SYNTHESIS OF BASEMENT MEMBRANE COLLAGEN BY CULTURED HUMAN ENDOTHELIAL CELLS*

By ERIC A. JAFFE,‡ C. RICHARD MINICK, BURT ADELMAN, CARL G. BECKER, AND RALPH NACHMAN

(From the Departments of Medicine and Pathology, Cornell University Medical College, New York 10021)

Endothelial cells in vivo rest on the subendothelium, a complex array of acellular, connective tissue structures. Ultrastructural studies of the subendothelium in vivo have shown that it is composed of at least five different components; amorphous basement membrane, microfibrils, elastic fibers, fibrillar collagen, and mucopolysaccharides (1). The subendothelium is thought to have several important physiologic functions. It provides most of the rigidity of capillary walls (2), serves as an anchor for the overlying endothelial cell layer, and may act as a filter (3). The subendothelium is also involved in pathologic processes. Loss of endothelial cells and exposure of the subendothelium to blood is an important step in the development of thrombosis since the subendothelium can initiate platelet adhesion and aggregation (1). Alterations in the structure and function of the subendothelium may contribute significantly to the pathogenesis of atherosclerosis (1) and the vascular disease of diabetes mellitus. For example, one of the earliest changes seen in diabetes mellitus is thickening of capillary basement membranes (4).

In this paper we report that cultured human endothelial cells synthesize material which is morphologically and immunologically like amorphous basement membrane and biochemically like basement membrane collagen. Cultured endothelial cells probably also synthesize material which is morphologically similar to microfibrils and elastic fibers.

Materials and Methods

Cell Culture Techniques and Culture Media. Human endothelial cells were derived from umbilical cord veins and cultured using methods and materials previously described (5). Endothelial cells were cultured in plastic 35 × 10-mm plastic Petri dishes or plastic T-25 or T-75 flasks (BioQuest Div., Becton, Dickinson & Co., Cockeysville, Md. or Corning Glass Works, Science Products Div., Corning, N. Y.). The culture medium consisted of Medium 199 containing either *(a)* 20% heat-inactivated fetal calf serum or horse serum, or *(b)* 20% heat-inactivated horse serum plus 10% heat-inactivated human serum (all Flow Laboratories, Inc., Rockville, Md.). The culture media also contained penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM,

* This work was supported by National Institutes of Health Grants HL-14810 and HL-1803 and grants from The Arnold R. Krakower Hematology Foundation and The Cross Foundation. A preliminary report of this work was presented at the 48th Annual Meeting of the American Heart Association, 1975. Anaheim, Calif., 1975 *Circulation*. 52(Suppl. II):61a.

‡ Recipient of an Irma T. Hirschl Career Scientist Award.

Grand Island Biological Co., Grand Island, N. Y.). Ascorbic acid (50 μg/ml, Sigma Chemical Co., St. Louis, Mo.) was added daily to the culture media because of rapid turnover of the vitamin in tissue culture (6). For some studies, endothelial cells were cultured in media which also contained β-aminopropionitrile (50 μg/ml, Sigma Chemical Co.).

Preparation of Cells and Tissues for Microscopy. Segments of umbilical vein were prepared for electron microscopy as previously described (5). Cultured endothelial cells to be viewed on edge were fixed *in situ* in plastic Petri dishes and processed as previously described (5).

Cultured endothelial cells to be examined as whole cell pellets were prepared by detaching them and their surrounding extracellular matrix from plastic Petri dishes or T-25 or T-75 flasks by incubation for 10 min at 37°C with 0.01% EDTA in cord buffer (0.14 M NaCl, 0.004 M KCl, 0.001 M phosphate buffer, pH 7.4, 0.011 M glucose). In some cases, this was followed by scraping the cells and extracellular matrix off the bottom of the culture vessel with a rubber policeman. The detached cells and extracellular matrix were pelleted by centrifugation and processed for electron microscopy as previously described (5). Thick sections of Epon blocks were stained for light microscopy with toluidine blue.

Semipurified extracellular matrix synthesized by cultured endothelial cells was prepared by detaching endothelial cells and extracellular matrix with 0.01% EDTA in cord buffer using a rubber policeman and processing the cell pellet by a modification of the method of Westberg and Michael (7). Detached cells and extracellular matrix were resuspended in phosphate-buffered saline (PBS;[1] 0.145 M NaCl, 0.01 M phosphate buffer, pH 7.4) containing 0.4 mM phenylmethyl sulfonyl fluoride and 0.5 mM *N*-carbobenzoxy-α-L-glutamyl-L-tyrosine (8). Resuspended cells and matrix were sonicated for eight 1-min bursts while suspended in a 4°C ice bath. 1 min of cooling was allowed between each 1-min burst of sonication. The sonicated suspension was centrifuged at 180 *g* for 15 min at 4°C. The pellet containing the extracellular matrix was washed twice by centrifugation with PBS and resuspended in PBS. This material was processed for electron microscopy similarly to whole cell pellets.

Specimens of both whole cell pellets and semipurified extracellular matrix were digested with purified collagenase, elastase, and trypsin. Purified collagenase was prepared by the method of Peterkofsky and Diegelmann (9) from partially purified collagenase (type CLSPA, Worthington Biochemical Corp., Freehold, N. J.). Samples to be digested with purified collagenase were transferred to a 1.5-ml polypropylene tube (Brinkmann Instruments, Inc., Westbury, N. Y.), washed three times by centrifugation at 8,000 *g* for 20 min at 4°C with 0.5 ml of 0.2 M Tris-HCl, pH 7.6 containing 1 mM $CaCl_2$, and resuspended in 0.5 ml of the same buffer. *N*-ethylmaleimide (final concentration, 2.5 mM) and purified collagenase (120 μg) were added and the mixture incubated with end over end rotation for 20 h at 37°C. After digestion, the suspension was pelleted at 8,000 *g* for 20 min at 4°C, washed twice with 0.2 M Tris-HCl, pH 7.4, and processed for electron microscopy. Samples to be digested with trypsin were prepared as above except that the buffer was 0.2 M Tris-HCl, pH 7.4, and 100 μg of twice crystallized trypsin (type III, Sigma Chemical Co.) were added. Specimens to be digested with elastase were also prepared as above except that the buffer was 0.2 M Tris-HCl, pH 8.8 to which 50 μg of elastase (type III, Sigma Chemical Co.) was added. Soybean trypsin inhibitor (250 μg/ml, Worthington Biochemical Corp.) was also added because it inhibits the contaminating proteolytic but not the intrinsic elastolytic activity of elastase (10). Controls for each digestion were processed as above except that the enzymes were omitted. Microscopy was performed with either a Siemens Elmiskop I (Siemens Corp., Iselin, N. J.) or a Philips EM 301 electron microscope (Philips Electronic Instruments, Inc., Mount Vernon, N. Y.).

Preparation of Antisera. Human dermal collagen, obtained from Drs. Sidney Rothbard and Robert Watson (Cornell University Medical College) and prepared by their method (11), was used with complete Freund's adjuvant to immunize rabbits. The resulting anti-collagen sera were heat inactivated at 56°C for 30 min. Goat antibody to human glomerular basement membrane was obtained from Dr. Charles Christian of Cornell University Medical College. Human glomerular basement membrane used to produce this antiserum was prepared by the method of Krakower and Greenspon (12) and further digested with bacterial collagenase (type CLSPA). After centrifugation and dialysis, the pellet was used to immunize a goat.

