Characterization of Cell Matrix Associated Collagens Synthesized by Aortic Endothelial Cells in Culture†

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ABSTRACT: Several collagen types have been isolated and characterized from bovine aortic endothelial cells and their associated extracellular matrix. Two collagens, which comigrated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with the α1(III), α1(V), and α2(V) collagen chains, were isolated by salt precipitation from pepsin digests of cell layer proteins. Two of these chains were further purified by molecular-sieve and ion-exchange chromatography and were identified as α1(III) and α1(V) by one-dimensional peptide maps generated with mast cell protease and cyanogen bromide. In contrast to type III collagen, which was found in both the culture medium and cell layer, type V collagen appeared to be restricted to the cell layer. In addition to their occurrence as cell layer constituents, both types III and V collagens were localized to an extracellular matrix after the cells had been removed from the culture dishes by detergent. Preliminary studies based on peptide maps comparing type III collagen from the cell layer and culture medium provide evidence for structural heterogeneity within this collagen type.

Endothelial cells line the inner surface of blood vessels and are directly involved in the maintenance of a nonthrombogenic surface, as a permeability barrier between blood and underlying connective tissue stroma, and in intimal repair following vascular injury. In vivo, these cells exist as a closely spaced, flattened monolayer which lies apposed to the subendothelium. Endothelial cells also form a contact-inhibited monolayer in vitro (Gimbrone, 1976) and continue to synthesize and/or bind several compounds which are integral to hemostasis, including plasminogen activator, prostacyclin, factor VIII antigen, angiotensin-converting enzyme, and thrombin [for review, see Mason et al. (1979)].

Biosynthetic studies on endothelial cells in culture have indicated that significant amounts of fibronectin, in addition to smaller quantities of collagens and noncollagenous proteins, are secreted into the culture medium (Macarak et al., 1978; Jaffe & Mosher, 1978; Howard et al., 1976; Sage et al., 1979a). A study by Jaffe et al. (1976) on human umbilical vein endothelium in vitro indicated that these cells elaborated an extensive extracellular matrix and synthesized many of the components of the subendothelium, including basement membrane. Further studies on endothelial cell layers have revealed the presence of elastin (Carnes et al., 1979), collagens (Barnes et al., 1978; Sage et al., 1979a), and fibronectin (Birdwell et al., 1978).

The extracellular matrix plays an integral role in determining cell shape and response to growth factors and in endothelial cell polarity and orientation (Gospodarowicz & Ill, 1980; Birdwell et al., 1978; Waxler et al., 1979). In addition, it has been shown that granulocytes migrate through endothelium in culture and that certain tumor cells are capable of metastatic invasion through endothelial monolayers (Beesley et al., 1979; Kramer & Nicolson, 1979), phenomena that may involve cell surface and subendothelial structures. Platelet adherence to basement membrane (Davis et al., 1979) and to the subcellular surface of endothelial cells (Wechezak et al., 1979) also suggests a direct involvement of cell layer constituents.

In a previous study we had reported that bovine aortic endothelial cells secrete principally type III procollagen into the culture medium, and preliminary data had suggested the presence of types IV and V[1] in the cell layer (Sage et al., 1979a). In this report we demonstrate that type III collagen is also present in endothelial extracellular matrices but that type V collagen appears to be restricted to the cell layer. On the basis of mast cell protease and CNBr peptide maps, the structure of type III collagen in the cell layer appears to differ from that in the culture medium.

Material and Methods

Cell Culture and Metabolic Labeling. Adult bovine aortic endothelial cells were isolated and subcultivated according to Schwartz (1978). Both [^{3}H]thymidine-selected and nonselected cultures were used (Schwartz, 1978), and further subculture was performed as previously described (Sage et al., 1979a), except that in some cases VSP[2] serum (Biocell Laboratories) was substituted for fetal calf serum.

For preparative-scale isolation of collagen, confluent cultures between the seventh and fourteenth passage were labeled for 21–24 h in serum-free DMEM containing 50 μg/mL sodium ascorbate, 80 μg/mL β-APN, and 50 μCi/mL L-[2,3-$^3H_2$]-proline (35 Ci/mmol; New England Nuclear) or L-[5-^{3}H]-proline (29 Ci/mmol; Amersham/Searle Co.) as previously described (Sage et al., 1979a).

Isolation of Cell Layer Collagen. The procedure is summarized in Figure 1. [^{3}H]Proline-labeled culture medium was removed from the cells, and the cell layers were washed vigorously 3 times with phosphate-buffered saline containing 0.2 mM $PhCH_2SO_2F$. The cells and their associated matrix were scraped with a rubber policeman into 0.1 M acetic acid containing 0.5 μg/mL pepstatin A at 0 °C and dialyzed vs. 0.1 M acetic acid. The dialysate was then centrifuged at

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[1] The nomenclature used for types IV and V collagen chains and fragments is described in Bornstein & Sage (1980). αB has been designated α1(V), αA as α2(V), and αC as α3(V).

