPA-dependent PKC activation in MC grown on plastic.

Quiescent RMC were treated with TPA (200 nM) for the indicated times and then exposed to VRKRTLRL oligopeptide, a PKC-specific substrate, together with digitonin and [γ-32P]ATP at 30°C. The reaction was stopped 10 min later with 5% TCA, and the supernatant was spotted onto phosphocellulose (p81) paper for counting the incorporated radioactivity. The PKC activity was expressed as nmol of 32P incorporated/min/μg DNA. Some wells were pretreated with TPA for 24 h in order to down-regulate PKC activity, indicated as T. In order to determine incorporation of non-specific radioactivity, some cells were exposed to the assay mixture containing no substrate (lane NS), and processed as indicated above. Values are mean ± SD of 3 experiments.
Fig. 16 – Effect of collagen type I on PKC activity in RMC.

RMC were seeded onto plastic or collagen type I-coated 24-well plates, rendered quiescent, and activated with TPA for the indicated times. The assay was performed as described in Fig. 15, including down-regulation of PKC on both surfaces (T). Values represent the range of 2 independent experiments.
Fig. 17 - Time course of PKC activity after cell attachment onto plastic or collagen type I.

RMC were detached from confluent 10 cm plates by trypsinization, passaged at 1:3 split ratio onto plastic (○) or collagen type I-coated (●) 24-well plates in the presence of 10% FBS, and PKC activity was determined at the indicated times after cell attachment as described in the legend to Fig 16. The level of PKC activity was corrected for the amount of DNA/well in both non-coated and collagen-coated plates, and also in resuspended cells (0 time point) prior to cell passage. Values are the mean of measurements from duplicate wells.
activity was blunted, staying in the range 0.2 - 0.5 nmol/min/μg DNA (Fig. 17). Such a suppressive effect of collagen type I is characterized by a lag of PKC activity up to 6 h, followed by several fold increase afterwards, and is maximal at 48 h during this experiment (Fig. 17). However, the highest PKC activity on collagen type I was still 10 times lower than in cells seeded on plastic, indicating that growth on collagen type I keeps PKC activity very low from the time of cell passage.

3.1.7. Effect of collagen type I on CaMK activity

Induction of c-fos can also occur through a CaMK-dependent pathway (589, 801). CaMK phosphorylates CREB and SRF which then bind to DNA elements, such as CRE or SRE (Fig. 45) (801). Furthermore, evidence is presented below that CaMK-II is involved in c-fos induction in RMC (section 3.2.5). CaMK-II activity was measured after activation with ionomycin (100 nM) in cells grown on collagen type I and compared to that in cells plated on plastic. To determine whether CaMK-II was activated by ionomycin in RMC, the ability of cell homogenates to phosphorylate the CaMK II-specific substrate, autocamtide, was measured by the amount of incorporated [γ-32P]ATP. CaMK-II activity was constant when saturable amounts of Ca²⁺ and calmodulin were present in the reaction mixture, representing total activity of the enzyme (Fig. 18). However, when Ca²⁺ and calmodulin were omitted, a time-dependent CaMK-II activity was observed, peaking at 30 s after ionomycin treatment (Fig. 18). This autonomous CaMK-II activity, independent of the continued presence of Ca²⁺ and calmodulin, corresponds to the activity present in the cells prior to lysis. Auto-CaMK-II activity increased 5-6 times from the basal level 30 s after activation with ionomycin in RMC grown on plastic (Fig. 18). The basal auto-CaMK-II activity in cells grown on collagen type I was twice as high as in cells grown on plastic (Fig 19). Ionomycin caused a very modest increase in auto CaMK-II activity at 30 s (Fig. 19). The peak of CaMK-II activity in cells grown on collagen type I was still several times lower than in cells grown on plastic. Pretreatment of cells grown on both substrates with KN-93 caused inhibition of
Fig. 18 - Time course of ionomycin-dependent CaMK-II activation

Quiescent RMC were treated with ionomycin (100 nM) at time zero and cytosolic extracts were obtained at intervals up to 10 min later. CaMK-II activity was determined as the ability of the cytosolic extracts to phosphorylate autocamtide-2 in the presence of [γ-32P]ATP, as described in Methods. Autonomous CaMK-II activity (■) was determined in the absence of free Ca²⁺ and calmodulin and is expressed as a percentage of total CaMK-II activity at the corresponding time point. Total activity (□) was determined in the presence of added Ca²⁺ and calmodulin and is expressed as nmol 32P incorporated into autocamtide-2 per min per mg protein. Values are mean ± SD from 4 independent experiments.
Fig. 19 – Time course of ionomycin-dependent CaMK-II activation on collagen type I.

RMC were seeded onto plastic or collagen type I-coated petri dishes, rendered quiescent, and treated with ionomycin for the indicated times. Cytosolic extracts were obtained as described in the legend to Fig. 10. The autonomous (■) and total (□) CaMK-II activities were determined also in cells pretreated with KN-93 (¶), a specific CaMK-II inhibitor, as described in legend to Fig. 18. Values are the range of measurements from 2 independent experiments.
auto-CaMK-II activity (Fig. 19, indicated by $\hat{\cdot}$), further suggesting a CaMK-II-dependent ($^{32}$P) incorporation into substrate. However, pretreatment of cells with KN-93 did not significantly affect total CaMK activity which was constant throughout the experiment (Fig. 19). These results suggest that growth on collagen type I suppresses accumulation of autonomous CaMK-II activity in response to ionomycin.

### 3.1.8. Effect of collagen type I on RMC contraction

Growth on collagen type I suppressed PKC and CaMK activity. Because, both kinases are involved in the process of contraction, we asked whether collagen type I would affect RMC contractility. To optimize experimental conditions for contraction on different surfaces, the effects of temperature and agonist were studied. Cells in buffered HBSS without agonist did not show a significant decrease in cross-sectional area (CSA) on collagen type I at 25°C or 30°C, but at 37°C decreased their CSA by ~20% (Fig. 20). In a similar experiment on plastic, RMC showed no significant decrease in CSA at any temperature (Fig. 20). With addition of endothelin-1 (ET-1), contraction of RMC was significantly lower at 25°C than at 30°C or 37°C on both collagen type I and plastic (for example, 93.2 ± 4.4% of starting area at 25°C vs. 67.6 ± 17.1% at 30°C by 20 min on collagen type I (P<0.005) and 93.2 ± 7.3% at 25°C vs. 86.0 ± 10.0% on plastic (P<0.001); Fig. 20). Because RMC showed a similar response to ET-1 at 30°C and 37°C and, in control experiments (no agonist), showed a spontaneous decrease in CSA at 37°C on collagen type I, additional contraction experiments were performed at 30°C.

ET-1 caused greater contraction of RMC plated on collagen type I than on plastic (e.g., P < 0.001 at 20 min; Fig. 21). On collagen, cells started to change shape 2 min after addition of ET-1 and significantly decreased their CSA as early as 5 min after addition of agonist (P<0.001). Minimum CSA (~70% of starting area) was reached after 20-25 min (Fig. 21a). AVP and ANG II caused lesser contraction (~75% and 80% of initial area, respectively).
Fig. 20 - Temperature dependence of ET-1-induced RMC contraction.

Cells were plated onto collagen type I-coated petri dishes or onto plastic dishes and allowed to attach. After 24 h, growth medium (RPMI 1640 + 10% FBS) was changed to Ca²⁺- and Mg²⁺-free Hanks' buffered salt solution (HBSS), and then 16 - 20 h later contraction was measured in fresh HBSS with (●) or without (○) ET-1 (0.1 μM). The time when HBSS alone or HBSS containing ET-1 was added to cells is indicated as time = 0. Experiments were carried out at 25°, 30°, or 37°C. Points represent cross-sectional area expressed as a percentage of that at time = 0. Each time point represents mean ± SD of triplicate area measurements of 225 cells on images captured in 3 - 5 experiments. Changes in area on collagen type I with ET-1 were significantly lower than controls by 5 min at 25° (P < 0.001), at 30° (P < 0.001), and at 37°C (P < 0.05) and remained so thereafter. On plastic, contractions were significant by 10 min at 25° (P < 0.01) and 37°C (P < 0.001) and by 2 min at 30°C (P < 0.001) and thereafter.
Fig. 21 – Effect of agonist and substratum on RMC contraction.

Cells were plated onto collagen type I-coated petri dishes (a) or onto uncoated dishes (plastic, b) and allowed to grow for 24 h prior to changing to Ca\(^{2+}\)-, Mg\(^{2+}\)-free HBSS for contraction studies as described in Fig. 20. Contraction was initiated 16-20 h later, as indicated time = 0 by addition of fresh HBSS with no additions (O), or with addition of 0.1 μM ET-1 (●), 2.5 μM ANG-II (■), or 20 nM AVP (▲) at 30°C. Each time point represents mean ± SD of triplicate measurements on captured images of 135 cells in 3 separate experiments. On collagen type I, contractions were significant by 5 min with all 3 agonists (all P < 0.001 vs. agonist-free controls) and thereafter. On plastic, contraction in response to ET-1 and ANG-II was significant by 2 min (P < 0.01) and 5 min (P < 0.01), respectively.
Less contraction occurred on plastic, where again endothelin was a slightly more effective agonist than ANG II (Fig. 21b). Thus, contrary to the decreased mitogenic response to serum elicited by growth on collagen type I, the contractile response to agonist is enhanced.

To ascertain whether these changes in cell shape represent true contraction, we examined the involvement of NO and MLCK. NO is a potent relaxant of RMC, and its production is inhibited by L-NMMA. Pretreatment with 2 mM L-NMMA caused an increase in the response to ET-1 (Fig. 22), consistent with attenuation of a relaxant stimulus unmasking greater contraction. In addition KT-5926, a MLCK inhibitor effectively blocked the ET-1-dependent decrease of > 30% in CSA (Fig. 22).

Concentrations of ET-1 that elicited this contractile response were by themselves insufficient to cause cell proliferation on either plastic or collagen type I. In experiments identical to those described in Fig. 7 but with 100 nM ET-1 substituted for serum in some wells, no increase in cell number or DNA content per well were observed at 72 h on either substratum (Table 4). ET-1 caused a modest increase in [³H]thymidine incorporation that again was small compared with that elicited by serum and was unaffected by substratum (Fig. 7).

3.1.9. Effect of collagen type I on myosin light chain phosphorylation

MLC phosphorylation is a prerequisite for activation of myosin ATPase by actin and thus for contraction. Myosin complex was separated by either immunoprecipitation with polyclonal antiserum to non-muscle myosin or by pyrophosphate PAGE followed by isoelectric focusing of the extracted complex. As shown in Fig. 23 isoelectric focusing revealed two major bands with apparent pI of 6.0 and 5.5. These two bands show a similar pattern of phosphorylation, dephosphorylation, and inhibition by the MLCK inhibitor KT-5926 (Fig. 23A), supporting the interpretation that they are monophosphorylated MLC, either free (pI = 5.5) or in association with another protein. To support this idea further, cutting the
Cells were grown on collagen type I as described in Fig. 20, and contraction was initiated after the 16 - 20-h exposure to Ca\textsuperscript{2+}. Mg\textsuperscript{2+}-free HBSS, as indicated time = 0, by addition of fresh HBSS ( ), HBSS with 0.1 μM ET-1 ( ● ), HBSS with 100 μM N\textsuperscript{O}-monomethyl-L-arginine (L-NMMA) ( □ ), HBSS with 0.1 μM ET-1 + 100 μM L-NMMA ( ■ ), or HBSS with 0.1 μM ET-1 + 10 μM KT5926 ( △ ), all at 30°C. Each time point represents mean ± SD of triplicate measurements of 180 cells on images captured in 3 - 4 experiments. All contractions with ET-1 are significant with respect to either control or ET-1 + KT5926 (P < 0.001 by 2 min). Contraction with L-NMMA is significantly different from control (P < 0.001) only after 15 min. Contraction with ET-1 + L-NMMA vs. ET-1 alone was significant only between 5 and 15 min inclusive (P < 0.01).
Table 4 – Effect of endothelin-1 on cell proliferation

Cells were passaged at a 1:4 split either on plastic or collagen type I-coated 6-well plates as described in Fig. 5. For growth stimulus, medium was supplemented either with 10% FBS or 100 nM ET-1. Cell number and DNA content were measured at 72 h, as described in Methods. Values are means ± SD of 6 wells.

<table>
<thead>
<tr>
<th>Substratum</th>
<th>Growth stimulus</th>
<th>Cell Number/well X 10^5</th>
<th>DNA content µg/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic</td>
<td>Serum</td>
<td>5.23 ± 0.21</td>
<td>4.68 ± 0.51</td>
</tr>
<tr>
<td>Plastic</td>
<td>Endothelin-1</td>
<td>0.99 ± 0.05</td>
<td>0.88 ± 0.1</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>Serum</td>
<td>1.01 ± 0.19</td>
<td>0.70 ± 0.14</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>Endothelin-1</td>
<td>0.94 ± 0.1</td>
<td>0.78 ± 0.15</td>
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</tbody>
</table>
Subconfluent RMC cultures were incubated with [32P]orthophosphate for 3 h and then treated with 0.1 μM ET-1 at time = 0. Autoradiography of isoelectric focusing gels of a 20-kDa component of myosin immunoprecipitates were performed as described in Methods. Known isoelectric points (pI) are carbonic anhydrase and soybean-trypsin inhibitor standards run on the same gel. They were interpolated to obtain pI values reported in text. A): cells grown on plastic. Lanes 1 and 4 - 8 are from extracts from cells collected at times = 0, 2, 5, 10, 20, and 40 min, respectively. Lane 2, extract at 5 min with inclusion of 10 μM KT-5926 from time = 0. Lane 3, no sample added (BLK). B): cells grown on collagen type I. Lanes 1 - 6 are from extracts of cells collected at times = 0, 2, 5, 10, 20, and 40 min, respectively. MLC indicates myosin light chain that comigrated with MLC standard, pI=5.0. Gels are typical of those from at least 3 repeats of each experimental condition.
bands from the isoelectric focusing gel and subjecting them to denaturing PAGE showed silver-staining bands at 20 kDa in both cases with a large protein of 250 kDa present in the pI 6.0 band (Fig. 24). The nature of this protein was not explored further; its apparent mass and pI value are consistent with those of filamin, known to associate with actin and separate from myosin only after prolonged electrophoresis in pyrophosphate gels (781).

Phosphorylation of MLC was quantitated by densitometry of both bands on the isoelectric focusing autoradiogram. A third band at pI ~ 4.95, appearing transiently at 2-10 min after stimulation with ET-1, was quantitated separately and plotted as a percentage of maximum activity (Fig. 23A). Based on its pI and ET-1 inducibility it likely represents diphosphorylated MLC (781, 783). Phosphorylation of all bands is maximal at 5-10 min after addition of ET-1 and declines thereafter (Fig. 25).

Cells grown on collagen type I show a quantitatively similar response to endothelin, i.e., three phosphorylated bands on isoelectric focusing of a myosin immunoprecipitate that have same pI to those from cells grown on plastic. In contrast to cells grown on plastic, there is no detectable basal phosphorylation, and maximal incorporation has already been attained before 5 min, consistent with the greater and more rapid contractile response of the cells on collagen. The band at pI < 5.0 is smeared and may arise from multiple phosphorylated forms.

3.1.10. Effect of collagen type I on Ca2+ signaling

Contraction in response to agonists is mediated by an observable increase in the concentration of cytosolic free ([Ca2+]i). We attempted to determine whether the greater changes in CSA on collagen type I paralleled an increase in Ca2+ signaling. Cells were grown on Acclar plastic coverslips with and without a collagen coating and loaded with fura 2/AM. Cells on both substrates had basal levels of intracellular Ca2+ of ~ 70 nM. Typical tracings of the response of [Ca2+]i to 100 nM ET-1 in cells on plastic show an initial rapid
Fig. 24. SDS-PAGE of isoelectric-focused myosin bands.

Cells were seeded onto plastic (lanes 1-4) or onto collagen type I (lanes 5-8) precoated petri dishes, loaded with [32P]orthophosphate, treated with ET-1 for 5 min, and subjected to isoelectric foccusing as described in the legend for Fig. 23. After silver-staining of the isoelectric-focused gel, bands running at pI 6.0-5.5 (lanes 1 and 5), 5.5-5.0 (lanes 2 and 6), 5.0-4.5 (lanes 3 and 6) and 4.5-4.0 (lanes 4 and 8) were cut from the gel, homogenized as described in Materials and methods and separated on 10% SDS-PAGE along with molecular weight markers indicated to the right. Two prominent bands were recovered at approximately 250 and 20 kDa corresponding to the molecular weights of filamin and MLC, respectively.
Fig. 25 – Incorporation of $^{32}$P into myosin light chain.

