Thrombospondin gene expression by endothelial cells in culture is modulated by cell proliferation, cell shape and the substratum

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Endothelial cells plated on the surface of a two-dimensional substratum (gelatin-coated dishes, dishes coated with native type I collagen or collagen gels) form a cobblestone monolayer at confluence, whereas cells plated within a three-dimensional gel matrix elongate into a sprouting morphology and self-associate into tube-like structures. In this study, we have compared the synthesis of thrombospondin by quiescent endothelial cells displaying (a) the same morphological phenotype (cobblestone) on different substrata (gelatin and collagen) and (b) different morphological phenotypes (cobblestone and sprouting) on the same substratum (collagen). We demonstrate that thrombospondin is a major biosynthetic product of confluent, quiescent cells cultured on dishes coated with either gelatin or collagen, and that the synthesis of this protein is markedly decreased when cells are plated on or in three-dimensional collagen gels. Moreover, we demonstrate that cells plated in gel (sprouting) secrete less thrombospondin than do cells plated on the gel surface (cobblestone). The regulation of thrombospondin synthesis is reversible and occurs at the level of transcription, as steady-state mRNA levels for thrombospondin decrease in a manner comparable with the levels of protein secreted by these cells. We also show that mRNA levels for laminin B₂ chains are increased when cells are cultured on and in collagen gels compared with on gelatin-coated dishes, suggesting that the syntheses of thrombospondin and laminin are regulated by different mechanisms. When cells are cultured on gelatin- or collagen-coated dishes, thrombospondin gene expression is directly proportional to the proliferative state of the cultures. By contrast, the synthesis of thrombospondin by cells cultured on collagen gels remains at equally low levels whether they are labelled when they are sparse and rapidly proliferating or when they are confluent and quiescent. Fibronectin synthesis was found to increase with increasing confluency of the cells plated on all three substrata. These results demonstrate that thrombospondin gene expression is modulated by cell shape, cell proliferation and the nature of the substratum used for cell culture.

INTRODUCTION

Thrombospondin is a high-*M*_r glycoprotein (*M*_r 420 000) which is composed of three identical subunits cross-linked by disulphide bonds (for reviews, see Lawler, 1986; Frazier, 1987; Majack & Bornstein, 1987). The complete amino acid sequence of human thrombospondin has been determined from cDNA clones (Dixit *et al.*, 1986; Lawler & Hynes, 1986). Thrombospondin was originally described as a protein released from platelet α -granules in response to activation by thrombin (Baenziger *et al.*, 1971, 1972), but it is now known to have a much wider distribution *in vivo*. Thus, thrombospondin has been identified in a variety of human tissues including blood vessels, skin, muscle, kidney and glandular epithelium (Wight *et al.*, 1985), and is known to be synthesized by many normal and transformed cells in culture (see reviews by Lawler, 1986; Majack & Bornstein, 1987; and recent reports by Miller & McDevitt, 1988; Gehron-Robey *et al.*, 1989). It has been shown that thrombospondin can bind to several components of the extracellular matrix, including collagen, fibronectin, fibrinogen, heparin, laminin, osteonectin and plasminogen (Lawler, 1986; Majack & Bornstein, 1987; Clezardin *et al.*, 1988) and to the cell surface (Roberts *et al.*, 1987; Lawler *et al.*, 1988). On the basis of this information, it has been proposed that thrombospondin is a multifunctional protein which is involved in a variety of cell-cell and cell-matrix interactions *in vivo*.

