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Collagens of Basement Membranes

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Recent biochemical and immunohistochemical studies have described several components of basement membranes including heparan sulfate proteoglycan, 2 high molecular weight glycoproteins (fibronectin and laminin), and 2 collagen types (IV and V). These collagens have several properties which distinguish them from other types that are located in the interstitium: (a) type IV forms an amorphous, felt-like matrix, and neither IV nor V is found in large, cross-banded fibrils, (b) both have an increased content of hydrophobic amino acids, (c) the precursor (pro) forms are larger than those of interstitial collagens, (d) type IV contains interruptions within the triple helix, and e) both IV and V are resistant to human skin collagenase but are substrates for selected neutral proteases derived from mast cells, macrophages, and granulocytes.

By immunofluorescence staining, type IV collagen has been localized to basement membranes at the dermal-epidermal junction, in capillaries, and beneath endothelial cells in larger vessels. Ultrastructurally it has been shown to be a specific component of the lamina densa. Type V collagen has been localized to the pericellular matrices of several cells types and may be specific for extramembranous structures which are closely associated with basal laminae.

Other collagenous proteins have been described which may be associated with the extracellular matrix. One of these is secreted by endothelial cells in culture and by peptide mapping represents a novel collagen type. It is secreted under ascorbate-free conditions and is highly sensitive to proteolytic degradation.

It has been proposed that a dynamic reciprocity exists between cells and their extracellular matrix which partially determines cell shape, biosynthesis, migration, and attachment. Examples of phenotypic modulation in several of these phenomena have been shown with endothelial cells grown on different substrates and isolated from different vascular environments.

Basement membranes have been identified as amorphous extracellular matrices which are interposed between both epithelial and endothelial cells and the underlying connective tissue stroma. Ultrastructurally, they appear most often as continuous sheets of electron-dense material that surround capillary endothelium and are disposed beneath respiratory and digestive epithelium. Specialized, highly distinct basement membranes (BM) include those of the kidney glomerulus, lens capsule, corneal endothelium (Descement's membrane), and the dermal-epidermal junction.

Figure 1 is an electron micrograph of the basement membrane as seen at the dermal-epidermal junction in human skin. Several component layers can be observed: (a) the plasma membrane of the epidermal keratinocyte, (b) the lamina lucida and associated anchoring filaments, (c) the basal lamina (also designated the basement membrane), consisting of a dense granular substance referred to as the lamina densa; and (d) the fibrous components of the subbasement membrane (in some tissues referred to as the reticular layer) in which are deposited anchoring fibrils, microfibrils, and collagen fibrils. These fibrillar structures will be discussed in a later section. For further details on the morphology, structure, and metabolism of basement membranes, the reader is referred to the excellent review by Kefalides, Alper, and Clark [1].

In the last few years, our understanding of the biochemical composition of basement membranes has improved considerably, due to the characterization of several integral components of this structure. Basement membranes contain a heparan sulfate-rich proteoglycan, of approximately $0.5–1 \times 10^6$ molecular weight, which is thought to regulate in part the permeability of this layer [2,3]. Fibronectin is a high molecular weight, disulfide-bonded glycoprotein with constitutent chains of approximately 250,000 daltons. It has specific domains within its structure which bind to collagen, fibrinogen, heparin, gangliosides, actin, and cell surfaces, and it mediates the attachment of certain cells to collagen-coated dishes *in vitro* (for a review, see reference 4). Fibronectin is probably not an ubiquitous component of basement membranes, although studies on this point are presently not conclusive. By indirect immunofluorescence, fibronectin has been localized in a broad zone around the dermal-epidermal junction and in the BM zone surrounding hair and sebaceous glands (Fig 2*A*, panels *a* and *e*) [6].

Laminin has been shown by immunoelectron microscopy to be an ubiquitous component of the lamina lucida [6]. In skin it has been localized by immunofluorescence staining to the dermal-epidermal junction and to basement membranes surrounding blood vessels and sweat glands (Fig 2*A*, panels *b* and *f*) [6]. This highly asymmetric molecule contains a large amount of carbohydrate and has a molecular weight of approximately 1×10^6 daltons. It is synthesized by epithelial and endothelial cells in culture and has been recently shown to interact specifically with heparan sulfate [7]. In addition, Terranova, Rohrbach, and Martin [8] have shown that laminin mediates the attachment of epithelial cells to type IV collagen. This sort of macromolecular interaction is undoubtedly important in maintaining the functional integrity of the basement membrane.

Type IV collagen has been localized by immunoelectron microscopy to the lamina densa [6]. Characterization of this collagen type was hindered by the extreme insolubility of the complex in the basement membrane. However, the use of a basement membrane-producing tumor and *in vitro* biosynthetic systems allowed the extraction of a soluble type IV procollagen without proteolytic and other degradative procedures (for a

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This report constitutes a review of studies from several laboratories concerned with the structure and location of basement membrane collagens, endothelial cell collagen, and macromolecules of the extracellular matrix.

Established Investigator of the American Heart Association

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Abbreviations:

BAE: bovine aortic endothelial
β-APN: β-aminopropionitrile
BM: basement membrane
DEAE: diethylaminoethyl
DTT: dithiothreitol
ECM: extracellular matrix
FITC: fluorocein isothiocyanate
SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis
SMC: smooth muscle cells