 Autoradiograms such as those shown in Fig. 23 were integrated by laser densitometry to obtain level of incorporation of $^{32}$P into myosin light chain as a function of time. Cells were grown on collagen type I (solid bars) or plastic (open bars) and harvested at indicated times. Intensity of $^{32}$P signal is expressed as a percent of that at time of maximum incorporation. Bars represent total intensity of all 3 bands on autoradiograms (see text for description). Horizontal divisions distinguish monophosphorylated (lower) from di- (poly-) phosphorylated (upper) product, as described in the text.
RMC were grown on Aclar coverslips with or without a collagen type I coating and allowed to attach for 48 - 72 h before loading with fura-2 acetoxy-methyl ester (AM). Cells were monitored by an epifluorescence microscope with excitation at 340 and 380 nm and emission at 500 nm after addition of 0.1 μM ET-1. Cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]i) peaks averaged 1,017±184 nM on collagen type I and 534±192 nM on plastic in 6 independent experiments and were significantly different by Student's t-test ($P < 0.001$; $n = 6$). Representative tracings are shown.
rise followed by a sustained second phase of elevation (Fig. 26). ET-1, ANG II, and AVP all caused an increase in \([\text{Ca}^{2+}]_i\) in cells on uncoated coverslips, with the greatest increase (up to ~500 nM) produced by ET-1 (Table 5). Growth on collagen type I had no effect on the increase in \([\text{Ca}^{2+}]_i\) elicited by ANG II or AVP but markedly enhanced the response to ET-1; in cells grown on collagen, 100 nM ET-1 caused an increase in \([\text{Ca}^{2+}]_i\) to ~1 μM. This increase was transient and \([\text{Ca}^{2+}]_i\) decreased to the same level as in cells on plastic after 200 s.

After binding to cell receptors serum growth factors cause an initial increase in \([\text{Ca}^{2+}]_i\) generated by PLCy-dependent IP3 release (802). Because collagen type I suppressed the serum-dependent mitogenic response and c-fos induction, we wondered whether growth on collagen type I would affect \(\text{Ca}^{2+}\) signaling after activation of cells with serum. The basal levels of \([\text{Ca}^{2+}]_i\) in RMC grown on plastic (n=4) and collagen type I (n=3) did not differ significantly and were 61 ± 33 and 86 ± 26, respectively (Table 6). Similarly, peak \([\text{Ca}^{2+}]_i\) after treatment with serum was approximately the same; 286 ± 27 in cells grown on collagen and 280 ± 47 in cells grown on plastic. This result indicates that the suppressive effect of collagen type I on mitogenesis of RMC does not involve \(\text{Ca}^{2+}\) signaling and points to an intact \(\text{Ca}^{2+}\) signaling pathway in cells grown on collagen type I.

3.2. EFFECT OF HEPARIN ON RMC

3.2.1. Heparin suppresses mitogenic response of RMC

Heparin was shown to suppress RMC proliferation in vivo and in vitro. To test the effect of heparin on the mitogenic responsiveness of RMC we studied \(^{[\text{H}]\text{thymidine}}\) incorporation as described above for collagen. When released from quiescence with serum RMC increased their \(^{[\text{H}]\text{thymidine}}\) incorporation peaking between 16 and 18 h, suggesting entry of cells into S phase of the cell cycle (Fig. 7). In the experiment shown in Fig. 27 quiescent RMC had a \(^{[\text{H}]\text{thymidine}}\) incorporation of 0.2 ± 0.07 cpm (n=3), indicating a very low level of
Table 5 – Agonist-dependent cytosolic free Ca²⁺ signaling in RMC.

RMC were grown on Aclar coverslips without or with collagen type I coating. When cultures reached 90% confluence, they were loaded with fura 2-AM (2 μM) and monitored for changes in [Ca²⁺]ᵢ after treatment with arginine vasopressin (AVP, 0.1 μM), angiotensin II (ANG-II, 2.5 μM), or endothelin-1 (ET-1, 0.1 μM). Values are mean ± SD from 4 independent experiments. * P = 0.001, significantly different from plastic.

<table>
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<th>Substratum</th>
<th>Baseline</th>
<th>AVP</th>
<th>ANG-II</th>
<th>ET-1</th>
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<tr>
<td>Plastic</td>
<td>76 ± 12</td>
<td>130 ± 6</td>
<td>138 ± 11</td>
<td>534 ± 192</td>
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<tr>
<td>Collagen type I</td>
<td>72 ± 14</td>
<td>135 ± 24</td>
<td>132 ± 28</td>
<td>1017 ± 184*</td>
</tr>
</tbody>
</table>
Table 6 – Effect of collagen type I on serum-dependent Ca^{2+} signaling.

RMC were passaged and grown on plastic or collagen type I-coated Aclar coverslips until they reached 90% confluence. Cells were then loaded with fura 2-AM, treated with 5% NuSerum, and [Ca^{2+}]_{i} was monitored as described in Table 5. NS, not significant.

<table>
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<th>SURFACE</th>
<th>[Ca^{2+}]_{i} (nM)</th>
<th>P vs. plastic</th>
<th>n</th>
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<tr>
<td></td>
<td>baseline</td>
<td>peak</td>
<td></td>
</tr>
<tr>
<td>Plastic</td>
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<td>280 ± 47</td>
<td>-</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>86 ± 26</td>
<td>286 ± 27</td>
<td>(NS)</td>
</tr>
</tbody>
</table>
DNA synthesis. Eighteen hours after activation with 5% serum, as expected, a 20-fold increase in \[^{3}H\]thymidine incorporation was observed (4.4 ± 0.2 cpm, P<0.001 versus quiescent; n=5). When heparin was present with serum it significantly suppressed DNA synthesis at most concentrations tested. At a concentration of 1.0 μg/ml, heparin suppressed \[^{3}H\]thymidine incorporation by about 75% (Fig. 27, lane 3, P<0.001 versus serum-treated cells; n=3). DNA synthesis was affected to a similar extent by 0.4 and 0.2 μg/ml heparin (Fig. 27; lane 4 & 5; P<0.001 versus serum-treated cells in both cases; n=3). Further dilution of heparin resulted in a lowering of the suppressive effect on DNA synthesis. At concentrations of 0.1 and 0.05 μg/ml, heparin suppressed \[^{3}H\]thymidine incorporation by 36% (Fig. 27, lane 6; P < 0.01 versus serum treated cells; n=3) and 32% (Fig. 27, lane 7; P = 0.010 versus serum treated cells; n=3), respectively, while at a concentration of 0.025 μg/ml heparin did not have a significant effect. These results suggest that heparin at a concentration of 1 μg/ml is a potent inhibitor of RMC mitogenic response and is still effective at a concentration as low as 50 ng/ml, although at lower potency.

3.2.2. Heparin suppresses c-fos induction of RMC

The initial genetic response of cells to growth stimuli is induction of immediate early response genes, such as c-fos and c-jun (Fig. 45, 579, 794-796). Because heparin suppressed the mitogenic response of RMC, as determined by measuring the \[^{3}H\]thymidine incorporation, we wondered whether heparin would affect the induction of immediate early genes such as c-fos.

Quiescent cells (Fig. 28, lane 1) showed some basal steady state level of c-fos mRNA, that was several-fold increased when cells were treated either with serum (lane 4) or TPA (lane 6), consistent with previous work from our lab (637). The induction of c-fos was greatest when cells were activated with serum and to a lesser extent after treatment with TPA. The steady state c-fos mRNA level was not affected significantly when quiescent cells were treated with vehicle (DMSO; lane 2) or with heparin (lane 3) only. Heparin (1 μg/ml)
Fig. 27 - Effect of heparin on serum-dependent $[^3]$H]thymidine incorporation in RMC.

RMC were passaged into 12-well plates and rendered quiescent by exposing to RPMI1640 containing 0.4% FBS for 48 h. Cells were left untreated (0 time point, lane 1) or treated with 5% NuSerum for 18 h in the absence (S, lane 2) or presence of various concentrations of heparin (lanes 3 - 8). Cells were processed as described in Fig. 7, and samples were counted for incorporated radioactivity. Values represent mean ± SD from 3 - 5 separate experiments. ** P < 0.001, and * P 0.01, significantly different from serum treated cells only, by unpaired Student's t-test.
Fig. 28 - Effect of heparin on serum- and TPA-dependent c-fos induction in RMC.

Quiescent RMC were left untreated (lane 1), or treated with serum (lanes 4 and 5), TPA (lanes 6 and 7), or treated with heparin (lane 3) or with vehicle (DMSO, lane 2) only. To examine the effect of heparin on c-fos induction cells were released from quiescence with serum or TPA in the presence of heparin (lanes 5 and 7, respectively). After 30 min total RNA was extracted, and the level of c-fos mRNA and 18S rRNA were determined as described in Fig. 8. Northern blots are representative of 2 similar experiments.
suppressed both serum- and TPA-dependent c-fos induction by about 50% (lanes 5 & 7) consistent with previous results from mitogenic studies.

Because growth factors and vasoactive agents elicit similar initial responses including induction of early response genes, we wondered whether heparin would affect ET-1- or PDGF-dependent c-fos induction. Quiescent cells again showed low steady state c-fos mRNA levels (Fig. 29, lane 1). When cells were treated either with serum (lane 2), PDGF (lane 4), or ET-1 (lane 8), several fold increases of c-fos mRNA were observed (Fig. 29). The induction of c-fos was greatest when cells were activated with serum and PDGF. A much lower response was observed after activation of cells with ET-1, a potent vasoactive agent. Once again, heparin presence suppressed both PDGF- and ET-1-dependent c-fos induction by half.

To study the involvement of PKC in PDGF-dependent c-fos induction, some plates were pretreated with TPA for 24 h, a process known to down-regulate PKC activity, and then exposed to PDGF alone or in the presence of heparin. The TPA pretreatment suppressed by about 70% PDGF-induced c-fos mRNA whether heparin was present or not (Fig. 29, lanes 4-7), also suggesting that other pathways could be involved in this process. One such pathway could be through an increase in [Ca^{2+}]; since pretreatment of cells with BAPTA totally abolished the serum-induced increase of steady state c-fos mRNA levels (Fig. 29, lanes 2 and 3; and Fig. 30, lanes 3 and 4). Likewise, BAPTA-pretreated cells did not show observable c-fos induction after exposure to TPA or PDGF (Fig. 30, lanes 5-8). To further determine whether sustained elevation of [Ca^{2+}] was able to bring about c-fos induction, a Ca^{2+}-ionophore, ionomycin, was used. At a concentration of 100 nM, ionomycin caused a plateau in [Ca^{2+}] comparable to growth-eliciting stimuli (~380 nM; Table 7) which was sufficient to cause a several fold increase in c-fos induction (Fig 31, lane 3). As expected, pretreatment of cells with BAPTA totally abrogated ionomycin-dependent c-fos induction (Fig 31, lanes 3 and 4). Similarly, BAPTA inhibited endothelin-dependent induction of c-
Fig. 29 - Effect of heparin and BAPTA on c-fos induction in RMC.

Quiescent RMC were left untreated (lane 1) or treated with serum in the presence (lane 3) or absence (lane 2) of BAPTA, or treated with PDGF in the presence (lanes 6 and 7) or absence (lanes 4 and 5) of heparin, or treated with ET-1 in the presence (lane 9) or absence (lane 8) of heparin. For the purpose of down-regulating PKC activity some cells were pretreated with TPA for 24 h prior to exposure to PDGF or PDGF + heparin (lanes 5 and 7, respectively). Total RNA was extracted, and c-fos mRNA and 18S RNA levels were determined as described in Fig. 8. Northern blots are representative of 2 similar experiments.
Fig. 30 — Effect of BAPTA on c-fos induction

Quiescent RMC were left untreated (lane 1), pretreated with BAPTA for 30 min (lanes 2, 4, 6, and 8), or treated with serum (lanes 3 and 4), TPA (lanes 5 and 6), or PDGF (lanes 7 and 8). Total RNA was extracted and c-fos mRNA and 18S RNA levels were determined as described in Fig. 8. Northern blots are representative of 2 similar experiments.
Table 7 – Effect of heparin, BAPTA and kinase inhibitors on ionomycin-dependent Ca²⁺ signaling.

RMC were seeded onto Aclar coverslips, rendered quiescent, and loaded with fura-2-AM, followed by BAPTA-AM where indicated, with or without 30 min pretreatment with kinase inhibitors [KN-93 (50 μM), KT-5926 (80 nM), or ML-7 (30 μM)], as described in Methods. Cells were then treated with 100 nM ionomycin in the presence or absence of heparin (1 μg/ml), and [Ca²⁺]ᵢ was calculated from calibrated fluorescence tracings such as those shown in Fig. 24. Values are mean ± SD of n measurements. P is the level of significance obtained after comparing [Ca²⁺]ᵢ peaks of other samples to the cells treated with ionomycin only, by Student’s t-test.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>[Ca²⁺]ᵢ (nM)</th>
<th>P</th>
<th>vs. ionomycin alone</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>baseline peak</td>
<td></td>
<td></td>
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<tr>
<td>Ionomycin</td>
<td>65 ± 153</td>
<td>376 ± 58</td>
<td>-</td>
<td>9</td>
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<tr>
<td>Ionomycin + heparin</td>
<td>51 ± 26</td>
<td>405 ± 119</td>
<td>(NS)</td>
<td>7</td>
</tr>
<tr>
<td>Ionomycin + BAPTA</td>
<td>69 ± 9</td>
<td>74 ± 14</td>
<td>&lt; 0.001</td>
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</tr>
<tr>
<td>Ionomycin + KN</td>
<td>68 ± 32</td>
<td>369 ± 55</td>
<td>(NS)</td>
<td>3</td>
</tr>
<tr>
<td>Ionomycin + KT</td>
<td>58 ± 5</td>
<td>356 ± 71</td>
<td>(NS)</td>
<td>3</td>
</tr>
<tr>
<td>Ionomycin + ML</td>
<td>49 ± 43</td>
<td>367 ± 78</td>
<td>(NS)</td>
<td>3</td>
</tr>
</tbody>
</table>
Fig. 31 – Effect of BAPTA on ionomycin- and ET-1-dependent c-fos induction.

Quiescent RMC were treated with ionomycin (lanes 3 and 4) or with ET-1 (100 nM, lanes 5 and 6) with (lanes 4 and 6) or without (lanes 3 and 5) BAPTA preloading, and total RNA was extracted 30 min later. Control cells were left untreated (lane 1) or pretreated with BAPTA (lane 2). The level of c-fos mRNA was determined by Northern blotting and autoradiography. The intensity of the signal representing hybridization of the c-fos cDNA was normalized to the intensity of the 18S rRNA probe, determined by laser densitometry. The autoradiograph is typical of 2 separate experiments.
fosl, also suggesting an involvement of [Ca\(^{2+}\)]\(_i\) in this process (Fig. 31, lanes 5 & 6). As a control, BAPTA alone did not significantly change c-fosl expression (Fig. 30 and 31, lanes 2).

The above results suggested a requirement for Ca\(^{2+}\) ions in c-fosl induction. Therefore, we determined the effect of EGTA on serum-dependent \(^{3}H\)thymidine incorporation. As indicated in Fig. 32 quiescent cells had a very low level of thymidine incorporation (1.0 ± 0.1 \(\times\) 10\(^3\) cpm, \(n=4\)). As reported earlier, a strong induction of DNA synthesis was observed 18 h after cells were released from quiescence with serum (9.3 ± 0.4 \(\times\) 10\(^3\) cpm, \(P < 0.001\) serum treated versus quiescent cells, \(n=4\)). Serum-stimulated \(^{3}H\)thymidine incorporation was strongly suppressed by heparin (3.4 ± 0.4 \(\times\) 10\(^3\) cpm, \(P < 0.001\) versus serum treated cells, \(n=4\)), and totally abolished by the presence of EGTA (0.5 ± 0.1 \(\times\) 10\(^3\) cpm, \(P < 0.001\) versus serum treatment only, \(n=4\)), consistent with previous observations.

### 3.2.3. MAPK activation is suppressed by heparin

To investigate the mechanism of heparin's action we studied the effect of heparin on MAPK activity. Cytosolic extracts were immunoprecipitated with an anti-Erk-2 antibody and immunoprecipitates were tested for their ability to phosphorylate MBP in the presence of \([\gamma-\text{P}]\text{ATP}\).

MAPK activity was tested in cells treated with serum, TPA, or ionomycin to determine whether MAPK is involved in signaling by each agonist. Similar amounts of immunoprecipitable Erk-2 were recovered before and after each treatment (Fig. 33A). However, as expected, both serum and TPA caused an increase in immunoprecipitable MAPK activity while ionomycin failed to produce measurable activity (Fig. 33B). Heparin caused a decrease in MAPK activity induced by either serum or TPA (Fig. 33B), consistent with blockade of c-fosl inducing signals at or upstream of MAPK. Likewise, increased phosphorylation of activated MAPK is observed as a band with reduced electrophoretic
Fig. 32 - Effect of heparin and EGTA on serum-dependent [3H]thymidine incorporation in RMC.

RMC were seeded into 24-well plates in 1:3 split ratio and rendered quiescent. Cells were left untreated (lane 1) or treated with serum alone (lane 2) or serum in the presence of either 1 μg/ml of heparin (lane 3) or 2 mM EGTA (lane 4), and processed as described in the legend of Fig. 26. Values are mean ± SD from 4 separate experiments. * P < 0.001, significantly different from value of serum treated cells only.
Fig. 33 – MAPK activity in heparin-treated RMC.