As controls for immunohistologic studies, antisera were absorbed with the eliciting antigens for

[1] *Abbreviations used in this paper:* PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

2 h at 37°C and overnight at 4°C. The antisera were centrifuged at 43,000 *g* at 4°C for 1 h and the supernate decanted. Rabbit anti-goat IgG and goat anti-rabbit IgG were prepared as previously described (5) and conjugated with fluorescein according to the method of Goldstein et al. (13) as previously described (14).

Immunofluorescence Microscopy. For examination by immunofluorescence microscopy, the IgG fractions of samples of appropriate antisera were conjugated with fluorescein isothiocyanate according to methods described above. Tissues and cultured cells were prepared for immunofluorescence microscopy and these tissues processed by previously described methods (5).

Incorporation of [^{3}H]Proline into Protein. Dose-dependent and time-dependent incorporation of [^{3}H]proline into TCA-precipitable protein by cultured endothelial cells was studied. Results are expressed in disintegrations per minute per milligram protein per hour.

For the time-dependent study, Petri dishes containing 10^6 cultured endothelial cells in the stationary phase were incubated with 2.5 μCi/ml of L-[3,4-^{3}H]proline, sp act, 38 Ci/mmol (New England Nuclear, Boston, Mass.). After the appropriate labeling periods, the cells were processed for liquid scintillation counting as previously described (15). For the dosage study, endothelial cells were incubated for 24 h with either 2.5, 0.25, or 0.025 μCi/ml [^{3}H]proline and then processed as above. For both studies, each point is the average of three or four replicate Petri dishes.

Collagenase Digestion of Radioactively Labeled Endothelial Cell Protein. Cultured endothelial cells in the stationary phase were incubated for 24 h with media containing 5 μCi/ml of either L-[^{3}H]tryptophan, sp act, 1.64 Ci/mmol (New England Nuclear) or [^{3}H]proline. The culture medium was removed and saved and the cells washed five times with ice-cold PBS containing either 10^{-4} M unlabeled proline or tryptophan. The cells and extracellular matrix were taken up by incubation in 0.02% EDTA in cord buffer containing 10^{-4} M tryptophan or 10^{-4} M proline for 15 min at 37°C. The detached cells and extracellular matrix were washed three times with digestion buffer (40 mM Tris-HCl, pH 7.6 containing 0.5 mM $CaCl_2$) by centrifugation at 200 *g* for 10 min at 20°C and resuspended in 1 ml of the same buffer. The particulate cell suspensions were frozen and thawed twice and then sonicated. The cell suspension was split into two parts to which *N*-ethylmaleimide (final concentration, 2.5 mM) and 25 μl of either purified collagenase (30 μg in 0.05 M Tris-HCl, pH 7.6, 5 mM $CaCl_2$) or buffer alone were then added. The mixtures were incubated at 37°C for 2 h. To insure that enough protein was present for precipitation by TCA, bovine serum albumin (Miles Laboratories Inc., Elkhart, Ind.) as carrier (final concentration, 1 mg/ml) was added. The undigested proteins and carrier bovine serum albumin were precipitated with 0.5 ml of 20% TCA at 4°C for 20 min. The tubes were centrifuged at 8,000 *g* for 20 min at 4°C and the supernate removed and saved. The precipitate was washed once by centrifugation with 0.5 ml of ice-cold 10% TCA and the supernate added to the first TCA supernate. The precipitate was solubilized by digestion with 0.8 ml of 1 N NaOH at 37°C overnight. Samples of the supernate and precipitate were added to 10 ml of PCS (Amersham/Searle Corp., Arlington Heights, Ill.) and counted in a liquid scintillation counter. Final results were expressed as percent digestion (disintegrations per minute TCA soluble/total disintegrations per minute) and were corrected for the radioactivity found to be TCA soluble in control experiments.

The radioactive postculture medium was dialyzed at 4°C against digestion buffer containing 10^{-4} M unlabeled proline or tryptophan until the dialysate was free of radioactivity. The dialyzed postculture medium was then digested with purified collagenase and further processed as above.

Conversion of [^{14}C]Proline to [^{14}C]Hydroxyproline. Cultured endothelial cells were incubated for 24 h with L-[^{14}C]proline, 1 μCi/ml of media, sp act, 225 mCi/mmol (New England Nuclear). The culture medium was removed and the cells washed five times with PBS containing 10^{-4} M unlabeled proline. The cells and extracellular matrix were taken up by incubation in 0.02% EDTA in cord buffer containing 10^{-4} M proline. The pellet containing the cells and extracellular matrix was washed twice with PBS containing 10^{-4} M proline, once with PBS, and three times with distilled H_2O. The washed pellet was hydrolyzed in 6 N HCl under vacuum at 108°C for 48 h. The proline and hydroxyproline in the hydrolysate were separated on a modified Beckman 120C amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif., performed by Worthington Biochemical Corp.) and the appropriate fractions counted in a liquid scintillation counter as above. The radioactivity applied to the amino acid analyzer was completely recovered in the proline and hydroxyproline fractions.

Characterization of Collagen Synthesized by Cultured Endothelial Cells. Cultured human endothelial cells were labeled with radioactive glycine and proline and digested sequentially with pepsin and purified collagenase. The fractions were analyzed by sodium dodecyl sulfate (SDS)-

polyacrylamide gel electrophoresis before and after collagenase treatment to demonstrate the presence of collagen-like polypeptides.

Cultured endothelial cells in the stationary phase were incubated with 5 μCi/ml of [^{3}H]proline and 5 μCi/ml of [2-^{3}H]glycine, sp act, 11.4 Ci/mmol (New England Nuclear) for 24 h. The radioactive postculture medium was removed and processed as detailed below. The cells were washed three times with warm (37°C) cord buffer containing 10^{-4} M proline and 10^{-4} M glycine. The cells and extracellular matrix were taken up with 5 mM EDTA in cord buffer containing 0.5% bovine serum albumin and 0.011 M glucose by incubating at 37°C for 10 min and then scraping the flask with a rubber policeman. The flasks were washed once with the same mixture and the washings added to the cells. The cells and extracellular matrix were centrifuged at 1,000 *g* for 10 min at 20°C, washed once with 0.5 M acetic acid, resuspended in 0.5 M acetic acid, and sonicated in an ice bath for three 1-min intervals at full power with a Kontes sonicator (Kontes Co., Vineland, N. J.). Pepsin (twice crystallized, Sigma Chemical Co.) was added to a final concentration of 0.5 mg/ml and the mixture incubated at 4°C for 6 days or at 20°C for 18 h. The suspension was centrifuged at 40,000 *g* for 10 min at 4°C and the supernate removed and processed as detailed below. The precipitate was washed twice with 0.1% acetic acid, split into equal portions, and one-half lyophilized for subsequent analysis by SDS-polyacrylamide gel electrophoresis. The remaining half was washed twice with 0.2 M Tris-HCl pH 7.6 containing 1 mM $CaCl_2$, resuspended in 0.5 ml of the same buffer, and sonicated in an ice bath with three 1-min bursts as above. *N*-ethylmaleimide (final concentration, 2.5 mM) and purified collagenase (120 μg) were added and the suspension incubated overnight at 37°C. The reaction was stopped by adding 10 μl of 0.1 M EDTA and centrifuging the suspension at 8,000 *g* for 20 min at 4°C. The supernate was removed, dialyzed against 0.1% acetic acid at 4°C, and lyophilized. These samples were analyzed by SDS-polyacrylamide gel electrophoresis. The supernate from the original pepsin digest was similarly studied after precipitation by 20% NaCl.