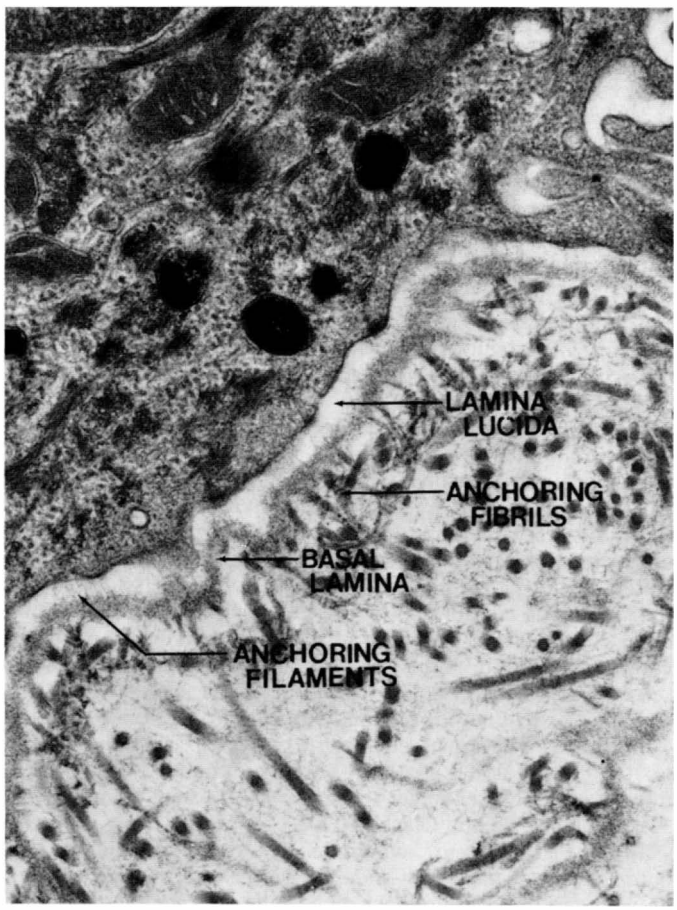

FIG 1. Dermal-epidermal junction of normal human skin. Transmission electron micrograph of dermal-epidermal junction showing lamina lucida and basal lamina (lamina densa) of basement membrane. Anchoring filaments are located between the plasma membrane of the epidermal keratinocyte and anchoring fibrils are associated with the dermal side of the basal lamina. Collagen fibrils can be seen throughout the dermis. (Courtesy of Dr. K. Holbrook, Department of Biological Structure, University of Washington, Seattle, WA.)

review, see reference 9). Concomitant studies which utilized limited pepsin digestion of basement membranes, followed by biochemical characterization of the larger collagenous fragments, established that type IV collagen contained (a) at least 2 unique chains, (b) a longer triple helix than the interstitial collagen types, (c) pepsin-sensitive sites within the triple helix, and (d) multiple disulfide bonds. The macromolecular structure of type IV collagen, however, was not understood until the isolation of a large, highly disulfide-bonded complex after proteolytic treatment of basement membranes, which was termed 7S. Studies of this fragment by the rotary shadowing technique have provided a definitive demonstration of the organization of the type IV collagen polymer [10]. The 7S form was found to be a domain of type IV collagen which interconnected 4 triple helical molecules by disulfide bonds and possibly other covalent crosslinks. The following section will discuss both the structure and localization of the collagenous components of basement membranes.

COLLAGENS ASSOCIATED WITH THE BASEMENT MEMBRANE

A brief summary of the collagen types that have been characterized as distinct gene products is presented in Table I. The interstitial collagens (types I, II, and III) form fibrillar structures *in vivo* which have not been observed for the basement membrane collagens (types IV and V). Large, cross-banded fibers are characteristic for type I collagen, while type III collagen forms fine "reticular" fibrils that are distributed most abundantly in tissues requiring a high degree of compliance.

Type IV collagen is an integral component of basement membranes by both biochemical and immunohistological criteria (for a review, see 1). Immunofluorescence studies have shown it to be distributed in the BM zone of the dermal-epidermal junction [6,14,15] (Fig 2*A*, panels *c* and *g*) and in the aortic subendothelium [13]. Figure 2*B* illustrates positive staining of the skeletal muscle endomysium which surrounds the individual fibrils. Immunoelectron microscopy using the peroxidase-antiperoxidase technique has localized type IV collagen to basal laminae of many tissues including those of alveoli and capillaries [11], aortic media (surrounding smooth muscle cells) [12], kidney glomeruli, tubules, and Bowman's capsule [15], and the dermal-epidermal junction [6].

In contrast, type V collagen has been localized by immunoelectron microscopy preferentially to pericellular matrices, as shown in Fig 3 for the smooth muscle cell exocytoskeleton [12]. It can be seen that antibodies to type V collagen did not stain between adjacent smooth muscle cells (SMC) (*arrowhead*) but were distributed on the adventitial side of the cell surface. Sano et al [11] have shown the preferential localization of type V collagen to bronchiolar, alveolar epithelial, alveolar septal, and capillary endothelial cell surfaces by a similar technique. These results are in agreement with those of a previous study [13] which demonstrated type V collagen on the luminal surface of endothelial cells. This protein was thought to be responsible for maintaining the endothelium as a nonthrombogenic layer [13]. Gay et al [14] were unable to demonstrate staining of the BM at the dermal-epidermal junction using antibodies directed against type V collagen. However, Roll et al [15] have presented evidence for codistribution of types IV and V collagen in the mesangial matrix and basement membranes of the kidney by immunoferritin labeling of ultrathin frozen sections. Further studies are needed to resolve the precise location of type V collagen. At present, results at the ultrastructural level support a cell surface-associated distribution of type V collagen that in some instances is associated with, but not necessarily a component of, the basal lamina.

In Table II are listed several structural characteristics of types IV and V collagen which distinguish them from the interstitial types. A detailed review of the biochemical properties of these collagens can be found in Bornstein and Sage [9]. In general, the basement membrane collagens have elevated levels of hydrophobic amino acids and decreased amounts of alanine and arginine, compared to the interstitial types. Two distinct α chains have been characterized in type IV collagen, and three in type V, the latter having been recently characterized as both a hetero-($[\alpha 1(V)]_2\alpha 2(V)$) and homopolymer ($[\alpha 1(V)]_3$) (16). Both types IV and V collagen are secreted as procollagens which are larger than those of types I, II, and III and which appear to undergo more limited processing [9,16]. The continuous triple helical conformation of the interstitial collagens imparts an unusually protease-resistant character to these molecules. It is especially important to note that type IV collagen contains interruptions within the triple helix [17]. These breaks in the repeating Gly-X-Y sequence, which result in a localized unfolding or relaxation of the triple helix, are most likely responsible for the lability of type IV collagen to several proteases, such as those from mast cells and granulocytes, which are normally noncollagenolytic [9].

Several laboratories have reported that neither type IV nor V collagen was a substrate for human skin collagenase [9]. Subsequently, a collagenase that was specific for type IV collagen was isolated from metastatic tumor cells by Liotta and associates [18], and it was proposed that tumor penetration could occur by degradation of the basement membrane.

