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A Unique, Pepsin-Sensitive Collagen Synthesized by Aortic Endothelial Cells in Culture†

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ABSTRACT: A unique collagen, designated EC, has been isolated from the culture medium of adult bovine aortic endothelial cells. After diethylaminoethylcellulose chromatography of [^{3}H]proline-labeled culture medium, three non-disulfide-bonded bacterial collagenase-sensitive components with apparent M_r of 177 000 (EC 1), 125 000 (EC 2), and 100 000 (EC 3) were demonstrated. Molecular sieve chromatography, cyanogen bromide cleavage, and two-dimensional peptide mapping of radioiodinated EC fragments produced by protease digestion suggest that the lower molecular weight components originate from EC 1. Both EC 1 and EC 2 were digested by pepsin within 10 min to products of less than 60 000 molecular weight, under conditions which supported only limited proteolysis of other native collagens. A pepsin-resistant fragment of M_r 50 000, derived from a digest of EC 2, contained equal amounts of hydroxyproline and proline, suggesting that at least a portion of the endothelial collagen contains a stable, collagen-like triple helix. Comparative mapping using mast cell protease and cyanogen bromide cleavage, followed by polyacrylamide gel electrophoresis, indicates that the primary structure of this collagen differs from that of other known collagen types.

Endothelial cells line vascular channels and provide an effective permeability barrier between the blood and connective tissue stroma. These cells are disposed as a contact-inhibited monolayer which adheres to the subendothelial matrix. Vascular endothelial cells from several species have been established in culture, and these cells maintain the typical morphology of endothelial cells in vivo [Lewis et al., 1963; Jaffe et al., 1973b; Booyse et al., 1975; for a review, see Gimbrone (1976)]. Synthesis and/or specific binding of several biologically important compounds by endothelial cells, including plasminogen activator (Loskutoff & Edgington, 1977), factor VIII antigen (Jaffe et al., 1973a), prostacyclin (Weksler et al., 1977), angiotensin-converting enzyme (Hial et al., 1979), low-density lipoprotein (Stein & Stein, 1976), and glycosaminoglycans (Buonassissi & Root, 1975) have been studied in detail. Recent studies have shown that, in addition to the major biosynthetic product, fibronectin (Macarak et al., 1978; Birdwell et al., 1978; Jaffe & Mosher, 1978), the principal collagenous component secreted into the culture medium by bovine aortic endothelial cells is type III procollagen (Sage et al., 1979a). Other collagen types including type V appear to be restricted to the cell layer (Sage et al., 1979a; Sage et al., 1981a). However, different results have been obtained by other workers studying both bovine aortic endothelial cells (Howard et al., 1976) and cells from other species (Jaffe et al., 1976; Barnes et al., 1978; Kay et al., 1979).

Conflicting reports of collagen biosynthesis by endothelial cells should be resolved since questions regarding this process have important implications for several cellular functions, such as (a) platelet adhesion and production of a thrombogenic surface following endothelial injury, (b) synthesis of basal lamina, (c) attachment to and elaboration of an extracellular matrix, (d) polarity of secretion, and (e) apparent modulation of protein synthesis in vitro, including collagen type switching (Cotta-Pereira et al., 1980). In this study we present evidence for the synthesis and secretion of a unique collagen type by cultures of bovine aortic endothelial cells.

Materials and Methods

Cell Culture and Metabolic Labeling. Adult bovine aortic endothelial cells were isolated, [^{3}H]thymidine selected, and subcultured according to procedures described by Schwartz (1978). Further subcultivation was performed as previously described (Sage et al., 1979a), except that in the later stages of this work the cells were maintained in DMEM[1] containing 16% VSP bovine serum (Biocell Laboratories). Cultures ranging from primary to fourteenth passage were labeled, but those used for preparative-scale procedures were between the seventh and twelfth passage. The effects of different sera including PDS, growth factors such as FGF, and [^{3}H]thymidine selection on collagen synthesis by these cells were tested as previously described (Sage et al., 1979a).

Cultures which had just reached confluence were labeled for 21–24 h by using 50 μCi/mL L-[2,3-^{3}H]proline (35 Ci/mmol; New England Nuclear) in serum-free DMEM containing 50 μg/mL sodium ascorbate and 80 μg/mL β-APN.

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[1] Abbreviations used: DMEM, Dulbecco–Vogt modified Eagle's medium; EC, endothelial collagen; VSP, viable serum protein; PDS, bovine serum which is deficient in the platelet-derived growth factor; FGF, fibroblast growth factor; β-APN, β-aminopropionitrile fumarate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; MalNEt, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; CM, carboxymethyl; DEAE, diethylaminoethyl.

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For experiments in which Hyp/Pro ratios were to be determined, either L-[^{14}C]proline (283 mCi/mmol; New England Nuclear) or L-[5-^{3}H]proline (29 Ci/mmol; Amersham/Searle Co.) was used.

Preliminary Fractionation of Culture Medium Protein. Immediately after removal of labeled culture medium from the cells, protease inhibitors were added to a final concentration of 0.2 mM $PhCH_2SO_2F$, 10 mM MalNEt, and 2.5 mM EDTA, pH 7.5. The culture medium was clarified and subsequently chilled to 0 °C. For screening and initial characterization, small aliquots of culture medium protein were precipitated in 10% trichloroacetic acid containing 50 μg/mL BSA and 1 μg/mL pepstatin A (Protein Research Foundation, Osaka, Japan). The remainder of the [^{3}H]proline-labeled medium was stirred overnight at 4 °C in 20% ammonium sulfate (w/v), and the resulting precipitate was removed by centrifugation for 30 min at 48000*g*. Ammonium sulfate was then added to the supernatant solution to a final concentration of 50% (w/v), and the precipitate was recovered by centrifugation. The 50% ammonium sulfate supernate was dialyzed against 0.1 M acetic acid containing 0.5 μg/mL pepstatin A and lyophilized.

The 20% and 20–50% ammonium sulfate precipitates were dissolved separately in, and dialyzed against, 6 M urea and 50 mM Tris-HCl, pH 8.0, containing 0.2 mM $PhCh_2SO_2F$ and 2.5 mM EDTA at 4 °C. Culture medium proteins were subsequently resolved on DEAE-cellulose (DE-52, Whatman) (Sage et al., 1979a). All column fractions were dialyzed against 0.1 M acetic acid containing 0.5 μg/mL pepstatin A and lyophilized.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis and Fluorescence Autoradiography. Proteins were resolved on discontinuous polyacrylamide slab gels containing 0.5 M urea (Laemmli, 1970; Studier, 1973), as described by Crouch & Bornstein (1978). The bands were visualized either after staining with Coomassie Brilliant Blue R or after processing for fluorescence autoradiography (Bonner & Laskey, 1974; Laskey & Mills, 1975). A scanning densitometer was used to measure protein-containing bands in the linear absorbance range.