Cell extracts were immunoprecipitated with anti-Erk-2 antibody after exposing quiescent cells to various reagents as described in the legend to Fig. 9. A): the Western blot of the immunoprecipitate run on a denaturing polyacrylamide gel shows a major band corresponding to Erk-2 (42 kDa) and a lesser band representing cross-reactivity to Erk-1 (44 kDa). The position of prestained molecular mass markers are indicated to the right. Cells were harvested untreated (lane 1) or after 2 min in the presence of 5% NuSerum (lane 3), 500 nM TPA (lane 5), or 100 nM ionomycin (lane 7) all with (lanes 2, 4, 6, 8, respectively) or without (lanes 3, 5, 7) heparin. The second lane was from an unstimulated control sample in which 1 μg/ml heparin was added to the immunoprecipitation reaction mixture to demonstrate that any residual heparin that was found in the samples from heparin treated cells did not interfere with immunoprecipitation. B): The immunoprecipitates were used to phosphorylate MBP in an in vitro assay as described in Fig. 9. The light band migrating ahead of the major band represents heterogeneity of MBP, as demonstrated on silver-stained gels of the starting material. C): Western blot of cytosolic extracts were probed with anti-Erk-2 antibodies. The prominent band at 42 kDa is Erk-2 and appears as a single band when it is not activated (lanes 1, 2, 4, 6 - 8), whereas enzyme activation causes appearance of an additional band due to increased phosphorylation and decreased mobility (lanes 3 and 5). The second, lighter band at 44 kDa arises from cross-reactivity of antibody with Erk-1. Control (lane 9) represents sample containing protein A-Sepharose + cytosolic extract of serum-treated cells without antibody. The figure is representative of 3 similar experiments.
mobility. Heparin inhibited the appearance of this second band in both serum- and TPA-treated cells (Fig. 33C). These results suggest that heparin suppresses c-fos induction through MAPK-dependent and independent pathways, because ionomycin failed to cause MAPK activation but did increase the steady state level of c-fos mRNA.

3.2.4. Effect of heparin on tyrosine phosphorylation

MAPK is activated in response to serum through activation of the p21ras complex after tyrosine phosphorylation of growth factor receptors (803, 804). Because heparin suppressed MAPK activation we asked whether heparin affects serum-dependent tyrosine phosphorylation.

After release from quiescence with 5% NuSerum RMC displayed a strong increase in tyrosine phosphorylation from basal levels of proteins running at > 200, 120, and 70 on SDS-PAGE, as determined by immunoblotting with an antiphosphotyrosine antibody (Fig. 34). A weak MAPK signal at 42 kDa is not obvious on the reproduction in Fig 34. Along with molecular mass markers a tyrosine-phosphorylated EGF receptor was included as a positive control [Fig. 34, EGFR, lane 1]. The intensity of phosphorylation was the highest between 2 and 5 min. (Fig. 34, lanes 4 - 8 ) and decreased thereafter (Fig. 34, lanes 10 - 13) in RMC grown on plastic. When cells were pretreated with genistein (50 μM) a tyrosine kinase inhibitor, the phosphorylation of proteins at > 200, and 70 kDa was inhibited, as was to a lesser extent that of a protein at 120 kDa, indicating a tyrosine kinase-dependent phosphorylation. The presence of heparin did not affect tyrosine phosphorylation of these three proteins (Fig. 34, lanes 3, 5, 8, 11, and 13).

3.2.5. Heparin affects CaMK activation

Another pathway to c-fos induction is through activation of CaMK-II. CaMK-II is quickly activated in response to elevations in intracellular Ca²⁺ and results in phosphorylation of CRE- and SRE-binding transcription factors (801). It was recently shown (749) that
Fig. 34 – Effect of heparin on tyrosine phosphorylation in RMC.

Quiescent RMC were activated with serum only (lanes 4 - 13) or in the presence of heparin (lanes 5, 8, 11, and 13), or after 30-min exposure to 50 μM genistein (lanes 6, and 9). Cells were harvested at the indicated times, and processed for SDS-PAGE and autoradiography as described in Fig. 13. Some cells were left untreated in the absence (lane 2) or presence (lane 3) of heparin, representing time = 0. Tyrosine-phosphorylated EGF receptor was included on the electrophoretic gel as a positive control (lane 1, EGFR) in addition to prestained molecular mass markers indicated to the right. The Western blot is representative of 3 similar experiments. For the purpose of comparison tyrosine-phosphorylated bands of each lane were subjected to densitometry and plotted as a bar graph. a) represents intensity of total tyrosine phosphorylation of all four bands in each lane, determined by densitometry.
Fig. 34

Intensity of all tyrosine phosphorylated bands

<table>
<thead>
<tr>
<th>Heparin</th>
<th>Genistein</th>
<th>time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>30</td>
</tr>
</tbody>
</table>

kDa 1 2 3 4 5 6 7 8 9 10 11 12 13 kDa

200
EGFR
120
70
48
205
118
48
heparin inhibited c-fos induction after cell activation with ionophores such as ionomycin and A23187. In addition, heparin suppressed serum- and TPA-dependent MAPK activation, while ionomycin did not affect MAPK activity. Therefore, we wanted to study the effect of heparin on CaMK-II activity, as a potential target of heparin's action. Heparin added to cells along with ionomycin suppressed the subsequent activation of CaMK-II (Fig. 35). However, it had no effect on total CaMK-II, or on the activation of CaMK-II by Ca^{2+} and calmodulin when present in the assay mixture in vitro (Fig. 35b). Therefore, while heparin inhibits the Ca^{2+}-dependent activation of CaMK-II in vivo, it does not appear to do so by interfering directly with activation of calmodulin by Ca^{2+}, or with association of the Ca^{2+}-calmodulin complex with CaMK-II.

We next examined a series of kinase inhibitors to implicate CaMK-II in c-fos induction. KT-5926 inhibits CaMK-II with a $K_i = 4$ nM, and also inhibits MLCK ($K_i = 18$ nM) and PKC ($K_i = 720$ nM) (805). It inhibited ionomycin-dependent c-fos induction in RMC with an IC_{50} of about 80 nM (Fig. 36). In contrast, the MLCK inhibitor, ML-7 ($K_i = 300$ nM for MLCK, $K_i = 42$ nM for PKC) has not been reported to have an inhibitory effect on CaMK (806), and did not affect ionomycin-dependent c-fos induction at the highest concentration tested (30 nM, Fig. 36 and 37). Consistent with their reported activities on PKC, KT-5926 (80 nM) did not affect phorbol ester-dependent c-fos induction, but ML-7 (30 nM) caused an approximately 50% decrease in c-fos mRNA expression after treatment with TPA (Fig. 37). After ionomycin treatment, KT-5926 inhibited CaMK-II activation (Fig. 35) and caused a strong decrease in the c-fos response, whereas ML-7 was almost without effect on c-fos (Fig. 37). KN-93, which shows high specificity for CaMK (807), inhibited the development of CaMK-II autonomous activity (Fig. 35). It did not affect TPA-induced c-fos expression, but strongly suppressed that due to ionomycin (Fig. 37). Interestingly, while KN-93 did not inhibit ionomycin-dependent total CaMK-II activity, KT-5926 inhibited both total and autonomous CaMK-II activity.
Fig. 35 – Effect of heparin and inhibitors on CaMK-II activity.

Autonomous (top panel) and total (bottom panel) CaMK-II activities was measured in quiescent and treated RMC as described in Experimental Procedures and the legend to Fig. 18. Without ionomycin, cells were treated with no additions (control), with DMSO (0.1 % v/v), solvent control for the inhibitors), or heparin (1 μg/ml). Other cells were treated with ionomycin (100 nM) alone or ionomycin with heparin (1 μg/ml). Some ionomycin-treated cells were pre-treated with the inhibitors KT-5926 (KT, 80 nM) or KN-93 (KN, 50 μM) for 30 min before addition of ionomycin. All cells were harvested 30 s after addition of DMSO, heparin, or ionomycin, for measurement of kinase activity. To determine whether heparin, potentially remaining as a contaminant in the cell extract of the heparin-treated cells, could interfere with substrate phosphorylation, it was added (1 μg/ml) directly to the assay reaction mixture of control ionomycin-treated cells (right-most bar, heparin (R)), and shown to be without effect. The values represent mean ± SD of 4 separate experiments.
Fig. 35

![Bar chart showing CaMK II activity (nmol/min/mg protein) for different treatments with and without ionomycin.](image)
Fig. 36 – Effect of kinase inhibitors on ionomycin-dependent c-fos induction.

Quiescent RMC were not pretreated or pretreated for 30 min with increasing concentrations of kinase inhibitors KN-93 (5 - 100 µM), KT-5926 (0.02 - 1 µM), and ML-7 (0.5 - 30 µM). Cells were then treated with ionomycin (IO, 0.1 µM) for 30 min, or left untreated, (C) and harvested for total RNA. The relative level of c-fos mRNA is presented as described in the legend to Fig. 8.
Fig. 37 — Effect of heparin and kinase inhibitors on induction of c-fos by ionomycin or TPA.

Quiescent RMC were pretreated for 30 min with kinase inhibitors [80 nM KT-5926 (KT), 50 μM KN-93 (KN), or 30 μM ML-7 (ML)] as indicated. Cells were then activated with either ionomycin (100 nM) or TPA (200 nM) in the presence (H, 1 μg/ml) or absence of heparin, or left untreated, and total RNA was collected 30 min later. The relative level of c-fos mRNA is presented as described in the legend to Fig. 8. The values are mean ± SD from 4 separate experiments. a, different from each other at P < 0.01; b, different at P < 0.001.
Because heparin and kinase inhibitors affected ionomycin-dependent c-fos induction, we
wanted to determine whether they would act by interfering with Ca^{2+} signaling. None of the
kinase inhibitors nor heparin affected ionomycin-dependent Ca^{2+} signaling (Table 7). None
of the inhibitors resulted in increased basal levels of c-fos mRNA or affected c-fos induction
by serum (Fig. 38), which is mediated by MAPK but does not involve activation of PKC
(749). Taken together, these studies show that under conditions of specific CaMK
inhibition, ionomycin fails to induce c-fos.

3.2.6. Effect of heparin on Ca^{2+} signaling

An increase in the concentration of cytosolic Ca^{2+}, elicited by extracellular stimuli, is
required for several cell functions including proliferation (4, 460) and contraction (Fig. 45,
588). The peak of [Ca^{2+}] is observed seconds after treatment with agonist and is
required for CaMK activation and increased c-fos state mRNA, as shown above. Because
growth factors and vasoactive agents cause an increase in cytosolic Ca^{2+} similar to that
achieved with ionophores, and because heparin was shown to suppress CaMK-II activity and
c-fos induction in response to the same agents, heparin's effect on cytosolic Ca^{2+} signaling
was tested.

For this purpose, cells were seeded onto Aclar coverslips and loaded with the cell-
permeable fluorescent dye fura 2/AM as in previous experiments. There is a characteristic
initial rise in [Ca^{2+}], followed by a plateau after agonist stimulation. The initial increase in
[Ca^{2+}] is due to the release of calcium from intracellular stores and is called transient or
phase I, whereas the sustained increase is dependent upon an increase in calcium entry into the
cell, and is called phase II. Stimulation of quiescent cells with serum caused a rapid rise in
[Ca^{2+}], from a base-line value of 111 ± 36 nM to a peak (phase I) of 316 ± 136 nM (Table
8). This was followed by a sustained signal (phase II), measuring 227 ± 73 nM at 75 s after
addition of serum. EGTA completely obliterated the sustained signal, [Ca^{2+}], returning to the
base-line value at 75 s, consistent with the interpretation that phase I represents mobilization
Quiescent RMC were pretreated with KT-5926 (80 nM), KN-93 (50 μM) or with ML-7 (30 μM) and then activated with 5% (v/v) NuSerum without or with heparin (1 μg/ml) for 30 min. In the absence of serum, DMSO (inhibitor solvent control), heparin, or inhibitors alone are shown not to induce c-fos. Autoradiograms of Northern blots and the relative c-fos mRNA level in histogram form are presented as described in the legend to Fig. 8. Values are mean ± S.D. (n=4). * different from serum-treated cells (P < 0.01).
Table 8 – Effect of heparin on serum- and TPA-dependent Ca²⁺ signaling

RMC were passaged onto Aclar coverslips, rendered quiescent, loaded with fura 2-AM, and [Ca²⁺]i was measured after treatment with NuSerum or TPA in the absence or presence of heparin (1 μg/ml), EGTA (2 mM), verapamil (VER, 8 μM), dextran sulfate (DS, 1 μg/ml), or chondroitin sulfate (CS, 1 μg/ml) as described in Table 5. Baseline refers to the value before any treatment. Phase I is the peak concentration reached after treatment, and the phase II concentration was calculated 75 s after the peak. The ratio of phase II to phase I is expressed as a percentage after subtracting the baseline from both values. All measurements are from at least 3 independent experiments and are expressed as mean ± SD. The P values were calculated by the unpaired Student’s t-test for comparison of the phase II/phase I ratio to that of serum- or TPA-treated cells.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>baseline</th>
<th>phase I</th>
<th>phase II</th>
<th>phase II/phase I (%)</th>
<th>P</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>111 ± 36</td>
<td>317 ± 136</td>
<td>227 ± 73</td>
<td>64 ± 21</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Serum + heparin</td>
<td>110 ± 40</td>
<td>304 ± 99</td>
<td>139 ± 40</td>
<td>25 ± 13</td>
<td>&lt; 0.001</td>
<td>12</td>
</tr>
<tr>
<td>Serum + EGTA</td>
<td>47 ± 1</td>
<td>132 ± 18</td>
<td>49 ± 1</td>
<td>2.0</td>
<td>&lt; 0.001</td>
<td>3</td>
</tr>
<tr>
<td>Serum + VER</td>
<td>78 ± 21</td>
<td>256 ± 19</td>
<td>131 ± 22</td>
<td>31 ± 9</td>
<td>&lt; 0.05</td>
<td>4</td>
</tr>
<tr>
<td>Serum + DS</td>
<td>97 ± 10</td>
<td>290 ± 123</td>
<td>174 ± 44</td>
<td>46 ± 15</td>
<td>(NS)</td>
<td>4</td>
</tr>
<tr>
<td>Serum + CS</td>
<td>137 ± 48</td>
<td>317 ± 172</td>
<td>228 ± 78</td>
<td>50 ± 17</td>
<td>(NS)</td>
<td>4</td>
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<tr>
<td>TPA</td>
<td>74 ± 7</td>
<td>173 ± 40</td>
<td>141 ± 39</td>
<td>88 ± 7</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>TPA + heparin</td>
<td>81 ± 6</td>
<td>178 ± 36</td>
<td>134 ± 31</td>
<td>52 ± 15</td>
<td>(NS)</td>
<td>3</td>
</tr>
</tbody>
</table>
of intracellular Ca\textsuperscript{2+} stores, while phase II depends on entry of extracellular Ca\textsuperscript{2+}. The dihydropyridine calcium channel antagonist, verapamil, diminished phase II without affecting phase I, also consistent with this interpretation. When heparin was added with serum, phase I did not change significantly (304 ± 89), but phase II was decreased from ~64% of the phase I value at 75 s to ~25% as compared to cells treated with serum alone. Dextran sulphate (DS), polyanionic control, and chondroitin sulphate (CS), a GAG control, did not have significant effects on the Ca\textsuperscript{2+} signal.

Stimulation of cells with TPA also caused a rapid and sustained increase in [Ca\textsuperscript{2+}], but of a more modest degree than produced by serum. Like the serum-dependent Ca\textsuperscript{2+} signal, the sustained phase at 75 s was quantitated against phase I. The presence of heparin did not significantly affect either the initial Ca\textsuperscript{2+} increase or phase II in TPA-treated cells (Table 8).

These results, in addition to those from Table 7 suggest that heparin suppresses serum-dependent Ca\textsuperscript{2+} influx, but does not affect the Ca\textsuperscript{2+} signal after treatment with ionophores.

To further assess the effect of heparin on Ca\textsuperscript{2+} signaling we used another ionophore, A23187 and the L-type Ca\textsuperscript{2+} channel agonist, BayK 8644. This time the sustained phase of the Ca\textsuperscript{2+} signal was not quantitated, because an increase in [Ca\textsuperscript{2+}] was due to influx of Ca\textsuperscript{2+} from the extracellular pool after addition of either agonist. Quiescent, fura-2-loaded RMC had basal [Ca\textsuperscript{2+}] between 48 ± 17 and 99 ± 5 nM (Table 9). Treatment with BayK 8644 caused a Ca\textsuperscript{2+} influx thereby raising [Ca\textsuperscript{2+}] to 295 ± 39 nM. When cells were pretreated with verapamil, an L-type Ca\textsuperscript{2+} channel blocker, the peak of [Ca\textsuperscript{2+}], was suppressed, averaging 136 ± 32 nM (P<0.001 versus BayK treated cells only, n=4), and almost identical to the effect of heparin: heparin added with BayK 8644 decreased the peak of [Ca\textsuperscript{2+}] to 137 ± 22 nM (P<0.001 versus BayK treated cells only, n=4). As expected, A23187-dependent [Ca\textsuperscript{2+}], peaking at 296 ± 82 was not suppressed by heparin. In fact, it was increased, averaging 448 ± 77 (P<0.05 versus A23187 treated cells only, n=4).
Table 9 — Effect of heparin on A23187- and BayK 8644-dependent Ca²⁺ signaling.