The resistance of type V collagen to human skin collagenase is shown in Fig 4*A*. Under conditions where type I collagen was cleaved by greater than 90% to produce the characteristic TC^A

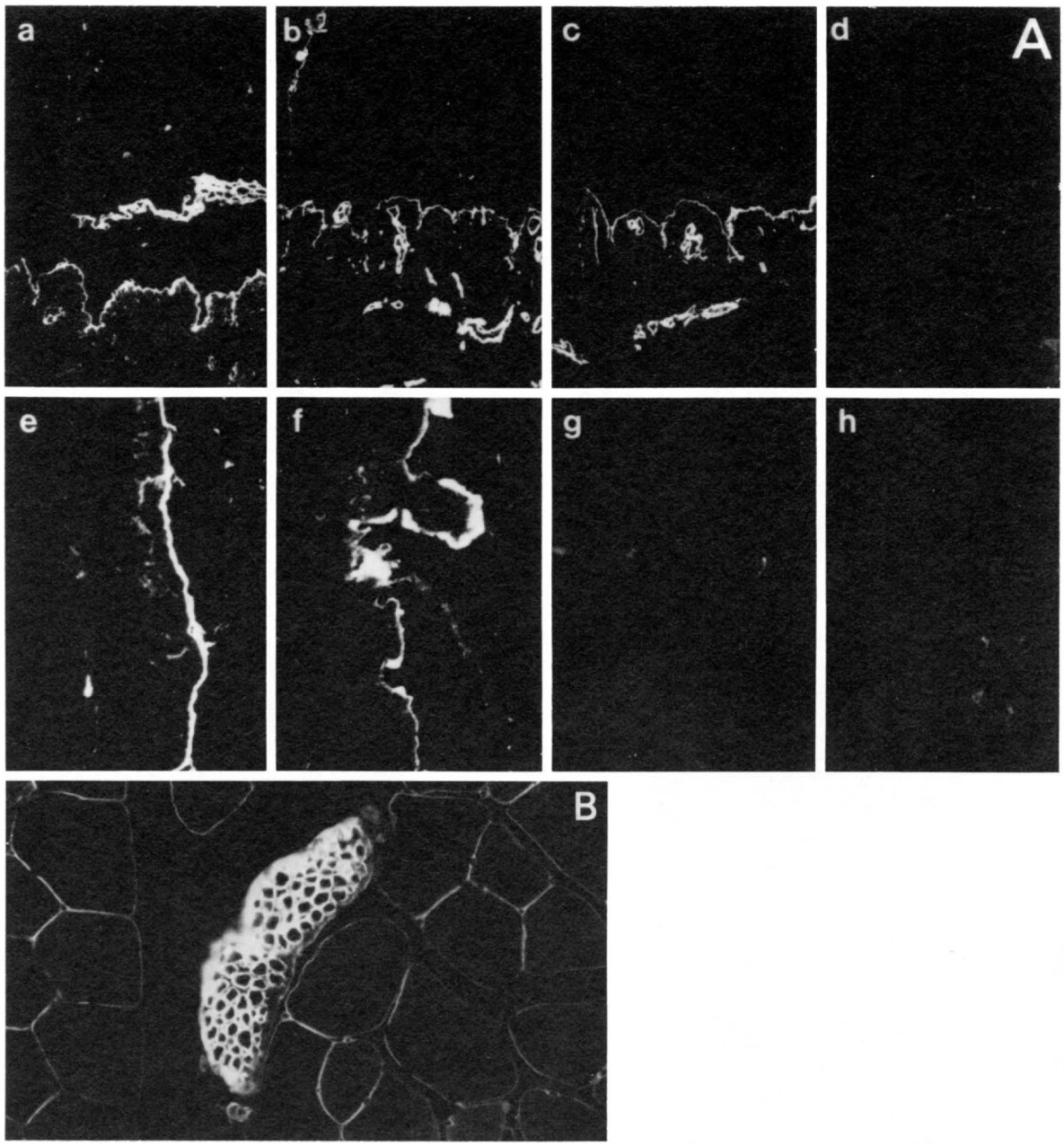

FIG. 2. Localization by indirect immunofluorescence of basement membrane components in (*A*) dermal-epidermal junction and (*B*) skeletal muscle. *A*, Human epidermis was separated from the underlying dermal layer by suction blistering which caused fragmentation within the lamina lucida. Panels *a–d* represent the floor of the blister with the attached lamina lucida and lamina densa; panels *e–h* represent the roof of the blister, with associated epidermis on the left side and torn lamina lucida on the right side (within the blister cavity). Tissues were incubated with rabbit antibodies to fibronectin (*a,e*), laminin (*b,f*), type IV collagen (*c,g*), and type II collagen (*d,h*), followed by exposure to FITC-conjugated goat anti-rabbit IgG. Fibronectin is contained within a clot which has formed in the blister cavity (*a*) and is associated with the lamina lucida (*a,d*). Laminin was localized to the lamina lucida and to basement membranes surrounding blood vessels and sweat glands (*b,e*). Type IV collagen was located specifically in the lamina densa (*c*). The control, with anti-type II collagen antibodies, was negative (*d,h*). (Reprinted from Saksela et al [5], with permission.) *B*, Mouse muscle, including a section through a peripheral nerve, was exposed to anti-type IV collagen antibodies, followed by fluorcein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (× 220). The endomysium stains positively for type IV collagen. (Photo courtesy of K. Alitalo and A. Vaheri, Department of Virology, University of Helsinki, Helsinki, Finland.)

and TC^B fragments, type V collagen was unaffected. The synthesis of type V collagen by endothelial cells *in vitro* [20] prompted us to test its susceptibility to thrombin, as shown in Fig 4*B*. At enzyme to substrate ratios between 1:40 and 1:400 by weight, approximately 90% of the type V collagen was cleaved within 2 hr at 34–37°C [21]. Type IV collagen was partially degraded by this enzyme, but type I was unaffected at temperatures below 39°C. It was suggested that thrombin could be involved during injury to the endothelium, since a disruption of the extracellular matrix could be accomplished by cleavage of type V collagen and would facilitate endothelial cell migration and possibly proliferation in wound repair. Selective susceptibility of type V collagen to a neutral protease from macrophages [22] and from a leiomyoma [23] has also recently been reported.