The radioactive culture medium was centrifuged to remove cells. Collagen and other proteins were precipitated with 40% saturated ammonium sulfate by incubation at 4°C overnight. The precipitate was collected by centrifugation, redissolved in 0.5 M acetic acid, and dialyzed against 0.5 M acetic acid for 3 days at 4°C. Pepsin (0.5 mg/ml) was added and the mixture incubated at 18°C for 24 h. Pepsin was irreversibly denatured by raising the pH to 8 and incubating for 20 min at 20°C. Half the media was dialyzed against 0.1% acetic acid and lyophilized and the other half dialyzed against 0.2 M Tris-HCl pH 7.6 containing 1 mM $CaCl_2$ and digested with collagenase as above.

Similar experiments were also performed on extracellular matrix prepared by methods described above from endothelial cells cultured and labeled in the presence of the lathyrogen, β-aminopropionitrile (50 μg/ml) to inhibit collagen cross-linking. In these experiments, however, the extracellular matrix was digested with pepsin for 2 days at 10°C and the material solubilized by pepsin was analyzed directly on SDS-polyacrylamide gel electrophoresis without being precipitated by NaCl.

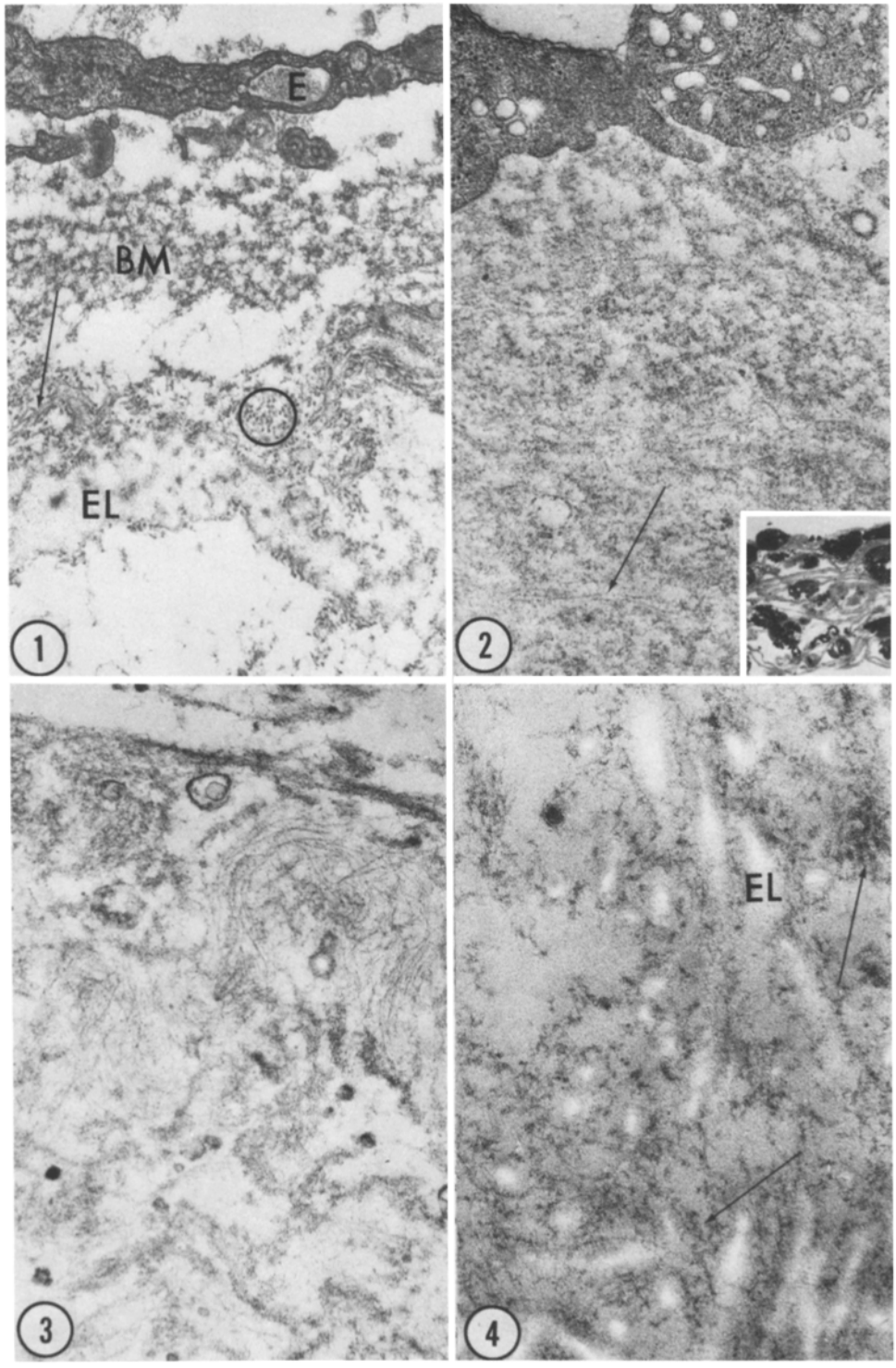
SDS-Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis using 5% gels was performed as described by Weber and Osborn (16). Lyophilized samples for analysis were dissolved in 8.5 M urea containing 2% SDS and 14 mM dithiothreitol and boiled for 5 min before

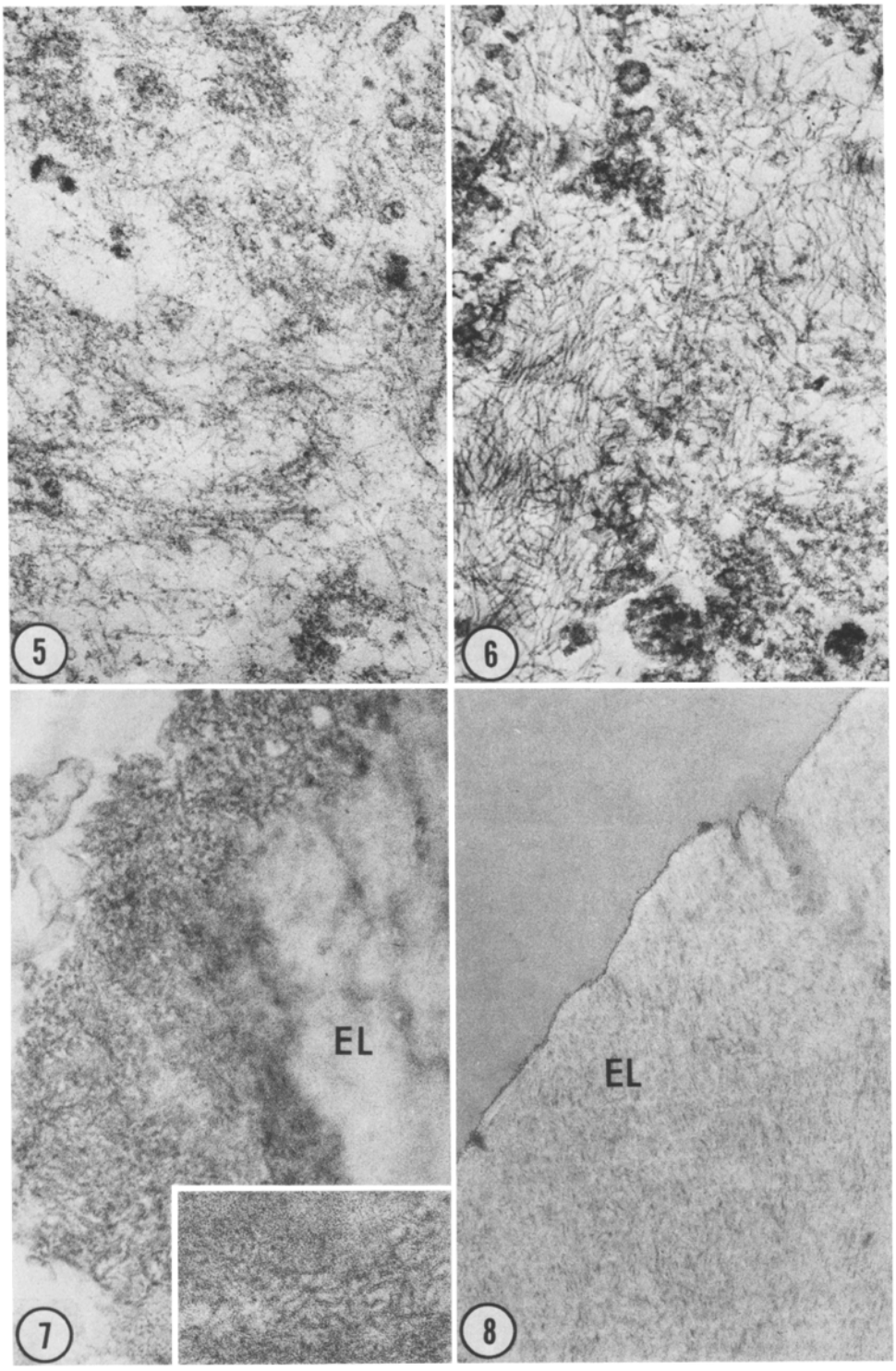
FIG. 1. Electron photomicrograph of wall of umbilical vein. Endothelial cells (E) cover subendothelium comprising amorphous basement membrane (BM), microfibrils cut both longitudinally (arrow) and in cross section (circle), and elastic fibers. Elastic fibers consist of amorphous elastin (EL) bordered by microfibrils. × 33,000.

