Collagen Synthesis by Bovine Aortic Endothelial Cells in Culture†

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ABSTRACT: Endothelial cells isolated from bovine aorta synthesize and secrete type III procollagen in culture. The procollagen, which represents the major collagenous protein in culture medium, was specifically precipitated by antibodies to bovine type III procollagen and was purified by diethylaminoethylcellulose chromatography. Unequivocal identification of the pepsin-treated collagen was made by direct comparison with type III collagen isolated by pepsin digestion of bovine skin, utilizing peptide cleavage patterns generated by vertebrate collagenase, CNBr, and mast cell protease. The type III collagen was hydroxylated to a high degree, having a hydroxyproline/proline ratio of 1.5:1.0. Pulse–chase studies indicated that the procollagen was not processed to procollagen intermediates or to collagen. Pepsin treatment of cell layers, followed by salt fractionation at acidic and neutral pH, produced several components which were sensitive to bacterial collagenase and which comigrated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis with αA, αB, and type IV collagen chains purified from human placenta by similar techniques. Bovine aortic endothelial cells also secreted fibronectin and a bacterial collagenase-insensitive glycoprotein which, after reduction, had a molecular weight of 135 000 on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (using procollagen molecular weight standards) and which was not precipitable by antibodies to cold-insoluble globulin or to α2-macroglobulin. Collagen biosynthesis by these cells provides an interesting model system for studying the polarity of protein secretion and the attachment of cells to an extracellular matrix. The presence of type III collagen in the subendothelium and the specific interaction of this protein with fibronectin and platelets suggest the involvement of this collagen in thrombus formation following endothelial cell injury.

Vascular endothelial cells are derived from mesenchyme and are disposed as a contact-inhibited monolayer between the lumen of the vessel and the subendothelial matrix. The "cobblestone" morphology seen in vivo is maintained in vitro. Questions concerning the nature of the subendothelium as a thrombogenic surface and as a matrix which anchors the endothelial cell layer have prompted investigations of the biosynthetic properties of endothelial cells in culture. Such studies have suggested that the matrix to which these cells adhere may be elaborated in part by the cells themselves, indicating that endothelial cells in vitro might prove useful for studying basement membrane synthesis and cellular attachment.

It has recently been shown that both bovine and human endothelial cells synthesize large amounts of fibronectin or CIG[1] (Macarak et al., 1978; Birdwell et al., 1978; Jaffe & Mosher, 1978), but studies on collagen biosynthesis by these cells have been controversial. Some investigators have reported the synthesis of a type IV (basement membrane) collagen (Jaffe et al., 1976; Howard et al., 1976; Kay et al., 1979), while other laboratories, working with porcine and guinea pig aortic endothelial cells, have suggested that the major biosynthetic products are types I and III (interstitial) collagens (Barnes et al., 1978; Mayne & Mayne, 1978).

We chose bovine aortic endothelial cells isolated and maintained as described by Schwartz (1978) as a system to study the biosynthesis and processing of the several collagen types, their disposition on the cell surface, and their incorporation into the extracellular matrix. This report describes the biochemical characterization of the collagens synthesized by these cells in culture.

Materials and Methods

Cell Culture. Endothelial cells were isolated from bovine thoracic aorta, [^{3}H]thymidine-selected, and subcultured according to methods described by Schwartz (1978). Confluent cells were passaged every 4–6 days at a ratio of 1:4. For biochemical studies, the cells were maintained in DMEM containing penicillin G (100 units/mL) and streptomycin sulfate (100 μg/mL) (Grand Island Biological Co., Inc.) in the presence of 0.0002% butyl *p*-hydroxybenzoate and 20% FCS (Irvine Scientific).

To ascertain whether certain growth conditions would affect the type of collagen(s) synthesized, we grew and labeled some cultures in the presence of 20% FCS, 10% calf serum, 10% PDS (Ross et al., 1978), FGF (50 ng/mL, Collaborative Research, or 12.5 ng/mL, a gift from Dr. Denis Gospodarowicz), and ECGF (1.1 μg/mL, Collaborative Research). In addition, bovine endothelial cells, cloned and maintained in the presence of FGF (12.5 ng/mL), were also checked at the fourth and sixth passages, as were cells which had not undergone [^{3}H]-thymidine selection. In each case the medium was changed daily with a fresh supplement of growth factor.

Metabolic Labeling. All labeling experiments were performed by using just-confluent cultures between the sixth and twelfth passage. Serum-free DMEM was supplemented with penicillin–streptomycin, antimycotic, sodium ascorbate (50 μg/mL), and β-APN (80 μg/mL). The cultures were preincubated for 30 min to 1 h and subsequently labeled for 18–24 h with 20–50 μCi/mL L-[2,3-^{3}H]proline (35 Ci/mmol; New England Nuclear) under conditions producing linear incorporation of isotope into the culture medium. Other isotopes used included D-[2-^{3}H]mannose (2 mCi/mmol) and L-[5-^{3}H]proline (29 Ci/mmol; Amersham/Searle Co.). In all cases unlabeled proline was omitted from the medium. For studies involving mannose incorporation, glucose was omitted and the medium

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[1] Abbreviations used: DMEM, Dulbecco–Vogt-modified Eagle's medium; FCS, fetal calf serum; PDS, bovine serum which is deficient in the platelet-derived growth factor; FGF, fibroblast growth factor; ECGF, endothelial cell growth factor; EDTA, ethylenediaminetetraacetic acid; β-APN, β-aminopropionitrile fumarate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; MalNEt, *N*-ethylmaleimide; $NaDodSO_4$, sodium dodecyl sulfate; CIG, cold-insoluble globulin; DTT, dithiothreitol; EGF, epidermal growth factor; DEAE, diethylaminoethyl; CM, carboxymethyl.

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was supplemented with 1.1 g/L sodium pyruvate.

For pulse–chase experiments, cells were plated at equal densities onto 60-mm plates, preincubated as described above, and pulsed for 30 min with 50 μCi/mL L-[2,3-^{3}H]proline. The cells were then washed twice with DMEM and chased in medium containing 20 mM L-proline. Duplicate plates were analyzed at time points from 0 to 30.5 h.

Preliminary Characterization of Radiolabeled Protein. Radiolabeled medium was harvested from the cultures into protease inhibitors at 4 °C, producing a final concentration of 0.2 mM $PhCh_2SO_2F$, 10 mM MalNEt, and 2.5 mM EDTA. The medium was clarified, and the cell layers were washed and stored, according to methods described by Crouch & Bornstein (1978).

Culture medium proteins were precipitated in 10% trichloroacetic acid containing 1 μg/mL pepstatin A (Protein Research Foundation, Osaka, Japan) at 0 °C (Crouch & Bornstein, 1978), prior to analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis. Alternatively, samples were precipitated at 20, 40, and 60% ammonium sulfate concentrations (w/v) at 4 °C and the resulting pellets were redissolved in and dialyzed vs. 0.1 M acetic acid containing 1 μg/mL pepstatin A, followed by lyophilization.