Bacterial Collagenase Digestion. Protein isolated by DEAE-cellulose chromatography was dissolved in 0.1 M acetic acid containing pepstatin A at 4 °C and dialyzed into 0.1 M Hepes, pH 7.4, containing 5 mM $CaCl_2$, 0.2 mM $PhCH_2SO_2F$, and 10 mM MalNEt. Alternatively, trichloroacetic acid precipitates of culture medium or column fractions were suspended in 50 μL of 1 N NaOH plus 100 μL of a 10× concentration of the above buffer. All samples were brought to 950 μL with 1× buffer or distilled water and spun briefly at room temperature in a microfuge (Beckman). Bacterial collagenase (50 μL) (Advance Biofactures, form III), which was dissolved in 0.33 M calcium acetate and 0.025 M Tris-HCl, pH 7.4, at a final concentration of 100 μg/mL, was added to each sample; controls received 50 μL of buffer. The samples were incubated at 37 °C for 2 h, after which they were chilled to 0 °C and precipitated in 10% trichloroacetic acid. To the pellets were added 5 μL of 1 N NaOH plus 45 μL of $NaDodSO_4$–polyacrylamide gel electrophoresis sample buffer containing 50 mM DTT (Laemmli, 1970), and aliquots were measured by liquid scintillation counting prior to analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis.

Pepsin Digestion. Lyophilized samples containing (1) salt-extracted lathyritic rat skin collagen, (2) type III procollagen isolated by DEAE-cellulose chromatography of endothelial cell culture medium, and (3) DEAE-cellulose fractions containing other collagenous components were dissolved at 0.1–0.5 mg/mL in 0.1 M acetic acid, pH 2.8, at 4 °C. The solutions were clarified in a microfuge and subsequently stirred in an ice bath. Aliquots were removed at 0 min, prior to addition of pepsin (Worthington, 2× recrystallized, dissolved in water at 10 mg/mL) at an enzyme to substrate weight ratio of 1:100. At designated time points, equal aliquots were removed and immediately frozen in a dry ice–ethanol bath prior to lyophilization. After 16 h, the remainders of the digests were spun, and both the pellets and supernates were lyophilized. The digestion products were then analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

A fragment of endothelial collagen (EC) which was stable after 18 h of pepsin treatment was purified from a clarified digest by precipitation in 10% NaCl at acidic pH, followed by dissolution in 0.15 M NaCl and 50 mM Tris-HCl, pH 7.5.

Hyp/Pro Determination of Endothelial Collagens. Different molecular weight forms of EC which had been labeled with either [5-^{3}H]proline or L-[^{14}C]proline were resolved on $NaDodSO_4$–polyacrylamide gel electrophoresis and the gel was stained with Coomassie blue. Bands corresponding to these proteins were cut from the gel and washed with four successive changes of 25% 2-propanol, followed by three changes of 10% methanol, by vigorous shaking at room temperature (Elder et al., 1977). The gel slices were lyophilized and subsequently suspended in 0.5 mL of 0.05 M ammonium bicarbonate, pH 8. Proteinase K (20 μg) (EM Biochemicals) was added to each preparation, and the incubation was continued for 18 h at 37 °C. The supernate was then lyophilized and hydrolyzed in 0.5 mL of 6 N HCl at 108 °C for 24 h. Alternatively, protein was extracted from the gel after repeated enzyme additions for up to 48 h, as well as by diffusion into bicarbonate buffer containing no enzyme. The residual gels were solubilized in 0.2 mL of 60% perchloric acid plus 0.4 mL 30% of hydrogen peroxide at 60 °C for 16 h and were subsequently counted to assess recovery (Limeback & Sodek, 1979). Type I procollagen labeled with [^{3}H]proline was also analyzed by this method.

Purification of EC by Molecular Sieve and CM-cellulose Chromatography. Lyophilized fractions from DEAE-cellulose chromatography which contained EC were dissolved in 0.1 M acetic acid containing pepstatin at 4 °C and dialyzed at room temperature into 1 M $CaCl_2$ and 50 mM Tris-HCl, pH 7.5, containing 0.2 mM $PhCH_2SO_2F$ and 2.5 mM EDTA. The sample was clarified by centrifugation and denatured by heating at 50 °C for 15 min, prior to chromatography on 6% agarose (Bio-Gel A-5m, BioRad Laboratories) at room temperature. The column (1.5 × 165 cm) was calibrated by using pepsin-treated bovine type I collagen and reduced and alkylated type IV collagen fragments, as well as with rat skin α1(I)CB7 and α1(I)CB6. CM-cellulose chromatography (CM-52, Whatman) was performed at 42 °C in 6 M urea and 40 mM sodium acetate, pH 4.8. The sample was denatured before chromatography and was eluted with a linear gradient of 0–80 mM NaCl in 200 mL.

Comparative Peptide Mapping. Chromatographically pure EC was cleaved with mast cell protease and with CNBr (Pierce) according to previously described procedures (Sage et al., 1979b). The digestion products were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis and the peptide patterns compared to those of types I, III, IV, and V collagens, the α1(IV)-140K fragment[2] (Sage et al., 1979b), and the

[2] The nomenclature used for type IV and type V collagen chains and fragments is described in Bornstein & Sage (1980).

α1(V) chain [purified by hydroxylapatite chromatography according to Hong et al. (1979)]. All collagens were isolated from pepsin digests of bovine placenta.

EC 1 and EC 2 were also compared by a two-dimensional peptide mapping technique as described by Elder et al. (1977) and further modified for collagens (Sage et al., 1981b). Briefly, the proteins were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis and the Coomassie blue stained bands were cut from the gel and radioiodinated with the Bolton–Hunter reagent (New England Nuclear). The gel slices were incubated for 18 h at 37 °C with 20 μg of proteinase K (EM Biochemicals), and the resulting digest was lyophilized and characterized by thin-layer electrophoresis, followed by thin-layer chromatography on precoated cellulose plates.

Immunochemistry. Immune precipitations using the double-antibody technique were performed on radiolabeled culture media and on chromatographically purified EC as described by Crouch et al. (1978). IgG fractions of antisera directed toward bovine α_2-macroglobulin (Behring Diagnostics), porcine angiotensin II converting enzyme (a gift from Dr. L. Slakey), bovine and sheep α-elastin (gifts from Drs. R. Mecham and J. Davidson, respectively), mouse laminin and type IV procollagen (gifts from Dr. G. R. Martin), and bovine type I, IV, and V collagens were tested, as well as affinity-purified antibodies to human cold-insoluble globulin and bovine type III procollagen.

