Schmer, G., & Kreil, K. (1969) *Anal. Biochem. 29*, 186–192.
Sherton, C. C., & Wool, I. G. (1972) *J. Biol. Chem. 247*, 4460–4467.
Steers, E. J., Craven, G. R., Anfinsen, C. B., & Bethune, J. L. (1965) *J. Biol. Chem. 240*, 2478–2484.
Takagi, T., & Doolittle, R. F. (1974) *Biochemistry 13*, 750–756.
Tsunasawa, S., & Narita, K. (1981) *Methods Enzymol. 91*, 84–92.
Waxdal, M. J., Konigsberg, W. H., Henley, W. L., & Edelman, G. M. (1968) *Biochemistry 7*, 1959–1966.
Wittmann-Liebold, B. (1973) *FEBS Lett. 36*, 247–249.

Type IV Collagen Synthesis by Cultured Human Microvascular Endothelial Cells and Its Deposition into the Subendothelial Basement Membrane†

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ABSTRACT: Cultured microvascular endothelial cells isolated from human dermis were examined for the synthesis of basement membrane specific (type IV) collagen and its deposition in subendothelial matrix. Biosynthetically radiolabeled proteins secreted into the culture medium were analyzed by sodium dodecyl sulfate gel electrophoresis after reduction, revealing a single collagenous component with an approximate M_r of 180 000 that could be resolved into two closely migrating polypeptide chains. Prior to reduction, the 180 000 bands migrated as a high molecular weight complex, indicating the presence of intermolecular disulfide bonding. The 180 000 material was identified as type IV procollagen on the basis of its (1) selective degradation by purified bacterial collagenase, (2) moderate sensitivity to pepsin digestion, (3) immunoprecipitation with antibodies to human type IV collagen, and (4) comigration with type IV procollagen purified from human and murine sources. In the basement membrane like matrix elaborated by the microvascular endothelial cells at their basal surface, type IV procollagen was the predominant constituent. This matrix-associated type IV procollagen was present as a highly cross-linked and insoluble complex that was solubilized only after denaturation and reduction of disulfide bonds. In addition, there was evidence of nonreducible dimers and higher molecular weight aggregates of type IV procollagen. These findings support the suggestion that the presence of intermolecular disulfide bonds and other covalent interactions stabilizes the incorporation of the type IV procollagen into the basement membrane matrix. Cultured microvascular endothelial cells therefore appear to deposit a basal lamina-like structure that is biochemically similar to that formed in vivo, providing a unique model system that should be useful for understanding microvascular basement membrane metabolism, especially as it relates to wound healing, tissue remodeling, and disease processes.

The basal lamina underlying most capillaries serves several important functions, such as acting as a selective permeability barrier and providing support and orientation for the endothelium (Vracko, 1974; Caufield & Farquhar, 1978). During wound healing and tissue remodeling, the microvasculature must invade surrounding tissue, a process that necessitates modifications in the basal lamina. The composition of the microvascular basal lamina has been determined to some degree by the use of immunohistochemistry (Kleinman et al., 1982; Timpl et al., 1979; Hassel et al., 1980). Accurate biochemical studies are hampered, however, by difficulties in obtaining sufficient quantities of purified material and by the high insolubility of these structures. The presence of aldehyde-derived and disulfide intermolecular cross-linking in the basement membrane has frustrated attempts to characterize individual components without resorting to proteolysis. However, the availability of cell cultures that retain the correct phenotypic expression of basal lamina production would provide an appropriate system for the detailed analysis of the process. Using such a system, it may be possible to delineate the steps involved in the secretion and assembly of matrix components into the basal lamina and to evaluate alterations that occur in diseases of the microvasculature.

Type IV collagen has been shown to be specific to basal laminae and is believed to act as the major structural protein in this matrix (Timpl et al., 1981; Yurchenco & Furthmayr, 1984). It would be expected, then, that if cultured microvascular endothelial cells maintain their correct differentiated phenotype, they would secrete type IV collagen as the primary collagen type. The present study describes the synthesis of type IV procollagen by cultured human microvascular cells and its deposition into the subendothelial matrix.

Experimental Procedures

Cell Culture. Microvascular endothelial cells were isolated from the dermis of newborn human foreskin as reported (Davison et al., 1980; Kramer et al., 1984, 1985). Cells were

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normally cultured in Iscove's modification of Dulbecco's minimal essential medium (Gibco) containing 10% heat-inactivated human serum supplemented with added growth factors as described (Kramer et al., 1985). Primary human umbilical cord vein cells were obtained and cultured to confluence as reported (Davison et al., 1983). Human HT1080 cells (Rasheed et al., 1974) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum.

Biosynthesis Labeling. For metabolic labeling, postconfluent cell monolayers were incubated for 18–24 h with 10–50 μCi/mL L-[2,3-^{3}H]proline (250 μCi/mmol), L-[^{14}C]proline (50 μCi/mmol), or L-[^{35}S]methionine (600 μCi/mmol). These isotopes were used in medium in which the unlabeled amino acid was omitted and serum concentration was reduced to 5%. In addition, fresh recrystallized ascorbate (50 μg/mL) was added every 24 h to the culture medium beginning the day following cell plating and continued during the labeling period. BAPN[1] (80 μg/mL) was normally added to the labeling medium.

At the end of the labeling period, the medium was harvested, cooled to 4 °C, and adjusted to 1 mM PMSF, 10 mM NEM, and 10 mM EDTA. Cellular debris was removed by an initial low-speed centrifugation at 500*g* for 10 min followed by centrifugation at 30000*g* for 30 min. The supernatant was either used immediately or stored at −20 °C. The cell layer was washed twice with cold PBS and either stored at −20 °C, immediately solubilized in SDS–PAGE buffer at 100 °C for 3 min, or processed for isolation of the subendothelial matrix. Type IV procollagen was purified from the culture medium of HT1080 cells labeled with [^{3}H]proline as described by Alitaro et al. (1980) using DEAE chromatography.

Matrices were isolated as previously reported (Kramer et al., 1984, 1985). Briefly, the washed cell layer was rinsed with hypotonic buffer (5 mM Tris-HCl, 0.5 mg/mL BSA, 0.1 mM $CaCl_2$, 1 mM PMSF, pH 7.5), warmed to 37 °C, and then incubated with the same buffer for 8–10 min. Cell lysis was completed by two brief extractions with 0.5% NP-40 detergent in hypotonic buffer for 1 min each at 37 °C. Under these conditions, the insoluble matrices remained attached to the dish and were either stored at −20 °C or processed for SDS–PAGE by solubilization in sample buffer at 100 °C for 3 min. Hydroxyproline/proline ratios were determined by high-performance liquid chromatography (HPLC) on a Spectra Physics system using a ODS-10-C18 reverse-phase column (Whatman).

