The biosynthesis of extracellular-matrix components by bovine retinal endothelial cells displaying distinctive morphological phenotypes

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Previous studies have indicated that the morphology and behaviour of bovine retinal microvessel endothelial cells are influenced by culture conditions *in vitro*. Data are presented here concerning the biosynthesis of matrix macromolecules by bovine retinal endothelial cells cultured under conditions in which the cells display either the 'cobblestone' or the 'sprouting' phenotype. Newly synthesized matrix proteins were identified by their characteristic electrophoretic mobilities, immunoprecipitation with specific antibodies, susceptibilities to enzymic digestions and chromatographic behaviour. Type IV procollagen was the major collagenous species synthesized by early-passage cells forming a 'cobblestone' monolayer. In contrast, cells displaying the 'sprouting' morphology switched to the predominant synthesis of interstitial fibrillar collagens (types I and III). Fibronectin was synthesized by retinal endothelial cells under all the experimental conditions studied. A non-collagenous glycoprotein of M_r approx. 47000 was also a major biosynthetic product of these cells. The synthesis of thrombospondin was very much dependent on the nature of the substratum on which the cells were cultured. This glycoprotein was synthesized in large amounts by 'cobblestone' endothelial cells cultured on gelatin-coated dishes, whereas its synthesis was markedly decreased by culturing the cells on collagen gels, and the protein appeared to be absent when the cells were plated within collagen gels ('sprouting' cells). Late-passage retinal cells synthesized predominantly type I procollagen, variable amounts of type III procollagen and only traces of type IV procollagen, irrespective of whether the cells displayed a 'cobblestone' or 'sprouting' morphology.

INTRODUCTION

The structural rigidity of the wall of small blood vessels is in large part attributable to the capillary basement membrane, which lies in close apposition to the endothelium and has embedded within it intramural pericytes. Basement membranes exhibit considerable morphological and biochemical variability, depending on their anatomical location (Heathcote & Grant, 1981; Canfield & Grant, 1984; Madri *et al.*, 1984). In spite of this variability, type IV procollagen, a number of specific glycoproteins (e.g. laminin, entactin and possibly fibronectin) and heparan sulphate proteoglycan appear to be common constituents of all basement membranes. It is likely that endothelial cells contribute to the synthesis of their basement membrane *in vivo*, but it is not clear whether the synthesis is affected by changes in the environment of the cells such as those that occur during angiogenesis.

The isolation and culture of endothelial cells derived from the retinal microvasculature has been reported (Frank *et al.*, 1979; Bowman *et al.*, 1982; Buzney *et al.*, 1983; Gitlin & D'Amore, 1983; Schor & Schor, 1986). When plated on a two-dimensional substratum, retinal endothelial cells proliferate and eventually form a contact-inhibited 'cobblestone' monolayer morphologically similar to the lining of the vessel wall (Bowman *et al.*, 1982; Gitlin & D'Amore, 1983; Schor & Schor, 1986). In contrast, the same cells plated within a three-dimensional gel of native type I collagen display an elongated or 'sprouting' phenotype; under these condi-

tions the cells do not divide, but migrate and self-associate, forming three-dimensional structures reminiscent of those seen in angiogenesis *in vivo* (Schor & Schor, 1986). In the present study we have investigated the proteins synthesized and secreted by bovine retinal endothelial cells under culture conditions in which the cells display these different morphological phenotypes. Particular attention has been paid to the collagenous molecules secreted by these cells.

EXPERIMENTAL

Materials

Culture medium, donor calf serum, sodium pyruvate, glutamine, non-essential amino acids and antibiotics were obtained from Gibco Bio-Cult, Paisley, Scotland, U.K. Ascorbic acid was obtained from BDH Chemicals, Poole, Dorset, U.K. β -Aminopropionitrile fumarate, tunicamycin, phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, 2-mercaptoethanol, trypsin, pepsin (1:60000, from pig stomach mucosa), and bacterial collagenase (type IA) used in the isolation of primary cell cultures were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Highly purified bacterial collagenase (form III) used in analytical procedures was obtained from Advanced Biofactures, New York, NY, U.S.A. Guanidinium chloride was obtained from Fluka Chemicals, Glossop, Derbyshire, U.K.

L-[U- 14 C]Proline (280 mCi/mmol), L-[35 S]methionine (1290 Ci/mmol) and [14 C]methylated protein mixture

comprising myosin (M_r 200 000), phosphorylase *b* (doublet of M_r 100 000 and 92 500), bovine serum albumin (M_r 69 000), ovalbumin (M_r 46 000), carbonic anhydrase (M_r 30 000) and lysozyme (M_r 14 300) were obtained from Amersham International, Amersham, Bucks., U.K. DEAE-cellulose (DE-52) was purchased from Whatman Chemical Separations, Maidstone, Kent, U.K., and protein A-Sepharose (CL-4B) was purchased from Pharmacia Fine Chemicals, Milton Keynes, U.K.