For incorporation studies in culture medium, Hyp/Pro analyses were performed as described by Crouch & Bornstein (1978) and protein concentration in sonicated cell layers was determined by the method of Lowry et al. (1951). Incorporation into cell layer proteins was determined on a soluble acetic acid extract, prepared by homogenizing the cell layers in a Dounce homogenizer in 0.1 M acetic acid containing 1 μg/mL pepstatin A, followed by dialysis, clarification, and lyophilization. A bacterial collagenase (Advance Biofactures, form III) assay as outlined by Peterkofsky & Diegelmann (1971) was utilized to quantitate collagenous [^{3}H]proline-labeled protein. Digestion of culture medium protein with bacterial collagenase was performed according to Crouch & Bornstein (1978).

Pepsin Digestion. Aliquots of whole medium, harvested in the presence of inhibitors as described above and containing 1 μg/mL pepstatin A, were dialyzed vs. 0.1 M acetic acid, lyophilized, and weighed. The material was suspended at 1 mg/mL in 0.5 M acetic acid and subsequently incubated with pepsin (Worthington Biochemicals) at an enzyme/substrate weight ratio of 1:50 for 24 h at 4 °C. Sodium chloride was added to the clarified pepsin supernatant to a final concentration of 0.9 M, and the resulting precipitate was collected by centrifugation, dissolved in 1 M NaCl and 50 mM Tris, pH 7.5, and reprecipitated at 4.5 M NaCl (Chung & Miller, 1974; Trelstad et al., 1972). Lyophilized cell layer material which had been solubilized in 0.1 M acetic acid was similarly treated, but at a 1:10 enzyme/substrate weight ratio, followed by neutralization of the incubation mixture for 30 min. After the pH was readjusted to 2.2, solid NaCl was added to a final concentration of 10% and the resulting precipitate was centrifuged, dissolved in and dialyzed vs. 0.1 M acetic acid, and lyophilized.

Pepsin digestion of chromatographically purified bovine endothelial medium collagen was performed at a 1:100 enzyme/substrate weight ratio for 18 h at 4 °C. The reaction was terminated by addition of pepstatin A in a 10-fold molar excess over pepsin, and the collagen was fractionated at 0.7 and 1.8 M NaCl at acidic pH (Rhodes & Miller, 1978) and at 1.7, 2.6, and 4.5 M NaCl at pH 7.5 (Burgeson et al., 1976).

Purification of Medium Collagen by Ion-Exchange and Molecular Sieve Chromatography. Whole culture medium labeled with [^{3}H]proline was stirred overnight at 4 °C in the presence of protease inhibitors and 20% ammonium sulfate (w/v), and the resulting precipitate was dissolved in and dialyzed vs. 6 M urea and 50 mM Tris, pH 8.0, containing 0.2 mM $PhCH_2SO_2F$ and 2.5 mM EDTA. DEAE-cellulose chromatography (DE-52, Whatman) was performed essentially as described by Burke et al. (1977), using a 0–200 mM NaCl linear gradient elution.

Molecular sieve chromatography was performed on whole [^{3}H]proline-labeled, pepsin-digested medium by using 6% agarose (Bio-Gel A-5m, Bio-Rad Laboratories), equilibrated with 1 M $CaCl_2$ and 50 mM Tris, pH 7.5, as described by Crouch & Bornstein (1979).

Pepsin-treated medium collagen was chromatographed prior to reduction on CM-cellulose (CM-52, Whatman) in 6 M urea and 40 mM sodium acetate, pH 4.8, using a 0–100 mM NaCl linear gradient, according to the method of Crouch & Bornstein (1978). Protein in the eluted radioactive peak was reduced and alkylated in CM-cellulose buffer adjusted to pH 8.0 as previously described (Sage et al., 1979) and subsequently rechromatographed on CM-cellulose.

Comparative Peptide Mapping. Bovine endothelial cell culture medium collagen and other collagens were compared by cleavage with CNBr (Eastman), human skin collagenase (a gift from Dr. A. Eisen), and rat mast cell protease (a gift of Dr. R. Woodbury, as prepared by Dr. N. Katunuma). Bovine type III collagen was prepared by limited pepsin digestion of type III procollagen extracted from fetal bovine skin (Timpl et al., 1975), followed by selective salt precipitation at 1.7 M NaCl at neutral pH.

CNBr digestion was performed as indicated by Crouch & Bornstein (1979), and digests were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing conditions. Cleavage of native collagens with vertebrate collagenase and cleavage of reduced and alkylated protein with mast cell protease were performed according to Sage et al. (1979). In both cases the digestions were terminated by adding an equal volume of sample buffer containing 50 mM DTT (Laemmli, 1970) prior to analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis and Fluorescence Autoradiography. Proteins were resolved on discontinuous methylenebis(acrylamide) slab gels containing 0.5 M deionized urea (Laemmli, 1970; Studier, 1973), as described by Crouch & Bornstein (1978). After electrophoresis, the gels were either stained with Coomassie Blue (Sage & Bornstein, 1979) or processed for fluorescence autoradiography by permeation with dimethyl sulfoxide and 2,5-diphenyloxazole (Bonner & Laskey, 1974) followed by exposure to sensitized X-ray film (Laskey & Mills, 1975). Protein bands were scanned in linear range with a scanning densitometer (Quick Scan, Helana Laboratories).

Interrupted electrophoresis for characterization of type III collagen was performed according to the method of Sykes et al. (1977).

Immunochemistry. Antibodies directed toward bovine type III procollagen were raised in rabbits, and serum IgG fractions were affinity purified by selective absorption on Sepharose CL-4B (Pharmacia) to which bovine type III procollagen was coupled. The antigen was judged to be of high purity by the criterion of $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing and nonreducing conditions after chromatography on DEAE-cellulose.

Immune precipitations using a double-antibody technique were performed on [^{3}H]proline-labeled culture media with

Table I: Collagen Synthesis by Endothelial Cells in Vitro[a]

	dpm/mg of cell protein ($\times 10^{-6}$)	dpm in collagen per mg of cell protein[c] ($\times 10^{-4}$)	% collagen synthesis[d]	Hyp/Pro ($\times 100$)	3-Hyp/total Hyp ($\times 100$)
medium	12.3 (3)[e]	37.7 (3)	3.11 ± 0.2	7.3	1.3
cell layer[b]	12.3 (3)	5.4 (3)	0.25 ± 0.017	1.3	2.6

[a] Measured by L-[5-^{3}H]proline incorporation; cells were labeled for 24 h with 50 μCi/mL, as described under Materials and Methods. [b] Acetic acid extract. [c] Based on hydroxyproline and proline analyses, assuming an equal content of these amino acids in collagen. [d] Mean ± standard deviation, based on bacterial collagenase digestion assay. [e] Number of determinations; the figures represent average values.

antibodies to bovine type III procollagen, α2-macroglobulin (Behring Diagnostics), and human CIG, essentially as described by Crouch et al. (1978). Background radioactivity using preimmune sera was from 5 to 7% of the total counts per minute. In all cases the radioimmune precipitate was analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis to establish the specificity of the reaction. Because varying amounts of fibronectin appeared to coprecipitate with the second antibody, [^{3}H]proline-labeled culture medium was passed over a gelatin–Sepharose column to remove this protein (Engvall & Ruoslahti, 1977) prior to generation of immune titration curves. Procollagen in [^{3}H]proline-labeled media was immune precipitated and identified by NaDodSO$_4$–polyacrylamide gel electrophoresis. Collagen production in pulse–chase experiments was quantitated by densitometric scanning of the band corresponding to procollagen.

