Secretory Phenotypes of Endothelial Cells in Culture: Comparison of Aortic, Venous, Capillary, and Corneal Endothelium

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Endothelial cells from different tissues display variations in morphology, intercellular junctions, cell surface and growth properties, and in production of basal lamina components, both in vivo and in vitro. We have investigated the spectra of extracellular proteins secreted by bovine endothelial cells cultured from large vessels, cornea, and capillaries. Aortic, venous, and corneal endothelial cells displayed highly similar patterns of protein synthesis as judged by analysis of the culture medium; the major products were fibronectin, a glycoprotein similar or identical to platelet thrombospondin, and Type III procollagen. Ion-exchange chromatography, followed by peptide mapping, confirmed the presence of EC, a novel endothelial collagen previously described in bovine aortic endothelial cell cultures. Minor variations were found in the collagens of the cell layers: Type III, the predominant interstitial collagen, was associated with the basement membrane Types IV and V and, in the case of corneal endothelium, with Type I.

In contrast, capillary endothelial cells secreted significantly more collagen than did the aortic, venous, and corneal cells. Approximately 50% of the protein in the culture medium was collagenous and consisted of Types I and III collagen in a ratio of 2:3. These interstitial collagens were the only types detected in capillary cell layers as well.

The pattern and overall rate of collagen synthesis by capillary endothelial cells in vitro contrasted significantly with that of the other endothelial cell types and closely resembled that described for cultures of sprouting endothelium. These alterations in secretory phenotype may reflect: 1) a true difference in cell type between capillary and other types of endothelium, 2) differences resulting from cell isolation and initial culture conditions, or 3) a correlation between growth regulation and protein synthesis. (Arteriosclerosis 1:427-442, November/December 1981)

The interaction of endothelial cells with the pericellular/extracellular matrix is now recognized as a fundamental phenomenon which plays an integral role in cell behavior and function. Endothelial cells in culture secrete an extracellular matrix, consisting of several distinct collagen types, other glycoproteins, and proteoglycans, which is interposed between the basal cell surface and the substratum. Several recent studies have demonstrated correlations between endothelial cell behavior and function and certain biosynthetic or artificial extracellular substrates with which the cells interact. Gospodarowicz and III concluded that the extracellular matrix produced by corneal endothelium in culture was able to sustain the growth rate of corneal and vascular endothelial cells in the absence of exogenous growth factors. Both the polarity and survival of endothelial cells in culture have been shown to result from interactions with secreted stromal components or collagen-coated surfaces. Other investigators have suggested that the response of capillary endothelial cells to tumor angiogenesis factor was made possible by growth on collagen gels and that levels of DNA synthesis were a function of aortic endothelial cell shape. These and related experiments emphasize the interrelationship between
endothelial cell-matrix interactions and cell function.

At this point, direct comparisons between sub-
endothelial basal laminae produced in vivo and
the pericellular/extracellular stromal compo-
nents that are synthesized by endothelial cells in
vitro must be drawn with caution. One approach
toward understanding the relationship between
these two structures has involved both morpho-
logic and biochemical characterization of their
constituent macromolecules. Types IV and V
collagen have been shown to be associated with
subendothelial structures both in vivo and in
vitro. In addition, the glycoproteins laminin
and fibronectin have been identified in subendo-
thelial matrices. These proteins mediate the
adhesion of certain cell types to collagenous
substrates in vitro (see the review by Kleinman et
al. Laminin is specific for the attachment of
certain epithelial cells to Type IV collagen, and
fibronectin may function in cell-cell and cell-
matrix attachment in certain tissues.

Such comparisons, however, have been com-
plicated by the morphologic and biochemical
heterogeneity of the cells and of the basal lami-
nae that underlie the endothelium in different ves-
sels. The basal lamina varies from a continuous,
intact structure in capillaries to a discontinuous,
poorly-defined component in muscular arteries.
In the aortas of certain mammals, it may be rud-
imentary or altogether absent. The shape of
endothelial cells has also been found to vary with
vascular origin: the cells have been described as
small and elongated in the rat aorta, rectangular
in vena cava, and polygonal with tessellated bor-
ders in the pulmonary trunk. Other variations
among endothelia that have been found to occur
both in vivo and in vitro include variations in inter-
cellular junctional complexes, concentration
of insulin receptors, and certain metabolic
functions.

To better understand endothelial cell-matrix in-
teractions, we have studied some of the major
proteins that are secreted by endothelial cells
derived from different sources in culture. In ear-
er studies using adult bovine aortic endo-

thelial cells, we identified three secreted glyco-
proteins of M, 150,000–250,000 after disulfide
bond reduction, comprising about 70% of total
protein secreted into the culture medium: fibro-
nectin, Type III procollagen, and a glycoprotein
recently identified as thrombospondin. In
addition, a novel collagen type (EC) was charac-
terized in the culture medium, and Types IV and
V collagen were described in the cell layer.
Using the aortic cells as a basis for comparison,
we have investigated whether other bovine endo-

thelial cells, including those from cornea, vena
cava, and adrenal capillary, secreted different
products. Studies on collagen synthesis by
endothelial cells have produced controversial
results, especially in the production of interstitial
(Types I and III) versus basement membrane-as-

sociated collagens (Types IV and V). Additional
studies have described phenotypic modulation
in endothelial cell cultures, in which loss of cell
polarity, thromboresistance, and the typical
"cobblestone" morphology, were associated
with changes in the profile of collagen biosyn-
thesis.