Guinea-pig anti-(bovine type IV collagen) was a gift from Dr. S. Ayad of this Department. Rabbit anti-(human plasma fibronectin) and rabbit anti-(human platelet thrombospondin) were generously supplied by Dr. M. J. Humphries, National Cancer Institute, Bethesda, MD, U.S.A., and Dr. D. S. Pepper, Scottish National Blood Transfusion Service Headquarters, Edinburgh, Scotland, U.K., respectively. Rabbit anti-(human Factor-VIII-related antigen) was purchased from Dakopatts, High Wycombe, Bucks., U.K.

Cell culture

Bovine retinal endothelial cells were isolated from the adult retina as previously described (Schor & Schor, 1986). Briefly, retinal cells (endothelial cells and pericytes) were released from bovine retinas by sequential digestion with bacterial collagenase and trypsin; primary cultures that appeared to contain predominantly endothelial cells were selectively farmed to obtain apparently homogeneous endothelial-cell populations (Schor & Schor, 1986). Cloned populations of endothelial cells were isolated by double cloning (Schor *et al.*, 1983). The identity of the isolated cells was confirmed by their positive staining with anti-(Factor VIII-related antigen). Stock cultures were routinely maintained on gelatin-coated dishes in Eagle's minimum essential medium supplemented with 15% (v/v) donor calf serum, ascorbic acid (50 μ g/ml), 2 mM-glutamine, 1 mM-sodium pyruvate, non-essential amino acids, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Cultures were incubated at 37 °C in a moist atmosphere of CO₂/air (1:19).

To investigate the synthesis of proteins by bovine retinal endothelial cells displaying the 'cobblestone' phenotype, the cells were cultured either on gelatin-coated Petri dishes or on the surface of gels of native type I collagen (Schor & Schor, 1986). In order to study the 'sprouting' phenotype, the cells were homogeneously dispersed within a type-I collagen gel matrix by mixing the cells with the collagen gelling solution before casting (Schor, 1980; Schor & Schor, 1986). In this latter case, a thin layer of collagen gel was used to underlay and overlay the collagen matrix in which the cells had been dispersed. The studies presented here were conducted with uncloned endothelial cells between passages 3 and 6 (referred to as early-passage cells) or between passages 9 and 11 (referred to as late-passage cells), except where reference is specifically made in the text to the use of cloned cells.

Radiolabelling of endothelial cell cultures

Endothelial cells cultured either on gelatin-coated dishes or on collagen gels were radiolabelled at confluence, when they had formed a contact-inhibited 'cobblestone' monolayer (usually by day 5). In experiments where the cells were plated within collagen gels, the cultures were radiolabelled when the cells had migrated within the gel to form three-dimensional networks as

described by Schor & Schor (1986) (usually by day 21). At this time, the cultures were incubated for 24 h at 37 °C with Eagle's minimum essential medium either lacking the non-essential amino acid supplement ([¹⁴C]proline labelling) or methionine ([³⁵S]methionine labelling) but containing 5% (v/v) donor calf serum, ascorbic acid (50 μ g/ml), β -aminopropionitrile fumarate (50 μ g/ml), 2 mM-glutamine, 1 mM-sodium pyruvate, antibiotics (100 units/ml) and either [¹⁴C]proline (3 μ Ci/ml) or [³⁵S]methionine (50 μ Ci/ml). Before labelling with [³⁵S]methionine, the cultures were preincubated for 2 h in methionine-free Eagle's minimum essential medium. In studies where the influence of tunicamycin (0.5–4 μ g/ml) was investigated, the cells were preincubated with this drug for 2 h before the addition of [³⁵S]methionine (Kurkinen *et al.*, 1984).

Isolation of newly synthesized proteins

At the end of the incubation period, the medium was collected and the attached cell layer/matrix was washed three times with cold Eagle's minimum essential medium. In experiments where the cells were cultured on gelatin-coated dishes, the cell layer/matrix was collected by scraping the dishes with a rubber policeman. When the cells were cultured on or within collagen gels, each washing was followed by centrifugation (2000 *g* for 5 min) in order to separate the interstitial fluid of the hydrated gel from the cells, their matrix and the collagen gel. The medium and washings were combined and the following proteinase inhibitors were added to the final concentrations indicated: phenylmethanesulphonyl fluoride (2 mM), *N*-ethylmaleimide (10 mM), 6-aminohexanoic acid (25 mM) and EDTA (25 mM).