[2] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; DMEM, Dulbecco–Vogt modified Eagle's medium; VSP, viable serum protein; β-APN, β-aminopropionitrile fumarate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; CM, carboxymethyl; DEAE, diethylaminoethyl; BAE, bovine aortic endothelial; FITC, fluorescein isothiocyanate.

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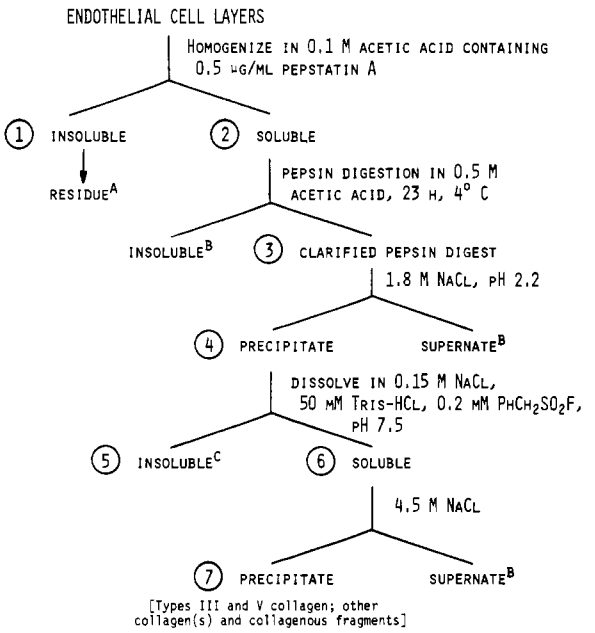

FIGURE 1: Scheme for isolation of collagen from endothelial cell layers. Superscript A indicates that the residue was obtained by pepsin digestion of the insoluble material in step 1 and contained no collagenous components > M_r 50 000 by NaDodSO$_4$–polyacrylamide gel electrophoresis analysis except aggregates which did not enter the gel. Superscript B indicates that this material contained <5% collagen by Hyp/Pro analysis. Superscript C indicates that this fraction contained from 10 to 40% of the total type V collagen which was recovered from the cell layer.

48000*g* for 30 min, and the pellet and supernate were lyophilized separately.

The lyophilized protein was suspended at 1 mg/mL in 0.5 M acetic acid at 4 °C. Pepsin (Worthington, 2× recrystallized, dissolved in water at 10 mg/mL) was added at an enzyme to substrate weight ratio of 1:10, and the digestion proceeded for 23 h at 4 °C. The digest was clarified by centrifugation at 48000*g* for 30 min, and collagenous protein was precipitated by addition of NaCl to 1.8 M, accompanied by stirring for several hours. Alternatively, the digest was neutralized with 6 N NaOH for 30 min, after which the pH was lowered to 2.2 and the proteins were precipitated at a concentration of 1.8 M NaCl by stirring overnight.

The precipitates containing cell layer collagen were isolated by centrifugation and were dissolved in and dialyzed extensively against 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5, containing 0.2 mM $PhCH_2SO_2F$. Insoluble material was removed by centrifugation, dissolved in and dialyzed against 0.1 M acetic acid, and lyophilized. Protein which was soluble in 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5, was precipitated at a concentration of 4.5 M NaCl, subsequently dissolved in and dialyzed against 0.1 M acetic acid, and lyophilized. Recovery of protein was quantitated at each step by amino acid analysis (Sage et al., 1979b).

Purification of Cell Layer Collagen by Molecular-Sieve and Ion-Exchange Chromatography. Collagens isolated by salt fractionation from clarified pepsin digests were denatured at 50 °C for 10 min and initially chromatographed on 6% agarose (Bio-Gel A-5m, Bio-Rad Laboratories) in 1 M $CaCl_2$, 50 mM Tris-HCl, 0.2 mM $PhCH_2SO_2F$, and 2.5 mM EDTA buffer, pH 7.5, at room temperature (Sage et al., 1979a). Column fractions containing α chain-sized or larger components were dialyzed against 6 M urea and 40 mM sodium acetate, pH 4.8, prior to denaturation and further chromatography on CM-cellulose (CM-52, Whatman) at 42 °C. Gradient elution was performed from 0 to 80 mM NaCl over 200 mL.

Fractions from CM-cellulose chromatography which contained putative type V collagen were dissolved in 4 M urea and 5 mM sodium phosphate buffer, pH 6.3, containing 0.1 mM $PhCH_2SO_2F$ and denatured by heating at 90 °C for 2 min. The sample was applied to an hydroxylapatite column at room temperature and washed with several column volumes of buffer. Stepwise gradient elution was performed with 0.1 M NaCl in the column buffer, followed by 0.5 M sodium phosphate, pH 6.5, in 4 M urea (Hong et al., 1979).