RMC were passaged onto Aclar coverslips, loaded with fura 2/AM, as described in the legend to Table 5, and [Ca²⁺]ᵢ was measured after treatment with BayK 8644 (10 µM) or A23187 (10 µM) in the presence or absence of heparin (1 µg/ml) or verapamil (VER, 8 µM), as indicated. All measurements are from at least 4 independent experiments and are expressed as mean ± SD. The P values were calculated by the unpaired Student’s t-test after comparison of the peak Ca²⁺ signal in the BayK 8644- or A23187-treated cells.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>[Ca²⁺]ᵢ (nM)</th>
<th>P</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>baseline</td>
<td>peak</td>
<td></td>
</tr>
<tr>
<td>BayK</td>
<td>75 ± 31</td>
<td>295 ± 39</td>
<td>-</td>
</tr>
<tr>
<td>BayK + heparin</td>
<td>48 ± 17</td>
<td>137 ± 22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BayK + VER (8µM)</td>
<td>58 ± 29</td>
<td>136 ± 32</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>A23187</td>
<td>93 ± 21</td>
<td>296 ± 82</td>
<td>-</td>
</tr>
<tr>
<td>A23187 + heparin</td>
<td>99 ± 5</td>
<td>448 ± 77</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
These results confirm our previous observations that heparin suppresses serum-dependent Ca\(^{2+}\) influx, and suggest that heparin does so by blocking L-type Ca\(^{2+}\) channels. On the other hand, heparin does not suppress ionophore-dependent Ca\(^{2+}\) signals, and may even increase the [Ca\(^{2+}\)]\(_{\text{in}}\) peak after activation with A23187 and to a lesser extent with ionomycin.

### 3.2.7. Effect of heparin on RMC contraction

Numerous growth factors and vasoactive agents promote both cell proliferation and elicit a contractile response. Commonly, both groups increase [Ca\(^{2+}\)]\(_{\text{i}}\) which leads to calmodulin-dependent activation of CaMK and MLCK, involved in proliferation and activation of actomyosin ATPase for contraction, respectively (Fig. 45). Because heparin suppressed the RMC mitogenic response, c-fos induction, and activation of CaMK-II we wondered whether heparin would affect RMC contractility. This was investigated with ET-1, a potent vasoactive agent treating MCs seeded onto collagen type I. Untreated cells or cells treated with heparin only, did not show a significant decrease in CSA (Fig. 39). Cells treated with ET-1 started decreasing their CSA as early as 2 min, reached a peak decrease of about 30% (Fig. 39, \(P<0.001; n=180\)) at 20 min, and then began to return to the original CSA. There was no difference in measured CSA between cells treated with ET-1 in the presence or absence of heparin. To test whether changes in CSA were true contractile responses, cells were exposed to KT-5926, an MLCK inhibitor. Pretreatment with KT-5926 totally obliterated the contractile response, indicating that MLCK-dependent contraction was occurring (Fig. 39).

### 3.2.8. Effect of heparin on myosin light chain phosphorylation

To further confirm that a contractile response was being observed, we tested the effect of heparin on MLCK phosphorylation, a prerequisite for activation of myosin ATPase and contraction. Cells were loaded with \(\text{[P}^{32}\text{P}\)orthophosphate and treated with ET-1 in the
RMC were plated onto collagen type I-coated culture dishes and allowed to attach. After preconditioning with Ca$^{2+}$-Mg$^{2+}$-free HBSS, contraction was measured in fresh serum-free HBSS, as described in Methods. Contraction was initiated at time zero by the addition of fresh HBSS (O), HBSS containing 1 µg/ml heparin (□), HBSS with 0.1 µM endothelin-1 (ET-1, ●), HBSS with 0.1 µM ET-1 plus 1 µg/ml heparin (■), or HBSS with 0.1 µM ET-1 plus 10 µM KT5926 (▲), all at 30°C. Each time point represents the mean ± SD of triplicate measurements of a total of 180 cells on images captured in 3 separate experiments.

**Fig. 39 – Effect of heparin on ET-1-induced RMC contraction.**
presence or absence of heparin or KT-5926. Cell extracts were subjected to pyrophosphate PAGE to isolate the myosin complex, followed by isoelectric focusing to determine phosphorylated MLC. Untreated cells showed a basal level of MLC phosphorylation (Fig. 40). Treatment with ET-1 caused an increase in MLC phosphorylation that was not suppressed by the presence of heparin but was strongly inhibited by pretreatment with KT-5926, again suggesting a MLCK-dependent phosphorylation.

Because cell contraction is a consequence of increased [Ca^{2+}]_{i} and subsequent MLCK activation by the Ca/calmodulin complex, we determined the effect of heparin on ET-1-dependent Ca^{2+} signals. ET-1 caused an abrupt increase in [Ca^{2+}]_{i} to a value of 570 ± 259 nM (Table 10). This transient increase was almost doubled when heparin was present (1039 ± 519 nM). In addition, the presence of heparin decreased the ratio of phase II/phase I, from 56% in control cells to 20% in the presence of heparin (P<0.005; n=4). This decrease is due to both a decrease in the second phase and an increase in the transient phase (Table 10). When EGTA was added with ET-1, it suppressed Ca^{2+} influx by 30%, as expected, bringing the phase II/phase I ratio to 27 ± 7% (p<0.03; n=3). Dextran sulfate (DexS, polyanionic control) and CS (GAG chain control) did not affect the ET-1-dependent Ca^{2+} signal (Table 10).

3.2.9. Does heparin scavenge oxygen radicals?

The above results indicate that heparin suppresses c-fos induction through at least two pathways, one MAPK-dependent, and the other CaMK-II-dependent. In both cases heparin acts by diminishing the activation of the corresponding kinase. This could be achieved in several ways. One possible mechanism is activation of phosphatases, which would in turn dephosphorylate kinases and thereby inactivate them. Because reactive oxygen species inactivate phosphatases, and because they were shown to be necessary for PDGF-dependent MAPK activation and subsequent c-fos induction (808), we postulated that heparin could scavenge oxygen radicals and thereby exert an inactivating check upon phosphatases.
Fig. 40 - Effect of heparin on ET-1-dependent myosin light chain phosphorylation.

RMC were grown in RPMI 1640 medium supplemented with 10% FBS. Subconfluent cultures were incubated with [32P]orthophosphate for 3 h and then treated with 100 nM ET-1 at time zero, with or without heparin (1 μg/ml) or KT-5926 (10 μM) as indicated. Myosin complex was extracted and myosin light chain was separated by isoelectric focusing and autoradiography (top panel) as described in Methods. Isoelectric point standards are shown by the arrows to the left of the Figure. The relative intensities of the phosphorylated myosin light chain bands, as determined by laser densitometry, are shown in the histogram, relative to the sample with ET-1 alone taken as 100%. The bars show the mean ± SD from three experiments.
Table 10 – Effect of heparin on endothelin-1-dependent Ca\textsuperscript{2+} signaling.

[Ca\textsuperscript{2+}]\textsubscript{i} was measured in fura 2-loaded RMC after treatment with ET-1 (100 nM) in the absence or presence of heparin (1 \mu g/ml), dextran sulfate (DexS, 1 \mu g/ml), chondroitin sulfate (CS, 1 \mu g/ml), or EGTA (2 mM) from tracings similar to those in Fig. 25 as described in Methods. Baseline, phase I, and phase II of the Ca\textsuperscript{2+} signal, and the ratio of phase II/phase I were calculated as described in the legend to Table 8. All measurements are from at least 3 independent experiments and are expressed as mean \pm SD. The P values were calculated by the unpaired Student's t-test for comparison of the phase II/phase I ratio to the ET-1-treated cells.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>baseline (nM)</th>
<th>phase I (nM)</th>
<th>phase II (nM)</th>
<th>phase II/phase I (%)</th>
<th>P</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>119 \pm 14</td>
<td>573 \pm 259</td>
<td>317 \pm 54</td>
<td>56 \pm 18</td>
<td>&lt;0.05</td>
<td>5</td>
</tr>
<tr>
<td>ET-1 + heparin</td>
<td>103 \pm 19</td>
<td>1040 \pm 519</td>
<td>266 \pm 94</td>
<td>19 \pm 7</td>
<td>(NS)</td>
<td>4</td>
</tr>
<tr>
<td>ET-1 + DexS</td>
<td>116 \pm 9</td>
<td>349 \pm 94</td>
<td>260 \pm 103</td>
<td>58 \pm 18</td>
<td>&gt;0.05</td>
<td>4</td>
</tr>
<tr>
<td>ET-1 +CS</td>
<td>128 \pm 1</td>
<td>735 \pm 349</td>
<td>425 \pm 178</td>
<td>50 \pm 8</td>
<td>(NS)</td>
<td>4</td>
</tr>
<tr>
<td>ET-1 + EGTA</td>
<td>224 \pm 119</td>
<td>527 \pm 325</td>
<td>227 \pm 32</td>
<td>28 \pm 7</td>
<td>&lt;0.03</td>
<td>3</td>
</tr>
</tbody>
</table>
3.2.9.1. Do H$_2$O$_2$ and catalase alter heparin's effect on RMC?

-on the mitogenic response of RMC

To test this idea H$_2$O$_2$ and catalase, an enzyme that destroys H$_2$O$_2$, were used in addition to heparin. First we determined the effect of H$_2$O$_2$ and catalase on the mitogenic response of RMC. As described earlier, in a typical experiment for studying RMC mitogenic response, serum activation of quiescent RMC caused a characteristic burst of DNA synthesis from $1.1 \pm 0.2 \times 10^3$ cpm (n=4) in quiescent cells to about $8.6 \pm 0.8 \times 10^3$ cpm at 18 h after serum treatment (Fig. 41, P<0.001 versus quiescent cells, n=4). H$_2$O$_2$ did not induce [H]thymidine incorporation in RMC at any concentration that was used, starting from 200 pM up to 30 mM (Fig. 41, P<0.001 for all concentrations versus serum treated cells, n=4). It even decreased [H]thymidine incorporation at 0.2 and 0.5 mM, consistent with induction of apoptosis in MC (809). Next, we tested the effect of H$_2$O$_2$ at the lowest concentration (200 μM) on serum-induced DNA synthesis. Once again, serum treatment caused a strong increase in [H]thymidine incorporation from $0.9 \pm 0.2 \times 10^3$ cpm (n=4) in quiescent cells to $9.3 \pm 0.4 \times 10^3$ cpm 18 h after activation with serum (Fig. 42, P<0.001, n=4). When heparin was present with serum, the characteristic suppressive effect on DNA synthesis was observed lowering [H]thymidine incorporation to $3.2 \pm 0.3 \times 10^3$ cpm (Fig. 42, P<0.001, n=4). H$_2$O$_2$ obliterated serum-dependent DNA synthesis averaging $0.2 \pm 0.05 \times 10^3$ cpm (Fig. 42, P<0.001, n=4). This suppressive effect of H$_2$O$_2$ was partially reversed by cell pretreatment with catalase (3000 U/ml) allowing $3.8 \pm 0.1 \times 10^3$ cpm of [H]thymidine incorporation (Fig 42, P<0.001 versus serum treated cells, n=4).

Overall, these results suggest that the ability of heparin to inactivate various kinase cascades is not due to scavenging H$_2$O$_2$. Furthermore, H$_2$O$_2$ inhibits DNA synthesis in RMC, possibly due to its ability to induce apoptosis (809). The partial reversal of H$_2$O$_2$-dependent inhibition of [H]thymidine incorporation by catalase pretreatment suggested some...
RMC were passaged into 24-well plates and rendered quiescent. Cells were left untreated (lane 1), or treated with increasing concentrations of \( \text{H}_2\text{O}_2 \) (0.2 - 30 mM, lanes 2 - 6), or with 5% NuSerum (S, lane 7) for 18 h and tested for \(^3\text{H}\)thymidine incorporation as described in Fig. 7. Values are the range of measurements from 2 experiments.
Fig. 42 – The effect of H$_2$O$_2$, catalase, and heparin on serum-dependent mitogenic response of RMC.

Quiescent RMC were left untreated (lane 1) or treated with serum (lanes 2 - 5) for 18 h and processed to test [H]$^3$[J]thymidine incorporation as described in Fig. 7. In addition to serum, some cells were exposed to heparin (H, lane 3), or to H$_2$O$_2$ (200 µM, lanes 4 & 5) to test their effect on serum-stimulated mitogenic response. Cells were also pretreated with catalase (3000 U/ml) for 2 h and then medium was changed with one that contains serum and H$_2$O$_2$ together as indicated in lane 5. Values are the range of measurements from 2 separate experiments.
uptake of the enzyme by RMC.

Because the inhibitory effect of H2O2 on serum-dependent DNA synthesis in RMC was partially reversed by cell pretreatment with catalase we sought to study the effect of catalase on MAPK activity and c-fos induction.

-on MAPK activity

As described above, the ability of anti-Erk-2 antibody immunoprecipitates to incorporate \(\gamma\)-\(^{32}\)P\)ATP into MBP was tested after different treatments of RMC. Cells were activated with serum or TPA in the presence of heparin and/or after pretreatment with 3000 U/ml catalase. Non-activated cells or cells treated with catalase only showed some basal level of MAPK activity (Fig. 43, lanes 1 and 2). Very little or no activity was observed when antibodies were absent from the reaction mixture, or when the assay was performed without cytosolic extract (Fig. 43, lanes 10 and 11). However, MAPK activity was increased several fold when cells were activated with serum or TPA (Fig. 43, lanes 3 and 7). Cell pretreatment with catalase did not change serum- or TPA-dependent MAPK activation (Fig. 43, lanes 5 and 9), while the characteristic decrease in MAPK activity was observed when heparin was present with either serum or TPA (Fig. 43, lanes 4 and 8). Although catalase pretreatment did not affect serum- or TPA-dependent MAPK activity, it seems that catalase enhanced heparin's suppressive effect on serum-dependent MAPK activity (Fig. 43, lane 6).

-on c-fos induction

Finally, the effect of catalase on c-fos induction was studied. Little or no increase in steady state c-fos mRNA levels was observed in quiescent cells or cells treated with catalase only (Fig. 44, lanes 1 and 2). Release from quiescence caused a strong induction of c-fos in serum- and TPA-treated RMC (Fig. 44, lanes 3 and 7). As expected, heparin suppressed c-fos mRNA levels by 50 % after both treatments (Fig. 44, lanes 4 and 8). When cells were pretreated with catalase c-fos induction was suppressed after activation with both serum and
Quiescent RMC were pretreated with catalase (3000 U/ml) for 2 h, or left without treatment. They were then exposed for 2 min to serum or TPA in the presence or absence of heparin as indicated, and processed as described in Fig. 9. Immunoprecipitates of cytosolic extracts with anti-Erk-2 antibody, from each sample, were tested for their ability to phosphorylate MBP in vitro, and visualized by autoradiography after protein separation on SDS-PAGE. In order to test for non-specific phosphorylation of MBP, protein-A-Sepharose and antibody together (lane 11) were tested, in addition to a sample containing protein-A-Sepharose plus cytosolic extract of cells treated with serum only (antibody was omitted, lane 10). The histogram was obtained by integration of the autoradiogram using laser densitometry. The autoradiogram is representative of 2 similar experiments.
Quiescent RMC were pretreated with catalase as described in Fig. 41 and then activated with 5% (v/v) NuSerum or TPA (200 nM) in the presence or absence of heparin (1 μg/ml) for 30 min. In the absence of serum or TPA, catalase alone is shown not to induce c-fos. Autoradiograms and the scanning of Northern blots and the relative c-fos mRNA level are shown as described in the legend to Fig. 8. The autoradiogram is representative of 2 experiments.
TPA, but to a lesser extent than in cells treated with heparin (Fig. 44, lanes 5 and 9). c-fos induction was not affected by pretreatment with catalase, and catalase did not affect heparin's suppressive effect on the steady state mRNA level of c-fos (Fig. 44, lanes 6 and 10). Therefore, catalase affected neither cytosolic events such as the activation of MAPK, nor nuclear events such as transcription, while heparin affected both, suggesting that heparin does not exert its effects by scavenging H$_2$O$_2$. 
4. DISCUSSION

The ECM expansion in human or rat glomerulonephritis is indicative of a disturbed balance between synthesis and catabolism. Numerous studies implicate TGF-β as a major contributor to the ECM expansion. TGF-β is expressed in a variety of experimental and human glomerular diseases in which ECM deposition is evident (5, 285). It stimulates collagen, fibronectin, proteoglycan, and PAI synthesis in cultured MCs (5). More importantly, the inhibition of TGF-β by neutralizing antibody (287) or decorin (289) prevented matrix expansion in the Thy-1 model. In addition to TGF-β, ECM synthesis can be initiated by other cytokines such as PDGF B-chain (4), platelet activating factor (296), IL-1 (292), IL-6 (293), and vasoactive agents such as ET-1 (295). There also might be inhibition of matrix-degrading proteases due to upregulation of TIMP (641) or PAI-1 (642). The composition and organization of ECM modulate cell phenotype including proliferation, adhesion, and gene expression (33). MC in culture possess similar phenotypic characteristics to MCs in vivo during glomerulonephritis (431). Therefore, cultured MCs have been used as a model for pathological matrix accumulation (772) and for studying the proliferative phenotype of MCs (431). Likewise, cultured RMC were used as a model for pathological changes of cell phenotype in this study. In the present study, MC growth on plastic was compared to that on collagen type I-coated dishes, or on plastic but in heparin-supplemented medium and sometimes in the presence of collagen type I. It was the aim of this study to establish the effects of these treatments on MCs, and to elucidate the mechanisms of the effects.