It has previously been shown that vascular cells (endothelial cells, smooth muscle cells and pericytes) synthesize thrombospondin *in vitro* (McPherson *et al.*, 1981; Mosher *et al.*, 1982; Hunter *et al.*, 1984; Mumby *et al.*, 1984; Majack *et al.*, 1985; Canfield *et al.*, 1986, 1989). This protein may be deposited into the extracellular matrix of the vessel wall (Wight *et al.*, 1985) and can also be released into the plasma (Dawes *et al.*, 1988). Within the vasculature, thrombospondin has been proposed to play a role in platelet aggregation (Leung, 1984; Tuszynski *et al.*, 1988), cell attachment (Tuszynski *et al.*, 1987b; Lawler *et al.*, 1988; Majack *et al.*, 1988), growth (Mumby *et al.*, 1984; Majack *et al.*, 1988) and tumour cell metastases (Tuszynski *et al.*, 1987a). This protein may also play an important role in modulating the response of vascular cells to growth factors, since Majack *et al.* (1986) have reported that thrombospondin facilitates the mitogenic response of vascular smooth muscle cells to epidermal growth factor. Furthermore, Mumby *et al.* (1984) have shown that the synthesis of thrombospondin by endothelial cells is directly proportional to the proliferative state of the cells, suggesting a role for this protein in tissue growth and reorganization. In the present study we have investigated the effect of culture conditions on the synthesis of thrombospondin by bovine aortic endothelial cells. We demonstrate that thrombospondin gene expression may be modulated not only by cell proliferation but also by cell shape and by the nature of the substratum used for tissue culture; moreover, this modulation is reversible.

Abbreviation used: MEM, minimum essential medium.

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EXPERIMENTAL

Cell culture

Cells and culture media. Bovine aortic endothelial cells were isolated from adult animals as previously described (Schor *et al.*, 1983). The cells were routinely cultured in Eagle's minimum essential medium (MEM) supplemented with 20% (v/v) donor calf serum, ascorbic acid (50 µg/ml), 2 mM-glutamine, 1 mM-sodium pyruvate, non-essential amino acids, penicillin (100 units/ml) and streptomycin (0.1 mg/ml); all tissue culture media were purchased from Gibco-Bio-Cult, Paisley, Scotland, U.K. This complete growth medium is referred to as 20%-DCS/MEM. Stock cultures were maintained on gelatin-coated dishes as previously described (Schor & Schor, 1986) and were incubated at 37 °C in a moist atmosphere of CO₂/air (1:19).

Substrata. For the various experiments, endothelial cells were plated (a) on gelatin-coated dishes, (b) on dishes coated with native type I collagen, (c) on the surface of three-dimensional gels of native type I collagen and (d) homogeneously dispersed within the three-dimensional collagen gel matrix. Gelatin-coated dishes and three-dimensional collagen gels were prepared as described in detail elsewhere (Schor, 1980; Schor & Schor, 1988). Collagen-coated dishes were prepared by incubating plastic tissue culture dishes with a solution of type I collagen (200 µg/ml) for 1 h and washing the dishes twice with Hanks balanced salt solution before use.

Experimental protocol. We have demonstrated previously that endothelial cells *in vitro* are capable of expressing two distinct morphological phenotypes, known as cobblestone and sprouting, depending on culture conditions (Schor *et al.*, 1983; Schor & Schor, 1986). To investigate the synthesis of thrombospondin by aortic endothelial cells displaying the cobblestone phenotype, the cells were plated on gelatin-coated Petri dishes, on collagen-coated Petri dishes or on the surface of gels of native type I collagen ('on gel') at a density of $(1.6-1.8) \times 10^6$ cells/90 mm dish. In order to study the sprouting phenotype, the cells were homogeneously dispersed within a type I collagen gel matrix ('in gel') by mixing the cells with the collagen gelling solution before casting. For the 'in gel' experiments, endothelial cells were plated at approximately three times the density used for monolayer cultures, as only between 30 and 50% of the cells remain viable under these conditions.

The different morphological phenotypes displayed by endothelial cells are reversible (Schor *et al.*, 1983; Schor & Schor, 1986). In order to reverse the endothelial phenotype, cells which were originally plated in gel and had formed three-dimensional associations were recovered by digestion of the gel with MEM containing bacterial collagenase (4 mg/ml) (Worthington, Freehold, NJ, U.S.A.) and Dispase (0.5 mg/ml) (BCL, Lewes, East Sussex, U.K.) for 40 min at 37 °C. The cells were collected by centrifugation (1000 g, 4 min), replated on to gelatin-coated dishes and grown to confluence in the usual manner.

The studies presented here were conducted with two different batches of aortic endothelial cells used between passage 5 and 22.