OTHER COLLAGEN TYPES: ENDOTHELIAL CELL COLLAGEN

Collagen types other than those listed in Table I have been described. One of these was purified from placental tissues and

TABLE I. *Distribution of collagen types*

Type	Tissue localization	Reference
I	Connective tissue interstitium: bone, tendon, skin	9
II	Cartilage	9
III	Similar to I; absent in bone, but prominent in blood vessels and fetal skin.	9
IV	Basement membranes (lamina densa)	1, 6
V	Pericellular matrices of epithelial and mesenchymal cells; subendothelium	11, 12, 13

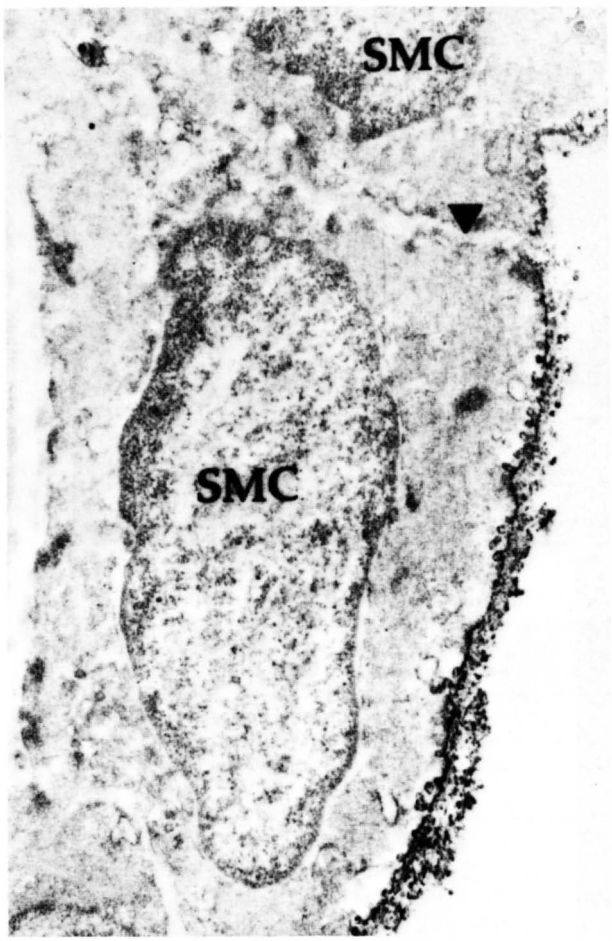

FIG 3. Localization of type V collagen to the exocytoskeleton of smooth muscle cells by immunoelectron microscopy. Section through rat renal arteriole showing 2 adjacent smooth muscle cells (SMC). The adventitial aspect of the SMC surface has been stained positively for type V collagen using a peroxidase-antiperoxidase technique. Staining did not occur in the basement membrane between the SMC (▼) nor on the side facing the internal elastic lamina (× 20,000). (Reproduced from Gay et al [12] with permission.)

TABLE II. *Properties of basement membrane-associated collagens: Types IV and V*

1. Cross-banded fibrils not observed; type IV forms amporhous, felt-like meshwork.
2. Procollagen molecules larger than those of interstitial types.
3. Increased content of hydrophobic amino acids.
4. Type IV contains interruptions within the triple helix.
5. Both resistant to human skin collagenase but are substrates for selected neutral proteases.

was termed intimal collagen [24,25]. Although in some respects it resembled type IV collagen, intimal collagen had a unique polypeptide structure and did not appear to originate from basement membranes [25].

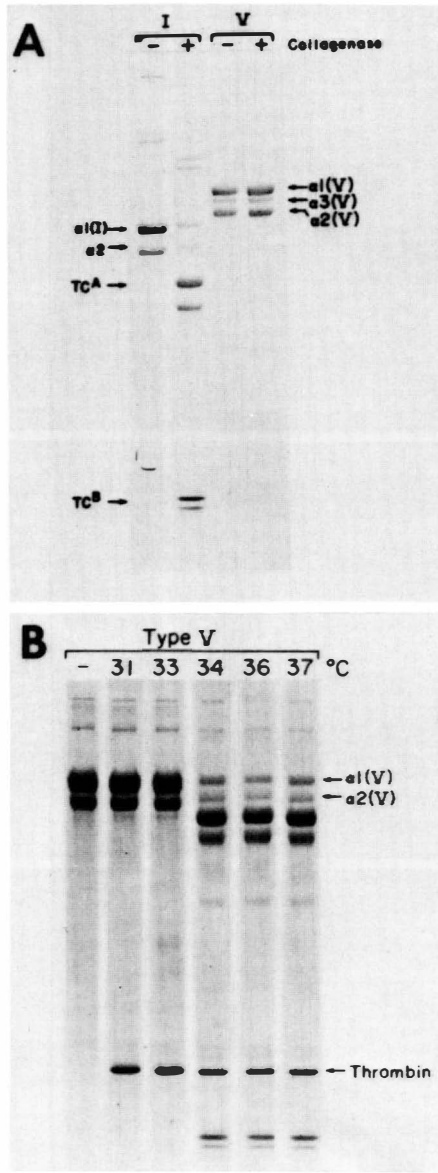

FIG 4. Sensitivity of type V collagen to neutral proteases. Type V collagen was incubated with human skin collagenase (*A*) or thrombin (*B*), and the reactivity was analyzed by SDS-PAGE. Protein was visualized by staining with Coomassie blue. *A*, Native types I and V collagen in the presence (+) or absence (−) of collagenase. Incubations occurred at 22°C for 48 hr, at an enzyme to substrate weight ratio of 1:50. Intact α chains and the reaction products of type I collagen (TC^A and TC^B) have been indicated; type V collagen was not cleaved. (Reproduced from Sage and Bornstein [19] with permission.) *B*, Type V collagen was incubated with highly purified thrombin at a 1:40 enzyme to substrate weight ratio for 2 hr at the temperatures indicated. At 34°C, type V collagen was cleaved to specific fragments as shown. (Reproduced from Sage, Pritzl, and Bornstein [21] with permission.)