FIG. 2. Electron photomicrograph of pellet of cultured endothelial cells. In toluidine blue-stained thick sections (inset), endothelial cells are surrounded by bands of dense, amorphous staining material. By electron microscopy, endothelial cells adhere to bands of dense granular material containing microfilamentous structures (arrow). × 33,000.

FIG. 3. Electron photomicrograph at higher magnification of extracellular matrix similar to that in Fig. 2. Granular material resembling amorphous basement membrane surrounds tangles of microfilamentous structures resembling microfibrils. × 42,000.

FIG. 4. Electron photomicrograph of extracellular matrix of cultured endothelial cells revealing electron-lucent islands of amorphous elastin (EL) bordered by microfibrils cut in cross section (arrows). × 78,000.


E
BM
EL
1
2
3
EL
4


5
6
EL
7
EL
8

electrophoresis. Gels were either stained with Coomassie Brilliant Blue by the method of Fairbanks (17) and scanned for protein, or sliced and assayed for radioactivity as previously described (18).

The molecular weights of the collagenase-digestible proteins were determined as described by Furthmayr and Timpl (19). Acid-soluble collagen derived from calf skin (Sigma Chemical Co.) was solubilized in 2% SDS-8.5 M urea-14 mM dithiothreitol by heating at 50°C for 1 h and run as the standard. Molecular weights were calculated assuming the α-chain to be 93,200 and the β-dimer 186,400 (20).

Results

Morphologic Studies of Subendothelium. Ultrastructural studies of the subendothelium in vivo have shown that it is composed of five different components. These are amorphous basement membrane, microfibrils, elastic fibers, fibrillar collagen, and mucopolysaccharides. These components are all present in the subendothelium of umbilical veins and three of them, amorphous basement membrane, microfibrils, and elastic fibers are illustrated in Fig. 1. Fibrillar collagen, which is seen in the subendothelium only in small quantities, was not present in the area shown in the photomicrograph. Mucopolysaccharides are not identifiable by electron microscopy using conventional techniques.

When cultured endothelial cells were detached from the surface of a Petri dish with 0.5 mM EDTA, the cells rounded up and floated in the buffer attached to a membranous structure visible to the naked eye. The cells and attached membranous structures were pelleted by centrifugation and prepared for electron microscopy. Thick sections (0.5 μm) of the embedded cells were stained with toluidine blue and examined by light microscopy. Endothelial cells were embedded in ribbons of dense, amorphous material (Fig. 2, inset).

The bands of dense, amorphous material seen in Fig. 2 (inset), when examined by electron microscopy, were composed largely of granular amorphous basement membrane. Microfilamentous structures varying from ~40 to 120 Å in size were embedded in the amorphous basement membrane and were present both as single fibrils and as meshworks (Figs. 2 and 3). Some of the larger fibrils (~100 Å) had a "hollow core" appearance on cross section and resembled elastic microfibrils. Amorphous elastin and structures resembling elastic fibers were also seen and were composed of a central area of relatively electron-lucent amorphous elastin bordered by microfibrils (Figs. 4 and 7).

When semipurified extracellular matrix synthesized by cultured endothele amorphous basement membrane component was digested, leaving exposed an extensive meshwork of microfibrils (Figs. 5 and 6). When pellets of cells and their surrounding extracellular matrix were treated with trypsin, the amorphous elastin component was left intact but the microfibrils were digested (Figs.

FIG. 5 and 6. Semipurified extracellular matrix prepared from cultured endothelial cells consisted of amorphous basement membrane with many microfibrils in the background (Fig. 5). Treatment with purified collagenase for 20 h resulted in digestion of amorphous basement membrane revealing tangles of ~100 Å microfibrils (Fig. 6). × 59,000; 50,000.

FIG. 7 and 8. Photomicrograph of extracellular matrix in endothelial cell pellet. Aggregates of ~100 Å microfibrils (inset) surround an island of relatively electron-lucent material resembling the amorphous component of elastin (EL) (Fig. 7). After treatment with trypsin, microfibrils were digested, leaving only amorphous elastin (EL) (Fig. 8). × 53,000.

7 and 8). When similar pellets were treated with elastase, the elastic fibers were no longer identified.

To determine the orientation of the extracellular matrix, cultured endothelial cells were fixed and embedded for electron microscopy *in situ* in Petri dishes. The block with its embedded cells was then rotated 90° and sectioned perpendicular to the surface of the Petri dish. Extracellular matrix was invariably present between the cells and the bottom of the dish or between overlapping cells. The extracellular matrix was never seen on the upper surface of the cells.

Immunofluorescence Studies. When sections of umbilical cord were treated with goat antiserum to human glomerular basement membrane and then with fluorescein-conjugated rabbit antiserum to goat IgG, fine, often coalescent immunofluorescent lines were observed in the subendothelium. Basement membrane of smooth muscle cells was also immunofluorescent (Fig. 9). In sections of kidney, the same antisera stained glomerular basement membrane, smooth muscle basement membrane, all endothelial cell basement membrane, and renal tubular basement membrane. The antiserum did not stain collagen in the adventitia of blood vessels or in the renal medulla.

When endothelial cells which had been cultured for 1 wk were treated with the same antiserum, intracellular and extracellular staining was observed (Figs. 10 and 11). The intracellular staining was granular and probably represents basement membrane components in vesicles awaiting extracellular release. The extracellular staining appeared as a delicate meshwork; in some areas the extracellular matrix had condensed and appeared as brightly staining ribbons. Immunofluorescent staining of endothelial and smooth muscle basement membranes in sections of umbilical vein was completely inhibited by previous absorption with human glomerular basement membrane (Fig. 12) as was immunofluorescent staining of the cytoplasm and extracellular matrix of cultured endothelial cells. Absorption of the antisera with human dermal collagen reduced the staining only slightly. Control studies using normal rabbit serum were negative.