SDS–Polyacrylamide Gel Electrophoresis. A modified discontinuous buffer system (Maizel, 1971) was used as previously reported. Slab gels were 1.5 mm thick and approximately 25 cm long and were constructed of a 3% stacking and either a 4%, 5%, or 6% running gel containing 0.5 M urea. All samples were prepared in SDS–solubilization buffer and heated at 100 °C for 3 min prior to electrophoresis. Samples of culture medium were diluted with an equal volume of water and an equal volume of a 3-fold concentrated solution of SDS–solubilization buffer.

Immunoprecipitation. Culture medium was dialyzed against immunoprecipitation buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% NP-40, 1 mM PMSF, 1 mM NEM, pH 7.5). Samples were incubated with affinity-purified rabbit antibodies to human type IV collagen for 1 h at 37 °C. Goat anti-rabbit IgG antibodies were then added, and the incubation was continued for 1 h at 37 °C. The immunoprecipitates were collected by centrifugation (2000*g*) after incubation overnight at 4 °C. The pellets were washed 3 times with immunoprecipitation buffer after suspension by brief sonication. Finally, the pellets were suspended in buffer, layered onto a solution of 0.1% SDS, 1% NP-40, 10% sucrose, and 10 mM Tris-HCl, pH 7.5, and centrifuged at 5000*g* for 20 min. The pellets were resuspended in SDS–solubilization buffer and heated at 100 °C for 3 min prior to electrophoresis. Affinity-purified antibodies to human type IV collagen with specificity for both α1(IV) and α2(IV) chains were generously provided by Dr. Heinz Furthmayr (Yale University). Antibodies specific to collagens I and III were a gift from Dr. Hynda Kleinman (NIH). Antibody to type V collagen was kindly provided by Dr. Helene Sage (University of Washington).

Bacterial Collagenase Digestion. Culture medium was dialyzed against 50 mM Tris-HCl buffer containing 10 mM $CaCl_2$ and 0.5 mM NEM. Purified bacterial collagenase (Biofractors) was added at a concentration of 30 units/mL and incubated at 37 °C. Isolated subendothelial matrices were digested with collagenase in a similar fashion. Matrices were isolated from radiolabeled cultures and washed extensively, first with PBS and finally with the Tris buffer indicated above. Collagenase was added at 50 units/mL, and the digestion was allowed to proceed. At selected time periods, samples were removed, and the reaction was stopped by boiling in the presence of SDS–electrophoresis solubilization buffer containing 10 mM EDTA and 5% 2-mercaptoethanol.

Pepsin Digestion. Culture medium was dialyzed against 50 mM Tris-HCl, 1 mM PMSF, and 1 mM NEM. The samples were adjusted to 0.5 M acetic acid, pH 3.0. Pepsin (3 times crystallized, Sigma) was added at a concentration of 25 μg/mL and incubated at 4 °C for time periods up to 72 h. The reaction was terminated by the addition of pepstatin (10 μg/mL), and the samples were immediately frozen on an acetone–dry ice bath and freeze-dried. For examination by electrophoresis, the samples were dissolved in SDS–solubilization buffer (pH 6.7) and immediately heated to 100 °C.

Enzyme-Linked Immunosorbent Assay (ELISA). Microvascular endothelial cells were seeded at confluent cell density onto tissue-culture 96-well cluster plates (6 mm in diameter) that were precoated with bovine plasma fibronectin as described (Kramer et al., 1985). After 8–10 days of culture, the substratum-attached subendothelial matrix was isolated as described above and processed for ELISA, with a modification of the previously described procedure (Kramer & Vogel, 1984). Briefly, the matrices were washed with 100 mM Tris-HCl (pH 7.5) and incubated with dilution buffer (1% BSA, 0.05% NP-40 in the same buffer) for 1 h. The wells were then incubated for 1 h at 22 °C with primary rabbit antibody (or antisera) serially diluted in the dilution buffer, followed by four washes with dilution buffer that also contained 1% goat serum. The samples were then reacted with the second antibody (goat anti-rabbit IgG peroxidase conjugate) as described (Kramer & Vogel, 1984).

Conventional competitive ELISA was also performed according to the procedure described by Engvall (1980). By this technique, the amount of type IV procollagen secreted into the culture medium was determined. In addition, a related assay was used in which the amount of type IV collagen present in the isolated subendothelial matrix was estimated

[1] Abbreviations: BAPN, β-aminopropionitrile; PMSF, phenylmethanesulfonyl fluoride; NEM, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DEAE, diethylaminoethyl; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; EHS, Engelbreth–Holm–Swarm.