Another unusual collagen has been isolated from endothelial cell cultures by Sage, Pritzl, and Bornstein [26] and by Benya [27]. This collagen, which has been termed EC, constitutes approximately 20% of the total collagen produced by cultures of bovine aortic endothelial (BAE) cells. Some properties of this collagen have been listed in Table III. Although EC can be radiolabeled with [^{3}H]-cysteine, none of the forms recovered from the culture medium contained interchain disulfide bonds. Peptide mapping of fragments produced by cyanogen bromide cleavage revealed a pattern on sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) which was different from those of other collagen types (Fig 5). An unusual feature

TABLE III. *Properties of EC, a novel collagen type*

1. Three forms recovered from endothelial cell culture medium:
   EC1-M_r 177,000
   EC2-M_r 125,000
   EC3-M_r 100,000
2. Interchain disulfide bonds absent
3. Ascorbate-independent mode of secretion
4. Degraded by pepsin to fragment of M_r 50,000
5. Labile to several neutral proteases including human skin collagenase
6. Unique primary structure indicated by peptide mapping

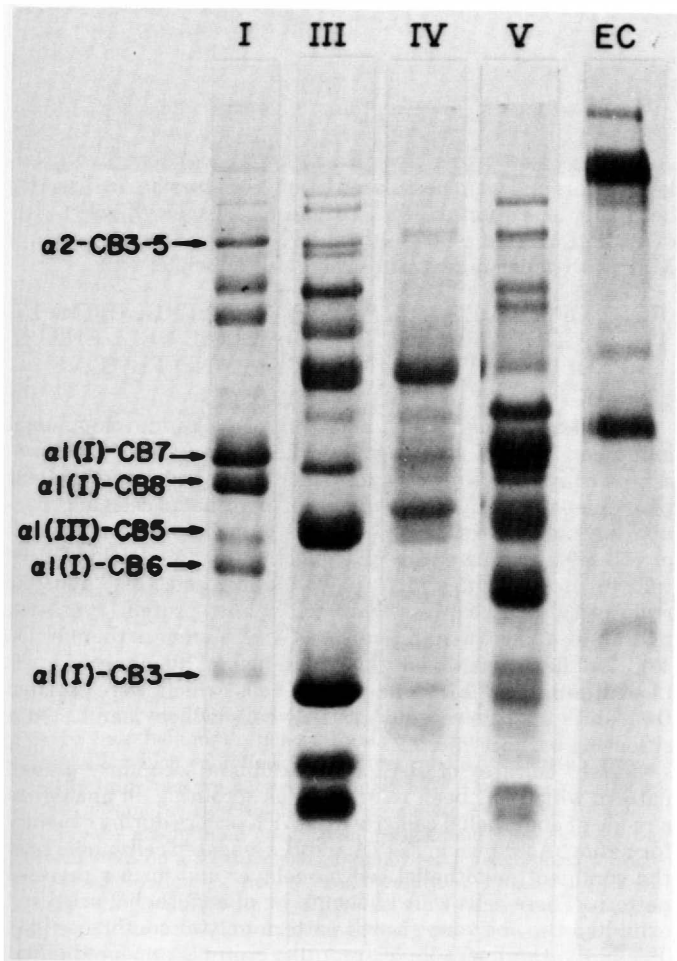

FIG 5. Comparison of CNBr peptides from different collagen types. Types I, III, IV, V, and EC collagen were cleaved with CNBr, and the products were resolved by SDS-PAGE under reducing conditions. Some of the major CNBr peptides of types I and III collagen have been indicated. The distribution of peptides from EC suggests that it is a unique collagen type. (Reproduced from Sage [28] with permission.)

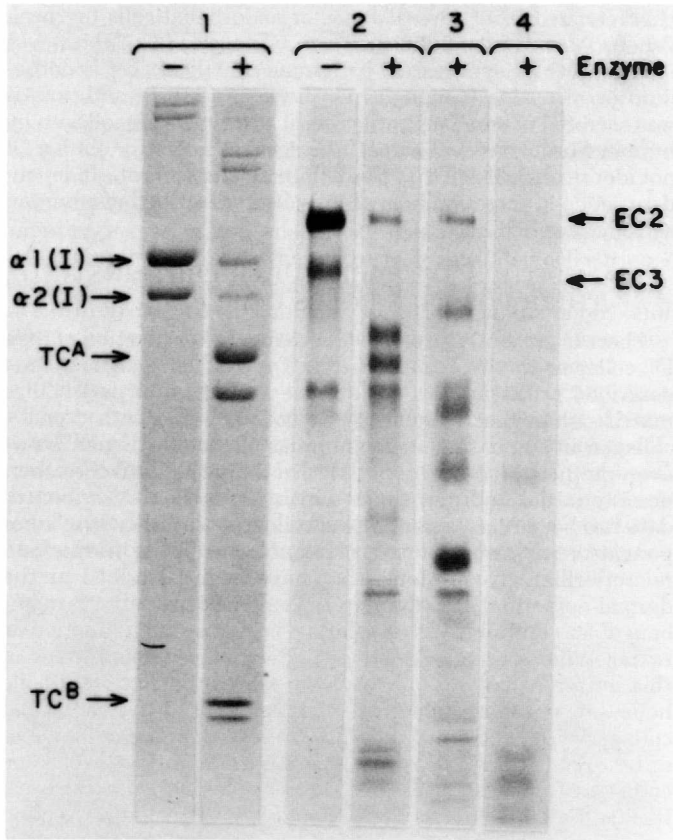

FIG 6. Sensitivity of EC to neutral proteases. Bovine aortic endothelial cells were incubated with [^{3}H]proline for 24 hr in serum-free medium which was supplemented with sodium ascorbate and β-aminopropionitrile (β-APN). Proteins were subsequently precipitated from the culture medium using 50% ammonium sulfate (w/v). The precipitate was dissolved in 6 M urea, 50 mM Tris-HCl, pH 8.0, containing protease inhibitors, and proteins were resolved by chromatography on diethylaminoethyl (DEAE)-cellulose at 4°C. The fraction containing EC, which did not bind to the DEAE-cellulose, was dialyzed into neutral salt buffers and was used for enzymatic studies. The reactions were terminated by adding an equal volume of electrophoresis buffer, and the products were analyzed by SDS-PAGE on a composite 7.5%/12.5% slab gel in the presence of 50 mM dithiothreitol (DTT). Proteins were visualized by staining with Coomassie blue (*lane 1*) or by fluorescence autoradiography (*lanes 2, 3, and 4*). *Lane 1:* type I collagen, incubated control (−) and with human skin collagenase (+), as described in the legend to Fig 4*A*. α chains and cleavage products (TC^A − M_r 70,000 and TC^B − M_r 25,000) have been indicated. *Lane 2:* EC, incubated control (−) and with human skin collagenase (+), at 22°C for 24 hr. The starting materials containing EC2 (M_r 125,000) and EC3 (M_r 100,000) have been identified. *Lane 3:* EC, incubated with thrombin, at 28°C for 1 hr. *Lane 4:* EC, incubated with trypsin, at 28°C for 1 hr.

of EC was its lability to several proteases, as shown in Fig 6. Unlike types IV and V collagen, EC was cleaved by human skin collagenase to fragments of M_r 65,000–80,000. The presence of several nontriple-helical domains in EC was suggested by its extreme sensitivity to the neutral proteases trypsin and thrombin at relatively low temperatures (28°C) (Fig 6). In addition, a fragment of M_r 50,000 was produced within 5 min after incubation with pepsin at 4°C; the triple helices of native types I, II, III, and V, and to a lesser extent type IV, are resistant to this enzyme [26].