When the cells were radiolabelled with [³⁵S]methionine, the culture medium was collected as described above and analysed immediately. When the cells were radiolabelled with [¹⁴C]proline, the medium proteins were precipitated at 4 °C by the addition of (NH₄)₂SO₄ to 30% saturation (0–30% precipitate) and recovered by centrifugation (27 000 *g* for 45 min). The precipitate was resuspended in 0.1 M-Tris/HCl buffer (pH 7.4) containing 0.4 M-NaCl and the proteinase inhibitors described above and dialysed extensively against this solution. The labelled proteins remaining soluble at 30% (NH₄)₂SO₄ saturation were precipitated by the further addition of (NH₄)₂SO₄ to 80% saturation (30–80% precipitate) and collected as described above. These samples were stored frozen at –20 °C until used for biochemical analysis.

Proteins present in the cell layer/matrix were extracted in 4 M-guanidinium chloride/50 mM-Tris/HCl, pH 7.4, for 24 h at 4 °C. Proteins solubilized by this treatment were recovered by centrifugation (27 000 *g* for 45 min) and dialysed extensively against 0.5 M-acetic acid. A small proportion of non-diffusible proteins precipitated during dialysis and were recovered by centrifugation as above. This extraction procedure was found to solubilize up to 95% of the total radioactivity deposited into the cell layer/matrix by cultured retinal endothelial cells.

Enzymic digestion procedures

Bacterial collagenase. Newly synthesized proteins were tested for their susceptibilities to digestion by bacterial collagenase. Samples were dialysed against 62 mM-Tris/HCl, pH 7.4, containing 5 mM-CaCl₂, 2 mM-*N*-ethylmaleimide and 1 mM-phenylmethanesulphonyl

Fig. 1. Fluorograms of [^{14}C]proline-labelled medium proteins synthesized by early-passage retinal endothelial cells cultured on gelatin, on collagen and within collagen gels

Cells were labelled for 24 h with [^{14}C]proline and the medium proteins precipitable at (a) 0–30% and (b) 30–80% saturation with $(\text{NH}_4)_2\text{SO}_4$ were analysed by electrophoresis on 6.5% polyacrylamide gels under reducing conditions. Samples from cultures grown on gelatin are in tracks 2 and 6, samples from cultures grown on collagen are in tracks 3 and 7 and samples from cells plated within collagen gels are in tracks 4 and 8. Tracks 1 and 5 contain a standard of [^3H]acetylated rat tail tendon collagen. The migration positions of fibronectin, type IV procollagen, thrombospondin and a protein of M_r 47000 are indicated. Electrophoresis was performed on different polyacrylamide gels. The migration positions of [^{14}C]methylated non-collagenous proteins of known M_r are indicated.

fluoride for 16 h at 4 °C, and divided into two equal portions. One portion was treated with 30 units of highly purified bacterial collagenase for 3 h at 37 °C, and the other was incubated without the enzyme as a control. Samples were then freeze-dried and resuspended in Laemmli (1970) sample buffer (see below) before analysis by SDS/polyacrylamide-gel electrophoresis and fluorography.

Pepsin. In some experiments, the radiolabelled proteins were digested with pepsin. Samples were first dialysed against 0.5 M-acetic acid for 16 h at 4 °C and pepsin was then added to give a final concentration of 100 $\mu\text{g}/\text{ml}$. After incubation for 4 h at 4 °C, digestion was terminated by the immediate freeze-drying of the samples.

DEAE-cellulose chromatography

Samples were analysed by ion-exchange chromatography on DEAE-cellulose essentially as described by Sage *et al.* (1979). Samples were dialysed into DE-buffer (50 mM-Tris/HCl, pH 8.0, containing 6 M-urea, 2.5 mM-EDTA and 10 mM-phenylmethanesulphonyl fluoride) for 2 h at 4 °C and were then applied to a column (10 cm $\times$ 1 cm) of DE-52 resin which had been pre-equilibrated with DE-buffer at 4 °C. The column was washed with DE-buffer, and bound proteins were eluted with a linear gradient (total volume 300 ml) of this buffer containing 0–0.2 M-NaCl. Fractions (5 ml) were collected and samples were analysed by liquid-scintillation spectrometry. Peaks of radioactivity were pooled, dialysed against distilled water and freeze-dried.

Electrophoretic analyses

The nature of the newly synthesized proteins was examined by discontinuous SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). Samples equivalent to 5000 c.p.m. of ^{14}C or 40000 c.p.m. of ^{35}S were denatured by heating at 100 °C for 2 min in Laemmli sample buffer containing 2% (w/v) SDS, with or without 5% (v/v) 2-mercaptoethanol. In order to distinguish between collagen types I and III, the interrupted reduction method described by Sykes *et al.* (1976) was used. Electrophoresis was performed on slab gels, which consisted of a separating gel (15 cm $\times$ 15 cm $\times$ 0.3 cm) and a stacking gel (15 cm $\times$ 1 cm $\times$ 0.3 cm), in a water-cooled apparatus at 30 mA/gel. For fluorography the gels were fixed in 10% (v/v) acetic acid/25% (v/v) methanol, impregnated with 2,5-diphenyloxazole, dried and exposed to pre-flashed X-Omat AR film (Bonner & Laskey, 1974; Laskey & Mills, 1975).