Since some of the reported biosynthetic differ-
ences among endothelial cells may be due to the
species or tissue of origin or to variations in tis-
sue culture methodology, we undertook this sur-
vey to provide an intraspecies comparison
among cells from alternate locations using simi-
lar biochemical techniques. By analyzing extra-
cellular protein synthesis by vascular and avascular
endothelial cells, and by cells from both large
vessels and the microvasculature, we hope to elu-
cidate some of the mechanisms involved in endo-

thelial cell growth, migration, and regeneration.

Methods

Cell Culture and Metabolic Labeling

Cultures of adult bovine aortic endothelial (BAE) cells were isolated, subcultured, and main-
tained as previously described. Alternatively, BAE cells which had not been subjected to
[3H]thymidine treatment were studied to provide a similar basis of comparison with endothelia ob-
tained from other tissues. Non-[3H]thymidine-selected BAE strains were obtained by gentle
scraping of the aortic endothelial layer without prior collagenase treatment. These cultures,
which displayed highly similar morphology, growth characteristics, and biosynthetic capac-
ity as compared to [3H]thymidine-selected cul-
tures, were homogeneous with respect to cell type and did not manifest the sprouting pheno-
type. BAE cells were usually metabolically
labeled between the fourth and 12th passages,
although in some experiments both primary cul-
tures and cells in later passage were used.

Endothelial cells from bovine superior vena
cava (BVE), a gift from Ulrich Delvos, University of
Washington, were isolated according to pub-
lished procedures. These cultures were hom-
geneous and uniformly positive for Factor VIII-
antigen, displayed the cobblestone morphology
and topo-inhibited monolayer at confluence
which have been described for BAE cells, and
exhibited a finite life span in vitro. BVE cells were
grown in Dulbecco modified Eagle’s medium
(DMEM) containing 10% to 20% fetal calf serum
(FCS) under the same conditions as previously
reported for BAE cells. Cells were passaged at
a split ratio of 1:4 and were used between the
fourth and sixth passages.

Bovine corneal endothelial (BCE) cells were
provided by J. W. Chandler and J. Funderberg,
University of Washington. Descemet's membrane was mechanically stripped from the posterior surface of adult bovine cornea and was placed in a 60 mm Petri dish containing MEM supplemented with antibiotics and 10% FCS. Cultures were initially passaged weekly at a split ratio of 1:2 and were later subcultured and grown in DMEM under the same conditions used for BAE and BVE cells. BCE strains were not contaminated by other cell types and exhibited a finite life span in vitro of approximately 25 to 30 population doublings. These cultures, which were found to be negative for Factor VIII-antigen, were metabolically labeled between the 12th and 16th passage.

Capillary (CAP) endothelial cells were a gift from Bruce Zetter. This culture was cloned from bovine adrenal gland capillary endothelium as described by Folkman et al.7 CAP cells, grown in tumor-conditioned medium, stained positively for Factor VIII-antigen and exhibited a mean doubling time of 28 hours.

All endothelial cultures were metabolically labeled just as the cells reached confluence (3 to 4 days after subculture) under the conditions previously described.30 After a 30 to 60 minutes preincubation in serum-free DMEM supplemented with antibiotics, β-aminopropionitrile (β-APN) (64 μg/ml) and sodium ascorbate (50 μg/ml), a fresh aliquot of this medium containing 50 μCi/ml L-[3,3H]-proline (16-35 Ci/mole; New England Nuclear) was added to the cultures. The cells were incubated with isotope for 21 to 24 hours, after which the culture medium and cell layers were initially recovered in the presence of protease inhibitors, before further processing.33

**Characterization of Collagen Types and Other Proteins**

**Collagen Production**

The incorporation of [3H] proline into total secreted protein in the culture medium was determined by precipitation with trichloroacetic acid32 and was subsequently normalized to 10⁶ cells for comparative purposes. The percentage of total medium protein which was collagenous was determined by selective precipitation at 0.7 M NaCl and, subsequently, at 1.8 M NaCl, at acidic pH. Collagens were dialyzed against 0.1 M acetic acid, lyophilized, and subsequently analyzed by SDS-PAGE and by peptide mapping using CNBr.

Collagens from BVE and BCE cell layers were also characterized by chromatography on CM-cellulose, using conditions previously described.11

**Immunofluorescence Studies**

Intact BVE and BCE monolayers were fixed and exposed to affinity-purified antibodies to bovine Type III procollagen, human Type IV collagen, and human plasma fibronectin, in a double-antibody procedure as previously used for BAE cells.11 In addition, antiserum to murine Type IV procollagen and to murine laminin (a gift from G. R. Martin, National Institutes of Health) were used. Extracellular matrices from which the cells had been removed by shaking for 10 minutes in 2% Triton X-100 in phosphate-buffered saline (PBS), followed by five 2-minute washes in PBS, were also examined by immunofluorescence using the same antibodies. The slides were photographed with a Zeiss Photomicroscope II equipped with epillumination.