Another unusual feature of EC was related to its mode of secretion. It was found that this collagen was secreted at normal levels in the absence of added sodium ascorbate [29]. Under these conditions, which result in a 90% inhibition of prolyl hydroxylation, endothelial cells were unable to secrete their principal collagenous product, type III procollagen [29]. It has been demonstrated that posttranslational hydroxylation is necessary for triple helix formation and subsequent secretion of the interstitial procollagens. However, secretion of type IV procollagen was also shown to be ascorbate-independent and therefore appeared to differ from that of types I–III [30]. This difference may be quantitative rather than qualitative since the interstitial collagens are secreted slowly in the absence of peptidyl hydroxylation [31]. The significance of a lack of requirement for ascorbate for secretion of both type IV procollagen and EC is presently not known, but it may reflect similarity in structure or intracellular distribution between these 2 collagens.

It must be stressed that the localization and function of EC as a basement membrane component has not been established, although its inclusion within the class of extracellular matrix proteins appears reasonable. This collagen-like protein has been

characterized from several types of endothelial cells (corneal, venous, aortic) from 2 different animal species [27, 32] but was found not to be synthesized by human umbilical vein endothelium or a murine hemangioendothelioma [33]. In addition, it was secreted in small amounts relative to type I procollagen by human fibroblasts and smooth muscle cells. A very similar, if not identical, collagen that also exhibited an ascorbate-independent mode of secretion has recently been described in a human astrocytoma cell line [34].

FILAMENTOUS EXTRACELLULAR PROTEINS AND THEIR POSSIBLE ROLES CELL BEHAVIOR

There is presently strong evidence for the localization of type IV collagen to the lamina densa. Type V collagen has been described primarily as a cell surface-associated or pericellular matrix component which may be codistributed with type IV collagen in the basement membranes of certain tissues. However, neither the function nor localization of EC and the other, noninterstitial collagen types which have been described to date has been ascertained. These collagens, together with components such as anchoring filaments, anchoring fibrils, and microfibrillar glycoproteins that have been described at the dermal-epidermal junction (Fig 1), could be considered as members of an additional class of fibrous proteins which function as extracellular structural elements. The collagenous proteins of this putative class do not form the long, uninterrupted triple helices or the fibrils which are characteristic of the interstitial collagen types and are, in addition, relatively protease labile. In order to consider the identity, function, and localization of some collagens that are neither interstitial types nor integral basal lamina (lamina densa) components, this concluding section summarizes the characteristics of several of these filamentous structures of the extracellular matrix (ECM). More complete descriptions of the microfilaments and basal lamina of the dermal-epidermal junction have been presented in reviews by Daróczy, Feldmann, and Királyk [35], Daróczy and Feldmann [36], and Briggaman [37].

Anchoring Fibrils

These extracellular fibrils were initially described by Palade and Farquhar [38] in amphibian skin. The fibrils (200–750 Å in diameter) were characterized as having a unique banding pattern and were polarized with respect to the basement membrane. The ends of these filaments form "fans," one of which is anchored in the matrix of the basal lamina and the other extends into the dermis [36,37]. Anchoring fibrils, which are arranged beneath the basal lamina in a continuous series of interconnecting arches, were found to be labile to bacterial collagenase but were insensitive to elastase, trypsin, and the reducing agent dithioerythritol [36,39]. Studies by Briggaman and his associates [37] have established that these fibrils were of dermal origin and were absent from the skin of patients affected with epidermolysis bullosa dystrophica, a severe blistering disorder in which a separation occurs between the epidermis and dermis. Anchoring fibrils are not present in the basement membranes of blood vessels and muscle [35].

Anchoring Filaments

These finely filamentous structures (20–80 Å in diameter) are interposed between the plasma membrane of the basal epidermal keratinocyte and the lamina densa of the basement membrane (Fig 1). Anchoring filaments were digested by bacterial collagenase and elastase and disappeared upon treatment of the tissue section with dithioerythritol [39]. Further information on the identity of this component is not presently available.

Dermal Microfibril Bundles

These fibrils extend perpendicularly through the dermis with an apparent epidermal attachment site at the basal lamina and a dermal attachment site at the elastic fiber [37]. They were shown to be sensitive to reduction but resistant to both elastase and collagenase [39] and have been equated with elastic fiber microfibrils.

Ultrastructurally, elastic fibers are composed of an amorphous core, consisting of the insoluble protein elastin, and a peripheral mantle of microfibrils that have a bead-like periodicity and a diameter of 100–120 Å. Initial analyses of the microfibrillar component from bovine ligamentum nuchae had suggested that the extracts contained neither elastin nor collagen [40]. More recent studies utilizing fibroblasts cultured from this tissue have demonstrated synthesis of at least 2 distinct components (MFP I and MFP II) which were specifically precipitated by anti-microfibrillar protein antiserum [41]. MFP I has been tentatively described as a novel collagenous glycoprotein.

Other structural glycoproteins have been described in the connective tissue matrix (for a review, see reference 42). In both cornea and skin, these glycoproteins were associated with collagen fibers and were postulated to act as a template in directing both the deposition and orientation of collagen. Kewley, Stevens, and Williams [43] also observed a close association of MFP with collagen fibers in ligamentum nuchae.