RESULTS

Effects of substrata on the nature of the proteins secreted into the medium by bovine retinal endothelial cells

Early-passage bovine retinal endothelial cells were cultured on gelatin-coated Petri dishes, on collagen gels and within three-dimensional collagen gels as described in the Experimental section. Cells plated on two-dimensional substrata (either gelatin or the surface of collagen gels) formed a 'cobblestone' monolayer at confluence. When they were grown within three-dimensional collagen gels,

Fig. 2. Immunoprecipitation of [^{35}S]methionine-labelled medium proteins with antibodies to fibronectin, type IV collagen and thrombospondin

Early-passage retinal endothelial cells were cultured on 35 mm gelatin-coated Petri dishes. At confluence the cells were radiolabelled with [^{35}S]methionine (50 $\mu\text{Ci}/\text{ml}$) for 24 h. At the end of the incubation period, samples (150 μl) of the medium were used for immunoprecipitation experiments (Cooper *et al.*, 1981). Briefly, samples were mixed with 1 ml of 50 mM-Tris/HCl, pH 8.0, containing 0.4 M-NaCl, 5 mM-EDTA and 1% (v/v) Nonidet P40, and 5 μl of antiserum was added. After incubation at room temperature for 1.5 h, 30 μl of a 50% (v/v) suspension of protein A-Sepharose in the above buffer was added and the incubation continued at 4 $^{\circ}\text{C}$ for a further 1.5 h. The complex of antigen, antibody and protein A-Sepharose was collected by centrifugation, washed twice with the above buffer and twice with 10 mM-Tris/HCl, pH 6.8. Bound proteins were released by heating at 100 $^{\circ}\text{C}$ for 3 min in double-concentration Laemmli sample buffer containing 10% (v/v) 2-mercaptoethanol. These proteins were separated by electrophoresis on 6.5% -polyacrylamide gels and detected by fluorography. Samples in tracks 1-4 are as follows: 1, total medium proteins; 2, proteins immunoprecipitated with anti-fibronectin serum; 3, proteins immunoprecipitated with anti-(type IV collagen) serum; 4, proteins immunoprecipitated with anti-thrombospondin serum. The migration positions of [^{14}C]methylated non-collagenous proteins of known M_r are indicated.

Fig. 3. Effect of bacterial collagenase on the newly synthesized [^{14}C]proline-labelled proteins secreted into the medium by retinal endothelial cells

Early-passage cells were labelled with [^{14}C]proline for 24 h. Medium proteins precipitable at 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ were digested with highly purified bacterial collagenase (30 units) for 3 h at 37 $^{\circ}\text{C}$ in 62 mM-Tris/HCl buffer, pH 7.4, containing 5 mM- CaCl_2 , 2 mM-*N*-ethylmaleimide and 1 mM-phenylmethanesulphonyl fluoride. The products were separated by electrophoresis under reducing conditions on 6.5% -polyacrylamide gels and detected by fluorography. Samples from cultures grown on gelatin are in tracks 2 and 3, samples from cultures grown on collagen are in tracks 4 and 5 and samples from cells plated within collagen gels are in tracks 6 and 7. Samples in tracks 2, 4 and 6 were incubated for 3 h without enzyme; samples in tracks 3, 5 and 7 were incubated for 3 h with bacterial collagenase. Track 1 shows a standard of [^3H]acetylated rat tail tendon collagen. The migration positions of fibronectin, type IV procollagen, thrombospondin, pro- $\alpha_1(\text{I})$ and pro- $\alpha_2(\text{I})$ chains and [^{14}C]methylated non-collagenous proteins of known M_r are indicated.

the cells displayed a typical elongated 'sprouting' morphology as described by Schor & Schor (1986). At this time, the cells were radiolabelled with either [^{14}C]proline or [^{35}S]methionine, and the newly synthesized proteins secreted into the medium were fractionated with $(\text{NH}_4)_2\text{SO}_4$ and analysed by SDS/polyacrylamide-gel electrophoresis.

