29

VASCULAR ENDOTHELIAL CELL-SURFACE PROTEOGLYCANS

by

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ABSTRACT

A predominant species of heparan sulfate proteoglycan that consisted of at least two subunits linked by disulfide bonding was isolated from cell layers of normal ("cobblestone") bovine vascular endothelial cells in culture. Treatment of the parent molecules with dithiothreitol caused their complete cleavage and permitted the subsequent separation of the larger and smaller subunits on Sepharose CL4B columns. Removal of dithiothreitol by dialysis resulted in the reformation of large disulfide-bonded molecules but such recombination of the subunits was prevented by prior reductive alkylation using iodoacetamide.

Buoyant density gradient analysis as well as gel chromatography on Sepharose CL6B columns, following alkaline borohydride and nitrous acid treatment of individual carbohydrate-rich subunits, showed that the latter consisted of core proteins associated solely with heparan sulfate glycosaminoglycans. The sizes of the latter were estimated by chromatographic techniques to be approximately 50 000 and 14 000 daltons in the case of the larger and smaller subunits, respectively. This is the first description of disulfide-bonded proteoheparan sulfates in bovine aortic endothelial cells.

Studies of the effects of various extracellular matrices on the proliferative behaviour of bovine aortic endothelial cells in culture revealed that extracellular matrix material from rat smooth muscle cells stimulated proliferation more than did other matrices. Bovine aortic endothelial cells also changed their morphology and cell-surface

proteoglycan profiles in response to particular extracellular matrices. Enzymic modifications of matrices did not, however, cause noticeable changes in the cell surface proteoglycans synthesized by bovine aortic endothelial cells. This discrepancy suggested that the observed differences in cell-surface proteoglycan profiles cannot be ascribed to any specific single constituent of the extracellular matrix but that its overall architecture may be the sole determinant of such differences.

When the turnover of endothelial cell proteoglycans was assessed, degradation of both intracellular and pericellular proteoglycans was inhibited by lysosomotropic agents. This indicated that these macromolecules may be degraded within the lysosomes; the cell layer proteoglycans are apparently internalized prior to their degradation in this location. Failure by both NH_4Cl and chloroquine completely to block the degradation of intracellular as well as pericellular proteoglycans suggested that other mechanisms of degradation also exist.

The results extend biochemical data on endothelial cell surface proteoglycans.

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ABBREVIATIONS AND SYMBOLS

AMP	=	adenosine monophosphate
BAE	=	bovine aortic endothelial
BSA	=	bovine serum albumin
°C	=	degrees Celsius
$CaCl_2$	=	calcium chloride
CHAPS	=	6-|(3-cholamidopropyl)dimethylammonia|-1-propanesulfonate
Ci	=	Curie (3.7×10^{10} disintegrations per second)
cm	=	centimeter
CPM	=	counts per minute
CS	=	chondroitin sulfate
D-Gal	=	D-galactose
D-GalN	=	D-galactosamine
D-Glc	=	D-glucose
D-GlcN	=	D-glucosamine
D-GalUA	=	D-galacturonic acid
D-GlcUA	=	D-glucuronic acid
DPM	=	disintegrations per minute
DS	=	dermatan sulfate
DTT	=	dithiothreitol
EDTA	=	ethylenediamine tetraacetic acid
ECM	=	extracellular matrix
g	=	acceleration due to gravity
GAG(S)	=	glycosaminoglycan(s)
GalNAc	=	N-acetylgalactosamine
GlcNAc	=	N-acetylglucosamine

h = hours

HCl = hydrochloric acid

HIFCS = heat-inactivated foetal calf serum

HS = heparan sulfate

HSPG(S) = heparan sulfate proteoglycan(s)

HUVE = human umbilical vein endothelial

IAA = iodoacetic acid

$K_{av} = \frac{V_e - V_o}{V_t - V_o}$

ℓ = litre

LDL = low density lipoprotein

L-IdUA = L-Iduronic acid

LPL = lipoprotein lipase

M = molar

MEM = minimum essential medium

mg = milligram

min = minutes

ml = millilitres

mM = millimolar

M_r = relative molecular mass

MW = molecular weight

n = number of determinations

$NaBH_4$ = sodium borohydride

$NaNO_2$ = sodium nitrite

NaOH = sodium hydroxide

NEM = N-ethylmaleimide

NH_4Cl = ammonium chloride

NH_4OH = ammonium hydroxide

nm = nanometer

PBS = phosphate-buffered saline

PG = proteoglycan

pH = negative logarithm of the hydrogen ion concentration

PMSF = phenylmethylsulfonyl fluoride

rpm = revolutions per minute

S.D. = standard deviation

SDS = sodium dodecyl sulfate

Tris = tris(hydroxymethyl)amino methane

UDP = uridine diphosphate

μ (prefix)= micro (10^{-6})

μg = microgram

μℓ = microlitre

μM = micromolar

V_e = elution volume

V_o = void volume

V_t = total volume

v/v = volume per volume ratio

w/v = weight per volume ratio

% = percent

CONTENTS

CHAPTER 1

1. INTRODUCTION

1.1. STRUCTURAL-FUNCTIONAL CONCEPT OF THE ARTERIAL WALL

The arterial wall consists of at least three distinct morphological layers. Fig. 1.1 is a diagrammatic representation of these layers. The innermost layer, the intima, consists of a continuous monolayer of endothelial cells supported by a layer of connective tissue which is bounded peripherally by an internal elastic lamina. The connective tissue matrix between the endothelial layer and the elastic lamina is made up of a small number of smooth muscle cells. The middle layer, however, the media, consists almost entirely of smooth muscle cells attached to one another by specialized junctions. These cells are surrounded by an extracellular matrix (ECM) composed of collagen, elastic fibres, glycoproteins and proteoglycans. There is an external elastic layer between the media and the adventitia, the latter being the outermost layer of the arterial wall. The adventitia consists predominantly of fibroblasts; however, some smooth muscle cells help to make up the cellular content. The adventitia also consists of a large amount of extracellular matrix material with relatively few cells in the same manner as the media.

Considerable advances have been made in understanding the connective tissue structural components of the arterial wall. Both the biological characteristics of the cellular elements and the ECM components are important in the understanding of arterial wall metabolism, especially in relation to the development of atherosclerosis.

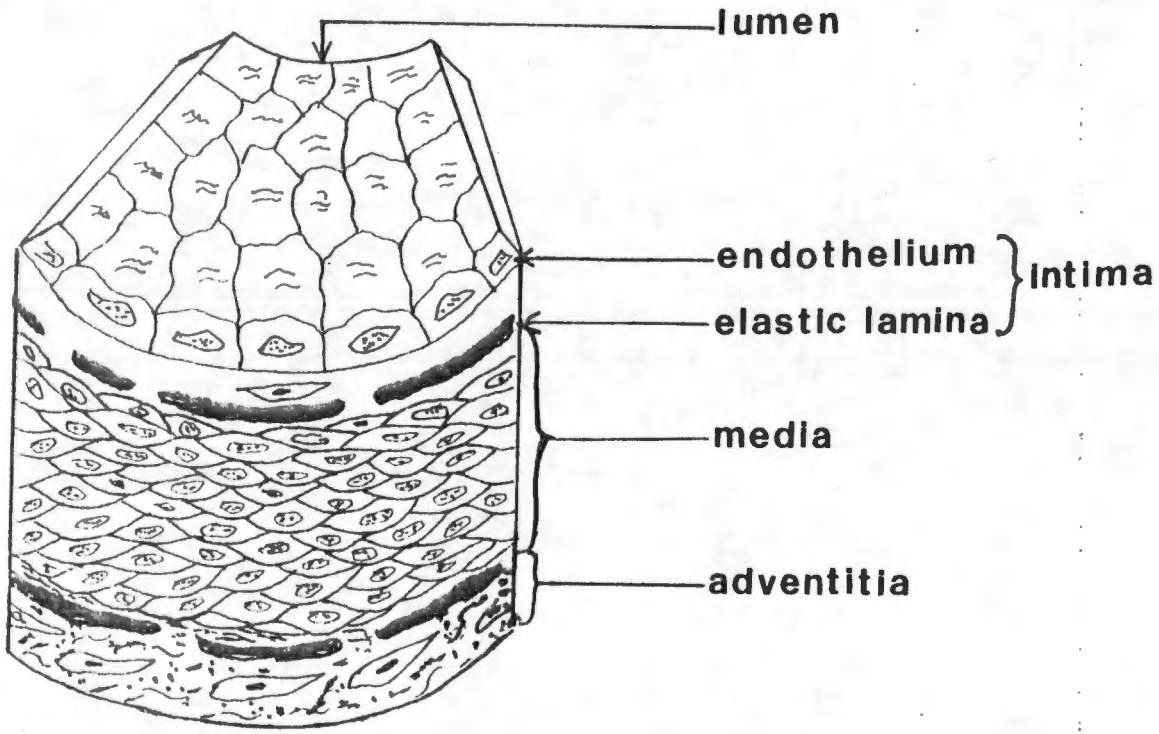

FIG. 1.1. The layers of an artery.

(After Ross, R. and Glomset, J.A. (1976) N. Eng. J. Med. 295(7): 369-376)

Observations on proteoglycans have improved our perspective of the role of connective tissue components in maintaining the integrity of the arterial wall. These complex substances have also been found to be important in pathological changes intrinsic to the arterial wall and in interactions with extrinsic factors related to blood and haemodynamic forces (5,52,71,90). The arterial wall has a well-differentiated structure adapted to be resilient and flexible for circulating blood and its components. As with other tissues that are subjected to various forms of stress and injury, arterial tissue must be able to respond to damage by repair and maintenance of its structure. This response involves hyperplasia and possible transformation of cells resulting in their metabolic alteration. These events probably occur partly as a result of attempts to preserve the integrity of the arterial wall. Thus cells respond in an adaptive manner to the circulating blood elements and also to various stimulating factors that result from wall damage. The interstitial matrix material and fibrous components of the arterial wall play a fundamental role in the pathogenesis of atherosclerotic lesions (5,52, 71,90).

The luminal surface of blood vessels is formed by the endothelium, a monolayer of endothelial cells which rest on a specialized basement membrane also known as the subendothelial extracellular matrix. The manner in which endothelial cells perform their normal function is reflected in part by the relation of these cells to their ECM. The endothelium in culture has been shown to be associated with a subcellular matrix which contains collagen, elastin, glycoproteins and proteoglycans (14,15,99,106). Studies of the composition of such an

ECM have helped establish the biosynthetic profile of endothelial cells and the corresponding effects on cellular behaviour. Several laboratories have established that the ECM plays an integral role in determining endothelial cell polarity, orientation, morphology and response to growth factors (8,11,13,16,26). Endothelial cells play important roles in controlling haemostasis and blood vessel permeability. Correspondingly, altered structure and function of endothelial cells might contribute significantly to the pathogenesis of vascular diseases. Detailed knowledge concerning the metabolism of endothelial cells under normal and pathological conditions would therefore be valuable for a better understanding of the pathophysiology of such diseases. The in vitro cultivation of endothelial cells has greatly facilitiated studies on the metabolic functions of these cells and their potential involvement in a wide variety of biologically interesting processes.

1.2. CULTURE AND MORPHOLOGY OF ENDOTHELIAL CELLS

The culture of bovine aortic endothelial (BAE) cells has become a very popular in vitro model for the study of some aspects of the blood vessel wall. Endothelial cells can be isolated free of contamination by smooth muscle cells, and even where initial isolates of BAE cells are contaminated with smooth muscle cells, the latter may be removed by one of two methods, viz., the "thymidine suicide" technique (129) or cloning (130).

Identification criteria for cultured BAE cells are similar to those for human umbilical vein endothelial (HUVE) cells (127), though there

are some significant differences. Both cell types form confluent monolayers of large, polygonal, closely apposed cells when examined by phase contrast microscopy (see plates in Chapter 3). However, when examined by transmission electron microscopy, cultured BAE cells, unlike HUVE cells, show no signs of Beibel-Palade bodies (rod-shaped cytoplasmic bodies) (127,129). The ordered monolayer of highly flattened, polygonal cells resembles a cobblestone pavement. Also, post-confluent BAE cells may show focal areas of a secondary growth pattern which has been called "sprouting" (129,131) and has been well-described by Schwartz (129). Such behaviour may be controlled by certain conditions, such as removal of overlying endothelial cells with collagenase, treatment of intact cultures with 8-butyrylcyclic AMP or cholera toxin (127). While the exact nature of "sprout" cells is not clear, they seem to be a subtype of endothelial cells (127). However, no in vivo correlate of this sprouter phenomenon has been documented.

The endothelium exhibits both morphological and functional polarity. The luminal side (the side exposed to the blood stream) has been shown to be non-thrombogenic, whereas the abluminal surface was thrombogenic (16). This is of great physiologic importance, as platelet adhesion to endothelium is thus prevented under normal circumstances and the possibility of thrombus formation thus minimized. Both events may play roles in the initiation of atherosclerotic plaque (71). In addition, various metabolites such as prostacyclin (PGI_2), factor VIII, plasminogen activator and angiotensin-converting enzyme are secreted from the luminal surface which all aid in the maintenance of a thrombo-resistant surface (129). A number of these

compounds have been used to identify and classify endothelial cells in culture (130).

When endothelial cells are removed from the inner lining of the vasculature and grown in vitro, in spite of the fact that they no longer exist in such an organized environment, they retain many important characteristics associated with the in vivo state cells.

1.3. ENDOTHELIAL CELL MOTILITY AND CONTACT INHIBITION

Monolayer morphology is fundamental to our concept of the endothelium as a continuous barrier. Available data suggest that endothelial cell growth is controlled by factors intrinsic to the endothelium resulting in contact inhibited cessation of growth. Such control of cell growth has been poorly characterized. Cell contact clearly inhibits cell movement since migrating cells cease movement when they encounter other migrating cells (127). Endothelial cell motility is one of several mechanisms which secure the integrity of the vascular intima during physiologic replacement of endothelial cells, and provide rapid initial re-endothelialization after a denuding intimal injury. The space underneath dying endothelial cells is occupied by adjacent cells moving into this area - followed by endothelial cell replacement (127).

1.4. ENDOTHELIAL CELL FUNCTION

The basic functions performed by endothelial cells are similar in blood vessels of all sizes (156). Endothelial cells must provide a non-thrombogenic surface to which platelets and other blood cells do

not adhere. They must mediate the passage of nutrients and other solutes from the blood to the tissues and they must maintain an open vessel lumen by growing as a single cell layer tightly adherent to the basement membrane of the vessel wall. Endothelial cells may also regulate vascular tone by synthesizing the vasoactive agent prostacyclin and angiotensin-converting enzyme. Despite these functional similarities there is some reason to believe that there may be significant physiological differences between endothelial cells in the smaller capillaries and those in the larger arteries and veins. For example, in several disease states including arthritis, psoriasis and neoplasia, there is a rapid proliferation of new capillaries with relatively little change in the large vessels. Other diseases, notably atherosclerosis, preferentially affect the large vessels. In addition, the endothelial cells of large and small vessels have markedly different morphologies. In large vessels, endothelial cells form the typical flat monolayer of tighly packed polygonal cells as already described, whereas in capillaries, individual endothelial cells may wrap around to form hollow cylindrical tubes through which blood flows (127).

In relation to their function, the surface components of vascular endothelial cells are important since they are in continuous contact with the circulating blood and its components. The outermost structural component of the endothelial cell is the pericellular coat or "glycocalyx". Any interaction between blood components and the endothelium involve this layer. Vascular endothelial cells synthesize and secrete sulfated glycosaminoglycans (19,30,50,67,72), some of which remain associated with the cell surface. The predominant proteoglycan

found on the surface of endothelial cells has been shown to be heparan sulfate proteoglycan (HSPG) (50), whereas other types of proteoglycans such as dermatan sulfate and chondroitin sulfates are found associated with the extracellular matrix. HSPGS with anticoagulant properties have been shown to be released by cultured endothelial cells (36,39, 50,67,72,74,116,120). Since vascular endothelial cells are in intimate contact with the plasma and thus form the initial barrier to transport of tissue nutrients as well as providing protection of the vascular wall from damaging macromolecules, it is likely that the specific glycosaminoglycans (GAGS) synthesized by endothelial cells and retained on their surface or in the subendothelial basement membranes determine, in part, the ability of the endothelium to sustain such functions.

Inspite of all these recent findings, little is known about the detailed biochemical characteristics of endothelial cell proteoglycans, their mode of attachment to cell membranes and/or to structural components of the subendothelial matrix. Their precise functional roles in vascular physiology and pathophysiology also remain unclear and demand increased attention.

1.5. THE SUBENDOTHELIAL MATRIX

Vascular endothelial cells rest on a subendothelium which performs a number of important physiological functions such as maintaining the mechanical integrity of the vessel and acting as a filtration barrier helping to control the passage of cells and molecules (99). The subendothelium also clearly provides a physical substratum for the growth and migration of endothelial cells and may indeed play

an important role in the control of these processes under both physiological and pathological conditions. Subendothelial matrices are made up of collagen, glycoproteins and proteoglycans (99). Vascular endothelial cells are believed to participate in the synthesis and deposition of their own subendothelial matrix *in vivo*. Cultured BAE cells have been shown to synthesize and deposit an ECM beneath their basal surface which is called subendothelial matrix - to indicate its anatomical similarity to the subendothelium *in vivo* (99). There is a continuous deposition of ECM by endothelial cells in culture, so that the thickness of the ECM increase as a function of time in culture. The sprouting cells may also contribute to the deposition of ECM in late confluent cultures (99). The subendothelial extracellular matrix is capable of supporting growth and proliferation of other cell types in culture (110). Although some progress has been made in studies on ECM-cell interactions, relatively little is known of the involvement of individual components of the ECM, and future research should clarify the very many enigmas that exist in this field.

1.6. IMPLICATION OF ENDOTHELIAL CELLS IN ATHEROSCLEROSIS

Atherosclerosis is the pathological alteration of the walls of blood vessels, in particular those of arteries (5,71). This pathology manifests itself by the presence of protruding lesions on the normally smooth luminal surface of the vessels. These lesions are called atherosclerotic plaques and the resultant thickening of the vessel wall may reduce blood flow sometimes seriously. These plaque sites often result in the formation of thrombi which cause complete occlusion of important blood vessels such as the coronary arteries.

Understandably, the pathophysiology of atherosclerosis has been much studied. A number of different mechanisms have been suggested for the deposition of atherosclerotic plaques (5,71,90), and there is evidence that atherosclerosis is a multifactorial disease in origin. Considerable advances have been made in understanding the connective tissue structural components of the arterial wall (5,90), since important functions of connective tissue components in normal and disease states are increasingly being recognized. The biologic characteristics of cellular elements, endothelial and smooth muscle cells, the fibrous structures, collagen and elastin, and the interstitial matrix proteoglycans are all of interest to understanding arterial wall metabolism and the development of atherosclerosis.

Endothelial cells also secrete extracellular matrix materials (99) that can influence the retention of plasma molecules within the arterial wall. Damage through injury to the endothelial layer exposes subendothelial connective tissue (5,127) to platelets and other blood components. The platelets interact with subendothelial collagen, aggregate and release platelet-derived growth factor (PDGF) and thromboxane A_2, a potent smooth muscle cell constrictor. The infiltration of PDGF and plasma constituents have been shown to result in the proliferation of smooth muscle cells and elaboration of connective tissue elements by smooth muscle cells. In the surrounding connective tissue it has been postulated that interaction of lipoproteins occurs with the anionic proteoglycans, particularly chondroitin-dermatin sulfate proteoglycans (85,90). The HSPG which is found on the cell surface and in association with internal elastic lamina has been shown to be a potent anticoagulant and may inhibit

platelet aggregation. In addition, the HSPG associated with the surface of endothelial cells acts as the receptor for lipoprotein lipase (LPL), a key enzyme in lipoprotein metabolism, resulting in its binding to the endothelium (206,208). Thus, HSPG may be considered as an antagonist to lesion development and as such may be an important factor in the pathogenesis of arterial disease (5,71,90).

1.7. STRUCTURE AND METABOLISM OF PROTEOGLYCANS

1.7.1. STRUCTURE OF GLYCOSAMINOGLYCANS

Proteoglycans are a ubiquitous family of macromolecules with unique physical properties (7,128,134). They are primarily extracellular, although intracellular as well as membrane-bound proteoglycans have also been described (2,4,15,17,19,21-25,30,31,36,40,46,48,50,54,55, 57,65-68,93-96,111,112,117,123,124). The basic structure of a proteoglycan consists of a peptide or protein core with covalently linked carbohydrate chains attached (Fig. 1.2). A small percentage of the latter is similar to the short sugar chains typical of glycoproteins (134), however, most of this component takes the form of long linear glycosaminoglycan (GAG) chains composed of repeating disaccharide units . The biochemical classification of GAGS is based on the structure of the repeating disaccharide units and the following distinct species have so far been characterized: hyaluronic acid, chondroitin sulfates, keratan sulfate, dermatan sulfate, heparin and heparan sulfates (Table 1.1). Except in the case of hyaluronic acid, these structures always exist covalently linked to a protein core, in the form of proteoglycans. Hyaluronic acid, which exists

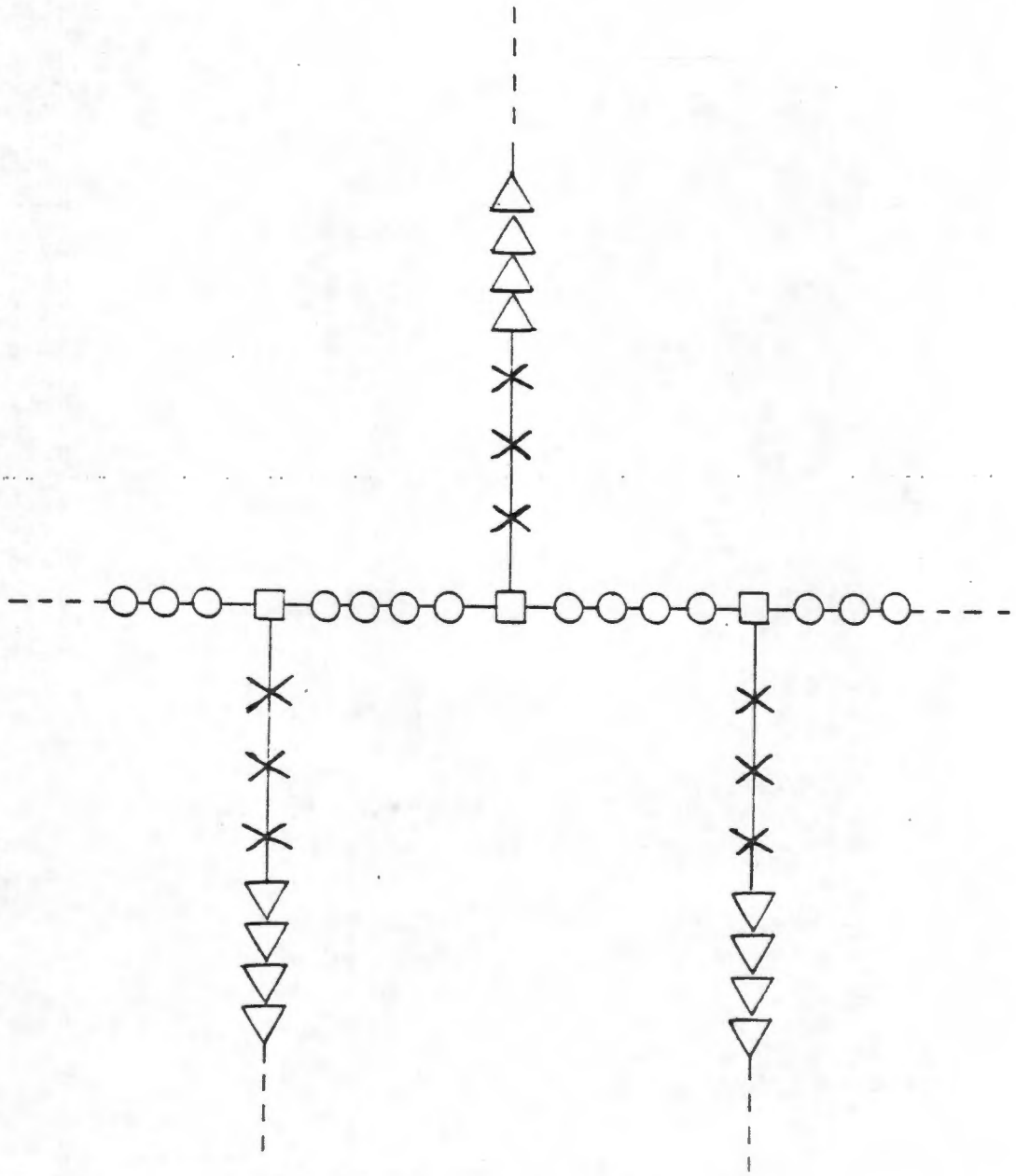

FIG. 1.2. Schematic diagram of the compositional structure of a proteoglycan. ○ = amino acids of the protein backbone not involved in glycopeptide linkage; □ = amino acids of the protein backbone which are involved in glycopeptide linkage; ✕ = monosaccharide units atypical to the glycosaminoglycan main structure and involved in glycopeptide linkage; ▽ = monosaccharide units of the glycosaminoglycan main chain. (Adapted from Ref. 134).

TABLE 1.1. MOLECULAR FEATURES, COMPOSITION AND DISTRIBUTION OF GLYCOSAMINOGLYCANS IN MAMMALIAN TISSUES

Polysaccharide	Molecular weight x 10^{-3} (Ref. 135)	Repeating disaccharide unit	Type of sulfation	Degree of sulfation	Distribution
1. Hyaluronic acid	4000 - 8000	D-GlcUA	-	-	Synovial fluid, cartilage, skin, vitreous humor, umbilical cord, connective tissue.
2. Keratan sulfate	4 - 19	D-Gal D-GalN-SO_4	O-SO_3^-	+++	Cartilage, cornea, bone intervertebral disc.
3. Dermatan sulfate	15 - 40	D-GlcUA L-IdUA D-GalN	O-SO_3^-	++	Skin, blood vessel, heart valves, cartilage, tendon, bone, aorta
4. Heparin	4 - 16	D-GlcUA L-IdUA D-GlcN D-GlcN-SO_4	O-SO_3^- N-SO_3^-	+++++	Lung, liver, mast cells, skin, intestinal mucosa
5. Heparan sulfate	75	D-GlcUA L-IdUA D-GlcN	O-SO_3^- N-SO_3^-	++++	Lung, liver, skin, blood vessel, thyroid, brain, cornea
6. Chondroitin-4-sulfate	5 - 50	D-GlcUA D-GalN	O-SO_3^-	++	Cartilage, skin, tendon, bone, blood vessels, cornea
7. Chondroitin-6-sulfate	5 - 50	D-GlcUA D-GalN	O-SO_3^-	+++	Cornea, bone, blood vessel, cartilage, aorta, intervertebral disc, umbilical cord.

as free GAG chains, is often very large and differs from other GAGS since it does not contain any sulfate groups.

Proteoglycans display a considerable heterogeneity with respect to a number of structural features, for example, the size of the polysaccharide chains, the ratio of iduronic acid (IdUA) to glucuronic acid (GlcUA) residues and to the amount and distribution of sulfate groups along the carbohydrate backbone (134). The structures of core proteins have not received much attention in the past. However, there is some evidence to suggest that different core proteins may exist and that these proteins may have different and discrete biological functions. Table 1.1 summarizes the molecular features, composition and distribution of GAGS in mammalian tissue. The work of Roden and Lindahl (136) demonstrated that the linkage between the sulfated glycosaminoglycans and protein is actually an O-glycosidic bond between serine and a xylose residue (128). Unlike the other sulfated glycosaminoglycans, heparin and heparan sulfate cannot be clearly distinguished according to structure since they exhibit a complex mixture of disaccharide units. They have been shown to be synthesized on different protein acceptors, however, and therefore represent distinct entities (136). In general, heparin is more highly charged than heparan sulfate. They are copolymers of two types of disaccharides, viz. D-GlcUA-D-GlcNAc, and L-IdUA-GlcNAc, and are the most complex of the GAGS. The number of such disaccharides in a chain usually varies from 10-60. Segments of one or several disaccharides, containing one type of uronic acid (L-IdUA or D-GlcUA) are interrupted by segments containing the other. The GlcN residues contain ester sulfate at position 6 and in some

instances also at position 3. Unique for heparin and heparan sulfate is that in many of the GlcN residues the N-acetyl group is replaced by an N-sulfate group. Heparin contains a higher proportion of N-sulfate and therefore also of IdUA residues than heparan sulfate. Heparan sulfate is also O-glycosidically linked to protein (136). Heparin, on the other hand, may be degraded after synthesis so that few molecules exhibit the linkage oligosaccharide (147). Heparan sulfate has been shown to be present on the surface of many cells, but may in addition be associated with the extracellular matrix in many fibrous connective tissues (83,136). Although a great deal is known about the structure of proteoglycans, many of the complexities of these molecules have yet to be studied and this has a bearing upon their isolation and separation.

1.7.2. BIOSYNTHESIS AND SECRETION OF PROTEOGLYCANS

Polysaccharide formation is initiated by the transfer of xylose units from UDP-xylose to serine or threonine residues in the core protein, and this transfer is catalyzed by xylosyltransferase (E.C.2.7.7.11). The xylosylated core protein serves as an acceptor for the successive transfer of two galactose units from their corresponding UDP-sugars, which is catalyzed by galactosyltransferase (E.C.2.7.7.10). In the case of chondroitin sulfates, polymer formation involves an alternating transfer of GalNAc and GlcUA units to the non-reducing end of the growing polysaccharide chain forming a chain of repeating disaccharides. The polymers of the other GAGS, consisting of their respective repeating disaccharide units, are formed in an analogous manner.

Subsequent to polymer formation, the repeating disaccharide is subjected to a number of enzymatically catalyzed polymer modification reactions. In the case of the chondroitin sulfates, these may involve GlcUA epimerization and O-sulfation of C-4 and C-6 of GalN units and C-2 of IdUA components. The polymer modification reactions involved in the biosynthesis of heparan sulfate and heparin include N-deacetylation and N-sulfation of the GlcNAc unit, C-5 epimerization of GlcUA units and O-sulfation at C-6 of GlcN units and at C-2 of IdUA residues.

The overall biosynthetic process as outlined above, is now well established (139), and recent observations suggest that in many, if not all of the post-translational modifications take place in the Golgi complex (72). However, there is still a paucity of direct evidence on the specific details of these modifications and their role in the secretory process (113). The mechanisms of proteoglycan secretion are still obscure. It appears that not all of the de novo synthesized proteoglycans are destined for export. Kimura et al. (140), using pulse-chase experimental techniques, have shown that in cultured chondrocytes the contents of secretory vacuoles were secreted into the matrix over a period of 10-30 min following a 5 min pulse with $^{35}SO_4^{2-}$ (140). One hour after the addition of radioactive label, most of the radioactivity was found in the matrix. They showed that once the proteoglycans were outside the cell they interacted with link protein, hyaluronic acid and matrix proteins to become an integral part of the matrix (140).