ROLE OF THE EXTRACELLULAR MATRIX (ECM) IN THE MODULATION OF ENDOTHELIAL CELL SHAPE, PROLIFERATION, MIGRATION, AND PROTEIN SYNTHESIS

Many studies performed on embryogenesis, developmental induction, and cell growth and proliferation *in vitro* have presented convincing evidence for the interdependence of cellular phenomena and an ECM. Studies on endothelial cells in particular have shown that cell-cell interactions and certain aspects of cell behavior were a direct reflection of the relationship of cells to the ECM to which they were exposed; this "dynamic reciprocity" was expressed in cell shape, protein synthesis, migration, and growth (reference 44 and references therein, 45–47). We have examined this relationship by analyzing the biosynthetic profile of endothelial cells which were isolated from different tissues and of BAE cells which manifested a sprouting pattern *in vitro*.

Certain cultures of BAE cells exhibit a secondary growth pattern which has been referred to as sprouting, in analogy to a mode of endothelial cell growth which occurs during capillary formation. As shown in Fig 7*A* and *B*, elongated cells undergrow the confluent endothelial cell monolayer and form a mycelial pattern. These cells were shown to be of endothelial orign and exhibited the aberrant growth pattern only at confluence [46]. The biosynthetic profile of sprouting cultures appeared different from that of BAE cells exhibiting the "cobblestone" morphology (Fig 7*B*). In addition to fibronectin and type III procollagen, sprouting cells secreted primarily type I procollagen in a pattern which was virtually indistinguishable from that of smooth muscle cells (Fig 7*C*) [46]. This phenotypic modulation in protein synthesis was directly correlated with the appearance of the sprouting growth pattern and was not observed when the same strain of BAE cells was examined before the sprouting occurred or at subconfluent densities when this altered growth state was not apparent.

The significance of the alteration in both morphology and protein biosynthesis observed in sprouting endothelial cell cultures is presently not known. Delvos et al [45] have described similar morphologic changes when BAE cells were plated within native collagen gels; these elongated cells, after trypsinization and replating, were subsequently able to grow and to migrate into a wound produced *in vitro*.

It has been proposed that differences in growth control can be reflected in the cellular biosynthetic profile [28,32]. When both secreted and cell layer-associated proteins were analyzed from bovine aortic, corneal, and venous endothelia, no major differences were found among the collagen types [32]. However, bovine capillary endothelial cells secreted large amounts of type

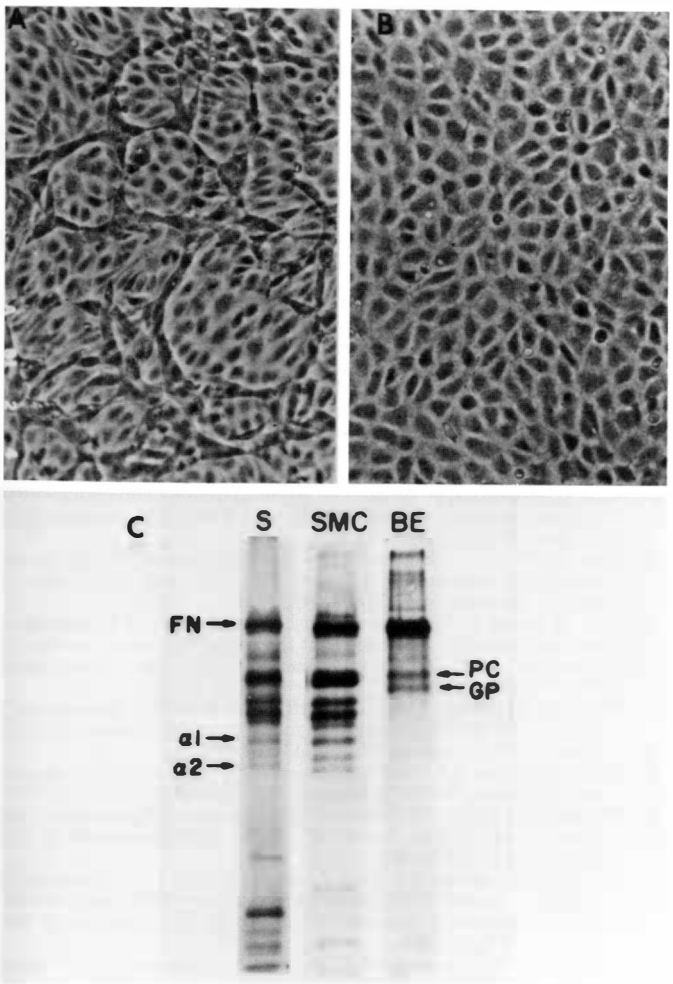

FIG 7. Comparison of proteins secreted by cultures of normal and sprouting endothelium. Protein synthesis was studied in a single strain of BAE cells which exhibited a normal morphology at early passages and a sprouting pattern between passages 8–12. Cells were incubated with [^{3}H]proline as described in the legend to Fig 6. Radiolabeled proteins were precipitated using trichloracetic acid and were resolved by SDS-PAGE on a 6%/10% slab gel under reducing conditions. Visualization was by fluorescence autoradiography. *A*, Phase-contrast micrograph of a sprouting culture of BAE cells. The sprouting phenotype consisted of a mycelial pattern of elongated cells which undergrew the monolayer. These cells eventually dominated the culture to the exclusion of contact-inhibited BAE cells. They stained positively for Factor VIII antigen and assumed an apparently normal morphology upon trypsinization and replating; however, at confluence the sprouting pattern reappeared (reduced from × 125). *B*, Phase-contrast micrograph of BAE cells exhibiting normal morphology. A stable monolayer was formed that consists of "cobblestone"-shaped cells. *C*, SDS-PAGE of radiolabeled proteins secreted by cultures of sprouting endothelium (*S*), smooth muscle cells (*SMC*), and endothelial cells exhibiting normal morphology (*BE*). Several proteins in the culture media have been identified: fibronectin (*FN*), type I collagen chains (*α1, α2*), type III procollagen (*PC*), and a noncollagenous glycoprotein, thrombospondin (*GP*). BAE cells which formed stable monolayers at confluence secreted 3 major components: fibronectin, type III procollagen, and thrombospondin. In contrast, sprouting endothelium displayed a secretory phenotype which was very similar to that of SMC, consisting of fibronectin and predominantly type I procollagen. The additional bands migrating between α1 and FN correspond to intact type I procollagen chains and partially processed intermediate forms. (Micrographs courtesy of Drs. C. Gajdusek and S. Schwartz, Department of Pathology, University of Washington, Seattle, WA. Reproduced in part from Bornstein, McPherson, and Sage [44] with permission.)