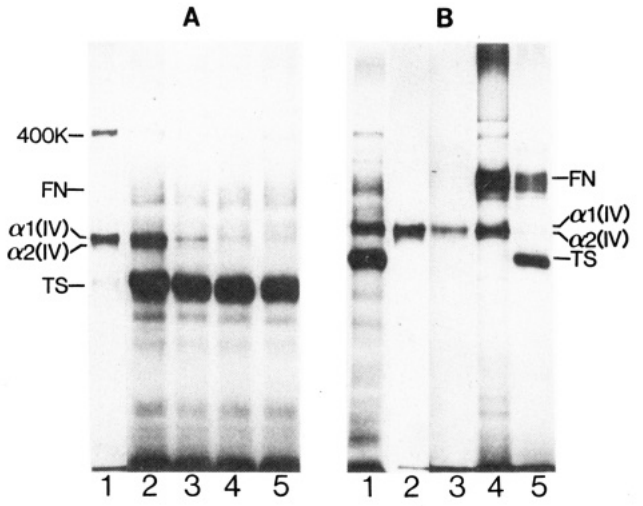

FIGURE 1: Identification of type IV procollagen in the culture medium of microvascular endothelial cells. Cells were labeled with [^{35}S]-methionine in the presence of BAPN, and the subendothelial matrix was isolated and processed for electrophoresis in 4% (panel A) or 5% (panel B) SDS gels under reducing conditions. In panel A, the isolated subendothelial matrix (lane 1) and culture medium (lane 2) are compared. A major protein is present in both the matrix and medium that migrates as two closely spaced bands with an apparent M_r of 180 000. The samples in lanes 1 and 2 represent approximately 10% and 2.5% of the radioactivity originally present in the matrix and medium compartment, respectively. Lanes 3–5 demonstrate the gradual loss of the 180 000-dalton band in the medium following digestion with bacterial collagenase after 30 min (lane 3), 2 h (lane 4), and 24 h (lane 5) of incubation. In panel B, culture medium (lane 1) was incubated with anti-type IV antibodies, and the resulting immunoprecipitate contained the M_r 180 000 material that corresponds to the α1(IV) and α2(IV) chains of type IV collagen (lane 2). An additional but minor band with a molecular weight of about 400 000 (visible only on the original autoradiogram) was also precipitated by the antibodies. The immunoprecipitated type IV procollagen comigrated with either human-type IV procollagen chain purified from the medium of the HT1080 cell line (lane 3) or type IV procollagen present in the isolated matrix (lane 4) and medium (lane 5) of cultured human umbilical cord vein endothelial cells. The positions of type IV procollagen chains [α1(IV) and α2(IV)], fibronectin (FN), thrombospondin (TS), and the 400 000-dalton band (400K) are indicated.

by overlaying the matrix with a predetermined concentration of antibody to type IV collagen. After overnight incubation, the supernatant solutions were then assayed as usual for a competitive ELISA. In this way, the type IV procollagen present in the matrix that was accessible for binding by the antibody could be determined.

Results

Analysis of Proteins Secreted into the Culture Medium. Previous work has shown that the foreskin microvascular endothelial cells secrete approximately 20% of newly synthesized protein into the culture medium, as measured by pulse labeling with radiolabeled amino acids (Kramer et al., 1985). A majority of the incorporated radioactivity was associated with the cell layer and about 4–5% of the labeled protein was incorporated into the insoluble subendothelial matrix, which can be readily isolated by a hypotonic-detergent extraction procedure. By ultrastructural and other criteria, the isolated microvascular matrix appears to be free of cellular debris (Kramer et al., 1985).

The typical SDS gel profile of radiolabeled proteins synthesized by the endothelial cells and secreted into the culture medium or deposited into the subendothelial matrix is shown in Figure 1A, lanes 1 and 2. The major radiolabeled proteins have been identified as fibronectin, type IV procollagen, and thrombospondin, with approximate M_r of 230 000, 180 000, and 150 000, respectively (Kramer et al., 1985). The α1(IV) and α2(IV) chains of type IV procollagen migrated on SDS gels as two closely spaced bands that were not always clearly resolved. The major portion of thrombospondin synthesized by the cells was found in the culture medium, and this distribution was the most striking difference seen when the protein patterns of the medium and matrix are compared (see Figure 1A, lanes 1 and 2).

The collagenous nature of the M_r 180 000 material secreted into the culture medium was confirmed by its sensitivity to bacterial collagenase digestion. The type IV procollagen was selectively lost during the time course of the digestion (Figure 1A, lanes 2–5). The failure of other radiolabeled proteins in the culture medium to disappear after prolonged collagenase digestion indicates that type IV procollagen is the predominant collagen produced by the microvascular endothelial cells.

Affinity-purified antibodies to human type IV collagen selectively immunoprecipitated the material from the culture medium with the expected M_r of 180 000, demonstrating its identity with the type IV collagen chains (Figure 1B, lane 2). In addition, a small amount of material with an apparent molecular weight of about 400 000 coprecipitated with the type IV collagen chains but is not well reproduced in this photograph. This M_r 400 000 band was sensitive to bacterial collagenase, moderately resistant to pepsin, and comigrated with the dimer of collagen type IV isolated from the mouse EHS sarcoma. Type IV procollagen purified by DEAE chromatography from culture media of human cell line HT1080 comigrated with the immunoprecipitated type IV procollagen synthesized by the microvascular endothelial cells (Figure 1B, lane 3). Human umbilical vein endothelial cells have also been shown to secrete type IV procollagen into their culture medium and to deposit it in the cell layer as an extracellular matrix (Sage & Bornstein, 1982; Jaffe et al., 1976; Madri et al., 1980). When culture medium and isolated extracellular matrix obtained from metabolically radiolabeled umbilical vein endothelial cells were compared with medium and matrix from the human microvascular endothelial cells, several similarities were noted. The type IV procollagen chains from the umbilical vein cells closely comigrated with the procollagen from the microvascular cells (Figure 1B, compare lanes 4 and 5 with lane 2). In addition, thrombospondin was present in large amounts in the culture medium of both cell types (Figure 1B, lane 1, and Figure 1B, lane 5) but was a minor component in the isolated matrices (Figure 1A, lane 1, and Figure 1B, lane 4). In contrast, fibronectin was a major constituent of the isolated matrix of the umbilical vein cells (Figure 1B, lane 4) but was present in variable but usually minor amounts in the microvascular cell matrix (Figure 1A, lane 1). In both cell types, fibronectin was a major secreted protein in the medium compartment (Figure 1B, lane 1, and Figure 1B, lane 5).

The pepsin sensitivity of the radiolabeled proteins secreted by the microvascular endothelial cells into the culture medium was examined at various times after pepsin was added. Analysis of samples under reduced conditions of electrophoresis indicated the persistence of a band at M_r 180 000 that corresponded to type IV procollagen chains (Figure 2, lanes 1–5). In addition, a very minor protein with a molecular weight of approximately 400 000 was observed also to exhibit resistance to pepsin digestion, but it was visible only in the original autoradiogram. Other secreted proteins, including fibronectin and thrombospondin, were susceptible to digestion and ultimately destroyed. As the type IV procollagen was gradually lost, polypeptides of M_r 175 000, 160 000, and 155 000 were