The use of drugs affecting the secretory process at distinct sites in

the cell has proved valuable for more detailed studies on the specific steps in the secretory pathway. Such secretion studies have been reported using cultured endothelial cells (39,72), smooth muscle cells (67) and rat ovarian granulosa cells (94). Colchicine and other antimicrotubular agents have been demonstrated to inhibit secretion in a variety of cells and tissues (113). Similarly, monensin (60) has been used to perturb the secretion of proteins, glycoproteins and proteoglycans. Available evidence has implicated the Golgi apparatus as the primary site of action of monensin as shown by the electron microscopic observation of Golgi structures (115). Monensin has been reported to cause undersulfation of proteoglycans and inhibit their secretion (40,41). However, still more detailed studies are necessary to delineate the pathway of secretion of proteoglycans.

1.7.3. CATABOLISM OF PROTEOGLYCANS

There is little information on the degradation of proteoglycans, although potential individual steps have been studied (128). Some data indicate that cartilage proteoglycans are gradually degraded by proteases (137). All connective tissue cells appear to have a capacity for synthesizing a variety of proteolytic enzymes (128). Kresse and coworkers (65,66,70) showed that arterial and skin fibroblasts in culture were able to specifically bind and internalize proteoglycans. The internalized GAG-peptide fragments were further degraded in the lysosomes. The enzymes responsible for the degradation of such peptides were cathepsins having pH optima of 3. The GAGS may be depolymerized by endoglycosidases (63).

Synthesis and degradation of endogenous proteoglycans have been studied in various culture systems, including smooth muscle cells (1,61,65-67,100,101), fibroblasts (10), ovarian granulosa cells (94) and hepatocytes (138). The fact, however, that proteoglycans are internalized prior to their degradation is now well established in several systems (65-70). Recently, Völker et al. (100,101) have studied the binding and degradation of proteoglycans by cultured arterial smooth muscle cells. Their study gave an ultrahistochemical insight into the catabolic pathway of proteoglycans, viz., proteoglycans were internalized by coated and non-coated vesicles, translocated to larger vesicles and finally to multivesicular bodies which were characterized by the presence of fuzzy coated vesicles in their interior. These vesicles developed into multilamellar bodies and secondary lysosomes. After degradation, the proteoglycans accumulated in residual vacuoles were released from the cell by exocytosis. This sequence of metabolic events has not been described biochemically before, but is supported by studies which have provided some data on binding, endocytosis and lysosomal degradation of proteoglycans (1,10,50,61,65-70,79,88,94).

1.7.4. INTERACTIONS OF PROTEOGLYCANS

A number of reports have dealt with in vitro interactions of various matrix components with proteoglycans and glycosaminoglycans (2,5,7,10, 11,15,18,20,29,52,55,58,89-92,104). A central problem has been in defining the specificity and physiologic relevance of such interactions. Many non-specific interactions can be mediated because of the strong polyanionic nature of GAGS. Available data suggest that proteoglycans may interact with other connective tissue elements under physiological

conditions and that some of these interactions play important roles in the structural organization and biological function of the ECM. Proteoglycans from cartilage have been shown to aggregate with hyaluronic acid in the presence or absence of link proteins, but the interaction is only stabilized by link proteins (141,142).

Electron microscopic studies have suggested that proteoglycans are arranged in an orderly manner along collagen fibres in a variety of tissues including cartilage, aorta (147) and tendon (148). The nature of proteoglycan-collagen interactions and their functional importance is still obscure. The cell-adhesion glycoproteins such as fibronectin and laminin have been shown by electron microscopy to occur in close association with HSPGs in ECMs in vivo and in culture systems (149). Self-association of IdUA-containing GAGS have been shown to occur at physiological ionic strength (150,151). The general rule of these interactions appears to be that GAG chains preferably associate with chains of their own kind (83), but hybrid-associations have also been reported (125). The physiological relevance of this type of interaction is presently unclear.

Information available on proteoglycan-elastin interactions is minimal. There is some evidence based mainly on electron-microscopic observations, that in both developing and mature elastin fibres (143,144) proteoglycan components are associated with elastin. Indirect evidence that the GAGS that associate preferentially with elastin were heparan sulfates has come from the observation that ruthenium red precipitates within lathyritic elastin were much smaller than those in the matrix, and more strictly similar to those recognized as heparan sulfate in glomerular and other basement membranes

(20,37,45,77,82,96,107-109,125). The general biological significance of elastin-proteoglycan association is not known (145).

1.7.5. PROTEOGLYCANS AND POTENTIAL MECHANISMS RELATED TO ATHEROSCLEROSIS

The implications of endothelial cells in atherosclerosis has already been dealt with in section 1.6. A number of specific physiological functions and alterations with ageing have been attributed to glycosaminoglycans (GAGS). These include providing structural support to the vessel wall and resistance to infection, influencing fibrillogenesis, regulating fluid and electrolyte balance, influencing blood coagulation, platelet aggregation, calcification, lipoprotein binding as well as facilitating lipid clearance and transfer of cholesteryl esters across the endothelium.

It has been known for many years that GAGS, particularly heparan sulfate, heparin and dermatan sulfate, possess anticoagulant activity. Vijayagopal studied the haemostatic properties of chondroitin sulfate-dermatan sulfate-proteoglycan versus heparan sulfate proteoglycan from bovine aorta, and demonstrated that the latter exhibited significantly more anticoagulant activity than the former (152,153). Both proteoglycans also exhibited thrombin-induced platelet aggregation but HSPG was more potent. They concluded from their studies that the core protein of the proteoglycans was not essential for their haemostatic properties and that the anticoagulant activity of aorta proteoglycans was due to the ability of GAG chains to accelerate the inactivation of serine proteases by antithrombin III. Proteoglycans might do this by increasing the binding affinity of these proteases

for antithrombin III (154). Bounassisi and Colburn (42) observed that HSPG isolated fromcultured rabbit aortic endothelial cells was capable of inhibiting blood coagulation. In atherosclerotic lesions from human aorta the anticoagulant activity of chondroitin sulfate was lower compared to that obtained from normal intima-media regions. Although the importance of arterial proteoglycans in relation to atherosclerosis is now well recongized, little attention has been paid to possible alterations in their biological properties in atherosclerosis (162).

Lipoprotein lipase (LPL), a key enzyme in the hydrolysis of plasma chylomicrons and very low density lipoproteins (VLDL)-triglycerides, is localized on the luminal endothelial surface of blood vessels (206). However, the exact nature of the association of LPL with the surface of endothelial cells is not clear, although it appears as if it probably involves ionic interaction of the enzyme with some component of the cell membrane, perhaps a HSPG (155). Exogenous heparin has been shown to release LPL from endothelial cells either by direct interaction with the enzyme or by competing for the binding sites on the cell surface (155). Proteoglycan-LPL binding may facilitate cholesteryl ester transfer into the arterial wall from chylomicron remnants, a hydrolytic product of chylomicron.

Lipids of atherosclerotic lesions originate from plasma apo B-containing lipoproteins such as low density lipoprotein (LDL) and very low density lipoprotein (VLDL) (5,85). The ECM components (elastin, collagen and GAG) in aorta are known to undergo qualitative and quantitative changes in atherosclerotic lesions that may affect retention

of plasma constituents especially apo B-containing lipoproteins (85). The increased retention may be due to altered matrix environment, both physically and biochemically. Histochemical studies have demonstrated a close relationship between arterial GAG, fibrous structures and lipid deposits in atheromatous lesions. These connective tissue macromolecules, especially proteoglycans, selectively interact and retain certain plasma lipoproteins (5), resulting in hypercholesterolaemia which causes atherosclerosis associated with increased LDL uptake by aorta, alterations in GAG metabolism and formation of LDL complexes of GAG in association with collagen and elastin (85). Although several studies now emphasize the role of lipoprotein-GAG complexes in lipid accumulation, the relationship of GAG metabolism to uptake and accumulation of apo B-containing lipoproteins remains to be explored.

1.8. EXTRACELLULAR MATRIX

Until recently the plasma membrane had always been described as the outer boundary of cell and as such separated the intracellular from the extracellular milieu. This in turn implied that the plasma membrane was always the site of interaction between the cell and the extracellular environment or between one cell and another. While this role for the plasma membrane is probably correct it has become increasingly apparent that cell behaviour is greatly influenced by a layer of macromolecules that interact directly with the plasma membrane. The layer of macromolecules that surrounds the plasma membrane is known as the extracellular matrix (ECM). The plasma membrane and the ECM should not be considered as entirely separate entities,

rather they should be seen as a functional complex. In molecular terms it is virtually impossible to make a clear delineation between the membrane and the matrix (119). A number of components extend through the plasma membrane into the layer of ECM, and many components of the matrix bind to the plasma membrane very tightly and extreme conditions have to be used to bring about separation (146).

In vivo, most if not all cells are surrounded by some form of ECM, the biochemical nature of which is now known to play important roles in cell shape, growth (7,8,13,16), migration differentiation (110), phenotypic modulation (28), cell-surface contacts (11,91) and gene expression (92). Other differential effects of the ECM on cellular behaviour include cell detachment (26) and secretion of proteins (12). Recent advances in cell biology have revealed the presence of a complex cytoskeleton that besides having an obvious role in cell shape and movement, is postulated to have regulatory functions as well. Moreover, there has been some elucidation of the composition and complexity of the molecules of the ECM as well as an appreciation of the interaction between cell and its ECM. The recognition of regulatory roles of the cytoskeleton and the ECM and the structural interrelationship between the two provides a foundation for conceivable mechanisms that may well be central to the many profound effects exerted by the ECM on the cell. A new era of research is beginning to emerge in view of the structural-functional continuum which exists between the cell and its ECM.

1.9. SELECTION OF RESEARCH AREA

The preceding review will have brought out that the endothelial cell surfaces, like all other cell surfaces, are intricately involved in a

number of important physiological as well as pathological processes. It is also evident that the manner in which endothelial cells are able to perform their normal function is reflected, in part, by the relation of their cell-surface macromolecules to the extracellular milieu. The cell-surface macromolecules, especially proteoglycans, are strategically positioned to regulate interactions between cells and their extracellular matrix (83).

A number of aspects of vascular wall proteoglycan metabolism had been studied in our department (1,49,215). Others have shown that vascular endothelial cells in culture synthesize proteoglycans which are either secreted into the medium or retained at the cell surfaces (50). Despite these, information on endothelial cell-surface proteoglycans is minimal compared to what has been documented for other cell types, particularly in regard to detailed biochemical characteristics and modes of attachment to cell membranes (2,10,17,21,48,51,57,84,93-95, 111,124-126,202).

In this thesis selective consideration has been given to investigate three areas of interest in vascular endothelium research. These include structural analysis and turnover studies of the proteoglycans associated with the surfaces of bovine aortic endothelial cells in culture, together with an assessment of the effects of various extracellular matrices on the growth, morphology and cell-surface proteoglycan profiles of these cells, with a view to establishing the special functional importance of these surface macromolecules in vascular endothelial cells.

CHAPTER 2

ISOLATION, FRACTIONATION AND CHARACTERIZATION OF PROTEOGLYCANS ASSOCIATED WITH THE CELL SURFACE OF BOVINE AORTIC ENDOTHELIAL CELLS IN CULTURE

2.1. INTRODUCTION

A class of sulfated macromolecules known as proteoglycans is receiving much attention in relation to its physiological roles both as cell surface molecules and as organizers of the extracellular milieu (1,2, 5,7,9,11,15,21,30,31,35,50,57,59,91,95,111,117,119,125,189). Of particular interest among these macromolecules are the subgroup of proteoglycans that contain heparan sulfate (HS) glycosaminoglycan (GAG) chains (196). Heparan sulfate proteoglycans (HSPGS) have been found to be present at the external surface of a number of different cultured cells including fibroblasts (2,10,46,48,81,117,121,126), human neuroblastoma cells (6), mouse mammary epithelial cells (17,51,124,125), rat hepatocytes (22,57,59), human colon carcinoma cells (21), endothelial cells (19,36,50,74,116,120), and they also appear in basement membranes and related extracellular matrices (24,25,77,82,107-109,125).

Proteoglycans (PGS) at cell surfaces have been implicated in such basic processes as cellular adhesion (2,11,15,42,58,76,83,111,146,152,182-189), control of cell growth (35,38,179,180), haemostasis (6,42,62,153) and thrombosis (120,154,200). Apart from this, HSPGS have been reported to play an important role in the organization of basement membranes and extracellular matrices (2,24,37,45,77,82,96,105,107,108,121,125). Furthermore, specific studies on PGS, especially HSPGS, have led to an

interest in these molecules in terms of their putative roles in angiogenesis (52,198), tumorigenesis and cell transformation (6,12,14, 21,22,27,76,77,79,82,91,105,119,179) as well as in the development of atherosclerosis (5,71,85,90,153,162).

The sizes of isolated PGS as well as those of the core proteins vary from one system to another (134). Variations in size and the degree of sulfate substitution of the polysaccharide chains also contributes to the heterogeneity of PGS (22,46,81,84,108,124,125). Although species and tissue-specific differences may account for such observations, different extraction procedures have been reported to yield cell surface HSPGS of varying molecular weights (203,204). Most recent reports (73,108,202) point to the fact that variations in sizes of HSPGS from some cell systems may be accounted for, in part, by the presence of disulfide bonds formed or destroyed under certain extraction conditions. No consensus in the literature currently exists on disulfide cross-linked PGS (124,125) despite the established notion that most cell surface receptors and trans-membrane molecules consist of identical or different subunits held together by non-covalent bonds or, very commonly, by disulfide bonds (207).

The mode of association of PGS with the cell surface has been clarified in the case of hepatocytes (22,57,59,111,204) and other cell types such as mouse mammary epithelial cells (17,51,95,124,125) and rat ovarian granulosa cells (93). The cell surface PG may be an integral membrane component or may be bound to the cell surface via GAG chains (57,59). HS has been identified as the major GAG of the surface of endothelial cells (50,74,116) and as such implicated as

the unique ligand for lipoprotein lipase (206). This is almost certainly not the sole function of surface-bound PGS of this class (as already mentioned), however, and their characterization in terms of structure and function remains a challenging task.

In view of these considerations and compelling evidence that HSPGS are important for the regulation of cell-cell and cell-matrix interactions (211), this chapter is devoted to the isolation and characterization of PGS associated with the surface of bovine aortic endothelial (BAE) cells in culture.

2.2. METHODS

2.2.1. ENDOTHELIAL CELL CULTURES

BAE cells were isolated and characterized as described previously (159-161). Cultures were initiated from cryopreserved stocks and seeded at initial densities of 5×10^5 cells/75 cm^2 tissue culture flasks (Falcon). Line E_8 was used up to passage 16. All cultures were maintained at 37°C in Eagle's minimum essential medium supplemented with 10% heat-inactivated foetal calf serum (HIFCS), 10% tryptose phosphate broth, 60 µg/ml penicillin G and 100 µg/ml streptomycin sulfate as bacteriostatic agents, in an atmosphere of 5% CO_2 : 95% air in a humidified incubator. Cells were routinely subcultured with 0.05% Trypsin-EDTA at a split ratio of 1:4. Such cultures generally reached confluence within 6-8 days.

2.2.2. RADIOLABELLING TECHNIQUE

Cells were plated into 75 cm^2 Falcon culture flasks in 10 ml medium. Labelling was initiated after 4-6 days as cultures reached confluency by replacing the medium used for plating with fresh medium supplemented with 5-20 µCi/ml $^{35}SO_4^{2-}$. Uptake of radioisotope was allowed to proceed for 20-24 h at 37°C in a 5% CO_2 : 95% O_2 humidified incubator.

2.2.3. EXTRACTION PROCEDURES

At the end of the labelling period, the medium was decanted and the cell layers washed 5 times with phosphate-buffered saline (PBS) prior to extraction by one of the following methods.

2.2.3.1. SDS Extraction

Washed monolayers were extracted with a solubilizing buffer containing 2% SDS (w/v) in 0.05 M Tris-HCl, pH 7.6, in the presence of the following protease inhibitors: EDTA, 20 mM; N-ethylmaleimide, 10 mM; benzamidinium-HCl, 5 mM; 6-aminohexanoic acid, 100 mM; PMSF, 2 mM; and soya bean trypsin inhibitor, 10 µg/ml. This solution was heated to 60°C prior to the addition to cell layers. This procedure was essentially as described by Oohira *et al.* (50), and was modified by the addition of fresh dithiothreitol (DTT) to a final concentration of 5-10 mM where indicated. The extract (approximately 5 ml) appeared clear but was very viscous and centrifugation at 1000 x g did not produce a pellet. Extracts were normally analyzed directly by Sepharose CL4B chromatography. Some samples were, however, passed

over Sephadex G-50 columns to remove any small molecular weight contaminants and unincorporated sulfate (see section 2.2.5).

2.2.3.2. Brief Trypsinization

The trypsin-removable cell surface fraction was obtained by treating the washed cell layer with 20 µg/ml trypsin (E.C. 3.4.21.4; bovine pancreatic type III) in 0.05 M Tris-HCl, 10 mM $CaCl_2$, pH 7.6, for a duration of 15 min at 37°C. This treatment did not cause detachment of cells from the substratum. This procedure extracted more than 90% of the radioactively labelled material and this was further checked by first washing the cell layers extensively with the buffer containing 50 µg/ml soya bean trypsin inhibitor to terminate trypsin activity and then extracting trypsin-resistant cell layers with SDS as described in section 2.2.3.1. Trypsinates were made to 2% SDS (w/v) by addition of solid detergent. Soya bean trypsin inhibitor and other protease inhibitors were added to give final concentrations as indicated in section 2.2.3.1.

2.2.4. REDUCTION AND ALKYLATION OF EXTRACTS

Reduction and alkylation of extracts were performed essentially as described by David et al. (125) with minor modifications. Briefly, SDS extracts of BAE cell layers were adjusted to 5-10 mM DTT, flushed with nitrogen and allowed to stand at room temperature for 5-24 h in the dark. Alkylation of reduced extracts was subsequently achieved by the addition of a molar excess of iodoacetamide (40 mM) over DTT and by flushing the solutions with nitrogen and by keeping them in the dark overnight at room temperature. Reduced and alkylated

extracts were dialyzed overnight against 0.1% SDS in 0.05 M Tris-HCl, pH 7.6 prior to chromatography on Sepharose CL4B.

2.2.5. GEL FILTRATION CHROMATOGRAPHY

Cell layer PG extracts were analyzed by gel filtration chromatography on columns prepared in and eluted at 37°C with 0.1% SDS (w/v) in 0.05 M Tris-HCl, pH 7.6, containing the various protease inhibitors already described (section 2.2.3.1). The void volume (V_o and total volume (V_t) of each column were calibrated with blue dextran 2000 and $^{35}SO_4^{2-}$, respectively. Fractions (see column details in this section) were collected on a LKB 2112 Redirac fraction collector (Bromma Sweden) and 0.5 ml aliquots of alternate fractions were assayed for radioactivity by addition of 5 ml scintillation cocktail (Beckman Ready-Solv EP or Packard 299 Instagel) and counting in a Packard Tricarb model 4640 counter. All samples were treated with SDS prior to chromatography unless already so treated and some samples were treated with DTT and iodoacetamide. When samples had been so treated, they were eluted with a 0.1% SDS buffer as described, with the addition of fresh DTT to a final concentration of 2 mM. The yield of material after column chromatography was greater than 90% of the material so applied.

Column details:

Gel Type	Dimensions (cm)	V_o (ml)	V_t (ml)	V_e (ml)
Sephadex G-25	0.5 x 100	20	32	1
Sephadex G-50	0.5 x 80	14	28	1
Sepharose CL4B	0.5 x 105	52	150	2
Sepharose CL6B	0.5 x 105	19.2	48	1.2

2.2.6. BUOYANT DENSITY CENTRIFUGATION

BAE cell layer extracts were chromatographed on Sepharose CL4B. Thepeak fractions containing PG were pooled separately and lyophilized. The lyophilized extracts were then dissolved in glass-distilled water and brought to a starting density of 1.52 g/ml by addition of solid caesium chloride. Samples were then centrifuged at 48 000 rpm in a Beckman VTi 60 rotor at 20°C for 20 h. Following gradient centrifugation, fractions of 1 ml were collected and the density and PG distribution in each fraction determined by refractometry (Refractometer, Hilger and Watts) and scintillation counting of measured volumes, respectively.

2.2.7. CHARACTERIZATION OF PROTEOGLYCANS

Peak proteoglycan (PG) fractions obtained after Sepharose CL4B chromatography were pooled separately and freeze-dried prior to further analysis on Sepharose CL6B columns. To study the ^{35}S-labelled GAGS, freeze-dried pools were dissolved in glass-distilled water and split into 3 portions. One portion was handled as control, one treated with alkaline borohydride, and the other with nitrous acid as detailed below. The sizes of the GAG chains as analyzed on Sepharose CL6B were compared with a molecular weight calibration curve generated with CS-GAG standards (63).

2.2.7.1. Alkaline Borohydride Treatment

Samples were treated with alkali under conditions which have been shown to cleave covalent protein-polysaccharide linkages (81) but which prevent "peeling" of long carbohydrate chains (81). Samples

were brought to 1 M $NaBH_4$ in 0.05 M NaOH and incubated at 45°C for 24 h. This was followed by neutralization of reaction mixtures with glacial acetic acid and subsequent analysis on a column of Sepharose CL6B to assess the release of GAG chains from the PG core protein.

2.2.7.2. Nitrous Acid Digestion

For nitrous acid treatments, 1 ml aliquots of pooled samples were incubated with equal volumes (125 µl) of 18% $NaNO_2$ and glacial acetic acid at room temperature for 90 min. This treatment cleaves HS by a β-elimination reaction, of the glycosidic bonds of N-sulfated and non-sulfated groups of glucosamine, releasing N-sulfates as $^{35}SO_4^{2-}$. The reaction was stopped by the addition of 150 µl concentrated NaOH. Neutralized samples were filtered through a 0.8 µm Millex-HA filter (Millipore) and then chromatographed on Sepharose CL6B. Samples sensitive to nitrous acid deamination were identified as HS.

2.3. RESULTS

When radiolabelled material extracted into hot 2% SDS-containing solution was chromatographed directly on Sepharose CL4B columns, a variety of profiles were obtained as shown in Fig. 2.1. The differences between the profiles depended on the growth conditions employed prior to extraction. When cells were grown to confluency and refed by medium change prior to labelling for 20 h by the addition of $^{35}SO_4^{2-}$ (see section 2.2.2), the extracted radioactivity chromatographed on Sepharose CL4B was resolved into a peak at V_o and another

at V_t (Fig. 2.1a). If the extracted material was first passed over a Sephadex G-50 column, however, only a single peak eluting at V_o was obtained on Sepharose CL4B (data not shown). Thus the low molecular weight material eluted at V_t both on Sepharose CL4B and on Sephadex G-50 and was largely made up of unincorporated sulfate.

When cultures were grown for 4 days post-confluency and incubated with radioactive sulfate without prior medium change, the profiles of extracted material were found to be as shown in Fig. 2.1b: Two peaks of macromolecular material were now observed, one eluting at the V_o of the Sepharose CL4B column and another as a peak with a K_{av} of 0.46. The peak of material that eluted at the V_t of the Sepharose CL4B column was also eliminated by prior chromatography on Sephadex G-50.

When BAE cells were grown on culture dishes coated with ECM derived from cultured rat smooth muscle cells (213), they reached confluency rapidly and exhibited atypical morphology termed "sprouting" (129). When these cells were cultured in the presence of $^{35}SO_4^{2-}$, they formed proteoglycans which gave rise to the Sepharose CL4B profiles shown in Fig. 2.1c. There were three distinct species of labelled macromolecules: One which eluted at V_o of the Sepharose CL4B column and two included species which had K_{av} values of 0.46 and 0.59, respectively. The large peak of material that eluted at the V_t of Sepharose CL4B columns was again shown to represent molecules that were very small in size when assessed by Sephadex G-50 chromatography, where they also eluted at the V_t position. Thus, depending on growth conditions, cultured BAE cells had associated with their cell layers either a single large proteoglycan which was always excluded from Sepharose

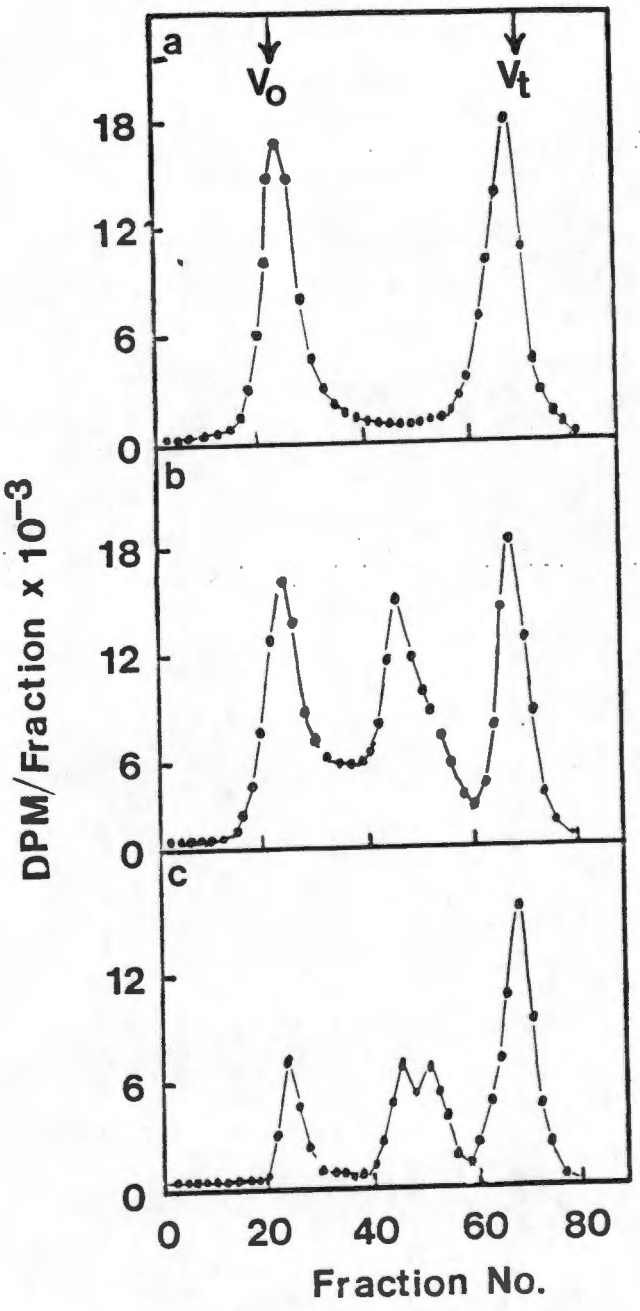

FIG. 2.1. Elution profiles of $^{35}SO_4^{2-}$-labelled material analysed by Sepharose CL-4B chromatography. Radioactive material was obtained by extraction of cell layers with solutions containing 2% SDS and protease inhibitors, as described in Methods. The profile shown in panel (a) represents material from cells refed prior to incubation with isotope; panel (b) shows cells incubated with radioisotope without prior medium change and panel (c) material from cells showing atypical (sprouter) morphology, extracted under the same conditions as described in the case of (b). Profiles were typical of those seen in numerous experiments and represent the total radioactivity present in each fraction. Recoveries of material applied were 90%.

CL4B columns or the same species together with some smaller proteoglycans (Fig. 2.1b and c).

Inclusion of detergents such as Triton X-100 or CHAPS, both at 2% final concentration, yielded profiles no different from those shown in Fig. 2.1, which suggested that differences in their interaction with the plasma membrane could not account for any of the observations described above.

In an attempt to further characterize the large molecular weight (V_o) species of proteoglycan, designated as peak I, redissolved freeze-dried samples of such material were treated with DTT to disrupt disulfide bonds. Samples were chromatographed on Sepharose CL4B before and after treatment with DTT (Figs. 2.2a and b). All the peak I material which eluted at V_o prior to treatment with DTT (Fig. 2.2a) was split into two species of different size which had K_{av} values of 0.29 and 0.54, respectively, after exposure to the thiol-reducing agent (Fig. 2.2b). These two peaks were designated DTT_1 and DTT_2 and were further studied as outlined below.

Extraction of confluent radiolabelled cells with 2% SDS solution containing 5 mM DTT gave two distinct peaks of macromolecular material on Sepharose CL4B, with K_{av} values of 0.29 and 0.54 identical to those mentioned above for DTT_1 and DTT_2. When such extracts were subsequently dialyzed to remove DTT the two included peaks DTT_1 and DTT_2 were no longer apparent and a single peak of material eluting at the V_o position of the column was again observed (Fig. 2.3b). When material extracted from confluent cells with 2% SDS solutions

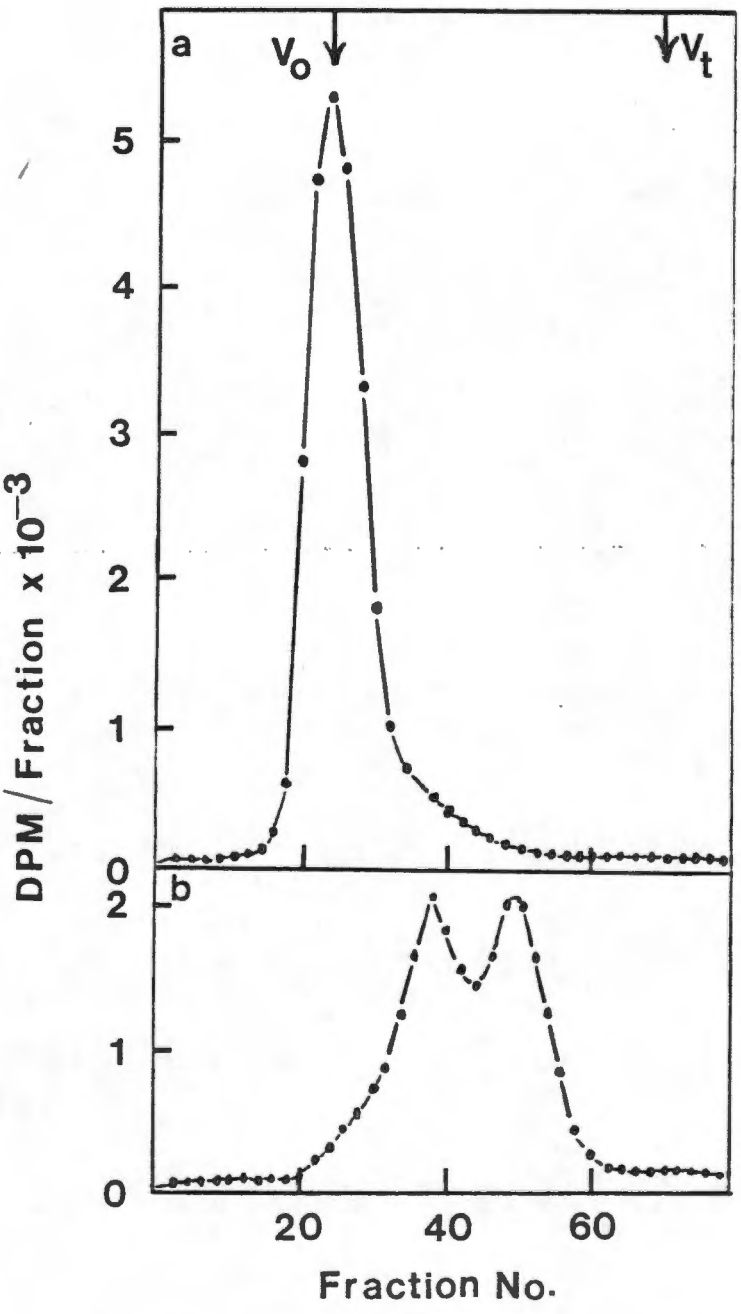

FIG. 2.2. Elution profile of $^{35}SO_4^{2-}$-labelled material analysed by Sepharose CL-4B chromatography. Material, extracted and analysed as described in the case of Figure 2.1a (peak I) was rechromatographed on Sepharose CL-4B columns either (a) before treatment with DTT or (b) after treatment with that reagent. Elution buffers included 5 mM DTT for all analysis carried out after DTT treatment. Recoveries of material applied were > 95% and analysis was performed on five individual extracts.

