The response of endothelial cells to TGFβ-1 is dependent upon cell shape, proliferative state and the nature of the substratum

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Summary

Endothelial cells plated on two-dimensional (2-D) substrata proliferate until they form a tightly apposed confluent monolayer of quiescent cells that display a typical 'cobblestone' morphology. When added to proliferating cultures TGFβ-1 (transforming growth factor β-1) inhibited cell growth and caused marked morphological changes, with the cells becoming enlarged and ragged. These effects were dose-dependent and reversible. TGFβ-1 also reduced the cloning efficiency and colony size of these cells, indicating that TGFβ-1 is cytotoxic and cytostatic for endothelial cells. By contrast, TGFβ-1 added to quiescent cobblestone cultures did not affect cell morphology or cell numbers. In the presence of 20% serum, the level of total protein synthesis per cell was significantly increased by TGFβ-1 in a dose-dependent manner when the cells were cultured on a 2-D substratum, regardless of whether the cells were proliferating or quiescent. Cobblestone morphology was retained in the presence of serum, and a similar change in protein synthesis was not observed when the cells were cultured on a 3-D collagen gel. These data suggest that the effects of TGFβ-1 upon endothelial cells depend on the shape (cobblestone or sprouting), the proliferative state of the cells, and on the nature of the matrix surrounding the cells. The response of these cells to TGFβ-1 in vivo may be similarly modulated during angiogenesis by changes in the cell phenotype and the composition of the surrounding matrix.

Key words: endothelial cells, TGFβ-1, angiogenesis.

Introduction

TGFβ-1 (transforming growth factor β1) is a homodimeric polypeptide of M, 25,000 that belongs to a large family of homologous proteins. This family includes the TGFβs 1-5 as well as a number of related peptides (approximately 30 % homologous) such as the bone morphogenetic proteins 2-7, activins, inhibins, Vg-1, Mullerian inhibiting substance and the decapentaplegic protein from Drosophila (for reviews see Massague, 1987; Barnard et al., 1987). TGFβ is capable of releasing additional growth factors such as tumour necrosis factor alpha (TNFα), itself a potent inducer of angiogenesis (Leibovich et al., 1987). The mechanism by which TGFβ induces angiogenesis remains obscure, as it has been reported to inhibit endothelial cell proliferation (Baird and Durkin, 1986; Frater-Schroder et al., 1986) and migration (Heinlein et al., 1986; Muller et al., 1987) in vitro. This anomaly between the in vivo and in vitro response may be due to a variety of factors, such as:

1 TGFβ may stimulate angiogenesis via a primary effect on a 'peri-endothelial' cell population rather than acting directly on the endothelial cells. For example, Wahl et al. (1987) and Wiseman et al. (1988) have reported that TGFβ is chemotactic for macrophages, which, once attracted to a site of inflammation/wound healing, are capable of releasing additional growth factors such as tumour necrosis factor alpha (TNFα), itself a potent inducer of angiogenesis (Leibovich et al., 1987).

2 The effect of TGFβ upon endothelial cells may be
modulated by environmental factors, such as the nature of the extracellular matrix (ECM). We have previously shown that the low Mr angiogenic factor ESAF stimulates endothelial cell proliferation when the cells are plated on a collagen substratum but not on plastic tissue culture dishes (Schor et al. 1980).

(3) The effects of TGFβ upon endothelial cells may depend on the phenotype of these cells. Endothelial cells in vitro may display two different phenotypes; on the surface of two-dimensional (2-D) substrata these cells form a cobblestone monolayer, whereas the same cells embedded within a three-dimensional (3-D) collagen gel adopt an elongated sprouting morphology. Madri and collaborators (Madri et al. 1988; Merwin et al. 1990) have reported that TGFβ-1 inhibited endothelial cell proliferation on 2-D substrata but stimulated tubule formation and gel contraction by endothelial cells within a 3-D collagen gel.

In order to clarify the role of TGFβ during angiogenesis we have investigated the last two possibilities. Thus we have studied the effects of TGFβ-1 upon large vessel (aortic) and microvascular (retinal) bovine endothelial cells, on different substrata, displaying different phenotypes (cobblestone or sprouting) and at different stages of proliferation and confluence. Our results indicate that the endothelial cell response to TGFβ-1 is dependent upon cell shape, proliferative state and the nature of the substratum.

Materials and methods

Materials

Eagle's minimum essential medium, donor calf serum, sodium pyruvate, glutamine, non-essential amino acids and antibiotics were obtained from Gibco Bio-cult, Paisley, Scotland, UK. Ascorbic acid was obtained from BDH Chemicals, Poole, Dorset, UK, and guanidinium chloride was obtained from Fluka Chemicals, Glossec, Derbyshire, UK. Thrombin and urokinase were purchased from Leo Laboratories, Aylesbury, Bucks, UK. Fibrinogen (grade L) was obtained from Kabivitrum, Stockholm, Sweden, and gel bond was obtained from ICN Biomedicals, High Wycombe, Bucks, UK.