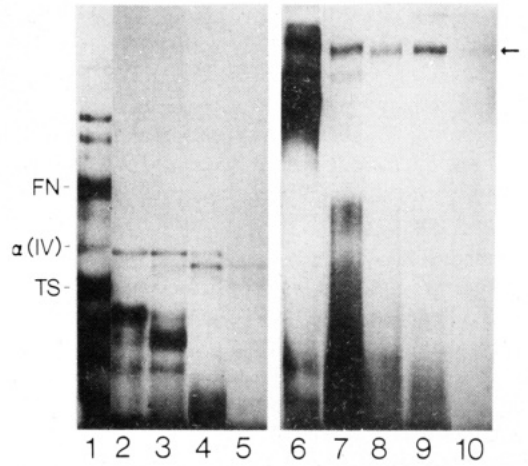

FIGURE 2: Pepsin digestion of culture medium proteins synthesized by microvascular endothelial cells. Cells were labeled with [^{35}S]-methionine and the culture medium was precipitated with 20% ammonium sulfate and adjusted to 0.5 M acetic acid. Pepsin digestion was carried out at 4 °C for 0 (lanes 1 and 6), 2 (lanes 2 and 7), 4 (lanes 3 and 8), 10 (lanes 4 and 9), and 20 h (lanes 5 and 10). The proteins were resolved in a 4% SDS gel under reduced (lanes 1–5) or nonreduced (lanes 6–10) conditions. The locations of fibronectin (FN), thrombospondin (TS), and type IV procollagen chains [α(IV)] are identified. Note the presence of a high molecular weight band (600 000) in lanes 7–10 (arrow) that is resistant to digestion but appears to be converted to type IV procollagen chains under reduced conditions.

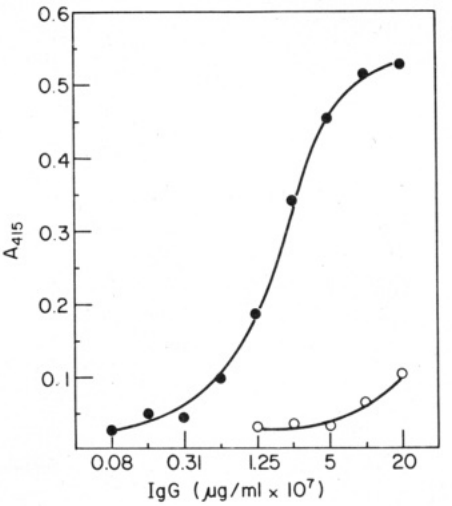

FIGURE 3: Detection of type IV collagen in the isolated subendothelial matrix by modified ELISA. Microvascular endothelial cells were cultured for 10 days in 6 mm diameter tissue culture dishes. The matrices were isolated and processed for modified ELISA using antibodies to type IV collagen (●) or nonimmune control antibodies (○), as described under Experimental Procedures. Bound peroxidase-conjugated second antibody was detected by measurement at 415 nm of chromogen generated in the presence of hydrogen peroxide. The binding of the specific, affinity-purified antibody against type IV collagen was saturable and specific.

concurrently formed and presumably were derived from the M_r 180 000 type IV procollagen chains. Extended exposure to pepsin resulted in the eventual loss of these bands.

If reduction of disulfide bonds was not performed prior to electrophoresis, a very high molecular weight component was observed that exhibited resistance to pepsin digestion but was eventually hydrolyzed after prolonged incubation (Figure 2, lanes 6–10). This protein, which barely penetrated the top of the 4% polyacrylamide running gel, had an apparent molecular weight in excess of 600 000 and was immunoprecipitated with antibodies to type IV collagen (not shown). The gradual disappearance of this 600 000 band proceeded at about the same rate as did the type IV procollagen chains seen in SDS gels under reducing conditions.

Analysis of Proteins in the Subendothelial Matrix. Using immunofluorescent and immunoperoxidase techniques, we have previously demonstrated that the subendothelial matrix produced by the microvascular endothelial cells is intensely stained by antibodies to type IV collagen (Kramer et al., 1984, 1985). In the current study, in an attempt to quantitate the amount of type IV collagen present in the isolated subendothelial matrix, we used a modified ELISA, which clearly demonstrated a specific and saturable binding of the anti-type IV collagen antibodies to the matrix (Figure 3). By employing a competitive ELISA, it was possible to approximate the amount of type IV procollagen present in the matrix. Using purified human type IV collagen isolated from the placenta as the standard, it was determined that about 175 ng of the antigen is accessible to binding by the anti-type IV collagen antibodies in the isolated matrices elaborated by 1.5×10^5 microvascular endothelial cells cultured for 5 days in 22 mm diameter dishes. Assuming a constant level of type IV synthesis, this would be equivalent to approximately 35 ng of type IV collagen deposited every 24 h. However, due to the possible sequestering of antigenic determinants in the matrix, this estimate of type IV procollagen in the matrix must be considered a minimum value. The amount of type IV procollagen secreted into the culture medium during a 24-h period by 1.5×10^5 cells was estimated by competitive ELISA to be approximately 60 ng/mL. Analysis by HPLC indicated that a high proportion of incorporated [^{14}C]proline was present in the matrix as hydroxyproline (≃26%).

Proteins present in the subendothelial matrix isolated from microvascular endothelial cells radiolabeled in the presence or absence of BAPN were studied. It was found that this inhibitor of lysyl oxidase increased the relative amounts of matrix-associated type IV collagen chains (180 000 daltons) in the 400 000 dalton band, as compared with the amount of fibronectin and thrombospondin present (Figure 4, lanes 2 and 3). Thus, in the absence of BAPN, a significant amount of the type IV procollagen appears to be cross-linked by lysyl oxidase derived bonds into higher molecular weight aggregates that are not resolved in conventional SDS gels. No obvious changes were noted in the radiolabeled profiles of the medium compartment obtained from cells exposed to BAPN (Figure 4, lanes 1 and 4). The fact that the amount of radiolabeled material present in the M_r 400 000 band was not diminished when the cells were incubated with BAPN suggests that other types of nonreducible cross-linking had occurred besides that produced by lysyl oxidase or that BAPN is not completely effective in inhibiting the lysyl oxidase.

Bacterial collagenase digestion of isolated subendothelial matrix confirmed the collagenous nature of the M_r 180 000 material that comigrated with authentic type IV collagen (Figure 4, lanes 5–7). In addition, the 400 000-dalton band was also sensitive to collagenase, indicating that it contains at least some collagenous domains. The 400 000-dalton material appeared to be more rapidly degraded by the collagenase than was the type IV procollagen. In addition, a faint, very high molecular weight band near the top of the gel was also lost after collagenase digestion. Curiously, after 1 h of incubation with the collagenase a number of new bands appeared: a major band of about M_r 160 000 and a ladderlike array of several closely spaced bands with molecular weights ranging from less than 400 000 to about 200 000. These intermediate bands were mostly digested when the collagenase treatment was extended to 3 h. The identity of these inter-