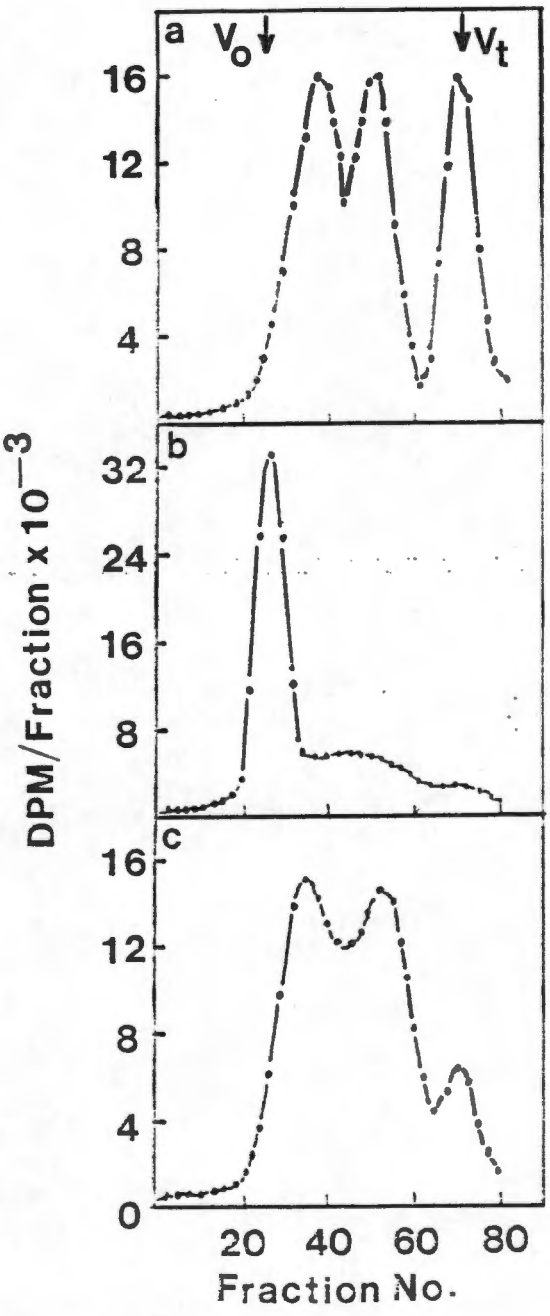

FIG. 2.3. Elution profile of $^{35}SO_4^{2-}$-labelled material analysed on Sepharose CL-4B columns before and after dialysis. Radioactively labelled material was extracted from cells under the same conditions as in the case of Figure 2.1a and as described in Methods, with the addition of 5 mM DTT to the extraction buffer. Extracted samples were chromatographed on Sepharose CL-4B columns either (a) before dialysis or (b) after dialysis to remove DTT. Some samples were treated with iodoacetamide prior to dialysis. The profile of such samples after Sepharose CL-4B chromatography is shown in panel (c). Recoveries of applied material were >90% and the experiments were performed twice with the same results.

containing DTT was treated anaerobically with iodoacetamide prior to exhaustive dialysis, the two peaks remained separate and thus the reassociation of the two species had clearly been prevented (Fig. 2.3c).

Further characterization of the three macromolecular species described above, namely peaks I, DTT_1 and DTT_2 was carried out using CsCl buoyant density gradient centrifugation (Fig. 2.4). All three peaks had similar buoyant densities ranging from 1.51 to 1.49 g/ℓ. These values are consistent with the molecules having a high content of GAG relative to core protein and this was confirmed by treatment of DTT_1 and DTT_2 with alkaline borohydride followed by chromatography on Sepharose CL6B (Fig. 2.5). The elution volumes of the split products were increased by a small amount only as compared with their untreated counterparts (Fig. 2.5a and c, and Fig. 5b and d). Estimation, using the data of Wasteson (63), of the molecular mass of the individual GAG chains of DTT_1 and DTT_2 from their K_{av} values of 0.26 and 0.6 indicated that their chain sizes were of the order of 50 000 and 14 000, respectively. Since the proteoglycans apparently consisted of two relatively small core proteins associated in the case of DTT_1 with large GAG chains in that of DTT_2 with shorter chains, the proportion of GAG representing HS was determined. Treatment of either DTT_1 or DTT_2 with nitrous acid prior to chromatography on Sepharose CL6B columns caused all the radioactively labelled material to be eluted at or close to the V_t of such columns (Fig. 2.5e and f). The GAG chains were thus shown to consist largely of heparan sulfate (HS).

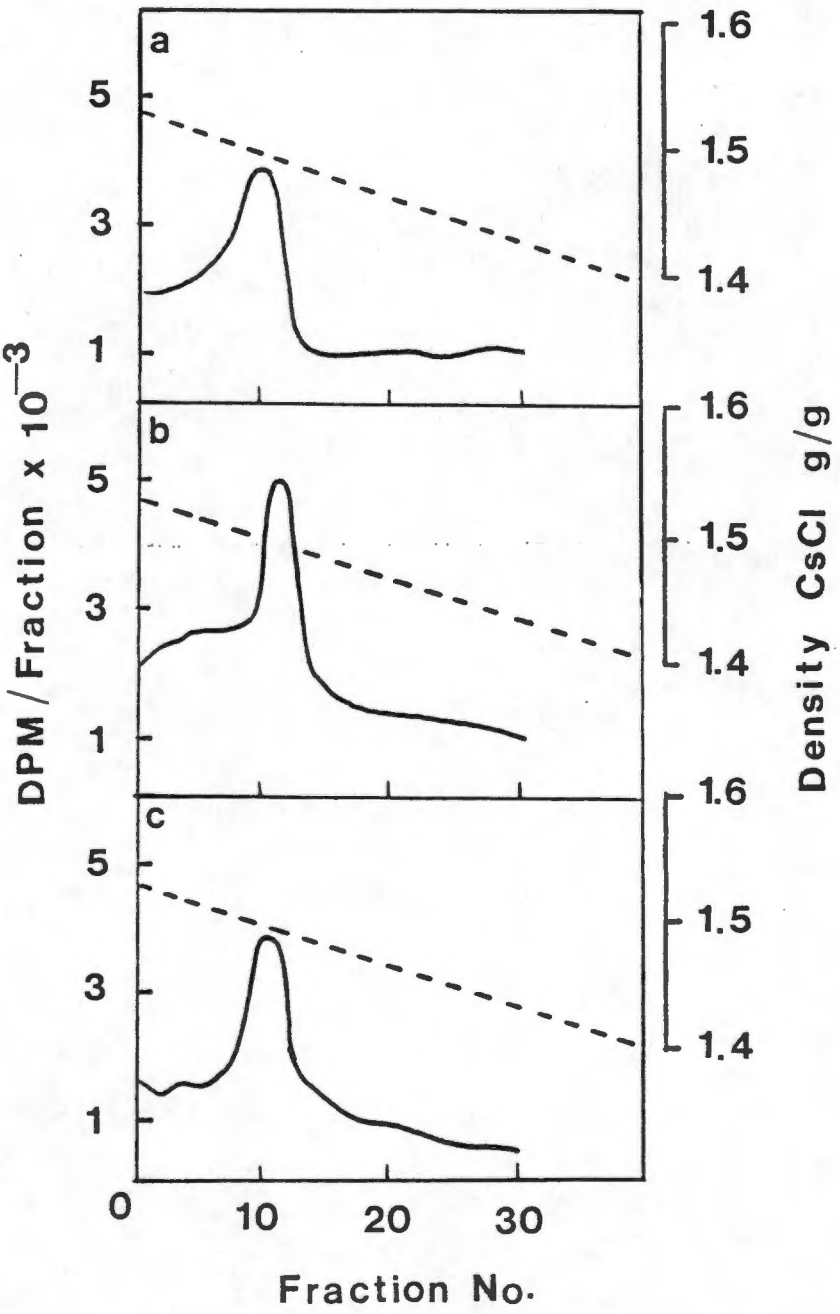

FIG. 2.4. Buoyant density gradient analysis of peak fractions collected after Sepharose CL-4B chromatography. Samples isolated from cells incubated with $^{35}SO_4^{2-}$ after a medium change were chromatographed as described in the case of Figures 2.1 and 2.2. Peak fractions were collected, freeze dried and dissolved as described under Methods. Profiles represent material from (a) peak I, (b) DTT_1 and (c) DTT_2 (see text for details). The density of CsCl was assayed on each fraction as described under Methods.

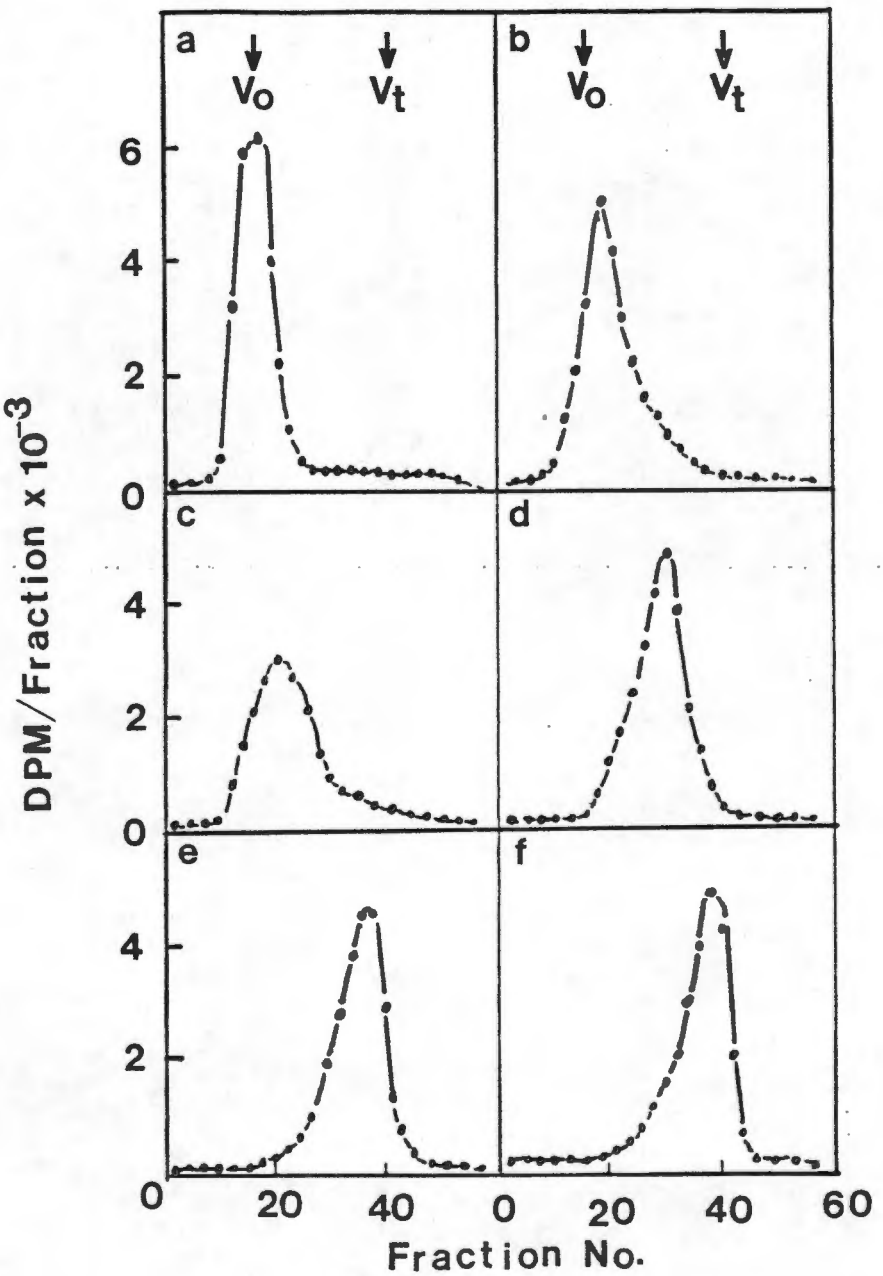

FIG. 2.5. Elution profiles of $^{35}SO_4^{2-}$-labelled material analysed by Sepharose CL-6B chromatography. Material obtained by collection of either peak DTT_1 (a,c,d) or peak DTT_2 (b,d,f) was analysed on Sepharose CL-6B columns before (a,b) and after (c,d) treatment with alkaline borohydride or nitrous acid (e,f). Recoveries from such columns were better than 95% of material applied and analysis was based on three individual samples (DTT_1 and DTT_2). The K_{av} values were used to estimate the approximate molecular weights of included material; for details, see text.

In order to better understand the molecular organization of the PGS described above and to attempt to explain the variability in the profiles in relation to the different growth conditions, labelled cell layers were extracted by means of mild trypsinization. The extent of enzymatic treatment was such that no release of cells occurred from their substrata but >90% of the radioactivity associated with the cell layers was solubilized by this procedure. When the extracted material was analyzed on Sepharose CL4B columns, three included peaks of radioactive material were observed (Fig. 2.6a). The large peak of radioactivity which eluted at V_t was composed mainly of unincorporated $^{35}SO_4^{2-}$ as revealed by Sephadex G-50 chromatography (data not shown); there was also some material that eluted just before V_t on such columns. The other two peaks that eluted from the Sepharose columns at K_{av} values of 0.39 and 0.63, respectively, were designated peaks T_1 and T_2. The residual radioactivity present in the cell layers after enzymatic extraction was solubilized by hot 2% SDS treatment and yielded the profile shown in Fig. 2.6b, after analysis on Sepharose CL4B. Thus mild exposure of cell layers to trypsin removed all signs of the large molecular weight proteoglycan(s) shown in Fig. 2.1a.

When confluent cultures were radioactively labelled after refeeding and the cell layers extracted with a hot solution containing protease inhibitors and 5 mM DTT but no detergent, the profiles obtained after Sepharose CL4B chromatography were as shown in Fig. 2.7a. Subsequent extraction of cell layers with the hot 2% SDS solution yielded profiles as shown in Fig. 2.7b; these were essentially the same as those shown in Fig. 2.1a and already discussed above. Treatment of such extracts

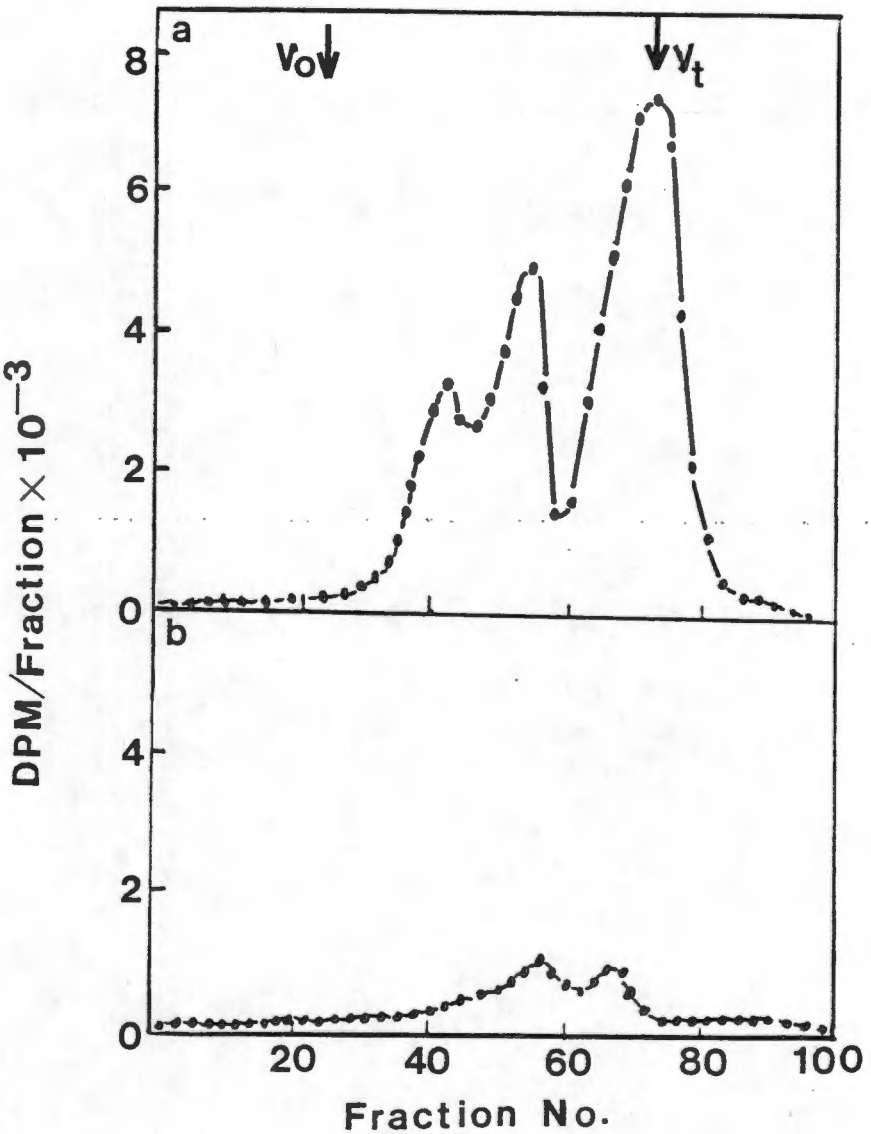

FIG. 2.6. Sepharose CL-4B analysis of $^{35}SO_4^{2-}$-labelled material (a) released by mild trypsin digestion of refed cells and (b) extracted by the hot 2% SDS solution, described under Methods, after removal of trypsinates. The combined extracts accounted for 98% of the total radioactivity associated with the cell layers and recoveries from columns were better than 90%. Experiments were performed on three occasions.

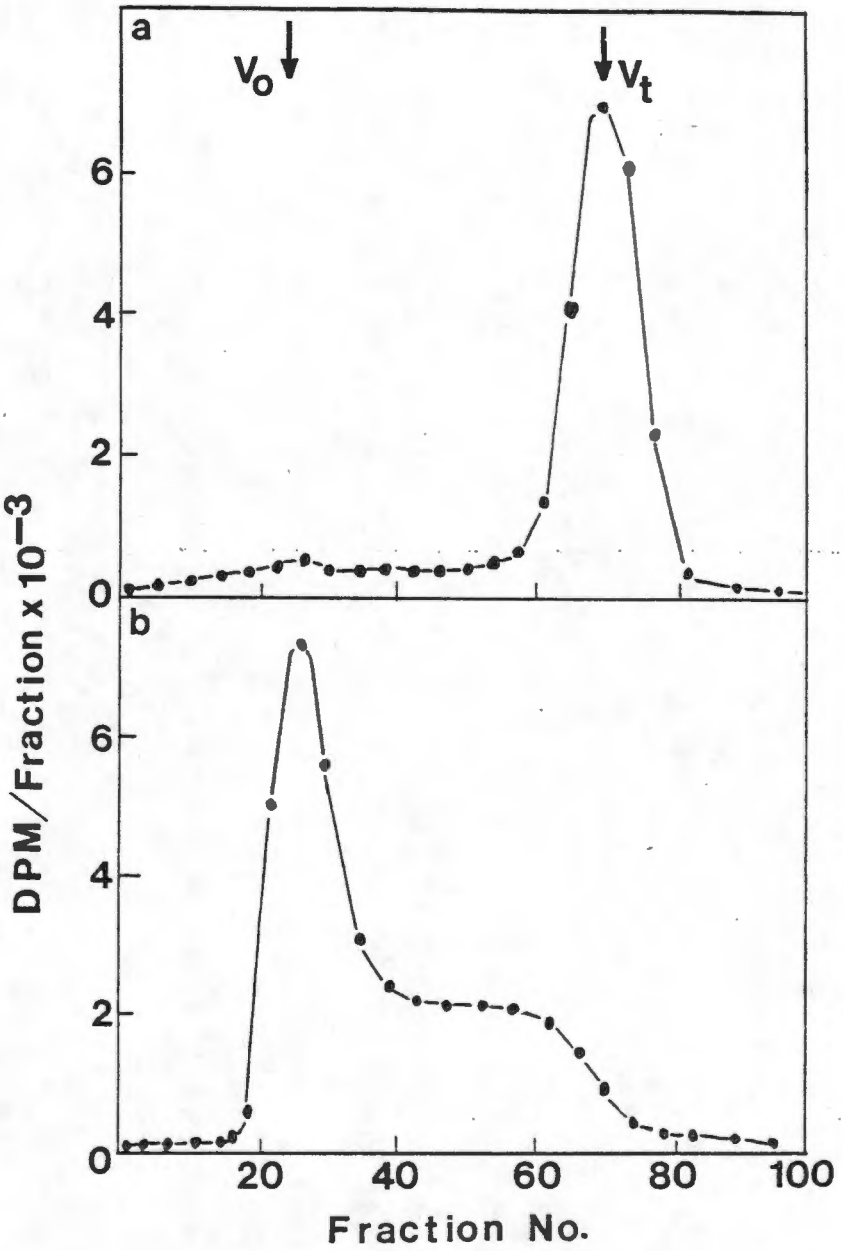

FIG. 2.7. Sepharose CL-4B analysis of $^{35}SO_4^{2-}$-labelled material (a) released from refed cells after extraction with a solution containing 5 mM DTT, 50 mM Tris-HCl, pH 7.6 and protease inhibitors as described under Methods and (b) released following subsequent extraction with the hot 2% SDS solution (see Methods). The combined extraction procedures accounted for 98% of the material associated with cell layers after radioactive labelling and recoveries from columns were 90%. Experiments were repeated thrice on cultures.

with DTT gave rise to profiles which were again the same as for Fig. 2.2b, namely two included peaks with K_{av} values of 0.30 and 0.59 (data not shown).

2.4. DISCUSSION

The Sepharose CL4B profile depicted in Fig. 2.1a and described in the Results section was obtained reproducibly in the case of cells showing typical "cobblestone" morphology and refed by medium change prior to incubation with $^{35}SO_4^{2-}$. This was the case both when the extractions were carried out using the hot detergent solution described (section 2.2.3.1) and when other detergents such as CHAPS and Triton X-100 were substituted for SDS. The profiles shown in Fig. 2.1b and c were obtained from cultures that had either not been refed before labelling (Fig. 2.1b) or that showed atypical or "sprouter" morphology (129). These profiles, which were subject to some minor variations between experiments, resemble those reported by others (50). Cultured endothelial cells are known to secrete plasminogen activator (PA) which accumulates in the culture medium between medium changes (214), and sprouting cells secrete up to ten times as much PA as do their quiescent counterparts. It is likely that the activation of serum plasminogen may be an explanation for the differences in the profiles of proteoglycans extracted from the two types of cell cultures.

However, of greater interest was the nature of the large molecular species of proteoglycan that was obtained by the careful procedure described in Fig. 2.1a. The smaller species discussed above were most likely artefactual and thus misleading to the overall understanding of the structure of HSPG.

The data obtained suggest that the HSPG molecules associated with the surfaces of cultured BAE cells exist as multimeric disulfide-bonded aggregates (Fig. 2.8). These are associated with the plamalemma via hydrophobic domains on two species of core proteins, as signified by the requirement for detergents in the extraction procedures. Treatment with DTT alone does not release any macromolecular material (Fig. 2.7), indicating that both core proteins are intercalated into the membrane. Reduction of disulfide bonds by DTT generates two distinct species of proteoheparan sulfate (Fig. 2.2) which accounts for all the macromolecular radioactivity. This reductive cleavage of the disulfide bonds is reversible when DTT is removed by dialysis, restoring the large molecular species (peak I, Figs. 2.1a, 2.2a and 2.3). The reaggregation is prvented if reduction by DTT is followed by irreversible alkylation with iodoacetamide.

The individual reduced subunits (DTT_1 and DTT_2) consist largely of HS chains attached to apparently rather small core proteins: Analysis both by buoyant density gradient centrifugation and Sepharose CL6B column chromatography after alkaline borohydride treatment indicates that the largest part of the molecules is GAG. In the case of DTT_2 an approximate M_r of the whole subunit can be estimated from thedata (Fig. 2.5) and those of Wasteson (63), and the size and number of HS chains can also be calculated. Subunit DTT_2 appeared to have a M_r of about 100 000 and is made up of 5 or 6 HS chains attached to small core proteins of M_r about 30 000. Presumably the core protein has a hydrophobic domain to facilitate the association of the subunits with the plasmalemma. A tentative model of this molecule shown in Fig. 2.8 is consistent with the data. One concern is that the

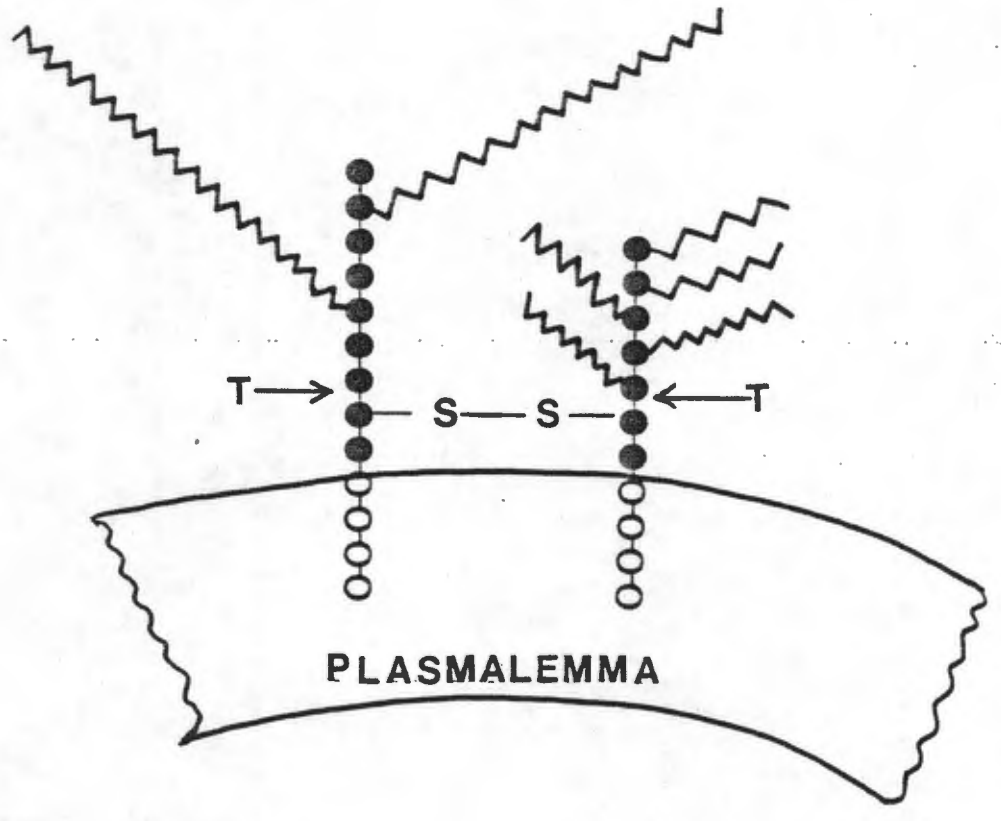

FIG. 2.8. Cartoon representation of the heparan sulfate proteoglycan described in this communication. The open circles (o) represent hydrophobic sequences of amino acids (hydrophobic domains) and glycosaminoglycan chains are represented as zigzag lines. The areas on the protein backbone sensitive to mild trypsinization are indicated as T.

inter-subunit disulfide arise artefactually during isolation and purification as "mixed" disulfides (108), but this is unlikely since N-ethylmaleimide was used in the extracting solutions as an inhibitor of such events.

Digestion of radiolabelled cells with trypsin yielded two distinct species of PG (Fig. 2.6), which were unaffected by treatment with DTT, suggesting that the trypsin-sensitive site(s) were most likely to be located on the extracellular side of the disulfide(s) bond(s) which link the two subunits (Fig. 2.8).

Since treatment of the cell layers with DTT in the absence of detergent does not result in any significant release of PG it can be assumed that both core proteins are inserted into the plasma membrane (see Figs. 2.7 and 2.8). Rather similar disulfide-bonded PGs have been observed before in the case of cultured fibroblasts (126,202), and these findings serve to support the notion of the generality of such entities (73). The special functional importance of these surface macromolecules in vascular endothelial cells makes the new finding potentially rather significant.

CHAPTER 3

THE EFFECTS OF EXTRACELLULAR MATRICES ON GROWTH, MORPHOLOGY AND CELL SURFACE PROTEOGLYCANS OF BOVINE AORTIC ENDOTHELIAL CELLS IN CULTURE

3.1. INTRODUCTION

In vivo, most cells produce and secrete an extracellular matrix (ECM), the natural substrate upon which they migrate, proliferate and differentiate. Although many aspects of the nature and composition of ECMs are still to be elucidated, these complex aggregates appear to be composed in large part of different types of collagen (8,55,69,91, 97-99, 107, 128,146,147), elastin (55,69,91,97,98,128,145,147), proteoglycans (2,5,7,9,24,37,45,82,91,96,105,108,109,128,146,147) and glycoproteins (5,8,29,58,69,91,98,99,107,110,146,147). Most, if not all, cells retain their ability to produce and deposit an ECM in vitro, indicating that attachment to a substrate is necessary, although not an absolute requirement, for their replication and proliferation. For many cell types the extent of proliferation in culture depends upon the availability and concentration of growth factors in the culture medium, but when maintained on specific substrata or on the ECM laid down by the cells, growth factors may not be required (8).