L-[5-3H]proline, L-[4,5-2H]thymidine and [methyl-3H]thymidine were purchased from New England Nuclear, DuPont (UK) Ltd., Stevenage, Herts, UK. [14C]Methylated standard protein mixture (Mr 14,300-200,000) was purchased from Amersham International, Amersham, Bucks, UK. Six different batches of human TGFβ-1 (purified from platelets) were used in the course of the experiments reported here. These were obtained from British Biotechnology Ltd., Cowley, Oxford, UK.

Cells and tissue culture conditions

Bovine retinal endothelial cells (BREC) and bovine aortic endothelial cells (BAEC) were isolated as previously described (Schor et al. 1983, Schor and Schor, 1986). Cells were identified as endothelial on the basis of their morphology and positive staining for Factor VIII-related antigen. Stock cultures were routinely maintained on gelatin-coated dishes in Eagle's minimum essential medium supplemented with 20 % donor calf serum, 50 μg ml⁻¹ ascorbic acid, 2 mm glutamine, 1 mm sodium pyruvate and non-essential amino acids. This growth medium will be referred to as 20 % DCS-MEM. Cultures were incubated at 37 °C in a humidified atmosphere consisting of 5 % CO₂, 95 % air.

For the various experimental investigations, cells were plated: (1) onto gelatin-coated dishes, (2) onto the surface of 3-D collagen gels (i.e. 'on gel'), (3) homogeneously within a 3-D collagen gel matrix (i.e. 'in gel') or (4) onto the surface of a 3-D collagen gel, grown to confluence and then embedded within a second, larger collagen gel (i.e. 'embedded monolayer') (Schor and Schor, 1986). Gelatin-coated dishes were prepared by incubating plastic tissue culture dishes with a gelatin solution (0.1 %, v/v, in distilled water) for 1 h at 37 °C, removing the gelatin solution and washing the dishes twice with Hank's balanced salt solution (HBSS). Type I collagen was extracted from rat-tail tendons with 0.5 M acetic acid, dialysed against distilled water and used to prepare an aqueous stock containing 2.0 mg ml⁻¹ collagen. The 3-D collagen gels were prepared as previously described (Schor et al. 1983). Cells within a 3-D collagen matrix were obtained by mixing the cells with the collagen gelling solution before casting (Schor et al. 1983).

Cloning efficiency

The effects of TGFβ-1 on cloning efficiency were examined using two different protocols: (1) Cells were plated at 25 cells cm⁻² on gelatinised dishes and TGFβ-1 was either added at the time of plating or 24 h later in order to allow the cells time to attach; the same results were obtained in both cases. (2) Proliferating cultures were incubated with 20 % DCS-MEM containing TGFβ. Parallel control cultures were maintained in either 20 % or 5 % DCS-MEM. Five days later the cells were trypsinised, their viability was assessed by Trypan Blue exclusion, and they were plated at 25 and 50 viable cells cm⁻² as in (1).

In both protocols, the cultures were fixed with formaldehyde (2 %, v/v) or sucrose (5 %, w/v) 11 days after plating and then stained with crystal violet (1 %, w/v) for 5 min. The number of colonies/dish were counted and expressed as a percentage of the number of cells plated. The number of cells per colony were also determined. In 4 experiments both 8 cm² and 20 cm² dishes were used.

Cell numbers and total thymidine incorporation

The number of cells present in cultures on gelatinised dishes, on the surface of 3-D collagen gels and within these gels were determined as previously described (Schor, 1980). In all experiments duplicate cultures were counted with a Coulter counter. Incorporation of thymidine was determined by incubating the cultures with [3H]thymidine (1 μCi ml⁻¹) for 1 h followed by 4 washes with ice-cold trichloroacetic acid (TCA). Cells cultured on gelatinised dishes were washed in situ, whilst cells on or within collagen gels were recovered by collagenase digestion of the gel prior to washing. After the final wash the cells were solubilised with 1 M NaOH, neutralised with an equal volume of 1 M HCl and the radioactivity was determined by scintillation counting. In all experiments duplicate cultures were analysed.

Labelling index

Endothelial cells were grown in the presence or absence of TGFβ-1 and the labelling index determined with a cell proliferation kit (Amersham International, Amersham) according to the manufacturer's instructions. The method is based on the incorporation of bromodeoxyuridine (BrdUrd) into DNA followed by its subsequent detection with a mouse monoclonal antibody against BrdUrd, a secondary anti-mouse peroxidase-linked antibody and diaminobenzidine as a substrate. The cells were counter-stained with haematoxylin prior to counting.

Labelling of cell cultures and isolation of newly synthesised proteins

Cells on gelatin-coated dishes were incubated with [3H]thymidine (20 μCi ml⁻¹) for 18-24 h in the absence or presence of TGFβ-1. After this period the medium was collected, and the cell layer matrix was washed twice with HBSS. Proteins present in the cell layer/matrix were extracted with 4 M guanidinium chloride/50 mM Tris-HCl, pH 7.4, for 24 h at 4 °C. Guanidinium chloride-insoluble material was removed by centrifugation (15,000 g for 20 min) and the supernatant dialysed extensively against 0.5 M acetic acid at 4 °C. The newly synthesised proteins from cells cultured on or in 3-D collagen gels were collected and analysed in the same way described for 2-D cultures, with the following differences. Each wash of the collagen gel with HBBS was followed by centrifugation (15,000 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 washes were combined. Second, the guanidinium chloride extraction of the cell
layer/matrix was carried out over 48 h at 4°C with gentle stirring. Medium and cell layer/matrix from all samples were stored at -20°C until analysed.