4.1. HEPARIN

4.1.1. mitogenic response

The effects of heparin on RMC growth were studied using serum-starved cells because they enter the cell cycle synchronously after stimulation. An exposure to 0.4% FBS for 48 h
was proven to render RMC quiescent. It was shown by Wang and Templeton (637) that 76% of cells were in G0/G1 phase 48 h after exposure to 0.4% FBS, and 72% of cells were in S phase 18 h after activation with 5% NuSerum. Consistent with these results, cells in the present study, when exposed to 0.4% FBS showed characteristics of a quiescent phenotype, that is, very low [3H]thymidine incorporation (at least five-fold lower than that of activated cells), a low steady state level of c-fos mRNA, and low levels of kinase activities and tyrosine phosphorylation. All these parameters increased at least two-fold upon cell activation. Therefore, after 48 h of serum starvation RMC are quiescent and re-enter the cell cycle with good synchrony upon stimulation. Using this serum-starved model to study synchronous cell cycling it was possible to investigate the antimitogenic properties of heparin.

The mitogenic response of activated MC was studied by measuring incorporation of radioactivity after exposing cells in different stages of the cell cycle to [3H]thymidine-supplemented medium. Heparin at a concentration as low as 50 ng/ml suppressed incorporation of [3H]thymidine by RMC by 30%, and suppressed nearly maximally at 1 μg/ml (about 70% inhibition). As suggested previously (637), this low concentration of heparin was added at the time of serum stimulation of growth-arrested RMC in this study to achieve maximal inhibition. These results are comparable to those of Wang and coworkers (636, 637) who showed that heparin at a concentration of 0.1 μg/ml suppressed [3H]thymidine incorporation by 25%. However, heparin was without effect on the mitogenic response at 50 ng/ml in their study. This observation, together with the fact that heparin at a concentration of 1 μg/ml suppressed the mitogenic response by about 50% in their study, suggests that some differences may arise from experimental design or cell sensitivity to heparin or different samples of heparin. In any case these results emphasize and support previous observations that MC and SMC are sensitive to heparin's antiproliferative effect (636, 653). Other cells were reported to require at least 200 μg/ml of heparin to show a similar effect (653). The present study, in addition to that of Wang and Templeton (637),
shows that heparin can exert an antimitogenic effect on RMC at a concentration as low as 0.05 - 0.1 μg/ml, which is comparable to the levels of heparin-like molecules occurring in vivo (810). At high concentrations (100 μg/ml), however, heparin could be added up to 18 h after serum stimulation and still show antiproliferative effect on SMC (653).

4.1.2. c-fos induction

Wright et al. (736) examined the effects of heparin on the expression of c-fos and c-myc in murine fibroblasts and reported that heparin decreased serum-induced expression of both mRNAs. In contrast, Reilly and colleagues (658) found that 100 μg/ml heparin had no significant effect on the induction of either gene in VSMC. However, it was subsequently shown that heparin in very high concentration (200 μg/ml) can suppress c-fos and c-myc mRNA levels in VSMC. In the present study, heparin at a concentration of 1 μg/ml suppressed induction elicited either by serum, PDGF, TPA, ET-1, or ionomycin by about 50%. That is comparable with results of Wang and Templeton (637) who established that heparin can suppress c-fos induction in SMC and RMC at a concentration lower than 10 μg/ml, and consistent with results of antimitogenic activity at this low concentration. This is also consistent with the K_d for binding of heparin to cells. According to previous reports heparin binds to most cells with K_d values between 10^{-8} - 10^{-10} M (637, 719, 734, 811, 812, 813), suggesting that the concentration of heparin that effectively inhibits c-fos should be lower than 10 μg/ml.

The ultimate target of many mitogen-activated pathways is transcriptional activation of a specific set of genes (814). c-fos, c-jun, and c-myc are immediate-early genes (IEG), members of a class of genes that are rapidly induced when resting cells are treated with mitogens, suggesting that they are involved in a cascade that initiates cycling. They are expressed during the G_0 → G_1 transition of the cell cycle and encode nuclear proteins that are thought to be necessary for cell proliferation (815-817). Antibody microinjection (818) and antisense experiments with fibroblasts (819, 820) suggest that the induction of both c-fos and
c-jun genes is important for mitogenesis, although the two genes are not always induced coordinately (821). Bruselbach et al. (822) showed a functional redundancy among AP-1 family members. They reported no difference in proliferation and reentry of the cell cycle by quiescent fibroblasts or embryonic stem cells lacking c-fos when compared to control cells (822). Induction of IEG expression is rapid, robust, and widespread, and does not require protein synthesis, indicating that posttranslational modification of preexisting proteins is sufficient to activate IEG expression. The distinct pattern of IEG expression leads to differential programs of secondary gene expression and distinct cellular responses. For instance, the link between the induction of c-fos and cell proliferation is shown by the timing of events after activation with serum. A half to one hour exposure of quiescent RMC to serum resulted in peak c-fos expression (present study and (637)). Continued exposure to serum caused a burst of [3H]thymidine incorporation between 16 and 18 h (present study and (636)), indicative of cells entering S phase, and was followed by at least a doubling of cell number after three days (685, 700, 766). Therefore, c-fos induction was used in this study as an indication of cells reentering the cell cycle after release from quiescence, and served as a means of studying the mechanisms by which extracellular signals generated at the plasma membrane activate transcription within the nucleus.

All the transcription factors that can lead to oncprotein induction are members of the regulatory class, able to induce transcription of genes whose promoters or enhancers carry appropriate response elements, but not necessary for basal level transcription (823). For example, the c-fos gene is regulated by calcium at the level of both transcriptional initiation and elongation. Using nuclear run-off transcription analysis, it has been shown that c-fos transcription is already initiated to some extent in unstimulated cells, but elongation of the nascent transcripts is blocked within the first intron. This block can be inductibly released by a calcium-dependent mechanism in response to a number of agents (824). AP-1 target sites are found in a large number of genes (823) and it is usually the case that an AP-1 target site confers upon a gene the ability to be activated by AP-1. The proteins encoded by c-fos and c-
jun are constituents of the AP-1 transcription factor complex that includes several other Fos-related (Fra-1, Fra-2, FosB and DFoxO) and Jun-related (JunB and JunD) proteins (825, 826). These proteins form dimeric complexes, Fos-Jun, heterodimer or Jun-Jun, homodimer. The highest binding affinity is between the heterodimeric complex and the TPAr responsive element. Dimer formation, which is essential for binding to DNA, is totally dependent on the leucine zipper dimerization motif on both proteins. Activated MAPK phosphorylates the Ets-like transcription factor Elk, forming part of the transcription factor complex that binds to the serum response element (SRE) in the c-fos promoter region (Fig. 45)(801, 827-830). The SRE, located at -300 relative to the transcription start site, is also involved in c-fos induction in response to Ca^{2+} (831). The inner core of the SRE, the CArG box, binds the transcription factor, serum response factor (SRF) (830). SRF binding is believed to be critical for serum stimulation of c-fos transcription because mutations within the SRE that abolish SRF binding also render the SRE unable to mediate serum induction of c-fos transcription (832). Elk-1 interacts with the c-fos SRE only if SRF is already bound through a CAGAT sequence, forming a ternary complex (830). In response to Ca^{2+}, CaMKII phosphorylates the SRF at Ser103 (833) enhancing its binding to the SRE (834). Miranti et al. (835) showed that the SRF complex bound at the SRE is the target of both CaMK-dependent and Ras-dependent signaling pathways and that they are distinct mechanisms by which activation of the SRF complex leads to c-fos induction. c-fos induction by increased [Ca^{2+}] through cAMP response element (CRE) however, is distinct from the response to growth factors (836); Ca^{2+} and cAMP activate c-fos transcription through CREB.

There are at least two post-receptor pathways involved in the induction of c-fos (837, 838). One pathway is dependent on PKC and is activated by bFGF, PDGF, bombesin, phorbol esters, and thrombin. The other pathway is dependent on cAMP and calcium, and is activated by EGF. Both are potential targets of heparin. Heparin effectively blocked the mitogenic response of PDGF, bFGF, and phorbol esters that act via PKC (654). It failed to inhibit c-fos expression in cells in which PKC was down-regulated (839), and selectively
inhibited PKC-dependent mechanisms of cell cycle progression in SMC (654, 659, 736, 839). Similarly, in the present study the induction of c-fos by PDGF was strong and comparable to that elicited by serum. Like serum, induction by PDGF was sensitive to heparin and BAPTA, and unlike serum, down regulation of PKC inhibited PDGF-dependent c-fos induction, consistent with PKC-dependence of PDGF-stimulated signal transduction pathways. In contrast, induction of c-fos by EGF has been shown not to involve PKC, and is insensitive to heparin (839), consistent with heparin acting on a PKC-dependent pathway. Heparin failed to block EGF-induced mitogenesis in SMC and its antiproliferative effects could be reversed by EGF (654, 840). This ability of EGF to reverse heparin inhibition was only observed when mitogen and heparin were added together. If SMC were pretreated with heparin for 48 h before addition of EGF, the number of EGF receptors on the cell surface was decreased and the protective effects of EGF were lost (655). On the other hand, Revis-Gupta et al. (841) found that heparin can stimulate EGF receptor-mediated phosphorylation of protein targets. Additional non-PKC-dependent pathways of c-fos induction were similarly insensitive to heparin, including those stimulated by dibutyril cAMP, 3-isobutyl-1-methyl-xanthine, and the Ca²⁺ ionophore A23187 (839).

Clapham et al. (802) showed that raised [Ca²⁺], was necessary for events associated with mitosis and cell division, and Perez-Terzic et al. (842) demonstrated that increased [Ca²⁺], was needed for the opening of nuclear membrane pores through which transcription factors and signaling peptides translocate to the nucleus. Collart et al. (824) suggested a key role of calcium-responsive element in the control of c-fos gene transcription. The induction of c-fos with any agent tested was abolished by BAPTA in the present study suggesting that calcium regulates a key mechanism either early in a signaling cascade, or later at the level of gene expression. Additionally, EGTA, an extracellular chelator of Ca²⁺, inhibited the serum-dependent mitogenic response. This result is comparable to that reported by Whiteside et al. (843) where we showed that BAPTA inhibited ET-1-, PDGF-, and serum-dependent [³H]thymidine incorporation. An increase in nuclear [Ca²⁺] followed the rise in cytosolic
[Ca^{2+}]_{i}\) after treatment of RMC with ET-1, PDGF, serum or ionomycin, and BAPTA inhibited both cytosolic and nuclear increases in [Ca^{2+}]_{i} (843). Collectively, this suggests that both transcription factors, CREB and SRE, induced c-fos expression in response to increased [Ca^{2+}]_{i}. CREB can function as a nuclear calcium-responsive transcription factor, whereas SRE mediates transcription by raising [Ca^{2+}]_{i} and does not require an increase in nuclear Ca^{2+} (844). While verapamil, an L-type Ca^{2+} channel blocker, did not affect serum-dependent c-fos induction (749), Pribnow et al. (845) reported that chelation of extracellular Ca^{2+} inhibited c-fos induction by ET-1 in fibroblasts. In the present study, BAPTA blunted c-fos induction in response to ET-1, and heparin suppressed it by 50%. Wang and Simonson (846) have demonstrated that Ca^{2+} influx through voltage-insensitive Ca^{2+} channels was essential for ET-1-elicited activation of CaMK-II and c-fos induction in MCs. This above observation together with the fact that heparin caused an increase in [Ca^{2+}]_{i} after treatment with ET-1, and that heparin suppressed CaMK-II activation stimulated by ionomycin, suggest that heparin suppresses ET-1-dependent c-fos induction through the CaMK pathway.

4.1.3. MAPK

The effect of heparin on MAPK activity was tested by an in vitro assay and also by its ability to prevent a decreased electroforetic mobility after activation of the enzyme. Yan and Templeton (847) and others (848, 849) showed that overexpression of constitutively active MEK decreased the electroforetic mobility of MAPK due to increased phosphorylation, with the appearance of a second band representing polyphosphorylated protein. A similar decrease in mobility of MAPK was observed by Bokemeyer et al. (850) in fibroblasts after activation with serum or TPA. The same phenomenon was observed in the present study. However, enzyme activation was inhibited by heparin. Bearing in mind the involvement of MAPK in c-fos induction, heparin's inhibition of MAPK activation by both serum and TPA can therefore account for the inhibition of c-fos induction by either stimulus. These results
are similar to those of others (748, 766) who reported that a high concentration of heparin (200 μg/ml) inhibited phosphorylation of MAPK stimulated by serum or TPA in VSMC. Because heparin does not affect activation of PKC with TPA, as measured by either histone H1 (637) or the EGF receptor peptide (749) as a substrate, it seems likely that heparin acts at a point after the serum and phorbol ester pathways converge, for instance at the level of Ras or below, to suppress activation of MAPK. Indeed, two recent publications on VSMC indicated that heparin was effective at points upstream of MAPK. One indicated that heparin suppressed serum-dependent MAPK activation by preventing activation of MAPKK-1 (851), and another reported that heparin suppressed PDGF-dependent raf activation (766). Heparin does not affect MAPK activity directly and in vitro, by adding it directly to kinase assay, can actually increase activation of MAPK at concentrations above 5 μg/ml (852).

In addition to affecting c-fos induction through the MAPK-dependent pathway, the present study also indicates that heparin is affecting it through a MAPK-independent pathway, but one dependent on an increase in [Ca^{2+}],. Calcium ionophores, at concentrations causing an increase in [Ca^{2+}], comparable to physiological agents, caused a rapid increase in c-fos mRNA that was sensitive to the presence of heparin, however, they do not activate PKC (749) or MAPK (present study). This result is comparable to that of Kröben et al. (853) who reported that an AVP-dependent increase in [Ca^{2+}], (700 nM) caused PKC-dependent, but Ca^{2+}-independent MAPK activation. Submaximal levels of ionomycin and thapsigargin, which resulted in intracellular [Ca^{2+}], levels comparable to those produced by AVP, did not induce comparable activation of MAPK (853). A higher concentration of ionomycin (1 μM) resulted in stimulation of MAPK comparable to AVP and was inhibited by down-regulation of PKC; AVP-dependent activation of MAPK was not affected by preloading cells with BAPTA (853).

The c-fos promoter contains a CRE 60 nucleotides upstream of the transcription start site that confers responsiveness to both cAMP and Ca^{2+} through CREB (801, 854, 855).
CaMK-II and cAMP/PKA signaling pathways converge at the level of CREB phosphorylation on serine 133 to activate c-fos transcription (855-857). A novel CREB kinase present in both cytoplasmic and nuclear extracts of PC12 cells can also phosphorylate serine 133 (858). CREB may also be phosphorylated by CREB kinase that is activated by Ca\(^{2+}\) entry through voltage-sensitive Ca\(^{2+}\) channels in some cells (801, 858). The present study and that of Wang and Templeton (749) show that induction of c-fos by ionomycin or by A23187 alone is heparin-sensitive. This suggests that inhibition of c-fos by heparin in MC could be through CaMK-dependent signaling since this enzyme readily responds to an increase in cytosolic [Ca\(^{2+}\)]. However, activation of PKA can also lead to phosphorylation of CREB. PKA-dependent c-fos induction was insensitive to the presence of heparin (839), suggesting to a different mechanism. Nevertheless, regulation through the SRE is sufficient to account for all observations noted above. In keeping with the idea that SRE is the more important element in these studies, it was noted that Ca\(^{2+}\) induction of c-fos via the CRE may be unique to neuronal cells (859) because CRE-containing c-fos promoters do not respond to Ca\(^{2+}\) in HeLa cells (860). Mutational analysis of the c-fos CRE showed that its loss was insufficient to prevent induction of c-fos by Ca\(^{2+}\) (836). Furthermore, CaMK-II also phosphorylates CREB at Ser\(^{142}\) which was shown to block the activation of CREB that would otherwise occur when Ser\(^{133}\) is phosphorylated (861). Therefore, it seems unlikely that heparin-sensitive induction of c-fos by Ca\(^{2+}\) is mediated through CRE in MCs. Once again, regulation of the SRE is sufficient to account for the observations in the present study.

4.1.4. CaMK-II

The only broad specificity kinases clearly established to respond directly to fluctuations in [Ca\(^{2+}\)] are the CaMKs (589), and CaMK-II leads to activation of transcription of a number of genes. c-fos is among the genes that are transcriptionally regulated by CaMK (801), through the ability of CaMK-II to phosphorylate both SRE (833) and CREB (Fig. 45) (857).
CaMKs are a family of kinases derived from four genes, consisting of homomultimers or heteromultimers of 6-12 kinase subunits each (589). Four distinct kinase subunits (α, β, γ, and δ) and a number of variants arising by alternative splicing have been cloned to date (589, 582). α and β isoforms are neuronal-specific, while γ and δ show a broader distribution including in brain (589). Schworer et al. (863) demonstrated by using immunoblotting and DNA sequencing that the δ CaMK-II subunit is a major CaMK subunit in SMC and other non-brain cells. In addition Wang and Simonson (846) demonstrated that MC do not express CaMK-IV, and that CaMK-II was responsible for ET-1-dependent activation of the mesangial cell c-fos promoter. Therefore, it seems likely that MCs in the present work expressed CaMK-II as their major CaMK.