Labelling of cell cultures and isolation and analysis of newly synthesized proteins

Cells grown on gelatin-coated dishes, on collagen-coated dishes, on collagen gels or in collagen gels were incubated for 20-24 h at 37 °C in 20%-DCS/MEM containing ascorbic acid (50 µg/ml) and either [³H]proline (20 µCi/ml) or [³⁵S]methionine (50 µCi/ml) (Amersham International, Amersham, Bucks., U.K.). At the end of the incubation period the medium was collected and the attached cell layer/matrix was washed twice with cold Eagle's MEM. When the cells were plated on or in

collagen gels, each washing was followed by centrifugation (2000 g for 10 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 phenylmethane-sulphonyl fluoride (2 mM) and *N*-ethylmaleimide (10 mM) were added to the final concentrations indicated. Samples were either analysed immediately or stored at -20 °C before analysis. In some experiments, mRNA was prepared from these cultures as described later. In other experiments, 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 as previously described (Canfield *et al.*, 1986). The samples were centrifuged (27 000 g for 45 min) and the solubilized proteins were dialysed extensively against 0.5 M-acetic acid at 4 °C. Samples were stored at -20 °C before biochemical analysis.

Electrophoretic analysis. Discontinuous SDS/PAGE was carried out on the conditioned medium after reduction with 5% (v/v) 2-mercaptoethanol as described elsewhere (Canfield *et al.*, 1986). Newly synthesized proteins were revealed by fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975). [¹⁴C]Methylated *M_r* standards were myosin (*M_r* 200 000), phosphorylase *b* (a 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), and were purchased from Amersham.

Identification of thrombospondin, fibronectin and laminin. Newly synthesized thrombospondin and fibronectin in the culture medium were identified by (a) immunoprecipitation with specific antibodies and analysis by SDS/PAGE and (b) affinity to heparin-Sepharose (thrombospondin and fibronectin) and gelatin-Sepharose (fibronectin) as previously described (Canfield *et al.*, 1986, 1987). The levels of mRNA for thrombospondin and laminin were determined by Northern blot hybridization as described below.

RNA isolation and Northern blot hybridization

RNA preparation. Endothelial cells were plated on gelatin-coated dishes, on the surface of collagen gels and within collagen gels as described in detail above. Total RNA was prepared from these cultures using a guanidinium thiocyanate/caesium chloride method (Chirgwin *et al.*, 1979). Quantification of RNA was based upon the absorbance of heat-denatured RNA at 260 nm. RNA was considered to be intact if ethidium bromide staining revealed discrete 28 S and 18 S ribosomal RNA bands after electrophoresis of denatured RNA through 1% agarose gels containing 2.2 M-formaldehyde (Maniatis *et al.*, 1982).

cDNA probes. The following human and mouse cDNAs were used in this study: *M₉*, the 1100-bp *Eco*RI insert in pGEM-2 coding for amino acids at the *N*-terminus of human endothelial thrombospondin (Lawler & Hynes, 1986; kindly donated by Dr. J. Lawler, St Elizabeth's Hospital of Boston, Tufts University School of Medicine, Boston, MA, U.S.A.), and the 675-bp *Eco*RI-*Hind*III in pPE9 coding for amino acids at the *C*-terminus of the *B₂* chain of mouse laminin and a small region of 3'-untranslated sequence (Barlow *et al.*, 1984; kindly donated by Dr. B. L. M. Hogan, Vanderbilt University, Nashville, TN, U.S.A.).

Northern blot hybridization. RNA was separated in 1% agarose gels containing 2.2 M-formaldehyde, transferred to nitrocellulose (pore size 0.45 µm) under vacuum, air-dried and then baked under vacuum at 80 °C for 2 h. Hybridization probes were prepared by random oligo-labelling with [³²P]dCTP using a Pharmacia kit (Pharmacia, Milton Keynes, U.K.) to specific radioactivities of 1×10^9 c.p.m./µg of DNA. Blots were pre-hybridized in a solution containing 50% formamide, 4× SSC

($1 \times \text{SSC} = 0.15 \text{ M-NaCl}/0.015 \text{ M-sodium citrate}$, pH 6.8), 1 mg of sheared salmon sperm DNA/ml, $1 \times \text{Denhardt's solution}$ (Maniatis *et al.*, 1982) and 0.1 % (w/v) SDS for 2–6 h at 37 °C. Hybridizations with heat-denatured ^{32}P -labelled cDNAs were conducted at 37 °C for 24 h in the same solution as described for prehybridizations. Filters were washed to a final stringency of $0.2 \times \text{SSC}$ and 0.1 % SDS at 55 °C. Autoradiograms were generated by exposure of the filters to X-ray film (X-Omat AR) in the presence of intensifying screens at -70°C .