Total incorporation of radioactivity into newly synthesised medium proteins was determined by scintillation counting of TCA-precipitated material. Briefly, 50 μl samples of the medium were incubated with 50 μl of 20% TCA for 30 min at 4°C. The precipitated proteins were recovered by centrifugation (15,000 g for 2 min) and the pellet washed twice with ice-cold acetone. The washed pellets were air dried and then solubilised with 1 M NaOH at 90°C for 2 min. The NaOH was neutralised with an equal volume of 1 M HCl and the radioactivity determined by scintillation counting. The radioactivity incorporated into the cell layer/matrix was determined directly by scintillation counting of 50 μl samples of the guanidinium chloride-extracted material. The guanidinium chloride-insoluble residue was solubilised with 1 M NaOH, neutralised and the radioactivity determined by scintillation counting.

Electrophoretic analysis
Newly synthesised proteins secreted into the medium and deposited into the cell layer/matrix were examined by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) using a water-cooled discontinuous slab gel system. The samples were run under reducing conditions (i.e. in the presence of 2-mercaptoethanol (5%, v/v)), on 6.5% polyacrylamide gels and an equal number of counts was loaded onto each track to permit comparison of the relative levels of specific proteins. The newly synthesised proteins were detected by fluorography (Bonner and Laskey, 1974; Laskey and Mills, 1975). The (14C)methylated M, standards were myosin (M, 200,000), phosphorylase b (a doublet of M, 100,000 and 92,500), bovine serum albumin (M, 69,000), ovalbumin (M, 46,000), carbonic anhydrase (M, 30,000) and lysozyme (M, 14,300).

PAI-1 characterisation
Reverse fibrin zymography. Culture medium samples were analysed for PAI-1 activity by reverse fibrin zymography, as originally described by Granelli-Piperno and Reich (1978) and modified by Booth et al. (1989). Samples were initially diluted in Tris/Tween buffer and then incubated in an equal volume of sample buffer (SDS (4%, v/v), 8 M urea, 40 mM iodacetate, 0.2 mM Tris–HCl, pH 8.0) for 30 min at 37°C. Samples were made 10% (v/v) with respect to glycerol and analysed by SDS–polyacrylamide gel electrophoresis, using 3% and 10% acrylamide stacking and resolving gels, respectively. The gels were washed with 90% in 2×500 ml aqueous Triton X-100 (2.5%, w/v), rinsed in distilled water and then applied to a detector gel. The detector gel contained fibrinogen (2 mg ml⁻¹), plasminogen (20–30 pg ml⁻¹, present as a contaminant of the fibrinogen), thrombin (2–30 pg ml⁻¹), urokinase (0.041 u ml⁻¹), 17.5 mM NaCl, 60 mM Tris–HCl, pH 6.8, and agarose (0.8%, w/v). After 16–20 h at 37°C the polyacrylamide gel was removed and the detector gel photographed.

Immunoprecipitation. The presence of PAI-1 in the medium was confirmed by immunoprecipitation with specific antibodies raised against bovine endothelial cell PAI-1 (generously provided by Dr D. J. Laskutoff, Scripps Clinical Research Foundation, La Jolla, CA, USA), as described by Canfield et al. (1989).

Experimental procedures
The experiments described in this paper were carried out with 3 batches of BAEC and 2 batches of BREC. In all cases experiments were performed a minimum of 3 times. The same results were obtained with both types of endothelial cells unless otherwise stated.

On 2-D substrata endothelial cells were plated at 1.5×10⁴ cells/cm² well in 20% DCS-MEM, and cultured for up to 20 days, changing the medium every 2–3 days during this period. TGFβ-1 (0.05–10 ng ml⁻¹) was added at different stages: namely, when the confluent cultures (point i in A), just confluent (point ii in A) and up to 7 days post-confluent (quiescent). The medium was subsequently replaced 3 days later, and the experiment was finished 5 days after the initial addition of TGFβ-1.

The effect of TGFβ-1 on endothelial cells within a 3-D environment was examined using 3 different protocols. (1) The cells were homogeneously dispersed within a type I collagen matrix. This was achieved by mixing 1×10⁵ cells with 0.5 ml of the collagen gelling solution before casting (Schor, 1980; Schor and Schor, 1986) into 2 cm² tissue culture wells. The cells were allowed to adopt an elongated 'sprouting' morphology over 2–3 days before being incubated with TGFβ-1 for a further 5 to 10 days. (2) Endothelial cells were grown to confluence on the surface of a 3-D collagen gel, which was then embedded within a second, larger collagen gel (Schor and Schor, 1988). The embedded monolayer was subsequently incubated in the absence or presence of TGFβ-1 for up to 15 days. (3) Endothelial cells were grown to post-confluence on 2-D substrata. Sprouting cells spontaneously appear under the monolayer and are embedded within the ECM of such cultures (Schor et al. 1983; Schor et al. 1984).