Several major differences were noted between the biosynthetic profiles of the cells cultured on the different substrata (Fig. 1). When the cells were cultured on gelatin or on the surface of collagen gels, the major high- M_r proteins identified in the culture medium were type IV procollagen, fibronectin and thrombospondin. The amount of thrombospondin synthesized was found to depend on the nature of the substratum, this glycoprotein being particularly abundant in the medium from cells grown on gelatin. Also present were several low- M_r

Fig. 4. Effect of pepsin on the newly synthesized [^{14}C]proline-labelled proteins secreted into the medium by retinal endothelial cells

Early-passage cells were labelled with [^{14}C]proline for 24 h, and the medium proteins precipitable at 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ were digested with pepsin (100 $\mu\text{g}/\text{ml}$) for 4 h at 4 $^\circ\text{C}$. The products were separated by electrophoresis on 6.5%-polyacrylamide slab gels and detected by fluorography. Samples in tracks 1–6 are as follows: 1 and 4, standard [^3H]acetylated rat tail tendon collagen; 2, sample from cells cultured on gelatin; 3, sample from cells cultured on collagen; 5 and 6, samples from cells cultured within collagen gels. Electrophoresis was performed under non-reducing conditions, except for track 6, in which the sample was reduced after it had entered the separating gel by approx. 1 cm (interrupted reduction). Electrophoresis was performed on different polyacrylamide gels. The migration positions of $\alpha_1(\text{I})$, $\alpha_2(\text{I})$ and $\alpha_1(\text{III})$ chains and [^{14}C]methylated non-collagenous proteins of known M_r are indicated.

bands; particularly interesting was a species of M_r 47000 which, like thrombospondin, was recovered predominantly in the 30–80% $(\text{NH}_4)_2\text{SO}_4$ fraction. When retinal endothelial cells were cultured within collagen gels, only trace amounts of type IV procollagen were synthesized, and type I procollagen was identified as the major collagenous species secreted, together with some type III procollagen (Fig. 1). Furthermore, under these conditions thrombospondin was not detected.

Fibronectin, thrombospondin and the procollagens described above were identified by a combination of immunological and biochemical techniques. Fig. 2 shows the immunoprecipitation of type IV procollagen, fibronectin and thrombospondin from the culture medium of early-passage endothelial cells grown on gelatin. It is noteworthy that, when analogous experiments were conducted using antiserum raised against mouse entactin

(generously supplied by Dr. B. L. M. Hogan, National Institute for Medical Research, Mill Hill, London N.W.7, U.K.), no radiolabelled proteins were precipitated. Fibronectin was also identified by its affinity for gelatin-Sepharose and heparin-Sepharose (Ruoslahti *et al.*, 1981; Yamada, 1983) (results not shown). The presence of thrombospondin in the culture medium was also detected by a sensitive radioimmunoassay (Hunter *et al.*, 1984); further confirmation of its identity was obtained by its ability to bind to heparin-Sepharose (Lawler *et al.*, 1978) (results not shown).

The effect of bacterial collagenase on the newly synthesized proteins secreted by retinal endothelial cells is shown in Fig. 3. The polypeptide bands identified as fibronectin and thrombospondin were resistant to digestion. The doublet which migrated just faster than the β chains of type I collagen, and which was shown in Fig. 2 to be precipitated by antibodies to type IV collagen, was digested by bacterial collagenase. The polypeptides migrating in the positions of pro- $\alpha_1(\text{I})$ and pro- $\alpha_2(\text{I})$ collagen chains, secreted when the endothelial cells were cultured within collagen gels, were also digested by this enzyme (Fig. 3).

When the culture medium from endothelial cells cultured on gelatin or on collagen was digested with pepsin, two major pepsin-resistant bands were identified (Fig. 4). These bands migrated more slowly than the α_1 chain of standard type I collagen, and were immunoprecipitated by antibodies to type IV collagen (results not shown). Analysis of the pepsin-digested culture medium obtained from cells cultured within collagen gels revealed a different pattern of collagenous peptides (Fig. 4). Under non-reducing conditions, the major bands co-migrated with the α_1 and α_2 chains of standard type I collagen; two minor bands were also present which corresponded to the partial degradation products of type IV procollagen described above. On interrupted reduction of the samples (Sykes *et al.*, 1976), a further minor polypeptide entered the polyacrylamide gel (Fig. 4). The migration and behaviour of this polypeptide was consistent with the presence of a small amount of type III procollagen in these samples.

Further evidence for the secretion of type IV procollagen by retinal endothelial cells was obtained by ion-exchange chromatography. Fig. 5(a) shows the profile obtained when the 0–30% $(\text{NH}_4)_2\text{SO}_4$ fraction of medium conditioned by endothelial cells grown on collagen was applied to a column of DEAE-cellulose. Most of the [^{14}C]proline-labelled polypeptides did not bind to the column (Fig. 5a, peak A). Electrophoresis of this unbound peak revealed the presence of two polypeptides which migrated slightly faster than the β chains of a type I collagen standard (Fig. 5b, track 2) and were immunoprecipitated by antibodies to type IV collagen (Fig. 5b, track 4). Fig. 5(b) also shows that the type IV procollagen polypeptides are disulphide-bonded into higher- M_r multimers, for under non-reducing conditions they did not enter a 6.5%-polyacrylamide gel (track 3).