Results

Collagen Synthesis by Endothelial Cells. A critical aspect of this work was the rigorous identification of cells used for biosynthetic studies as homogeneous cultures of bovine aortic endothelial cells. In all cases the cultures maintained a stable, contact-inhibited monolayer and exhibited no overgrowth by other cell types, including smooth muscle cells. Factor VIII antigen was uniformly detected by immunofluorescence (Jaffe et al., 1973). Cultures exhibiting the secondary growth pattern ("sprouting") described by Schwartz (1978) were not used in the present studies. The endothelial cells had a replicative life span in vitro of 35–40 doublings, during which they retained a stable karyotype (Schwartz, 1978). Cells were used at low passage numbers in these experiments, and senescent multinucleate cells were not apparent.

Bovine aortic endothelial cells incorporate [^{3}H]proline into collagenous protein which subsequently accumulated in the cell layer and was secreted into the medium. Table I summarizes the distribution of proteins containing [^{3}H]proline and estimates the proportion of [^{3}H]hydroxyproline-containing protein synthesized by these cells. In comparison to fibroblasts, the amount of proline-labeled collagenase-sensitive protein was quite low and comprised ~3% of the total protein in the medium, a value in agreement with that previously published for bovine aortic endothelial cells (Howard et al., 1976). Collagen synthesis in the cell layer comprised only 0.25% of total radiolabeled protein by collagenase assay. Another estimate of percent collagen synthesis, based on hydroxyproline and proline analyses, yields a value for the medium of 3.1% and for the cell layer of 0.44%. 3-Hydroxyproline comprised 1–3% of the total hydroxyproline in these cultures.

A typical profile on NaDodSO$_4$–polyacrylamide gel electrophoresis of [^{3}H]proline-labeled protein which had been secreted into the culture medium by endothelial cells is shown in Figure 1, lanes 1 and 2. Three biosynthetic products appeared in the molecular weight range 100 000–250 000 after reduction. The major secreted product has been identified as fibronectin (or CIG) on the basis of its mobility on NaDodSO$_4$–polyacrylamide gel electrophoresis (Mosesson & Umfleet, 1970) and its quantitative precipitation with affinity-purified antibodies to human CIG (lane 3). Precipitation of whole medium in 20% ammonium sulfate enriched for a component of ~160 000 molecular weight (lane 4) which was digested by bacterial collagenase (lane 5). This protein migrated slightly more slowly on NaDodSO$_4$–polyacrylamide gel electrophoresis than fibroblast proα1(I) chains (M_r 140 000) after reduction (data not shown) and in the absence of reducing agents migrated as a high molecular protein, indicating the presence of interchain disulfide bonds (lane 1).

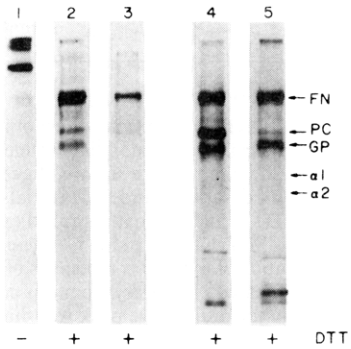

FIGURE 1: Fluorescent autoradiogram of [^{3}H]proline-labeled proteins in bovine endothelial cell culture medium. Nearly confluent cultures were labeled with L-[2,3-^{3}H]proline for 24 h in serum-free DMEM supplemented with ascorbate and β-APN. NaDodSO$_4$–polyacrylamide gel electrophoresis was performed on composite (6 and 10%) slab gels in the presence and absence of 50 mM DTT. Lanes 1 and 2, 10% trichloroacetic acid precipitates of culture medium; lane 3, immune precipitate of lane 2 with antibodies to human CIG; lane 4, 20% ammonium sulfate precipitate of culture medium; lane 5, protein in lane 4 after digestion with bacterial collagenase. Fibronectin (FN), procollagen (PC), and glycoprotein (GP) are indicated, as well as the positions of migration of bovine α1(I)- and α2-chain standards.

A third component, labeled GP in Figure 1, contained disulfide bonds and was not susceptible to bacterial collagenase (lane 5). When cells were labeled with radioactive cystine, glucosamine, and mannose, the glycoprotein was synthesized with a high specific activity, whereas tryptophan, methionine, valine, and tyrosine were all incorporated to approximately the same extent as that seen for proline. This glycoprotein migrated similarly to $p_C\alpha$1(I) chains after reduction on NaDodSO$_4$–polyacrylamide gel electrophoresis; it was not precipitated by antibodies to CIG (Figure 1, lane 3), to α2-macroglobulin, or to a variety of procollagens and collagens prepared in our laboratory. The glycoprotein had a similar apparent molecular weight on NaDodSO$_4$–polyacrylamide gel electrophoresis to the angiotensin-converting enzyme which is synthesized by human umbilical vein endothelial cells in culture, although this protein appears not to be secreted into the culture medium (Hial et al., 1979). However, the presence of large amounts of this enzyme in culture media from porcine aortic endothelial cells (Hayes et al., 1978) suggests that further investigation of the relationship between converting enzyme and the unidentified glycoprotein is necessary.

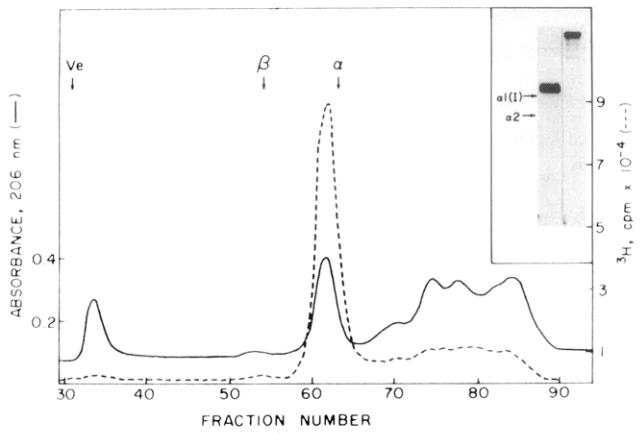

FIGURE 2: Molecular sieve chromatography of endothelial cell culture medium collagen after pepsin digestion. [^{3}H]Proline-labeled protein was precipitated with 20% ammonium sulfate, digested with pepsin and salt fractionated as described under Materials and Methods, reduced with 100 mM DTT, and chromatographed on Agarose A-5m in 1 M $CaCl_2$ and 50 mM Tris-HCl, pH 7.5. The excluded volume (V_e) and positions of elution of α and β components of type I lathyritic rat skin collagen are indicated. Recovery from this column was 90%. Inset: protein in the major radioactive peak was dialyzed vs. 0.1 M acetic acid, lyophilized, and subsequently analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis on a 6% separating slab gel in the presence (left lane) and absence (right lane) of 50 mM DTT. The positions of migration of marker α1(I) and α2 chains are indicated.