Results
Cell growth and cloning efficiency
We have previously reported (Schor et al. 1983) that endothelial cells plated on 2-D substrata (i.e. gelatine-coated tissue culture dishes or the surface of collagen gels) proliferate to form a cobblestone monolayer at confluence. These cultures appear confluent under the light microscope when they reach a density of 1×10⁶ cells/cm² dish. After confluence the cells continue to divide until a saturation density of approximately 2×10⁶ to 3×10⁶ cells/dish is obtained (Fig. 1A). The effect of TGFβ-1 on cell proliferation was examined by incubating cells at various stages of confluence (indicated by arrows in...
Fig. 1A) with the growth factor. Cells were sub-confluent (proliferating) at point i, just-confluent (still-proliferating) at point ii, and post-confluent (quiescent) at point iii. In one representative experiment measurement of (3H)thymidine incorporation at points i, ii and iii showed levels of 37 000, 16 500 and 3000 cts min\(^{-1}\) h\(^{-1}\) per 10\(^6\) cells, respectively. At each stage cells were incubated with TGF/β-1 (0.05–5.0 ng ml\(^{-1}\)), and cell numbers and (3H)thymidine incorporation determined in duplicate dishes after a period of 5 days. The addition of TGF/β-1 to sub-confluent cells (point i in Fig. 1A) resulted in a dose-dependent inhibition of proliferation (Fig. 1B) that was paralleled by a 7-fold reduction in (3H)thymidine incorporation when TGF/β-1 was added at 1 ng ml\(^{-1}\). This response was the same irrespective of whether the cells were grown on the surface of gelatinised dishes or on 3-D collagen gels. Furthermore, in sub-confluent cultures a labelling index (0.05-5.0 ng ml\(^{-1}\)) and cell numbers and (3H)thymidine incorporation in this case was slightly increased in the presence of TGF/β-1 at 1 ng ml\(^{-1}\), indicating that the control cultures had become contact-inhibited. At higher doses of TGF/β-1 (2.5 ng ml\(^{-1}\)) the final cell numbers were lower than those before the addition of TGF/β-1, indicating that this factor is cytotoxic as well as cytostatic for endothelial cells. The addition of TGF/β-1 to post-confluent cells (point iii in Fig. 1A) had no effect on cell numbers (Fig. 1D) or (3H)thymidine incorporation.

In order to determine whether the lack of effect of TGF/β-1 on the number of cells in post-confluent cultures was because the cells were quiescent and/or contact inhibited, endothelial cells were plated within 3-D collagen gels. We have previously reported that under these conditions the endothelial cells are quiescent but not contact-inhibited. The cells initially elongate over a 2- to 3-day period and assume a sprouting phenotype; an extensive network of sprouting cells and tubule-like structures is produced within the collagen gel over the subsequent days in culture. These morphogenetic events occur by cell migration in the absence of proliferation (Schor et al. 1983; Schor and Schor, 1986). The addition of TGF/β-1 to cells within a 3-D collagen gel (for 5 days) after the initial period of elongation resulted in a small reduction in the number of cells present, compared to control cultures. However, when the culture time was extended for up to 10 days, the number of cells in control cultures remained constant, whereas cell numbers in the presence of TGF/β-1 were reduced to less than 25 % of control levels (results not shown). This effect on cell numbers was clearly confirmed by microscopic observation (Fig. 6, see below). These results indicate that quiescent cells are still responsive to TGF/β-1 and that this factor may be toxic for sprouting endothelial cells. It should be noted that the same batches of TGF/β-1 were not toxic for other cells such as fibroblasts (not shown) or for the same endothelial cells under different conditions (e.g. post-confluent cultures on 2-D substrata; Figs 1D and 4F).

There are contradictory reports in the literature regarding the reversibility of the effects of TGF/β-1 on endothelial cell proliferation (Frater-Schroder et al. 1986; Takehara et al. 1987). In order to investigate this point, sub-confluent cultures of endothelial cells were incubated with 1 ng ml\(^{-1}\) and 2.5 ng ml\(^{-1}\) TGF/β-1 for a period of 5 days; the cultures were then washed extensively and incubated for up to 15 days in the absence of TGF/β-1. These cultures proliferated and achieved final densities approaching those of control cultures, suggesting that the effect of TGF/β-1 on proliferation is reversible (Fig. 2). Cells that were incubated continuously with TGF/β-1 at 2.5 ng ml\(^{-1}\) failed to proliferate. Indeed, the number of cells present at the end of the experiment was less than when TGF/β-1 was added, again indicating that TGF/β-1 is cytotoxic to endothelial cells.

The cloning efficiency of endothelial cells in the presence of TGF/β-1 was examined using two different protocols. In the first, the cells were plated at a density of 25 cells cm\(^{-2}\) on gelatinised dishes and incubated in 20% DCS-MEM. The final density in the TGF/β-1-treated cultures on 2-D substrata; Figs 1D and 4F)

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cultures fell between that of the two types of control cultures. In all cultures the cell viability, as assessed by Trypan Blue exclusion, was similar (i.e. approximately 95%); however, the cloning efficiency and colony size of cultures pre-treated with TGFβ-1 were markedly reduced compared to both 5% and 20% DCS-MEM control cultures (see Table 1). In the experiment shown (Table 1) cultures treated with TGFβ-1 at 1 and 2.5 ng/ml reached the same cell density; subsequent cloning efficiency was, however, reduced to a greater extent in cultures pre-treated with 2.5 ng/ml. These results indicate a cytotoxic effect of TGFβ-1, independent of the cell density of the cultures used for the clonogenic assay.