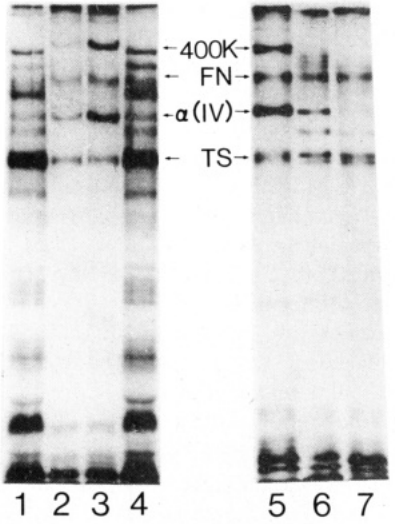

FIGURE 4: Identification of collagenous proteins in the isolated extracellular matrix. Microvascular endothelial cells were labeled with [^{35}S]methionine in the absence (lanes 1, 2, and 5–7) or in the presence (lanes 3 and 4) of BAPN; the subendothelial matrices and culture media were then processed as described under Experimental Procedures. The proteins in the isolated matrix (lanes 2 and 3) are compared with those in the culture medium (1 and 4). The positions of fibronectin (FN), thrombospondin (TS), and type IV procollagen chains [α(IV)] are indicated. Samples were resolved in 6% SDS gels under reducing conditions. Note the apparent increase in the amount of procollagen type IV chains and the 400 000-dalton band (400K) associated with the matrix when the cells were labeled in the presence of BAPN (compare lanes 2 and 3). The effect of bacterial collagenase digestion of the isolated but attached subendothelial matrix after 0 (lane 5), 1 (lane 6), and 3 h (lane 7) confirms that the 400 000-dalton band and the procollagen type IV chains are collagenous.

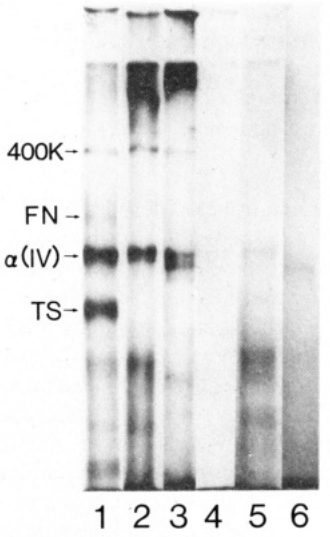

FIGURE 5: Digestion of matrix polypeptides with plasmin and pepsin. Cell-free extracellular matrices were prepared from cells labeled in the absence of BAPN with [^{14}C]proline. The matrices were incubated for 24 h at 4 °C in Tris-HCl buffer alone (lanes 1 and 4) or with 0.1 mg/mL plasmin (lanes 2 and 5) or 0.1 mg/mL pepsin (lanes 3 and 6) as described under Experimental Procedures. After addition of protease inhibitors, the residual matrices (lanes 1–3) and the supernatant of the digests (lanes 4–6) were processed for electrophoresis on a 4% SDS gel. The electrophoretic migrations of fibronectin (FN), thrombospondin (TS), type IV procollagen chains [α(IV)] and the 400 000-dalton band (400K) are indicated. Note the appearance of high molecular weight material just penetrating the running gel in the residual matrices after protease digestion (lanes 2 and 3).

mediate bands is unknown, but they may correspond to partial degradation fragments of the M_r 400 000 component or higher molecular weight cross-linked aggregates of type IV procollagen [such as the 7S region (Kuhn et al., 1981)].

To compare the relative susceptibility of the matrix polypeptides to proteolytic digestion, isolated subendothelial matrices were subjected to digestion with pepsin and plasmin (Figure 5). As expected from previous studies (Liotta et al., 1981; McKeown-Longo et al., 1984), fibronectin and thrombospondin were completely lost from the matrices and were not detectable in the supernatants of the digests. After the digestion, several lower molecular weight polypeptides were visible in the matrices and in the supernatants; these could represent fragments of the original protein. Type IV procollagen and its presumed dimer at M_r 400 000 were resistant to both proteases and remained immobilized in the matrix. Only minor amounts of the type IV procollagen were detected in the supernatants at the end of the digestions. The most unusual finding was the appearance of significant amounts of high molecular weight material (>600 000) in the matrix that originally was not detectable. After proteolysis, this high molecular weight material was observed just penetrating the top of the stacking and running gels and was not observed in the supernatant fractions. This material presumably represents highly cross-linked matrix proteins that prior to proteolysis were too large to enter the 3% stacking gel.

The stability and integrity of the isolated subendothelial matrix was examined by extraction with various reagents such as high salt, urea, and Gdn-HCl (Table I). Most of the radiolabel in the isolated subendothelial matrix was resistant to solubilization with relatively harsh solvents such as 8 M urea or 4 M Gdn-HCl. Furthermore, high salt or 0.5 M acetic acid also failed to elute much radioactivity. Moreover, extraction with 1% SDS was effective in solubilizing most of the radiolabeled material. The combination of chaotropic agents and reducing agent extracted significantly more material than the chaotropic agents alone. However, the degree of extraction in the presence of DTT mirrored the relative effectiveness of each chaotropic agent alone to solubilize the matrix. Even after extraction with SDS and DTT, a significant amount of radiolabel remained insoluble and could only be extracted after hydrolysis with NaOH. These results indicate a highly insoluble matrix that is stabilized by disulfide bonding. In addition, other types of covalent nonreducible bonds are apparently present. Including the lathyritic agent BAPN during the labeling period did not have a major impact on the extent of the extractability of the subendothelial matrix (not shown). When the extracts were examined by SDS–polyacrylamide gel electrophoresis, it was found that in the absence of reducing agent Gdn-HCl solubilized only small amounts of type IV collagen while thrombospondin and fibronectin were the major

Table I: Extraction of Radiolabeled Protein from the Subendothelial Matrix[a]

extraction conditions[b]	radioactivity solubilized (%)
1 N NaOH[c]	100
1.7 M NaCl	1
0.5 M HOAc	6
8 M urea	13
8 M urea + 2 mM DTT	30
4 M Gdn-HCl	16
4 M Gdn-HCl + 2 mM DTT	50
1% SDS[d]	68
1% SDS + 2 mM DTT[d]	89

[a] Subendothelial matrices were isolated from confluent cultures of foreskin MEC biosynthetically labeled with [^{3}H]proline as described under Experimental Procedures. The isolated matrices from 22 mm diameter dishes contained approximately 2×10^5 cpm. Data are the average of duplicate dishes and are representative of three separate experiments. [b] Matrices were extracted with the indicated reagent solution for 25 h at 22 °C. [c] Hydrolysis of isolated matrix was performed at 60 °C for 15 h. [d] Samples were heated to 100 °C for 3 min.

extracted components along with several unidentified low molecular weight proteins (<50 000) (Kramer et al., 1985). In contrast, the combination of urea or Gdn-HCl with DTT increased the solubilization of the type IV collagen and the M_r 400 000 complex.