Characterization of the proteins deposited in the cell layer/matrix by cultured bovine retinal endothelial cells

The newly synthesized proteins deposited in the cell layer/matrix by retinal endothelial cells cultured on the different substrata represented approx. 60% of the total radioactivity incorporated by these cells. These proteins

Fig. 5. Ion-exchange chromatography of [^{14}C]proline-labelled collagenous polypeptides

Early-passage cells were cultured on collagen gels and labelled with [^{14}C]proline for 24 h. The proteins precipitable at 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ were subjected to ion-exchange chromatography on DEAE-cellulose as described in the text. (a) Elution profile of radioactivity. The arrow indicates the point at which the linear gradient of 0–0.2 M NaCl was applied to the column. (b) Electrophoretic and fluorographic analyses of samples recovered from the column. Samples in tracks 1–4 are as follows: 1, [^3H]acetylated rat tail tendon collagen; 2, unbound fraction eluted in peak A, electrophoresed in the presence of 2-mercaptoethanol; 3, unbound fraction eluted in peak A, electrophoresed in the absence of 2-mercaptoethanol; 4, unbound fraction eluted in peak A, immunoprecipitated with anti-(type IV collagen) serum, electrophoresed in the presence of 2-mercaptoethanol. The migration positions of type IV procollagen and [^{14}C]methylated non-collagenous proteins of known M_r are indicated.

were solubilized with guanidinium chloride and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography. Fig. 6(a) shows the effects of bacterial collagenase on the proteins solubilized from the cell layers of cells cultured on gelatin and on collagen. The major collagenase-sensitive species was identified as a closely spaced doublet which migrated slightly faster than type I collagen β chains (Fig. 6a). These polypeptides were immunoprecipitated by antibodies to type IV collagen (results not shown). Fibronectin and the protein of M_r 47000 were identified as major collagenase-resistant species deposited in the cell layer/matrix.

When the retinal endothelial cells were cultured within a collagen gel, the major collagenous species extracted from the cell layer/matrix with 4 M-guanidinium chloride were types I and III procollagens. These species were identified by pepsin digestion and SDS/polyacrylamide-gel electrophoresis of non-reduced and interrupted-reduced samples (Fig. 6b).

Influence of tunicamycin on the synthesis of [^{35}S]methionine-labelled polypeptides by retinal endothelial cells cultured on gelatin-coated dishes

The ability of tunicamycin to inhibit the addition of asparagine-linked oligosaccharide side chains was used to identify the presence of such glycosylated chains in the proteins synthesized by retinal endothelial cells. Comparative analyses were made of the electrophoretic mobilities of polypeptides synthesized in the absence and presence of tunicamycin (4 $\mu\text{g}/\text{ml}$). As expected, the major high- M_r glycoproteins, fibronectin and thrombospondin, showed a marked increase in their electrophoretic mobilities in the presence of tunicamycin (Fig. 7), consistent with the presence of N-linked oligosaccharides. In comparison, the doublet of type IV procollagen showed only a slight

increase in mobility, indicating that the pro- $\alpha_1(\text{IV})$ and pro- $\alpha_2(\text{IV})$ chains also contain asparagine-linked side chains, albeit to a much lesser extent than either fibronectin or thrombospondin. A marked shift in electrophoretic mobility was also observed for the protein of M_r 47000 (Fig. 7). In the presence of tunicamycin, the apparent M_r of this species was decreased to approx. 42000, suggesting that this protein is particularly enriched in carbohydrate. The function of this glycoprotein remains to be determined.

Influence of passage number on collagen gene expression

During the course of our experiments it became apparent that cells which had undergone several passages *in vitro* synthesized a different spectrum of collagenous proteins compared with primary or early-passage cultures. As discussed above, type IV procollagen was the major collagenous species secreted by early-passage cells cultured on gelatin and on collagen. As the passage number of the cells increased, there was a gradual switch to the synthesis of predominantly interstitial collagens. Pepsin digestion revealed that late-passage retinal endothelial cells secreted predominantly type I procollagen and a variable amount of type III procollagen (results not shown). The switch was observed in the proteins secreted into the medium as well as in those associated with the cell layer, and was not due to an increase in the relative proportion of non-endothelial cells (e.g. pericytes) in uncloned cultures, since this phenomenon was also observed with cloned cells (results not shown).