**Morphology and morphogenesis**

Marked morphological changes were induced in BRECs and BAEC cultures by TGFβ-1. On 2-D substrata the addition of TGFβ-1 to sub-confluent cultures produced enlarged and ragged cells that failed to form a typical cobblestone monolayer (compare Fig. 4A and B). Similarly, TGFβ-1 added to just-confluent (still-proliferating) cultures resulted in disruption of the monolayer. A number of cells became detached from the substratum whilst the remaining adherent cells drew away from each other and became enlarged and ragged (compare Fig. 4C and D). These changes were discernible after two to three days in the presence of TGFβ-1. In contrast post-confluent (quiescent) cultures showed no morphological changes in the presence of TGFβ-1 (compare Fig. 4E and F).

We have previously reported that endothelial cells in vitro display a sprouting phenotype under the following conditions. (1) In post-confluent cultures on 2-D substrata a network of sprouting cells forms spontaneously under the surface of the original cobblestone monolayer. These sprouting cells are embedded within the sub-endothelial matrix (Schor et al. 1984), (Fig. 4G). (2) A cobblestone monolayer on the surface of a collagen gel may be embedded within a larger gel. Under these conditions the cells migrate out of the monolayer as elongated sprouting cells and associate within the new gel, forming loops and cords (Schor and Schor, 1986), (Fig. 5A). (3) When cells are plated directly within a collagen gel they elongate and associate into networks of sprouting cells (Schor et al. 1983; Schor and Schor, 1986), (Fig. 6A and C).

It should be noted that under the first two conditions both sprouting and cobblestone cells are present in the same cultures whereas only a homogeneous population of sprouting cells is present in the third system. Despite the apparent morphological similarity between the sprouting cells produced under these three conditions, they responded differently to TGFβ-1. In post-confluent cultures TGFβ-1 (at 1, 2.5, 5 and 10 ng/ml) did not prevent sprouting cells appearing under the monolayer, and it had no apparent effect on the morphology of the sprouting cells that were already present at the time of addition (compare Fig. 4E and F). In contrast, in the embedded monolayer system TGFβ-1 (1, 2.5 ng/ml) markedly reduced the number of cells migrating out of the monolayer, and decreased the average distance the cells migrated into the new gel (compare Fig. 5A and B). Similarly, cells embedded directly within a collagen gel,

**Table 1. Effect of TGFβ-1 pre-treatment on endothelial cell cloning efficiency and colony size**

<table>
<thead>
<tr>
<th>Pre-incubation</th>
<th>Final density (no. of cells/culture, x10^6)</th>
<th>Number of colonies (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&gt;11 cells/colony</td>
</tr>
<tr>
<td>20% DCS-MEM (control)</td>
<td>13.20</td>
<td>520 (100)</td>
</tr>
<tr>
<td>5% DCS-MEM (control)</td>
<td>3.52</td>
<td>349 (67)</td>
</tr>
<tr>
<td>20% DCS-MEM +TGFβ-1 (1.0 ng/ml^-1)</td>
<td>7.98</td>
<td>204 (39)</td>
</tr>
<tr>
<td>20% DCS-MEM +TGFβ-1 (2.5 ng/ml^-1)</td>
<td>7.80</td>
<td>230 (44)</td>
</tr>
</tbody>
</table>

BAEC were grown in 20% DCS-MEM on gelatin-coated dishes until they were 30% confluent, and then incubated in the presence of TGFβ-1 (1 and 2.0 ng/ml^-1) for 6 days. Control cultures were maintained for this period in either 20% or 5% DCS-MEM in order to determine whether a reduction in the final culture density affected the subsequent cloning efficiency. Cultures were then washed, trypsinised, their viability assessed by Trypan Blue exclusion, and plated at 50 viable cells/cm^2 on 20 cm^2 gelatin-coated dishes in 20% DCS-MEM. After 11 days in culture the total number of colonies (minimum size of 11 cells/colony) and the size of each colony were determined in duplicate dishes. Results are expressed as number of colonies/dish and as a percentage of the control cultures in 20% DCS MEM. Standard deviation was less than 5% for all determinations.
Fig. 4. The effects of TGFβ-1 on endothelial cell morphology on 2-D substrata: dependence on cell confluence. BAEC at different stages of confluence were incubated with TGFβ-1 (1 ng ml⁻¹) and photographs were taken 5 days later. Cells that were sub-confluent at the time of TGFβ-1 addition formed a regular cobblestone monolayer in control cultures (A) and became enlarged and ragged in the presence of TGFβ-1 (B). Cells that were just-confluent at the time of TGFβ-1 addition maintained a regular monolayer in control cultures (C), whereas in the presence of TGFβ-1 some cells were seen floating in the culture medium and the cells that remained attached drew away from each other and became large and ragged (D). Addition of TGFβ-1 to post-confluent cultures did not change the morphology of the cells. Control cultures (E) and TGFβ-1-treated cultures (F) remained as tight contact-inhibited cobblestone monolayers. The presence of sprouting cells in these cultures was not affected by TGFβ-1 (E and F). Bar, 150 μm.

rounded up over a period of 5 days in the presence of TGFβ-1 (1, 2.5 ng ml⁻¹) (compare Fig. 6A and B). With extended time in culture (i.e. up to 10 days) cell death occurred and very few cells were visible in the presence of TGFβ-1 (compare Fig. 6C and D).