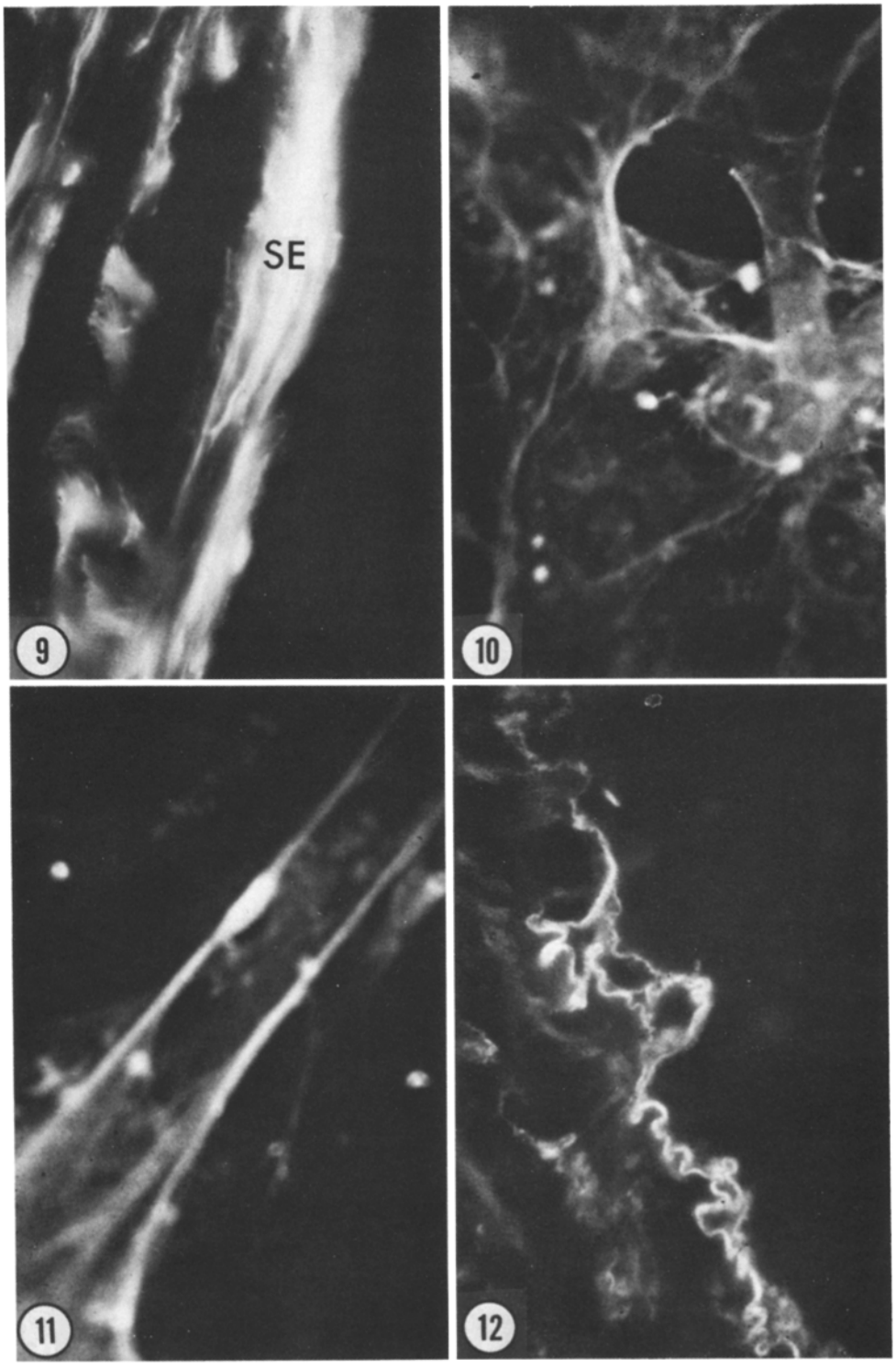
When sections of human skin were treated with rabbit antiserum to human dermal collagen and then with fluorescein-conjugated goat antiserum to rabbit IgG, dermal and adventitial collagen stained brightly. In sections of kidney treated similarly, glomerular mesangium stained brightly but glomerular base-

FIG. 9. Section of human umbilical vein treated with goat antiserum to glomerular basement membrane followed by fluoresceinated rabbit antibody to goat IgG. Delicate, often coalescent immunofluorescent lines are seen in the subendothelium (SE). The basement membranes of smooth muscle cells are also immunofluorescent. Lumen is to the right. × 500.

FIG. 10. Cultured endothelial cells treated with the same reagents as in Fig. 9. A meshwork of fine and coarse immunofluorescent extracellular fibrils is seen as well as less intense immunofluorescent staining of the cytoplasm. × 500.

FIG. 11. Cultured endothelial cells treated as above. Coarse laminae are immunofluorescent. These are similar to the bands illustrated in Fig. 2, inset. × 500.

FIG. 12. Section of human umbilical vein treated with goat antiserum to glomerular basement membrane previously absorbed with glomerular basement membrane followed by fluoresceinated rabbit antibody to goat IgG. Immunofluorescence is abolished. Elastic tissue is autofluorescent. × 500.


SE
9
10
11
12

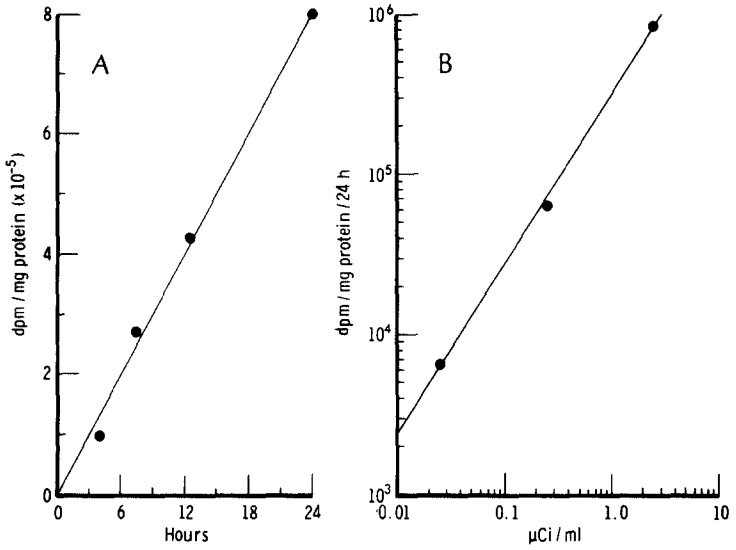


FIG. 13. Incorporation of [^{3}H]proline into protein. (A) Time-dependent incorporation, (B) Dose-dependent incorporation.

ment membrane stained only slightly. When cultured endothelial cells were treated with the same antisera, only weak staining was seen. Staining of endothelial cells by antiserum to human dermal collagen was completely inhibited by previous absorption with human dermal collagen.

Biochemical Studies

INCORPORATION OF [^{3}H]PROLINE INTO PROTEIN BY CULTURED ENDOTHELIAL CELLS. Both collagen and elastin contain large amounts of proline and glycine. The following studies were performed to establish that cultured endothelial cells could incorporate [^{3}H]proline into TCA-precipitable cellular and insoluble extracellular protein. Cells incubated with [^{3}H]proline at 37°C incorporated radioactivity into TCA-precipitable protein. Incorporation varied linearly with time (Fig. 13A) and with [^{3}H]proline concentration (Fig. 13B).