Other Procedures. Comparative peptide mapping was performed by using CNBr and mast cell protease cleavage, followed by NaDodSO$_4$–polyacrylamide gel electrophoresis as previously described (Sage et al., 1979a,b). Collagens which were used as standards were purified by salt fractionation and column chromatography from pepsin digests of bovine placenta (Sage et al., 1979b; Sage & Bornstein, 1979). BAE type III collagen was purified following pepsin digestion of a 20% ammonium sulfate fraction of [^{3}H]proline-labeled culture medium protein (Sage et al., 1979a).

NaDodSO$_4$–polyacrylamide gel electrophoresis was performed on discontinuous slab gels containing 0.5 M urea (Laemmli, 1970) essentially as described by Crouch & Bornstein (1978). Protein-containing bands were visualized by Coomassie blue staining or by fluorescence autoradiography (Sage et al., 1979a). Digestion with bacterial collagenase (Advance Biofactures, Form III) and Hyp/Pro determinations were carried out as previously described by Crouch & Bornstein (1978).

Radioimmune precipitation of type III collagen isolated from the cell layer was performed with affinity-purified antibodies to bovine type III procollagen (Sage et al., 1979a). Immunofluorescence studies were performed on intact BAE monolayers or on extracellular matrices from which the cells had been removed by treatment with detergent (Birdwell et al., 1978). Affinity-purified antibodies to bovine type III procollagen (Sage et al., 1979a) were used in a double-antibody procedure essentially as described by Bornstein & Ash (1977). In some experiments the cells were rendered permeable to the antibody by immersion in absolute ethanol at −70 °C for 15 s.

Results

Collagen Production by BAE Cells. The endothelial cells used in this study were free of smooth muscle cell contamination and maintained a stable, contact-inhibited monolayer at confluence for extended periods of time, as previously described (Schwartz, 1978; Sage et al., 1979a). These cells stained positively for factor VIII antigen (Jaffe et al., 1973) and did not exhibit a secondary growth pattern (Schwartz, 1978; Gospodarowicz et al., 1978; McAuslan & Reilly, 1979). Several different strains of cells, from the seventh to fourteenth passage, were utilized for studies of collagen production, including some cultures which had not been [^{3}H]thymidine-selected. BAE cells become senescent after ~40 population doublings but maintain a stable, unaltered karyotype in vitro until that time (Schwartz, 1978). The spectrum of collagen types which was observed in the cell layer was found not to vary with different cell culture conditions.

Figure 2 is a comparison of collagens found in the culture medium and cell layer of BAE cells. Pepsin treatment of cell layer protein which was soluble in acetic acid, followed by salt fractionation and NaDodSO$_4$–polyacrylamide gel electrophoresis, produced two non-disulfide-bonded components with mobilities of type V collagen chains plus two other reducible components, one migrating as an α chain (Figure 2, lanes 3 and 4). All the bands in lane 4 (Figure 2) were susceptible

Table I: Characterization of Collagen from Endothelial Cell Layers[a]

fraction[b]	dpm in collagen/mg of cellular protein ($\times 10^{-5}$)[c,d]	% collagen[d]	3Hyp/4Hyp	total Hyp/Pro
acetic acid insoluble (1)	8.15	0.41	6.3/100	1.05/100
acetic acid soluble (2)	7.45	0.84	4.0/100	2.12/100
collagen purified from acetic acid soluble fraction (7)				
(A) 6% agarose[e]			6.9/100	94/100
(B) hydroxylapatite[f]			5.8/100	100/100

[a] Measured by L-[5-^{3}H]proline incorporation. [b] Numbers in parentheses refer to purification steps as outlined in Figure 1. [c] Average of triplicate determinations. [d] Calculated according to Diegelmann & Peterkofsky (1972); protein quantitated by amino acid analysis. [e] Figure 2, lanes 3 and 4. [f] Figure 5 (inset) represents α1(V) chain.