Ionomycin at a concentration of 100 nM was sufficient to increase steady state c-fos mRNA levels after an increase of cytosolic [Ca²⁺], and activation of CaMK-II. CaMK-II activity was determined by the ability of cytosolic extract to phosphorylate a highly specific substrate for CaMK-II, derived from the autophosphorylation site of α CaMK-II subunit encompassing Thr²⁸⁶ (791, 864). The CaMK-II activity in the presence of non-limiting amounts of calmodulin and Ca²⁺ in the kinase assay mixture (Ca²⁺/calmodulin-dependent) thus would represent total activatable CaMK-II, and it was constant throughout the experiments. While in quiescent RMC basal auto-CaMK activity was only 2% of its total activity, in ionomycin treated cells that value climbed to 18% at the peak of activation. Similar results were found by Abraham et al. (865) who reported auto-CaMK-II activity over 50% of total activity in SMC treated with 1 μM ionomycin. In the present study, CaMK-II from MC also showed similar sensitivity to [Ca²⁺]. For instance an increase in [Ca²⁺] from a basal level to 400 nM in ionomycin-treated cells caused a six-to-seven-fold increase in auto-CaMK-II activity. While this result agrees with a report by Abraham et al. on SMC (865), other reports such as those by Wenham et al. on rat pancreatic islets treated with glucose or with K⁺ (866) and Yano et al. on rat cortical astrocytes treated with glutamate (867) found much smaller increases in auto-CaMK-II activity. These differences could be due to either
different cell types used or different experimental conditions.

Heparin did not interfere with the ionomycin-dependent elevation of \([\text{Ca}^{2+}]_i\). Heparin neither decreased myosin light chain phosphorylation nor RMC contraction, events dependent on activation of MLCK by \(\text{Ca}^{2+}/\text{calmodulin}\) (868). Therefore, heparin does not inhibit the activity of the \(\text{Ca}^{2+}/\text{calmodulin}\) complex per se, but does suppress the development of autonomous CaMK-II activity. This does not appear to arise as a direct effect of heparin on the kinase or its activation complex, because heparin added \textit{in vitro} to assay mixtures containing autonomous activity is without effect. Furthermore, heparin \textit{in vitro} does not interfere with the ability of added \(\text{Ca}^{2+}\) and calmodulin to activate CaMK-II autophosphorylation activity. Rather, heparin appears to initiate signals in the intact cell that prevent accumulation of autonomous CaMK-II activity in response to active \(\text{Ca}^{2+}/\text{calmodulin}\). The total amount of CaMK-II capable of activation \textit{in vitro} remains constant throughout the time course of the experiments and is unaffected by heparin.

Electron microscopic analysis of CaMK-II suggests a flower-with-petals structure where association domains of all subunits in a holo-enzyme assemble into a single globular hub from which radiate 8 - 12 smaller particles containing catalytic and regulatory domains of individual subunits (869). The active site Thr\textsubscript{305/306} is positioned in an auto-inhibitory segment (281-309), sterically blocking access to its substrates. The \(\text{Ca}^{2+}\)-bound form of calmodulin displaces this segment by wrapping around it and thereby activates the enzyme, dramatically increasing its affinity for Mg\textsuperscript{2+}/ATP, leading to phosphorylation of substrates and of the enzyme itself (autophosphorylation) (589). Both inter- and intrasubunit phosphorylation occurs within the holoenzyme (870, 871). Intersubunit autophosphorylation includes a site identified as Thr\textsubscript{286} in the \(\alpha\) subunit, which traps calmodulin (870, 871) by reducing the rate of dissociation causing CaMK activity to become independent of \(\text{Ca}^{2+}/\text{calmodulin}\), the so-called autonomous activity (872). Intrasubunit autophosphorylation occurs on Thr\textsubscript{305/306} in the center of the calmodulin binding domain. Phosphorylation of
these sites keeps CaMK inhibited until calmodulin releases the block by preventing their phosphorylation (589, 870, 871). The development of autonomous CaMK activity, assayed by phosphorylation of autocamtide-2 (791, 864) in the presence of EGTA is, therefore, an indicator of the extent to which Ca²⁺/calmodulin-dependent activation has occurred.

Ionomycin induced c-fos in a [Ca²⁺]-dependent fashion that was inhibited by CaMK-specific inhibitors, KT-5926 and KN-93. Neither KT-5926 nor KN-93 inhibited c-fos induction when cells were treated by serum or phorbol ester. ML-7 did not inhibit CaMK-II activity nor did it inhibit serum- or ionomycin-dependent c-fos induction but it did suppress the induction of c-fos elicited by phorbol esters. None of the inhibitors affected CaMK-I activity nor did it inhibit serum- or ionomycin-dependent c-fos induction but it did suppress the induction of c-fos elicited by phorbol esters. None of the inhibitors affected Ca²⁺ signaling, a result comparable with a report by Tansey et al. (873) who showed that KN-62, a very similar inhibitor to KN-93, did not affect Ca²⁺ signaling in response to ionomycin. With a reported $K_i = 4$ nM (805) for KT-5926, and from the results of the present study CaMK-II is more sensitive to this inhibitor than to KN-93. This may be because KT-5926 has a different mechanism of action. While KN-93 interferes with the autophosphorylation site on the kinase subunit and inhibits autonomous-CaMK activity without affecting total CaMK activity, KT-5926 strongly inhibits both activities. KT-5926 binds to both the calmodulin-binding and autophosphorylation site of CaMK subunit, encompassing approximately amino acids from Met²⁸¹ through Leu³⁰⁹. This span of amino acids may be needed for access of ATP, calmodulin, and protein substrates (589, 874-876). Nakanishi et al. (805) showed that KT-5926 inhibited MLCK by interacting with the ATP-binding site in the catalytic domain. The mode of inhibition was competitive with ATP and noncompetitive with the substrate. Similarly, inhibition by KT-5926 was not reversed by a higher concentration of calmodulin in the present study. Ionomycin failed to induce c-fos in experiments where specific inhibition of CaMK-II was achieved. Because heparin suppressed both CaMK activation and c-fos induction after stimulation by ionomycin, and because kinase inhibitors or heparin alone did not affect c-fos steady state mRNA or ionomycin-dependent Ca²⁺ signaling, the present study strongly suggests that heparin is
affecting c-fos induction through CaMK-dependent in addition to MAPK-dependent pathways.

Failure of heparin to interfere with MLCK-dependent phosphorylation of myosin light chain or suppress endothelin-mediated contraction argues for a site of action at the level of CaMK-II rather than upstream at calmodulin-related events. Again, this suggests that heparin's mechanism of action is other than through interference with one or more of the conserved domains of CaMK-II and MLCK, i.e., Ca$^{2+}$/calmodulin-binding, ATP-binding, or catalytic domains (868).

4.1.5. Effect on MLC phosphorylation and contraction

Similar to the mechanism of activation of CaMKs, activated calmodulin binds and removes a pseudosubstrate inhibitory region from MLCK's active site allowing MLCK to phosphorylate MLC (877-879). Phosphorylation of MLC is closely correlated with an increase in actin-activated myosin ATPase activity and initiation of cell contraction (880). This process can be inhibited by caldesmon and calponin (881, 882), and PKC (882, 883) or Ca$^{2+}$/calmodulin (881, 882) can reverse it. As [Ca$^{2+}$]$_i$ falls, the calmodulin-MLCK and calmodulin-caldesmon complexes dissociate and caldesmon returns to the inhibitory state. MLC phosphatases are also involved. They are Ca$^{2+}$-insensitive, and can be activated by PKA (884) to dephosphorylate MLC and to inhibit the interaction of actin with myosin (885).

ET-1 caused an increase in [Ca$^{2+}$]$_i$ to levels sufficient to produce MC contraction, and pretreatment with KT-5926 inhibited both MLC phosphorylation and cell contraction, consistent with a MLCK-dependent process. Consistent with the inhibition constants of KT-5926 for MLCK, CaMK-II, and PKC (18 nM, 4 nM, and 753 nM, respectively (805)) a concentration of 5-10 μM inhibited signals leading to MLCK activation (886). Heparin did not suppress cell contractility in spite of inhibiting activation of CaMK-II. This is consistent with reports suggesting that heparin inhibits cultured SMC proliferation but induces a
contractile phenotype (103, 887, 888). Kohno et al. (696) have shown that heparin lowers blood pressure. However, that effect of heparin was probably based on suppression of ET-1 production associated with long-term exposure rather than an effect on SMC contractility. In the present study, MLC phosphorylation in heparin-treated cells is of even higher intensity than in control cells. Similarly, the presence of the CaMK-II inhibitor KN-62 resulted in potentiation of Ca2+-dependent MLC phosphorylation. Heparin may act similarly to KN-62 by inhibiting CaMK activity and in turn phosphorylation of MLCK at the regulatory site. This phosphorylation increases the concentration of Ca2+/calmodulin required for MLCK activity in cells (889). If inhibited, for instance by heparin, then the potentiation of MLC phosphorylation is observed in response to agonist. Less phosphorylation of MLCK by CaMK-II lowers the requirement of MLCK for Ca2+/calmodulin to phosphorylate MLC (889). It may be that under our experimental conditions, heparin alone does not increase contractility because an additional stimulus is still required to initiate a Ca2+ signal, whereas the response observed to endothelin may already be maximal. Distinct effects of heparin on CaMK-II and MLCK may be important when contemplating its therapeutic use in mesangioproliferative renal disorders (671) or in arterial neointimal proliferation (890).

A mechanism that could explain heparin's effect on both MAPK and CaMK is activation of phosphatases. Phosphatases are inhibited by oxygen radicals (891), shown to be necessary for growth stimuli elicited by PDGF in SMC (808), and MC can produce reactive oxygen species (ROS) (892). If heparin scavenges ROS (893, 894) it could release the inhibitory block from phosphatases, causing dephosphorylation of corresponding kinases. However, several results suggest that heparin does not scavenge oxygen radicals nor act by this mechanism to suppress kinase activities: i) the inhibitory effect of H2O2 on the mitogenic response of RMC is partially reversed by preloading cells with catalase; ii) serum- and TPA-dependent MAPK activation are not significantly affected by catalase, while they are suppressed in the presence of heparin; iii) c-fos induction is inhibited by heparin but is not
affected by the presence of catalase, following treatment with serum or TPA; and iv) recently
Pukac et al. (766) reported that treatment with heparin inhibited the orthovanadate-stimulated
phosphorylation of MAPK. Because catalase did not affect either steady state c-fos mRNA
levels, or MAPK activity it is possible that MC were not loaded with catalase. Catalase
loading of different cell lines varies. For instance, intracellular catalase activity after 2 h-
exposure of VSMC to 3000 U/ml increased approximately 50-fold, while that was not
observed with HUVEC and HeLa cells (808). Although our preliminary experiments
suggested very modest loading of MC with catalase (not shown) diffusibility of ROS through
biological membranes would allow catalase, without entering the cell, to lower intracellular
ROS levels by decomposition of extracellular ROS and enhance the efflux gradient across the
plasma membrane (Pinkus, 1996 #1446).

In the present study, H2O2 at concentration 0.2 mM totally abolished mitogenic response
of RMC after activation with serum, consistent with ROS-dependent induction of apoptosis.
Exposure to low concentrations of H2O2 induces apoptosis in a variety cell types (895), and
expression of Bcl-2 protein prevents apoptosis induced by oxidative stress (896). In cultured
tubular epithelial cells, H2O2 induces endonuclease activation and subsequent DNA damage
and cell death (897). Recently, Sugiyama et al. (809) demonstrated on cultured HMC that
H2O2 at concentration ten times lower than in present study was able to induce apoptosis.

4.1.6. Effect on Ca2+ signaling

Ca2+ release from the ER is initiated by G protein-coupled, and tyrosine kinase-linked
receptors (898) that are expressed in MC (431). Both receptors activate PLC, which in turn
splits PIP2 and generates IP3, that releases intracellular Ca2+, and DAG which activates
PKC.

BayK8644 activated L-type Ca2+ channels and increased cytosolic [Ca2+]; to a level
comparable to those reported by McDermot et al. (780). Heparin inhibited Ca2+ entry after
cell activation with serum or with BayK8644. This effect is probably due to blocking of L-type Ca²⁺ channels, known to be present in MC (780, 899), because heparin blocked serum-stimulated Ca²⁺ uptake to about the same extent as verapamil in the present study. Furthermore, Knaus et al. demonstrated that heparin binds with high affinity to the L-type Ca²⁺ channel (754) and blocks BayK8644-dependent Ca²⁺ uptake (755). However, it is also possible that heparin prevents agonist-induced Ca²⁺ uptake by binding it in the extracellular medium. Heparin is a highly sulfated GAG with anionic character, and can bind Ca²⁺. However, the results from the present study argue for an effect on Ca²⁺ uptake rather than simple sequestration of extracellular Ca²⁺. A heparin concentration of 1 μg/ml is 2 μM in disaccharide units based on an average formula weight of the disaccharide subunit of ≈ 500 Da. At pH > 5 each disaccharide carries about four negative charges, so heparin can only bind 4 μM Ca²⁺ at complete neutralization of charge. Because the interaction of GAGs with small counteranions obeys a polyelectrolyte model (900), the amount of Ca²⁺ bound to heparin is only about half this amount. Therefore, a 2-4 μM reduction compared to the 1.2 mM Ca²⁺ extracellular pool is insignificant. This is supported by spectrophotometric measurements, indicating only minor effects on Ca²⁺ activity in solution at much higher concentrations of heparin than used here (901).

Several results indicated that interference with Ca²⁺ uptake through L-type Ca²⁺ channels is not responsible for inhibition of the mitogenic response. Verapamil blocked serum- and BayK8644-dependent Ca²⁺ uptake to a degree comparable to heparin (present study), but otherwise was without effect on c-fos induction (749). Heparin blocked c-fos induction by TPA and Ca²⁺ ionophores, although it did not suppress changes in [Ca²⁺]i caused by these agents. While the increase of c-fos steady state mRNA was strongly suppressed, Ca²⁺ signaling was increased following ET-1 or A23187 treatment in the presence of heparin. These observations suggest that extracellular heparin has mechanisms that inhibit mitogenic signaling at an intracellular level and independent of any of heparin’s effects on Ca²⁺.
4.1.7. Tyrosine phosphorylation

Because heparin does not affect PKC activity, but suppresses serum- and TPA-dependent MAPK activation and c-fos induction it is likely that heparin affects events preceding MAPK but after PKC and receptor activation. One of these events could involve tyrosine phosphorylation, necessary early in mitogen stimulated signal transduction. For instance serum stimulation of growth factor receptors results in recruitment and activation of ras/raf-1 and MEK that activates MAPK by phosphorylating it on threonine and tyrosine residues (902). Pukac et al. (766) reported that there was no effect of heparin on tyrosine phosphorylation of proteins about 180 kDa including PDGF receptor. A similar result is reported in the present work which together with Pukac et al. (766) indicates that heparin does not inhibit the binding of serum growth factors to the cell surface and does not block the initiation of growth factor-dependent signaling. This corroborates reports of Wang and Templeton (637) who showed little difference in magnitude of mitogenic response after passing serum through a heparin-Sepharose column, and of Reilly et al. (655) reporting that heparin does not inhibit PDGF binding to the cell surface. Heparin did not effect the pattern of tyrosine phosphorylated proteins. This is comparable to reports that used serum (748) and PDGF (766) to stimulate VSMC, however the authors observed that heparin was suppressing tyrosine phosphorylation of MAPK after 30 min. This difference of heparin's effect could be attributed to the experimental approach used in those two studies or to different type of cells.

Several lines of evidence point to an important role for cell surface binding in achieving the maximum antimitogenic activity of heparin. In this case extracellular heparin may act by neutralizing mitogenic growth factor activity, by interfering with growth factor-receptor binding, by direct interaction with heparin-dependent signaling receptors, or by altering the properties of the cell membrane. Heparin and HS assist in the delivery of active bFGF to its high affinity receptor in fibroblasts (903, 904), but also inhibit bFGF binding and mitogenesis in VSMC (905). Heparin also blocks binding of thrombospondin to the cell
surface, binding required for SMC proliferation (906). However, these mechanisms may not be as important as heparin binding itself. Removal of heparin binding proteins from serum by chromatography on heparin-Sepharose does not alter the antiproliferative properties of heparin on SMC (765) and MC (637). Furthermore, heparin did not affect PDGF-dependent tyrosine phosphorylation of PDGF-β receptor, suggesting both intact ligand binding and receptor activation in the presence of a high concentration of heparin (766). More direct evidence for a role of heparin independent of growth factors or growth factor receptors is provided by the experiments with TPA. Under serum-free conditions direct activation of PKC with phorbol ester leads to an induction of c-fos that is still inhibited by heparin.

Heparin may initiate its own signaling pathway upon binding to receptor. Heparin has high affinity binding sites on SMC (733, 907), MC (637) and fibroblasts (734). There is a qualitative correlation between heparin surface binding and its effect on the mitogenic response. Quiescent cultures of A10 cells had only half the number of binding sites as RMC and were about half as sensitive to suppression of c-fos induction at 30 min after immediate addition of heparin (637). Both the affinity and number of heparin binding sites decreased in proliferating RMC cultures and these cultures were less sensitive than quiescent cells to suppression of [3H]thymidine incorporation by heparin (637). A similar decrease in specific heparin-binding sites of about eight-fold was found in exponentially growing VSMC as compared to growth-arrested cells (719).