The lack of effect of TGFβ-1 on sprouting cells appearing under a cobblestone monolayer (Fig. 4E and F) was observed irrespective of whether the cells were cultured on gelatin-coated dishes or on the surface of collagen gels. In the latter case the sprouting cells initially migrate into the subendothelial ECM and later continue to migrate into the underlying type I collagen matrix (Schor et al. 1983, Schor et al. 1984); TGFβ-1 concentrations of up to 10 ng ml⁻¹ did not affect the morphological appearance of the monolayer or the sprouting cells immediately under it. However, TGFβ-1 reduced the number of sprouting cells that had migrated down into the type I collagen gel. It should be noted that these post-confluent cultures responded to TGFβ-1 by increasing protein synthesis, in the absence of an effect on the monolayer morphology (see below, and Table 2).

The possibility that TGFβ-1 may be sequestered by the subendothelial ECM and therefore may not be available to the sprouting cells embedded in it was tested as follows: gelatin-coated dishes, type I collagen gels, cell-free subendothelial ECMs (Schor et al. 1984) and post-
confluent cultures were incubated with TGFβ-1 (1–10 ng ml⁻¹) in 20% DCS MEM for 3 days. At this point the medium was collected and diluted (assuming that no loss of TGFβ-1 had occurred) to final concentrations of 0.5, 1.0 and 2.5 ng ml⁻¹ TGFβ-1 in 50% fresh medium. Medium from cultures without TGFβ-1 was also diluted in a similar manner and used as controls. These various media were then tested for the presence of TGFβ-1 by their effects on sub-confluent endothelial cultures. The solutions of TGFβ-1 that had been incubated on gelatin-coated dishes, collagen gels, endothelial ECMs and post-confluent endothelial cell cultures showed reduced activity compared with freshly made TGFβ-1; however, they retained sufficient activity (at 1 ng ml⁻¹ or higher) to affect the morphology and cell numbers of proliferating endothelial cell cultures.

Response of endothelial cells to TGFβ-1
Modulation of matrix biosynthesis by TGFβ-1

We have examined the effects of TGFβ-1 on protein synthesis by endothelial cells cultured on 2-D substrata or within 3-D collagen gels. TGFβ-1 was present for a total of 5 days, and [3H]proline was added for the last 18-24 h of this period. The newly synthesised proteins in the medium and cell layer/matrix were collected and analysed as described in Materials and methods. Table 2 clearly shows that TGFβ-1 induced a dose-dependent increase in the incorporation into newly synthesised proteins was determined, as described in Methods and methods. The results are from a typical experiment and are expressed as total incorporation into newly synthesised proteins per 10^6 cells. The same cells were plated within 3-D collagen gels and allowed to adopt a proliferative state. The newly synthesised proteins in the medium and cell layer/matrix were collected and analysed as described in Materials and methods. The results are from a typical experiment and represent the mean of duplicate cultures with standard deviations of less than 5%. The data clearly show that TGFβ-1 increased protein synthesis by endothelial cells on collagen-coated dishes in a dose-dependent manner, irrespective of the proliferative state of the cultures. By contrast, TGFβ-1 had no effect on the amount of protein synthesis by endothelial cells within 3-D collagen gels.

Table 2. Effect of TGFβ-1 on total protein synthesis by endothelial cells

<table>
<thead>
<tr>
<th>Culture</th>
<th>Substratum</th>
<th>TGFβ-1 (ng/ml⁻¹)</th>
<th>Cts/min⁻¹ incorporated/10⁶ cells</th>
<th>% of control</th>
</tr>
</thead>
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BREC were grown in 20% DCS MEM on gelatin-coated dishes until they were sub-confluent, just-confluent or post-confluent and TGFβ-1 (0.05-5.0ng/ml⁻¹) was added at each stage for a period of 5 days. The same cells were plated within 3-D collagen gels and allowed to adopt a sprouting morphology. TGFβ-1 (1 and 2.5ng/ml⁻¹) was then added for 5 days.

In all cases the cultures were incubated with [3H]proline (20μCi/ml) during the last 24 h of this 5 day period and the amount of radioactivity incorporated into newly synthesised proteins was determined, as described in Materials and methods. The results are from a typical experiment and are expressed as total incorporation into newly synthesised proteins per 10^6 cells. The same cells were plated within 3-D collagen gels and allowed to adopt a proliferative state. The newly synthesised proteins in the medium and cell layer/matrix were collected and analysed as described in Materials and methods. The results are from a typical experiment and represent the mean of duplicate cultures with standard deviations of less than 5%. The data clearly show that TGFβ-1 increased protein synthesis by endothelial cells on collagen-coated dishes in a dose-dependent manner, irrespective of the proliferative state of the cultures. By contrast, TGFβ-1 had no effect on the amount of protein synthesis by endothelial cells within 3-D collagen gels.