The components of the ECM and cell surfaces have been implicated in the attachment of cells to the substratum or ECM. Many cultured cells including fibroblasts, myoblasts, hepatocytes, chondrocytes and certain epithelial cells do not bind directly to either collagen substrata or plastic surfaces of culture dishes (163-168); instead,

extracellular glycoproteins bind the cells to the substrate (163-168). Of these glycoproteins, fibronectins (8,58,87,146,163,165,167,168) produced by fibroblasts (169-171) and laminin from endothelial cells (172,173) as well as some other cells (8), have been shown to be involved in multiple interactions with collagen and proteoglycans (8,11,58,76,83,87,91,96,104,107,110,128,146,147,158,163,168).

It has been observed that collagen substrates alter the morphology, adhesion and in some cases, differentiation of cells (8). Cells such as hepatocytes (174), corneal endothelial cells (175) and epidermal cells (176) are maintained in a viable state longer on collagenous substrata than on plastic.

The profiles of cell surface proteoglycans have been observed to vary from cell to cell and these molecules have also been implicated in the control of cell growth (179,180) and adhesive activities (181-183). Heparan sulfate proteoglycan (HSPG) has been included as a control factor in the assembly of ECM through its ability to interact with other matrix components such as fibronectin and collagen (58,184-188). Heparan sulfate is associated with increased cell-substratum adhesion and is involved in the spreading of cells onto fibronectin and other substrata (76,189).

Cell surfaces in organized tissues play critical roles in adhesion and recognition among cells and in cellular interactions with the extracellular milieu. A great deal of consideration has been given recently to the biochemical characterization of cell surface and ECM components such as collagens, glycoproteins and proteoglycans as well

as the putative roles of these molecules in cell adhesion and growth. In the light of observations on the intimate physical association that exists between cells and their ECM together with the ability of the ECM to modulate, in part, the synthetic profiles in a variety of cell culture systems (12,127,128), the practice of growing cells on ECMs instead of naked plastic may have far-reaching implications in many fields of biology (119). In this chapter I have attempted to analyze the effects various ECMs have on the proliferative behaviour, morphology and cell-surface proteoglycan profiles of bovine aortic endothelial cells in culture.

3.2. METHODS

3.2.1. ENDOTHELIAL CELL CULTURES

BAE cell cultures were set up as described under section 2.2.1.

3.2.2. RADIOLABELLING OF ENDOTHELIAL CELLS

Radiolabelling was as described under section 2.2.2.

3.2.3. GELATINIZATION OF DISHES

An aqueous solution of gelatin (Difco) 1% was prepared by allowing the gelatin to swell at 4°C in distilled water for 30 min. This solution was centrifuged at 2000 rpm and washed 7 times with cold distilled water at low speed centrifugation (1000xg) for a duration of 15 min each. Finally, the gelatin pellet was resuspended in the correct volume of distilled water and sterilized by autoclaving. Before use, the gelatin solution was heated at 37°C until all the

granules were in solution. Dishes were gelatinized by incubating them with 4 ml of sterile gelatin for at least 1 h at 37°C. The unbound material was removed and dishes allowed to dry overnight in a laminar flow hood. Dishes were washed twice with medium before plating cells onto gelatin-coated dishes.

3.2.4. PREPARATION OF ECM-COATED DISHES

Bovine aortic endothelial cells, line E8 (159-161), human skin fibroblasts, FG_o (190), and rat smooth muscle cells, line R9 and R22 C1F (69) were isolated and cultured as previously described. Cultures were initiated from cryopreserved stocks and seeded at $5x10^4$ cells/ 60 mm plastic dish (Falcon). Culture medium was changed every fourth day with daily additions of 50 µg/ml ascorbic acid to promote the deposition of a collagenous matrix (69). After 15-20 days in culture, medium was removed and the cultures washed once in phosphate-buffered saline (PBS). Cell layers were lysed by the addition of 2 ml of sterile 0.05 M NH_4OH for 30 min at room temperature in a laminar flow hood. After lysis, ECM-coated dishes were washed extensively with PBS to remove remaining nuclei and cytoskeleton elements, leaving an intact ECM firmly attached to the culture dish. Such matrix-coated dishes could be stored for up to 4 months at 4°C under sterile conditions.

3.2.5. MODIFICATION OF ECM-COATED DISHES

Dishes coated with ECM were modified by enzymatic hydrolysis with bovine pancreatic trypsin (EC. 3.4.21.4; type III, pretreated with elastin, 5 mg/ml, to adsorb contaminating elastase activity), elastase (EC. 3.4.21.11; type III from porcine pancreas), and collagenase

(E.C. 3.4.24.3; high purity type VII). All enzymes were used at a final concentration of 10 µg/ml in 0.05 M Tris-HCl, 0.01 M $CaCl_2$, pH 7.6. The ECMs were exposed to the enzyme (2 ml/60 mm dish) for 3 h at 37°C in 5% CO_2:95% air. At the end of the incubation period, enzyme solutions were removed and matrix layers washed extensively with PBS containing the following protease inhibitors (EDTA, 20 mM; NEM, 10 mM; Benzamidine-HCl, 5 mM; PMSF, 2 mM; 6-Aminohexanoic acid, 100 mM and 2% w/v Trypsin inhibitor). Finally, dishes were washed 4 times with medium and then incubated with medium overnight before seeding of BAE cells onto such modified matrices.

3.2.6. ENZYMATIC COMPOSITIONAL ANALYSIS OF ECM

The ECMs produced by E_8, FG_o, R22C1F and R9 may be analyzed by sequential enzyme digestion with Trypsin, Elastase, Collagenase (69). Trypsin removes matrix glycoprotein, elastase removes elastin and matrix collagen is sensitive to collagenase. Cells were seeded into 35 mm dishes at a density of 10^4. At confluence, medium was changed with fresh medium containing 0.5 µCi/ml $|^3H|$-proline and 50 µg/ml ascorbic acid. After 5-7 days labelling in the presence of ascorbic acid (15-20 days after seeding), cultures were harvested exactly as described in section 3.2.4. The composition of the matrices was determined by sequential enzyme digestion with trypsin, elastase and collagenase, using the buffer system described in section 3.2.5. Cultures were washed three times with the buffer between enzyme treatments. Residual radioactivity was determined by 2 M NaOH solubilization overnight at 37°C. The amount of radioactivity released by each treatment was expressed as a percentage of the total radioactivity in cell-free matrices.

3.2.7. GROWTH AND MORPHOLOGIC STUDIES

The proliferative behaviour and morphology of BAE cells grown on different substrata, viz., plastic, gelatin and ECM produced by rat smooth cells were investigated. BAE cells were seeded at an initial density of $5x10^4$ cells/60 mm diameter culture dish. Growth was measured by serial sampling over an 11-day period and triplicate plates were trypsinized and cell suspensions counted in Isoton^R using a Coulter electronic counter (Coulter Electronics, Inc., Hialeah, Florida). Cellular morphology was assessed by phase contrast microscopy and pictures taken with a Nikon AF inverted microscope on which was mounted a Nikon M-35 S camera using Pan F Ilford ASA 50 film

3.3. RESULTS

3.3.1. GROWTH OF BAE CELLS

The nature and organization of the substratum has previously been shown to affect the growth and morphology of cells (8,13,16,18,26, 110,122,146,157,158,164,175,177). In the present study the proliferative behaviour of BAE cells was analyzed when cultured on different substrata. The results of such growth analysis has been depicted in Fig. 3.1. A growth-promoting effect on BAE cells was observed with the different substrata tested. Matrix material from R_9 cells showed the most promotion of proliferation followed by R_{22}C1F, FG_o, E_8, gelatin and plastic. Cells maintained on ECM produced by R_9 rat smooth muscle cells reached a terminal density (28.76×10^5 cells/60 mm dish) within 5 days which was twice the number of cells obtained using R_{22}C1F- and FG_o-ECMs, 3-fold that of cells seeded onto an E_8-ECM, 5-fold that of cells seeded onto plastic, and 7-fold that of cells grown on gelatin.

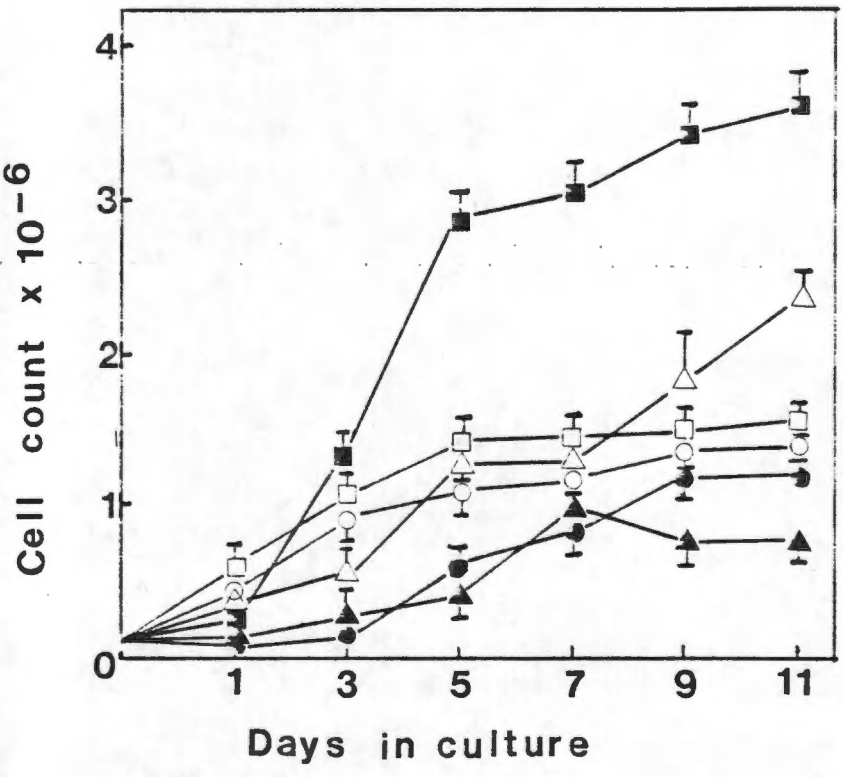

FIG. 3.1. Growth kinetics of BAE cells grown on plastic (●), gelatin (▲), R_9-ECM (■), R_{22}C1F-ECM (△), E_8-ECM (O), FG_o-ECM (□). Values are means ± S.D. (n=3).

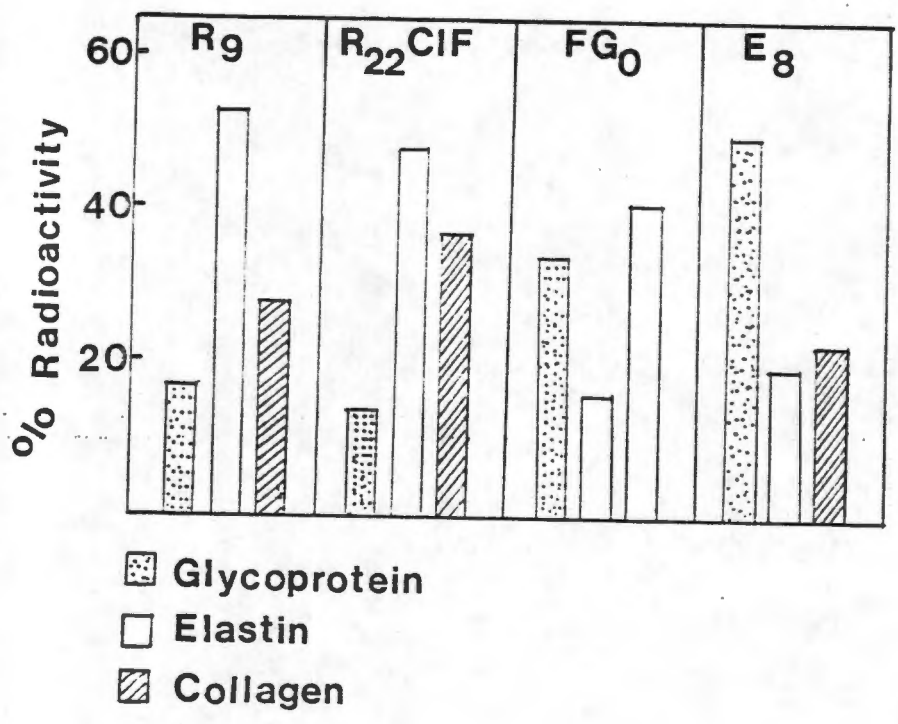

FIG. 3.2. Compositional analysis of ECMs produced by different cell lines in culture. See section 3.2.6 of Methods for details.

BAE cells plated onto plastic and gelatin-coated dishes proliferated slowly in comparison with cells plated onto ECM-coated dishes. E_8- and FG_o-ECMs had similar growth-stimulating effects on BAE cells although a higher final density was reached with FG_o-ECMs. The final cell density reached by BAE cells maintained on rat smooth muscle cell matrices (R_9 and $R_{22}C1F$) was not seen when BAE cells were grown on either plastic or gelatin nor when cells were maintained on FG_o- and E_8-ECMs. However, the initial proliferation rate was higher for cells on the latter two matrices which also gave greater plating efficiencies. These results are in agreement with the results of others (13,16,110,122,175) and indicate that the substratum upon which the cells grow, indeed affects their proliferative behaviour.

3.3.2. PHASE-CONTRAST MICROSCOPY AND MORPHOLOGY

BAE cells have been shown to exhibit normal and abnormal morphologies (127,129,131). The morphology of BAE cells in culture as assessed by phase-contrast microscopy is shown in Fig. 3.3. The cells assumed typical sparse (Fig. 3.3A), subconfluent (Fig. 3.3B) and confluent (Fig. 3.3C) morphologies varying from large polygonal (Fig. 3.3A and B) to smaller polygonal, closely apposed cells (Fig. 3.3C) or the so-called "cobblestone" morphology. Post-confluent BAE cells sometimes exhibited a "sprouting" morphology (Fig. 3.3F) in which fusiform cells were connected end-to-end to form a mycelial or spherical pattern. This atypical morphology was normally observed when BAE cells were kept in culture for prolonged periods of time. The morphology of BAE cells was best appreciated after they have been confluent for 5-10 days at which time they adopted a relatively stable morphological appearance (Fig. 3.3C). As yet, very little is known about "sprouting"

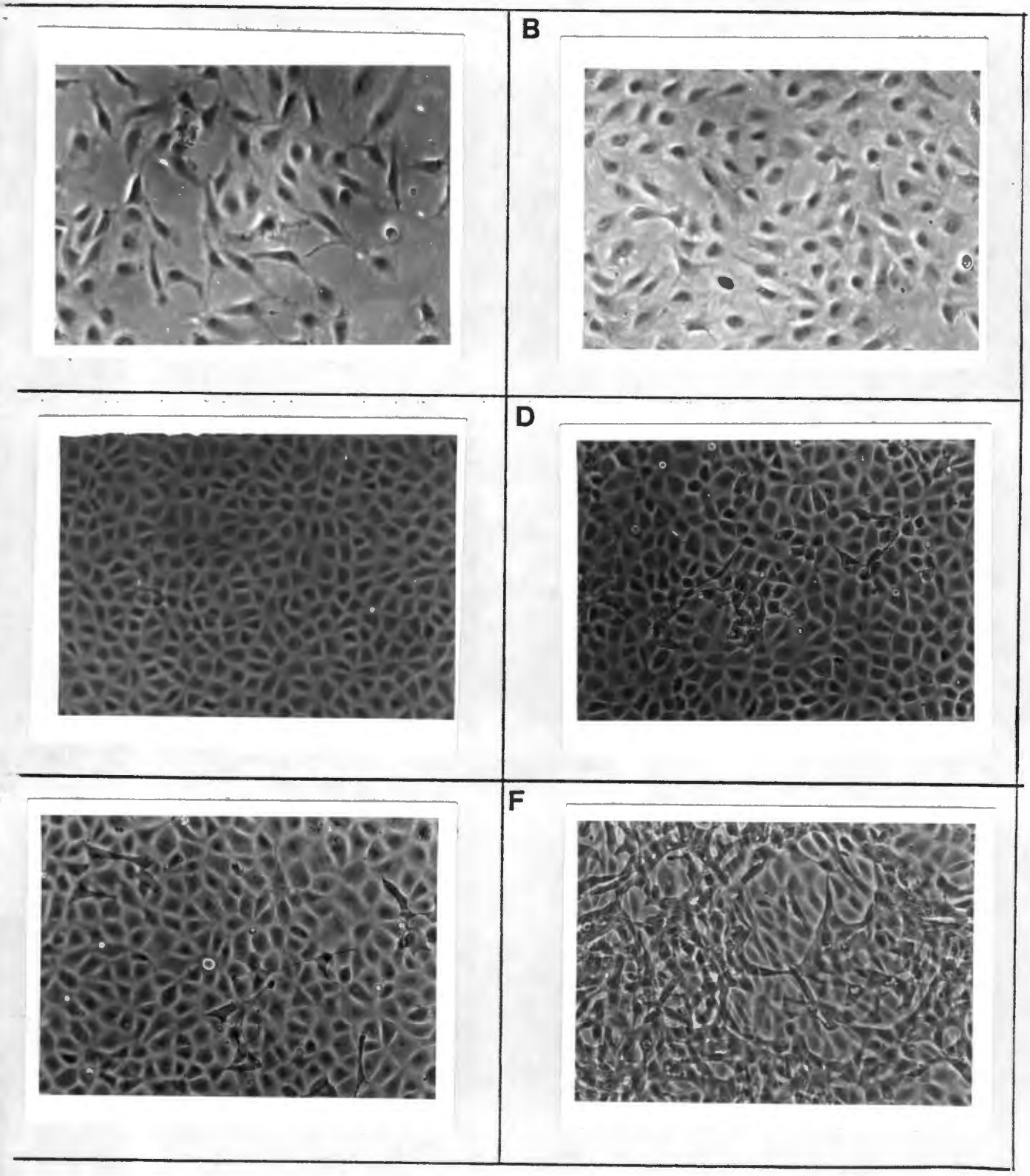

FIG. 3.3. Morphology of BAE cells in culture as assessed by phase-contrast microscopy. A, sparse cultures; B, subconfluent; C, confluent monolayer; D, late confluent; E, sparse sprouters; F, bulk sprouters. Magnification: x100.

cells and no *in vivo* correlate of this phenomenon has so far been reported.

There have been numerous reports that vascular endothelial cells maintained on ECMs undergo morphological changes (8,13,16,26,28,122,131, 157-159,175). The morphological changes caused by the ECMs may be attributed to differences in the tridimensional scaffolding and composition of ECMs which vary from cell to cell (8,13,16,69,97,98, 106,107,110,122,146,158,164,175,177). The results presented here agree with these reports and support the contention that matrix organization may indeed have profound effects on many aspects of cellular biology. Matrices differed morphologically (Fig. 3.4A-D) as well as in composition (Fig. 3.2). Rat smooth muscle cell (R_9 and R_{22}C1F) ECMs contained large amounts of elastin and collagen whereas fibroblastic (FG_o) and endothelial (E_8) ECMs contained higher amounts of glycoprotein(s), lesser quantities of elastin and an appreciable amount of collagen (Fig. 3.2). BAE cells produced a relatively fine matrix which contained high quantities of glycoprotein and equal proportions of elastin and collagen. The ECM produced by BAE cells (Fig. 3.4A) was very "basement membrane-like" and special care had to be taken in handling of such matrices since they detached easily from the plastic surface of the dish. The FG_o-ECM contained mainly collagen and glycoproteins and only small amounts of elastin. The latter also remained more firmly attached to its plastic substratum than the E_8-ECM. Smooth muscle cell (R_9 and R_{22}C1F) ECMs were much thicker than both the endothelial and fibroblast matrices and also showed the least tendency to lift from culture dishes.

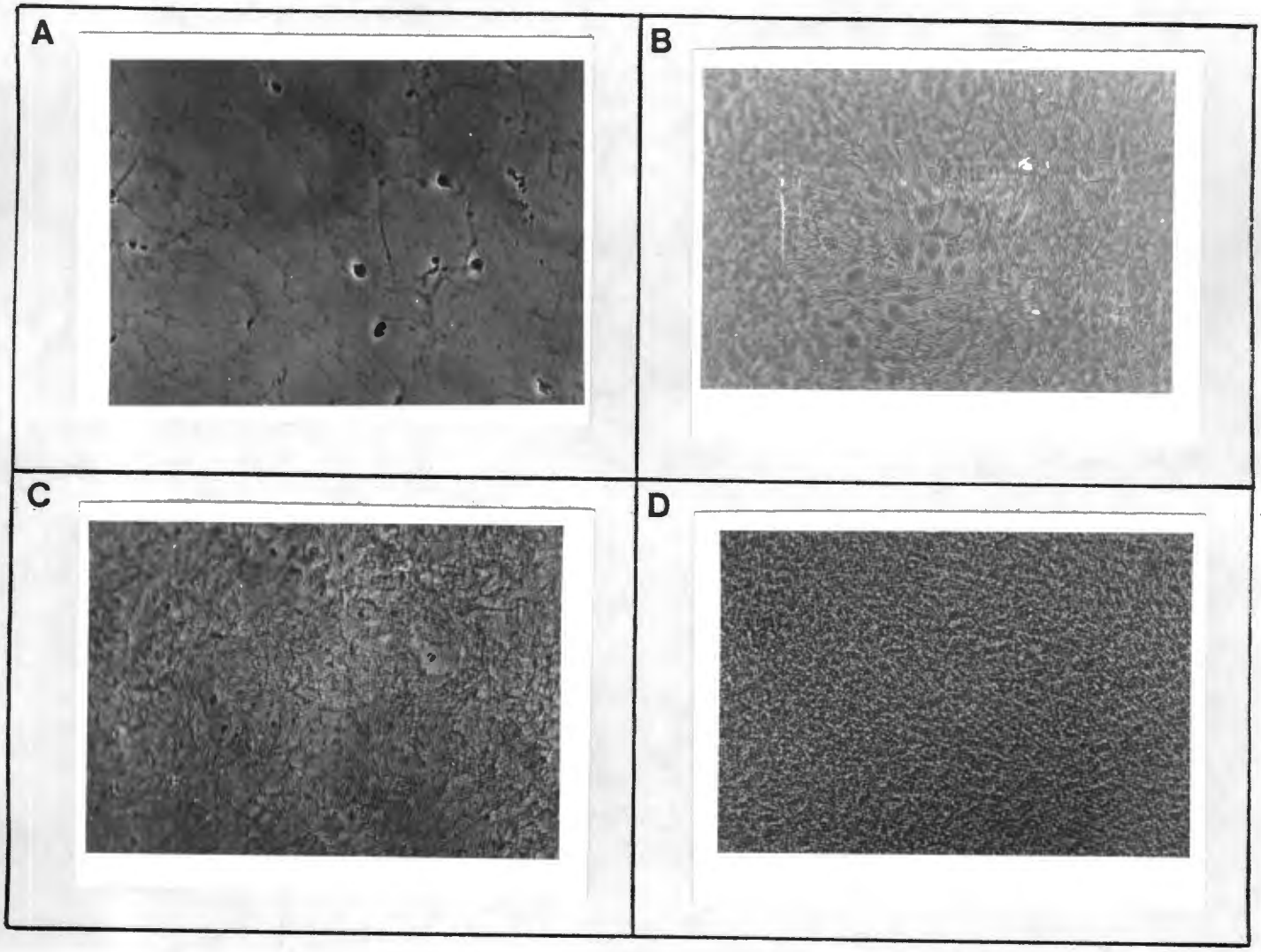

FIG. 3.4. Phase-contrast micrographs showing part of the ECMs produced by A, BAE cells (E_8); B, FG_o fibroblasts; C, R_{22}C1F-; D, R_9 - rat smooth muscle cells. ECMs were prepared, and pictures taken as described in Methods (section 3.2.7). Original magnification: x100.

As a general consideration it might be expected in view of these comments made above that differences in morphology of ECMs would also have effects on the morphology of cells grown on them. Consequently, the influence of different ECMs on endothelial cell morphology was studied. The appearance of BAE cells cultured on different substrata as examined by phase-contrast microscopy is shown in Fig. 3.5. On plastic, BAE cells had a polygonal morphology (Fig. 3.5A) forming the typical monolayer pattern. Compared with plastic, cells cultured on gelatin-coated dishes appeared more elongated and larger than those on plastic, whereas cells maintained on fibroblastic and endothelial matrices also appeared polygonal, but seemed to have integrated into the matrices. BAE cells cultured on ECMs from rat smooth muscle cell origin, exhibited a polygonal morphology and have also appeared to be cells integrated into the matrix. The cells were more tightly packed on these matrices, probably because they proliferated more rapidly than those on plastic, gelatin, FG_o- and E_8-ECMs. Also, sprouting cells were more prevalent on smooth muscle cell matrices than on other substrata (Fig. 3.6G,H). A good correlation between growth and morphology has been observed by comparing BAE cells grown on plastic with BAE cells grown on an R_9-ECM (Fig. 3.6A-G). Cultures on R_9 matrices were more densely packed, representative of a higher cell number, than were cultures maintained on plastic. This confirmed the notion that cells proliferate more rapidly on ECM-coated dishes than on naked plastic substrata.

Modification of ECMs, especially those produced by rat smooth muscle cells, had an effect on the growth and morphology of BAE cells. On

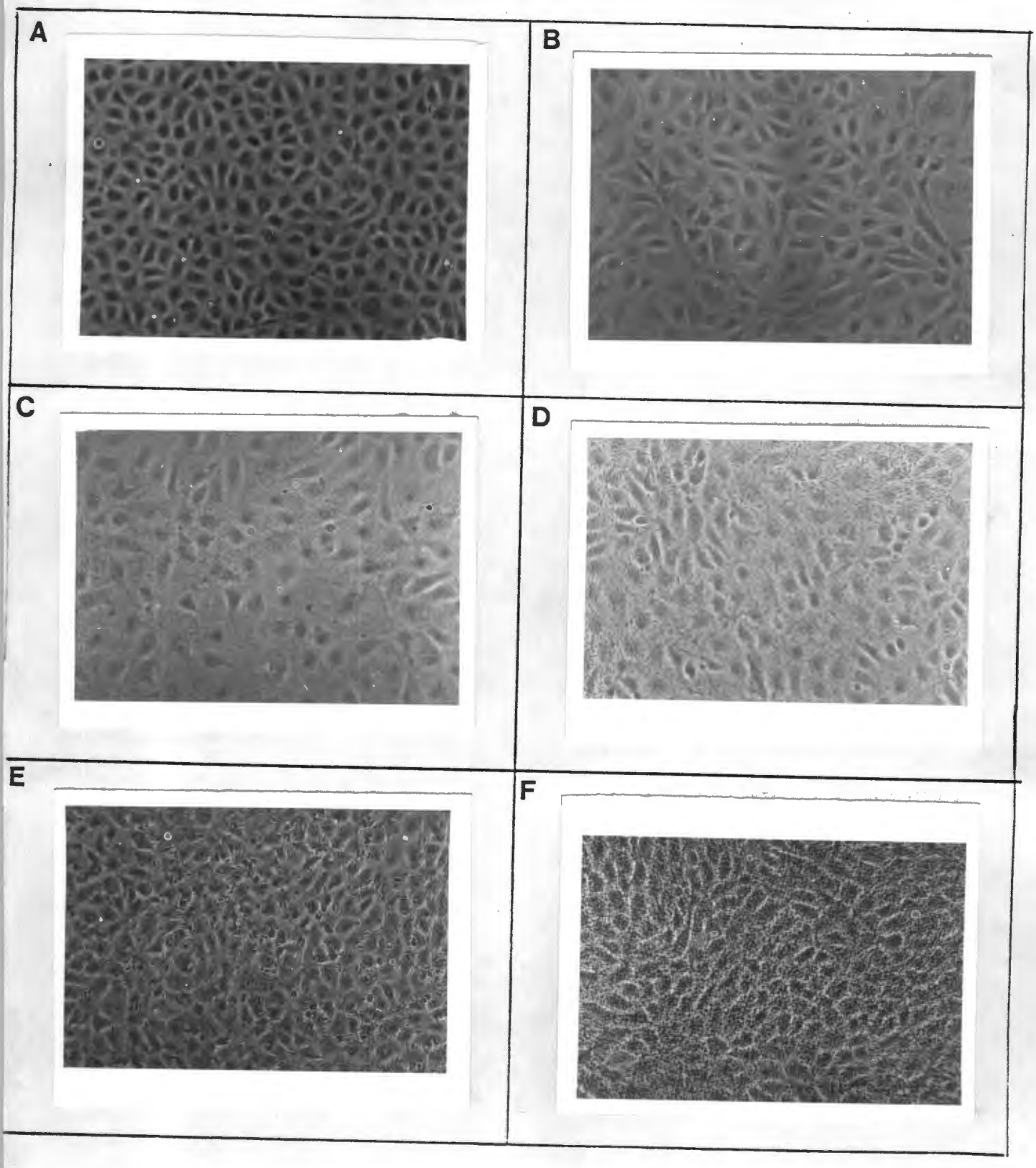

FIG. 3.5. Comparison of the morphological appearance of BAE cells cultured on different substrata. Phase-contrast micrographs were taken a few days after seeding. Magnification: x100. A, plastic; B, gelatin; C, E_8-ECM; D, FG_o-ECM; E, $R_{22}C1F$-ECM; F, R_9-ECM.