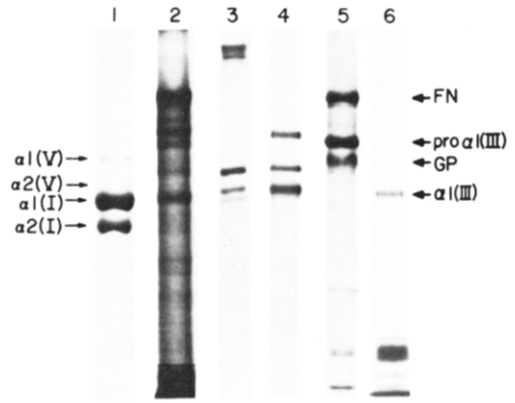

FIGURE 2: Comparison of endothelial cell layer and culture medium proteins after pepsin treatment. [^{3}H]Proline-labeled culture medium protein was precipitated in 20% ammonium sulfate, and cell layer protein was solubilized in 0.1 M acetic acid, prior to pepsin digestion. Proteins were resolved on composite 6 and 10% polyacrylamide slab gels and visualized by fluorescence autoradiography. All samples contained 50 mM DTT with the exception of that in lane 3. (Lane 1) Type I and type V collagen reference standards from pepsin-treated bovine smooth muscle cell culture medium; (lane 2) cell layer proteins prepared as in step 2 of Figure 1; (lane 3) cell layer proteins prepared by incubation with pepsin and salt precipitation (step 7, Figure 1), followed by chromatography on Agarose A-5m; (lane 4) material in lane 3 after reduction with 50 mM DTT; (lane 5) culture medium protein after precipitation in 20% ammonium sulfate; (lane 6) culture medium protein after pepsin treatment and salt fractionation as described for the cell layer material. The positions of migration of type I, III, and V collagen chains are indicated, and several biosynthetic products in endothelial cell culture medium, fibronectin (FN), type III procollagen (PC), and a noncollagenous glycoprotein (GP), are identified.

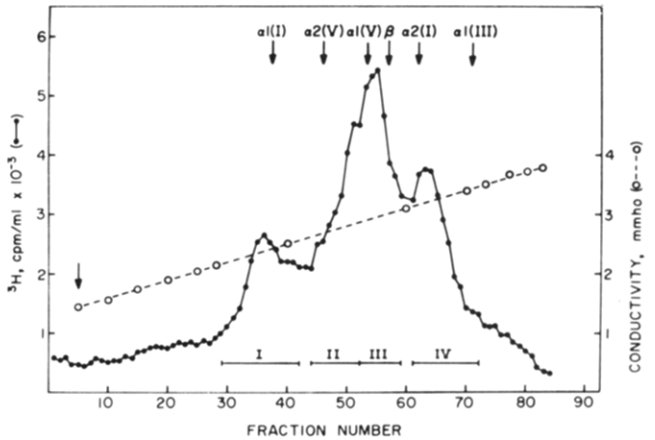

FIGURE 3: Chromatography of cell layer collagens on CM-cellulose. Collagens were isolated from clarified pepsin digests by salt precipitation and further purified by molecular-sieve chromatography. The sample (Figure 2, lane 3) was denatured and applied to a CM-cellulose column in 6 M urea and 40 mM sodium acetate, pH 4.8, at 42 °C. Gradient elution (arrow) from 0 to 80 mM NaCl was performed over 200 mL. Conductivities were read at room temperature. The positions of elution of several bovine collagen chain types are indicated. Roman numerals represent pooled column fractions.

to digestion with bacterial collagenase (data not shown). Similar treatment of culture medium protein, however, produced only an α chain-sized component after reduction, which has been identified as type III collagen (Figure 2, lane 6; Sage et al., 1979a).

Collagen production by BAE cells is low and comprises approximately 3–7% of total [^{3}H]proline-labeled protein in the culture medium after 24 h (Howard et al., 1976; Sage et al., 1979a). Most of this collagen is type III procollagen with as much as 25% present as a unique, pepsin-sensitive collagen (Sage et al., 1980). In the cell layer, collagen production as measured by [^{3}H]proline incorporation comprised ~1% of the total labeled protein (Table I; Sage et al., 1979a). Two-thirds of the cell layer collagen could be solubilized in 0.1 N acetic acid (Figure 1, step 2). When the acetic acid insoluble fraction was examined by $NaDodSO_4$–polyacrylamide gel electrophoresis, only higher molecular weight aggregates and material less than M_r 50000 were seen; after pepsin treatment, no bands were evident in the 100000–200000 molecular weight range.

Purification and Identification of Cell Layer Collagens. After salt precipitation (Figure 1, step 7), the cell layer collagens were chromatographed on Agarose A-5m to remove lower molecular weight fragments (Figure 2, lanes 3 and 4). At this stage of purification, the protein had a Hyp/Pro ratio of approximately 1:1, and 7% of the hydroxyproline was the 3 isomer (Table I). Recoveries of collagen were difficult to assess in this type of procedure but were consistent with a content of 1% collagen (w/w) in the starting acetic acid soluble fraction.

Collagens which had initially been chromatographed on 6% agarose were denatured and fractionated on CM-cellulose (Figure 3). $NaDodSO_4$–polyacrylamide gel electrophoresis of the pooled fractions is shown in Figure 4 and illustrates that several collagenous components could be resolved, although some degradation to lower molecular weight polypeptides had occurred. Peak I contained a non-disulfide-bonded α-sized collagen chain which currently remains unidentified. The second peak contained collagen chains which comigrated on $NaDodSO_4$–polyacrylamide gel electrophoresis with α1-(IV)–140K fragment after reduction (Sage et al., 1979a,b) with α1(V) and α2(V) chains. The putative type IV collagen chain has been difficult to isolate in sufficiently large quantities to permit further characterization. A collagen chain with the mobility of α1(V) on $NaDodSO_4$–polyacrylamide gel electrophoresis was partially resolved from the α2(V) chain in peak III, and this fraction was used for further purification and structural studies. A band with the mobility of an α2(I) collagen chain was also observed in peak III and was not further characterized due to its presence in limiting amounts. Since we were unable to precipitate any radioactive native cell