Discussion

In this paper, we present a detailed study of the effects of TGFβ-1 on endothelial cells under various tissue culture conditions. These conditions were selected in order to test the possible modulating effects of cell phenotype and environmental factors. We show that the response of endothelial cells to TGFβ-1 is dependent upon: (1) their proliferative state (proliferating or quiescent), (2) their shape (cobblestone or sprouting), (3) the nature of the matrix surrounding the cells (type I collagen or endothelial-cell produced ECM) and (4) the source of the cells (microvascular or large vessel). Previous studies have shown that TGFβ-1 inhibits the proliferation of endothelial cells in sub-confluent cultures (Baird and Durkin, 1986) and disrupts the endothelial monolayer when added to confluent cultures (Frater-Schroder et al. 1986). Our results confirm the inhibitory effect of TGFβ-1 on the growth of endothelial cells plated on 2-D surfaces (including the surface of a 3-D type I collagen gel). We show, however, that disruption of an endothelial monolayer only occurs when the cells are confluent but still proliferating; addition of TGFβ-1 to a confluent (quiescent) monolayer has no effect on the number of cells or their morphology. Nevertheless these cells remain responsive to TGFβ-1 regarding protein synthesis (see later). The inhibition of cell proliferation was paralleled by an inhibition of total [3H]thymidine incorporation and a reduction in the labelling index. These data were further supported by our results, which showed a dose-dependent reduction in endothelial cell cloning efficiency and mean colony size by TGFβ-1 (Fig. 3, Table 1). The reduction in cloning efficiency, together with the reduction in cell numbers seen with higher concentrations of TGFβ-1 and when cells were plated in gel clearly shows that TGFβ-1 is both cytostatic and cytotoxic for endothelial cells.
Contradictory reports have been published regarding the reversibility of TGF/β's effects in vitro (Frater-Schroder et al. 1986; Takehara et al. 1987). We found that the inhibition of endothelial cell proliferation was associated with marked morphological changes and both were reversible in sub-confluent cultures (Fig. 2). The different responses of proliferating and quiescent cells were further investigated using the latter under different tissue culture conditions. Thus, quiescent cultures were obtained by either growing endothelial cells to post-confluence on 2-D substrata (cobblestone phenotype) or by plating the cells directly within 3-D collagen gels (sprouting phenotype).

As previously mentioned TGF/β-1 added to quiescent cells on 2-D substrata (cobblestone) had no effect on cell numbers or morphology (Figs 1 and 4), suggesting that under these conditions the response to TGF/β-1 is dependent upon the proliferative state of the cells. By contrast, quiescent cells plated within a 3-D collagen gel (sprouting) showed dramatic morphological changes (Fig. 6) and a decrease in cell numbers in response to TGF/β-1, indicating that the effects of this growth factor are not merely modulated by the proliferative state of the cells but also by the cell shape and/or the matrix in contact with the cells. It is believed that the effects of TGF/β-1 occur through interaction of the growth factor with cell surface receptors of which three types have been described, and designated type I, type II and type III (Cheifetz et al. 1987). Muller et al. (1987) have demonstrated that the presence of these receptor types in endothelial cells depends on the state of confluence of the cells; type III receptors are found on proliferating cells, and receptors of types I and II on quiescent cells. In addition, Newman et al. (1989) have shown that the number of TGF/β receptors decreases as cells approach confluence. It is possible therefore that the differential effects of TGF/β-1 on proliferating and quiescent cells seen in this study may be due to different numbers and/or types of receptors being present, resulting in different signals being conveyed within the cell (Cheifetz et al. 1986). To our knowledge there are no data concerning the types of TGF/β receptors expressed on sprouting endothelial cells.

To investigate further whether the different responses of quiescent cobblestone and sprouting cultures were due to the shape of the cells, we examined the effects of TGF/β-1 upon sprouting endothelial cells using two additional systems. In one of these, sprouting cells were allowed to form under a post-confluent monolayer on 2-D substrata. These sprouting cells are embedded within the ECM deposited by the endothelial cells themselves (Schor et al. 1983; Schor et al. 1984). Addition of TGF/β-1 to these cultures had no apparent effect upon the morphology or numbers of sprouting cells present (Fig. 4E and F). In the second system, a confluent cobblestone monolayer was embedded within a type I collagen matrix. Under these conditions TGF/β-1 inhibited both the elongation and the migration of the sprouting cells (Fig. 5), suggesting that the nature of the matrix surrounding the sprouting cells is important in determining the response to TGF/β-1. The lack of response to TGF/β-1 by sprouting and cobblestone cells in post-confluent cultures (in terms of morphology and cell number) is not likely to result from lack of accessibility of the growth factor, since: (1) the same cultures are responsive in terms of protein synthesis (Table 2); (2) there is a reduction in the number of sprouting cells migrating through the ECM into the underlying collagen gel; (3) TGF/β-1 pre-incubated on these cultures remains active when tested on proliferating cultures; and (4) the presence of a cobblestone monolayer and its ECM embedded in a collagen gel (Fig. 6) did not prevent the inhibitory effects of TGF/β on the sprouting cells that migrated into the collagen gel.