RESULTS

Effects of substratum and cell shape on the biosynthesis of thrombospondin by endothelial cells

Cells plated on the surface of a two-dimensional substratum (gelatin-coated dishes, collagen-coated dishes or collagen gels) form a cobblestone monolayer at confluence. By contrast, cells plated in a three-dimensional collagen gel elongate to a sprouting morphology and self-associate into tube-like structures (Schor *et al.*, 1983; Schor & Schor, 1986). The effects of substratum and cell shape on the biosynthesis of thrombospondin by aortic endothelial cells were examined under the following non-proliferative conditions: (a) confluent cultures plated on gelatin-coated dishes, on dishes coated with native type I collagen and on collagen gels and (b) sprouting cells plated within collagen gels, as described in the Experimental section. In these experiments we have therefore compared the synthesis of thrombospondin by quiescent endothelial cells displaying (a) the same morphological phenotype (cobblestone) on different substrata (gelatin and collagen) and (b) different morphological phenotypes (cobblestone and sprouting) on the same substratum (collagen). Fig. 1 shows the total [^3H]proline-labelled proteins secreted into the medium by aortic endothelial cells cultured on the different substrata. This Figure clearly demonstrates that the synthesis of a protein identified as thrombospondin (see below) is modulated by substratum and cell shape. Thus thrombospondin is a major biosynthetic product of endothelial cells forming a cobblestone monolayer on gelatin-coated Petri dishes (Fig. 1, track 1). By comparison, this protein is secreted in much lower amounts by cells plated on gel (Fig. 1, track 2) and only trace amounts were ever detected in the culture medium of cells displaying the sprouting morphology in gel (Fig. 1, track 3). It is of interest that cells plated on a film of type I collagen secreted similar levels of thrombospondin as cells plated on gelatin-coated dishes (results not shown), suggesting that a three-dimensional collagen gel is required for the modulation of thrombospondin gene expression. The high- M_r glycoprotein was identified as thrombospondin by immunoprecipitation with specific antibodies (Fig. 1, tracks 5 and 6), and by its affinity for heparin–Sephacrose (results not shown). The observed decrease in thrombospondin secretion by endothelial cells plated on gel and in gel does not appear to be due to an overall decrease in protein synthesis by these cells, since (a) the incorporation of [^3H]proline/ 10^5 cells was actually found to be higher on gel than on gelatin-coated dishes (Schor *et al.*, 1984), (b) the same amount of trichloroacetic acid-precipitable radioactivity was loaded on to the SDS/polyacrylamide gels, and (c) mRNA levels for this protein were found to decrease in a comparable manner (see below).

We have previously shown that the cobblestone and sprouting morphologies displayed by endothelial cells *in vitro* are reversible (Schor *et al.*, 1983). In order to examine whether thrombospondin synthesis was also reversible, cells which had originally been plated in gel were recovered and replated on gelatin-coated dishes. These cultures were then radiolabelled when they had

Fig. 1. Fluorograms of newly synthesized proteins secreted into the medium by aortic endothelial cells

(a) Endothelial cells were plated on or in the different matrices as described in the Experimental section. After 6 days in culture, the cells were incubated with [^3H]proline for 24 h, and the newly synthesized proteins secreted into the medium were separated by electrophoresis under reducing conditions and detected by fluorography. Samples in tracks 1–4 are as follows: 1, sample from cells cultured on gelatin-coated dishes; 2, sample from cells cultured on collagen gels; 3, sample from cells cultured in collagen gels; 4, sample of cells initially grown in collagen gels and then replated on to gelatin-coated dishes. (b) Endothelial cells grown to confluence on gelatin-coated dishes were incubated with [^{35}S]methionine for 24 h. At the end of the incubation period, samples ($150 \mu\text{l}$) of the medium were used for immunoprecipitation experiments. 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 $5 \mu\text{l}$ of anti-serum was added. After incubation at room temperature for 1.5 h, $30 \mu\text{l}$ of a 50 % suspension of Protein A–Sephacrose in the above buffer was added and the incubation was continued at 4 °C for a further 1.5 h. The complex of antigen, antibody and Protein A–Sephacrose was collected by centrifugation and washed twice with the above buffer and twice with 10 mM-Tris/HCl, pH 6.8. Bound proteins were released by heating at 100 °C for 3 min in double-concentration Laemmli (1970) sample buffer containing 10 % (v/v) 2-mercaptoethanol, separated by electrophoresis on 6.5 % polyacrylamide slab gels and detected by fluorography. Samples in tracks 5 and 6 are as follows: 5, total medium proteins; 6, proteins immunoprecipitated with anti-thrombospondin serum. The migration positions of thrombospondin (Tsp) and [^{14}C]methylated non-collagenous proteins of known M_r are indicated.