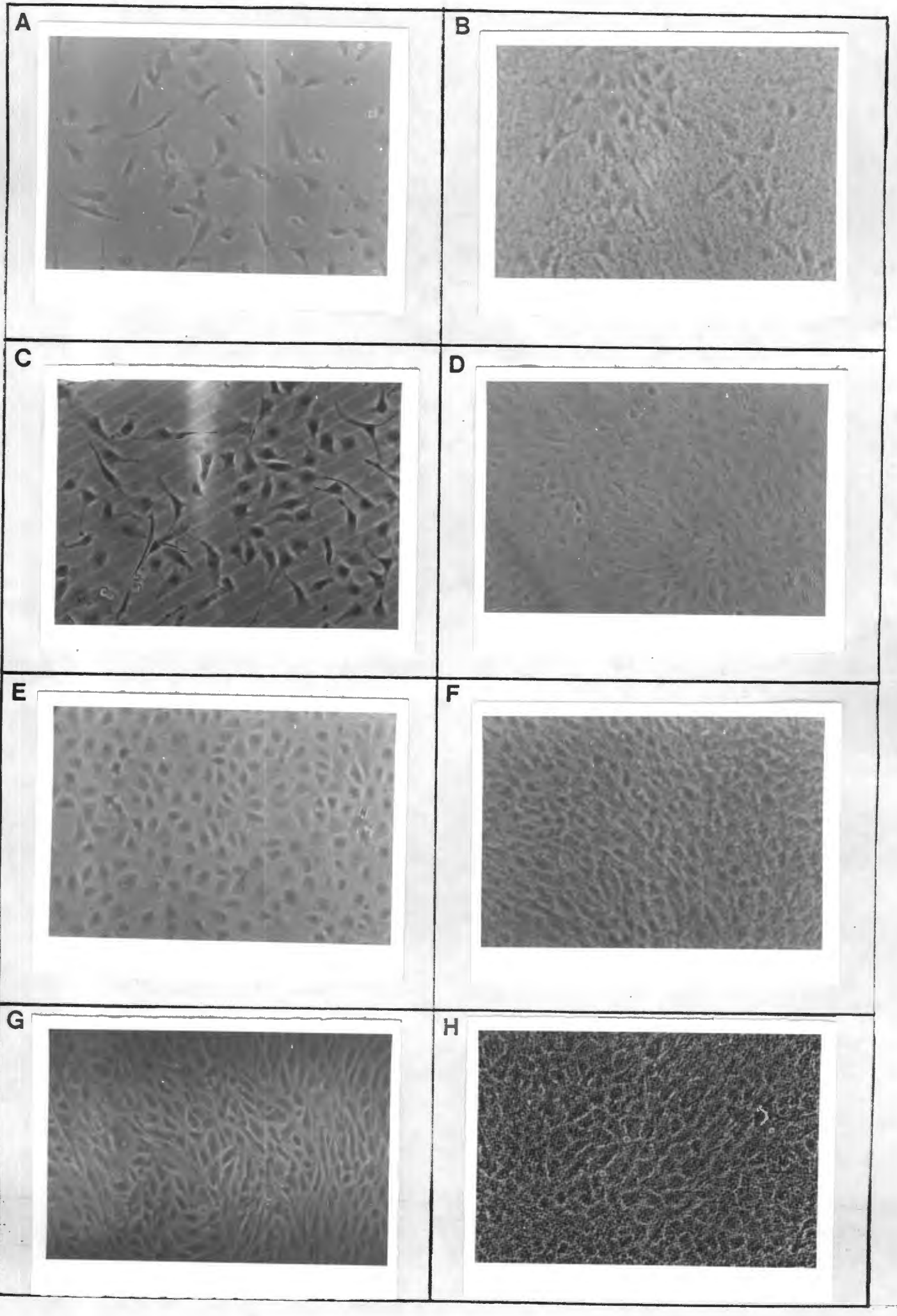
A
B
C
D
E
F
G
H

FIG. 3.6. Comparison of proliferation and morphology of BAE cells when maintained on plastic versus R_9-ECM. Pictures were taken after 24, 48, 72 and 96 h in culture. A, C, E and G represent the respective times for BAE cells on plastic, whereas B, D, F and H represent the respective times for BAE cells on R_9-ECM. Magnification: x100.

an untreated R_{22}C1F matrix, the cells grew rapidly and were tightly packed (Fig. 3.7A). Trypsin pretreatment of the matrix (Fig. 3.7B) altered the morphology of BAE cells in that they were more elongated than cells grown on an untreated matrix. Pretreatment of the matrix with elastase (Fig. 3.7C) abolished the growth-stimulating effect of the matrix and considerably fewer cells were present than on both control and trypsin-pretreated matrices. Collagenase treatment (Fig. 3.7D) of the matrix, however, did not have any discernible effects on the morphology of BAE cells, and also the growth-promoting effect of the matrix was unaffected by pretreatment with collagenase. These observations were compatible with similar recently reported data (122). The same results were obtained when R_9 ECMs were used.

3.3.3. EFFECTS OF ECMs ON THE PROFILE OF CELL SURFACE PROTEOGLYCANS OF BAE CELLS IN CULTURE

In view of the current observations and interest concerning the proposed role of the ECM in the regulation of cellular growth (8,13, 16,18,110,122,146,164), biosynthetic phenotype (2,5,9,12,15,28,29, 74,91,103,158,175), it was considered of interest to assess what effects different ECMs and modifications thereof might have on the cell-surface proteoglycan profile of BAE cells. The profiles obtained for material after analysis by Sepharose CL4B as described in Chapter 2 are shown in Fig. 3.8. BAE cells plated onto an untreated R_{22}C1F matrix (control) exhibited two distinct peaks of macromolecular material(Fig. 3.8A). The first peak was more or less coincident with that obtained for BAE cells cultured on plastic although it eluted with a K_{av} of 0.07 and not at V_o. However, the second peak (K_{av} 0.46), which was not apparent when E_8 were grown on plastic

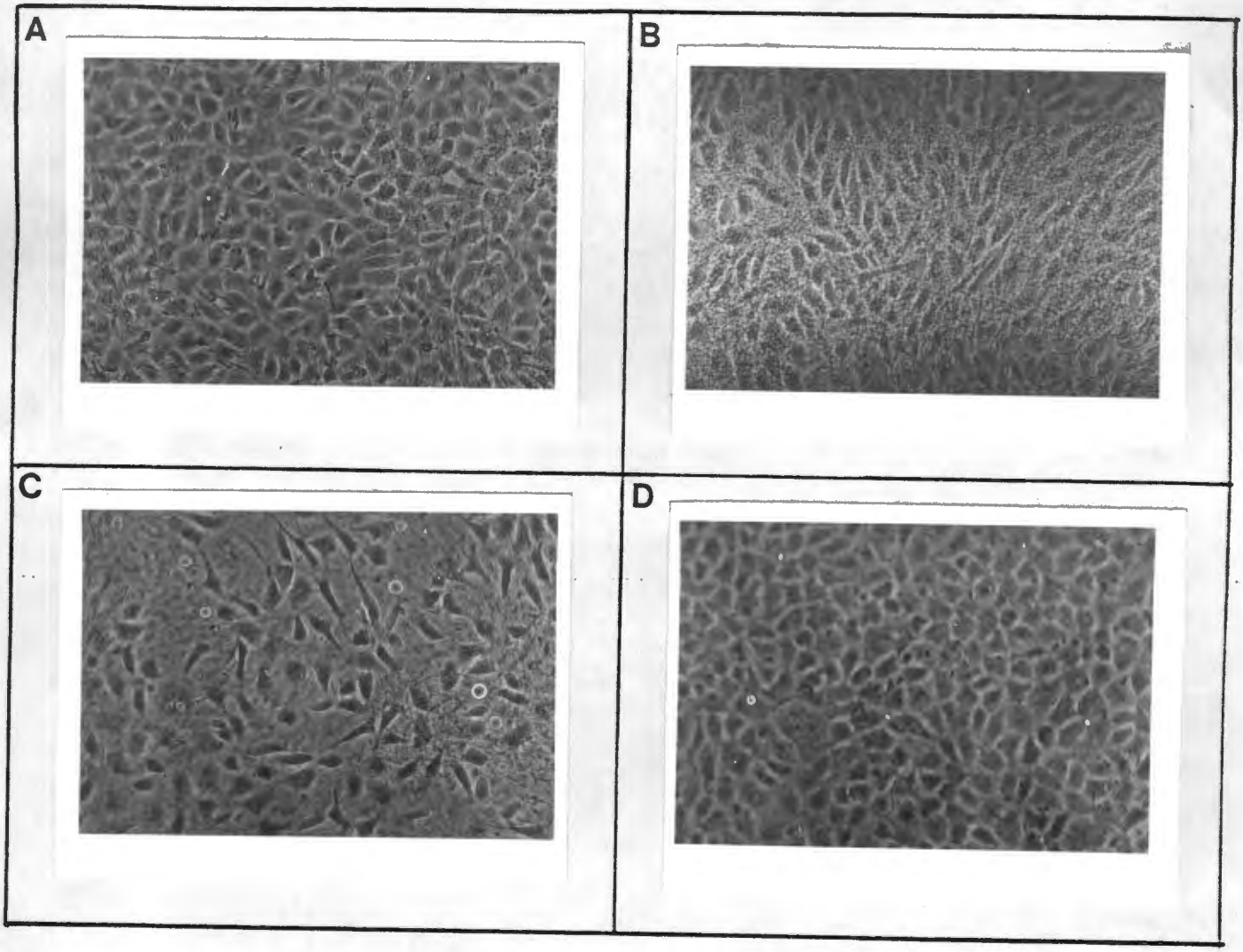

FIG. 3.7. Morphologic effect of different pretreated R_{22}C1F-ECMs on BAE cells. BAE cells were plated on A, control; B, trypsin-treated; C, elastase-treated and D, collagenase-treated matrices. Pictures were taken 5 days after plating. Magnification: x100.

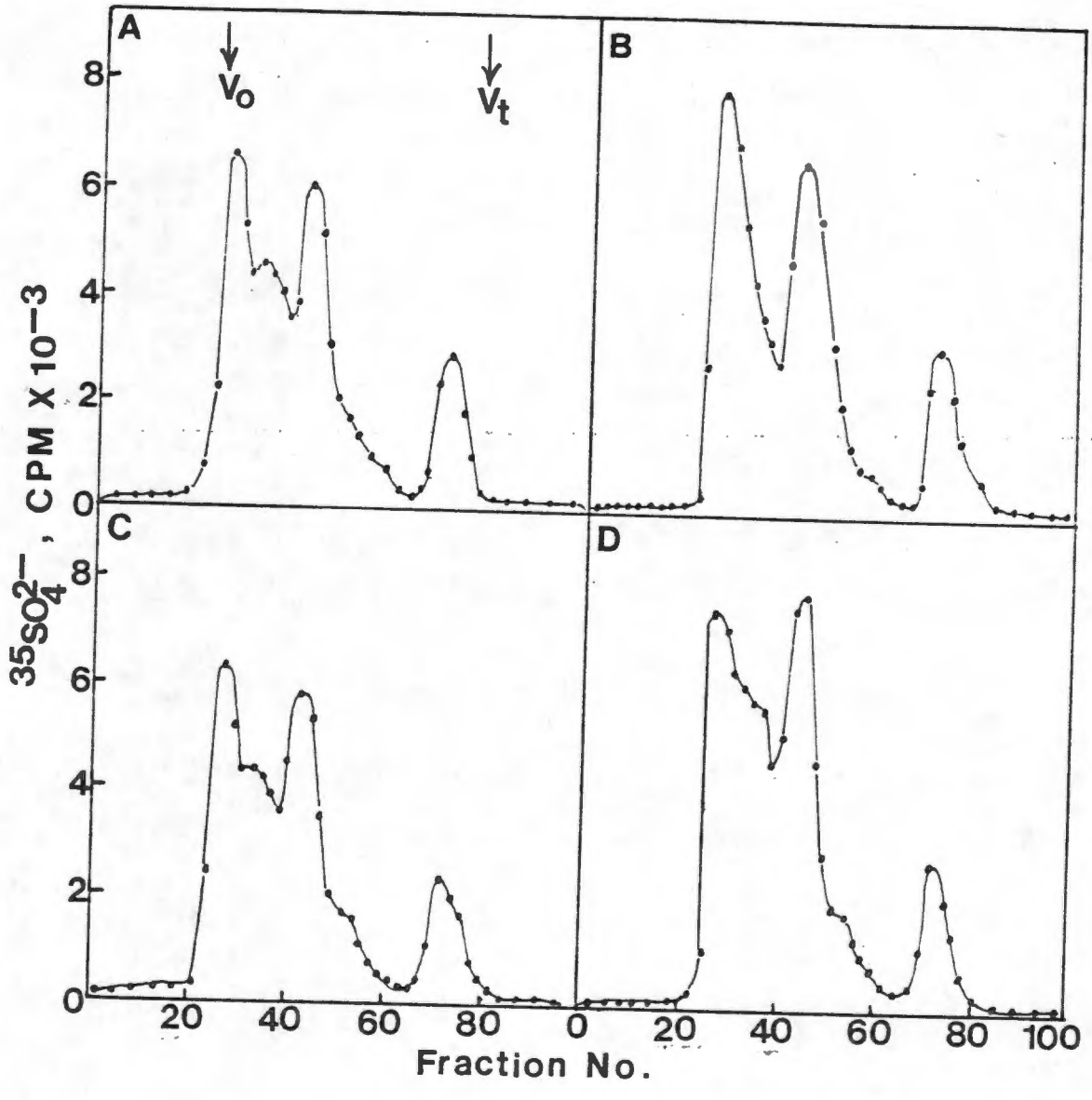

FIG. 3.8. Effect of different pretreated R_{22}C1F-ECMs on BAE cell surface proteoglycans. A, control; B, trypsin-treated; C, elastase-treated; D, collagenase-treated. Cell layers were extracted as described in section 2.2.3.1.

Chapter 2). This second peak could have represented a new species of proteoglycan associated with the endothelial cell surface. On the other hand, it could have been a degradation product of the first peak or even have represented material transiting the membrane for the extracellular matrix compartment. Similar peak patterns were obtained for trypsin-pretreated (Fig. 3.8B), elastase-pretreated (Fig. 3.8C), and collagenase-pretreated (Fig. 3.8D) R_{22}C1F ECMs and the same arguments for their respective profiles.

When the cell surface proteoglycans extracted from BAE cells grown on native and enzymatically-modified FG_o-ECMs (Fig. 3.9,A-D) were examined, a single peak was obtained in all cases. This peak was representative of the peak obtained for BAE cells cultured on plastic, but was strikingly different from profiles obtained for BAE cells grown on R_{22}C1F matrices which were cognate to the DTT_1 and DTT_2 peaks obtained in Chapter 2. Taken together, these results indicated that the observed differences probably cannot be ascribed to any specific single constituent of the ECM. As predicted, cells respond differently to a given type of matrix (i.e. results for R_{22}C1F versus FG_o-ECMs), but removal of glycoprotein, collagen or elastin by enzymatic digestion of the matrix does not allow one to assume that it is the removed component that determines the synthesis of a given cell surface proteoglycan. There is no doubt, however, that these constituents do play a role in cell growth and morphology (Fig. 3.7). Whether the changes in BAE cell morphology, induced by the presence of an ECM, caused concerted changes in the cell surface proteoglycan profile remains to be demonstrated. The lack of response to enzymic modifications of the ECM with respect to cell surface proteoglycans

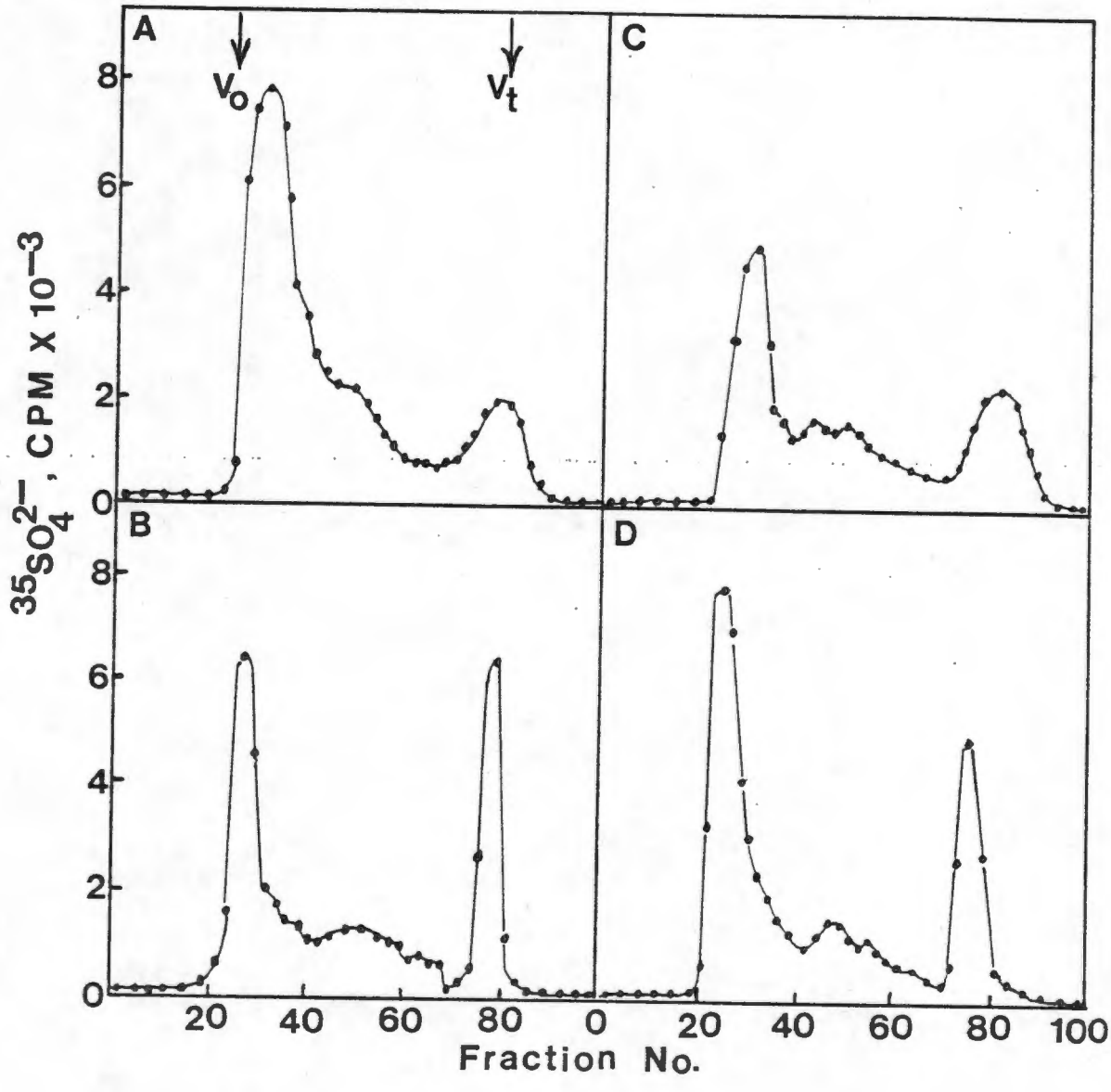

FIG. 3.9. Effect of different pretreated FG_o-ECMs on BAE cell surface proteoglycans. A, control; B, trypsin; C, elastase; D, collagenase. Details as for Fig. 3.8.

is puzzling since morphological changes have been observed, although morphology need not bear any relation to patterns of metabolism.

3.4. DISCUSSION

The ability of different substrata, particularly extracellular matrices produced by endothelial cells (line E_8), fibroblasts (FG_o), and rat smooth muscle cells (R_9 and $R_{22}C1F$), to support the *in vitro* proliferation of bovine aortic endothelial (BAE) cells was investigated. The experimental results indicated that the ECM, regardless of its cell-type origin, stimulated growth of BAE cells in culture better than plain naked plastic or plastic coated with gelatin. The smooth muscle cell (R_9 and $R_{22}C1F$) ECMs were found to be rich in elastin which constituted about 50% of their composition. The higher growth rate observed for cells plated onto ECMs could be ascribed to a permissive effect of the ECM on the cells via the matrix components. This effect of the ECM on the proliferation of BAE cells maintained in culture can also be reconciled with the ratio of the different components which is unique for a given type of ECM. ECMs differ in their three-dimensional structure as well as in biochemical composition. The ratio of elastin to collagen to glycoprotein was highest in ECMs produced by rat smooth muscle cells (R_9 and $R_{22}C1F$) and lowest in ECMs from fibroblastic- (FG_o) and endothelial-cell origin. It is therefore possible to include elastin as a likely candidate among other ECM components, such as collagen and glycoprotein, that is responsible for the higher growth rate observed in the case of BAE cells maintained on mainly elastin-ECMs (R_9 and $R_{22}C1F$). Although this reasoning is highly speculative since the overall matrix content may in no way reflect the type of component (e.g. elastin type I or

II; different types of collagen; glycoproteins like laminin, fibronectin, thrombospondin) responsible for the promoting effect on cell growth, it may, nevertheless support reports of elastin-proteoglycan interactions (145) and the implication of proteoglycans as control elements in cell division (35). That proteoglycans, in particular heparan sulfate (HS), may play a role in attachment and growth was demonstrated by the observation that BAE cells adhered to untreated and modified ECMs and still proliferated. The attachment of BAE cells to the ECM could be mediated via interactions between elastin in the matrix and HS located at the cell surface. This does not, of course, exclude the possibility of HS interacting with other components of the matrix to mediate attachment as has previously been suggested (58,76,181-189). Also, when the elastin and glycoprotein constituents of the ECM had been removed by pretreatment of the ECM with elastase, BAE cells still adhered to the substratum but fewer cells were present after a few days in culture as compared to control matrices. This indicated that the extent of proliferation was affected by the presence or absence of elastin or glycoproteins in the matrix. Although the observed effects cannot be regarded as a concrete and decisive indication of the involvement of HS and elastin in cell attachment and growth, they nevertheless point to the fact that elastin-proteoglycan interactions may exist (145).

Specific collagen substrates have also been reported to enhance the growth (177,191-193) of many cells in culture. Collagen can interact with glycoproteins and proteoglycans (147,148) and as such may regulate cell growth. Growth-promoting effects have been observed in culture

with ascorbic acid (194) and were attributed to the ability of ascorbate to increase the production of a collagenous matrix (56, 69,195). Not only the collagen and elastin components of the ECM are involved in the control of cell growth, but also glycoproteins which have been shown to occur in association with collagen, elastin and proteoglycans, especially HSPGS, in the ECM (149). From the results presented here, it is evident that although ECMs produced by E_8 and FG_o had growth-promoting effects on BAE cells, a more striking effect was observed with ECMs from rat smooth muscle-cell origin. The extent of proliferation of BAE cells was greatest in ECMs with a high elastin and collagen content in contrast to ECMs with high glycoprotein and collagen content. It is therefore reasonable to infer that both collagen and elastin are responsible for the higher growth rate observed with R_9-and R_{22}C1F-ECMs. Trypsin and collagenase pretreatment of the ECMs did not have any effect on the ability of ECMs to support the growth of BAE cells. Thus the elastin component of the ECM still supported good growth of BAE cells even when the other matrix components had considerably been reduced.

When BAE cells were seeded onto dishes coated with heterologous ECMs, they adhered, integrated into the matrix and assumed variable morphologies from roughly polygonal to fusiform cells. They proliferated and eventually formed confluent monolayers. The observation of variable endothelial cell morphologies may be ascribed to differences in the composition and organization of the ECM which vary with the cell type. In addition to the morphological data, changes in the profile of cell surface proteoglycans associated with BAE cells cultured onto different ECMs have also been observed. The endothelium

in culture has been shown both ultrastructurally and biochemically to be associated with the subendothelial matrix (5,14,18,99,105,106, 122,127). The manner in which cells perform their normal function is reflected in part by their relation to an ECM (127). Differences in composition and organization of such ECMs may cause alterations in the biosynthetic programmes of cells (9,28,91,110). Compatible with these reports were the observations that BAE cells changed their morphology and profile of cell surface proteoglycans and that these alterations may be directly linked to the ECM upon which the cells were grown. A structural and functional continuum is formed between the ECM, the cell membrane and the cell interior (7), suggesting that the matrix organization and composition in the microenvironment surrounding the cells may play important roles in directing responses of cells to a given modification of the ECM. Therefore, the implications of growing cells on an ECM instead of conventional tissue culture surfaces are enormous in the light of current interest in the proposed roles of the ECM in the regulation of cellular growth, biosynthetic phenotype, cancer and gene expression (13,16,26,91,92, 96,103,107).

CHAPTER 4

TURNOVER OF ENDOTHELIAL CELL SURFACE PROTEOGLYCANS IN CULTURE

4.1. INTRODUCTION

Some aspects of catabolism of proteoglycans have been discussed in Chapter 1 (section 1.7.3). The pathways for the degradative processing of proteoglycans by cells are still largely unknown (94). The major degradation of GAGS appears to occur in lysosomes: enzymes are present in these organelles which degrade GAGS, and the partially degraded GAGS accumulate in lysosomes in the various types of mucopolysaccharidoses, a disease associated with a deficiency of the GAG degradative enzymes (136,205).

Potential steps in the synthesis and degradation of endogenous proteoglycans have been studied in various culture systems including smooth muscle cells (1,61,65-67,100,101), fibroblasts (10), ovarian granulosa cells (94,123) and hepatocytes (138). Some of these studies have clearly demonstrated internalization of endogenous as well as exogenous proteoglycan molecules (94,100,101) and their subsequent intracellular degradation involving multiple pathways (94, 100). However, the exact intracellular localization of these degradation steps remains to be elucidated despite evidence for lysosomal involvement in the process. The possibility that non-lysosomal degradation pathways may exist in some cell systems cannot be totally excluded in view of the failure by specific lysosomal inhibitors to completely block degradation of proteoglycans (94,123).

Despite the enormous increase in interest over the past ten years in the vascular endothelium, details regarding the metabolism of GAGS by these cells have been largely absent from the literature. Bovine aortic endothelial (BAE) cells have been shown to distribute newly formed sulfated GAGS in a distinct manner into three main compartments, viz., extra-, peri- and intracellular pools, respectively (67). Generally, the metabolism of sulfated GAGS by endothelial cells resembles, in principle, that of other vascular and non-vascular cells but a more rapid turnover of intra- and pericellular GAGS has been observed with endothelial cells (67). The intracellular GAGS disappeared by degradation and exocytosis, and pericellular GAGS were shed into the culture medium (67). Endothelial cells are able to secrete proteoglycans (50). It is likely that the elucidation of specific steps, like intracellular localization, kinetics of migration and degradation of these easily labelled and identified macromolecules in endothelial cells will prove valuable for studies in vascular physiology and pathophysiology.

Pulse-chase experiments were subsequently designed to determine various aspects of proteoglycan metabolism in BAE cells, particularly the kinetic relationship between proteoglycans in the medium (extracellular pool), on the cell surface (pericellular pool), and inside the cell (intracellular pool). To investigate possible mechanisms for turnover of proteoglycans, the lysomotropic inhibitors, chloroquine and ammonium chloride (NH_4Cl) were used to assess the relative contributions of lysosomal and non-lysosomal degradation pathways. Drugs affecting the secretory process, colchicine and monensin, were used to assess the relationship between degradation of proteoglycans and

secreted macromolecules which exit from the cells. The molecular properties of pericellular and extracellular proteoglycans were investigated only in control endothelial cell cultures, i.e. cultures not exposed to drugs.

4.2. METHODS

4.2.1. CELL CULTURE AND METABOLIC LABELLING

BAE cells were isolated and cultured as described in Chapter 2 (section 2.2.1). Confluent cultures in 60 mm petri dishes (Greiner) were prelabelled with 10 µCi $|^{35}S|$-sulfate per ml culture medium for 20 h. After labelling, cultures were washed extensively 4 times with isotope-free medium. The labelled monolayers were then cultured for various lengths of time with complete medium containing 10 mM Na_2SO_4 (chase medium) in the presence or absence of the requisite drug at the indicated concentrations. Monensin, taken from a 10 mM stock in 96% ethanol, was added to the chase medium at a final concentration of 1 µM. Colchicine dissolved in chase medium was used at a final concentration of 50 µM. Chloroquine and NH_4Cl were added at final concentrations of 100 µM and 10 mM, respectively, to chase medium. Control cultures were exposed to the same concentration of solvents used for drug solutions.

4.2.2. HARVESTING OF CELL CULTURES

After each chase period, triplicate dishes were harvested and media, pericellular and cellular fractions prepared in the following manner. Media were quantitatively removed in the presence of protease inhibitors (section 2.2.3.1) and centrifuged briefly at 1000 x g for 5 min

to remove any free floating cells and the cell pellets retained for later analysis with cells released from the culture dishes. The cell monolayers were washed 4 times with phosphate-buffered saline (PBS). Some cell layers were extracted directly with a 2% (w/v) SDS solubilizing buffer as described in Chapter 2 (see section 2.2.3.1), while others were exposed to Trypsin (E.C. 3.4.12.4, 0.1% in PBS) for 3 min at 22°C. Under these conditions most of the cells detached from dishes. Non-adherent material was rapidly transferred to tubes containing soybean trypsin inhibitor at 1 mg/ml final concentration. The trypsin-treated cells were centrifuged at 1000 x g for 5 min. The supernatants were decanted into tubes containing protease inhibitors (see section 2.2.3.1) and represented pericellular fractions. The cell pellets were suspended in PBS containing protease inhibitors (section 2.2.31) and represented the cellular fractions which were then homogenized by ultrasonication for 30 sec with a Branson Sonifier, microtip 50 W (Branson Sonic Company, USA).

4.2.3. DESCENDING PAPER CHROMATOGRAPHY

The separation of macromolecular sulfated molecules from unincorporated precursor isotope was routinely carried out as follows. Aliquot portions (100 µl) of samples from all compartments were streaked onto Whatman 3 MM paper (25 x 45 cm) and subjected to descending paper chromatography, using 1-butanol : glacial acetic acid : 1 M NH_4OH, 2:3:1.5 (v/v/v) as solvent (67). After overnight runs, chromatograms were dried and the macromolecular material that remained at the origin was cut out and counted in scintillation cocktail (Beckman Ready-Solv EP). Under these conditions, precursor isotopes were eluted from

the origin but macromolecular labelled material remained. Corrections for background counts due to non-specific binding of the labelled precursor to macromolecules at the origin were routinely performed on each chromatogram by counting 100 µl aliquots of fresh medium containing the requisite amount of radioactive isotope as used in pulse-chase experiments. The values obtained for radioactivity remaining at the origin under these conditions were subtracted from those for test samples. Background values were always less than 5% of the values for macromolecular labelled material.

4.2.4. ANALYTICAL PROCEDURES

Protein determinations were carried out on cellular fractions using the method of Lowry et al. (210), with bovine serum albumin (Fraction V, Seravac Fine Chemicals Corporation, RSA) as standard.

4.2.5. GEL FILTRATION CHROMATOGRAPHY

Sepharose CL4B and Sephadex G-25 chromatography were performed as described in Chapter 2 (section 2.2.5).