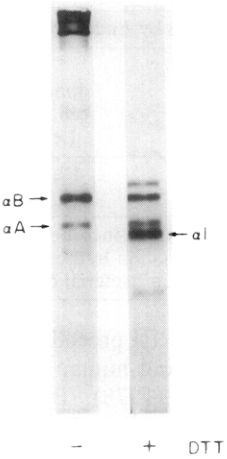

FIGURE 3: Fluorescent autoradiogram of endothelial cell layer after pepsin digestion. Cells were labeled as described in Figure 1, and cell layers were digested with pepsin and salt fractionated as described under Materials and Methods. Proteins were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis on composite (6 and 10%) slab gels in both the presence and absence of 50 mM DTT. The positions of migration of αA, αB, and α1 chains, isolated from human placenta, are indicated.

Pepsin digestion of whole medium followed by salt fractionation, using 0.9 M NaCl at acidic pH and 4.5 M NaCl at neutral pH, yielded a disulfide-bonded component which, after reduction, migrated more slowly than α1(I) chains on $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 2, inset). Molecular sieve chromatography of the reduced protein indicated an apparent molecular weight ~10% larger than that for an α1(I) chain (Figure 2).

For identification of the small amounts of collagen in endothelial cell layers, cultures containing radiolabeled protein were treated with pepsin and the soluble, digested material was precipitated with 10% NaCl at acidic pH. $NaDodSO_4$–polyacrylamide gel electrophoresis of this material (Figure 3) showed two nonreducible bands which were observed to migrate identically with pepsinized αB and αA collagen chains isolated from human placenta (Sage & Bornstein, 1979). After reduction, three additional bands were observed. Two bands comigrated with the 140 000 and 70 000 components of type IV collagen isolated from human placenta (Sage et al., 1979). The α-chain sized component (95 000 molecular weight) has been tentatively identified as type III collagen. All of the bands observed in Figure 3 were digested by bacterial collagenase.

Chromatographic Purification of Endothelial Cell Culture Medium Procollagen and Collagen. [^{3}H]Proline-labeled culture medium proteins were precipitated in 20% ammonium sulfate and chromatographed under native conditions on DEAE-cellulose. The elution profile is depicted in Figure 4, and $NaDodSO_4$–polyacrylamide gel electrophoresis analysis of pooled fractions is shown in Figure 5. Fraction I (Figure 4) contained several proteins which did not bind to the column and which did not appear to be disulfide bonded (data not shown). These bands were sensitive to bacterial collagenase and were degraded to components of less than 60 000 molecular weight after pepsin treatment at 4 °C for 24 h. The Hyp/Pro determined for this fraction was 0.98:1.00, with ~1% of the total Pro as the 3-Hyp isomer. Preliminary structural studies suggest the presence of a unique chain type in this fraction (H. Sage and P. Bornstein, unpublished experiments).

Fraction II (Figure 4) contained principally fibronectin (Figure 5, lane II), and fraction III was composed largely of a disulfide-bonded component which was sensitive to bacterial collagenase.

Pepsin digestion of fraction III, followed by salt fractionation, produced a single, reducible band on $NaDodSO_4$–polyacrylamide gel electrophoresis, identical with that depicted in the inset to Figure 2. Salt fractionation of the native protein indicated preferential precipitation at 0.7 M NaCl at acidic pH and at 1.7 M NaCl at pH 7.5. However, this precipitation was not complete, probably due to the presence of the radiolabeled collagen in low concentrations (less than 100 μg/mL). Since the salting out behavior and the elution position of the native protein on DEAE-cellulose suggested a relation to type III procollagen (Burke et al., 1977; Byers et al., 1974), the material in fraction III was subjected to pepsin treatment followed by interrupted $NaDodSO_4$–polyacrylamide gel electrophoresis. Figure 6 indicates that the endothelial cell medium collagen migrated more slowly than a similarly prepared sample of bovine skin type III collagen. The Hyp/Pro ratio determined for this collagen was 1.53:1.00, but there was variability in this ratio among different cell strains; a low level of 3-Hyp was detected. This level of hydroxylation is in considerable excess over that reported for type III collagen (1.12:1) prepared from tissues (Timpl et al., 1975). This observation, coupled with the more diffuse nature of the band on $NaDodSO_4$–polyacrylamide gel electrophoresis, suggested that the slower mobility of the protein in comparison with the α1(III) chain could be due to changes in posttranslational modifications, as was observed by Crouch & Bornstein (1978) for the type I trimer collagen isolated from amniotic fluid cells.

The protein was further characterized by chromatography on CM-cellulose, before and after reduction and alkylation (Figure 7). Each peak was found to be homogeneous by $NaDodSO_4$–polyacrylamide gel electrophoresis. The position of elution of the trimeric and monomeric chains is consistent with the identification of the protein as type III collagen. The shift in chromatographic position which is observed after reduction of disulfide bonds has been described before for type III collagen (Chung & Miller, 1974).

Structural Comparison of Endothelial Cell Culture Medium Collagen to Other Collagen Types. In view of earlier

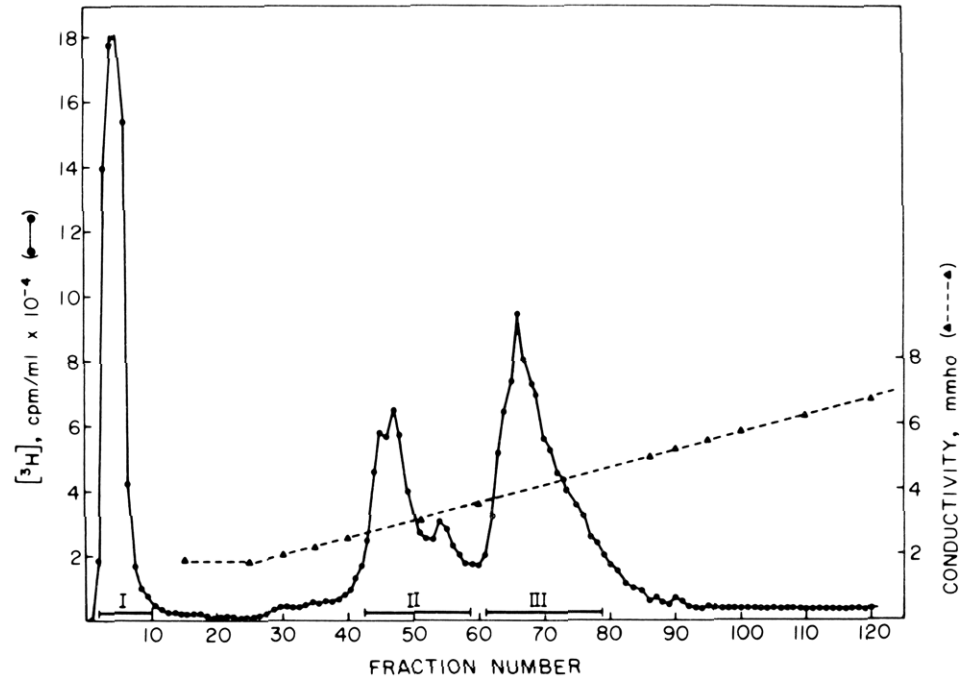

FIGURE 4: DEAE-cellulose chromatography of [^{3}H]proline-labeled endothelial cell medium proteins. [^{3}H]Proline-labeled protein was precipitated from culture medium with 20% ammonium sulfate in the presence of protease inhibitors and chromatographed on DE-52 cellulose in 6 M urea and 50 mM Tris-HCl, pH 8.0, containing $PhCH_2SO_2F$ and EDTA. Conductivities were read at 4 °C. Gradient elution (0–200 mM NaCl over 400 mL) was started at fraction 25. Roman numerals indicate pooled fractions.