formed a contact-inhibited cobblestone monolayer (usually 6 days after replating). As shown in Fig. 1 (track 3), endothelial cells plated in gel displayed a sprouting phenotype and secreted very low levels of thrombospondin compared with cells plated on gelatin-coated dishes (Fig. 1, compare tracks 1 and 3). When these cells were released from the gel and replated on to gelatin-coated dishes, the endothelial cells not only re-formed a confluent cobblestone monolayer but also restored the synthesis of thrombospondin to the levels previously noted (Fig. 1, track 4). These results demonstrate that the modulation of thrombospondin synthesis by the endothelial cells is reversible.

In order to determine whether the regulation of thrombospondin synthesis occurred at the level of transcription or translation, total RNA was prepared from endothelial cells

Fig. 2. Northern blot analysis of bovine aortic endothelial cell total RNA hybridized with ^{32}P -labelled cDNAs

Total RNA (10 μg) from aortic endothelial cells cultured on gelatin (G; tracks 1), on collagen gels (IC; tracks 2) and in collagen gels (OC; tracks 3) was electrophoresed in a formaldehyde-containing agarose gel, blotted, hybridized, washed and exposed to X-ray film as described in the Experimental section. The same filter was successively probed with ^{32}P -labelled cDNAs coding for thrombospondin (a, b) and laminin B_2 chains (c, d). In each case, the previously ^{32}P -labelled cDNA was removed by incubating the filter in boiling water for 20 min. The autoradiograms are shown in (a) and (c); the positions of the 28 S and 18 S ribosomal RNA bands are indicated as size markers. Scanning densitometric tracings of these autoradiograms are shown in (b) and (d). The ethidium-bromide-stained agarose gel (e) and densitometric scan (f) are included to confirm amounts of RNA were applied to the gel.

plated on gelatin (Fig. 2, tracks 1), on gel (Fig. 2, tracks 2) and in gel (Fig. 2, tracks 3). Samples were subjected to Northern blot hybridization as described in the Experimental section. The levels of mRNA for laminin B_2 chains in these samples were also examined for comparison. Fig. 2 shows the results obtained by successively probing the same filter with cDNAs coding for thrombospondin (Figs. 2a and 2b), and laminin B_2 chains (Figs. 2c and 2d). Figs. 2(e) and 2(f) also show the original ethidium-

bromide-stained agarose gel and confirm that the same amount of RNA was applied to each track of gel. These results clearly demonstrate a marked alteration in the level of mRNA for thrombospondin in endothelial cells cultured under different conditions (Figs. 2a and 2b). Densitometric scanning of the autoradiograms revealed that the levels of thrombospondin mRNA in endothelial cells cultured on and in collagen gels were decreased to 50 % and 25 % respectively of that found in the cells plated on gelatin-coated dishes. When the cells were removed from within collagen gels and replated on to gelatinized dishes, the level of mRNA for thrombospondin was restored to that seen for cells plated originally on to gelatin-coated dishes (results not shown). The same filter used for measuring the levels of thrombospondin mRNA was then probed with cDNA coding for the B_2 chain of laminin (Figs. 2c and 2d). These results show that mRNA specific for laminin B_2 chains was present in cells cultured under all three conditions. Scanning of the autoradiograms revealed that approximately twice as much mRNA for laminin B_2 chains was present when the cells were cultured on and in collagen gels compared with on gelatin-coated dishes. These results demonstrate that the regulation of thrombospondin expression occurs at the level of transcription and is reversible; furthermore, it is modulated by substratum and cell shape and is under different regulatory control than laminin.