**Results**

**Proteins in Endothelial Cell Culture Medium**

The biosynthetic profiles of BAE, BCE, and BVE cells were found to be strikingly similar. Figure 1 shows the proteins secreted into the culture medium over a 21- to 24-hour period that were precipitated in 10% trichloroacetic acid. In
**Figure 1.** Comparison of proteins secreted into the culture medium by bovine endothelial cells. Nearly confluent cultures were incubated with [3H] proline for 24 hours in serum-free medium supplemented with β-APN and sodium ascorbate. Radiolabeled proteins were precipitated from the culture media using 10% trichloroacetic acid, and equal aliquots were incubated with bacterial collagenase or buffer, as described in the Methods section. The samples were subsequently resolved by SDS-PAGE on composite 6%/10% slab gels in the presence of 50 mM DTT and were visualized by fluorescence autoradiography. Culture medium protein from each cell type is shown both before and after collagenase treatment. BAE = bovine aortic endothelial; BCE = bovine corneal endothelial; BVE = bovine venous endothelial; CAP = bovine capillary endothelial. Several biosynthetic products of endothelial cells are identified: fibronectin (FN), Type III procollagen (proα1(III)), and a noncollagenous glycoprotein (GP) which in BAE cells has been characterized as thrombospondin. Type I procollagen chains [(proα1(I), proα2(I)] are also designated.

In addition, certain proteins were specifically digested by the enzyme bacterial collagenase, indicating that each cell type produced a collagenous moiety.

BAE cells primarily secrete three proteins in the molecular weight range 150,000–250,000 after disulfide bond reduction, all of which have been identified and characterized (for a review, see Sage). These proteins are designated in figure 1 as fibronectin (FN, M, 240,000), Type III procollagen (proα1 (III)), and a glycoprotein (GP, M, 190,000) which has been identified as platelet thrombospondin. Note that both BCE and BVE cells synthesized three components of similar mobility on SDS-PAGE, both in the absence (data not shown) and presence (figure 1) of reducing agent. Between 35% (BCE) and 45% (BAE) of the total radioactive protein in the culture medium was precipitated by affinity-purified antibodies to plasma fibronectin; the mobility of these radiocimmune precipitates on SDS-PAGE coincided with that of bovine plasma fibronectin (data not shown). While the identification of the M, 190,000 protein (GP) as thrombospondin in both BCE and BVE culture media is tentative at this point, the collagenase-sensitive band has been further characterized following purification (see below).

In contrast to BAE, BCE, and BVE cells, CAP cells exhibited a somewhat different pattern of protein synthesis, as shown in figure 1. The three major components found in the other cell types (fibronectin, Type III procollagen, and thrombospondin) appeared to be present in CAP culture medium, by the criterion of mobility on SDS-PAGE; approximately 30% of the radioactive protein was specifically precipitated by antibodies to fibronectin (data not shown). However, two additional, collagenase-sensitive bands were evident in CAP culture medium; these have been designated in figure 1 as Type I procollagen chains [proα1(I) and proα2(I)] based on their...
mobilities on SDS-PAGE. This collagen type was further characterized.

A comparison of the overall protein synthesis and percent collagen production in the culture medium among BAE, BCE, BVE, and CAP cells is presented in Table 1. CAP cells exhibited the highest rate of incorporation of [3H]-proline into protein, approximately twice that of the most biosynthetically active BAE cultures. However, measurements of protein synthesis by endothelial cells in culture must be interpreted with caution. Although the precision of the measurement may be quite high, it is difficult to optimize growth conditions simultaneously for all cells without introducing additional variables which could influence biosynthetic rates. In addition, although the spectrum of proteins did not change, the passage number and interstrain variability have reportedly caused quantitative differences in protein synthesis.23,30 For this reason, we reported a range for both protein and collagen synthesis for BAE cells in Table 1.

The corneal endothelial cells used in this study (Table 1) were not senescent but had undergone more population doublings than the BAE and BVE cultures. Although in early passages they resembled the monolayers of ovoid cells isolated from rabbit cornea as described by Yue et al,39 in later passages they became progressively larger and flatter. This correlation between age in culture or donor age and corneal endothelial cell morphology has also been noted by other investigators.35,36 Experiments with earlier passage BCE cells (representing 12 population doublings), in which the levels of secreted protein were measured after ammonium sulfate precipitation, indicated that these cells may optimally have a biosynthetic rate very similar to that of BAE cells, with approximately 7% of protein synthesis represented by collagen.

BAE, BVE, and BCE cells secreted similar amounts of collagen (3% to 10%) into the culture medium (Table 1). In contrast, CAP cells secreted at least five times more collagen, relative to total protein synthesis, than the other cell types and approximately 10 times more collagen on a per cell basis (Table 1). As was evident in Figure 1, virtually half of all protein in CAP culture medium was collagenous (Table 1).

Characterization of Proteins in the Culture Medium

Ion-exchange chromatography on DEAE-cellulose at 4°C has been a useful technique for the initial fractionation of culture medium protein from many different kinds of cells. In addition, we have found that ammonium sulfate precipitation in two successive stages, followed by DEAE-cellulose chromatography, provides an effective characterization of proteins in endothelial cell culture medium. It should be noted, however, that the high molecular weight glycoprotein, thrombospondin, is recovered in very low yields from DEAE-cellulose and has therefore been purified by other techniques.24

Figure 2 represents the chromatographic profile of BCE culture medium protein, and Figure 3 shows the profile for BVE; these were initially fractionated in 20% ammonium sulfate (figures 2 A and 3 A) and subsequently in 20% to 50% ammonium sulfate (figures 2 B and 3 B). These profiles should be compared to those generated on DEAE-cellulose from similar ammonium sulfate fractions of BAE culture medium protein.30 Quantitative differences were seen among the three major peaks in the 20% ammonium sulfate fraction and the four major peaks in the 20% to 50% ammonium sulfate fraction, for BAE, BCE, and BVE media; however, variations of similar magnitude have often been observed with culture media from different strains of BAE cells. The most important observation was that, qualitatively, the same major protein species were present in BAE, BCE, and BVE culture media, as assessed by SDS-PAGE analysis of the peak fractions obtained from DEAE-cellulose chromatography (Figures 2 and 3 insets). These proteins are described below, by analogy to those from BAE cells.