COLLAGENASE DIGESTION OF [^{3}H]PROLINE-LABELED PROTEINS SYNTHESIZED BY CULTURED ENDOTHELIAL CELLS. To demonstrate that cultured endothelial cells synthesize collagen, endothelial cells were incubated with [^{3}H]proline, digested with purified collagenase, and the amount of [^{3}H]proline incorporated into protein and subsequently solubilized was quantitated. To demonstrate the specificity of the purified collagenase, endothelial cells were incubated with [^{3}H]tryptophan and the heavily labeled postculture medium digested with purified collagenase. Under these circumstances, no radioactivity above control levels was solubilized.

When [^{3}H]proline-labeled endothelial cells and their attached extracellular matrix were digested with purified collagenase, 18% of the [^{3}H]proline incorporated into TCA-precipitable material was digested and solubilized (Table I). When [^{3}H]proline-labeled endothelial cell postculture medium was digested with purified collagenase, 2.2% of the [^{3}H]proline incorporated into TCA-precipitable material was digested and solubilized (Table I).

TABLE I
Collagenase Digestion of [^{3}H]Proline-Labeled Cultured Endothelial Cells and Extracellular Matrix

Material tested*	Digestion‡
	%
Endothelial cells and extracellular matrix	18.0
Postculture medium	2.2

* Each experiment was performed in duplicate on two separate cell lines.
‡ Digestion represents the percentage of the total incorporated [^{3}H]proline made TCA soluble by digestion with purified collagenase. Blank values obtained by omission of the collagenase have been subtracted.

CONVERSION OF [^{14}C]PROLINE TO [^{14}C]HYDROXYPROLINE BY CULTURED ENDOTHELIAL CELLS. Hydroxyproline is an unusual amino acid which is found in only a few proteins, i.e., collagen, elastin, and the C1q component of complement (21, 22). However by immunofluorescence, antisera to C1q does not stain normal endothelial cells, glomerular basement membrane, or other connective tissue components (23).[2] Hydroxyproline in these proteins is formed by the post-translational hydroxylation of incorporated proline. To demonstrate that endothelial cells can form this amino acid, presumably by synthesizing collagen and/or elastin, the following experiment was performed. Cultured endothelial cells were incubated with [^{14}C]proline for 24 h, washed extensively, and the cells and extracellular matrix hydrolyzed in 6 N HCl. [^{14}C]Proline and [^{14}C]hydroxyproline were separated with an amino acid analyzer. When these fractions were analyzed for radioactivity, 7.1% of the [^{14}C]proline incorporated into proteins was present as [^{14}C]hydroxyproline (Table II).

CHARACTERIZATION OF THE COLLAGEN SYNTHESIZED BY CULTURED ENDOTHELIAL CELLS. Cultured endothelial cells were incubated with radioactive amino acids, digested with pepsin, and analyzed by SDS-polyacrylamide gel electrophoresis. The digested cell pellet contained two major peaks in the collagen region (Fig. 14). These peaks had mol wt of 120,500±3,300 and 94,200±1,500 (average of six experiments ± SEM, each performed on a different endothelial cell culture). When the pepsin-treated cell pellet was digested with purified collagenase, both peaks disappeared. In some experiments, small amounts of collagenase-digestible polypeptides of approximate mol wt 159,000 and 180,000 were seen.

Pepsin digestion was used in these cell culture experiments to solubilize the newly synthesized collagen. In addition, noncollagen, pepsin-sensitive polypeptides were digested. In five of six experiments, however, only about 10% of the radioactivity present in the collagen bands in the cell pellet was solubilized by digestion with pepsin. In one experiment, in which the endothelial cells were cultured and labeled in the presence of β-aminopropionitrile and extracellular matrix prepared, the collagen proteins were partially solubilized by digestion with pepsin and both peaks were identified by SDS-polyacrylamide gel electrophoresis. In this experiment, however, the bulk (~60%) of the radioactive collagens were still present in the particulate extracellular matrix.

[2] Koffler, D. Personal communication.

TABLE II
Conversion of [^{14}C]Proline to [^{14}C]Hydroxyproline by Cultured Human Endothelial Cells

cpm			
Applied to amino acid analyzer	[^{14}C]Proline (A)	[^{14}C]Hydroxyproline* (B)	Proline hydroxylated (B/[A + B])
			%
12,400	11,458	876	7.1

* The [^{14}C]proline used to label the cells contained <0.1% [^{14}C]hydroxyproline.

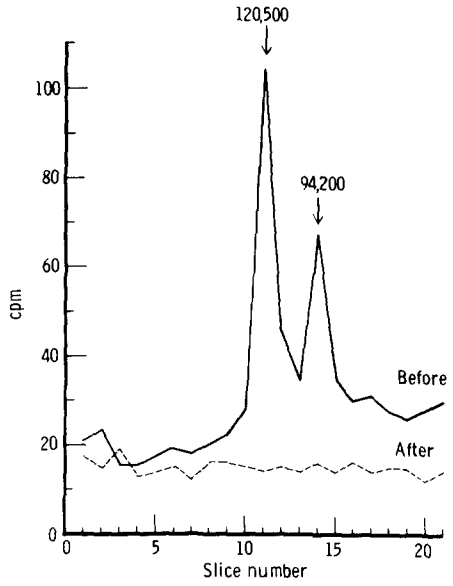


FIG. 14. SDS-polyacrylamide gel electrophoresis of pepsin-digested cell pellet before and after digestion with purified collagenase. Two collagenase-digestible polypeptides were detected. The upper portion of the sliced gel is shown.

When labeled postculture medium was digested with pepsin and analyzed by SDS-polyacrylamide gel electrophoresis, peaks of mol wt 183,000, 152,000, and 115,000 were observed (Fig. 15). All three peaks, but not the rest of the radiolabeled protein, were digested by purified collagenase.

Discussion

The studies reported here demonstrate that cultured human endothelial cells synthesize and release collagen which is incorporated into the extracellular matrix. In vivo, the extracellular matrix is a component of the subendothelium. Cultured endothelial cells synthesized two collagen polypeptides, one with a mol wt of 120,500, and the other with a mol wt of 94,200. Since these molecular weights were obtained after pepsin digestion of the proteins, they probably represent two different types of collagen chains rather than one type of chain and one of its higher molecular weight precursors (24). Four different types of

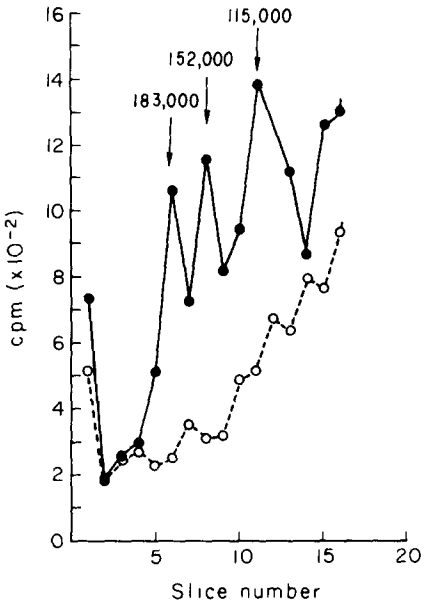


FIG. 15. SDS-polyacrylamide gel electrophoresis of pepsin-digested postculture medium. Three collagenase-digestible polypeptides were detected. Before collagenase digestion (●), after collagenase digestion (○). The upper portion of the sliced gel is shown.