Effect of proliferation on thrombospondin synthesis by aortic endothelial cells

Sprouting cells plated in a three-dimensional collagen gel differ from those plated on a two-dimensional substratum not only in their morphology but also in their proliferative potential. We have previously reported that endothelial cells do not proliferate when they are plated in collagen gels (Schor *et al.*, 1983; Schor & Schor, 1986). By contrast, cells plated on a two-dimensional substratum (gelatin or on gel) proliferate to form a cobblestone monolayer at confluence. When the cells reach confluency, as assessed by light microscopy, they are still capable of undergoing one or two population doublings before becoming quiescent. In late post-confluent cultures, sprouting cells appear underneath the cobblestone monolayer (Schor *et al.*, 1983). Previous studies have shown that endothelial cells grown on plastic tissue culture dishes synthesize more thrombospondin when they are rapidly proliferating than when they are confluent (Mumby *et al.*, 1984). In our studies we have investigated the effects of proliferation and substratum on thrombospondin gene expression. Accordingly, endothelial cells were plated on gelatin-coated dishes and radiolabelled with [^3H]proline when the cells were (a) rapidly proliferating (usually 1 day after plating, when the cells were 30–50 % confluent), (b) just confluent (day 6 or 7) and (c) 12 days post-confluent. Fig. 3 shows the [^3H]proline-labelled proteins secreted by these cells at different stages of growth. Thrombospondin is a major biosynthetic product of endothelial cells which are rapidly proliferating (Fig. 3, track 1), and is decreased when the cells have reached confluency (Fig. 3, track 2), confirming previously published data (Mumby *et al.*, 1984). However, the most marked effect is the down-regulation of thrombospondin secretion by endothelial cells which have been maintained in culture for 12–20 days post-confluency (Fig. 3, track 3). Fig. 3 (track 3) shows the proteins secreted by cells which were radiolabelled at 12 days post-confluency. At this stage, the cultures contain cells displaying both morphologies: there is a compact cobblestone monolayer with an extensive network of elongated sprouting cells underneath (results not shown). The results shown in Fig. 3 were confirmed using Northern blot hybridization as previously described. The mRNA levels for thrombospondin were highest when the cells were rapidly proliferating, were decreased at confluency and could

Fig. 3. Fluorograms of the [^3H]proline-labelled proteins secreted into the medium by endothelial cells at different stages of growth

Endothelial cells were plated on gelatin-coated dishes and incubated with [^3H]proline for 24 h when the cells were rapidly proliferating (day 1; track 1), just confluent (day 7; track 2), and very post-confluent (day 19; track 3). The proteins secreted into the medium were collected and analysed by SDS/PAGE and fluorography. The migration positions of thrombospondin (Tsp), fibronectin (Fn) and [^{14}C]methylated proteins of known M_r are indicated.

barely be detected after 23 days in culture (results not shown). It is noteworthy that the secretion of fibronectin by endothelial cells plated on gelatin-coated dishes appears to be regulated in the opposite manner to thrombospondin; that is, rapidly proliferating cells produced very little fibronectin whereas cells which have reached confluence or are post-confluent secrete substantial amounts of fibronectin (Fig. 3, compare tracks 1–3).

We next investigated the effect of proliferation on thrombospondin synthesis by endothelial cells plated on collagen gels. The results shown in Fig. 4 clearly demonstrate that the synthesis of thrombospondin by cells plated on three-dimensional collagen gels was not affected by the proliferative state of the cultures, and remained at relatively low levels if the cells were

Fig. 4. Comparison of the [^3H]proline-labelled proteins secreted by endothelial cells on gel at different stages of growth

Endothelial cells were plated on the surface of collagen gels and were incubated with [^3H]proline for 24 h when the cells were rapidly proliferating (day 1, track 1) or just confluent (day 6, track 2). The newly synthesized proteins secreted into the medium were analysed as described in the Experimental section. The migration positions of thrombospondin (Tsp) and [^{14}C]methylated proteins of known M_r are indicated.

labelled when they were sparse and rapidly proliferating or when they were confluent and quiescent (Fig. 4, tracks 1 and 2). It should be noted that the presence of a three-dimensional collagen gel is required for this effect, as cells plated on collagen-coated dishes behave in the same manner as cells on gelatinized dishes (results not shown). In contrast, fibronectin synthesis was regulated in the same way as when cells were grown on gelatin-coated dishes; that is, confluent endothelial cells secreted more fibronectin than did rapidly proliferating cells (Fig. 4). These results indicate that thrombospondin secretion is not merely associated with the proliferative state of the cells but is also dependent on the physical state of the extracellular matrix in contact with the cells.