Endothelial Collagen (EC)

A novel endothelial collagen (EC) was recovered after DEAE-cellulose chromatography in three forms: EC1 (M, 177,000), EC2 (M, 125,000), and EC3 (M, 100,000). These forms, which do not contain interchain disulfide bonds, are all derived from the same precursor protein. EC2,

| Table 1. Comparison of Levels of Total Protein and Collagen Secreted by Bovine Endothelial Cells Into the Culture Medium |
|-----------------|-----------------|-----------------|
| Cell type       | dpm × 10^-6 in  |
|                 | total protein/10^6 | % collagen†     |
| Aortic‡         | 1.2-2.4          | 3-8             |
| Venous          | 2.8              | 10              |
| Corneal         | 0.40             | 6.8             |
| Capillary       | 4.6              | 49              |

Total protein and collagen levels were measured by incorporation of L-[2, 3-H] proline; cells were incubated with 50 μCi/ml for 24 hours, as described in Methods.

†Expressed as percent of dpm which were solubilized after incubation with bacterial collagenase, corrected for the increased proline content of collagen.

‡Range represents data from a minimum of six different cell strains from primary passage to approximately 30 population doublings (see ref. 30).
Figure 2. DEAE-cellulose chromatography of culture medium protein from BCE cells. \([{}^3\text{H}]\) proline-labeled protein was precipitated from culture medium using 20% ammonium sulfate (A) and subsequently 50% ammonium sulfate (B). Chromatography was performed on DEAE cellulose at 4°C in 6 M urea, 50 mM Tris-HCl, pH 8.0, in the presence of protease inhibitors. Gradient elution (arrow) was from 0-200 mM NaCl in 400 ml. Roman numerals indicate pooled fractions. A Inset. SDS-PAGE was performed on 6%/10% gels under reducing conditions, and protein bands were visualized by fluorescence autoradiography. Roman numerals refer to pooled fractions designated in accompanying chromatogram. Fibronectin (FN), type III procollagen (pro\(\alpha\)1(III)) and a partially processed form (pro\(\alpha\)1(III)) are identified, as well as two forms of endothelial collagen (EC2 and EC3). B Inset. Conditions as described for A. An additional molecular weight form of EC (EC1) is shown. Lane on the far left contains a Type I procollagen standard.
Figure 3. DEAE-cellulose chromatography of culture medium protein from BVE cells. \[^{3}H\] proline-labeled protein was processed, chromatographed, and analyzed by SDS-PAGE as described in figure 2. Roman numerals refer to pooled fractions. A. 20% ammonium sulfate precipitate. Inset. Fibronectin (FN), Type III pro- and p-collagen, and EC. B. 20% to 50% ammonium sulfate precipitate. Inset. FN, Type III procollagen, and EC are identified after SDS-PAGE under reducing conditions.
the predominant form, does not bind to DEAE-
cellulose under the conditions used in these
experiments, and it was recovered in peak I (fig-
ures 2 and 3). EC1, which is preferentially pre-
cipitated at higher concentrations of ammonium
sulfate and elutes at the beginning of the salt
gradient, was recovered in peak II of BCE
medium (figure 2 B). However, from BVE
medium it was precipitated in 20% ammonium
sulfate and did not bind to the DEAE-
cellulose (figure 3 A, peak I).

Fibronectin

Fibronectin was eluted from DEAE-cellulose at
a characteristic conductivity for both BCE
(figure 2 A, peak II; figure 2 B, peak III) and BCE
cell culture medium (figure 3, peak II). This was
the major radiolabeled protein in the culture
media.

Type III Procollagen

In the bovine system, Type III procollagen and
the partially processed form are characteris-
tically eluted from DEAE-cellulose somewhat
later in the linear NaCl gradient than fibronectin
and/or Type I procollagen. Chromatography of
both BCE and BVE cell culture media revealed
distinct peaks containing Type III pro- and p-
collagen, which were preferentially precipitated
at the lower ammonium sulfate concentra-
tion (figure 2 A, peak III and figure 3 A, peak III). This
procollagen was identified as Type III by: 1) spe-
cific precipitation with affinity-purified anti-
bodies to bovine Type III procollagen; 2) pro-
duction of a collagenous molecule, after pepsin
treatment, which migrated as a γ-component on
SDS-PAGE before reduction and as a single α-
chain after reduction; and 3) mapping studies of
the pro- and p-collagens utilizing CNBr cleavage
(data not shown). We have applied similar
criteria to the collagen which was purified after
pepsin treatment of unfractionated culture
media; results from these experiments are de-
scribed later.