I procollagen, therein resembling the sprouting cultures. The capillary cells used in this study have been characterized by Folkman and his associates [47] as forming tubes in culture, and this phenomenon was termed "*in vitro* angiogenesis." Although the tissue source and/or method of subculture could account for the biosynthetic difference between the cultures of capillary and sprouting endothelial cells and those from aorta, vein, and cornea which formed contact-inhibited, closely-apposed monolayers, studies by Ausprunk, Boudreau, and Nelson [48,49] have shown a correlation between extracellular matrix components and the endothelial cell growth pattern.

As shown in Fig 8*A*, several components of the ECM surrounding a resting endothelial cell could be distinguished by ruthenium red staining. These structures included cell surface-associated glycocalyces, components of the basal lamina, granules within the basal laminae (10–20 nm in diameter) and associated with collagen fibers (20–50 nm), and an interconnecting network of fine filaments. However, when a neovascular response was elicited from the limbal vessel of the cornea by an implant containing angiogenic factor(s), a redistribution of some of the ECM components was observed in the region of proliferating endothelial cells. In proliferating capillaries, the basal lamina contained smaller granules (8 nm in diameter) which were located only on the plasmalemma side of the endothelium. At the tip of the growing capillary, the basal lamina was absent

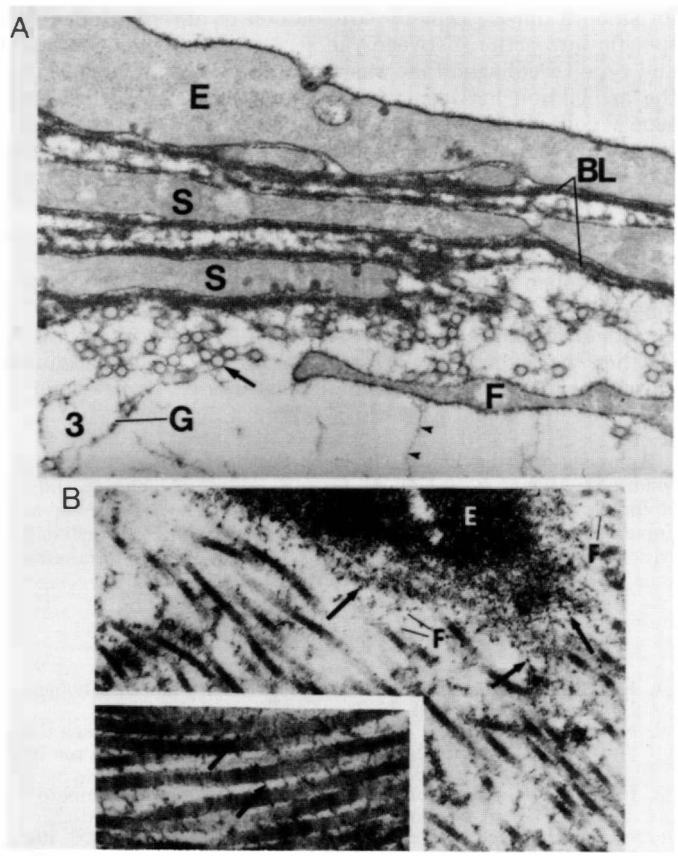

FIG 8. Ultrastructural organization of anionic components within the rabbit corneal microvasculature. *A*, Limbal blood vessel, located at the junction of the cornea and sclera, was fixed only in ruthenium red. Ruthenium red-positive materials include: (1) surface coats (glycocalyces) on endothelial cells (*E*), smooth muscle cells (*S*), and fibroblasts (*F*); (2) basal laminae (BL); (3) surfaces of collagen fibers (*arrow*); (4) granules (*G*) (20–50 nm) between collagen fibers in the adventitia; and (5) a network of fine filaments (3–5 nm in diameter) (*arrowheads*) which interconnect basal laminae, small collagen fibers, large granules, and glycocalyces (reduced from × 43,000). *B*, Endothelial cells (*E*) at the tips of proliferating capillaries after fixation in ruthenium red. Tangential section shows small ruthenium red-positive granules (8 nm in diameter) (*arrows*) adjacent to the plasmalemma and on collagen fibers in the corneal stroma. Thin filaments (3–5 nm in diameter) (*F*) interconnect these granules (reduced from × 54,000). *Inset*, Ruthenium red-positive granules, connected by thin filaments (*arrows*), are closely associated with collagen fibers in the cornea (reduced from × 84,000). (Reproduced from Ausprunk, Boudreau, and Nelson [48, 49] with permission.)

(Fig 8B). Some of these ruthenium red-positive components are likely to be products of the endothelial cells themselves [50] and may function in anchoring the proliferating cells, reorganizing the capillary basal lamina, or providing an anionic surface along the advancing luminal front, as suggested by Ausprunk, Boudreau, and Nelson [49]. Such phenotypic modulation in cell shape, proliferation, distribution of ECM material, and secreted proteins is strongly suggestive of a reciprocity between cell behavior and biosynthesis of extracellular macromolecules.

The relationship of basement membrane collagens, as components of the ECM, to cellular function has been supported by several recent studies. Acinar cells from a rat pancreatic carcinoma, which were unable to synthesize and maintain a complete basal lamina containing laminin and type IV collagen, displayed a disorganized growth pattern that was characteristic of neoplasia [51]. Salomon, Liotta, and Kidwell [52] showed that the response of rat mammary epithelial cells to different growth factors was determined by the substratum upon which the cells were plated. These authors in addition demonstrated that such potentiation could be linked to the synthesis of type IV collagen and its accumulation in the ECM. A role for type V collagen as an attachment protein has recently been described for smooth muscle cells by Grotendorst et al [53]. Unlike the specific interaction between the secreted component laminin and type IV collagen that was reported for epithelial cells [8], the association between the smooth muscle cell surface and type V collagen appeared to be mediated by an intrinsic membrane glycoconjugate. An additional role for type V collagen was proposed by Stenn, Madri, and Roll [54], who observed that sustained synthesis of this protein was required for epidermal cell migration.

Studies of this type underscore the importance of the extracellular milieu in processes involving proliferation, association, migration, and biosynthesis, and they provide a rationale for investigating the reciprocal nature of cell-matrix interactions. Further characterization of basement membrane/extracellular matrix macromolecules, including several novel collagen types, will assist in elucidating some of these mechanisms.

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