DISCUSSION

In this paper we present a detailed investigation of the effects of cell proliferation, cell shape and substratum on thrombospondin gene expression by bovine aortic endothelial cells. In the first series of experiments we investigated the effects of three types of substrata on the synthesis of thrombospondin by confluent endothelial cells. The three substrata consisted of type I collagen, either heat-denatured (gelatin) or native. In the latter case the collagen was either coated on to plastic dishes or made into three-dimensional gels. In these experiments the cells were morphologically identical, i.e. on all substrata tested they displayed a compact, contact-inhibited cobblestone monolayer at confluence. We demonstrated that thrombospondin was a major biosynthetic product of confluent, quiescent endothelial cells cultured on gelatin-coated dishes or on dishes coated with native type I collagen, and that the production of this protein was markedly decreased when the same cells were plated on the surface of a three-dimensional gel of native type I collagen (Fig. 1). We also found that this regulation occurred at the level of transcription, as steady-state mRNA levels for thrombospondin decreased in a manner comparable with the levels of protein secreted by these cells (Fig. 2). These results therefore demonstrated that the nature of the substratum on which endothelial cells were cultured, and moreover the physical state of this substratum, had a dramatic influence on thrombospondin gene expression by these cells. Mumby *et al.* (1984) have demonstrated that the synthesis of thrombospondin was directly proportional to the proliferative state of cells, and therefore suggested that this protein played a role in tissue growth and reorganization *in vivo*. In their study, they compared the biosynthesis of thrombospondin by subconfluent, proliferating cells with that by confluent, quiescent cells cultured on plastic tissue culture dishes. Our studies confirm and extend their findings and suggest an alternative explanation for the results. We show that the synthesis of thrombospondin by cells cultured on gelatin-coated and collagen-coated dishes is higher in proliferating compared with confluent cultures (Fig. 3). We also demonstrated that this regulation occurred at the level of transcription. However, when the same cells were plated on the surface of a three-dimensional collagen gel, the synthesis of thrombospondin by these cells was the same whether they were confluent or rapidly proliferating (Fig. 4). By comparison, fibronectin synthesis was found to be directly proportional to the states of confluence of the cells, whether they were maintained on gelatin or on collagen gels (Figs. 3 and 4). This confirms a recent report showing increasing immunostaining of endothelial cell matrices for fibronectin with increasing cell densities (Dreyfus & Lahav, 1988). Our results therefore support the proposition that the synthesis of thrombospondin is related to cell proliferation, but that this relationship may be dependent upon the nature of the matrix in contact with the cells.

Interestingly, the levels of thrombospondin gene expression by endothelial cells were also found to be dependent upon cell shape. Thus cells plated on the surface of a collagen gel formed a cobblestone monolayer at confluence, whereas cells plated within the gel matrix elongated into a sprouting cell morphology and self-associated into three-dimensional networks (Schor *et al.*, 1983; Schor & Schor, 1986). This process occurs in the absence of cell proliferation (Schor *et al.*, 1983; Schor & Schor, 1986). During the course of angiogenesis, endothelial cells undergo morphological changes similar to those we have observed in tissue culture. The cells adopt an elongated fibroblastoid phenotype and migrate through the surrounding connective tissue stroma forming cords and sprouts, eventually leading to the formation of a patent tubule (Folkman, 1984). Our studies demonstrated that sprouting cells in gel produced significantly less thrombospondin than did quiescent, cobblestone cells on the surface of collagen gels, and this modulation was reversible (Figs. 1 and 2). The ability of cells to rapidly modulate thrombospondin gene expression in response to cell morphology, growth and substratum (this study, Mumby *et al.*, 1984), heat shock (Ketis *et al.*, 1988) and growth factors (Majack *et al.*, 1986) suggests that this protein plays a key role in the control of various physiological and pathological processes *in vivo*, such as wound healing and angiogenesis.

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