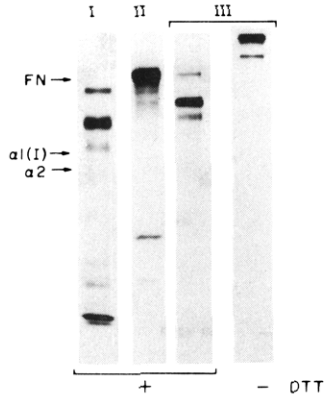

FIGURE 5: Fluorescent autoradiograms of [^{3}H]proline-labeled culture medium protein which was fractionated on DEAE-cellulose. Proteins were resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis on a composite (6 and 10%) slab gel in the presence and absence of 50 mM DTT. Roman numerals correspond to the pooled fractions indicated in Figure 4. The positions of migration of fibronectin (FN) and α1(I) and α2 chains are indicated.

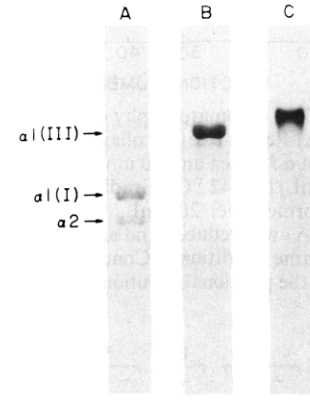

FIGURE 6: Interrupted electrophoresis of endothelial cell culture medium collagen. Fluorescent autoradiogram of pepsin-treated bovine skin type III collagen (B) and culture medium collagen (C) run on $NaDodSO_4$–polyacrylamide gel electrophoresis (6% separating slab gel) after delayed reduction with 50 mM DTT. A type I collagen standard (A) was stained with Coomassie blue.

reports that endothelial cells synthesize and secrete type IV (basement membrane) collagen as the major collagen in the culture medium (Jaffe et al., 1976; Howard et al., 1976; Macarak & Kefalides, 1978), we performed more detailed structural studies in an attempt to identify the secreted protein.

Figure 8 illustrates $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of native endothelial cell collagen, bovine type III collagen, and lathyritic type I rat skin collagen after incubation with vertebrate collagenase. Under conditions where greater than 90% of the type I collagen was cleaved to the characteristic doublets TC^A and TC^B, the endothelial collagen and type III produced a similar cleavage pattern, generating single TC^A and TC^B peptides. It can be seen, however, that the endothelial collagen TC^A and TC^B fragments both retained the characteristic diffuse, slower migration pattern which had been observed for the uncleaved protein. It has recently been shown that neither human type IV nor AB collagens are cleaved by this collagenase (Sage et al., 1979; Sage & Bornstein, 1979).

The results of CNBr digestion of types I and III and endothelial cell collagens are shown in Figure 9, which depicts the resolution of the major fragments by $NaDodSO_4$–polyacrylamide gel electrophoresis. The patterns produced by the endothelial and bovine type III collagens were very similar, although many of the bands from the endothelial collagen migrated more slowly and diffusely than did their counterparts in the tissue-derived type III collagen. Virtually all the CNBr peptides could be accounted for as being type III derived; α1(III)-CB3B, -3C, and -7 were not resolved in this gel system (Fietzek et al., 1977).

Further structural identification was made by using an enzyme with chymotrypsin-like activity, mast cell protease (Woodbury et al., 1978). The specific cleavages produced by this enzyme with different collagens, when incubated at 37 °C for short time periods, result in unique peptide "maps" for

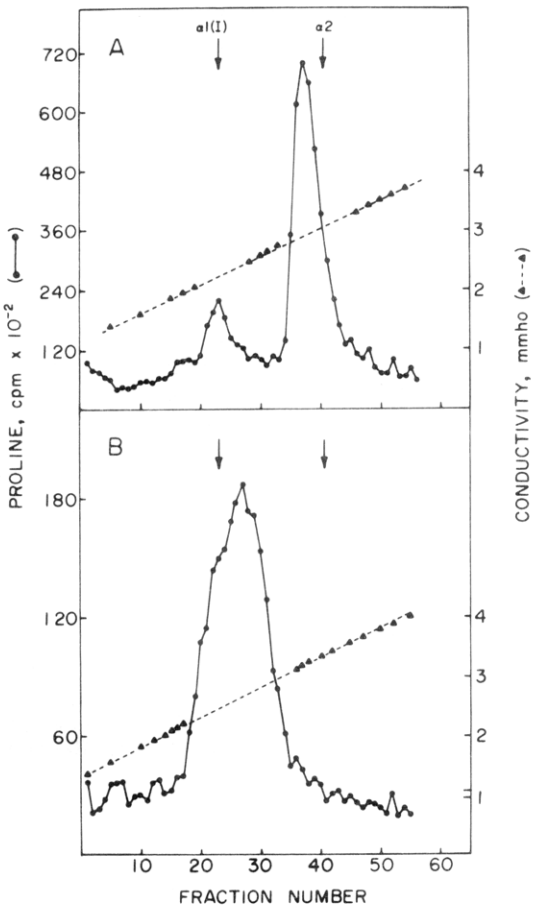

FIGURE 7: CM-cellulose chromatography of endothelial cell culture medium collagen. (A) Pepsin-treated collagen was chromatographed on CM-52 cellulose in 6 M urea and 40 mM sodium acetate, pH 4.8, at a flow rate of 30 mL/h at 42 °C. Gradient elution from 0 to 100 mM NaCl was performed over 200 mL. (B) Protein in the major radioactive peak in (A) was reduced and alkylated and rechromatographed under the same conditions. Conductivities were read at 4 °C. Arrows indicate the positions of elution of α1(I) and α2 collagen chains.

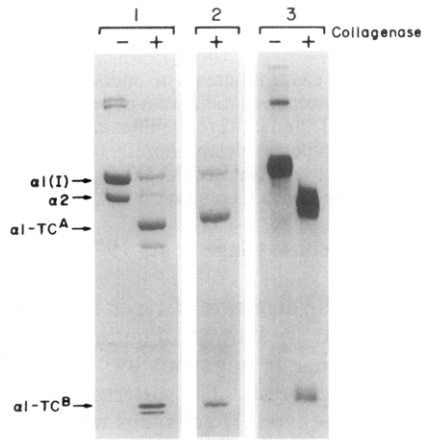

FIGURE 8: NaDodSO$_4$–polyacrylamide gel electrophoresis of collagens after incubation with vertebrate collagenase. Pepsin-treated samples were digested as described under Materials and Methods, reduced with 50 mM DTT, and analyzed on a composite (7.5 and 12.5%) slab gel. Protein was visualized by staining with Coomassie blue or by fluorescence autoradiography. Controls were incubated at 22 °C for 48 h. (1) Type I human skin collagen. (2) Bovine skin type III collagen. (3) Endothelial cell culture medium collagen.