It is also possible that internalized heparin affects signal transduction pathways. While uptake and processing of heparin do occur in SMC (733) and in MC (657), the appearance of intracellular fragments may be too slow to account for the full antimitogenic effect seen with addition of heparin at the time of stimulation, or the lesser effect observed when heparin is added 15 min later. Nevertheless, intracellular processing has been shown previously to affect the subsequent antimitogenic potency of heparin-like species. Herbert and Maffrand (732) isolated heparin-derived oligosaccharides from the media of endothelial cell cultures
after incubation with commercial heparin and found that they were 10 - 15-fold more potent inhibitors of SMC proliferation than heparin itself. However, several lines of evidence argue against an antiproliferative effect of heparin after its internalization. Labeled heparin is taken up by cells and degraded with half-time of several hours (637, 733). Although, heparin is an effective inhibitor of intracellular IP_3-receptors, sufficient heparin to achieve this effect does not enter the cell unless aided by permeabilization or microinjection (751, 752). It is possible to select heparin resistant SMC by long exposure of cells to heparin (733). Furthermore, in cells selected for resistance to heparin by growth in heparin-containing medium, uptake of heparin is not affected, but binding to the cell surface is decreased (733). Dextran sulfate reduced by 50% the antiproliferative action of heparin, while uptake of heparin was not affected (734). The process of internalization of heparin is too slow to account for an effect on c-fos induction observed 15 min after addition of heparin (637) and also for its effect on MAPK and CaMK in present study, observed after 0.5 and 2 min, respectively. Therefore it is reasonable to conclude that the effects of heparin on MAPK and CaMK and c-fos induction arise from interaction of heparin with the cell surface rather than uptake or metabolism, although the mechanism remains unknown.

4.2. COLLAGEN TYPE I

Results from the present study confirm the hypothesis that ECM composition modulates MC phenotype. Adhesion-dependent signaling provides the basis for localized signals from matrix, which may control patterns of morphogenesis and growth (908, 909). Moreover signals generated by adhesion receptors can regulate signal transduction pathways stimulated by locally released growth factors (910, 911). Indeed, the capacity of collagen type I to inhibit cell proliferation has been appreciated for some time (910, 912) and is consistent both with a low proliferative index of SMC in the media of the normal artery wall (913) and with acellularity of fibrosclerotic tissue.
4.2.1. Proliferation and mitogenic response

Adhesion and spreading of normal diploid cells (36, 914) are prerequisite for proliferation and for cells to respond to growth stimuli. However, adhesion of fibroblasts and SMC to type I collagen results in a dramatic reduction in cell proliferation as compared with cell culture on plastic (912, 915). When SMCs were cultured on polymerized collagen type I, basal and growth factor-stimulated [3H]thymidine incorporation into DNA were more than five-fold lower than on monomer collagen (plates coated with collagen from 0.1 M acetic acid solution) and were associated with several-fold lower cell number three days after treatment with growth factors (916). Accordingly, growth on collagen type I, in the present study, decreased MC proliferation and DNA synthesis, in keeping with earlier observations of lower stimulation of DNA synthesis on collagen type I and laminin than on collagen type IV or fibronectin (14). Three-dimensional cultures of RMC in collagen type I gels also showed markedly reduced [3H]thymidine incorporation in comparison with cultures on glass (23). In these three-dimensional cultures of RMC, down regulation of PDGF receptor expression on the cell surface may account for the decreased mitogenic responsiveness, but no such down regulation was observed on two-dimensional collagen type I gels (546).

Several lines of evidence argue against an artifact produced during the experiments with collagen type I. First, the number of cells attaching to plastic or collagen type I coated plates was similar. Second, cell density on collagen type I was comparable to that on plastic, as determined by DNA content per well. Third, a similar number of viable cells was observed on both surfaces, consistent with a report by Marx et al. (546) on MC and by Koyama et al. (916) on SMC. Fourth, the Ca²⁺ signal elicited by serum growth factors was similar on plastic and collagen type I. Therefore, the antiproliferative effect of collagen cannot be attributed to lower cell viability or to fewer cells attaching to collagen type I, but rather it is the result of a specific effect of collagen type I.
The intact collagen type I molecule is required for its antiproliferative effect on MC. Dedhar et al. (917) have identified a cell surface receptor complex in osteosarcoma cells that recognizes RGD sequences in collagen type I. It does not bind RGD in denatured collagen, so the conformation around the RGD sequence may be important. On the other hand, specific binding of collagen type I to fibroblasts is dependent on the primary sequence and not higher order structure and is competitively inhibited by cyanogen bromide-derived peptides from various regions of the \( \alpha_1(II) \) chain (918, 919). The present results indicate, however, that growth on collagen type I rather than binding of soluble collagen affects RMC proliferation and contraction. He et al. (762) have shown that growth on collagen type I gel suppressed the proliferative phenotype of mouse mesangial cells compared to growth on a thin film of collagen type I or fibronectin. This is consistent with our observation of decreased proliferation of RMC on a collagen type I gel relative to soluble collagen or fibronectin-rich mesangial matrix.

Collagen type I may exert its antiproliferative effects through integrins. Although, signaling molecules can localize to cell-matrix contact sites after integrin aggregation without ligand occupancy (354), the recruitment of cytoskeletal molecules to focal adhesion sites is extremely limited without integrin occupancy and associated phosphorylation. SMCs appear to require integrin-mediated signaling (cell-cell or cell-matrix interactions) for their survival (916). When human SMC are kept in suspension and cell-cell interactions are prevented, more than 50% become apoptotic within 24 h, whereas no apoptosis is observed in SMC on polymerized collagen. Koyama et al. (916) demonstrated that suppression of cyclin E-cdk2 activity and induction of cdk2 inhibitors by polymerized collagen is mimicked by monovalent Fab antibody fragments of a blocking antibody to \( \alpha_2 \) integrin, but not by Fab fragments of non-blocking antibody. MC express integrins of the \( \beta_1 \) and \( \beta_3 \) classes (763) and the organization of specific sub-types is dependent on adhesion to specific matrices. On collagen type I, MC organize integrins \( \alpha_1\beta_1, \alpha_2\beta_1 \) and \( \alpha_3\beta_1 \) into focal adhesions (763). Therefore, focal adhesion formation represents a way in which substrate-specific signals can arise. For
instance, FAK can become phosphorylated (763) and PKC can be activated (920) upon integrin clustering. The absence of both FAK phosphorylation and PKC activation on collagen may be a clue to changes in integrin-based structures. However, in MC phosphorylation of FAK appears to involve PKC (921), so the absence of PKC activity may account for the observed lack of FAK phosphorylation. Decreased FAK phosphorylation may lead to decreased MAPK activation, although recently β1-integrin-mediated activation of MAPK has been demonstrated to be independent of FAK in fibroblasts (922).

There is increasing evidence that receptor tyrosine kinases and integrins act in a coordinated fashion to modulate cellular responses involving adhesion, spreading, locomotion, proliferation, survival, and differentiation state (370). While the integrins most often mediate binding and attachment to components of the ECM, receptor tyrosine kinases recognize and respond to peptides by transducing signals (923). Recently, collagens were reported to bind to and activate two receptors, discoidin domain receptor 1 and 2 (DDR1 and DDR2), which are tyrosine kinases (924, 925). DDR1 is activated by collagen type I, II, III, V, and XI, and DDR2 is activated mainly by type I and III (924), and for their activation the triple-helical structure of collagen is essential. DDR1 and DDR2 were independently isolated by many laboratories from human, mouse, and rat tissues (924, 925), and they are expressed in brain, gastrointestinal tract, lung, heart, muscle and kidney as well (924, 925). Although downstream signaling from DDR receptors awaits to be elucidated, they may be important mediators of signaling in glomerular mesangial cells. It is proposed that DDR receptors may function as signaling components of a multi-integrin complex that transmits the intracellular signal initiated at the cell surface by engagement of integrins by collagen (926).

The polymerized actin network is essential for the function of both PKC-dependent and independent pathways of FAK tyrosine phosphorylation (927). It has been suggested that many signaling molecules induced by integrins and growth factors may function on insoluble cytoskeletal scaffolds (928). These cytoskeletal platforms are probably very different in cells
on plastic versus collagen type I gel. Although it was not part of the present study, rearrangement of cytoskeleton and low expression of rho or low rho-GTP activity is predicted in MC grown on collagen type I. Koyama et al. (916) observed in SMC a loss of stress fibers and focal adhesions after plating on collagen type I polymer surface comparable to collagen gel in the present study. The level of DNA synthesis in normal diploid cells is tightly coupled to cell attachment, shape and spreading (914, 929) and it can be controlled either by substratum adhesiveness or by cell-cell attachment. As cells are brought from an extremely flat shape towards a spheroid shape, fewer cells synthesize DNA (914, 929). Furthermore, suspended cells fail to activate cyclin E-cdk2 kinase activity (383, 384). The actin cytoskeleton participates in regulating cell shape. The principal actin structures include the cortical actin network, actin stress fibers, and cell surface protrusions such as membrane ruffles and microspikes (930). Talin localizes first to the newly forming adhesion before vinculin, and stress fibers polymerize later from these adhesions (931, 932). Rho regulates the assembly of cytoplasmic components such as talin and vinculin into a membrane-bound complex with integrin receptors to form focal adhesions and stress fibers. Ridley and Hall (364) reported that serum-starved Swiss 3T3 cells on plastic have very few stress fibers, but within 2 min of adding serum back to cells, actin is polymerized and assembles into stress fibers. Simultaneously, new focal adhesions are formed and associated with the ends of stress fibers at the plasma membrane. When rho function was blocked, both focal adhesion and stress fiber assembly induced by extracellular factors were completely inhibited (364). Thus, MC could possess fewer focal adhesion sites and less stress fibers, like SMC on collagen type I. In addition, they could suffer further loss of these structures due to starvation and if accompanied by the loss of kinase activities such as shown in the present study, they could lose their responsiveness to growth stimuli.

c-fos is an immediate-early response gene that is transiently expressed in order for the cell to pass through G1 phase and enter the cell cycle. Serum-dependent c-fos induction is inhibited by growth of MC on collagen type I, consistent with low kinase activities,
suppressed mitogenic response, and reduced cell proliferation on this surface. Likewise, SMC are arrested in G1 on polymerized collagen type I (916). The inhibition of active cyclin E-associated kinase complexes, possibly together with regulation of cyclin A expression, are likely targets in prevention of DNA synthesis in SMCs on polymerized collagen (916). Proto-oncogenes that are involved in the G0/G1 transition can cooperate with cyclins (933). For instance, c-myc is implicated in the expression of several cyclin genes (934). However, continuous overexpression of Myc inhibits expression of cyclins, suggesting a complex regulatory process (935).

In the healthy glomerulus, mesangial matrix consists mostly of collagen types IV and V, laminin, fibronectin, and proteoglycans (14, 543, 936, 937). In glomerulosclerosis, the occurrence of interstitial collagens (761, 938, 939) accompanies de novo synthesis of collagen type I (298, 639). The increased deposition of interstitial collagens may represent pathological reversion of MC to myofibroblasts (5) and may facilitate platelet adhesion (940, 941), cause release of inflammatory and mitogenic platelet factors, or alter cytokine receptor expression (546). However, deposition of collagen type I may also function to suppress RMC proliferation, joining heparin and heparan sulfates as endogenous substances with antiproliferative effects on MC (636, 670, 671). However, unlike heparin, collagen type I is shown to enhance ET-1 sensitivity in MC.

4.2.2. Kinases

Several signal transduction pathways that lead to immediate-early gene expression were strongly inhibited by growth of MC on collagen type I. Collagen inhibited restoration of PKC activity after plating trypsinized cells, inhibited MAPK activation and MAPK-dependent c-fos induction through PKC-independent pathways, prevented the autophosphorylation of CaMK II, suppressed tyrosine phosphorylation of MAPK and FAK, and decreased and delayed serum-dependent phosphorylation of several unidentified tyrosine kinase substrates.
4.2.2.1. PKC

In cells attaching to plastic PKC activity was several-fold increased from that of cells in suspension and was sustained through 48 h, consistent with a requirement for PKC in cell spreading (942) and involvement in focal adhesion formation (349). In contrast, PKC activity in cells attaching on collagen type I was strikingly low suggesting that collagen keeps PKC activity low from the beginning of cell-collagen contact. Furthermore, cells attached and starved on collagen type I were not sensitive to addition of TPA. However, caution should be exercised while interpreting present results, because PKC-ζ which was implicated in mitogenic signal transduction (943) cannot be activated by TPA or detected by oligopeptide from EGF receptor (944), and it was shown to be present in MCs (945, 946). Results from the present study are opposite to reports of collagen type I increasing membrane-bound PKC in osteoblasts (947), activating the PKC-ζ isoform in fibroblasts (948), and triggering translocation of PKC-ε in HeLa cells (949). Recently, it has been shown that syndecans are required for the localization and activation of PKC (352, 950). Furthermore, fibroblasts attach to and migrate upon plates coated with the cell binding domain of fibronectin, but require addition of the heparin (syndecan) binding domain to form focal adhesions (951), a process accompanied by FAK activation (349). The cell surface syndecans may be cleaved by trypsinization when cells are passaged, and indeed are shed even when cells are detached from cultures plates by lowering the Ca^{2+} concentration of the medium (952). Since syndecans are involved in attachment of the heparin-binding domain of fibronectin, their expression may be disfavored on a pure collagen substrate, and this may provide a mechanism for the lack of reactivation of PKC upon attachment to collagen.

Several lines of evidence suggest the effect of collagen on PKC activity is not simply due to interference with the assay, e.g., binding of [γ-32P]ATP or PKC substrate to collagen. The amount of radioactivity was approximately similar when the reaction mixture was collected from plastic or collagen-coated dishes without cells, or with cells but without
substrate. TPA, an agent that acts inside the cell to activate PKC directly, modestly increased PKC and MAPK activity in cells grown on collagen type I. The activity of both kinases was abolished by down regulation of PKC, suggesting that collagen did not interfere with availability of the phorbol ester to cells nor with availability of substrate and \(^{32}\text{P}\)ATP in the case of PKC assay. Thus, the inhibitory effect of collagen on PKC activity is not produced by interference with the assay but rather, appears to be a direct effect of matrix on the cell.

4.2.2.2. MAPK

The amount of \(^{32}\text{P}\)ATP incorporated into MBP by an immunoprecipitate of cytosol with anti-Erk-2 antibody, in the presence of a cAMP-dependent protein kinases inhibitor, is an indication of MAPK activity. Although the method is somewhat lengthy, it gives comparable results to an in-gel assay (953, 954) with very little or no basal activity in quiescent cells. The advantage of this in-vitro assay is that it uses far less MBP than the in-gel method and avoids high nonspecific radioactivity found in assays using p81 paper. Two lines of evidence, besides the immunoprecipitation, suggest that MAPK-dependent phosphorylation of MBP was being observed: i) the use of a MEK-specific inhibitor, PD98059, totally abolished serum-dependent enzyme activity; and ii) 24-h pretreatment with TPA suppressed PKC-dependent enzyme activation.

Sustained activity of MAPK observed in cells grown on plastic, is consistent with the notion that the duration of activation of MAPK is essential for enzyme translocation into the nucleus. Huwiler et al. (955) reported that PDGF-BB caused a potent and sustained phosphorylation and activation of MAPK in MCs. Serum (850) and TPA (850, 956) also caused sustained activation of MAPK in fibroblasts (850) and adult ventricular myocytes (956). Stimulation of PC12 cells with nerve growth factor stimulates sustained activation of MAPK and MEK and causes translocation of MAPK from cytosol to the nucleus (957, 958). However, EGF (958) and A-II (955), induced a transient activation of both enzymes that is not accompanied by nuclear translocation of MAPK (958).
A weak MAPK activity in MC grown on collagen type I is consistent with the effect of collagen on MC proliferation and serum-dependent c-fos induction. Very low MAPK activity after treatment with either serum or TPA suggests that the intracellular MAPK cascade is affected, although effects of collagen type I on the cell membrane could also be involved, e.g., altered expression of growth factor receptors (546). However, cells grown on collagen type I or on plastic responded with similar Ca^{2+} signaling upon stimulation with serum. Whether through reorganization of microfilaments, impaired integrin signaling (916) or increased phosphatase activity, PKC activity is kept very low on collagen type I, which may contribute to low MAPK activity. Another factor leading to decreased MAPK activity could be increased cAMP. Collagen type I was shown to augment cAMP production stimulated by PTH or a nonreceptor mechanism in osteoblasts (947). Increased levels of intracellular cAMP cause a large reduction in the PKC-dependent promotion of angiogenesis (959). cAMP also inhibits activation of CaMK and blocks signals from ras and raf (960, 961). However, involvement of cAMP, an agent that suppresses contraction, is not likely because cells grown on collagen type I have a greater contractile response to ET-1 than those grown on plastic.