collagen are known; types I, II, III, and IV (24, 25). Using antisera specific for types I, II, and III collagen, Gay et al. showed that the subendothelium in human aorta contained type III collagen but not types I or II (26). Type IV collagen has been identified in whole human aorta (27) as well as in basement membrane (25). It is thus probable that subendothelium in vivo contains types III and IV collagen but lacks types I and II collagen. Type IV collagen, as found in glomerular basement membrane and chick anterior lens capsule, is composed of α_1 (IV) chains which have a mol wt of ~120,000 (28, 29). Type III collagen is composed of polypeptide chains with a mol wt of ~95,000 (30). It is likely, therefore, that the larger collagen chain (mol wt 120,500) synthesized by cultured endothelial cells is α_1 (IV) collagen and the smaller collagen chain (mol wt 94,200) is α_1 (III) collagen. Types III and IV collagen are known to contain cysteine in their helical sections and to have extensive interchain disulfide bonds (25, 27, 30). These cross-links, not susceptible to lathyrogens, make types III and IV collagen difficult to solubilize by digestion with pepsin or other proteolytic enzymes and several authors have reported inability to solubilize more than 20% of the total vessel wall collagen with a 3-day pepsin digestion (25, 27). This probably accounts for our difficulty in solubilizing the endothelial cell collagens.

The pepsin digested, radiolabeled postculture medium contained three collagenase-digestible polypeptides. The polypeptide of mol wt 183,000 probably represents a dimer, β_{11}, of the smaller mol wt (94,200) collagen molecule. These dimers have been described in purified preparations of type III collagen (26, 31, 32). The polypeptide of mol wt 152,000 may represent a precursor of the α_1 (IV) chain, pro α_1 (IV) (33). This molecular weight collagen polypeptide was infre-

quently detected in collagen preparations obtained from cultured cells and extracellular matrix due to its susceptibility to pepsin digestion with resultant conversion to an α_1 (IV) collagen chain of mol wt 120,000 (29). It is of interest that pro α_1 (IV) chains were clearly identified in the postculture medium; the reasons for the lack of conversion by pepsin of pro α_1 (IV) chains to α_1 (IV) chains are not clear. The polypeptide of mol wt 115,000 probably represents the α_1 (IV) collagen chains (mol wt 120,500) seen in the particulate fractions.

When cultured endothelial cells labeled with [^{3}H]proline were digested with purified collagenase, 18% of the [^{3}H]proline incorporated into protein was solubilized by purified collagenase. When endothelial cells were labeled with [^{14}C]proline, hydrolyzed with 6 N HCl, and [^{14}C]proline and [^{14}C]hydroxyproline separated by amino acid analyzer, 7.1% of the ^{14}C was present as [^{14}C]hydroxyproline. Since 70% of the amino acids in purified basement membrane collagen are present as hydroxyproline (25), approximately 10% (7.1%/70%) of the total protein synthesized by the cultured endothelial cells was collagen. These two estimates (10 and 18%) are consistent with culture to culture variations in the amount of amorphous basement membrane (and presumably basement membrane collagen) noted by transmission electron microscopy.

It has previously been shown for other systems (Descemet's membrane, glomerular basement membrane) that amorphous basement membrane is a complex made up of collagen and one or more glycoproteins (25, 34). Cultured endothelial cells and extracellular matrix stained weakly with an antisera to human dermal collagen and this staining was completely abolished by absorption of the antisera with human dermal collagen. Thus, endothelial cells and extracellular matrix contained proteins immunologically related to collagen. Endothelial cells were stained brightly with antiserum to glomerular basement membrane. Absorption of the anti-glomerular basement membrane sera with dermal collagen reduced the staining only slightly, whereas absorption with glomerular basement membrane completely inhibited the staining. These immunofluorescent observations can be explained by the presence of additional antigens associated with basement membrane collagens and/or by differences between dermal and basement membrane collagens. By analogy with glomerular basement membrane, these other components could be high and low molecular weight glycoproteins (25, 34).

Extracellular matrix synthesized by cultured endothelial cells also contained two other morphologically recognizable components, microfilamentous structures varying in diameter from ~40 to 120 Å and amorphous elastin. Microfilamentous structures measuring approximately 100 Å in diameter had a tubular profile and a "hollow core" in cross section. These fibrils resemble those described by Palade and Bruns in the outer portion of capillary basement membranes (35) and those described as elastic microfibrils (36, 37). Similar microfibrils often bordered areas of amorphous elastin thus forming structures like elastic fibers. Like their in vivo counterparts, the amorphous elastin was digestible by elastase but not by trypsin or purified collagenase whereas the microfibrils were digested by trypsin but not by purified collagenase. Despite the absence of direct biochemical evidence for synthesis, the cell culture technique used in these studies suggests that these proteins were probably synthe-

sized by the cultured endothelial cells. Endothelial cells were initially isolated and later passed using a crude preparation of collagenase which contains, in addition, a variety of proteases other then collagenase. The enzyme preparation is capable of digesting the endothelial cell extracellular matrix, leaving behind bare cells which are then passed. An initial isolation and two passages by this technique plus a 10- to 20-fold increase in cell number and area covered by the cells make it unlikely that much of the extracellular matrix was carried along with the cells from the umbilical vein wall.

Type IV collagen has been found in other tissues in structures that morphologically resemble amorphous basement membrane. It is thus probable that the type IV collagen synthesized by endothelial cells is present in the amorphous basement membrane component of the subendothelium. The fact that the amorphous basement membrane is digestible by purified collagenase strongly suggests that at least one of its components is a collagen and it may be that both the collagens are associated with this particular structure. Fibrillar collagen was not seen in the subendothelium synthesized by cultured endothelial cells. This may be due either to the fact that endothelial cells do not synthesize fibrillar collagen in vivo or to lack of the proper conditions in culture. The last component of the subendothelium, mucopolysaccharides, is not identifiable by electron microscopy using standard techniques and thus was not observed in our studies. It is reasonable to expect that cultured human endothelial cells synthesize mucopolysaccharides and incorporate them into the extracellular matrix since it has been shown by Buonassisi that cultured rabbit aortic endothelial cells synthesize cellular mucopolysaccharides (38, 39).

Summary

Studies were performed to determine if cultured human endothelial cells synthesized basement membrane collagen. In culture, endothelial cells were attached to grossly visible membranous structures which on light microscopy were composed of ribbons of dense, amorphous material. On transmission electron microscopy, these membranous structures consisted of amorphous basement membrane, and material morphologically similar to microfibrils and elastic fibers. By immunofluorescence microscopy, these membranous structures stained brightly with antisera to human glomerular basement membrane. Cultured endothelial cells incorporated [^{3}H]proline into protein; 18% of the incorporated [^{3}H]proline was solubilized by purified collagenase. When endothelial cells were cultured with [^{14}C]proline, 7.1% of the incorporated counts were present as [^{14}C]hydroxyproline. Cultured endothelial cells were labeled with [^{3}H]glycine and [^{3}H]proline and digested with pepsin. The resulting fractions on analysis by SDS-polyacrylamide gel electrophoresis contained two radioactive protein peaks of mol wt 94,200 and 120,500. Both these peaks disappeared after digestion with purified collagenase. The peak of mol wt 120,500 corresponds to that of α_1 (IV) collagen; the peak of mol wt 94,200 probably corresponds to that of α_1 (III) collagen.