Discussion

Microvascular endothelial cells isolated from neonatal or adult skin have been successfully introduced into culture. The endothelial origin of these cells has been verified by a number of criteria, including the presence of Weibel–Palade bodies, positive staining with antibodies to factor VIII antigen, typical endothelial cell morphology, and, finally, as described in the present study, the synthesis of type IV collagen. The presence of contaminating cell types, such as fibroblasts or smooth muscle cells, was usually negligible, as measured by cellular morphology and staining for factor VIII antigen. In addition, the failure to detect significant amounts of interstitial procollagen types I or III in the culture medium suggests minimal contamination by non-endothelial cells.

In vivo, microvascular endothelial cells lie on a continuous basal lamina that is rich in type IV procollagen (Laurie et al., 1982; Timpl et al., 1984). It is not surprising, then, that the same microvascular endothelial cells would synthesize large amounts of type IV collagen, which is subsequently assembled into a subendothelial matrix. Other investigators have reported the synthesis of type IV procollagen by human endothelial cells isolated from umbilical cord vein, aorta, and vena cava (Fry et al., 1984). However, this is the first documentation of type IV procollagen production by cultured human microvascular endothelial cells and its deposition into a subendothelial matrix. Synthesis of type IV procollagen by cultured rat epididymal capillary cells has been shown to be modulated by the nature of the culture substratum (Madri & Williams, 1983). In contrast, Sage et al. (1981) showed that bovine adrenal gland capillary cells secrete a combination of type I and III procollagens but not type IV procollagen.

That type IV procollagen appears to be the principal procollagen in the medium as well as in the subendothelial matrix of microvascular endothelial cells is based on the following observations: (1) A protein with a M_r of 180 000 comigrates with authentic type IV collagen standards purified from the human HT1080 cell line and the mouse EHS tumor matrix; the same protein frequently demonstrated splitting into a closely migrating pair of bands corresponding to pro-α1(IV) and pro-α2(IV) chains, with the expected predominance of the pro-α1(IV) chain. (2) The M_r 180 000 protein, whether in the matrix or the medium, was sensitive to bacterial collagenase and was selectively precipitated from the medium with affinity-purified antibodies to human type IV collagen. (3) The M_r 180 000 protein was resistant to limited pepsin digestion but eventually was broken down into fragments characteristic of type IV collagen (Tryggvason et al., 1980). The microvascular endothelial cells do not appear to produce significant amounts of other collagen types, since attempts at immunoprecipitation of radiolabeled culture medium with antibodies specific to collagen types I, III, or V were unsuccessful (unpublished data). Furthermore, bacterial collagenase or pepsin digestion of proteins secreted into the culture medium or deposited into the matrix did not suggest the presence of significant amounts of other collagen types. Studies are currently under way to determine if the microvascular endothelial cells synthesize type VIII collagen discovered by Sage et al. (1980).

Under nonreduced conditions, type IV procollagen in the culture medium and matrix was present as a very high molecular weight disulfide-linked complex (>600 000), which was effectively cleaved by reducing agents under denaturing conditions. The resistance of this disulfide-linked complex to pepsin digestion indicates triple helical domains and suggests it represents the heterotrimer of two pro-α1(IV) and one pro-α2(IV) chains, as found in other systems (Timpl et al., 1978; Robey & Martin, 1981; Bachinger et al., 1982). The electrophoretic mobility of this complex in SDS–polyacrylamide gels was similar to that reported by Duncan et al. (1983) for triple helical procollagen IV molecules. The same group also observed the formation of tetramers consisting of four individual triple helical procollagen molecules that are joined by disulfide bonding. It is unclear if tetramers are formed by the microvascular endothelial cells, since the presence of millimolar amounts of cystine in the culture medium may inhibit this process (Duncan et al., 1983).

Studies of the extractability of the subendothelial matrix indicate an extremely stable structure that is resistant to significant solubilization by high salt, acetic acid, 8 M urea, or 4 M Gdn-HCl. The stabilizing contribution of disulfide bonding is evident in this matrix, since reduction by DTT resulted in an increased solubilization with urea or Gdn-HCl extraction media. Basement membranes isolated from in vivo sources have demonstrated the role of disulfide bonds in the stabilization of the component macromolecules (Spiro, 1973; Kefalides, 1973; Bailey et al., 1984). In the present studies, extraction with SDS plus reducing agent was found to be the most effective method, resulting in the solubilization of nearly 90% of the labeled protein. This compares favorably to the results of Taylor & Price (1980), who reported that approximately 80% of glomerular basement membrane protein was brought into solution by extraction with SDS plus reducing agent.

Substantial high molecular weight material cross-linked by nonreducible bonds is present in the subendothelial matrix, and further analysis is necessary to determine its composition. However, it is evident that at least some of this material is collagenous, since a portion of it was sensitive to bacterial collagenase. The exact nature of the 400 000-dalton collagenous band visualized in SDS gels is uncertain but could represent a nonreducible cross-linked dimer of type IV procollagen or, alternatively, type IV procollagen cross-linked to other matrix proteins. The fact that small amounts of this material in culture medium could be specifically immunoprecipitated with antibodies to type IV collagen suggests a complex of type IV procollagen. The apparent resistance of this material to pepsin and plasmin would imply that it is a dimer of type IV procollagen and does not contain substantial amounts of noncollagenous proteins. It has been shown that type IV procollagen is present in basement membranes as high molecular weight complexes that contain disulfide and aldehyde-derived cross-links (West et al., 1980; Minor et al., 1981; Heathcote et al., 1978; Taylor & Price, 1980; Tanzer & Kefalides, 1973; Crouch & Chang, 1984).

The apparent inability of BAPN to completely inhibit the formation of nonreducible cross-links in the type IV collagen secreted into the matrix of the microvascular endothelial cells suggests either that other forms of cross-linking have occurred or that the BAPN is not effective in inhibiting lysyl oxidase. The former possibility has been proposed previously by Weber et al. (1984), who suggests that the nonreducible cross-links between the globular domains of type IV collagen (NC1) isolated from the EHS tumor may be due to transglutaminase-mediated covalent bonds. The latter possibility should also be considered since the microvascular endothelial cells

form a continuous monolayer of cells that are probably connected by tight junctions (Davison et al., 1980), thus limiting access of the reagent to the site of basal lamina formation.