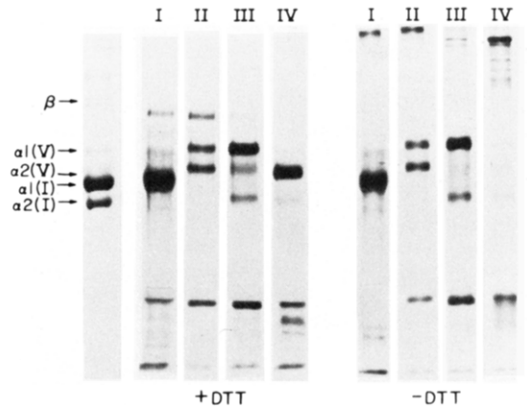

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of cell layer collagens fractionated by ion-exchange chromatography. Collagen chains, pooled as indicated in Figure 3, were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis on composite 6 and 10% slab gels with and without reducing agent and were visualized by fluorescence autoradiography. Roman numerals refer to pooled fractions from Figure 3. A collagen reference standard containing type I and V has been included.

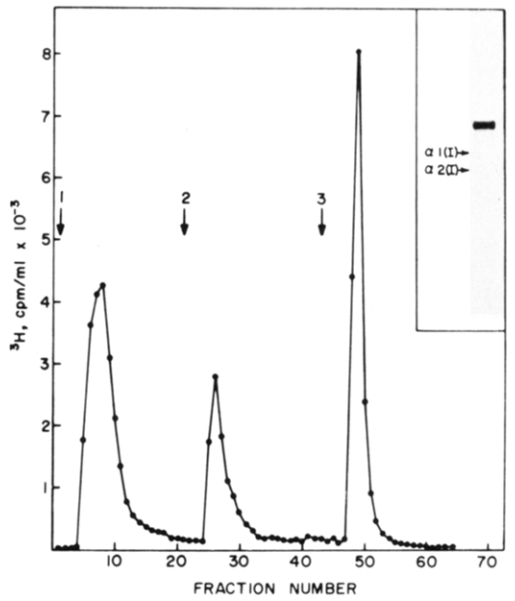

FIGURE 5: Chromatography of cell layer collagens on hydroxylapatite. The sample (Figure 3, fraction III) was denatured and applied to a hydroxylapatite column in 4 M urea and 5 mM sodium phosphate buffer, pH 6.3, containing 0.1 mM $PhCH_2SO_2F$ at room temperature. Gradient elution was stepwise. Arrows indicate elution conditions. (1) Sample loaded and column washed in buffer as described above; (2) application of 0.1 M NaCl in column buffer; (3) application of 0.5 M sodium phosphate, pH 6.5, in column buffer. (Inset) $NaDodSO_4$–polyacrylamide gel electrophoresis of material in peak 3. The positions of migration of bovine type I collagen chains are indicated.

layer proteins by using affinity-purified antibodies to type I procollagen or collagen, we conclude that this component, which was not in the starting material prior to CM-cellulose chromatography, represents a partial degradation product of another collagen chain.

The components in CM-cellulose peak III (Figure 4) were denatured and further chromatographed on hydroxylapatite as described by Hong et al. (1979). The stepwise gradient elution profile is shown in Figure 5. $NaDodSO_4$–polyacrylamide gel electrophoresis of the pooled fractions revealed that a reducible, collagenase-sensitive component which comigrated with α1(IV)–140K did not bind to hydroxylapatite (data not shown). A pure collagen chain was eluted with 0.5 M sodium phosphate, as evidenced by $NaDodSO_4$–polyacrylamide gel electrophoresis of fraction 3 (Figure 5, inset). This component had an Hyp/Pro ratio of 1:1 and contained 6% of the hydroxyproline as 3-hydroxyproline (Table I). When this chain was cleaved by mast cell protease and the digest resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis, the cleavage products comigrated with those generated by mast cell protease cleavage of the α1(V) chain purified from bovine placenta (Figure 6, compare lanes 4 and 5).