Results

Preliminary Characterization of Endothelial Collagen (EC). The endothelial cells used in this study were free of smooth muscle cell contamination and maintained a stable, contact-inhibited monolayer at confluence which was uniformly positive for factor VIII antigen as determined by immunofluorescence staining (Jaffe et al., 1973a; Schwartz, 1978). The cells typically undergo 35–40 doublings, during which they manifest a stable karyotype, before reaching senescence (Schwartz, 1978). At no time was a secondary growth pattern observed (Schwartz, 1978; McAuslan & Reilly, 1979; Gospodarowicz et al., 1978a,b), and cultures containing senescent cells were not used in these experiments.

Previous experiments have established that collagen production by bovine aortic endothelial cells comprises 3–7% of the total [^{3}H]proline-labeled protein in the culture medium (Howard et al., 1976; Sage et al., 1979a). Most of this collagen is type III procollagen, with ~10% as types IV and V in the cell layer (Sage et al., 1979a). However, metabolic labeling of these cells with [^{3}H]proline, followed by ammonium sulfate fractionation of the culture medium, revealed an additional collagenous component which migrated similarly to type IV procollagen on $NaDodSO_4$–polyacrylamide gel electrophoresis but which did not contain disulfide bonds (Figure 1, lanes 3–5 and 8–10). This protein was sensitive to bacterial collagenase under conditions in which neither fibronectin nor a noncollagenous glycoprotein was cleaved (Figure 1, lanes 6 and 7). Unlike most procollagens and collagens which are precipitated at 20% (w/v) ammonium sulfate, e.g., type III procollagen (Figure 1, lane 3), the endothelial collagen was found in approximately equal amounts in 20% and 20–50% ammonium sulfate fractions and in saturated solutions of ammonium sulfate (Figure 1, lanes 3–5 and 8–10). Labeling experiments utilizing [2-^{3}H]mannose indicated that EC 1 was a glycoprotein. Immune titration of dialyzed culture medium protein with antibodies to angiotensin-converting enzyme, laminin, elastin, and α_2-macroglobulin failed to precipitate any radioactivity over background under conditions in which antibodies to cold-insoluble globulin specifically precipitated fibronectin (data not shown).

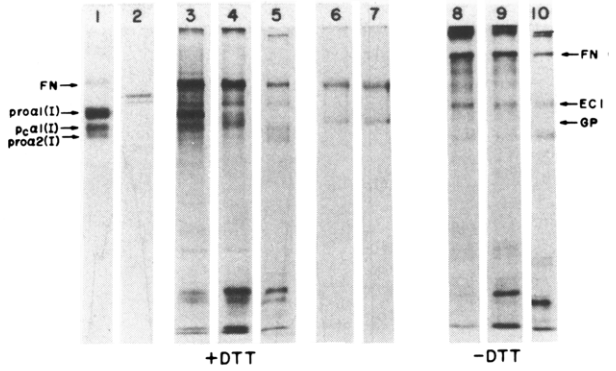

FIGURE 1: Fluorescence autoradiograms of [^{3}H]]proline-labeled proteins in endothelial cell culture medium. Culture medium proteins were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis on composite (6 and 10%) slab gels before and after reduction with DTT. Lane 1, culture medium from human amniotic fluid fibroblasts after precipitation in 10% trichloroacetic acid; lane 2, type IV procollagen standard, isolated chromatographically from human amniotic fluid AF cell culture medium (Crouch & Bornstein, 1979); lane 3, endothelial cell culture medium, after precipitation in 20% ammonium sulfate; lane 4, 20–50% ammonium sulfate precipitate; lane 5, 50% ammonium sulfate supernate; lane 6, collagenase digest of protein in lane 3; lane 7, collagenase digest of protein in lane 4; lane 8, lane 3 unreduced; lane 9, lane 4 unreduced; lane 10, lane 5 unreduced. EC 1 is identified as well as the positions of migration of fibronectin (FN), a noncollagenous glycoprotein (GP), and type I procollagen chains.

Pulse–chase studies using a 1-h pulse indicated the presence of EC 1 in the culture medium 2 h after the initiation of a chase (data not shown). Processing to lower or higher molecular weight components was not observed, either after a 20-h chase or during longer pulse periods.

Purification of Endothelial Collagen. Since type III procollagen and its conversion products could effectively be removed by precipitation in 20% ammonium sulfate, most of the EC was purified from 20 to 50% ammonium sulfate fractions. When these culture medium proteins were chromatographed on DEAE-cellulose under conditions in which procollagens and collagens retain their native conformation, the elution profile shown in Figure 2 was generated. $NaDodSO_4$–polyacrylamide gel electrophoresis indicated that a large proportion of the radioactivity which did not bind to the DEAE-cellulose was comprised of a band migrating slightly faster than pro α1(I); a less prominent band, which was not always apparent, migrated like an α chain (Figure 3, fraction I). These components which are designated EC 2 and EC 3, respectively, do not contain disulfide bonds (Figure 3). EC 1 was eluted at the beginning of the salt gradient (Figure 2; Figure 3, fraction II), although in some cases due to minor fluctuations in buffer conductivity, it did not bind to DEAE-cellulose (see Figure 7, lane 1). The band designated EC 1 was not affected by reduction (Figure 3). Both EC 1 and EC 2 were degraded by bacterial collagenase under conditions in which collagenous proteins but not fibronectin were specifically cleaved (Figure 4). Peak III in Figure 2 contained fibronectin (Figure 3, fraction III) and peak IV contained a noncollagenous protein of M_r 43 000 (H. Sage and P. Bornstein, unpublished experiments). As we will show below, our evidence indicates that EC 2 and EC 3 are derived from EC 1.

Difficulties were encountered in the purification of endothelial collagen using limited pepsin digestion until it became apparent that both native EC 1 and EC 2 were labile to this enzyme. Figure 5 shows a time course of pepsin digestion of EC 2. Within 1 min an α-chain-sized component was pro-