each type (Sage et al., 1979; Sage & Bornstein, 1979). It is important in this type of mapping, however, that the collagens be totally denatured for strict comparison. Accordingly, the

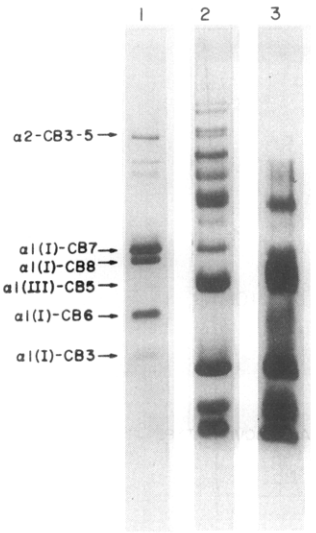

FIGURE 9: NaDodSO$_4$–polyacrylamide gel electrophoresis of collagens after cleavage with CNBr. Proteins were digested with CNBr as described under Materials and Methods, and the cleavage products were dissolved in NaDodSO$_4$–urea sample buffer containing 50 mM DTT, resolved on a 12.5% slab gel, and visualized by Coomassie blue staining or fluorescence autoradiography. Lane 1, type I collagen; lane 2, bovine skin type III collagen; lane 3, endothelial cell culture medium collagen. The major fragments derived from type I collagen are identified, as well as α1(III)-CB5 from bovine type III collagen.

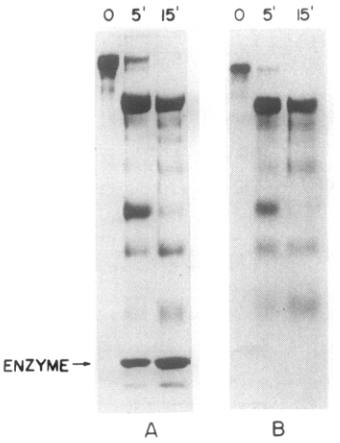

FIGURE 10: NaDodSO$_4$–polyacrylamide gel electrophoresis of collagens after incubation with mast cell protease. Collagens were digested with rat mast cell protease at 37 °C for 5 and 15 min, and the reactions were terminated by addition of NaDodSO$_4$–polyacrylamide gel electrophoresis sample buffer containing 50 mM DTT. The cleavage products were analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis on a 10% slab gel and visualized by Coomassie blue staining or fluorescence autoradiography. Equal aliquots of digest were loaded in each lane. (A) Bovine skin type III collagen and (B) endothelial cell collagen.

endothelial and bovine type III collagens were reduced, alkylated, and subjected to a time course of digestion. The cleavage products at two time points, as resolved by NaDodSO$_4$–polyacrylamide gel electrophoresis, are shown in Figure 10. The two patterns are essentially the same. The low molecular weight band migrating below the enzyme is derived from the enzyme by autocatalysis.

Immunological and Pulse–Chase Studies. Further corroboration of the structural identity of the endothelial cell and type III collagens was obtained by immune precipitation of dialyzed [^{3}H]proline-labeled culture medium with antibodies directed toward bovine type III procollagen. Approximately 8% of the total nondialyzable counts per minute were precipitated by this antibody, as contrasted to 34% when anti-CIG

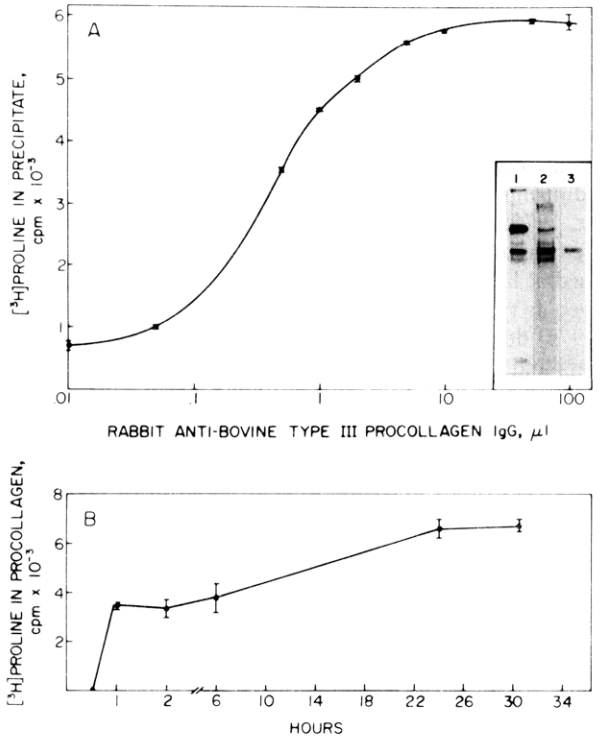

FIGURE 11: Immune precipitation of [^{3}H]proline-labeled endothelial cell culture medium with antibovine type III procollagen antibody. (A) Dialyzed medium, absorbed over gelatin–Sepharose. A double-antibody procedure as described under Materials and Methods was used. Inset: fluorescent autoradiograms analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis on composite (6 and 10%) slab gels in the presence of 50 mM DTT. Lane 1, trichloroacetic acid precipitate of whole culture medium; lane 2, dialyzed culture medium after passage over gelatin–Sepharose; lane 3, immune precipitate generated from medium in lane 2 by using 2.5 μL of rabbit antibovine type III procollagen IgG. (B) Pulse–chase medium. Confluent cultures were labeled for 30 min with [^{3}H]proline and chased for 30.5 h in serum-free DMEM containing 20 mM proline, as described under Materials and Methods. Equal aliquots of culture medium were dialyzed and incubated with 2.5 μL of rabbit antibovine type III procollagen IgG, and the immune precipitates were subsequently analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis in the presence of 50 mM DTT. The curve was generated by densitometry of the fluorescent autoradiogram.

antibodies were used. Figure 11A shows an immune titration curve of culture medium with antibovine type III procollagen antibodies. The concentration of IgG at the inflection point of the curve was taken for the radioimmune precipitation which was analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis (Figure 11, inset, lane 3).

Surface immunofluorescence studies on endothelial cells using antibodies directed toward human types I, III, and IV collagens were negative. However, antibodies to bovine type III procollagen produced an intense and extensive staining reaction (data not shown).

From pulse–chase studies, the synthesis and secretion time for endothelial type III procollagen was estimated to be less than 1 h, as quantitated by radioimmune precipitation of culture medium at several time points during the chase and subsequent densitometry of fluorescent autoradiograms (Figure 11B). Processing of the type III procollagen was not observed during the chase period (up to 30.5 h) nor in experiments in which the cells were labeled for 48 h (data not shown).

Discussion

Bovine aortic endothelial cells in vitro synthesize and secrete three major proteins into the culture medium: fibronectin, a noncollagenous glycoprotein, and type III procollagen. That the principal procollagen found in endothelial cell culture medium is type III has been established by several criteria. (1) The protein was sensitive to bacterial collagenase and was quantitatively precipitated by affinity-purified antibodies to bovine type III procollagen. (2) A protein eluting from DEAE-cellulose at a position corresponding to type III procollagen from other species was converted, after limited pepsin digestion, to a disulfide-bonded trimer which, after reduction, migrated on NaDodSO$_4$–polyacrylamide gel electrophoresis in the position of α chains. (3) The reduced chain was eluted from CM-cellulose slightly after α1(I) chains, as has been described for human type III collagen (Chung & Miller, 1974). (4) Structural comparison of this collagen with type III collagen from fetal bovine skin, isolated by the same procedures of pepsin treatment and salt fractionation, revealed the same peptide cleavage patterns generated by vertebrate collagenase, CNBr, and mast cell protease.