In contrast to our observations, Langholz et al. (962) showed activation of ERK1 and ERK2 in human fibroblasts 2 h after cell-collagen type I contact, irrespective of two-dimensional or three-dimensional culture conditions. Zhu and Assoian (378) reported the activation of MAPK in fibroblasts within 1 h on fibronectin and at 3 h on collagen type IV. Koyama et al. (916) reported no difference between MEK and MAPK activities 0.5 - 6 h after plating SMCs on monomeric or polymerized collagen type I. In the present study, 48 h exposure to low levels of serum caused such irreversible changes that MAPK activity in cells grown and starved on collagen type I could not be recovered with serum or with phorbol esters. The basis for these differences is not clear, but they could result from the different cell lines used or from the cells being in different stages of the cell cycle before stimulation.
Ionomycin-dependent autonomous CaMK-II activity was also inhibited in MC grown on collagen when compared to those grown on plastic. This was unexpected since cell growth on collagen type I produced a higher Ca²⁺ signal and more rapid MLC phosphorylation in response to ET-1 than in cells on plastic. CaMK activity could be regulated by interactions between PKC and calmodulin, so that decreased PKC on collagen type I could result in decreased CaMK activation. The activation of PKC in PC12 cells resulted in an elevated cytosolic concentration of calmodulin (965). Because activation of calmodulin-dependent enzymes by Ca²⁺ and calmodulin is a mass action phenomenon, the elevation of free calmodulin, at either basal Ca²⁺ or stimulated Ca²⁺ levels, would serve to further activate these enzymes. The interaction of calmodulin and PKC could be mediated through myristoylated alanine-rich C kinase substrate (MARCKS). MARCKS colocalizes with PKC (964) and binds calmodulin in focal contacts (964, 965). Activation of PKC during chemotaxis results in the phosphorylation-dependent cycling of MARCKS between plasma membrane and the cytosol (966). Furthermore, phosphorylation of MARCKS by PKC causes release of bound calmodulin from MARCKS (967). Thus, MARCKS could serve to release calmodulin near the plasma membrane upon activation of PKC. With decreased kinase activities MARCKS may remain bound to calmodulin and keep it inactive.

Koyama et al. (916) reported that polymerized collagen suppressed p70S6K activity to levels below those found in suspended cells. A similar phenomenon was observed with PKC activity after attachment to collagen type I in the present study. Thus it seems unlikely that the effect of fibrillar collagen can be explained solely by a lack of integrin signaling and lack of formation of focal adhesions. Collagen type I mediated inactivation of p70S6K was associated with rapid dephosphorylation of the enzyme raising the possibility that fibrillar collagen may regulate a phosphatase upstream of p70S6K (916). In the present study, growth on collagen type I suppressed tyrosine phosphorylation and the activity of several kinases,
consistent with the idea of increased phosphatase activity.

4.2.3. Contraction

RMC responded to vasoactive agents with a decrease in CSA. Consistent with earlier authors considering a decrease of > 7% to be significant (768) 40 - 60% of the cells responded to stimulation with any of the agonists on either plastic or collagen type I matrix. These results are comparable to several earlier studies that demonstrated a mean decrease of 25 - 40% in area at 30 min in response to ET-1 (491) and ANG-II (625, 777). On the other hand several authors have found a faster response to ANG-II that may be due to the use of primary cells (968) or early subcultures (9, 10). This response is generally considered to represent contraction; the delay between exposure and contraction may then represent the time needed for remodeling of the cytoskeleton or stress fibers (20) or adhesive contacts with substratum. Consistent with this latter view are results obtained on teflon (777), where more cells responded to agonist than on glass, and on poly-2-hydroxyethyl methacrylate (969), which allowed a faster response of more cells than plastic. Results obtained on silicon rubber sheets further support the idea that the shape change represents contraction rather than rounding up upon detachment. Isometric contraction of MC (626) and fibroblasts (544) is observed as a baseline wrinkling of the silicone rubber, and addition of ANG-II to MC or trypsin to fibroblasts leads to an increase in wrinkling. Therefore, cultured MC have been widely used (424, 777, 969, 970) as a model for studying factors that may modulate capillary surface area or wall tension (423) through MC contraction in vivo.

Several lines of evidence suggest contraction is occurring in the present study. Each agonist caused an increase in $[\text{Ca}^{2+}]_i$. The order of the size of the calcium response is the same as the order of the change in area among cells grown on collagen or plastic and stimulated with various agonists. When observations were continued long enough, surface area was seen to reach a minimum and then increase, consistent with relaxation back to a
resting state. Phosphorylation of MLC precedes the change in area, and the MLCK inhibitor KT-5926 suppresses myosin phosphorylation and prevents the CSA change. L-NMMA, an inhibitor of production of the vasorelaxant NO*, enhances the response to agonist. Taken together, these observations strongly suggest that dynamic, myosin-dependent contraction is observed.

Incorporation of $^{32}P$ into MLC in RMC is linear for at least 6 h and does not attain equilibrium at the time (20) so shorter term experiments allow inferences about changes in the rate of phosphorylation. Agents that affected cell shape by increasing adenosine-3',5'-cyclic monophosphate levels were associated with a decreased rate of phosphorylation at 20 and 40 min, whereas prevention of loss of stress fibers and shape change was accompanied by an increased rate of phosphorylation (20). This suggests that phosphorylation of the light chain may be necessary for maintaining stress fibers and that these fibers must break down before shape changes can occur (20). In contrast, 10 nM ANG-II was found to stimulate RMC contraction and increase MLC phosphorylation by 90 % after 30 min (625). In the present study, ET-1-induced contraction is observed following a transient increase in phosphorylation that peaks as early as 2 min after addition of agonist and declines thereafter. The difference between the present study together with those of Garcia-Escribano et al. (625) on the one hand and those of Kreisberg et al. (20) on the other may reflect the agonists used, the time course of the phenomenon, or the balance of kinase and phosphatases activities in individual experimental circumstances.

MLC phosphorylation is prerequisite for activation of myosin ATPase by actin and thus for actomyosin-based contraction (971, 972). SMC MLC contains five sites for phosphorylation, two of which are specifically phosphorylated by Ca$^{2+}$-dependent MLCK (973). The inhibitory constant of KT-5926 for MLCK is 12 nM in in vitro assays, compared with 753 nM for PKC, and selective inhibition of MLCK is achieved at 5 - 10 μM in cultured cells (805, 886). Therefore inhibition of phosphorylation by KT-5926 supports productive
MLCK-dependent phosphorylation in the present study that was required for ET-1-induced contraction. Basal phosphorylation may represent phosphorylation at sites used by other kinase(s) that are not directly involved in contraction (973). The absence of detectable basal phosphorylation in cells on collagen type I may indicate decreased activity of such kinases in these nonproliferating cells that are active in proliferating cells on the other substratum.

RMC growing on collagen type I show a greater change in CSA in response to agonists than those on plastic. Two factors may contribute. Because ANG-II causes greater change in CSA on collagen type I [e.g., 77.8 ± 13.3 % on collagen type I versus 91.5 ± 11.5 % on plastic at 30 min (P < 0.01)] but elicits similar Ca²⁺ signals on both substrata, it appears that the degree of contraction depends on cell-substrate contacts. Phenotypic changes on collagen type I that produce a greater degree of contraction at the same level of signaling seem less likely but could include easier remodeling of the cytoskeleton on collagen. On the other hand, collagen type I facilitates a greater contractile response to ET-1 by enhancing Ca²⁺ signaling and producing a more rapid increase in MLC phosphorylation. Although we have not measured ET-1 receptors, an increase in receptor number is a plausible explanation.

Vasoactive agents and growth factors generally initiate both mitogenic and contractile response in RMC, perhaps through a common signaling intermediate such as PLC (424). Growth on collagen type I dissociates these responses, suppressing proliferation and serum-stimulated DNA synthesis while increasing the contractile response to ANG-II, AVP, and ET-1. This appears to be opposite to the effect of collagen type I on VSMC where collagen was shown to facilitate the transition from a contractile to a synthetic, proliferative phenotype (634). Whether this represents a true difference between VSMC and MC or arises from specific experimental conditions remains to be determined. Nevertheless, if these changes in MC behaviour occur in response to collagen type I in vivo, the appearance of this collagen in pathological lesions might be a reparative response to suppress MC proliferation and maintain contractility. However, continued deposition of type I collagen may interfere with cell
renewal and contribute to eventual acellularity in the sclerotic glomerulus.

4.3. SUMMARY AND SIGNIFICANCE

The conclusions that can be drawn from this work are summarized in Fig 45.

Heparin

(i) Heparin is a potent suppressor of RMC mitogenic responsiveness, acting at concentration as low as 50 ng/ml. (ii) It suppresses c-fos induction elicited by serum, phorbol esters or ionophores. (iii) It suppresses serum- and TPA-dependent MAPK activation. (iv) It suppresses ionomycin-dependent CaMK autophosphorylation. (v) It also inhibits serum-and BayK 8644-dependent Ca²⁺ influx probably due to its high affinity to L-type Ca²⁺ channels. (vi) Heparin does not affect ET-1-dependent MC contractility;

Collagen type I

(vii) A collagen type I surface also suppresses RMC proliferative and mitogenic responsiveness. (viii) Cell attachment to intact molecules of collagen type I is required for this effect. (ix) Collagen type I suppresses serum-dependent c-fos induction in RMC, and probably does so by suppressing (x) MAPK activation, (xi) CaMK-II activation, (xii) PKC activation, and (xiii) tyrosine phosphorylation. (xiv) However, growth on collagen type I increases ET-1-dependent contractile response of RMC (Fig 45).

This study therefore, sheds more light on mechanisms of the effect of ECM on MC, and has practical implications. Based on the above observations, we propose a model for a role of ECM components such as heparin/HSPG and collagen type I in regulating MC phenotypes. In the healthy kidney MC are quiescent with a low level of growth factor synthesis and growth factor receptor expression. During injury of the mesangium, platelets and macrophages appear at the site of damage and release several stimulatory growth factors. Growth factor receptors expression is increased and signaling events are initiated that can lead
Fig. 45 - Summary of effects of collagen type I and heparin on rat mesangial cells
to hyperproliferation, and hypercellularity. Increased production of HSPGs and collagen type I among the other ECM components may then serve to limit cell activation. A balance between the elevated concentrations of mitogenic agents and the increased amounts of HSPGs and collagen type I may then determine whether MC continue to proliferate. However, continued deposition of type collagen type I may interfere with cell growth and contribute to acellularity in the sclerotic glomerulus.

4.4. FUTURE DIRECTIONS

The present study sheds new light on mechanisms by which heparin exerts its antiproliferative effect on MC. In spite of the significance of this work it leaves unanswered several crucial questions.

1. At what point does heparin suppress MAPK activation?

2. At what point does heparin suppress CaMK activation?

3. Does heparin affect the level of cAMP or cGMP?

4. Do MC possess heparin receptors?

1) The present study suggested that heparin suppresses c-fos induction through a MAPK-dependent pathway by suppressing activation of MAPK. Heparin does not seem to affect MAPK activation directly. Rather, heparin may affect enzymes upstream of MAPK, such as MEK, raf etc. MEK is a dual-specificity kinase that phosphorylates MAPK on both Thr and Tyr residues, and thereby activates it. MEK activation could be tested either by immunoprecipitation with an appropriate antibody followed by SDS PGAE after \([^{32}P]orthophosphate cell loading, or by a change in electrophoretic mobility of MEK, after activation of cells with an appropriate agonist in the presence or absence of heparin. Appearance of a band at 52 kDa would represent the phosphorylated form of the enzyme, in addition to a 50 kDa band where inactive MEK migrates (847). MEK activity also could be
tested by an in vitro kinase assay, where recombinant p42-MAPK-dependent MBP phosphorylation in the presence of [γ-32P]ATP would be an indication of the anti-MEK antibody immunoprecipitable activity. In addition a constitutively activated form of MEK (ΔMEKK, arising from mutations at S218E and S222E (847)) could be used. ΔMEKK is epitope-tagged with an ‘EE’ tag from polyoma virus middle T antigen to facilitate its detection, and is under control of the T7 promoter. If heparin blocks the action of MEK, transfection of RMC with ΔMEKK should lead to direct activation of MAPK and c-fos expression. If heparin has a direct affect on MAPK, this construct should not overcome heparin blockade.

If heparin acts upstream of MEK its effect on Raf-1 could be examined using a similar approach by preloading cells with [32P]orthophosphate followed by immunoprecipitation. In addition the effect of heparin on Ras could be tested by using immunoprecipitation and Western blotting with anti-Ras and anti-phosphotyrosine antibodies to quantify Tyr phosphorylation of Ras at 21 kDa. In order to increase specificity, BZA-SB, an inhibitor of farnesyl transferase (974) could be used. It blocks attachment of a farnesyl moiety to Ras, and thereby inhibits normal Ras function.

MAPK is also controlled by dual specificity phosphatases, PAC1 (975) and MKP-1, whose gene transcription can be induced by SAPK (976). Both are immediate early response genes induced by similar parallel stimuli to those that activate MAPK (850). Their mRNAs appear only 30 min after stimulation and their de novo synthesis is required before inactivation of MAPK can occur (850, 975, 976). Experiments using catalase in mitogenic studies, suggested that heparin does not operate by scavenging oxygen species and therefore does not activate phosphatases by that mechanism. Furthermore, heparin was effective as early as 0.5 - 2 min while inhibiting CaMK and MAPK activation, respectively, arguing against its involvement in activating MKP-1 and PAC1. However, a membrane associated phosphotyrosine phosphatase called PTPaseG has been identified and its expression appears
to be constitutive (977, 978). It can be activated within 1 min with GTP and
dephosphorylates Raf-1 in vivo and in vitro, suppressing activation of MAPK pathways (979). It is pertussis toxin-sensitive and its substrate specificity beyond Raf is unknown. Its plasma membrane association makes it potentially accessible to cell surface-bound heparin. Therefore, the effect of heparin on PTPaseG activity should be tested.

2) Uninhibited activation of MLCK in the presence of heparin suggests that heparin acts at the level of CaMK-II and does not affect calmodulin activation. However, it is possible that heparin partially decreases calmodulin activation and that CaMK-II just requires a higher level of activated calmodulin than MLCK. To distinguish these possibilities, measurement of calmodulin activity will be necessary. According to Flik et al. (980) calmodulin-dependent phosphodiesterase (PDE) converts cAMP to AMP, whose concentration can be measured indirectly by the amount of inorganic phosphate liberated by alkaline phosphatase. In order to address specificity of the assay, trifluoperazine, a calmodulin antagonist should be used in addition to heparin. Decreased PDE activity in heparin-treated cells would indicate that calmodulin is a target of heparin, and that MLCK is less sensitive to calmodulin levels than CaMK-II. If heparin does not affect PDE activity, that would suggest that the effect of heparin is on CaMK-II autophosphorylation.

Although the present study indirectly implicated CaMK-II in c-fos induction, direct evidence is lacking. For that purpose constitutively active CaMK-II may be used. By converting Thr286, a phosphorylation site in CaMK, to negatively charged Asp, a constitutively active T286D mutant has been prepared (981). Transient over expression of active CaMK-II should induce c-fos transcription, and it should be resistant to heparin.

3) Recently, PKA was shown to inhibit activation of CaMK-IIV in COS-7, PC12, and Jurkat cells (961). PKA was shown to block signals from Ras to Raf-1 (960). Heparin increases cellular levels of cAMP while inducing capacitation of sperm (982) and increases cGMP in endothelial cells by activating guanylyl cyclase (983). Therefore, activated PKA
due to increased concentration of cAMP could inhibit auto-CaMK activity in the presence of heparin. To test this, cAMP concentration could be measured in heparin-treated cells, in addition to measuring PKA activity. The application of forskolin, or cell-permeant dibutryl cAMP (to increase cAMP levels) on the one hand, and KT5720, a PKA inhibitor, on the other, would increase specificity of the test.

4) The presence of specific heparin receptors is still awaits to be determined. There are cell surface low affinity (734) in addition to high affinity (637, 734) heparin-binding sites. To identify them heparin could be iodinated, tritiated or labeled by FITC. Binding of heparin should occur at the cell membrane site.

The studies with collagen type I raise several questions.

1. What is the organization of the cytoskeleton in MC grown on collagen type I?
2. Does collagen type I affect development of focal adhesions and stress fibers in MC?
3. Does collagen type I affect expression of ET-1 receptors on MC?
4. How does collagen type I regulate PKC activity?

1) Collagen type I has distinct and specific effects on MC morphology, growth, and signal transduction systems. The polymerized actin network is essential for the function of both PKC-dependent and independent pathways of PAK tyrosine phosphorylation (927). The state of actin polymerization in cells grown on collagen type I could be determined by a DNase I inhibition assay (984), and compared to those grown on plastic.

2) In addition to cytoskeletal organization, the development of focal adhesions and stress fibers could be changed. Adhesion could be detected by immunocytochemistry with anti-vinculin, anti-talin, and anti-actin antibodies. These observations could be supplemented by phalloidin staining of actin filaments in cells grown on both collagen type I and plastic.
3) Down-regulation of PDGF receptor expression is observed on the surface of MC grown in three-dimensional collagen gels (546). ET receptor subtypes are modulated in a pathophysiological setting. It is possible that collagen type I regulates expression of ET receptors in MC. ET receptors on MC grown on collagen type I and plastic could be detected by determining binding of $^{125}$I-labeled ET-1 and $^{125}$I-labeled ET-3 to MC. The expression of the ET-receptor message could be determined by using RT-PCR or Northern blotting. Furthermore, the inhibitory effect of ET$_A$ antagonists (BQ-123 and FR 139317) on ET-1 induced effects could also be used.

4) Expression of PKC isoforms on MC grown on collagen type I could be determined by Western blotting by using isoform-specific antibodies, or by doing RT-PCR with appropriate primers for individual isoforms. Activation and translocation of specific isoforms could be followed by confocal microscopy using an isoform-specific fluorescent antibody.
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