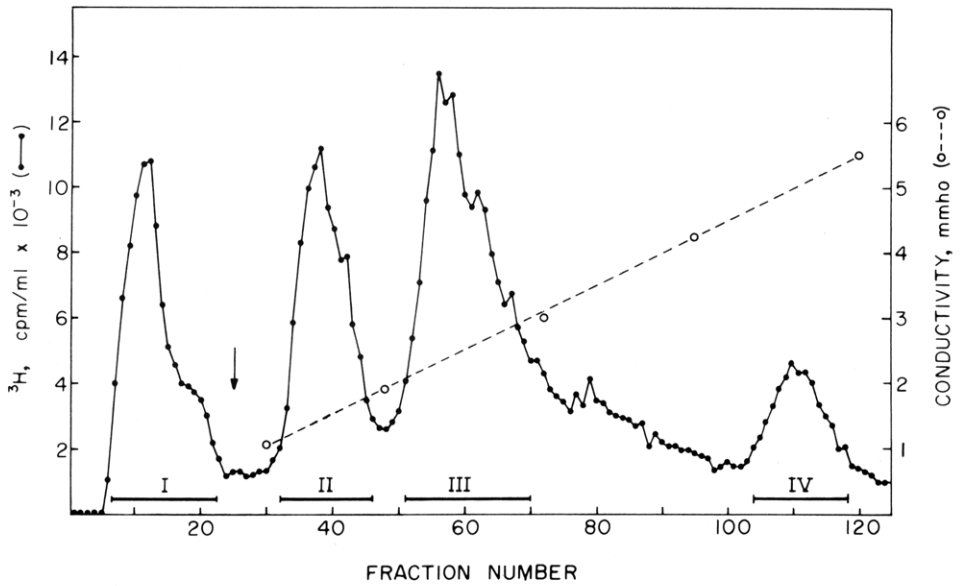

FIGURE 2: DEAE-cellulose chromatography of endothelial cell culture medium protein. [^{3}H]Proline-labeled medium protein which was fractionated by precipitation with 20% followed by 50% ammonium sulfate was chromatographed on DEAE-cellulose in 6 M urea and 50 mM Tris-HCl, pH 8.0, containing protease inhibitors. Conductivities were read at 4 °C. The arrow indicates start of gradient elution (0–200 mM NaCl over 400 mL). Roman numerals indicate pooled fractions.

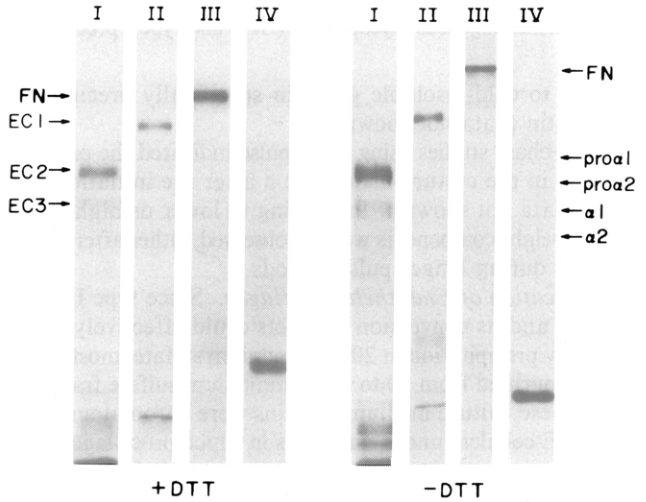

FIGURE 3: NaDodSO$_4$–polyacrylamide gel electrophoresis of culture medium proteins (20–50% ammonium sulfate fraction) which were separated by DEAE-cellulose chromatography. Proteins were resolved on a 6 and 10% composite slab gel both in the presence and in the absence of DTT. Roman numerals correspond to the peak fractions indicated in Figure 2. Fibronectin (FN) and endothelial collagens (EC 1, 2, and 3) are identified, and the positions of migration of type I procollagen and collagen chains are indicated.

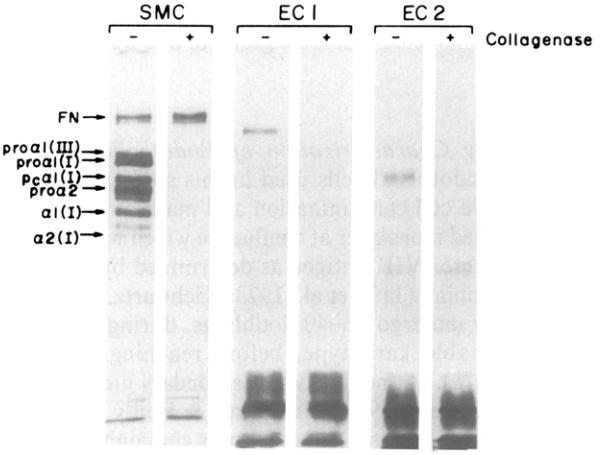

FIGURE 4: Fluorescence autoradiograms of EC after digestion with bacterial collagenase. [^{3}H]Proline-labeled bovine smooth muscle cell (SMC) culture medium was precipitated in 10% trichloroacetic acid; EC 1 and EC 2 were DEAE-cellulose fractions (Figure 2, peaks II and I, respectively). Samples were incubated with enzyme, and equal aliquots of the digestion products, precipitated in 10% trichloroacetic acid, were resolved on 6 and 10% composite slab gels under reducing conditions. Fibronectin (FN) and procollagen and collagen chains are identified.

duced which was degraded after 5 min to fragments less than M_r 60000. The same results were obtained with EC 1, except that the transitory fragment produced was slightly larger than an α chain (data not shown). During incubation with the enzyme, some material aggregated and became insoluble; however, NaDodSO$_4$–polyacrylamide gel electrophoresis showed that these aggregates all consisted of low (<60000) molecular weight components (data not shown). Pepsin treatment of either type I or type III procollagen, which had also been isolated from cell culture medium by DEAE-cellulose chromatography, produced α chains (Figure 5, lanes 3B and 4).

After digestion of EC 2 with pepsin for 18 h, a stable product was generated. Purification of this fragment was achieved by chromatography of the digest on 6% agarose (Figure 6). A molecular weight calibration curve plotted from the data in Figure 6 indicated an M_r of 50000 for the EC 2 pepsin-resistant fragment, shown eluting as peak I, which was judged pure by the criterion of NaDodSO$_4$–polyacrylamide gel electrophoresis (Figure 6, inset).

Incubation of several preparations of both EC 1 and EC 2 with human skin collagenase and with mast cell protease resulted in less than 2% cleavage. Under the same conditions, the collagenase cleaved type I collagen and type III procollagen to the extent of 90% or more, but these collagens in their native conformation were also resistant to mast cell protease.