4.3. RESULTS

Pulse-chase experiments were undertaken in order to study the metabolism of ^{35}S-labelled macromolecules, particularly proteoglycans, in cultures of bovine aortic endothelial (BAE) cells. The kinetics and distribution of ^{35}S-labelled proteoglycans at different chase times after a 20 h labelling period in cultured BAE cells is depicted in Fig. 4.1. At zero chase time (i.e. after a 20 h pulse with $^{35}SO_4^{2-}$ and replacement

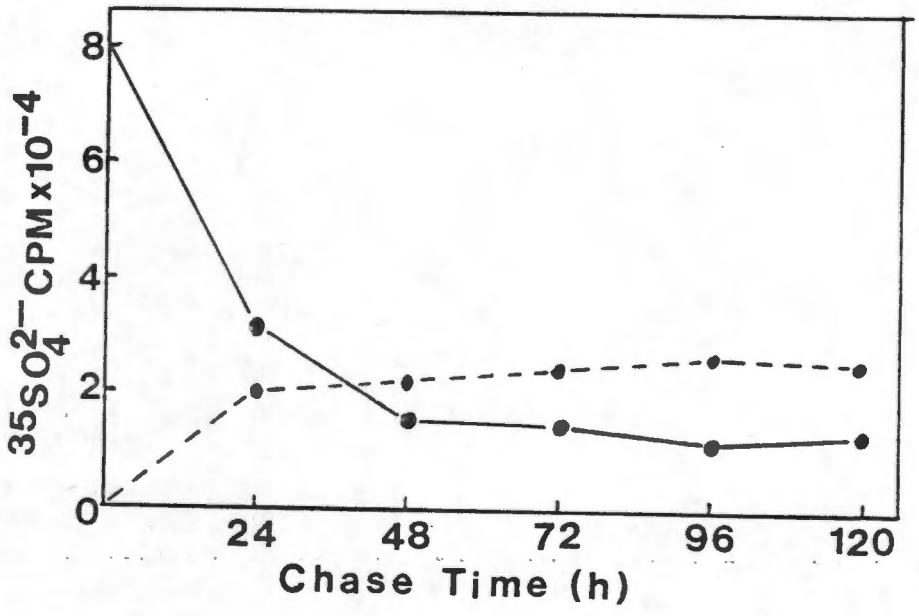

FIG. 4.1. Kinetics and distribution of ^{35}S-labelled macromolecules during pulse-chase experiments (see section 4.2.2 for details). The initial total macromolecular radioactivity present in the medium (----) and cell layer (——) compartments at each time point was calculated.

with chase medium), the bulk of the macromolecular radioactivity was associated with the cell layer, whereas the medium contained only free and unincorporated $^{35}SO_4^{2-}$, i.e., material left after washing. During the first 24 h of the chase period, with isotope-free medium containing 10 mM Na_2SO_4, the amount of radioactive material associated with the cell layer compartment decreased by approximately 58% and, after 48 h chase, the decrease amounted to 80%. During the remaining chase period (72-120 h) of the experiment, the cell layer compartment showed smaller decreases in macromolecular sulfate content, with little or no loss after 48 h chase. The decrease in macromolecular $^{35}SO_4^{2-}$ content of the cell layer was proportional to the amount of ^{35}S-labelled macromolecules appearing in the medium after the various chase times (Fig. 4.1), thus indicating a kinetic relationship between cell-associated and medium ^{35}S-labelled proteoglycans. These results were further substantiated by gel chromatography on Sephadex G-50 of 2% SDS-extracts of ^{35}S-labelled proteoglycans from the extracellular (medium) and pericellular (cell layer) compartments of cultured BAE cells (Fig. 4.2). At the end of the 20 h pulse with $^{35}SO_4^{2-}$, labelled proteoglycans associated with the cell layer eluted as a large peak in the V_o region of the column and free unincorporated isotope eluted at V_t (Fig. 4.2A). The ^{35}S-labelled proteoglycans present in the medium at chase time 0 h eluted as a very small peak at V_o of the column, again indicating that the bulk of the macromolecular ^{35}S-label was present in the cell layer of that particular time and that the medium contained free $^{35}SO_4^{2-}$ (Fig. 4.2A) which eluted at V_t. After 24 h and 48 h chase times (Fig. 4.2 B and C, respectively), the proportions of the V_o peaks were both 25% of the total ^{35}S-labelled proteoglycans for chase time 0 h. The proportions of the V_t cell layer peaks were 27% and 13%

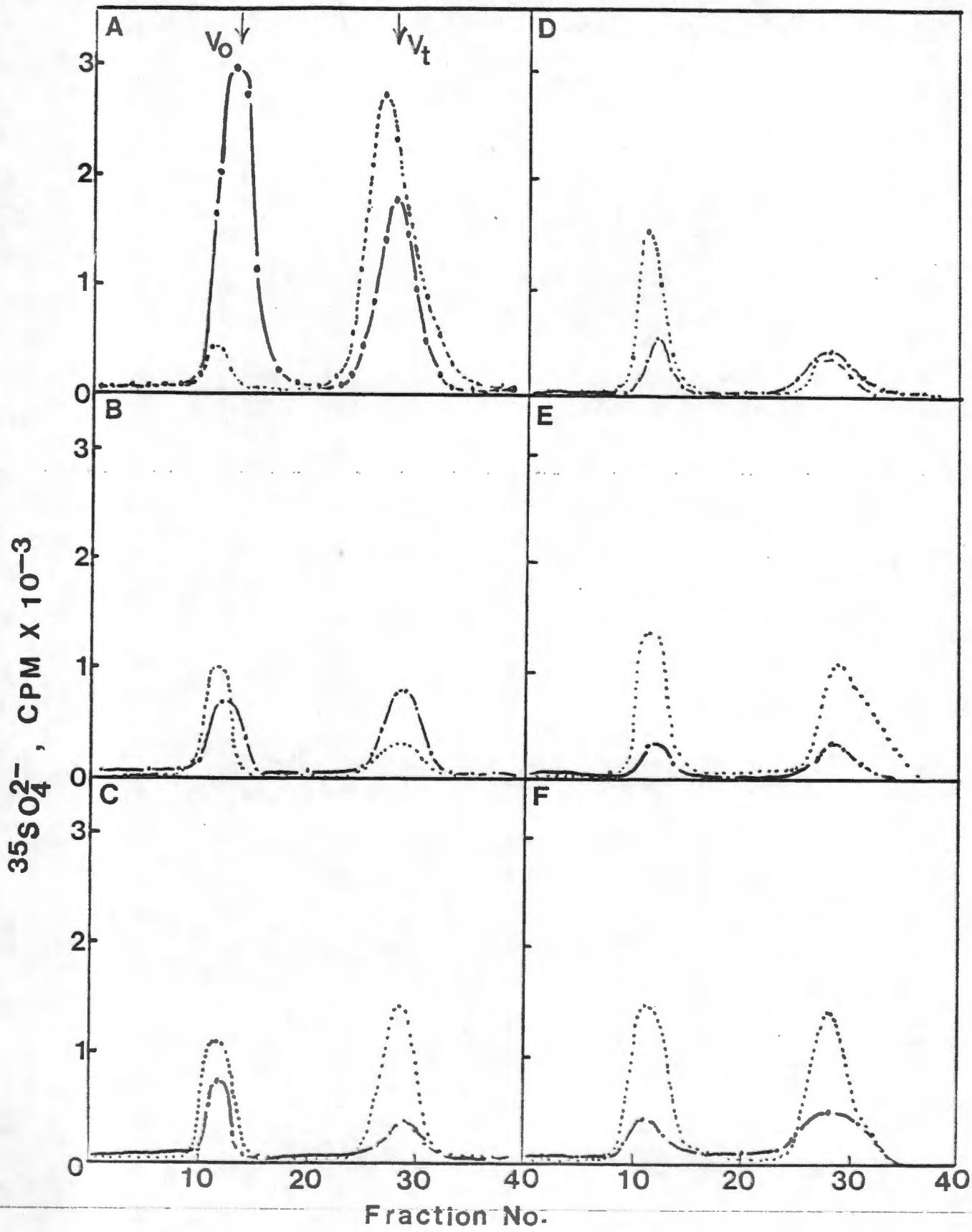

FIG. 4.2. Gel chromatography on Sephadex G-50 of ^{35}S-labelled proteoglycans/glycosaminoglycans from the extracellular (---) and pericellular (——) compartments of cultured BAE cells, pulse-labelled for 20 h (A). Medium and cell layer fractions were processed after 24 h (B), 48h (C), 72 h (D) and 120 h (F) of chase as described in Methods.

of the total ^{35}S-labelled macromolecules present at 0 chase time, after 24 h and 48 h chase times, respectively, and these proportions did not change to any significant extent throughout the chase period (Fig. 4.2 D, E and F). The ^{35}S-labelled proteoglycans present in the chase medium eluted as V_o peaks which were approximately 33% and 37% of the macromolecular ^{35}S-label associated with the cell layer at chase time 0 h, after 24 h and 48 h chase times, respectively (Fig. 4.2 B, C). The amount of V_o material appearing in the chase medium reached a maximum at 72 h chase, after which no further increase was observed (Fig. 4.2 D, E, F). The increase in the amount of labelled material associated with V_t, which either represented free $^{35}SO_4^{2-}$ or degraded products in the chase medium, was noted at each time point during the chase period. Taken together these results indicate that about 87% degradation of cell layer ^{35}S-labelled proteoglycans occurred within 48 h and that the degradation products were shed into the medium. Also, at each time point of the chase period, the total radioactivity (cell layer and medium ^{35}S-label in the V_o and V_t peaks) was the same as that for the cell layer at chase time 0 h (i.e., after 20 h pulse with $^{35}SO_4^{2-}$) within experimental error (range 92-125%) confirming the quantitative recovery of ^{35}S-label during extraction and chromatography steps; and hence, indicated that the loss of ^{35}S-label in macromolecules from the cell layer could be accounted for by the generation of macromolecular and free radiosulfate which accumulated in the medium.

The size of ^{35}S-labelled proteoglycans associated with the medium and cell layer compartments of BAE cells was further analyzed by Sepharose CL4B gel chromatography as described in Chapter 2 (section 2.2.5).

At the end of the 20 h pulse-labelling period with $^{35}SO_4^{2-}$, labelled proteoglycans in the cell layer compartment eluted as a single V_o peak which represented macromolecular $^{35}SO_4^{2-}$, while unincorporated $^{35}SO_4^{2-}$ eluted in the V_t region of the column profile (Fig. 4.2A). This peak pattern was similar to that obtained for BAE cell layer heparan sulfate proteoglycans (HSPGS) in Chapter 2. The medium contained mainly free $^{35}SO_4^{2-}$ which eluted at V_t of the column. After the 24 h chase time, the medium contained an intermediate form of HSPG which eluted between V_o and V_t, thus indicating a reduction in size of the HSPGS which may have been due to some proteolytic activity at the cell surface. The exact nature of the proteolytic attack on cell layer HSPG was not investigated further. The cell layer peak, after 24 h chase, was diminished by about 56% (Fig. 4.3B) - exactly the same result as that obtained for Sephadex G-50 chromatography (Fig. 4.2B). After 48 h chase, the medium contained ^{35}S-labelled HSPGS which eluted as a truncated peak between V_o and V_t on Sepharose CL4B (Fig. 4.3C). This double peak pattern has previously been observed (Chapter 2) with dithiothreitol-treated cell layer extracts - implying that a cleavage of disulfide bridges had occurred to release the two different sized species (see Chapter 2) into the medium. These two species were slightly smaller than those described in Chapter 2.

The effects of various drugs on the degradation of ^{35}S-labelled proteoglycans in the intracellular and pericellular compartments of cultured BAE cells were examined by using various chase protocols after radiolabelling the cells with $^{35}SO_4^{2-}$ for 20 h (see Methods). The time courses of pulse-chase experiments carried out with BAE cells

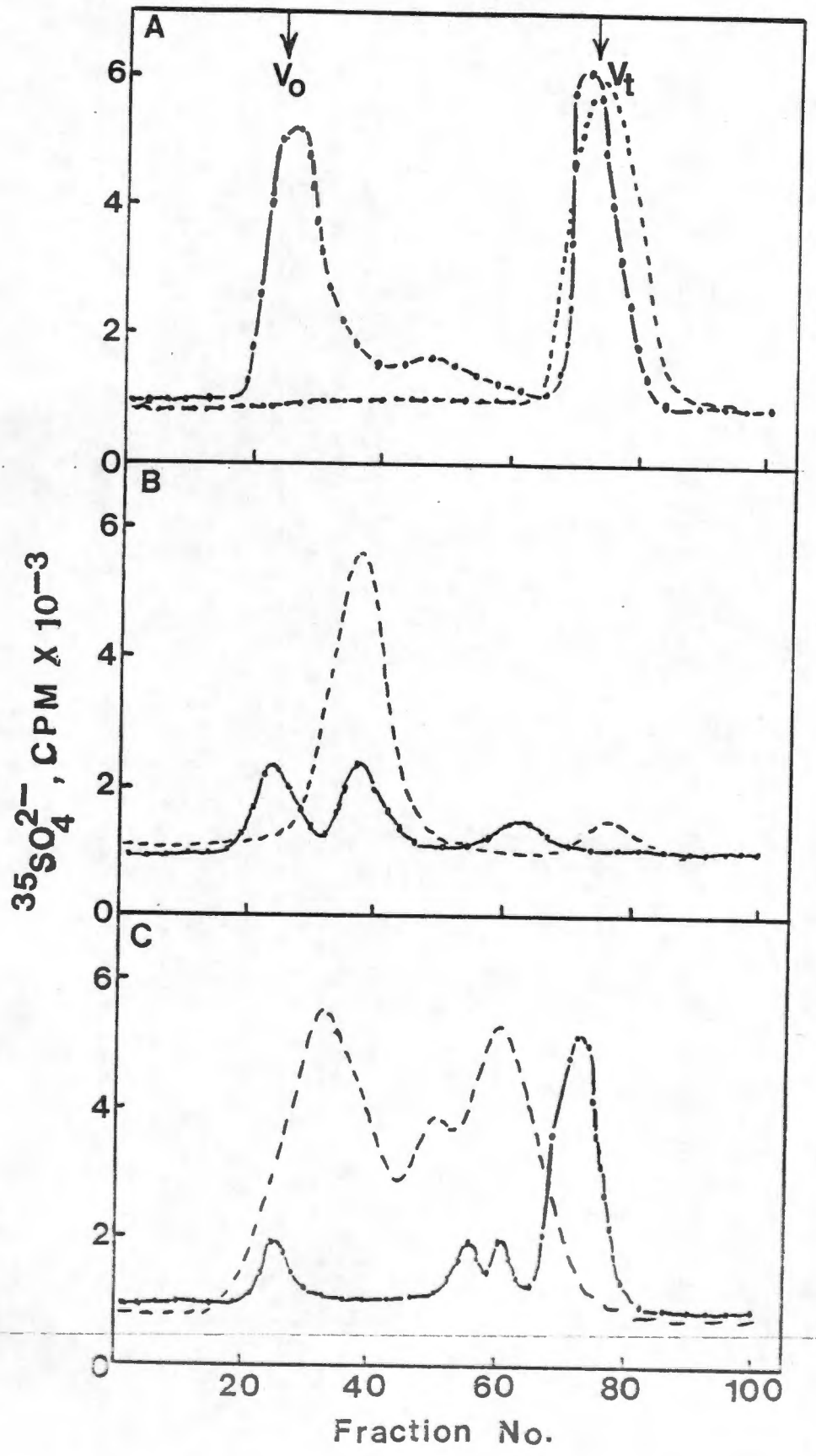

FIG. 4.3. Sepharose CL-4B chromatography of cell layer (——) and medium (---) ^{35}S-labelled proteoglycans at A, 20 h pulse; B, 24 h chase and C, 48 h chase times. The areas of the peaks are proportional to the total radioactivity in the original extracts.

in the absence or presence of various drugs are depicted in Fig. 4.4, A-E. In all experiments, the total macromolecular radioactivity present in the extra-, intra- and pericellular compartments at the different chase times, was the same as that for chase time zero within experimental error (range: Fig. 4.4: A, 98-113%; B, 97-105%; C, 81-112%; D, 100-142%; E, 73-100%), indicating the quantitative recovery of macromolecular $^{35}SO_4^{2-}$ in the different compartments. In control cultures, ^{35}S-labelled proteoglycans associated with the cell layer (pericellular compartment), were rapidly degraded (approximately 60% and 92% of the total macromolecular material present at 0 h chase time being degraded after 6 h and 24 h chase times, respectively (Fig. 4.6A)). The intracellular compartment of control cultures also exhibited rapid degradation of ^{35}S-labelled macromolecules, but in this case degradation was constant for up to 12 h chase after which no further degradation occurred since, at this time point, 80% of the labelled material was already degraded (Fig. 4.6B). The degradation of ^{35}S-labelled proteoglycans in the intra- and pericellular compartments was accompanied by a steady increase in the appearance of macromolecular ^{35}S-labelled material in the extracellular compartment (Fig. 4.4A), which underlined the kinetic relationship between the three compartments. The kinetics of degradation of macromolecular $^{35}SO_4^{2-}$ was the same in drug-treated cultures as in controls (Fig. 4.4), except that variations in the amounts of ^{35}S-label associated with each compartment (Fig. 4.5) were noted. Treatment of radiolabelled cultures with 10 mM NH_4Cl almost completely inhibited intracellular loss (Fig. 4.4B and 4.5C) of ^{35}S-labelled proteoglycans whereas the decrease in macromolecular material associated with the pericellular compartment was inhibited throughout the

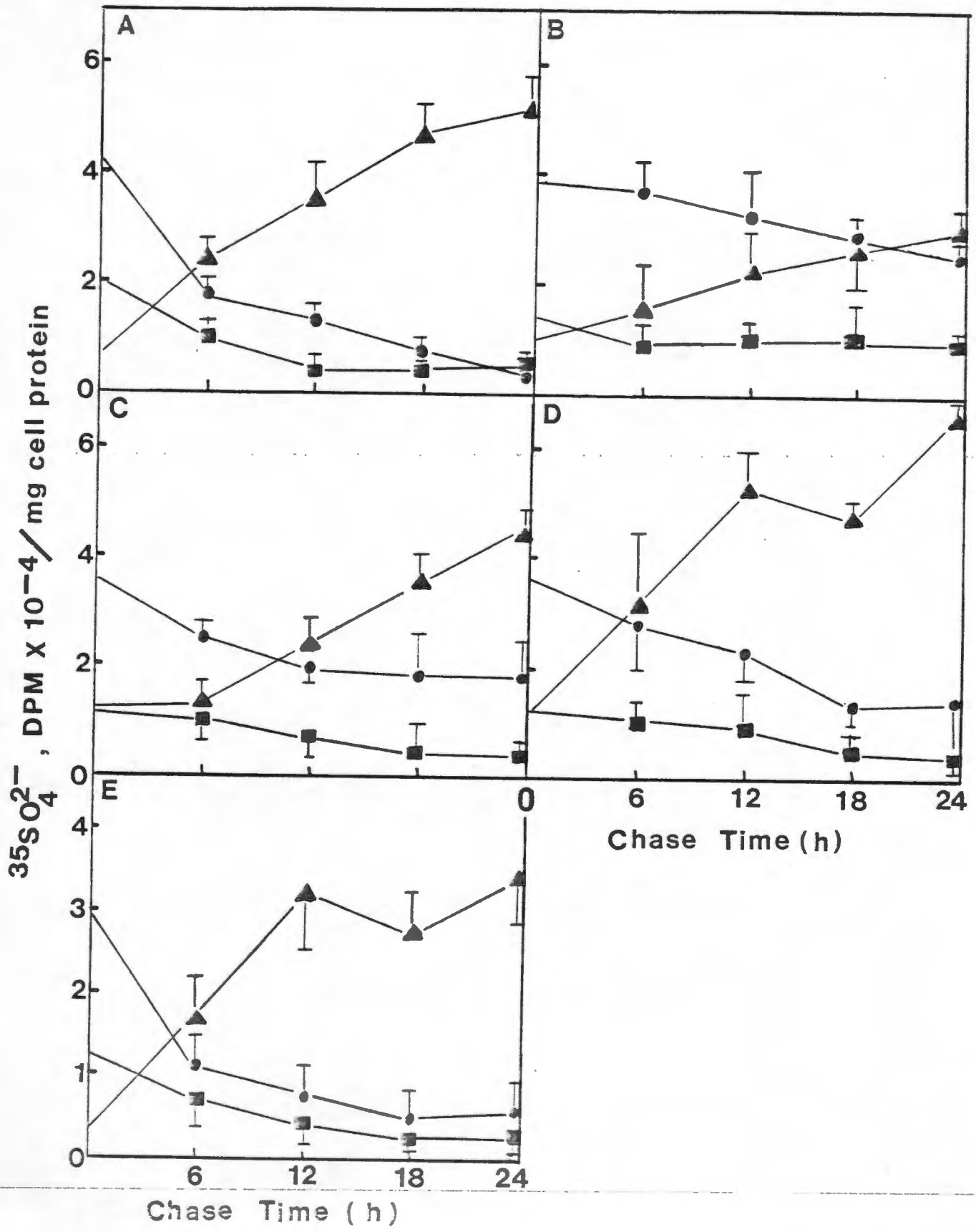

FIG. 4.4. Time course of pulse-chase experiments carried out with BAE cells in the absence (A) and presence of 10 mM NH_4Cl (B), 100 μM chloroquine (C), 1 μM monensin (D) and 50 μM colchicine (E). The data represent the radioactivity in the intracellular (■), pericellular (●) and extracellular (▲) compartments. Values are means ± S.D. of triplicate culture dishes from one of three experiments.

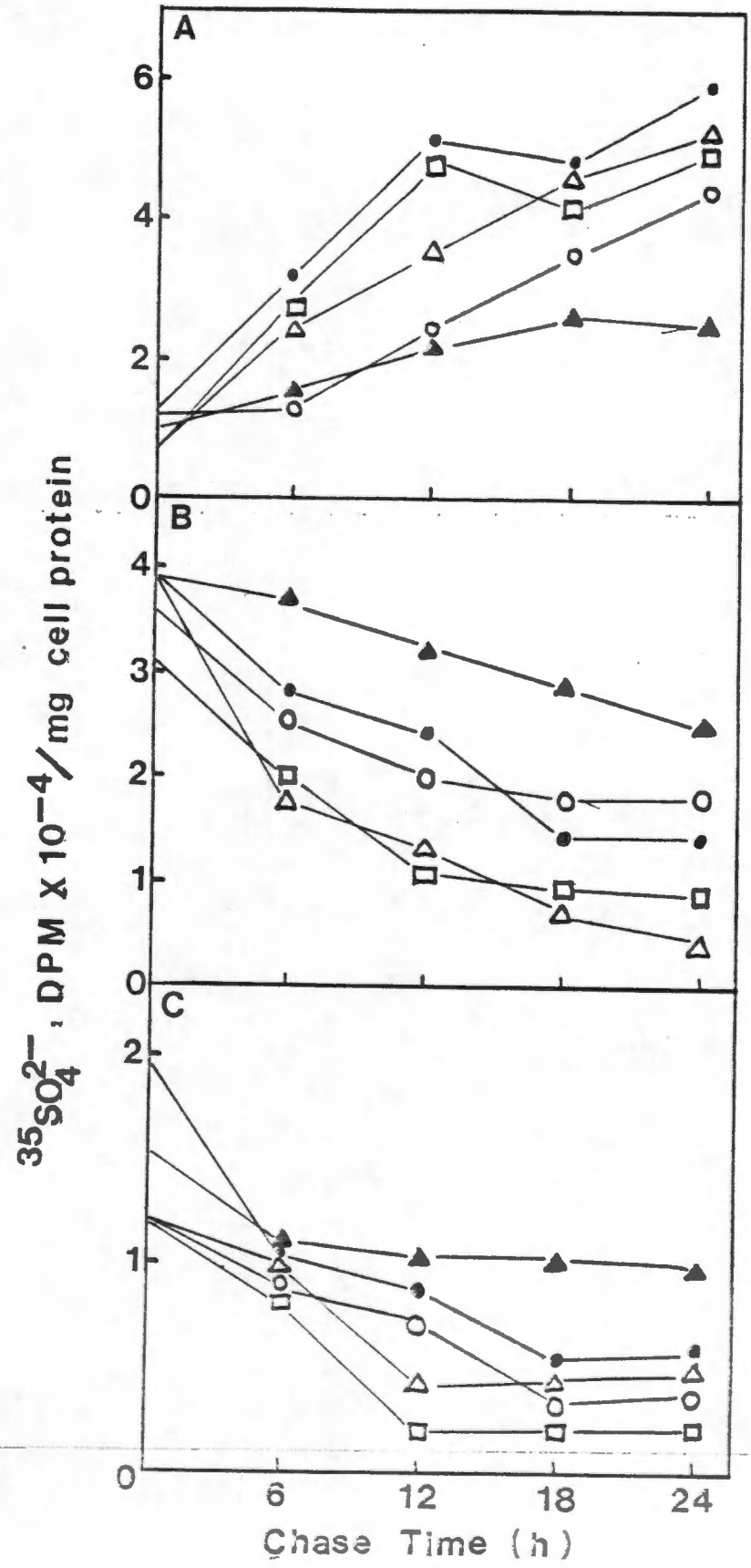
A
B
C
6
4
2
0
3
1
6
12
18
24
Chase Time (h)
$^{35}SO_4^{2-}$: DPM X 10^{-4}/mg cell protein

FIG. 4.5. Kinetics of degradation of ^{35}S-labelled macromolecules by BAE cells in the absence (△) and presence of 10 mM NH_4Cl (▲), 100 μM chloroquine (o), 1 μM monensin (●) and 50 μM colchicine (□). Data are presented in the same way as in Fig. 4.4, except that extracellular (A), pericellular (B) and intracellular (C) values were plotted separately.

chase period by about 52% (Fig. 4.6A). Intracellular degradation of ^{35}S-proteoglycans in NH_4Cl-treated cultures was inhibited to the same extent (Fig. 4.5C and 4.6B). NH_4Cl also inhibited the release of ^{35}S-proteoglycans into the medium by approximately 40-50% (Fig. 4.5A). As a result, the net decrease of labelled proteoglycans in NH_4Cl-treated cultures was much less than in the controls. Chloroquine, at a concentration of 100 μM (Fig. 4.5 B,C and 4.6 A,B) had the same inhibitory effect on the degradation of ^{35}S-labelled proteoglycans in the peri- and intracellular compartments although the degradation was affected to a much greater extent in NH_4Cl-treated cells. The decrease of ^{35}S-labelled macromolecules in the cell layer compartment was much slower in chloroquine-treated cultures than in the control, reflecting inhibition of the degradation of proteoglycans by chloroquine. Thus, the decreased degradation of both intra- and pericellular proteoglycans in the presence of these lysomotropic agents must be due, at least in part, to the blocking of the lysosomal pathway involved in the degradation of proteoglycans.

The secretion-blocking agents, monensin and colchicine, at concentrations of 1 μM and 50 μM, respectively, affected the degradation of ^{35}S-labelled proteoglycans in the intra- and pericellular compartments rather differently: Both colchicine and monensin caused an increase in the rate of ^{35}S-labelled macromolecules appearing in the medium with chase time (Fig. 4.5A). Monensin-chased cell cultures secreted more ^{35}S-labelled material than did control cultures. Colchicine treated cultures also secreted more ^{35}S-labelled macromolecules than did control cultures during 6 h and 12 h chase periods, after which time the extent of secretion was the same for up to 24 h chase times.

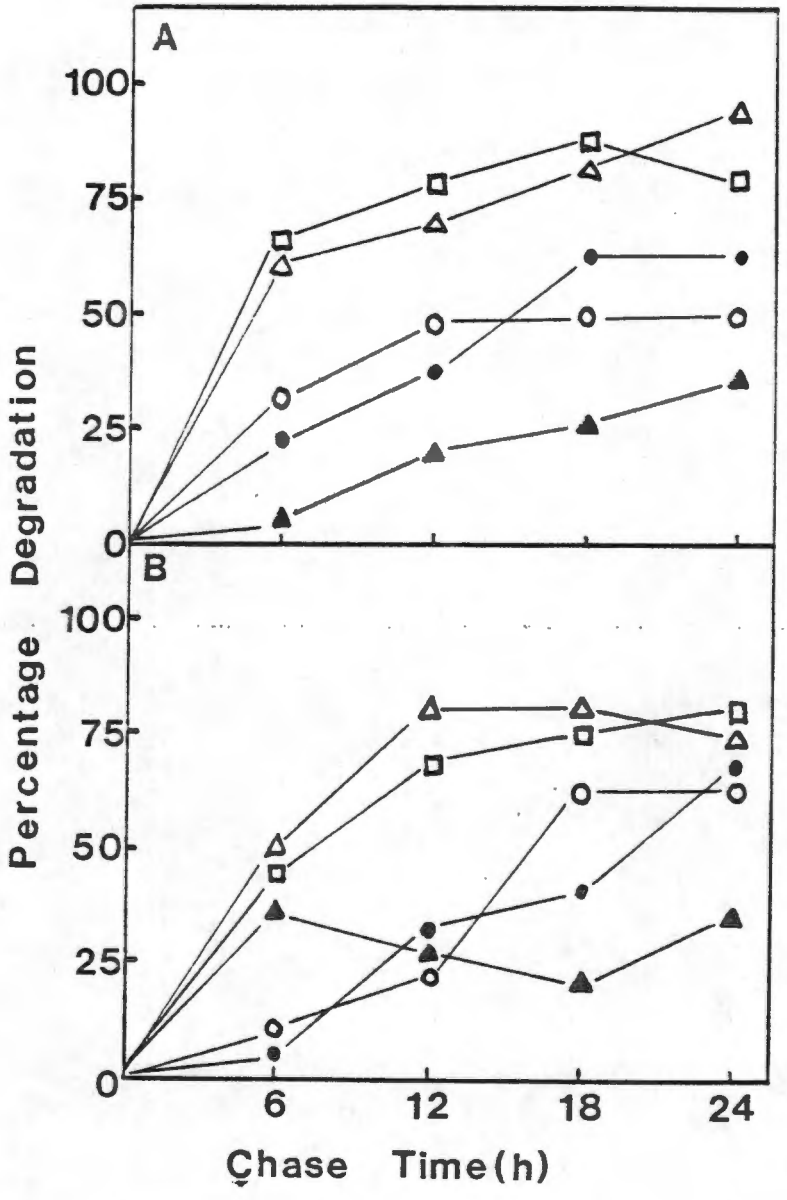

FIG. 4.6. Effects of inhibitors on the degradation of pericellular (A) and intracellular (B) ^{35}S-labelled proteoglycans in cultured BAE cells. Cells were chased in the absence (△) and presence of NH_4Cl (▲), chloroquine (o), monensin (●) and colchicine (□) at concentrations indicated in Figs. 4.4 and 4.5. The values represent the percentages of initial mean macromolecular radioactivity present at each time point.

These results were unexpected in view of the known effects of these agents (40,41,60,113-115), and secretion of ^{35}S-labelled macromolecules *per se* was not affected by either monensin or colchicine by virtue of the fact that the amount of ^{35}S-labelled macromolecules increased in the medium throughout the chase period (Fig. 4.5A). As expected, the pericellular compartment was not affected to any significant extent by the presence of colchicine (Fig. 4.5B and 4.6A) because the decay of macromolecular ^{35}S-label paralleled that obtained for control cultures (Fig. 4.5B and 4.6A). The same observation was made of the intracellular compartment (Fig. 4.5C and 4.6B). In contrast, monensin inhibited the degradation of pericellular ^{35}S-labelled proteoglycans by 20-33% (Fig. 4.5B and 4.6A), and that of intracellular ^{35}S-labelled proteoglycans by approximately 43% between chase times 6-18 hours, and by about 8% at 24 h chase (Fig. 4.5C and 4.6B). It would appear, therefore, that both monensin and colchicine did not inhibit the secretion of ^{35}S-label from such treated cultures but degradation of intra- and pericellular labelled material was partially inhibited by monensin only.