43K Protein

This component, a monomeric glycoprotein
containing intrachain disulfide bonds, migrates
on SDS-PAGE with an apparent Mₐ of 43,000,
based on a calibration curve using several
globular proteins in the molecular weight range
of 20,000 to 94,000. It is preferentially precipi-
tated at the higher concentration of ammonium
sulfate and has been purified to homogeneity by
chromatography on DEAE-cellulose, followed by
Sephadex G-200. In addition to its presence in
the culture media of BAE cells, bovine aortic
smooth muscle cells, human fetal fibroblasts,
and a murine hemangiendothelioma27 (Sage,
Pritzl, and Bornstein, unpublished observations),
it was evident that both BCE and BVE culture
media contained this protein as well (figures 2 B
and 3 B, peak IV). Peptide mapping studies using
several different proteinases have shown that the
43K protein is not related to fibronectin, α2-
macroglobulin, thrombospondin, or actin (Sage,
Pritzl, and Bornstein, unpublished observations).

Identification of Collagen Types

To identify the collagenous component which
did not bind to DEAE-cellulose in BCE and BVE
culture media (figures 2 A and 3 A, peak I), these
fractions were dialyzed against 0.1 N acetic acid,
lyophilized, and subjected to cleavage using
CNBr. Resolution of the peptides by SDS-PAGE,
as shown in figure 4, confirmed that an EC which
appeared structurally identical to that produced
by BAE cells (lane 4) was also synthesized by
BVE cells (lane 2) and by BCE cells (lane 3).

A limited supply of culture medium from CAP
cells precluded analysis of protein by DEAE-
cellulose chromatography. Since the presence of

Figure 4. CNBr cleavage of EC synthesized by venous,
corneal, and aortic endothelial cells. Fractions from
DEAE-cellulose chromatography containing EC were
incubated with CNBr, and the cleavage products were
resolved by SDS-PAGE on a 12.5% gel in the presence
of DTT. EC from BAE cell culture medium was also
purified by CM-cellulose chromatography, before
cleavage.34 Peptides were visualized by staining with
Coomassie blue (lane 1) and by fluorescence autoradi-
ography (lanes 2–4). Lane 1. Type I collagen. Lane 2.
EC from BVE cells. Lane 3. EC from BCE cells. Lane 4.
EC from BAE cells. The major cleavage
products of Type I collagen are identified.
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EC, which comprises approximately 20% to 25% of the total collagen in BAE, BCE, and BVE culture media, becomes readily apparent only after chromatography on DEAE-cellulose, we do not know whether CAP cells synthesized this novel collagen type.

In order to account for collagens that were present in endothelial cell culture medium but might not be recovered by ion-exchange chromatography, unfractionated radiolabeled culture medium protein was incubated with pepsin, a technique which produces extensive fragmentation of most proteins and converts procollagen molecules to native collagen molecules composed of α-chains (M, 95,000). The pepsin-resistant collagens, purified by NaCl precipitation and molecular sieve chromatography, are shown in figure 5. EC has been shown to be pepsin-labile and was therefore not recovered using this method.

In BAE, BCE, and BVE culture media, we recovered a single collagen type, which comigrated on SDS-PAGE with standard bovine Type III collagen both before and after reduction. The presence of disulfide bonds distinguished this collagen from Type I, which is not affected by reduction and which contains an additional chain, α2(I). In contrast to the other endothelial cells, CAP cells also synthesized Type I collagen. Based on densitometric scanning of the fluorescence autoradiogram, CAP cell medium contained Type III and Type I collagen in a ratio of approximately 3:2. CNBr cleavage of the collagens from BAE, BCE, and BVE media produced peptide patterns on SDS-PAGE which were indistinguishable from that of a standard Type III collagen prepared from bovine skin (data not shown).

**Identification of Collagen Types in the Cell Layer**

Several collagen types have been characterized from BAE cell layers. These include, in addition to Type III, Types IV and V which appear to be associated with basal laminae. When the pepsin-resistant collagens from BAE and BCE

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** Comparison of collagen types secreted into the culture medium by bovine endothelial cells. Culture media from BAE, BCE, BVE, and CAP cells were incubated with pepsin, and resistant proteins were subsequently purified by precipitation using NaCl (see Methods). All pepsin-resistant proteins, except those from CAP cells, were further purified by molecular sieve chromatography on 6% agarose and were resolved by SDS-PAGE on 5%/10% slab gels in both the presence and absence of DTT. Proteins were visualized by fluorescence autoradiography. The source of each of the collagens is indicated. Type III collagen (unreduced) and Types I and III collagen chains are identified.
cell layers were directly compared by SDS-PAGE, two differences were apparent (figure 6, lanes 1 and 2). BCE cells synthesized significantly less Type IV collagen, as assessed by the relative amounts of \( \alpha(IV) \) (M, 140,000 after reduction). In addition, Type I collagen represented approximately 10% of the total collagenous protein in the cell layers of BCE cells.

Since Types III and V collagen were described as the major collagenous species in BAE cell layers,\(^1\) BCE and BVE cell layer collagens were fractionated at NaCl concentrations that enrich for these two types. Type III collagen was precipitated at 0.7 M NaCl from both BCE cell layers (figure 6, lane 3) and BVE cell layers (figure 6, lane 5). In addition, Type I collagen was observed in BCE cultures (figure 6, lane 3) but not in BVE cultures. Type V collagen was precipitated at NaCl concentrations of 1.8 M from both BCE and BVE cell layers (figure 6, lanes 4 and 6, respectively). There was less Type IV collagen in these cultures than in BAE cell layers.

In contrast, CAP cell layers were found to contain both Types I and III collagen in a ratio of approximately 1:1 (figure 6, lanes 7 and 8). Fractionation of CAP cell layer collagens by NaCl precipitation results in a greater than 90% recovery of cpm in the 0.7 M precipitate; analysis of the 1.8 M precipitate by SDS-PAGE did not reveal any bands with the mobility of \( \alpha(IV) \) or \( \alpha(II) \). These results indicate that the synthesis of Type V collagen by CAP cells is very low (less than 2% of total collagenous protein in both the cell layer and the culture medium).