Relationship of EC 1, EC 2, and EC 3. To this point we did not have evidence that the EC components were all derived from a common molecule (possibly EC 1). Pulse–chase studies had not revealed any processing of higher molecular weight components to EC 1 or EC 2, and careful quantitation of the purification steps for EC involving ammonium sulfate fractionation, dialysis, and DEAE-cellulose chromatography had shown that there was no artifactual proteolysis which could

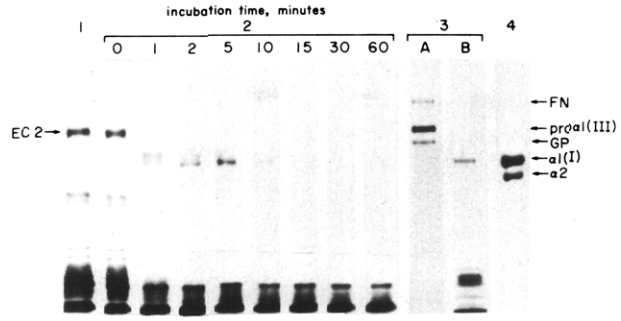

FIGURE 5: Fluorescence autoradiograms of a time course of pepsin digestion of EC 2 isolated by DEAE-cellulose chromatography. The digestion products were analyzed on a 5 and 8% composite slab gel in the presence of DTT. Lane 1, EC 2 (starting material); lanes within section 2 represent enzymatic digestions for the indicated times; section 3, lane A, fraction from DEAE-cellulose chromatography containing fibronectin (FN), type III procollagen, and a noncollagenous glycoprotein (GP); lane B, lane A after digestion with pepsin for 16 h; lane 4, type I procollagen after digestion with pepsin for 16 h. α1(I) and α2 chains are identified.

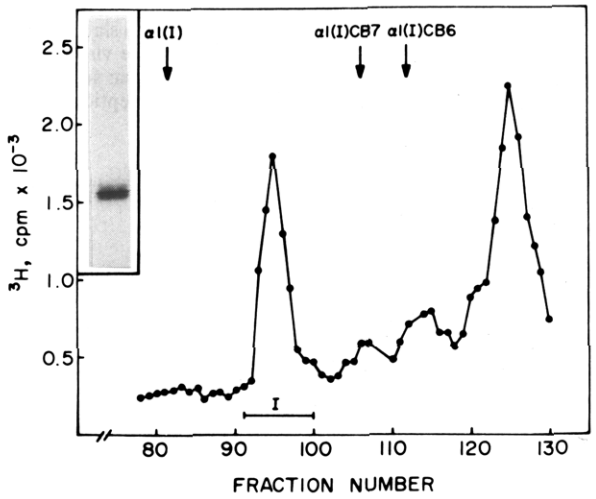

FIGURE 6: Molecular sieve chromatography of an 18-h pepsin digest of EC 2. The digest was chromatographed on agarose A-5m in 1 M $CaCl_2$, pH 7.5; molecular weight markers are shown. Peak I was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as shown in inset. Inset: $NaDodSO_4$–polyacrylamide gel electrophoresis of peak I performed on a 6–10% composite slab gel under reducing conditions.

account for conversion of one form of EC to another during these initial isolation procedures (data not shown).

To address this question, we applied a fraction containing EC 1, EC 2, and EC 3, obtained by DEAE-cellulose chromatography (Figure 7, lanes 1 and 4) to a 6% agarose column equilibrated in 1 M $CaCl_2$, pH 7.5 buffer, containing protease inhibitors. The major peak which eluted between collagen standards of 140 000 and 95 000 molecular weight contained an equal mixture of EC 2 and EC 3 (Figure 7, lanes 2 and 5), even though the starting material contained much more EC 2 than EC 3. When this peak was rechromatographed on 6% agarose, an α-chain-sized component was recovered which contained principally EC 3 (Figure 7, lanes 3 and 6). This experiment suggests that EC 3 may be derived from EC 2. In several chromatographic runs, EC 1 was never recovered, but we cannot exclude conversion of EC 1 to EC 2 or EC 3.

Hydroxyproline to proline ratios for EC 1, EC 2, and the EC 2 pepsin-resistant fragment were determined (Table I). Due to problems of poor recovery and lability during molecular sieve chromatography, the method of digestion of the protein directly from the gel slice prior to hydrolysis was utilized. This technique gave reliable values for [^{3}H]proline-labeled α2(I)

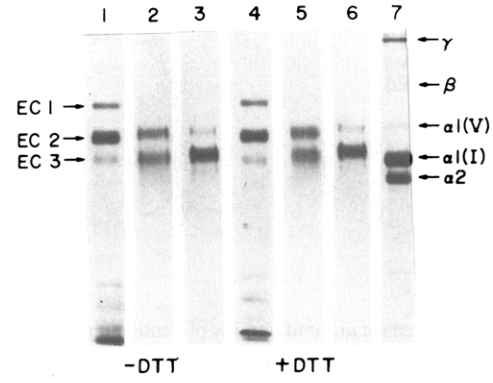

FIGURE 7: $NaDodSO_4$–polyacrylamide gel electrophoresis of [^{3}H]-proline-labeled endothelial cell culture medium collagens fractionated by molecular sieve (6% agarose) chromatography. Proteins were resolved on 6 and 10% composite slab gels in the presence and absence of DTT. Lane 1, starting material containing a mixture of EC 1, EC 2, and EC 3; lane 2, major peak from molecular sieve chromatography; lane 3, material in lane 2 rechromatographed on 6% agarose; lanes 4–6, lanes 1–3 after reduction; lane 7, a reference standard of collagen chains.

Table I: Hydroxyproline/Proline Ratios for Endothelial Collagen[a]

protein	3-Hyp/4-Hyp	total Hyp/Pro	recovery[f] (%)
α2(I)[b]	2.7:100	91:100	54
EC 1[c,d]	9.5:100	54:100	67
EC 2[c]	8.8:100	97:100	63
EC-PF[e]	6.6:100	105:100	

[a] Cells were labeled with 50 or 20 μCi/mL L-[5-^{3}H]proline or [^{14}C]proline, respectively, for 21–24 h, as described under Materials and Methods. [b] Type I collagen was isolated from culture medium of human amniotic fluid fibroblasts (Crouch & Bornstein, 1978), and α2(I) was purified by CM-cellulose chromatography. [c] Eluted from the gel slice by using proteinase K. [d] Weighted average of values determined from two separate preparations. [e] Pepsin-resistant fragment of EC 2 (M_r 50 000), purified by molecular sieve chromatography. [f] Calculated as (cpm solubilized × 100)/(cpm solubilized + cpm remaining in gel slice).

and permitted calculation of highly reproducible ratios among the collagens tested. However, the error in quantitating recoveries from the gel slices is high due to the difficulty in counting accurately the solubilized gel which is highly fluorescent. The values for percent recovery reported in Table I are therefore artificially low. EC 1 contained ~10% of hydroxyproline as the 3-Hyp isomer, while the value for EC 2 was 9% and that for the pepsin-resistant fragment, ~7%. There was a significant increase in the total hydroxyproline to proline ratio for EC 2 as compared to EC 1, and the pepsin-stable, 50 000 molecular weight fragment also contained nearly equal amounts of the two imino acids. To ascertain that enzymatic digestion of the gel slice did not result in selective solubilization of certain sequences of the denatured protein, we eluted EC 1 from the gel using only bicarbonate buffer, prior to hydrolysis. Although the recovery was quite low (<10%), both the 3-Hyp/4-Hyp and the total Hyp/Pro ratios were identical (less than 2% variation) with those derived from protease-treated samples.