The fact that chains of type IV procollagen, its dimer, and higher molecular weight material remained associated with the matrix even after prolonged protease digestion with pepsin or plasmin indicates that the collagens are associated in an insoluble network that does not require the presence of other matrix components such as laminin, fibronectin, or thrombospondin for their integrity. Indeed, it has been shown that in native basement membranes type IV collagen is resistant to plasmin whereas laminin is completely digested (Liotta et al., 1981). In the present study, digestion of isolated matrices with pepsin or plasmin caused the appearance of new high molecular weight material that previously was absent in the upper regions of the SDS gels but that still remained associated with the matrix. Chromatography of reduced SDS-extracted matrix material on Sepharose CL-4B columns confirmed the presence of a smear of material of extremely high molecular weight, estimated to be in excess of 10×10^6; nearly 90% of this high molecular weight material was sensitive to degradation by bacterial collagenase (not shown). These results suggest that the subendothelial matrix is composed of a mixture of high molecular weight proteins that are formed by intermolecular cross-linking. The cross-links include both disulfide and aldehyde-derived bonds, and possibly other as yet unidentified bonds. Recently, other workers have reported the presence of high molecular weight cross-linked type IV collagen that remains unaffected in the presence of BAPN and cannot be cleaved by reducing agents (Crouch & Chang, 1984).

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References

Alitalo, K., Vaheri, A., Kreig, T., & Timpl, R. (1980) *Eur. J. Biochem. 109*, 247–255.

Bachinger, H. P., Fessler, L. I., & Fessler, J. H. (1982) *J. Biol. Chem. 257*, 9796–9803.

Bailey, A. J., Sims, T. J., & Light, N. (1984) *Biochem. J. 218*, 713–723.

Bonner, W. M., & Laskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.

Bornstein, P., & Sage, H. (1980) *Annu. Rev. Biochem. 49*, 957–1003.

Caufield, J. P., & Farquhar, M. G. (1978) *Lab. Invest. 39*, 505–512.

Crouch, E., & Chang, D. (1984) *J. Cell Biol. 99*, 402a.

Davison, P. M., Bensch, K. G., & Karasek, M. A. (1980) *J. Invest. Dermatol. 75*, 316–321.

Davison, P. M., Bensch, K. G., & Karasek, M. A. (1983) *In Vitro 19*, 937–942.

Duncan, K. G., Fessler, L. I., Bachinger, H. P., & Fessler, J. H. (1983) *J. Biol. Chem. 258*, 5869–5877.

Engvall, E. (1980) *Methods Enzymol. 70*, 419–439.

Fessler, L. I., & Fessler, J. H. (1982) *J. Biol. Chem. 257*, 9804–9810.

Fry, G., Parsons, T., Hoak, J., Sage, H., Gingrich, R. D., Erolani, L., Nghiem, D., & Czevionke, R. (1984) *Arteriosclerosis (Dallas) 4*, 4–13.

Hassell, J. R., Robey, P. G., Barrach, H. J., et al. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4494–4498.

Heathcote, J. G., & Grant, M. E. (1981) *Int. Rev. Connect. Tissue Res. 9*, 191–264.

Heathcote, J. G., Sear, C. H. J., & Grant, M. E. (1978) *Biochem. J. 176*, 283–294.

Jaffe, E. A., Minick, C. R., Adelman, B., Becker, C. G., & Nachman, R. (1976) *J. Exp. Med. 144*, 209–225.

Kefalides, N. A. (1981) *Collagen Relat. Res. 1*, 295–312.

Kefalides, N. A., Alpen, R., & Clark, C. C. (1979) *Int. Rev. Cytol. 61*, 167–228.

Kleinman, H. K., McGarvey, M. L., Liotta, L. A., Robery, P. G., Tryggvason, K., & Martin, G. R. (1982) *Biochemistry 21*, 6188–6193.

Kleinman, H. K., McGarvey, M. L., Hassel, J. R., & Martin, G. R. (1983) *Biochemistry 22*, 4969–4974.

Kramer, R. H., & Vogel, K. G. (1984) *JNCI, J. Natl. Cancer Inst. 72*, 889–899.

Kramer, R. H., Davison, P., & Karasek, M. (1983) *J. Invest. Dermatol. 80*, 341C.

Kramer, R. H., Bensch, K. G., Davison, P. M., & Karasek, M. A. (1984a) *J. Cell Biol. 99*, 692–698.

Kramer, R. H., Davison, P. M., Bensch, K. G., & Karasek, M. A. (1984b) *J. Cell. Biochem. 8B*, 284.

Kramer, R. H., Fuh, G. M., Davison, P. M., Bensch, K. G., & Karasek, M. A. (1985) *J. Cell. Physiol. 123*, 1–9.

Kuhn, K., Wiedemann, H., Timpl, R., Risteli, J., Dieringer, H., Voss, T., & Glanville, R. W. (1981) *FEBS Lett. 125*, 123–128.

Laurie, G. W., Lebond, C. P., & Martin, G. R. (1982) *J. Cell Biol. 95*, 340–344.

Liotta, L. A., Goldfarb, R. H., Brundage, R., Siegal, G. P., Terranova, V., & Garbisa, S. (1981) *Cancer Res. 41*, 4629–4636.

Madri, J. A., & Williams, S. K. (1983) *J. Cell Biol. 97*, 153–165.

Madri, J. A., Dreyer, B., Pitlick, F. A., & Furthmayr, H. (1980) *Lab. Invest. 43*, 303–315.

Madri, J. A., Pratt, B. M., Yurchenco, P. D., & Furthmayr, H. (1984) in *Basement Membranes and Cell Movement*, pp 6–24, Pitman, London.

Maizel, J. V. (1971) *Methods Virol. 5*, 179–246.

Mans, R. J., & Novelli, G. D. (1961) *Arch. Biochem. Biophys. 94*, 48–52.

McKeown-Longo, P. J., Hauning, R., & Mosher, D. (1984) *J. Cell Biol. 98*, 22–28.

Minor, R. P., Clark, C. C., Koszalka, T. R., Brent, R. L., & Kefalides, N. A. (1982) *Int. Cell Biol.*, 109a.

Rasheed, S., Nelson-Rees, W. A., Toth, E. M., et al. (1974) *Cancer 33*, 1027–1033.