BCE and BVE cell layer collagens were further characterized by chromatography on CM-cellulose under denaturing conditions and by cleavage with CNBr. Figure 7 depicts the elution profile of BCE cell layer collagen after CM-cellulose chromatography. The major peak which coeluted with a bovine type III collagen standard migrated as an homogeneous \( \alpha \)-chain on SDS-PAGE after disulfide bond reduction (figure 7, peak IV and inset). SDS-PAGE analysis revealed that peak I contained only low molecular weight material, peak II contained primarily a component with the mobility of \( \alpha(IV) \) chains, and peak III contained a mixture of \( \alpha(IV) \) and \( \alpha(II) \) chains, together with additional bands presumably derived from degraded \( \alpha(II) \) chains (data not shown).

CNBr cleavage provided further identification of the major collagen in BCE and BVE cell layers as Type III (figure 8). The major fragments pro-
duced from BCE and BVE cell layer collagen (figure 8, lanes 1 and 2, respectively) comigrated with the peptides generated by CNBr cleavage of Type III collagen purified from BAE culture medium (figure 6, lane 3). Some of the additional bands in the BCE preparation, which comigrated with α1(I)-CB7, CB6, and CB3, resulted from the presence of Type I collagen in the cell layer, as noted earlier (figure 6, lanes 2 and 3).

The distribution of collagen and fibronectin on the BCE cell surface and in the extracellular matrix can be seen from the immunofluorescence studies summarized in figure 9. Antibodies to Type III procollagen produced two distinct staining patterns on BCE cell surfaces: 1) a punctuate pattern which appeared to be confined to the cell surface, and 2) a pattern of fine filaments which spanned several individual cells (figure 9 A). The latter distribution was predominant in BVE cultures and was similar to that reported for Type III procollagen on the surface of BAE cells. In contrast, antibodies to both human and mouse Type IV collagen revealed a hazy pattern over BCE cells (figure 9 B) but did not produce any immunofluorescence when incubated with BAE or BVE cells.

After BCE cells were removed from the coverslips by detergent, the insoluble residue stained intensely when exposed to anti-Type III procollagen (figure 9 C) and antifibronectin antibodies (figure 9 D). BAE and BVE cells exhibited a similar staining intensity and pattern with anti-Type III procollagen antibodies; however, staining with antibodies to fibronectin produced a considerably weaker reaction which, in the case of BAE cells, was partially dependent on the strain tested. Very weak, diffuse staining was observed on BAE, BCE, and BVE matrices using antibodies to Type IV collagen and to laminin (data not shown). These immunofluorescence studies support the biochemical data which identify Type III collagen and fibronectin as two major proteins which are secreted by aortic, corneal, and venous endothelial cells. A summary of collagen production by the four types of bovine endothelial cells is presented in table 2.

Figure 7. Chromatography of BCE cell layer collagens on CM-cellulose. Collagens isolated by NaCl precipitation from pepsin-solubilized BCE cell layers (figure 6, lane 2) were denatured and chromatographed on CM-cellulose at 42°C in 6 M urea and 40 mM sodium acetate, pH 4.8. Gradient elution (arrow) was performed from 0-80 mM NaCl in 200 ml. Roman numerals refer to pooled fractions. The elution positions of collagen α-chains from a standard preparation of bovine placental collagens are indicated. Inset. Protein in peak Fraction IV was analyzed by SDS-PAGE before (A) and after (B) reduction. Arrows indicate the positions of migration of α1(I) and α2(I) chains.

Figure 8. CNBr cleavage of the major collagen type in bovine endothelial cell layers. Collagens were isolated from BCE (figure 6, lane 3) and BVE cell layers (figure 6, lane 5) after pepsin treatment and precipitation by 0.7 M NaCl at acidic pH. BAE Type III collagen was purified from culture medium by CM-cellulose chromatography. The samples were incubated with CNBr, and the cleavage products were resolved on 12.5% SDS-gels under reducing conditions. Lane 1. BCE cell layer collagens. Lane 2. BVE cell layer collagens. Lane 3. BAE Type III collagen. The positions of migration of major CNBr-derived peptides from Types I and III collagen are indicated.
Table 2. Collagen Synthesis by Bovine Endothelial Cells in Culture

<table>
<thead>
<tr>
<th>Tissue of Origin</th>
<th>Culture medium</th>
<th>Cell layer</th>
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<tbody>
<tr>
<td>Aorta</td>
<td>III, EC</td>
<td>III, IV, V</td>
</tr>
<tr>
<td>Vena cava</td>
<td>III, EC</td>
<td>III, V</td>
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<tr>
<td>Cornea (Descemet's membrane)</td>
<td>III, EC</td>
<td>I, III, IV, V</td>
</tr>
<tr>
<td>Adrenal capillary*</td>
<td>I, III</td>
<td>I, III</td>
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</table>

*This cell type was not tested for the presence of EC.