Molecular weight calibration curves based on type I procollagen and its intermediates indicated an M_r of 177 000 for EC 1 and 125 000 for EC 2 (data not shown). EC 1 exhibited a slightly higher mobility on $NaDodSO_4$–polyacrylamide gel electrophoresis than reduced type IV procollagen, while EC 2 comigrated with pro α2(I) (Figures 1 and 3) and with pepsin-treated α1(V) (Figure 7). Although differences in apparent molecular weight between EC 1 and EC 2 would be

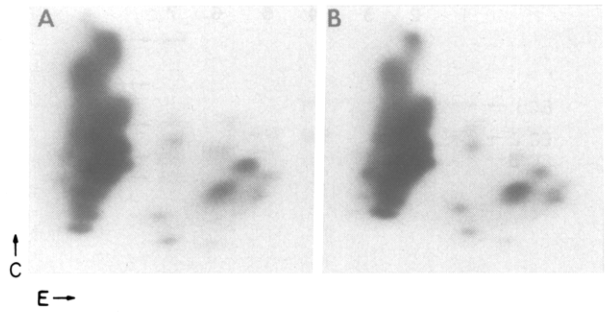

FIGURE 8: Fluorescence autoradiograms of peptide maps of EC 1 and EC 2. Preparations containing EC 1 and EC 2 were resolved on $NaDodSO_4$ gels, and the Coomassie blue staining bands corresponding to these proteins were cut from the gel and radioiodinated. The gel slices were digested with proteinase K and the digestion products mapped by two-dimensional thin-layer electrophoresis and chromatography on cellulose plates. (A) EC 1; (B) EC 2. The origin is in the lower left corner. Electrophoresis was performed in the first dimension (E) and chromatography in the second dimension (C).

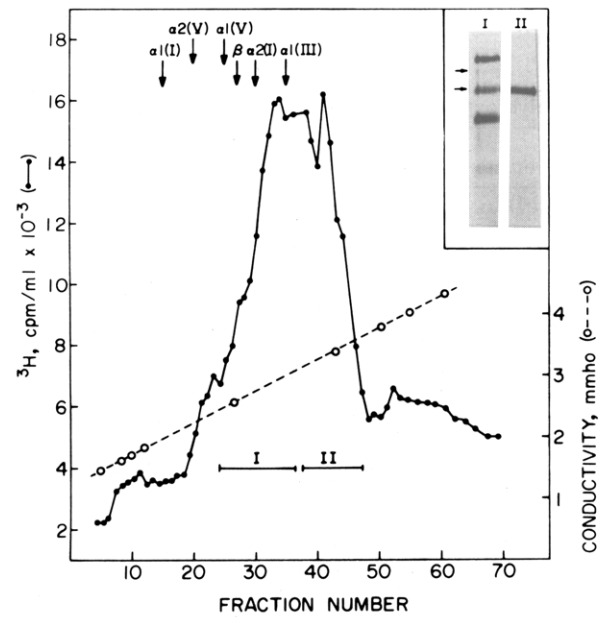

FIGURE 9: Purification of EC 2 by CM-cellulose chromatography. A sample containing primarily EC 2 (Figure 7, lane 1) was chromatographed on CM-cellulose in 6 M urea and 40 mM sodium acetate, pH 4.8, at 42 °C. Conductivities were read at room temperature. The arrow indicates start of gradient elution (0–80 mM NaCl over 200 mL). Roman numerals refer to pooled peak fractions. Positions of elution of standard bovine collagen chains are indicated. Inset: $NaDodSO_4$–polyacrylamide gel electrophoresis of EC 2 purified by ion-exchange chromatography. Proteins in peak fractions I and II were resolved on a 6 and 10% composite slab gel under reducing conditions. Arrows indicate position of migration of pro α1(I) and pro α2(I), respectively.

expected to cause some dissimilarity in a two-dimensional peptide map of each chain, the resulting "fingerprints" after radioiodination and protease cleavage exhibited a high degree of identity, suggesting that EC 2 is a derivative of EC 1 (Figure 8). Other collagen chain types are readily distinguishable from one another when mapped by this technique (Sage et al., 1981b).

Structural Comparison of EC to Other Collagen Types. EC 2 was purified to homogeneity by chromatography on CM-cellulose, as shown in Figure 9. The leading half of the broad peak contained a mixture of EC 1, EC 2, and EC 3, while the trailing half contained only EC 2, which eluted relatively late in the salt gradient after bovine α1(III) (Figure 9 and inset). This preparation of denatured EC 2 was cleaved with mast cell protease, and its cleavage pattern was compared by $NaDodSO_4$–polyacrylamide gel electrophoresis with those of other bovine collagen types. As shown in Figure 10, the EC 2 pattern differed from those of type III, α1(IV)-140K, and α1(V). Bovine types I, II, and V were also compared, and longer incubation times with the enzyme were examined; in all cases, the EC 2 pattern was unique (data not shown).

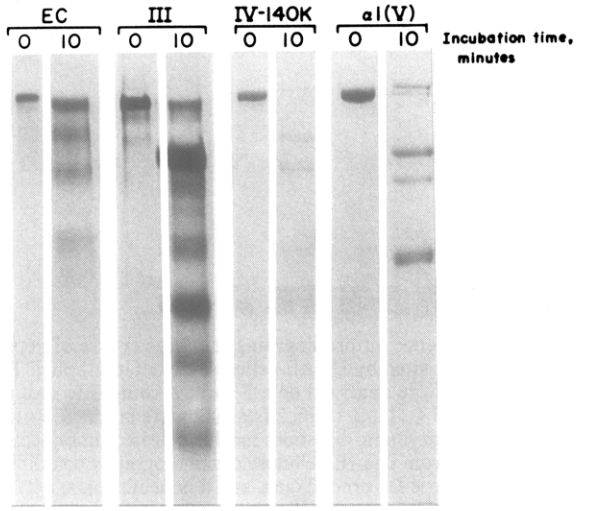

FIGURE 10: Mast cell protease cleavage of EC 2 and other bovine collagen types. Collagens were denatured and incubated both with and without mast cell protease for 10 min. The digestion products were precipitated in 10% trichloroacetic acid and resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis on a 10% slab gel under reducing conditions. Protein-containing bands were visualized by staining with Coomassie blue. The dried gel was then subjected to fluorescence autoradiography in order to visualize peptides derived from EC 2.