Robey, P. G., & Martin, G. R. (1981) *Collagen Relat. Res. 1*, 27–38.

Rouslahti, E., Vuento, M., & Engvall, E. (1978) *Biochim. Biophys. Acta 534*, 210–215.

Sage, H. (1982) *J. Invest. Dermatol. 79*, 515–595.

Sage, H., & Bornstein, P. (1982) *Arteriosclerosis 2*, 27–36.

Sage, H., Woodbury, R. G., & Bornstein, P. (1979) *J. Biol. Chem. 254*, 9893–9900.

Sage, H., Pritzl, P., & Bornstein, P. (1981) *Arteriosclerosis 1*, 427–433.

Salo, T., Liotta, L. A., & Tryggvason, K. (1983) *J. Biol. Chem. 258*, 3058–3063.

Spiro, R. G. (1967) *J. Biol. Chem. 242*, 1915–1922.

Tanzer, M. L., & Kefalides, N. A. (1973) *Biochem. Biophys. Res. Commun. 51*, 775–780.

Taylor, S. A., & Price, R. G. (1980) *FEBS Lett. 115*, 293–296.

Timpl, R., Martin, G. R., Bruckner, P., et al. (1978) *Eur. J. Biochem. 84*, 43–52.
Timpl, R., Martin, G., Brucker, P., Wick, G., & Wiedemann, H. (1979) *Eur. J. Biochem. 84*, 43–52.
Timpl, R., Wiedemann, H., Van Delden, V., Furthmayr, H., & Kuhn, K. (1981) *Eur. J. Biochem. 120*, 203–211.
Timpl, R., Fujiwara, S., Dziadek, M., Aumailley, M., Weber, S., & Engel, J. (1984) in *Basement Membranes and Cell Movement*, pp 25–43, Pitman, London.
Tryggvason, K., Gehran Robey, P., & Martin, G. R. (1980) *Biochemistry 19*, 1284–1289.
Vracko, R. (1974) *Am. J. Pathol. 77*, 314–346.
Weber, S., Engel, J., Wiedemann, H., Glanville, R. W., & Timpl, R. (1984) *Eur. J. Biochem. 139*, 401–410.
West, T. W., Fox, J. W., Jodlowski, M., Fretag, J. W., & Hudson, B. G. (1980) *J. Biol. Chem. 255*, 10451–10459.
Yurchenco, P. D., & Furthmayr, H. (1984) *Biochemistry 23*, 1839–1850.

Low Molecular Weight Fibroblast Collagen: Structure, Secretion, and Differential Expression as a Function of Fetal and Cellular Age†

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ABSTRACT: A unique low molecular weight collagen that was highly resistant to proteolytic degradation was originally isolated from fetal calf ligamentum nuchae fibroblasts and hence termed FCL-1 [Sage, H., Mecham, R., Johnson, C., & Bornstein, P. (1983) *J. Cell Biol. 97*, 1933–1938]. The differential expression of this protein was studied as a function both of fetal (donor) age and of subcultivation in vitro. Concomitant isolation, subculture, and metabolic radiolabeling experiments performed on cell strains from fetal calf ligament (FCL) and fetal bovine skin (FBS) representing different gestational ages (85–270 days in utero) showed that (a) FCL-1 was synthesized preferentially by fibroblasts from younger animals and (b) expression of FCL-1 diminished as a function of increased passage in culture. Levels of FCL-1, measured as percent of total radiolabeled culture medium protein that precipitated in a concentration range of 20–50% ammonium sulfate, ranged from 22% in FCL 85 cells to 7.7% in FCL 270 (term) cells. FBS fibroblasts at passages 6–10 secreted from 13% to 6% FCL-1, respectively. When cells from an 85-day fetal ligament were allowed to accumulate copious extracellular matrix in vitro, the production of FCL-1 was increased to 32%. FCL-1 was not immunoreactive with polyclonal antibodies directed toward most of the sequences of the interstitial type I and type III procollagens. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the apparent molecular weight of FCL-1 was 13 000 (on the basis of collagen peptide standards) and approximately 30 000 (on the basis of globular protein standards). Incubation with bacterial collagenase produced a stable cleavage product of M_r 8000 (by collagen standards) or 17 000 (by globular standards). In contrast, pepsin removed a small peptide of approximately 1000–2000 in molecular weight from FCL-1, and a gradual but progressive proteolysis of the collagen was observed over a period of 1–6 h. Pulse–chase studies revealed a secretion time of approximately 60 min for FCL-1, without the appearance of any processed, intermediate forms. These studies confirm that FCL-1 represents a novel member of the collagen gene family that manifests differential expression as a function of development.

The collagen gene family encompasses 10 distinct types that, collectively, include a minimum of 18 structurally unique α chains (Bornstein & Sage, 1980; Miller, 1985). In view of recent data confirming several unusual variations in the primary structure of the collagen triple helix, Miller (1985) has proposed a new classification scheme for these proteins based on three groups: group I collagens have a continuous triple helix with α chains of $M_r \geq 95\,000$; group II collagens, also composed of polypeptide chains of $M_r \geq 95\,000$, have triple helical regions interrupted by nontriple helical sequences; group III collagens contain triple-helical segments with chain lengths of $M_r < 95\,000$. Studies on the structure of collagen genes have also indicated several different groups on the basis of nucleotide sequence homology, codon usage, conservation of exon size, frequency of introns, and the total size of the gene [for a review, see Boedtker et al. (1983)]. Among the vertebrate collagens, Lozano et al. (1985) have shown that type IX collagen belongs to a novel class of collagen genes that is distinct from the class of genes encoding the fibrillar collagen types I–III.

Type I collagen, the most abundant protein in connective tissue, is essential for the later stages of normal embryonic development (Löhler et al., 1984). This requirement is due, at least in part, to the specific interactions between mesenchymal cells and their extracellular matrix (ECM),[1] of which type I and other collagens form a substantial proportion. The fetal calf ligamentum nuchae is an example of a developmental system in which the component fibroblastic mesenchymal cells

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[1] Abbreviations: BSA, bovine serum albumin; FCL, fetal calf ligament; FBS, fetal bovine skin; DMEM, Dulbecco–Vogt-modified Eagle's medium; DEAE, diethylaminoethyl; CM, carboxymethyl; DTT, dithiothreitol; ECM, extracellular matrix; β-APN, β-aminopropionitrile fumarate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris–saline, 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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