Thus, cultured human endothelial cells synthesize material which is morphologically and immunologically like amorphous basement membrane and biochemically like basement membrane collagen. Cultured endothelial cells probably also synthesize material which is morphologically similar to microfibrils and elastic fibers.

We wish to thank Christine Baranowski, Liliane Bronson, John Shuman, Maria Litrenta, and Arlene Hardy for their invaluable technical assistance.

Received for publication 24 March 1976.

References

1. Stemerman, M. B. 1975. Vascular intimal components: precursors of thrombosis. *In* Progress in Hemostasis and Thrombosis. T. H. Spaet, editor. Grune & Stratton, Inc., New York. 2:1.
2. Murphy, M. E., and P. C. Johnson. 1975. Possible contribution of basement membrane to the structural rigidity of blood capillaries. *Microvasc. Res.* 9:242.
3. Caulfield, J. P., and M. G. Farquhar. 1974. The permeability of glomerular capillaries to graded dextrans. *J. Cell Biol.* 63:883.
4. Siperstein, M. D., R. H. Unger, and L. L. Madison. 1968. Studies of muscle capillary basement membranes in normal subjects, diabetic and prediabetic patients. *J. Clin. Invest.* 47:1973.
5. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* 52:2745.
6. Peterkofsky, B. 1972. The effect of ascorbic acid in collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts. *Arch. Biochem. Biophys.* 152:318.
7. Westberg, N. G., and A. F. Michael. 1970. Human glomerular basement membrane. Preparation and composition. *Biochemistry.* 9:3837.
8. Nachman, R. L., A. Hubbard, and B. Ferris. 1972. Iodination of the human platelet membrane. *J. Biol. Chem.* 248:2928.
9. Peterkofsky, B., and R. Diegelmann. 1971. Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. *Biochemistry.* 10:988.
10. Walford, R. L., and B. Kickhöfen. 1962. Selective inhibition of elastolytic and proteolytic properties of elastase. *Arch. Biochem. Biophys.* 98:191.
11. Rothbard, S., and R. F. Watson. 1972. Demonstration of collagen in human tissues by immunofluorescence. *Lab. Invest.* 27:76.
12. Krakower, C. A., and S. A. Greenspon. 1951. Localization of the nephrotoxic antigen within the isolated renal glomerulus. *Arch. Pathol.* 51:629.
13. Goldstein, G., I. S. Slizys, and M. W. Chase. 1961. Studies in fluorescent antibody staining. I. Nonspecific fluorescence with fluorescein-coupled sheep antirabbit globulins. *J. Exp. Med.* 114:89.
14. Becker, C. G., and R. L. Nachman. 1973. Contractile proteins of endothelial cells, platelets and smooth muscle. *Am. J. Pathol.* 71:1.
15. Jaffe, E. A., L. W. Hoyer, and R. L. Nachman. 1973. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J. Clin. Invest.* 52:2757.
16. Weber, K., and M. Osborn. 1975. Proteins and sodium dodecyl sulfate molecular weight determination on polyacrylamide gels and related procedures. *In* The Proteins. H. Neurath, R. L. Hill, and C. L. Boeder, editors. Academic Press, Inc., New York. 3rd edition. 1:180.
17. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606.
18. Jaffe, E. A., and R. L. Nachman. 1975. Subunit structure of factor VIII antigen synthesized by cultured human endothelial cells. *J. Clin. Invest.* 56:698.
19. Furthmayr, H., and R. Timpl. 1971. Characterization of collagen peptides by sodium dodecylsulfate-polyacrylamide electrophoresis. *Anal. Biochem.* 41:510.

20. Butler, W. T., K. A. Piez, and P. Bornstein. 1967. Isolation and characterization of the cyanogen bromide peptides from the α1 chain of rat skin collagen. *Biochemistry.* 6:3771.
21. Grant, M. E., and D. J. Prockop. 1972. The biosynthesis of collagen. *N. Engl. J. Med.* 286:194.
22. Reid, K. B. M., D. M. Lowe, and R. R. Porter. 1972. Isolation and characterization of C1q, a subcomponent of the first component of complement, from human and rabbit sera. *Biochem. J.* 130:749.
23. Lewis, E. J., G. J. Busch, and P. Schur. 1970. Gamma G globulin subgroup composition of the glomerular deposits in human renal disease. *J. Clin. Invest.* 49:1103.
24. Martin, G. R., P. H. Byers, and K. A. Piez. 1975. Procollagen. *Adv. Enzymol. Relat. Areas Mol. Biol.* 42:167.
25. Kefalides, N. A. 1973. Structure and biosynthesis of basement membranes. *Int. Rev. Connect. Tissue Res.* 6:63.
26. Gay, S., L. Balleisen, K. Remberger, P. P. Fietzek, B. C. Adelmann, and K. Kuhn. 1975. Immunohistochemical evidence for the presence of collagen Type III in human arterial walls, arterial thrombi, and in leukocytes, incubated with collagen in vitro. *Klin. Wochenschr.* 53:899.
27. Trelstad, R. L. 1974. Human aorta collagens: evidence for three distinct species. *Biochem. Biophys. Res. Commun.* 57:717.
28. Grant, M. E., J. D. Schofield, N. A. Kefalides, and D. J. Prockop. 1973. The biosynthesis of basement membrane collagen in embryonic chick lens. III. Intracellular formation of the triple helix and the formation of aggregates through disulfide bonds. *J. Biol. Chem.* 248:7432.
29. Grant, M. E., R. Harwood, and I. F. Williams. 1975. The biosynthesis of basement-membrane collagen by isolated rat glomeruli. *Eur. J. Biochem.* 54:531.
30. Chung, E., and E. J. Miller. 1974. Collagen polymorphism: characterization of molecules with the chain composition of $(\alpha 1\ (III))_3$ in human tissues. *Science (Wash. D. C.).* 183:1200.
31. Piez, K. A., E. A. Eigner, and M. S. Lewis. 1963. The chromatographic separation and amino acid composition of the subunits of several collagens. *Biochemistry.* 2:58.
32. Byers, P. H., K. H. McKenney, J. R. Lichtenstein, and G. R. Martin. 1974. Preparation of Type III procollagen and collagen from rat skin. *Biochemistry.* 13:5243.
33. Minor, R. R., C. C. Clark, T. R. Koszalka, R. L. Brent, and N. A. Kefalides. 1975. Synthesis and deposition of basement membrane procollagen. *J. Cell Biol.* 67:287*a*.
34. Kefalides, N. A. 1975. Basement membranes: structural and biosynthetic considerations. *J. Invest. Dermatol.* 65:85.
35. Palade, G. E., and R. R. Bruns. 1964. Structure and function of normal muscle capillaries. *In* Small Blood Vessel Involvement in Diabetes Mellitus. M. D. Siperstein, A. R. Colwell, and K. Meyer, editors. American Institute of Biological Sciences, Washington. 39.
36. Ross, R., and P. Bornstein. 1969. The elastic fiber. I. The separation and partial characterization of its macromolecular components. *J. Cell Biol.* 40:366.
37. Haust, M. D., R. H. More, S. A. Bencosme, and J. U. Balis. 1965. Elastogenesis in human aorta: an electron microscopic study. *Exp. Mol. Pathol.* 4:508.
38. Buonassisi, V. 1973. Sulfated mucopolysaccharide synthesis and secretion in endothelial cell cultures. *Exp. Cell Res.* 76:363.
39. Buonassisi, V., and M. Root. 1975. Enzymatic degradation of heparin-related mucopolysaccharides from the surface of endothelial cell cultures. *Biochim. Biophys. Acta.* 385:1.