**Discussion**

Bovine endothelial cells, cultured from aorta, vena cava, and cornea (Descemet's membrane), synthesized a very similar spectrum of proteins. Fibronectin, comprising 35% to 45% of the total protein secreted into the culture medium over a 24 hour period, was identified by radioimmune precipitation using affinity-purified antibodies, followed by SDS-PAGE. Two other high molecular weight glycoproteins, thrombospondin and laminin, have been tentatively identified in BCE and BVE cultures by their mobility on SDS-PAGE, both in the presence and absence of reducing agent and by immunoprecipitation/immunofluorescence studies. These proteins have been characterized in greater detail in BAE cells.2, 24

The principal collagen secreted by these three endothelial cell types was identified as Type III procollagen, which comprised 3% to 8% of the total protein in the culture medium (Table 1). This procollagen was characterized by: 1) mobility on SDS-PAGE both before and after reduction; 2)
Approximately 50% of the total protein secreted also synthesized fibronectin, Type III procollagen, in both the culture medium and cell layer. Venous, arterial, pulmonary, and corneal, adrenal capillary cells. Analysis of the pepsin-solubilized collagens in the endothelial cell layers by NaCl fractionation, SDS-PAGE, ion-exchange chromatography, and (in the case of Type III) CNBr cleavage, revealed that Type III was the predominant collagen species incorporated into the extracellular matrix. In addition, the basement membrane-associated Types, IV and V, were deposited in this compartment in lesser amounts. However, in contrast to Type III, these collagens were confined exclusively to the cell layer. The presence in the cell layer of an alternate form of Type III collagen, which exhibited a different CNBr peptide pattern from that in the culture medium, was recently reported in BAE cells. The maps shown in figure 8 suggest that the extra peptide, which migrated between α1(I)-CB6 and α1(I)-CB3, may be present in other endothelial cell layers as well. A precedent for structural polymorphism between a secreted and a membrane-bound form of the same protein was described for the IgM molecule by Kehry et al. Interestingly, a novel collagen, EC, which had previously been characterized in BAE culture medium, was also found to be secreted by BCE and BVE cells (figures 2 and 3). This collagen, accounting for approximately 25% of the total collagen in the culture medium, was isolated after passage over DEAE-cellulose (to which it does not bind) and was identified by its characteristic CNBr peptide pattern after SDS-PAGE (figure 4). The presence of EC in endothelial cell layers has not been established due to its extreme lability to most proteases, including pepsin.

In contrast to the endothelial cells from aorta, vena cava, and cornea, adrenal capillary cells synthesized Type I procollagen, which was identified in both the culture medium and cell layer by mobility on SDS-PAGE before and after pepsin treatment (figures 1, 5, and 6). The cells also synthesized fibronectin, Type III procollagen, and, possibly, thrombospondin (figure 1). Approximately 50% of the total protein secreted into the culture medium was collagenous, a value more similar to that observed for smooth muscle cells or fibroblasts than for endothelial cells. Studies on collagen biosynthesis in BAE and BCE cell cultures by Gospodarowicz and coworkers have yielded results very similar to those described in this survey. Type III was the major procollagen produced by both cell types, Types IV and V were found to be preferentially associated with the cell layer; the ratio of collagen types secreted by BCE cells was 3:16:1 (Types I:III:IV + V). One might have predicted that corneal endothelium, which is avascular and covers a continuous, morphologically distinct basement membrane (Descemet's membrane), would synthesize extracellular proteins different from those of aortic endothelium, which faces the bloodstream and covers a poorly defined basement membrane. Both cell types, however, have similar embryonic origins and arise from primary mesenchyme. Since these cells synthesize collagen types other than Type III, and some of these types could contribute to extracellular basal laminae, it is possible that a synthetic modulation occurs in culture that favors the quantitative increase of one collagen type. This notion is not inconsistent with the production of basement membrane (Type IV) collagen by rabbit corneal endothelial cells, as described by Kefalides et al. and by Sundar Raj et al. However, these researchers based their identification of this type largely on posttranslational modifications of proline and lysine residues, a characteristic which often displays tissue or cell-dependent variation (see Bornstein and Sage for a review).

Other investigators have described both interstitial and basement membrane collagens in aortic endothelial cells cultured from different species. Madri et al. localized Types IV and V collagen to BAE extracellular matrices, but no collagen was identified in the culture medium. The source of this discrepancy remains unresolved but may have arisen from differences in the initial isolation and subculturing of the cells, in the number of passages, or in cell density at the time of labeling. In this regard, Kay et al. have reported that normal human umbilical vein (HUV) endothelial cells preferentially sequester Type IV procollagen in the cell layer but release it into the culture medium upon viral transformation. In addition, Laurie et al. presented immunohistochemical evidence that secretory organelles of endothelial cells stained positively for Type IV procollagen during the early stages of tooth development but became negative for this antigen at later stages after cellular migration. Unlike the bovine endothelial cells in this study, which did not secrete Type IV procollagen into the culture medium, we have found that cultures of both HUV cells and of a murine hemangioendothelioma did secrete this collagen type, which could be purified from the culture medium by ion exchange chromatography. In terms of secretory phenotype, therefore, these endothelial cells do appear to be different from those of large vessels, capillary, and cornea; these observations confirm earlier suggestions.
that a Type IV procollagen was secreted by HUV cells.1,48