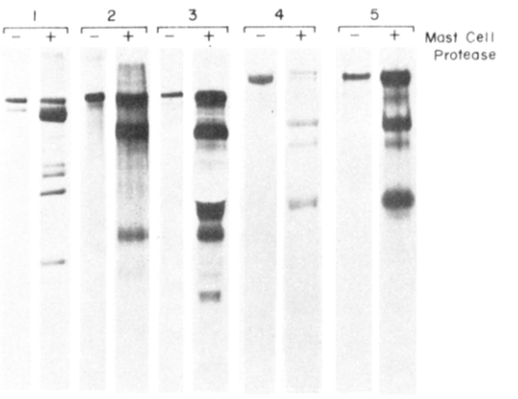

FIGURE 6: Mast cell protease cleavage of collagen purified from endothelial cell layers. Collagens were cleaved with mast cell protease, and the products were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis on a 10% slab gel under reducing conditions and visualized by staining with Coomassie blue followed by fluorescence autoradiography. Starting material is shown as incubated control. (Lane 1) Bovine type I collagen; (lane 2) type III collagen purified from BAE cell culture medium; (lane 3) cell layer collagen which was purified on CM-cellulose (Figures 3 and 4, fraction IV); (lane 4) bovine placental α1(V), purified by hydroxylapatite chromatography; (lane 5) cell layer collagen purified on hydroxylapatite (Figure 5, fraction 3).

Peak IV (Figure 4) contained an α-sized collagen chain after disulfide bond reduction which was precipitable by affinity-purified antibodies to bovine type III procollagen. This collagen chain was further purified by molecular-sieve chromatography (data not shown) and mapped after digestion with mast cell protease. When compared to the cleavage patterns of bovine types I, III, and α1(V), the cell layer collagen was found to resemble type III collagen most closely, with the exception of one major peptide and one or two minor ones which were present in the cell layer protein but not in the culture medium protein (Figure 6, compare lanes 2 and 3). The type III reference standard used was isolated from BAE culture medium and was purified by CM-cellulose chromatography after pepsin digestion. Because of the differences between the two maps, the cell layer and culture medium collagens were cleaved with CNBr, and the products were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 7). The two patterns were very similar with the exception of an additional major peptide in the cell layer collagen which migrated in a position between that of α1(I)–CB6 and that of α1(I)-CB3. However, the overall CNBr peptide pattern of the cell layer collagen was clearly related to that of type III collagen.

Immunofluorescence Localization of Type III Collagen in BAE Cell Cultures. The results of exposing BAE cells to affinity-purified antibodies to bovine type III procollagen, followed by the addition of a second antibody to rabbit IgG which was conjugated to FITC, are shown in Figure 8. While background staining using preimmune sera was negligible (Figure 8a), intercellular matrix reacted positively with the antibody, and type III collagen was visualized as fine filaments exhibiting some coalescence on certain areas of the coverslip (Figure 8b). When the cells were made permeable to antibody

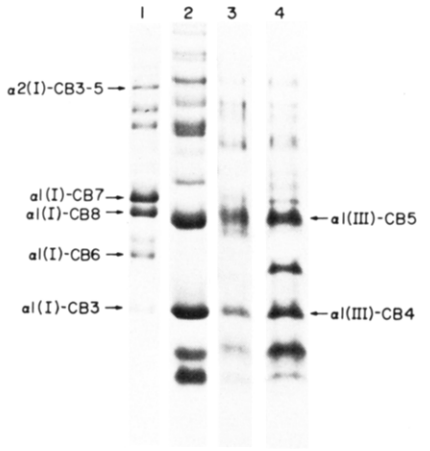

FIGURE 7: Cleavage of cell layer collagen by cyanogen bromide. Collagen isolated by CM-cellulose chromatography (Figures 3 and 4, fraction IV) and further purified by molecular-sieve chromatography was cleaved with CNBr, and the products were resolved by NaDodSO$_4$–polyacrylamide gel electrophoresis on a 12.5% slab gel in the presence of 50 mM DTT. Bovine type I and III collagens were cleaved in the same manner. The cleavage products were visualized by staining with Coomassie blue, followed by fluorescence autoradiography. (Lane 1) Bovine type I collagen; (lane 2) type III collagen purified from bovine skin; (lane 3) type III collagen purified from BAE culture medium after pepsin treatment; (lane 4) cell layer collagen. The positions of migration of certain CNBr peptides of type I and III collagen are indicated.

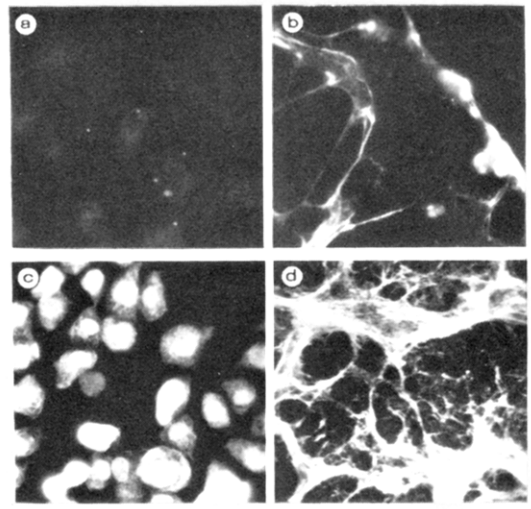

FIGURE 8: Immunofluorescence localization of type III procollagen on bovine aortic endothelial cells. Cells were seeded onto cover slips, fixed briefly in paraformaldehyde, exposed to rabbit anti-bovine type III procollagen antibodies, and stained by reaction of the immune complex with goat anti-rabbit IgG which was conjugated to FITC. (a) Intact cells exposed to preimmune serum; (b) cells as in (a) but treated with anti-type III procollagen antibodies; (c) sparsely plated cells made permeable by a brief immersion in absolute ethanol at −70 °C, followed by exposure to anti-type III procollagen antibodies; (d) confluent cells removed from the underlying matrix by gentle shaking in detergent buffer with the material remaining on the dish treated with anti-type III procollagen antibodies.