4.4. DISCUSSION

The metabolism of sulfated macromolecules in cultures of BAE cells was studied by pulse-chase experiments using $^{35}SO_4^{2-}$ as a precursor isotope to preferentially label proteoglycans/glycosaminoglycans. Maximum incorporation of the ^{35}S-label occurred within 20 h, with the bulk of the macromolecular radioactivity associated with the cell layer. The medium contained considerably less macromolecular material and mainly free or unincorporated sulfate. These results are consistent with published reports that the accumulation of

^{35}S-labelled material in the cell layer reaches a steady state after 20-24 h (19,50,212). The results also showed that the ^{35}S-labelled proteoglycans in the cell layer were rapidly degraded within 24-48 h and that the degradation products were shed into the medium. Furthermore, when medium samples collected at different time points during the chase period were subjected to Sephadex G-50 chromatography, it was seen that there was an increase in the labelled material associated with both V_o and V_t, which represented macromolecular and free or degraded radioactive sulfate, respectively, both arising from the cell layer. There was, however, no apparent accumulation of intermediate degradation products in the culture medium, but size analysis of the ^{35}S-labelled proteoglycans by Sepharose CL4B chromatography revealed an intermediate sized proteoglycan which appeared in the medium after the 24 h chase period. Also, an important observation was the appearance of two different sized species of proteoglycan in the culture medium after 48 h chase. These molecules had K_{av} values similar to those found for the two HSPG molecules obtained after DTT reductive cleavage of the disulfide-bonded membrane-intercalated species of BAE cells as described in Chapter 2. A possible explanation of this phenomenon could be that the two species were separated by some enzymatic activity at the cell surface and subsequently secreted into the culture medium. This possibility and the exact nature of the phenomenon has not been investigated further.

The degradation of both intracellular and pericellular proteoglycans was shown to be inhibited by the lysomotropic agents, chloroquine and NH_4Cl, thus indicating that such degradation occurred intracellularly within the lysosomes, since these agents exert their effect by entering

the lysosomes and raising the intralysosomal pH to inhibit the lysosomal hydrolases which function at acid pH optima (113). The inhibition of degradation of pericellular proteoglycans by lysomotropic agents would seem to indicate that cell layer proteoglycans are apparently internalized prior to their degradation inside the lysosomes. This does not, however, preclude any other mechanisms of extralysosomal degradation since both NH_4Cl and chloroquine failed to completely block the degradation of both intracellular and pericellular proteoglycans. The results of these inhibition studies are in agreement with reports of multiple degradative pathways for proteoglycans (67,94,100,101,123), but cannot explain the significance of different mechanisms of turnover in relation to their structure. More detailed studies are thus required to clarify the mechanisms as well as the sites and enzymes of catabolism of these molecules.

The carboxylic monovalent ionophore, monensin, has been used to inhibit secretion of molecules (proteins, glycoproteins, proteoglycans) destined for export in a wide variety of cells and tissues (49,41,60,113-115). In all cases, monensin has, either partially or completely, inhibited secretion but has not affected the molecular properties of the secreted products, except in the case of chondrocytes which secreted undersulfated proteoglycans when cultured in the presence of monensin (60). The use of colchicine has also proved to be effective in inhibiting secretion (113). In the BAE culture system, neither monensin nor colchicine inhibited the secretion of ^{35}S-labelled macromolecules. On the contrary, these secretion-blocking agents had somewhat unexpected effects in that

they both caused an increase in the rates of macromolecular ^{35}S-label secreted into the medium compared with control cultures. Monensin inhibited the degradation of pericellular and intracellular ^{35}S-labelled proteoglycans but to a lesser extent than did the lysomotropic inhibitors. The inhibition of degradation by monensin can be conveniently explained by assuming that this ionophore exerts some of its inhibitory effects by raising the pH inside intracellular vesicles in a manner similar to lysomotropic agents (113). Unlike monensin, colchicine had no effect on the degradation of ^{35}S-labelled proteoglycans associated with either compartment, a phenomenon certainly hard to explain.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The vascular endothelium has evoked intense interest over the past decade (156) although its involvement in various physiologic and pathologic processes had been recognized before the turn of the century. The use of cultured endothelial cells as a tool to study their potential involvement in biological processes has tremendously increased our current understanding of their functions. Nevertheless, there are certain areas where endothelial cells have yet to yield their perplexities. Perhaps the more elusive of these involve cell-cell communication, cell-ECM interplay and surface (barrier) properties. Most pertinent to all these interests are the molecules of the endothelial pericellular coat known as proteoglycans. Information on endothelial cell surface proteoglycans is minimal compared with what has been documented for other cell types, particularly in regard to detailed biochemical characteristics and modes of attachment to cell membranes. Therefore, part of the work presented in this thesis was conducted with the objective of characterizing proteoglycans associated with the surfaces of bovine aortic endothelial (BAE) cells in culture. Additionally, the relation of BAE cells to different extracellular matrices (ECMs) with regard to morphology, growth and profiles of cell surface proteoglycans was also studied. Some attempt has also been made to study the turnover of plasma-membrane proteoglycans in the BAE cell-culture system.

My observations revealed that BAE cells have associated with their cell surface a dominant pool of proteoglycans which was consistently

demonstrable after SDS extraction. These proteoglycans contained large GAG chains and relatively small core proteins as shown by alkaline borohydride treatment and CsCl density gradient centrifugation. The majority of the ^{35}S-labelled macromolecular material of the BAE cell surface was identified as heparan sulfate by virtue of its susceptibility to nitrous acid deamination. When SDS extracts of BAE cell layer proteoglycans were chromatographed on Sepharose CL4B, the proteoglycans eluted as aggregates in the void volume in the presence of detergent. When this peak fraction was treated with the thiol-reducing agent, DTT, and rechromatographed on a column of Sepharose CL4B in the presence of both SDS and DTT, the aggregates disappeared; they now eluted as two included peaks with the total radioactivity which was initially present in the V_o peak equally distributed between them. Thus, no loss of macromolecular ^{35}S-label was evident after reductive cleavage of the large aggregated HSPG species. Exclusion of DTT in the elution buffer resulted in re-aggregation of the two HSPG species which again eluted as a single peak in the V_o region of the column. These observations permit the conclusion that BAE cell layer proteoglycans consist of at least two polypeptide chains joined together by disulfide bonds and having large GAG entities. Although this finding of a disulfide-bonded proteoglycan structure is the first reported for endothelial cells, other reports of a similar nature for basement membrane and fibroblast proteoglycans have appeared in the literature (73,202). In this regard, Hassel et al (198) reported contamination of basement membrane proteoglycans with the 230 000 MW chain of laminin and other high MW proteins that could only be removed by reduction with DTT and ion-exchange chromatography. It seems likely that association of laminin

and other proteins with proteoglycans could have occurred as a result of disulfide exchange but this was negated by the inclusion of N-ethylmaleimide in the extraction and purification. These authors have attributed disulfide-dependent association to occur as a means of stabilizing the configuration of the participating molecules in a manner analogous to link protein-cartilage interaction. Disulfide-dependent association between collagen and proteoglycans has been reported for glomerular basement membranes (201).

Extraction of ^{35}S-labelled BAE monolayers by mild trypsinization released more than 90% of the radioactivity associated with the cell layers. The trypsin-extractable material eluted as three peaks on Sepharose CL4B: two peaks at included positions and one peak at V_t. Following trypsin treatment the remaining cell layer was extracted with SDS as usual and eluted as a greatly diminished broad profile. This was to be expected since the trypsinization released the majority of the ^{35}S-labelled HSPGS. The question of how the trypsin-removable HSPGS were attached to the BAE cell surface required clarification. Repeated attempts to remove cell surface HSPGS with either DTT or high salt or both were unsuccessful and suggested that the HSPGS were firmly (covalently) attached. This viewpoint was further corroborated by the need for detergent (SDS, Triton X-100 or CHAPS) to achieve solubilization of membrane-anchored HSPGS. Furthermore, the quantitative release of two distinct species of HSPG by mild trypsin treatment, together with the failure by either DTT or NaCl or both to alter the elution positions of the two species, implied that the trypsin-labile sites were most likely located on the extracellular side of the disulfide bonds which linked the two

proteoglycans. The latter two species are apparently associated with the lipophilic domain of the endothelial cell surface via hydrophobic segments of their small core proteins bearing large GAG chains exposed to the extracellular side. This structural finding is presumably of functional significance in view of the strategic positioning of HSPGS, both on plasma membranes and ECMs and may have important future implications in relation to the broad spectrum of biological functions (both physiological and pathological) which include control of cell growth, cellular adhesion, gene expression, cancer, blood-vessel disorders, diabetes, nephritis and inherited and acquired dysfunctions of the nervous system (211).

The ability of different substrata to promote cell growth has been amply documented. The results obtained in the case of studying the growth of BAE cells on extracellular matrices were very striking and in accordance with the reported effects of ECMs on cell growth and morphology. Although growth-promoting effects have been ascribed to components of the ECM, their precise individual roles remain obscure. The growth rate of BAE cells was higher on all types of ECM studied compared to conventional plastic substrata. Permissive effects of the ECM on BAE cells may be reconciled with the ratio of the different constituents which was found to be almost unique for a given type of ECM. In this respect, elastin was found to be the single most important component among other ECM components such as collagen and glycoprotein responsible for the higher growth rate observed in the case of BAE cells maintained on mainly elastin-ECMs (R_9 and R_{22}C1F). These ECMs also contained high quantities of collagen, a substrate known to enhance the growth of many cells in

culture. Trypsin and collagenase pretreatment of ECMs, however, did not have any appreciable inhibitory effect on ECMs' ability to support the growth of BAE cells; thus the remaining elastin component of the ECM removed the need of glycoprotein and probably collagen in the matrix to enhance or sustain growth. Also, when the elastin constituent of the ECM had been removed by pretreating the ECM with elastase, BAE cells still attached to the remaining substratum but fewer cells were found to be present after a few days in culture as compared to control (untreated) matrices. This clearly indicated that the extent of proliferation was markedly diminished in the absence of elastin. Therefore, it seems not improbable that elastin-proteoglycan interactions are important for cell growth (145); with HSPGS at the surface of BAE cells intricately involved in cell-substratum adhesion and regulation of cellular growth. Changes in the profile of cell surface proteoglycans have also been noted when BAE cells were cultured on various ECMs. These changes may be attributed to differences in the types of ECM produced by various cells and in the extreme case to the overall composition of the ECM, since the observed changes could not be attributed to any single component of the ECM and removal of any of the ECM constituents by enzyme pretreatment did not affect the profile of cell surface HSPGS. It should be mentioned, however, that this aspect has not extensively been investigated and that no clear-cut conclusions of how the ECM caused a change in the cell surface HSPG profile can be made. A partial answer to this question may be that the ECM dictates to the cell what phenotype to express. In the light of these observations and considerations further studies need to be carried out to gain further insight into cell-ECM interactions.

Pulse-chase studies revealed that ^{35}S-labelled HSPGS in the cell layer were rapidly degraded within 24-48 h and that the degradation products were shed into the medium. The observation that two different sized HSPGS appeared in the culture medium after the 48 h chase period was particularly striking. These two HSPG species eluted in the same positions as the two HSPG molecules which was obtained by reductive cleavage with DTT of the disulfide-bonded membrane-intercalated species of BAE cells. It might be expected that the two species of HSPG present in the medium after 48 h resulted from some form of proteolytic activity at the cell surface in a manner similar to trypsin cleavage of core proteins; only this time, the attack on the protein core was more specific and the HSPGS released from the cell surface were slightly reduced in size, i.e. native cell surface proteoglycan. This specific proteolytic cleavage of cell surface HSPGS analogous to protease (trypsin) release may occur in vivo, generating free extracellular proteoglycans. The significance of a protease-sensitive site is unclear but it may function during deposition of proteoglycans into the ECM.

The degradation of both intracellular and pericellular proteoglycans in the BAE culture system was shown to be inhibited by the lysomotropic agents, chloroquine and NH_4Cl, which indicated that such degradation occurred within lysosomes. The fact that the degradation of pericellular proteoglycans was inhibited by these agents would seem to indicate that cell layer proteoglycans are apparently internalized in order to be degraded. However, non-lysosomal mechanisms are also involved - presumably at the cell surface. Monensin had effects similar to those of the lysosomotropic inhibitors.

Thus the proteoglycans associated with the surface of BAE cells have been characterized. In addition, it was shown that BAE cells have in common with many other types of cell a dominant pool of HSPGS which may participate in interactions with ECMs and may account for the many functional properties described for the vascular endothelium.

REFERENCES

1. Diehl, T., Scott-Burden, T. and Gevers, W. (1983) Sulphate Turnover of Surface Proteoglycans in Cultured Rat Smooth Muscle Cells. Biochemistry International 6 (1): 29-41.

2. Woods, A., Höök, M., Kjellén, L., Smith, C.G. and Rees, D.A. (1984) Relationship of Heparan Sulfate Proteoglycans to the Cytoskeleton and Extracellular Matrix of Cultured Fibroblasts. J. Cell. Biol. 99: 1743-1753.

3. Kanwar, Y.S., Hascall, V.C., Jakubowski, M.L. and Gibbons, J.T. (1984) Effect of β-D-xyloside on the Glomerular Proteoglycans. I. Biochemical Studies. J. Cell. Biol. 99: 715-722.

4. Klein, D.J., Dehnel, P.J., Oegema, T.R. and Brown, D.M. (1984) Alterations in Proteoglycan Metabolism in the Nephrotic Syndrome Induced by the Aminonucleoside of Puromycin. Lab. Invest. 50 (5): 543-551.

5. Berenson, G.S., Radhakrishnamurthy, B., Srinivasan, S.R., Vijayagopal, P., Dalferes, E.R. and Sharma, C. (1984) Recent Advances in Molecular Pathology: Carbohydrate - Protein Macromolecules and Arterial Wall Integrity - A Role in Atherogenesis. Exp. Mol. Pathol. 41: 267-287.

6. Maresh, G.A., Chernoff, E.A. and Culp, L.A. (1984) Heparan Sulfate Proteoglycans of Human Neuroblastoma Cells: Affinity Fractionation on Columns of Platelet Factor-4^{+}. Arch. Biochem. Biophys. 233 (2): 428-437.

7. Muir, H. (1984) Proteoglycans as organizers of the intercellular matrix. Biochem. Soc. Trans. 11: 613-621.

8. Kleinman, H.K., Klebe, R.J. and Martin, G.R. (1981) Role of Collagenous Matrices in the Adhesion and Growth of Cells. A Review. J. Cell. Biol. 88: 473-485.

9. Robinson, J. and Gospodarowicz, D. (1984) Effect of ρ-Nitrophenol-β-D-xyloside on Proteoglycan Synthesis and Extracellular Matrix Formation by Bovine Corneal Endothelial Cell Cultures. J. Biol. Chem. 259 (6): 3818-3824.

10. Lark, M.W. and Culp, L.A. (1984) Turnover of Heparan Sulfate Proteoglycans from Substratum Adhesion Sites of Murine Fibroblasts. J. Biol. Chem. 259 (1): 212-217.

11. Sugrue, S. (1984) The Interaction of Extracellular Matrix with the Basal Cell Surface of Embryonic Corneal Epithelia. Prog. Clin. Biol. Res. 154: 77-87.

12. Prysor-Jones, R.A., Silverlight, J.J. and Jenkins, J.S. (1985) Differential effects of Extracellular Matrix on secretion of Prolactin and Growth Hormone by Rat Pituitary Tumour Cells in vitro. Acta Endocrin. 108 (2): 156-160.

13. Gospodarowicz, D. and Ill, C. (1980) Extracellular Matrix and Control of Proliferation of Vascular Endothelial Cells. J. Clin. Invest. 65: 1351-1364.

14. Savion, N., Vlodavsky, I. and Fuks, Z. (1984) Interaction of T lymphocytes and Macrophages with Cultured Vascular Endothelial Cells: Attachment, Invasion and Subsequent Degradation of the Subendothelial Extracellular Matrix. J. Cell Physiol. 118: 169-178.

15. Montesano, R., Mossaz, A., Ryser, J.-E., Orci, and Vassalli, P. (1984) Leukocyte Interleukins induce Cultured Endothelial Cells to produce a highly-organized, Glycosaminoglycan-rich Pericellular Matrix. J. Cell Biol. 99 (5): 1706-1715.

16. Gospodarowicz, D., Vlodavsky, I. and Savion, N. (1980) The Extracellular Matrix and the Control of Proliferation of Vascular Endothelial and Vascular Smooth Muscle Cells. J. Supramol. Struct. 13: 339-372.

17. Koda, J.E. and Bernfield, M. (1984) Heparan Sulfate Proteoglycans from Mouse Mammary Epithelial Cells. J. Biol. Chem. 259 (19): 11763-11770.

18. Polansky, J.R., Wood, I.S., Maglio, M.T., Alvarado, J.A. (1984) Trabecular Meshwork Cell Culture in Glaucoma Research: Evaluation of Biological Activity and Structural Properties of Human Trabecular Cells in vitro. Opthalmology 91 (6): 580-595.

19. Bar, R.S., Stueck, S., Dake, B., Spanheimer, R. (1984) Multiplication-stimulating Activity stimulates Proteoglycan Synthesis in Cultured Endothelial Cells. Endocrinol. 115 (6): 2487-2489.

20. Pasquali-Ronchetti, Bressan, G.M., Fornieri, C., Baccarani, M., Castellani, I. and Volpin, D. (1984) Elastin Fiber-associated Glycosaminoglycans in β-Aminoproprionitrile-induced Lathyrism. Exp. Mol. Pathol. 40: 235-245.

21. Jozzo, R.V. (1984) Biosynthesis of Heparan Sulfate Proteoglycan by Human Colon Carcinoma Cells and its Localization at the Cell Surface. J. Cell Biol. 99: 403-417.

22. Robinson, J., Viti, M. and Höok, M. (1984) Structure and Properties of an Under-sulfated Heparan Sulfate Proteoglycan Synthesized by a Rat Hepatoma Cell Line. J. Cell Biol. 98: 946-953.

23. Schmidt, A., Von Teutul, A. and Buddecke, E. (1984) Characterization of Proteoglycans synthesized by Cultured Arterial Smooth Muscle Cells of the Rat. Hoppe-Seyler's Z. Physiol. Chem. 365: 445-456.

24. Kanwar, Y.S., Veis, A., Kimura, J.H. and Jakubowski, M.L. (1984) Characterization of Heparan Sulfate-Proteoglycan of Glomerular Basement Membranes. Proc. Natl. Acad. Sci. 81: 762-766.

25. Miller, R.R. and Waechter, C.J. (1984) Structural Features and some Binding Properties of Proteoheparan Sulfate Enzymatically labeled by Calf Brain Microsomes. Arch. Biochem. Biophys. 228 (1): 247-257.

26. Gordon, P.B., Levitt, M.A., Jenkins, C.S.P. and Hatcher, V.B. (1984) The Effect of the Extracellular Matrix on the Detachment of Human Endothelial Cells. J. Cell. Physiol. 121 (3): 467-475.

27. Diglio, C.A., Wolfe, D.E. and Meyers, P. (1983) Transformation of Rat Cerebral Endothelial Cells by Rous Sarcoma Virus. J. Cell Biol. 97: 15-21.

28. Madri, J.A. and Williams, S.K. (1983) Capillary Endothelial Cell Culture: Phenotypic Modulation by Matrix Components. J. Cell Biol. 97: 153-165.

29. Grover, A., Andrews, G. and Adamson, D.E. (1983) Role of Laminin in Epithelium Formation by F9 Aggregates. J. Cell Biol. 97: 137-144.

30. Heathcote, J.G. and Orkin, R.W. (1984) Biosynthesis of Sulfated Macromolecules by Rabbit Lens Epithelium. I. Identification of the Major Macromolecules Synthesized by Lens Epithelial Cells in vitro. J. Cell Biol. 99: 852-860.

31. Heathcote, J.G., Bruns, R.R. and Orkin, R.W. (1984) Biosynthesis of Sulfated Macromolecules by Rabbit Lens Epithelium. II. Relationship to Basement Membrane Formation. J. Cell Biol. 99: 861-869.

32. Van Kuppevelt, T.H.M.S.M., Cremers, F.P.M., Lomen, J.G.W. and Kyper, C.M.A. (1984) Staining of Proteoglycans in Mouse Lung Alveoli. II. Characterization of the Cuprolinic Blue-positive, Anionic Sites. Histochem. J. 16: 671-686.

33. Chen, K. and Wight, T.N. (1984) Proteoglycans in Arterial Smooth Muscle Cell Cultures: An Ultrastructural Histochemical Analysis. J. Histochem. Cytochem. 32 (4): 347-357.

34. Clowes, A.W., Clowes, M.M., Gown, A.M. and Wight, T.H. (1984) Localization of Proteoheparan Sulfate in Rat Aorta. Histochemistry 80: 379-384.

35. Dietrich, C.P. (1984) A Model for Cell-Cell Recognition and Control of Cell Growth Mediated by Sulfated Glycosaminoglycans. Brazilian J. Med. Biol. Res. 17 (1): 5-15.

36. Robinsin, J. and Gospodarowicz, D. (1983) Glycosaminoglycans Synthesized by Cultured Bovine Corneal Endothelial Cells. J. Cell Physiol. 117: 366-376.

37. Rohrbach, D.H., Hassel, J.R., Kleinman, H.K. and Martin, G.R. (1982) Alterations in the Basement Membrane (Heparan Sulfate) Proteoglycan in Diabetic Mice. Diabetes 31 (2): 185-188.

38. Castellot, J.J., Favreau, L.V., Karnovsky, M.J. and Rosenberg, R.D. (1982) Inhibition of Vascular Smooth Muscle Cell Growth by Endothelial Cell-derived Heparin. J. Biol. Chem. 257 (19): 12256-11260.

39. Heifetz, A., Watson, C., Johnson, A.R. and Roberts, M.K. (1982) Sulfated Glycoproteins Secreted by Human Vascular Endothelial Cells. J. Biol. Chem. 257 (22): 13581-13586.

40. Kajiwara, T. and Tanzer, M.L. (1982) Monensin-induced Accumulation of β-D-xyloside-initiated Glycosaminoglycans. FEBS Letters 149 (1): 17-21.

41. Nishimoto, S.K., Kajiwara, T. and Tanzer, M.L. (1982) Proteoglycan Core Protein is Accumulated in Cultured Chondrocytes in the Presence of the Ionophore Monensin. J. Biol. Chem. 257 (18): 10558-10561.

42. Buonassisi, V. and Colburn, P. (1982) Biological Significance of Heparan Sulfate Proteoglycans. Ann. N.Y. Acad. Sci. 401: 76-84.

43. Franzén, A., Björnsson, S. and Heinegard, D. (1982) Zonal Rate Centrifugation of Proteoglycans in Sucrose Gradients. Anal. Biochem. 120: 38-45.

44. Shishiba, Y. and Yanagishita, M. (1983) Presence of Heparan Sulfate Proteoglycan in Thyroid Tissue. Endocrinol. Japon. 30 (5): 637-641.

45. Oohira, A., Wight, T.N., McPherson, J. and Bornstein, P. (1982) Biochemical and Ultrastructural Studies of Proteoheparan Sulfates synthesized by PYS-2, a Basement Membrane-producing Cell Line. J. Cell Biol. 92: 357-367.

46. Edward, M. and Oliver, R.F. (1983) Changes in the Synthesis, Distribution and Sulphation of Glycosaminoglycans of Cultured Human Skin Fibroblasts upon Ascorbate Feeding. J. Cell Sci. 64: 245-254.

47. Nilsson, J., Ksiazek, T., Thyberg, J. and Wasteson, A. (1983) Cell Surface Components and Growth Regulation in Cultivated Arterial Smooth Muscle Cells. J. Cell Sci. 64: 107-121.

48. Fransson, L., Carlstedt, I., Cöster, L. and Malmström, A. (1983) Proteoheparan Sulfate from Human Skin Fibroblasts. J. Biol. Chem. 258 (23): 14342-14345.

49. Scott-Burden, T., Murray, E., Diehl, T. and Gevers, W. (1983) Glycosaminoglycan Synthesis by Smooth Muscle Cells Cultured in the Absence and Presence of Ascorbic Acid. Hoppe-Seyler's Z. Physiol. Chem. 364: 61-70.

50. Oohira, A., Wight, T.N. and Bornstein, P. (1983) Sulfated Proteoglycans Synthesized by Vascular Endothelial Cells in Culture. J. Biol. Chem. 258: 2014-2021.

51. Rapraeger, A.C. and Bernfield, M. (1983) Heparan Sulfate Proteoglycans from Mouse Mammary Epithelial Cells. J. Biol. Chem. 258 (6): 3632-3636.

52. Folkman, J. (1983) Angiogenesis: Initiation and Modulation. Symp. Fundam. Cancer Res. 36: 201-208.

53. Robinson, H.C. and Lindahl, U. (1981) Effect of Cycloheximide, β-D-xylosides and β-D-galactosides on Heparin Biosynthesis in Mouse Mastocytoma. Biochem. J. 194: 575-586.

54. Mitchell, D. and Hardingham, T. (1981) The Effects of Cycloheximide on the Biosynthesis and Secretion of Proteoglycans by Chondrocytes in Culture. Biochem. J. 196: 521-529.

55. Oakes, B.W., Batty, A.C., Handley, C.J. and Sandberg, L.B. (1982) The Synthesis of Elastin, Collagen and Glycosaminoglycans by High Density Primary Cultures of Neonatal Rat Aortic Smooth Muscle. An Ultrastructural and Biochemical Study. Eur. J. Cell Biol. 27: 34-46.

56. Murad, S., Grove, D., Lindberg, K.A., Reynolds, G., Sivarajah, A. and Pinnell, S.R. (1981) Regulation of Collagen Synthesis by Ascorbic Acid. Proc. Natl. Acad. Sci. 78 (5): 2879-2882.

57. Kjellén, L., Pettersson, I. and Höök, M. (1981) Cell-surface Heparan Sulfate: An Intercalated Membrane Proteoglycan. Proc. Natl. Acad. Sci. 78 (9): 5371-5375.

58. Johansson, S. and Höök, M. (1980) Heparin Enhances the Rate of Binding of Fibronectin to Collagen. Biochem. J. 187: 521-524.

59. Kjellén, L., Oldberg, A. and Höök, M. (1980) Cell-surface Heparan Sulfate. J. Biol. Chem. 255 (21): 10407-10413.

60. Tajiri, K., Uchida, N. and Tanzer, M.L. (1980) Undersulfated Proteoglycans are Secreted by Cultured Chondrocytes in the Presnece of the Ionophore Monensin. J. Biol. Chem. 255 (13): 6036-6039.

61. Robinson, H.C., Horner, A.A., Höok, M., Ögren, S. Lindahl, U. (1978) A Proteoglycan Form of Heparin and its Degradation to Single-Chain Molecules. J. Biol. Chem. 253 (19): 6687-6693.

62. Casu, B. (1979) Structure and Biological Activity of Heparin and other Glycosaminoglycans. Pharmacol. Res. Commun. 11 (1): 1-17.

63. Wasteson, A. (1971) A Method for the Determination of the Molecular Weight and Molecular Weight Distribution of Chondroitin Sulphate. J. Chromatogr. 59: 87-97.

64. Salisbury, B.H.J. and Wagner, W.D. (1981) Isolation and Preliminary Characterization of Proteoglycans Dissociatively Extracted from Human Aorta. J. Biol. Chem. 256 (15): 8050-8056.

65. Kresse, H., Von Figura, K., Buddecke, E. and Fromme, H.G. (1975) Metabolism of Sulfated Glycosaminoglycans in Cultivated Bovine Arterial Cells. I. Characterization of Different Pools of Sulfated Glycosaminoglycans. Hoppe-Seyler's Z. Physiol. Chem. 356: 929-941.

66. Kresse, H., Tekolf, W., Von Figura, K. and Buddecke, E. (1975) Metabolism of Sulfated Glycosaminoglycans in Cultivated Bovine Arterial Cells. II. Quantitative Studies on the Uptake of $^{35}SO_4$-labeled Proteoglycans. Hoppe-Seyler's Z. Physiol. Chem. 356: 943-952.

67. Gamse, G., Fromme, H.G. and Kresse, H. (1978) Metabolism of Sulfated Glycosaminoglycans in Cultured Endothelial Cells and Smooth Muscle Cells from Bovine Aorta. Biochim. Biophys. Acta 544; 514-528.

68. Prinz, R., Schwermann, J., Buddecke, E. and Von Figura, K. (1978) Endocytosis of Sulfated Proteoglycans by Cultured Skin Fibroblasts. Biochem. J. 176: 671-676.

69. Jones, P.A., Scott-Burden, T. and Gevers, W. (1979) Glycoprotein, Elastin and Collagen secretion by Rat Smooth Muscle Cells. Proc. Natl. Acad. Sci. 76 (1): 353-357.

70. Truppe, W. and Kresse, H. (1978) Uptake of Proteoglycans and Sulfated Glycosaminoglycans by Cultured Skin Fibroblasts. Eur. J. Biochem. 85: 351-356.

71. Ross, R. and Glomset, J.A. (1976) The Pathogenesis of Atherosclerosis. N. Eng. J. Med. 295 (7): 369-376.

72. Buonassisi, V. (1973) Sulfated Mucopolysaccharide Synthesis and Secretion in Endothelial Cell Cultures. Exp. Cell Res. 76: 363-368.

73. Lowe-Krentz, L.J. and Keller, J.M. (1984) Disulfide-bonded Aggregates of Heparan Sulfate Proteoglycans. Biochemistry 23: 2621-2627.

74. Winterbourne, D.J., Schor, A.M. and Gallagher, J.T. (1983) Synthesis of Glycosaminoglycans by Cloned Bovine Endothelial Cells cultured on Collagen Gels. Eur. J. Biochem. 135: 271-277.

75. Smalley, J.W., Shuttleworth, C.A. and Grant, M.E. (1984) Synthesis and Secretion of Sulphated Glycosaminoglycans by Bovine Periodontal Ligament Fibroblast Cultures. Archs. Oral Biol. 29 (2): 107-116.

76. Turley, E.A. (1984) Proteoglycans and Cell Adhesion. Their Putative Role during Tumorigenesis. Cancer Metastasis Reviews 3 (4): 325-339.

77. Fujiwara, S., Wiedemann, H., Timpl, R., Lustig, A. and Engel, J. (1984) Structure and Interactions of Heparan Sulfate Proteoglycans from a Mouse Tumour Basement Membrane. Eur. J. Biochem. 143: 145-157.

78. Masuda, H. (1984) The Preparation of Heparan Sulfate from the Mitral Valve of the Human Heart. Int. J. Biochem. 16 (1): 99-103.

79. Nakajima, M., Irimura, T., Di Ferrante, N. and Nicolson, G. (1984) Metastatic Melanoma Cell Heparanase. Characterization of Heparan Sulphate degradation Fragments produced by B16 Melanoma Endoglucuronidase. J. Biol. Chem. 259 (4): 2283-2290.

80. Purvis, J.A., Embery, G. and Oliver, W.M. (1984) Molecular size distribution of proteoglycans in human inflamed gingival tissue. Archs. Oral Biol. 29 (7): 513-519.