DISCUSSION

Vascular endothelial cells *in vivo* may display two distinctive phenotypes. Endothelial cells which line the

Fig. 6. Fluorograms of the [^{14}C]proline-labelled proteins deposited in the cell layer/matrix by retinal endothelial cells

Early-passage cells were labelled with [^{14}C]proline for 24 h, and the proteins deposited into the cell layer/matrix were solubilized by incubation with 4 M-guanidinium chloride/50 mM-Tris/HCl, pH 7.4 for 24 h at 4 °C. Samples thus solubilized were digested with either (a) highly purified bacterial collagenase or (b) pepsin as described in the text, and the products were analysed by electrophoresis on 6.5%-polyacrylamide slab gels and fluorography. Track 1 contains a standard of [^3H]acetylated rat tail tendon collagen. Samples from cells grown on collagen are in tracks 2 and 3, samples from cells grown on gelatin are in tracks 4 and 5, and samples from cells cultured within collagen gels are in tracks 6 and 7. Samples in tracks 3 and 5 were incubated with bacterial collagenase, and samples in tracks 2 and 4 were incubated without the enzyme as controls; electrophoresis was performed after reduction of these samples. Samples in tracks 6 and 7 were digested with pepsin; electrophoresis was performed either without reduction (track 6) or by interrupted reduction (track 7) of the samples. The migration positions of fibronectin, type IV procollagen, thrombospondin, the protein of M_r 47000, the $\alpha_1(\text{I})$, $\alpha_2(\text{I})$ and $\alpha_1(\text{III})$ chains and [^{14}C]methylated non-collagenous proteins of known M_r are indicated. The electrophoretic profiles of the samples from cells cultured on collagen gels are distorted because of the large amounts of unlabelled type I collagen, which was also solubilized with 4 M-guanidinium chloride.

Fig. 7. Effect of tunicamycin on the synthesis of [^{35}S]methionine-labelled proteins by retinal endothelial cells cultured on gelatin

Early-passage cells were cultured on gelatin-coated Petri dishes. Confluent cultures were preincubated for 2 h in methionine-free Eagle's minimum essential medium, with or without tunicamycin (4 $\mu\text{g}/\text{ml}$) as described in the text. At this time [^{35}S]methionine (50 $\mu\text{Ci}/\text{ml}$) was added and the incubation continued for a further 24 h. The culture medium was collected and centrifuged to precipitate any cell debris. The supernatants were analysed by electrophoresis under reducing conditions on 6.5%-polyacrylamide slab gels, followed by fluorography. Track 1: total medium proteins from cultures incubated without tunicamycin. Track 2: total medium proteins from cultures incubated with tunicamycin. The migration positions of fibronectin, type IV procollagen, thrombospondin and the protein of M_r 47000 are indicated, together with [^{14}C]methylated protein standards.

inner surface of blood vessels form a contact-inhibited 'cobblestone' monolayer. In contrast, during angiogenesis the endothelial cells adopt an elongated fibroblastoid phenotype, and form cords and sprouts, which lead eventually to the formation of patent tubules (Folkman, 1984). Retinal-microvessel endothelial cells *in vitro* may also display these two distinct phenotypes. On a two-dimensional substratum the cells form a contact-inhibited monolayer of 'cobblestone' appearance. When plated within a three-dimensional collagen gel, the cells display the elongated 'sprouting' phenotype (Schor & Schor, 1986). In the present paper we show evidence that type I and III procollagens are the major collagenous

species synthesized by 'sprouting' cells, whereas type IV procollagen is the predominant species synthesized by cells displaying the 'cobblestone' phenotype (Figs. 1, 3, 4 and 6). It is also noteworthy that preliminary studies using retinal pericytes in monolayer culture indicate that these cells synthesize the same matrix proteins as 'sprouting' endothelial cells (A. E. Canfield & A. M. Schor, unpublished work).

The collagenous species secreted by 'sprouting' aortic endothelial cells have previously been shown to be different from those secreted by monolayer cultures (Cotta-Pereira *et al.*, 1980; Levene *et al.*, 1984). Changes include a switch from the secretion of predominantly type III procollagen ('cobblestone' cells) to type I procollagen ('sprouting' cells) (Cotta-Pereira *et al.*, 1980), or changes in the relative proportions of types I, III and V collagens synthesized (Levene *et al.*, 1984). These studies were conducted with post-confluent cultures of aortic endo-

thelial cells in which the 'sprouting' cells grow underneath the intact 'cobblestone' monolayers, and therefore cells displaying both morphologies were present. In this regard it is important to note that, by culturing the retinal endothelial cells either on the surface of or within three-dimensional collagen gels, we were able to examine the proteins synthesized by pure populations of either 'cobblestone' or 'sprouting' cells.