by treatment with absolute ethanol at −70 °C after fixation, type III collagen was observed to be inside the cells as well (Figure 8c). The brightly staining cells contrasted strongly with the weakly staining matrix which was sparse in these subconfluent cultures. After removal of confluent cells with detergent, the subcellular matrix reacted strongly with antibodies to type III procollagen, as shown in Figure 8d. These results support the biochemical data which have shown that type III related (pro)collagens are both secreted and cell matrix associated proteins.

Although type V collagen was not identified in this study by immunohistochemical techniques, pepsin treatment of the extracellular matrix, isolated as described in the legend of Figure 8d, produced two collagenous chains which comigrated on NaDodSO$_4$–polyacrylamide gel electrophoresis with the bovine type V collagen standard (data not shown).

Discussion

Structural characterization of the collagenous component of bovine aortic endothelial cell layers has shown that the major collagens present are types III and V. Pepsin digestion of cell layer material which was soluble in dilute acetic acid, followed by salt precipitation, produced several proteins which were sensitive to bacterial collagenase. Further fractionation of these components by molecular-sieve and ion-exchange chromatography led to the purification of α1(III) and α1(V) collagen chains which were identified by one-dimensional peptide mapping on NaDodSO$_4$–polyacrylamide gels after cleavage with mast cell protease and/or CNBr (Figures 6 and 7). Two minor collagen chains were eluted early from CM-cellulose. One, with an M_r of 140 000 after disulfide bond reduction, could be a fragment of the α1(IV) chain; the other, which was of α chain size and did not contain disulfide bonds, is currently unidentified.

When the cell layer α1(III) chain was compared to pepsin-treated α1(III) isolated from the culture medium, an additional major peptide was found in CNBr digests of the cell layer collagen (Figure 7). In addition, a different major cleavage product was observed in mast cell protease digests of cell layer type III collagen (Figure 6, lanes 2 and 3), which migrated on NaDodSO$_4$–polyacrylamide gel electrophoresis with an approximate molecular weight of 60 000 using globular protein standards. The CNBr peptide, which was slightly smaller by NaDodSO$_4$–polyacrylamide gel electrophoresis than bovine α1(I)–CB6 (216 residues) and migrated between α1(III)–CB5 and α1(III)–CB4 (Fietzek et al., 1977), could result from a consistent incomplete cleavage or could represent an alteration in primary structure. The extra peptide was present in all preparations of cell layer collagen which were analyzed, comprising several different strains of BAE cells. CNBr cleavage of type III procollagen isolated from culture medium did not produce a peptide of similar mobility on NaDodSO$_4$–polyacrylamide gel electrophoresis, indicating that this fragment was not derived from an NH_2- or COOH-terminal, non-triple-helical extension. It is also unlikely that this cleavage product arose from a contaminating low molecular weight fragment since the cell layer collagen was purified by molecular-sieve chromatography prior to digestion. Since the expected cleavage products of α1(III) were all present in the digest (Figure 7, lane 3), it is possible that the cell layer type III collagen is a mixture of two closely related forms. A small proportion of this alternate form may also exist in the culture medium [see Figure 9 of Sage et al. (1979a)]. Precedence for the existence of collagen subtypes in other tissues is provided by the observations of Kao & Foreman (1980).

Overall collagen production by BAE cells comprised <10% of the total labeled protein in these cultures (Sage et al., 1979a). Approximately 10% of the total labeled collagen was found in the cell layer, indicating that most of the endothelial cell collagen is secreted. These figures may be deceptively low in estimating the content of collagen in the extracellular matrix, however, since they are based on incorporation of radioactivity over a 24-h period and do not take into consideration the accumulation of collagen over longer periods of time. Immunofluorescence studies suggest an enrichment of type III collagen in this compartment (Figure 8). Most of the

collagen in the culture medium has been characterized as type III procollagen, with about 20–25% contributed by a unique, pepsin-sensitive collagen (Sage et al., 1979a, 1980).

In the cell layer, one-third of the counts in collagen could not be solubilized in dilute acetic acid. After pepsin digestion of this residue, only nonreducible, high molecular weight aggregates, which could be composed partially of cross-linked types III and IV collagen, and polypeptides of M_r <50 000 were obtained. Studies by Crouch et al. (1980) using amniotic fluid cells in culture have shown that type IV (pro)collagen is highly insoluble as a matrix component and is not extracted with dilute acetic acid.