81. Lark, M.W. and Culp, L.A. (1984) Multiple classes of heparan sulfate proteoglycans from fibroblast substratum adhesion sites. J. Biol. Chem. 259 (11): 6773-6782.

82. Fenger, M., Wewer, U. and Albrechtsen, R. (1984) Basement membrane heparan sulfate proteoglycan from the L2 rat yolk sac carcinoma. FEBS LETTERS 173 (1): 75-79.

83. Höök, M., Kjellén, L. and Johansson, S. (1984) Cell-surface glycosaminoglycans. Ann. Rev. Biochem. 53: 847-869.

84. Tyree, B., Horigan, E.A., Klippenstein, D.L. and Hassell, J. (1984) Heterogeneity of heparan sulfate proteoglycans synthesized by PYS-2 cells. Arch. Biochem. Biophys. 231 (2): 328-335.

85. Srinivasan, S.R., Vijayagopal, P., Dalferes, E.R., Abbate, B., Radhakrishnamurthy, B. and Berenson, G.S. (1984) Dynamics of lipoprotein-glycosaminoglycan interactions in the atherosclerotic rabbit aorta in vivo. Biochim. Biophys. Acta 793: 157-168.

86. Fleischmajer, R. and Perlish, J.S. (1984) The pathophysiology of the fibrosis in scleroderma skin. Prog. Clin. Biol. Res. 154: 381-404.

87. Clemmensen, I. (1984) Significance of plasma fibronectin. Haematologia 17 (1): 101-106.

88. Beck, M., Lücke, R. and Kresse, H. (1984) Dyggve-Melchior-Clausen syndrome: Normal degradation of proteodermatan sulfate, proteokeratan sulfate and heparan sulfate. Clinica Chimica Acta 141: 7-15.

89. Yamada, K., Shimizu, S., Brown, K.S. and Kimata, K. (1984) The histochemistry of complex carbohydrates in certain organs of homozygous brachymorphic (bm/bm) mice. Histochemical Journal 16: 587-599.

90. Barnes, M.J. (1985) Collagens in Atherosclerosis. Collagen Rel. Res. 5: 65-97.

91. Hay, E.D. (1983) Cell and extracellular matrix: Their organization and mutual dependence. In Modern Cell Biology Vol. 2 (Ed. Birgit H. Stair) pp. 509-548.

92. Bissell, M.J., Hall, H.G. and Parry, G. (1982) How does the extracellular matrix direct gene expression? J. Theor. Biol. 99: 31-68.

93. Yanagishita, M. and Hascall, V.C. (1984) Proteoglycans synthesized by rat ovarian granulosa cells in culture: Isolation, fractionation and characterization of proteoglycans associated with the cell layer. J. Biol. Chem. 259 (16): 10260-10269.

94. Yanagishita, M. and Hascall, V.C. (1984) Metabolism of proteoglycans in rat ovarian granulosa cell culture: Multiple intracellular degradative pathways and the effect of chloroquine. J. Biol. Chem. 259 (16): 10270-10283.

95. Rapraeger, A. and Bernfield, M. (1985) Cell surface proteoglycan of mammary epithelial cells. J. Biol. Chem. 260 (7): 4103-4109.

96. Höök, M., Couchman, J., Woods, A., Robinson, J. and Christner, J.E. (1984) Proteoglycans in basement membranes. Ciba Found. Symp. 108: 44-59.

97. De Clerck, Y.A and Jones, P. (1980) The effect of ascorbic acid on the nature and production of collagen and elastin by rat smooth muscle cells. Biochem. J. 186: 217-225.

98. De Clerck, Y.A. and Jones, P. (1980) Effect of ascorbic acid on the resistance of the extracellular matrix to hydrolysis by tumor cells. Cancer Res. 40: 3228-3231.

99. Schor, A.M., Schor, S.L. and Allen, T.D. (1984) The synthesis of subendothelial matrix by bovine aortic endothelial cells in culture. Tissue and Cell 16 (5): 677-691.

100. Völker, W., Schmidt, A., Robenek, H. and Buddecke, E. (1984) Binding and degradation of proteoglycans by cultured arterial smooth muscle cells. I. Endocytosis and intracellular translocation of proteoglycan-gold conjugates. Eur. J. Cell Biol. 34: 110-117.

101. Völker, W., Schmidt, A., Buddecke, E., Themann, H. and Robenek, H. (1985) Binding and degradation of proteoglycans by cultured arterial smooth muscle cells. II. Binding sites of proteoglycans on the cell surface. Eur. J. Cell Biol. 36: 58-65.

102. Arumugham, R. and Anderson, J.C. (1984) Distribution in caesium chloride gradients of proteoglycans secreted by bovine aorta smooth muscle cells and study of their aggregation with hyaluronate and cartilage proteoglycan. Biochim. Biophys. Acta 797: 128-135.

103. Parry, G., Lee, E.Y., Farson, D., Koval, M. and Bissell, M.J. (1985) Collagenous substrata regulate the nature and distribution of glycosaminoglycans produced by differentiated cultures of mouse mammary epithelial cells. Exp. Cell Res. 156: 487-499.

104. Turksen, K., Aubin, J.E., Sodek, J. and Kalnins, V.I. (1984) Changes in the distribution of laminin, fibronectin, type IV collagen and heparan sulfate proteoglycan during colony formation by chick retinal pigment epithelial cells in vitro. Collagen Rel. Res. 4(6): 413-426.

105. Bar-Ner, N., Kramer, M.D., Schirrmacher, V., Ishai-Michaeli, R., Fuks, Z. and Vlodavsky, I. (1985) Sequential degradation of heparan sulfate in the subendothelial extracellular matrix by highly metastatic lymphoma cells. Int. J. Cancer 35: 483-491.

106. Kramer, R.H., Fuh, G.M., Bensch, K.G. and Karasek, M.A. (1985) Synthesis of extracellular matrix glycoproteins by cultured microvascular endothelial cells isolated from the dermis of neonatal and adult skin. J. Cell. Physiol. 123 (1): 1-9.

107. Timpl, R. (1985) Molecular aspects of basement membrane structure. Prog. Clin. Biol. Res. 171: 63-74.

108. Hassel, J.R., Leyshon, W.C., Ledbetter, S.R., Tyree, B., Suziki, S., Kato, M., Kimata, K. and Kleinman, H.K. (1985) Isolation of two forms of basement membrane proteoglycans. J. Biol. Chem. 260 (13): 8098-8105.

109. Heickendorff, L. and Ledet, T. (1984) Glycosaminoglycans of arterial basement membrane-like material from cultured rabbit aortic myomedial cells. Biochim. Biophys. Acta 798: 276-282.

110. Fridman, R., Fuks, Z., Ovadia, H. and Vlodavsky, I. (1985) Differential structural requirements for the induction of cell attachment, proliferation and differentiation by extracellular matrix. Exp. Cell Res. 157: 181-194.

111. Stow, J.L., Kjéllen, L., Unger, E., Höok, M. and Farquhar, M.G. (1985) Heparan sulfate proteoglycans are connected on the sinusoidal plasmalemmal domain and in intracellular organelles of hepatocytes. J. Cell Biol. 100 (3): 975-980.

112. Kjéllen, L., Pertoft, H., Oldberg, A., Höök, M. (1985) Oligosaccharides generated by an endoglucuronidase are intermediates in the intracellular degradation of heparan sulfate proteoglycans. J. Biol. Chem. 260 (14): 8416-8422.

113. Oda, K., Misumi, Y. and Ikehara, Y. (1983) Disparate effects of monensin and colchicine on intracellular processing of secretory proteins in cultured rat hepatocytes. Eur. J. Biochem. 135: 209-216.

114. Nishimoto, S.K., Kajiwara, T., Ledger, P.W. and Tanzer, M. (1982) Effects of the ionophore monensin on type II collagen and proteoglycan synthesis and secretion by cultured chondrocytes. J. Biol. Chem. 257 (19): 11712-11716.

115. Ueda, M., Robinson, F.W., Smith, M.M. and Kono, T. (1985) Effects of monensin on insulin processing in adipocytes. J. Biol. Chem. 260 (7): 3941-3946.

116. Bar, R.S., Dake, B.L. and Spanheimer, R.G. (1985) Sulfate glycosaminoglycans in cultured endothelial cells from capillaries and large vessels of human and bovine origin. Atherosclerosis 56: 11-26.

117. Vogel, K.G. and Peterson, D.W. (1981) Extracellular, surface and intracellular proteoglycans produced by human embryo lung fibroblasts in culture (IMR-90). J. Biol. Chem. 256 (24): 13235-13242.

118. Schor, S.L., Schor, A.M., Allen, T.D. and Winn, B. (1985) The interaction of melanoma cells with fibroblasts and endothelial cells in three-dimensional macromolecular matrices: A model for tumour cell invasion. Int. J. Cancer 36: 93-102.

119. Knox, P. (1984) The cell surface in health and disease. Molec. Aspects Med. 7: 177-311.

120. Shimada, K. and Ozawa, T. (1985) Release of heparan sulfate proteoglycans from cultured aortic endothelial cells by thrombin. Thrombosis Res. 39: 387-397.

121. Woods, A., Couchman, J.R. and Höök, M. (1985) Heparan sulfate proteoglycans of rat embryo fibroblasts. A hydrophobic form may link cytoskeleton and matrix components. J. Biol. Chem. 260 (19): 10872-10879.

122. Hsieh, P. and Baum, J. (1985) Effects of fibroblastic and endothelial extracellular matrices on corneal endothelial cells.

123. Yanagishita, M. (1985) Inhibition of intracellular degradation of proteoglycans by leupeptin in rat ovarian granulosa cells. J. Biol. Chem. 260 (20): 11075-11082.

124. Rapraeger, A., Jalkamen, N., Endi, E., Koda, J. and Bernfield, M. (1985) The cell surface proteoglycan from mouse mammary epithelial cells bears chondroitin sulfate and heparin sulfate glycosaminoglycans. J. Biol. Chem. 260 (20): 11046-11052.

125. David, G. and Van den Berghe (1985) Heparin sulfate-chondroitin sulfate hybrid proteoglycan of the cell surface and basement membrane of mouse mammary epithelial cells. J. Biol. Chem. 260 (20): 11067-11074.

126. Carlstedt, I., Cöster, L., Malmström, A. and Fransson, L. (1983) Proteoheparan sulfate from human skin fibroblasts. Isolation and structural characterization. J. Biol. Chem. 258 (19): 11629-11635.

127. Jaffe, E.A. (1984) In: Biology of Endothelial Cells (Ed. E.A. Jaffe). Martinus Nijhoff Publishers. Boston. The Hague. Dordrecht. Lancaster.

128. Piez, K.A. and Reddi, A.H. (eds.) (1984) Extracellular Matrix Biochemistry. Elsevier. New York. Amsterdam. Oxford.

129. Schwartz, S.M. (1978) Selection and characterization of bovine aortic endothelial cells. In vitro 14: 966-980.

130. Gospodarowicz, D., Moran, J. and Birdwell, C. (1976) Clonal growth of bovine vascular endothelial cells: Fibroblast growth factor as a survival agent. Proc. Natl. Acad. Sci. USA 73: 4126-4124.

131. Cotta-Pereira, G., Sage, H., Bornstein, P., Ross, R. and Schwartz, S.M. (1980) Studies of morphologically atypical ('sprouting') cultures of bovine aortic endothelial cells. Growth characteristics and connective tissue protein synthesis. J. Cell. Physiol. 102: 183-191.

132. Vogel, A., Ross, R. and Raines, E. (1980) Role of serum components in density-dependent inhibition of growth of cells in culture. PDGF is the major serum determinant of saturation density. J. Cell Biol. 85: 377-385.

133. Wall, R.T., Harker, L.A., Quadracci, L.J. and Striker, G.E. (1978) Factors influencing endothelial cell proliferation in vitro. J. Cell Physiol. 96: 203-214.

134. John F. Kennedy (1979) Proteoglycans - Biological and Chemical Aspects in Human Life. Elsevier. Amsterdam. Oxford. New York.

135. Comper, W.D. and Laurent, T.C. (1978) Physiological function of connective tissue polysaccharides. Physiol. Rev. 58 (1): 255-315.

136. Rodén, L. (1980) Structure and metabolism of connective tissue proteoglycans. In: Lenarz, W.J., ed. The Biochemistry of Glycoproteins and Proteoglycans. New York. Plenum, pp. 267-371.

137. Sandy, J.D., Brown, H.L.G., Lowther, D.A. (1978) Degradation of proteoglycans in articular cartilage. Biochim. Biophys. Acta 543: 536-544.

138. Prizn, R., Klein, U., Sudhakaran, P.R., Sinn, W., Ullrich, R. and Von Figura, K. (1980) Metabolism of sulfated glycosaminoglycans in rat hepatocytes. Synthesis of heparan sulfate and distribution into cellular and extracellular pools. Biochim. Biophys. Acta 630: 402-413.

139. Palade, G. (1975) Intracellular aspects of protein synthesis. Science 189: 347-358.

140. Kimura, J.H., Hardingham, T.E. and Hascall, V.C. (1980) Assembly of newly synthesized proteoglycan and link protein into aggregates in cultures of chondrosarcoma chondrocytes. J. Biol. Chem. 255 (15): 7134-7143.

141. Hascall, V.C. and Sajdera, S.W. (1969) Protein-polysaccharide complex from bovine nasal cartilage. The function of glycoprotein in the formation of aggregates. J. Biol. Chem. 244: 2384-2396.

142. Hardingham, T.E. and Muir, H. (1974) Hyaluronic acid in cartilage and proteoglycan aggregation. Biochem. J. 139: 565-581.

143. Ehrich, K.C., Radhakrishnamurthy, B. and Berenson, G.S. (1975) Isolation of CS-DS-PG from bovine aorta. Arch. Biochem. Biophys. Acta 171: 361-369.

144. Radhakrishnamurthy, B., Ruiz, H.A., Berenson, G.S. (1977) Isolation and characterization of proteoglycans from bovine aorta. J. Biol. Chem. 252: 4831-4841.

145. Contri, M.B., Fornieri, C. and Ronchetti, I.P. (1985) Elastin-proteoglycans association revealed by cytochemical method. Connective Tissue Res. 13 (3): 237-249.

146. Ruoslahti, E., Hayman, E.G. and Pierschbacher, M.D. (1985) Extracellular matrices and cell adhesion. Arteriosclerosis 5: 581-594.

147. Hascall, V.C., Hascall, G.K. (1981) In: Cell Biology of Extracellular Matrix (ed. E.D. Hay) pp. 36-64. New York: Plenum.

148. Scott, J.E., Orford, C.R. (1981) Dermatan sulphate-rich proteoglycan associates with rat tail tendon collagen at the d band in the gap region. Biochem. J. 197: 213-216.

149. Laurie, G.W., Leblond, C.P., Martin, G.R. (1983) Light microscopic immunolocalization of type IV collagen, laminin, heparan sulfate proteoglycan and fibronectin in the basement membranes of a variety of rat organs. Am. J. Anat. 167: 71-82.

150. Fransson, L.-Å., Havsmark, B., Sheehan, J.K. (1981) Self-association of heparan sulfate. Demonstration of binding by affinity chromatography of free chains on heparan sulfate-substituted agarose gels. J. Biol. Chem. 256: 13039-13043.

151. Fransson, L.-Å., Cöster, L., Malmström, A., Sheehan, J.K. (1982) Self-association of scleral proteodermatan sulfate. Evidence for interaction via dermatan sulfate side chains. J. Biol. Chem. 257: 6333-6338.

152. Vijayagopal, P., Radhakrishnamurthy, B., Srinivasan, S.R. and Berenson, G.S. (1980) Studies of biologic properties of proteoglycans from bovine aorta. Lab. Invest. 421: 191-196.

153. Vijayagopal, P., Radhakrishnamurthy, B., Srinivasan, S.R. and Berenson, G.S. (1983) Hemostatic properties and serum lipoprotein binding of a heparan sulfate proteoglycan from bovine aorta. Biochim. Biophys. Acta 758: 70-83.

154. Hatton, M.W., Berry, L.R. and Regaeczi, E. (1978) Inhibition of thrombin by antithrombin III in the presence of certain glycosaminoglycans found in mammalian aorta. Thromb. Res. 13: 655-670.

155. Olivecrona, T., Bengtsson, G., Marklung, S.-E., Lindahl, U. and Höök, M. (1977) Heparin-lipoprotein interactions. Fed. Proc. 36: 60-65.

156. Hammersen, F. and Hammersen, E. (1985) Some structural and functional aspects of endothelial cells. Basic Res. Cardiol. 80 (5): 491-501.

157. Gordon, P.B., Conn, G. and Hatcher, V.B. (1985) Glycosaminoglycan production in cultures of early and late passage human endothelial cells: The influence of an anionic endothelial cell growth factor and the extracellular matrix. J. Cell Physiol. 125 (3): 596-607.

158. Kramer, R.H. (1985) Extracellular matrix interactions with the apical surface of vascular endothelial cells. J. Cell Sci. 76: 1-16.

159. Jaffe, E.A., Nachman, R.L., Becker, C.G., Minick, C.R. (1973) Culture of human endothelial cells derived from unbilical veins. J. Clin. Invest. 52: 2745-2756.

160. Macaroak, E.J., Howard, B.V., Kefalides, N.A. (1977) Properties of calf endothelial cells in culture. Lab. Invest. 36: 62-67.

161. Sanan, D.A., Strümpfer, A.E.M., Van der Westhuyzen, D.R., Coetzee, G.A. (1985) Native and acetylated low density lipoprotein metabolism in proliferating and quiescent bovine endothelial cells in culture. Eur. J. Cell Biol. 36: 81-90.

162. Wagner, W.D. (1985) Proteoglycan structure and function as related to atherosclerosis. Ann. N.Y. Acad. Sci. 454: 52-

163. Chicquet, M., Puri, E.C. and Turner, D.C. (1979) Fibronectin mediates attachment of chicken myoblasts to a gelatin-coated substratum. J. Biol. Chem. 254: 5475-5482.

164. Grinnell, F. (1978) Cellular adhesiveness and extracellular substrata. Int. Rev. Cytol. 53: 65-144.

165. Grinnell, F., Feld, M. and Minter, D. (1980) Fibroblast adhesion to fibrinogen and fibrin substrates: Requirement for cold-insoluble globulin (plasma fibronectin). Cell 19: 517-525.

166. Hewitt, A.T., Kleinman, H.K., Pennypacker, J.P. and Martin, G.R. (1980) Identification of an adhesion factor for chondrocytes. Proc. Natl. Acad. Sci. USA 77: 385-388.

167. Höök, M., Rubin, K., Oldberg, A. and Vaheri, A. (1977) Cold-insoluble globulin mediates the adhesion of rat liver cells to plastic petri dishes. Biochem. Biophys. Res. Commun. 79: 726-731.

168. Pearlstein, E. (1976) Plasma membrane glycoprotein which mediates adhesion of fibroblasts to collagen. Nature (Lond.) 262: 497-500.

169. Baum, B., McDonald, J.A. and Crystal, R.G. (1977) Metabolic fate of the major cell surface protein of normal human fibroblasts. Biochem. Biophys. Res. Commun. 79: 8-15.

170. Ruoslahti, E. and Vaheri, A. (1974) Novel human serum protein from fibroblast plasma membranes. Nature (Lond.) 248: 789-791.

171. Yamada, K.M. and Weston, J.A. (1974) Isolation of a major cell surface glycoprotein from fibroblasts. Proc. Natl. Acad. Sci. USA 71: 3492-3496.

172. Jaffe, E.A. and Mosher, D.F. (1978) Synthesis of fibronectin by cultured human endothelial cells. J. Exp. Med. 147: 1779-1791.

173. Macarak, E.J., Kirby, E., Kirk, T. and Kefalides, N.A. (1978) Synthesis of cold-insoluble globulin by cultured calf endothelial cells. Proc. Natl. Acad. Sci. USA 75: 2621-2625.

174. Sattler, C.A., Michalopoulos, G., Sattler, G.A. and Pitot, H.C. (1978) Ultrastructure of adult rat hepatocytes cultured on floating collagen membranes. Cancer Res. 38: 1539-1549.

175. Gospodarowicz, D., Greenburg, G. and Birdwell, C.R. (1978) Determination of cellular shape by the extracellular matrix and its correlation with the control of cellular growth. Cancer Res. 38: 4155-4171.

176. Murray, J.C., Stingl, G., Kleinman, H.K., Martin, G.R. and Katz, S.I. (1978) Epidermal cells adhere preferentially to type IV (basement membrane) collagen. J. Cell Biol. 80: 197-201.

177. Wicha, M., Liotta, L.A., Garbissa, S. and Kidwell, W.R. (1979) Basement membrane collagen requirements for attachment and growth of mammary epithelium. Exp. Cell Res. 124: 181-190.

178. Hauschka, S.D. and Konigsberg, T.R. (1966) The influence of collagen on the development of muscle colonies. Proc. Natl. Acad. Sci. USA 55: 119-126.

179. Kawakami, H. and Teryama, H. (1981) Liver plasma membranes and proteoglycans prepared therefrom inhibit the growth of hepatoma cells in vitro. Biochim. Biophys. Acta 646: 161-168.

180. Rolin, R., Albert, S.O., Gelb, N.A. and Black, P.H. (1975) Cell surface changes correlated with density-dependent growth inhibition. Glycosaminoglycan metabolism in 3T3, SV3T3 and Con A-selected revertant cells. Biochemistry 14: 347-357.

181. Bamhart, B.J., Cox, S.H. and Kraemer, P.M. (1979) Detachment variants of Chinese hamster cells. Hyaluronic acid as a modulator of cell detachment. Exp. Cell Res. 119: 327-332.

182. Culp, L.A., Murray, B.A. and Rollins, B.J. (1979) Fibronectin and proteoglycans as determinants of cell-substratum adhesion. J. Supramol. Struct. 11: 401-427.

183. Knox, P. and Wells, P. (1979) Cell adhesion and proteoglycans. I. The effect of exogenous proteoglycans on the attachment of chick embryo fibroblasts to tissue culture plastic and collagen. J. Cell Sci. 40: 67-78.

184. Jilek, F. and Hörman, H. (1979) Fibronectin. IV. Influence of heparin and hyaluronic acid on the binding of native collagen. Hoppe-Seyler's Z. Physiol. Chem. 360: 597-603.

185. Laterra, J., Ansbacher, R. and Culp, L.A. (1980) Glycosaminoglycans that bind cold-insoluble globulin in cell-substratum adhesion sites of murine fibroblasts. Proc. Natl. Acad. Sci. USA 77: 6662-6666.

186. Lindahl, U. and Höök, M. (1978) Glycosaminoglycans and their binding to biological macromolecules. Ann. Rev. Biochem. 47: 385-417.

187. Perkins, M.E., Ji, T.H. and Hynes, R.O. (1979) Cross-linking of fibronectin to sulfated proteoglycans at the cell surface. Cell 16: 941-952.

188. Ruoslahti, E. and Engvall, E. (1980) Complexing of fibronectin, glycosaminoglycans and collagen. Biochim. Biophys. Acta 631: 350-358.

189. Laterra, J., Ansbacher, R. and Culp, L.A. (1983) Cell surface heparan sulfate mediates some adhesive responses to glycosaminoglycan-binding matrices, including fibronectin. J. Cell Biol. 96: 112-123.

190. Coetzee, G.A., Van der Westhuyzen, D.R., Berger, G.M.B., Henderson, H.E. and Gevers, W. (1982) Low density lipoprotein metabolism in cultured fibroblasts from a new group of patients presenting clinically with homozygous familial hypercholesterolaemia. Arteriosclerosis 2 (4): 303-311.

191. Gey, G.O., Svotelis, M., Foard, M, and Bang, F.B. (1974) Long-term growth of chicken fibroblasts on a collagen substrate. Exp. Cell Res. 84: 63-71.

192. Gospodarowicz, D. and Ill, C.R. (1980) Do plasma and serum have different abilities to promote cell growth? Proc. Natl. Acad. Sci. USA 77: 2727-2730.

193. Karasek, M.A. and Charlton, M.E. (19) Growth of post-embryonic skin epithelial cells on collagen gels. J. Invest. Dermatol. 51: 247-252.

194. Rowe, D.W., Starman, B.J., Fujimoto, W.Y. and Williams, R.H. (1977) Differences in growth response to hydrocortisone and ascorbic acid by human diploid fibroblasts. In Vitro 13: 824-830.

195. Peterkofsky, B. (1972) The effect of ascorbic acid on collagen-polypeptide-synthesis and proline hydroxylation during the growth of cultured fibroblasts. Arch. Biochem. Biophys. 152: 318-328.

196. Höök, M., Robinson, J., Kjellen, L., Johansson, S. and Woods, A. (1982) In: Extracellular Matrix (Hawkes, S.P. and Wang, J.L., eds.) pp. 15-22. Academic Press.

197. Bretscher, M.S. (1985) Heparan sulfate proteoglycans and their polypeptide chains from BHK cells. The EMBO Journal 4 (8): 1941-1944.

198. Madri, J.A. and Pratt, B.M. (1986) Endothelial cell-matrix interactions: In vitro models of angiogenesis. J. Histochem. Cytochem. 34 (1): 85-91.

199. Laug, W.E., Weinblatt, M.E. and Jones, P.A. (1985) Endothelial cells degrade extracellular matrix proteins produced in vitro. Thrombosis and Haemostasis 54 (2): 498-502.

200. Hatton, M.W.C. and Moar, S.L. (1985) A role of pericellular proteoglycan in the binding of thrombin or antithrombin III by blood vessel endothelium? The effects of proteoglycan-degrading enzymes and glycosaminoglycan-binding proteins on ^{125}I-thrombin binding by the rabbit thoracic aorta in vitro. Thrombosis and Haemostasis 53 (2): 228-234.

201. Parthasarathy, N. and Spiro, R.G. (1981) Characterization of the glycosaminoglycan component of the renal glomerular basement membrane and its relationship to the peptide portion. J. Biol. Chem. 256 (1): 507-513.

202. Cöster, L., Malström, A., Carlstedt, I. and Fransson, L.-Å. (1983) The core protein of fibroblast proteoheparan sulphate consists of disulphide-bonded subunits. Biochem. J. 215: 417-419.

203. Oldberg, Å., Höök, M., Öbrink, B., Pertoft, H. and Rubin, K. (1977) Structure and metabolism of heparan sulfate. Biochem. J. 164: 75-81.

204. Oldberg, Å., Kjellén, L. and Höök, M. (1979) Cell surface heparan sulfate: Isolation and characterization of a proteoglycan from rat liver membranes. J. Biol. Chem. 254 (17): 8505-8510.

205. McKusik, V.A. and Neufeld, E.F. (1983) In: The Metabolic Basis of Inherited Diseases (Stanbury, J.B., Wyngaarden, J.B., Frederickson, D.S., Goldstein, J.L. and Brown, M.S., eds.) pp. 751-777. McGraw-Hill, New York.

206. Cheng, C.-F., Oosta, G.M., Bensadoun, A. and Rosenberg, D. (1981) Binding of lipoprotein lipase to endothelial cells in culture. J. Biol. Chem. 256 (24): 12893-12898.

207. Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russell, D.W. and Schneider, W.J. (1985) Receptor-mediated endocytosis. Ann. Rev. Cell Biol. 1: 1-35.

208. Williams, M.P., Streeter, H.B., Wusteman, F.S. and Cryer, A. (1983) Heparan sulphate and the binding of lipoprotein lipase to porcine thoracic aorta endotheliam. Biochim. Biophys. Acta 756: 83-91.

209. Berenson, G.S., Radhakrishnamurthy, B., Srinivasan, S.R., Vijayagopal, P. and Dalferes, E.R. (1985) Proteoglycans and potential mechanisms related to atherosclerosis. Ann. N.Y. Acad. Sci. 454: 69-76.

210. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with folin phenol reagent. J. Biol. Chem. 192: 265-275.

211. Gallagher, J.T., Lyon, M. and Steward, W.P. (1986) Structure and function of heparan sulfate proteoglycans. Biochem. J. 236 (2): 313-325.

212. David, G. and Bernfield, M.R. (1979) Collagen reduces glycosaminoglycan degradation by cultured mammary epithelial cells: Possible mechanism for basal lamina formation. Proc. Natl. Acad. Sci. USA 76 (2): 786-790.

213. Scott-Burden, T., Bogenmann, E. and Jones, P.A. (1986) Exptl. Cell Res. (in press).

214. Levin, E.G. and Loskutoff, D.J. (1980) Cell 22: 701-707.

215. Murray, E., Scott-Burden, T., Ferguson, P. and Gevers, W. (1983) Increased sulphation level and altered composition of glycosaminoglycans synthesized by cultured smooth muscle cells in the presence of β-D-xylosides. Biochim. Biophys. Acta 763: 299-308.

APPENDIX

Chemicals, Biochemicals, Materials and Culture Ware were obtained from the following suppliers:

BDH Chemicals, Poole, England	: Ethylenediamine tetraacetic acid, sodium dodecyl sulfate, dithriothreitol
Coulter Electronics Inc., Hialeah, Florida, USA	: Isoton II
Difco Laboratories, Detroit, Michigan, USA	: Tryptose phosphate broth, gelatin
Gibco Laboratories, New York, USA	: Eagle's minimum essential medium buffered with Earle's salts
Glaxo (Pty) Ltd, Wadeville, Transvaal, RSA	: Penicillin G, streptomycin sulfate
E. Merck, A.G. Darmstadt, Germany	: All laboratory chemicals used (analytical grade), L (+) ascorbic acid
Seravac, Division of Fine Chemicals Corporation (Pty) Ltd, Epping, Cape, RSA	: Bovine serum albumin fraction IV
Packard Instruments, Downers Grove, USA	: Scintillation mixture 299 Instagel
Pharmacia, Uppsala, Sweden	: Sepharose CL2B, Sepharose CL4B, Sepharose CL6B, Sephadex G-50, Sephadex G-25, Blue dextran 2000

Sigma Chemical Co., St Louis, MO, USA	: Collagenase (EC.3.4.24.3), elastase (EC.3.4.21.11), trypsin (EC.3.4.21.4), N-ethylmaleimide
State Vaccine Institute, Cape Town, RSA	: Foetal calf serum
Whatman Ltd., Kent, England	: 3 MM chromatography paper
Boehringer Mannheim, GmbH, W. Germany	: Caesium chloride
Beckman Instruments (Pty) Ltd, Cape Town, RSA	: Beckman Ready-Solv EP scintillation cocktail

Radioactive Isotopes

New England Nuclear, Boston, USA	: L-I$^{5-3}$HI-Proline, 20-40 Ci/nmole
The Radiochemical Centre, Amersham, Bucks, England	: Sulfur-^{35}, 25-40 Ci/mg
Millipore Filters, Millipore Corporation, Bedford, MA 01730 USA	: Millex-HA filters
Corning Ltd, Staffordshire, England	: Tissue culture plastic ware