The significance of the synthesis of Type I procollagen by CAP cultures is presently unknown. However, this secretory phenotype is particularly reminiscent of descriptions of variant or "modulated" endothelial cell cultures. BAE cells that displayed a secondary growth pattern, referred to as sprouting, synthesized Type I procollagen and lesser amounts of Type III procollagen and fibronectin.26 These cells are elongate, stain positively for Factor VIII-antigen, and grow under the monolayer in a mycelial pattern which eventually dominates the culture.26,49 An apparently similar alteration in growth control occurred when BAE cells that were initially cloned and grown in the presence of FGF were subcultured in the absence of the growth factor. These cells displayed alterations in morphology, polarity, thromboresistance, and protein synthesis, as manifested by the production of Type I collagen.25

The capillary endothelial cells used in this study have been characterized as forming tubes in culture, a phenomenon termed "angiogenesis in vitro."50 Although the mechanisms responsible for inducing such an angiogenic response have not been delineated, it is likely that factors extrinsic, as well as intrinsic, to the cell are involved. If CAP cells are indeed responsive to a different sort of growth control than that which regulates endothelial cells in, for example, larger vessels, this difference could be a function of the external substrate, the presence of receptors for certain growth factors, or the absence of "control macromolecules" which prevent proliferation. The temporal and spatial involvement of the secreted, extracellular proteins during capillary tube formation is presently not known. CAP cells have been shown to require a substrate composed of Type I collagen for growth.7 Bowman et al.51 reported that brain capillary endothelial cells in primary cultures secreted significantly reduced quantities of fibronectin when compared to capillary pericytes and required the addition of exogenous matrix components. This level of fibronectin synthesis, which approaches that of smooth muscle cells and is four- to sixfold lower than that reported for BAE cells (Sage, Pritzl, and Bornstein, unpublished observations), could be insufficient for CAP cell attachment and growth.

It may also be relevant that the extracellular distribution of fibronectin has been correlated with collagen type synthesis by cells in culture,52 and fibronectin was recently shown by the immunoperoxidase technique to be distributed in an axial repeat pattern along native Type I procollagen fibrils.53 That fibronectin and interstitial collagen types do interact in vivo has been shown by their codistribution in granulation tissue during wound healing.54 It is therefore likely that one or both of these proteins may provide extracellular substrates during cellular migration. In this regard it has been suggested that variant BAE cells (resembling the sprouting cultures described by Schwartz29 and Gospodarowicz et al.49) function as "lead cells" to provide a fibronectin substrate for endothelial cells involved in a neovascular response.55

The production of Type III procollagen by endothelial cells may have significance in hemostasis. The distribution of this collagen type is very similar to that of reticulin and has been localized around small blood vessels in skin using immunoelectron microscopy.56 Immunofluorescence studies have shown this collagen type to be a major component of the subendothelium,57 although this distribution was not confirmed in a more recent report.3 Although all the interstitial collagen types induce platelet aggregation,58 Type III collagen was preferentially bound by both fibronectin and Factor VIII-related antigen.59,60 The presence of fibronectin on the surface of thrombin-stimulated platelets further suggested that platelet adhesion to the subendothelial collagen could be mediated by this glycoprotein after the endothelial surface had been disturbed.91

Synthesis of Type IV collagen by endothelial cells provides circumstantial evidence that these cells secrete at least some components of the basal lamina which underlies them. This collagen type is an integral component of all basement membranes and has been localized to endothelial extracellular matrices both in vivo and in vitro.2,3,10 In the present study, it was present only as a cell layer constituent.

While Type IV collagen has been characterized specifically as a structural component of the lamina densa, Type V is now considered to be a basement membrane-associated, or pericellular, collagen.62 Type V collagen has been localized in vitro to the extracellular matrix of endothelial cells from several different tissues but has not been detected in the culture medium.3,39 It has been identified in vivo in vascular subendothelia3,10 and in the avascular cornea.63 Recent work by Madri et al.3 has attributed the thromboresistance of the endothelium to the presence of Type V collagen on the luminal surface. In contrast to results obtained from earlier experiments in which soluble basement membrane types did not elicit platelet aggregation, more recent investigations have shown that this activity was dependent on the quaternary structure of the collagen and that fibrillar forms of Types IV and V were active in promoting platelet aggregation.64

The extracellular matrix/basal lamina is of fundamental importance to several processes involving endothelial cell injury, regeneration, and repair. Vracko et al.65 have suggested that the accumulation of basal lamina in muscle capil-
laries, which is exaggerated in diabetics and aged individuals, results from the process of cell renewal. A similar process could be involved in certain corneal disorders in which a retrocorneal fibrous membrane is formed. This structure is comprised of a thickened Descemet's membrane which contained several layers of endothelial cells embedded within an abundant extracellular matrix.66

The involvement of distinct basement membrane structures, such as the alveolar and glomerular basement membranes and Descemet's membrane, in the regulation of permeability has been established. However, studies on nonrenal vascular basement membranes or basal laminae have been less conclusive. A recent report has proposed a role for granulocytes in altering vascular permeability during acute vascular response to inflammation.67 One mechanism could involve secretion of enzymes which specifically degrade matrix components.30 Alteration of the extracellular matrix would not only result in changes in the permeability barrier but could also promote cell detachment and migration; for example, removal of collagen from fibroblast extracellular matrices increased cell motility.68 In addition, the elaboration of certain factors by cells associated with the inflammatory response could promote cell migration; in this regard, mast cell heparin has been shown to stimulate migration of capillary endothelial cells in vitro.69 Elucidation of these and other processes that function in endothelial cell and in microvessel regeneration will contribute to an understanding of angiogenesis and intimal repair in vivo.

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Secretory phenotypes of endothelial cells in culture: comparison of aortic, venous, capillary, and corneal endothelium.

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