A few studies have reported the synthesis of type IV collagen by 'cobblestone' endothelial cells derived from human umbilical veins (Madri *et al.*, 1980; Sage & Bornstein, 1982), rabbit cornea (Kefalides *et al.*, 1976) and bovine aorta (Howard *et al.*, 1976; Madri *et al.*, 1980). However, most studies using monolayer cultures indicate the secretion of predominantly interstitial fibrillar collagens by large-vessel (Barnes *et al.*, 1978; Sage *et al.*, 1979, 1981a,b; Sankey & Barnes, 1984; Levene *et al.*, 1984), corneal (Sage *et al.*, 1981a; Sankey *et al.*, 1981; Tseng *et al.*, 1981) and microvessel (Sage *et al.*, 1981a; Madri & Williams, 1983) endothelial cells. The disparity in the results obtained from different laboratories may reflect differences in the origin of the cells (Levene *et al.*, 1984), in cellular heterogeneity (Sankey & Barnes, 1984; Schor *et al.*, 1984) and in culture conditions and substratum (Madri & Williams, 1983). The number of doublings that the cells have undergone *in vitro* may also be an important experimental parameter, since our results demonstrate that late-passage retinal-microvessel endothelial cells synthesize a different population of collagenous polypeptides compared with early-passage cells. A switch from the synthesis of primarily type IV collagen to the synthesis of a mixture of collagen types I, III and IV has previously been reported for bovine lens epithelial cells which have undergone several subcultures *in vitro* (Laurent *et al.*, 1981). Furthermore, these workers also reported that the initial phenotype could be partially restored by culturing the cells on an extracellular matrix (produced by bovine corneal endothelial cells) in the presence of an eye-derived growth factor (Kern *et al.*, 1983).

Bovine retinal endothelial cells in culture also synthesized several non-collagenous glycoproteins, in particular fibronectin, thrombospondin and a protein of M_r 47000 (Figs 1, 2, 6 and 7). Fibronectin was identified in both the medium and cell layer/matrix of retinal endothelial cells cultured on gelatin, on collagen and within collagen gels. This glycoprotein has previously been shown to be synthesized by capillary (Sage *et al.*, 1981a), large-vessel (Jaffe & Mosher, 1978; Birdwell *et al.*, 1978; Sage *et al.*, 1979, 1981a; Sage & Bornstein, 1982) and corneal endothelial cells (Sage *et al.*, 1981a). These cells may therefore be a source of both circulating and matrix fibronectins *in vivo* (Ruoslahti *et al.*, 1981).

A second high- M_r , disulphide-bonded, glycoprotein was identified as thrombospondin by radioimmunoassay and by immunoprecipitation experiments (Fig. 2). Thrombospondin has also been identified in the culture medium of other endothelial cells (McPherson *et al.*, 1981; Sage *et al.*, 1981a; Mosher *et al.*, 1982; Raugi *et al.*, 1982; Kramer *et al.*, 1985), and immunofluorescence studies have shown the presence of this glycoprotein in the extracellular matrix deposited by endothelial cells, smooth-muscle cells and fibroblasts (Raugi *et al.*, 1982; Jaffe *et al.*, 1983). It is therefore possible that, like fibronectin, thrombospondin is present as both a circulating protein (in blood platelets) and a matrix protein

in vivo. The synthesis of thrombospondin by retinal endothelial cells was found to be dependent on the substratum on which the cells were cultured, for its synthesis was markedly decreased when cells were grown on collagen gels, and it was not detected when the cells were cultured within collagen gels (Fig. 1). It is noteworthy that Cotta-Pereira *et al.* (1980) also reported that the production of thrombospondin was diminished in 'sprouting' cultures of endothelial cells.

A third, non-collagenous, glycoprotein with M_r approx. 47000 was secreted in large amounts by retinal endothelial cells cultured on all three matrices. Experiments with tunicamycin have revealed that this protein contains asparagine-linked side chains (Fig. 7). The relationship of this glycoprotein to species of similar M_r synthesized by parietal endodermal cells (Kurkinen *et al.*, 1984), endothelial cells and smooth-muscle cells (Sage *et al.*, 1984) requires investigation, although preliminary results suggest they are not related (A. E. Canfield, unpublished work). Our experiments with tunicamycin (Fig. 7) also confirmed the presence of N-linked carbohydrate side chains in fibronectin and thrombospondin, and a small number in type IV procollagen (Kurkinen *et al.*, 1982; Taylor & Grant, 1985).

It has previously been shown that the 'cobblestone' and 'sprouting' morphologies represent reversible phenotypes of aortic (Makarski, 1982; Schor *et al.*, 1983) and retinal (Schor & Schor, 1986) endothelial cells *in vitro*. Retinal cells displaying these two morphological phenotypes also show distinctive behavioural (Schor & Schor, 1986) and biosynthetic (the present paper) characteristics. In the course of angiogenesis, endothelial cells undergo morphological changes similar to those observed in tissue culture. Whether the synthesis of collagen by endothelial cells *in vivo* also switches from type IV to types I/III has not been determined, but remains an intriguing possibility.

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