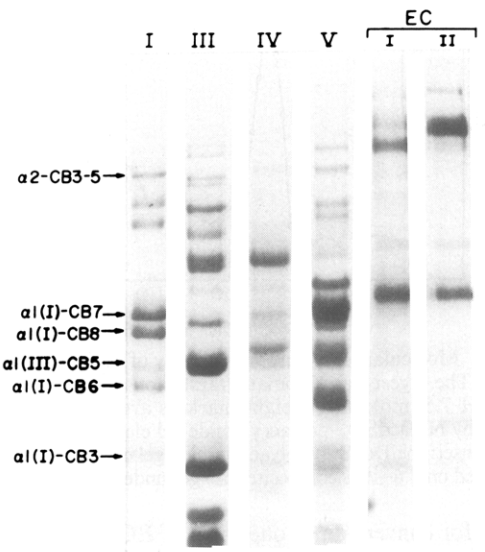

FIGURE 11: CNBr cleavage of EC and other bovine collagen types. Peaks I and II from CM-cellulose chromatography of EC (Figure 9) and bovine collagen types I, III, IV, and V were cleaved with CNBr. The products were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis on a 12.5% slab gel in the presence of DTT. Proteins were visualized by staining with Coomassie blue, followed by fluorescence autoradiography. Some of the major CNBr peptides of α1(I), α2(I), and α1(III) are indicated.

Figure 11 shows one-dimensional peptide maps produced by cleavage of collagen types with CNBr, followed by $NaDodSO_4$–polyacrylamide gel electrophoresis. The EC 2 pattern again appeared to be unique (EC, lane II). This figure also

suggests a close structural relationship among EC 1, EC 2, and EC 3, as EC lane I contained as starting material a mixture of approximately equal amounts of the three proteins (Figure 9, inset, lane II). With the exception of a pair of large, cleaved peptides which appear to shift in intensity, EC lanes I and II (Figure 11) appear very similar.

Immune precipitation of either purified EC or dialyzed culture medium protein with antibodies to bovine type I, III, IV, and V collagens and to bovine type III and mouse type IV procollagens supported the results of structural studies, indicating that EC collagen differed from other known collagen types. Background radioactivity by using preimmune sera was less than 5% of the total counts, and no more than 2% of radioactivity above background was precipitated by using any of the anticollagen antibodies except for anti type III procollagen, which specifically reacted with 7–8% of the nondialyzable counts in the culture medium. The material that reacts with this antibody has been characterized previously as type III procollagen (Sage et al., 1979a).

Discussion

Bovine aortic endothelial cells synthesize and secrete into the culture medium a previously undescribed collagen which has been designated EC. This protein is distinct from the type III procollagen that has been characterized as the major secreted collagenous product of these cells (Sage et al., 1979a). Evidence attesting to the unique primary structure of EC comes from several types of peptide mapping studies. Analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis of fragments produced by CNBr and mast cell protease cleavage showed that EC differed from all the other known bovine collagen types. In addition, two-dimensional maps of radioiodinated EC peptides produced by cleavage with proteinase K confirmed that EC represented a unique collagen gene product. The Bolton–Hunter reagent labels collagen with a high specific activity, and the enzyme produces many highly reproducible cleavages in collagen chains, resulting in peptide maps which are true "fingerprints" for each collagen type (Sage et al., 1981b). The EC map is characterized by a greater proportion of larger, more hydrophobic peptides than is found in the other types. Finally, antibodies to other collagen types including bovine type III procollagen did not precipitate EC 1 or EC 2.

Calculations based on recoveries of labeled collagen after ammonium sulfate fractionation of culture medium indicate a ratio of type III procollagen to EC of ~4:1. However, the recovery of EC is significantly lower than that of type III procollagen due to its tendency to be distributed among several ammonium sulfate fractions, the resulting complexity of its purification from additional noncollagenous proteins, and its lability to degradation, particularly when denatured. Preliminary studies involving extraction of endothelial cell layers, both with acetic acid under nondenaturing conditions and with detergents, have suggested that EC is also present in this fraction.

Screening of other endothelial cells indicated that all the following strains have retained the capacity to synthesize this novel collagen: (a) porcine aortic endothelial cells, (b) human umbilical vein endothelial cells, (c) at least eight strains of [^{3}H]thymidine-selected bovine aortic endothelial cells, (d) primary cultures of (c) before [^{3}H]thymidine selection, (e) non-[^{3}H]thymidine-selected bovine aortic endothelial cells grown in serum lacking the platelet-derived growth factor, a mitogen for smooth muscle cells (Ross et al., 1978), (f) cells as in (d) except cloned and grown in 20% fetal calf serum, and (g) two strains of adult bovine aortic endothelial cells cloned in the presence of FGF (gifts of Dr. D. Gospodarowicz and Dr. C. Birdwell). The presence of EC in all of these different populations suggests that synthesis of this collagen is not a reflection of the methods of isolation or maintenance of these cells. We found it interesting that EC appeared to be synthesized by endothelial cells of various origins, since several of these strains have been shown to secrete type I or type IV procollagens as their major collagenous product (Kay et al., 1979; Barnes et al., 1978; Jaffe et al., 1976) rather than type III procollagen as reported for the bovine aortic cells. EC was not observed in endothelial cell cultures that had been overtaken by the "secondary growth pattern" (Schwartz, 1978), in which cells of apparent endothelial origin undergo gross changes in morphology and growth patterns with a concomitant switching to type I collagen synthesis (Cotta-Pereira et al., 1980). Whether EC is unique to endothelial cells must at present remain an open question; however, we have not observed it in culture media from fibroblasts, amniotic fluid cells, or bovine aortic smooth muscle cells.

EC was purified by standard techniques utilizing DEAE-cellulose chromatography at 4 °C and could be recovered in three forms: EC 1 (M_r 177 000), EC 2 (M_r 125 000), and EC 3 (M_r 100 000). The molecular weights are approximations determined relative to type I procollagen and procollagen intermediate standards. All of these forms were sensitive to bacterial collagenase and did not contain disulfide bonds. Attempts to resolve the EC components by molecular sieve chromatography on 6% agarose resulted in a loss of EC 1 with a concomitant increase in EC 2 and EC 3, despite inclusion of several types of protease inhibitors in the elution buffer. Rechromatography of an ~1:1 mixture of EC 2 and EC 3 produced a significant increase in the amount of EC 3 relative to that of EC 2 (Figure 7). These experiments suggest conversion of higher molecular weight to lower molecular weight EC components.