It was noted, however, that the reduced collagen, after pepsin digestion, consistently migrated as a slower, more diffuse band on NaDodSO$_4$–polyacrylamide gel electrophoresis, as compared to type III collagen purified from bovine skin. The effect was especially apparent on interrupted NaDodSO$_4$–polyacrylamide gel electrophoresis and after cleavage with vertebrate collagenase, although some of the CNBr peptides retained this behavior as well (Figures 6, 8, and 9). Similar results have been reported for the type I trimer chains synthesized by amniotic fluid cells in culture; these chains are highly hydroxylated and migrate more slowly than type I procollagen chains on NaDodSO$_4$–polyacrylamide gel electrophoresis (Crouch & Bornstein, 1978). It is therefore of interest that the endothelial cell type III collagen has a Hyp/Pro ratio of 1.5:1 as compared to a ratio of 1.12:1 for bovine dermal type III collagen (Timpl et al., 1975). Furthermore, cultures of bovine aortic smooth muscle cells (a gift from Dr. S. Schwartz), labeled and analyzed in parallel with culture medium from bovine endothelial cells, were observed to synthesize, in addition to type I procollagen, a type III procollagen which was essentially identical with that produced by endothelial cells in mobility on NaDodSO$_4$–polyacrylamide gel electrophoresis and in reactivity toward antibovine type III procollagen antibody. These results differ from the finding of Macarak & Kefalides (1978) that smooth muscle and endothelial cells synthesize different collagens.

At no time have we observed processing of the procollagen to collagen α chains. However, after 24 h of labeling, a reducible bacterial collagenase-sensitive component was observed which coeluted with type III procollagen from DEAE-cellulose (Figures 4 and 5). The protein migrated on NaDodSO$_4$–polyacrylamide gel electrophoresis with a reduced molecular weight of 140 000 and near the position expected for reduced pα(III) chains. This finding is in agreement with reports from several laboratories which indicate that both in tissues (Timpl et al., 1975; Fessler & Fessler, 1979) and in cell culture (Goldberg, 1977) type III procollagen is neither processed rapidly (i.e., in comparison to type I procollagen) nor completely.

Endothelial cells in vitro also produce other collagen types. Pepsin treatment of cell layers, followed by salt fractionation and NaDodSO$_4$–polyacrylamide gel electrophoresis (Figure 3), revealed two non-disulfide-bonded, collagenase-sensitive bands which corresponded in migration to αA and αB chains isolated from human tissues (Burgeson et al., 1976; Rhodes & Miller, 1978; Sage & Bornstein, 1979). Further salt fractionation revealed that these molecules were selectively precipitated at 1.0–1.8 M NaCl at acidic pH and at 3.5–4.5 M

NaCl at pH 7.5; this solubility behavior has been described for AB collagen by Burgeson et al. (1976) and Rhodes & Miller (1978). Jaffe et al. (1976) reported that human umbilical vein endothelial cells synthesized types III and IV collagens, as demonstrated by the appearance of two species after pepsin digestion which migrated with apparent molecular weights of 120000 and 94000 on $NaDodSO_4$–polyacrylamide gel electrophoresis. Since these collagenase-sensitive proteins were isolated from the cell layer, they might correspond to αA and αB chains, which have been characterized in smooth muscle cell layers as well (Mayne et al., 1978). The pepsin-resistant moieties in the endothelial cell culture medium, including the major species of M_r 115000 described by Jaffe et al. (1976), could all be derived from type III procollagen.

The endothelial cells used in this study were factor VIII positive and always exhibited the cobblestone appearance typical of the contact-inhibited monolayer growth pattern described for these cells (Gospodarowicz et al., 1976; Schwartz, 1978). It was also important to ascertain whether the synthesis and expression of a certain collagen phenotype could be altered or modulated as a result of routine culture or labeling procedures; accordingly, we analyzed several different growth conditions and methods of cell preparation and assessed in vitro collagen production. The preparations included endothelial cells which had not been [^{3}H]thymidine-selected but were grown in plasma-derived serum and cells which had been cloned as described by Schwartz (1978) in the presence of FGF. [^{3}H]Thymidine-selected bovine endothelial cells were grown and labeled in the presence of FCS, calf serum, PDS, and several growth factors and at both high and low density. [^{3}H]Proline-labeled culture medium protein was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis after both trichloroacetic acid precipitation and fractionation at different saturations of ammonium sulfate. Although in some instances we observed different proportions of labeled proteins in the culture medium, the collagen type did not appear to be altered, nor were any new collagens observed in either the medium or cell layer by the criteria of mobility on $NaDodSO_4$–polyacrylamide gel electrophoresis, susceptibility to bacterial collagenase, and immune precipitation characteristics. Cells cloned and maintained in the presence of FGF also continued to produce type III procollagen, although some differences were observed in labeling efficiency and in the relative proportions of secreted products. It is highly probable that these endothelial strains do not respond to growth factors in the manner which has been described for other endothelial cells (Gospodarowicz et al., 1978a) or for 3T3 cells (Nilsen-Hamilton & Hamilton, 1979). However, the apparent modulation of fibronectin, procollagen, and glycoprotein synthesis is of interest, and further studies in this area are in progress.

Other laboratories have reported the production of interstitial collagens by endothelial cells in vitro. Barnes et al. (1978) described the synthesis of types I and III collagen by porcine aortic endothelial cells, and Mayne & Mayne (1978) have characterized type I collagen from guinea pig aortic endothelial cells. The present studies were conducted with six different strains of bovine endothelial cells, only one of which was found to synthesize equal amounts of types I and III procollagen. This particular strain, however, was highly prone to sprouting, a secondary growth pattern described in some confluent cell cultures by Gospodarowicz et al. (1978b) and by Schwartz (1978). Experiments in this laboratory have shown that these sprouting cells synthesize type I collagen (Cotta-Pereira et al., 1979). We therefore suggest that synthesis of significant proportions of type I procollagen is not truly indicative of the biosynthetic profile of endothelial cells.

The observation that endothelial cells synthesize and secrete predominantly type III procollagen poses some interesting possibilities regarding the source and function of this protein in the vascular system. It has been reported that type III collagen constitutes from 40 to 70% of the total collagen in the adult human blood vessel wall (Rauterberg & von Bassewitz, 1975; McCullagh & Balian, 1975). Presumably, the source of most of this collagen is the smooth muscle cell of the medial layer which secretes as much as 68% of its total collagen as type III (Burke et al., 1977). A study by Gay et al. (1975) demonstrated the presence of type III collagen in thrombi and the subendothelium by indirect immunofluorescence, whereas type I collagen was absent. Subendothelial type III collagen could therefore be responsible for the thrombogenicity observed when the endothelial surface is perturbed. Several reports have indicated the effectiveness of both types I and III collagen, in fibrillar form, as inducers of platelet aggregation (Balleisen et al., 1975), and a primary sequence within the cyanogen bromide peptide CB4 of type III collagen was found to be the specific active site (Fauvel et al., 1978). In contrast, neither of the basement membrane collagens, types IV and AB, have been found to elicit platelet aggregation or serotonin release (Trelstad & Carvalho, 1979). Recent reports have suggested that fibronectin is the receptor on platelets which binds collagen (Bensusan et al., 1978; Plow et al., 1979), and several laboratories studying collagen–fibronectin interactions have shown that both native and denatured type III collagens demonstrate a high specificity for fibronectin (Engvall et al., 1978; Dessau et al., 1978; Hörmann & Jilek, 1978). These observations suggest that type III collagen may play a major role in both platelet aggregation and thrombus formation that may follow endothelial injury.