Type III (pro)collagen has been localized in the culture medium, in the extracellular matrix, and within BAE cells (Figure 8). In contrast, type V collagen was found only in the cell layer and the extracellular matrix; its limits of detection in the medium, after pepsin treatment, have been estimated to be <0.1% of the radioactivity in type III collagen. Type V collagen has been characterized in smooth muscle cell and subcultured chondrocyte cell layers (Mayne et al., 1978; Benya et al., 1977), but it was not detected in either the medium or cell layer of cultures of morphologically altered ("sprouting") BAE cells (Cotta-Pereira et al., 1980). The α3(V) chain, which has been reported to occur in type V collagen isolated from vascular tissues (Brown et al., 1978; Sage & Bornstein, 1979), was observed in approximately half of the BAE strains used in this study by the criterion of mobility on NaDodSO$_4$–polyacrylamide gel electrophoresis.

Other investigators have reported different results regarding collagen types in endothelial cell layers. Barnes et al. (1978) described type I and III collagens, in addition to other unidentified chains which could have been derived from type IV collagen, in porcine aortic endothelial cell layers; these cells also secreted type I and III collagens into the culture medium. Jaffe et al. (1976) isolated two collagen chains of M_r 120 000 and 94 000 by NaDodSO$_4$–polyacrylamide gel electrophoresis after pepsin digestion of human umbilical vein cell layers. Although these were identified as type III and IV collagen, it is probable that they corresponded to α1(V) and α2(V). Normal human venous endothelial cells, which synthesize type IV procollagen, incorporate this protein preferentially into the cell layer, while their SV40-transformed counterparts secrete it into the culture medium (Kay et al., 1979).

The presence of type III and V collagen in the endothelial extracellular matrix suggests several possibilities regarding their function as basement membrane components and in phenomena related to thrombosis. Type V collagen, originally isolated from tissues rich in basement membranes (Burgeson et al., 1976; Chung et al., 1976), has been implicated as a basement membrane collagen, and immunohistochemical data have indicated a preferential association of this collagen with cell surfaces and morphologically distinct basement membranes [Madri & Furthmayr (1979); for review, see Bornstein & Sage (1980)]. However, this issue is controversial, and precise localization of type V collagen to basement membranes has not yet been demonstrated (Gay et al., 1979). Secretion of this collagen may be involved in directed cellular migration (Stenn et al., 1979) and has been shown to be under independent regulation from the synthesis of other collagen types in the chondrocyte system (Benya et al., 1978). Studies on the interaction between type V collagen and platelets have been somewhat controversial, although it appears that this collagen in fibrillar but not monomeric form is able to elicit platelet aggregation and serotonin release (Trelstad & Carvalho, 1979; Chiang et al., 1980).

The presence of type III collagen in the subendothelium (Gay et al., 1975) and its interaction with platelets [for review, see Beachey et al. (1979)] is particularly relevant to the synthesis of this collagen by BAE cells and its deposition in the extracellular matrix. Wechezak et al. (1979) have shown in an electron microscopic study that platelets adhered specifically to a trypsin- and collagenase-sensitive extracellular network of microfilaments which was exposed when endothelial cells were caused to retract from the monolayer. Fibrillar type III collagen is a potent inducer of platelet aggregation, although its effect may not be greater than that of the other interstitial collagens (types I and II) (Balleisen et al., 1979). Fibronectin has been shown to be present on the surface of platelets (Plow et al., 1979) and is known to interact with interstitial collagens (Engvall et al., 1978). Recent studies have also suggested a specific interaction between type III collagen (but not type I) and factor VIII related antigen which was followed by induction of platelet aggregation (Nyman, 1980).

The role of the extracellular matrix in certain cellular phenomena has aroused considerable interest. Experiments by Gospodarowicz & Ill (1980) have indicated that cellular proliferation and response to growth factors are influenced by the external substrate upon which the cells are maintained. Bovine aortic endothelial cells appear to be oriented with respect to their secreted extracellular material; this imposition of polarity could in turn dictate the spectrum of proteins, proteoglycans, and other components which are elaborated by these cells (Waxler et al., 1979). Conversely, a modulation in protein synthesis could be responsible for alterations in endothelial cell polarity and growth characteristics (Cotta-Pereira et al., 1980).

The interaction of endothelial cells with pericytes in capillaries could provide additional insight into the relationship between endothelial cells and their surface-associated matrix. Pericytes, which encircle capillaries and interdigitate extensively with endothelial cells (Tilton et al., 1979), have been recently described as synthesizing type III collagen (Cohen et al., 1980). Since this type of pericyte–endothelial cell interaction is greatly diminished, or possibly absent, in large vessels such as aorta, comparisons of the biosynthetic profiles of capillary and aortic endothelium would be of interest in determining the influence of extracellular matrix on protein synthesis.

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