The strongest evidence for the derivation of the EC components from a common precursor was found in peptide mapping studies. When EC 1 and EC 2 were examined by a two-dimensional proteinase K peptide mapping technique, the maps showed conspicuous overlaps, indicating extensive structural identity (Figure 8). In addition, EC 2 could be purified to homogeneity by ion-exchange chromatography on CM-cellulose (Figure 9). When the CNBr cleavage products of EC 2 and a mixture composed of approximately equal amounts of EC 1, EC 2, and EC 3 were compared by $NaDodSO_4$–polyacrylamide gel electrophoresis, the one-dimensional maps were highly similar (Figure 11). It is interesting that, of all the EC components, EC 2 occurred with the highest frequency and could be readily recovered from that fraction of culture medium protein which does not bind to DEAE-cellulose at pH 8.0. However, we were unable to demonstrate a precursor–product relationship between any other collagen and EC 2, either in pulse–chase studies or during the initial purification.

An unusual feature of EC is its sensitivity to pepsin. Within 10 min both EC 1 and EC 2 were degraded to products of less than 60 000 molecular weight (Figure 5). One of these products (M_r 50 000, from an EC 2 pepsin digest) was purified by molecular sieve chromatography and was found to contain approximately equal amounts of hydroxyproline and proline, suggesting that at least part of EC is composed of a stable triple helix. The hydroxyproline to proline ratio for EC 1 (0.54) was somewhat lower than that reported by Monson et al. (1975) for type I procollagen (0.67) and considerably lower than values determined for type IV procollagen from amniotic

fluid cells (1.44) (Crouch & Bornstein, 1979) and from a murine tumor (1.69) (Timpl et al., 1978).

The EC 2 form (M_r 125 000) contained hydroxyproline and proline in nearly equal amounts, suggesting the possibility of a triple helical structure throughout much of the molecule. However, the majority of EC 2 was degraded by pepsin, leaving the M_r 50 000 fragment which had a very similar hydroxyproline to proline ratio. Pepsin-sensitive sites within the triple helix of type IV procollagen and collagen have been reported by several laboratories [for a review, see Bornstein & Sage (1980)]. The pepsin sensitivity of EC appears to be different, as EC was considerably more labile to the enzyme and fewer specific, higher molecular weight fragments such as have been recently described for type IV procollagen and collagen (Crouch et al., 1980) were produced.

Precautions were taken to ensure that EC was in a native conformation during the course of these studies. Other (pro)collagens synthesized by these cells, including type V in the cell layer and type III in the culture medium, were converted only to α-chain-size components by pepsin. When EC and type III procollagen from the same culture medium were cochromatographed on DEAE-cellulose and subjected to pepsin digestion under identical conditions, EC was degraded in less than 10 min, while type III collagen chains first appeared after 4 h. Conversion of type III procollagen to type III collagen was complete after 16 h with a 94% recovery. EC was stable in acetic acid at 4 °C (Figure 5) and at 37 °C in physiologic buffers containing either $PhCH_2SO_2F$ or, as in the case of an incubated control for mast cell protease digestion, no inhibitors. The best evidence for a native conformation for EC 1 was its resistance to mast cell protease digestion prior to heating at 50 °C for 20 min. All native collagens, with the exception of type IV, are resistant to this enzyme, but they are readily degraded following denaturation (Sage et al., 1979b).

The role of the basal lamina and its constituents in directing endothelial cell related functions such as vascular permeability and platelet adherence is not yet clear. The disposition of endothelial cells in relation to the subendothelium varies, depending on the type of vessel (Gabbiani & Majno, 1976), and the basal lamina varies from a distinct continuous structure in capillaries to a discontinuous, fibrillar network in vessels such as the aorta (Zweifach, 1973). There is evidence that endothelial cell attachment to the basal lamina may involve fibronectin (Stenman & Vaheri, 1978) and, secondarily, collagen, as interactions between these two proteins have been shown in some cell culture systems to be necessary for cell adhesion (Kleinman et al., 1978), and collagenase treatment effectively removes endothelial cells from basal lamina (Gimbrone, 1976). A recent study by Wechezak et al. (1979) has suggested that platelet adherence to the subendothelium elaborated in vitro is specifically to an extracellular meshwork of microfilaments produced by the cells.

The role of EC in any of these processes remains to be defined, and our current work involves the in vivo localization of this unique collagen. EC synthesis and deposition could occur in response to arterial injury. It has been proposed that collagen accumulation in atherosclerotic plaques is the result of an interaction between endothelium and smooth muscle cells which have migrated into the intimal area (Bierman & Ross, 1977; Chidi et al., 1979). In a disease such as scleroderma, the duplication of basement membrane is thought to be related to the presence of proliferating intimal cells which have smooth muscle-like properties (Sinclair et al., 1976). If EC is indeed unique to endothelium, it could be used as a marker to define the involvement of these cells in pathological conditions.

Acknowledgments

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Characterization of a Platelet Endoglycosidase Degrading Heparin-like Polysaccharides†

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ABSTRACT: An endoglycosidase (heparitinase) acting on heparin and heparan sulfate was partially purified (~300 times) from human platelets by affinity chromatography on heparan sulfate substituted Sepharose. Only heparin-like polysaccharides were degraded by the enzyme. The susceptibility of various biosynthetic heparin intermediates indicated that the platelet heparitinase had a requirement for sulfamino but not ester sulfate groups. No activity toward other uronic acid containing glycosaminoglycans could be demonstrated. Glucuronidic but not glucosaminidic linkages in heparin or heparan sulfate were attacked by the enzyme as shown by analysis of the reducing sugar moiety in oligosaccharide products. The anticoagulant activity of heparin, determined in an antithrombin III activation assay, was markedly reduced after treatment with the heparitinase. The enzyme was released from its storage site in platelets after induction of the platelet release reaction. The physiological function of platelet heparitinase is not known but may be to modify extracellular heparin-like polysaccharides in the vascular system.

A number of exoenzymes have been demonstrated to participate in the degradation of glycosaminoglycans in mammals [for review, see, e.g., Dorfman & Matalon (1976)]. By the concerted action of these enzymes, inorganic sulfate and monosaccharide units are released from the nonreducing terminal of the polysaccharide. An endoglycosidase would presumably facilitate the degradation process by cleaving internal linkages in the polysaccharide and thus increase the number of nonreducing terminals which can serve as potential substrates for the exoenzymes. Recent work in our laboratory (Höök et al., 1975a, 1977; Wasteson et al., 1976) has demonstrated the presence of a family of endoglycosidases degrading heparin and heparan sulfate. By analyzing the reducing termini of heparan sulfate oligosaccharides stored intracellularly in cultured human skin fibroblasts, Klein and co-workers suggested that these oligosaccharides had been formed by endoenzymatic cleavage of both glucosaminidic and glucuronidic linkages in heparan sulfate chains (Klein & von Figura 1976a; Klein et al., 1976), and, later, the presence of an endoglucuronidase degrading heparan sulfate was demonstrated in human placenta (Klein & von Figura, 1976b). This work deals with a heparan sulfate degrading endoglycosidase

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