The predominance of type III procollagen as the major extracellular collagen produced by bovine aortic endothelial cells is perhaps inconsistent with the supposition that cells which secrete and adhere to a basement membrane should preferentially synthesize type IV procollagen. However, ultrastructural studies have indicated that, in aortas of larger mammals, the endothelial basement membrane, as a morphologically discernible structure, is either discontinuous or totally absent (French, 1966). Nevertheless, Trelstad (1974) has shown that, in addition to types I and III collagen, human aortas contain type IV collagen, and preliminary reports indicate that human (Kay et al., 1979; H. Sage and P. Bornstein, unpublished experiments) and guinea pig (Mayne & Mayne, 1978) endothelial cells synthesize type IV procollagen. In agreement with these findings, our data also suggest the presence of type IV collagen in the bovine endothelial cell layer (Figure 3). Type IV procollagen may therefore still play a role in the attachment of endothelial cells to the subcellular interstitium.

One of the most intriguing aspects of endothelial cell growth is the polarity exhibited by cells both in vivo and in vitro. Birdwell et al. (1978) have shown that fibronectin accumulates in an extracellular matrix on the basal surface of confluent cultures, and this polarity has also been observed with respect to collagen synthesis and accumulation (Jaffe et al., 1976). Studies in this laboratory have shown that reversible morphological changes between endothelial cells and sprouting cultures are correlated with modulation in synthesis of secreted proteins, including collagen and fibronectin (Cotta-Pereira et al., 1979). We are currently examining collagen production in wounded endothelial cell cultures, in which the cells have temporarily lost their polarity. Studies like these, in conjunction with

biochemical analyses of cell surface associated matrix proteins synthesized by endothelial cells, should provide interesting insights into properties of endothelial cells as well as illuminate some other basic cellular phenomena.

Acknowledgments

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Hydrodynamic Characterization of Highly Purified and Functionally Active Liver Microsomal Cytochrome P-450†

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ABSTRACT: The subunit molecular weight of highly purified phenobarbital-treated rat liver microsomal cytochrome P-450 (P-450) was estimated to be 55 000 ± 2000 by sedimentation velocity and equilibrium measurements in the presence of sodium dodecyl sulfate. Sedimentation equilibrium studies indicate that, in 0.1 M KCl at its isoelectric pH of 6.5, P-450 undergoes self-association with an apparent weight-average molecular weight (M_w) of about 500 000 at high dilution. The dependence of M_w on concentration could be described by a model in which six to nine P-450 monomers associate with high affinity to form a basic aggregate P_1 which further self-associates isodesmically. Values for k [$\sim 10^{11}$ M^{-1}, where $k = [P_{n+1}]/([P_n][P_1])$; Teller, D. C. (1973) *Methods Enzymol. 27*, 346–411] were found not to change appreciably for P-450 in the presence of the substrate *d*-benzphetamine, phospholipid monomers, or NADPH–cytochrome P-450 reductase. The molecular weight of the P_1 aggregate was decreased by ~100 000 in the presence of phospholipid monomers and increased about twofold in the presence of NADPH–cytochrome P-450 reductase plus phospholipid monomers. The presence of phospholipid micelles raised k about threefold, while 6 M guanidine hydrochloride and 0.1% (w/v) Triton N-101 lowered k to 10^{10} and 10^9 M^{-1}, respectively, and decreased the molecular weight of P_1 to ~150 000. Monomeric P-450 was detected only in the presence of amphipathic detergents. For the isodesmic self-association of the ~400 000-dalton aggregate of P-450 at pH 6.5 and 20 °C, $\Delta G = -14.4 \pm 0.1$ kcal mol^{-1}, $\Delta H = -4.8 \pm 1.3$ kcal mol^{-1}, and $\Delta S = 33 \pm 5$ eu. The results suggest that hydrophobic interaction is important for the isodesmic association of the P_1 aggregate. Several lines of evidence also suggest that P-450 activity is not very dependent upon the state of aggregation of the enzyme. Other P-450's isolated from rats and rabbits were also found to aggregate in a similar manner.

A number of enzymes have been shown to depend upon lipids for activity, as first reviewed by Fleischer & Fleischer (1967) and more recently by Sandermann (1978). While a number of these enzymes have been purified, relatively few detailed studies have been carried out to describe the interactions of such enzymes with lipids and detergents or how these proteins associate and dissociate in solution (McIntyre et al., 1978).

P-450,[1] the terminal oxidase of the microsomal mixed-function oxidase system that metabolizes a variety of endogenous and exogenous chemicals (Lu & West, 1978; Guengerich, 1979a; Coon et al., 1977), is associated with the flavoprotein NADPH–cytochrome P-450 reductase in the endoplasmic reticulum. Both enzymes have been isolated with detergents and purified to apparent homogeneity; a variety of evidence exists for the multiplicity of P-450 in various species (Coon et al., 1976, 1977; Guengerich, 1979a), and three different forms have been purified to apparent homogeneity from rat liver in this laboratory (Guengerich, 1978a).

Rates of P-450-dependent activities of microsomal preparations can be decreased by lipid extraction and restored by the addition of phospholipids (Vore et al., 1974). The activity of the reconstituted enzyme system has been shown to be stimulated by phosphatidylcholine (Strobel et al., 1970). The lipid appears to function at least at two levels. For example, di-12 GPC promotes association of P-450 with its organic substrates and with NADPH–cytochrome P-450 reductase (Guengerich & Coon, 1975; Coon et al., 1976), and these increased affinities are responsible for enhancement of the rate of electron flow (Strobel et al., 1970) and possibly other steps. The presence of phospholipid does not, however, appear to influence the oxidation–reduction potential of P-450 (Guengerich et al., 1975). Previous work suggests that P-450 exists as an aggregate in its active state (Autor et al., 1973), in a complex with NADPH–cytochrome P-450 reductase (Coon et al., 1976; French et al., 1978), but others have suggested that P-450 exists in an active monomeric state (Ingleman-Sundberg, 1977).

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[1] Abbreviations used: P-450, liver microsomal cytochrome P-450; $NaDodSO_4$, sodium dodecyl sulfate; di-12 GPC, L-α-dilauroylglyceryl-3-phosphorylcholine; NaDOC, sodium deoxycholate; EDTA, (ethylenedinitrilo)tetraacetic acid; cmc, critical micelle concentration; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; M_w, weight-average molecular weight; R_S, Stokes' radius; Gdn, guanidine.

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