Previous reports have shown that TGF/β-1 inhibits (1) endothelial cell migration on 2-D substrata (Heinmark et al. 1986; Muller et al. 1987; Sato and Rifkin, 1989), (2) migration in a Boyden chamber (Muller et al. 1987), and (3) PMA- or bFGF-stimulated migration into 3-D matrices (Muller et al. 1987; Mignatti et al. 1989). On the basis of our results, we speculate that TGF/β-1 would have no effect.
on the morphology, cell number or migration of the quiescent endothelial cells lining a blood vessel in vivo. If the initial migration of endothelial cells into and through their own ECM is induced by another angiogenic factor, the (now sprouting) endothelial cell response to TGF/β-1 will depend on the nature of the ECM surrounding the cells; thus further migration and sprouting into a type I collagen matrix may be inhibited whereas sprouting cells in contact with other matrix macromolecules (such as those present in the subendothelial ECM) may not be affected.

Madri et al. (1988) have previously shown that rat epididymal fat pad endothelial cells plated within 3-D collagen gels proliferated, contracted the gel and formed tubules. The two latter processes were stimulated in the presence of TGF/β-1. Somewhat contradictory results were obtained by Pepper et al. (1990); they demonstrated that TGF/β-1 (5 ng ml⁻¹) inhibited bFGF-induced invasion and tube formation by endothelial cells within a 3-D fibrin gel. Others, however, have reported from work of Madri et al. (1988) in that we have previously demonstrated that aortic and retinal endothelial cells plated within a 3-D collagen gel neither proliferate nor contract the gel (Schor et al. 1983; Schor and Schor, 1986). We now demonstrate that TGF/β-1 appears to be toxic to these cultures (Fig. 6). These apparently contradictory results indicate that endothelial cells from different sources have different properties and respond to TGF/β-1 in different ways. Indeed endothelial heterogeneity has been amply documented (for reviews see Fajardo, 1989; Machovich, 1988; Palade, 1988). The endothelial cells used by Madri et al. expressed α-smooth muscle actin (Kocher and Madri, 1989), a marker of smooth muscle cells and pericytes, which is not present in our endothelial cultures (Schor et al. 1990). It is therefore possible that the overall vascular response to TGF/β-1 may depend on the vascular bed or on the particular endothelial cell line examined. The local concentration of TGF/β-1 may also be important, as Plouet and Gospodarowicz (1989) have shown that low concentrations of this growth factor (0.1–1.0 ng ml⁻¹) can increase bovine corneal endothelial cell proliferation by increasing intracellular bFGF levels and Pepper et al. (1990) have indicated that low concentrations of TGF/β-1 can potentiate bFGF-induced invasion of a collagen gel. However, in the present study inhibition of endothelial cell proliferation was still observed with concentrations of TGF/β-1 (0.05 ng ml⁻¹) as low as those used in the studies cited above (Fig. 1B).

Concomitant with inhibition of endothelial cell growth on 2-D substrata TGF/β-1 markedly increased total protein synthesis in a dose-dependent manner (Table 1). This increase was also seen when TGF/β-1 was added to post-confluent quiescent cells cultured on 2-D substrata, suggesting that increased matrix deposition is independent of any effect on cell morphology or growth. The TGF/β-1-induced increase in total protein synthesis by BAEC and BREC on 2-D substrata was accompanied by a specific increase in PAI-1 synthesis, except in the case of sub-confluent BREC cultures. This finding indicates that TGF/β-1 may induce matrix accumulation by decreasing matrix degradation as previously suggested by Saksela et al. (1987); however, a different mechanism must also be involved, since sub-confluent BREC increase total protein synthesis in the presence of TGF/β-1 without PAI-1 levels being specifically elevated. This was the only difference found between aortic (BAEC) and retinal (BREC) endothelial cells. Since both types of cells are able to form new blood vessels in vivo (Wise et al. 1971; Suedehi et al. 1990), this finding may not be relevant to angiogenesis but it rather supports the theory that endothelial cells from different sites may respond to TGF/β-1 in different ways. Several groups have reported that TGF/β specifically increases fibronectin synthesis by endothelial cells (Madri et al. 1988) and various fibroblasts (Ignotoz and Massague, 1986; Varga et al. 1987) on 2-D substrata. However, whilst our data show that TGF/β-1 increased the overall protein synthesis, including fibronectin, this protein was not specifically increased. This apparent contradiction of previously published data may again be attributed to endothelial cell heterogeneity, but may equally well reflect difference between tissue culture conditions; for example, in this study TGF/β-1 activity was assayed in the presence of 20 % serum, compared with 10 % (Muller et al. 1987; Merwin et al. 1990) in other studies. Our data showing that there was no change in the amount of matrix synthesis or specific matrix molecules by endothelial cells cultured within 3-D collagen gels in the presence of TGF/β-1 agree with those of Madri et al. (1988).

In summary, our data indicate that the effects of TGF/β-1 upon endothelial cells depend on the cell phenotype, which in turn is determined by the extracellular matrix in contact with the cells. Overall, TGF/β-1 is able to inhibit endothelial cell proliferation, migration and sprouting and to stimulate protein synthesis. This latter response may be particularly important during the maturation of newly formed blood vessels. In view of the effects of TGF/β on endothelial cells in culture, the angiogenic activity of this factor has been explained in terms of a secondary response mediated by macrophages and TNFα. Thus, Wahl et al. (1987) and Wiseman et al. (1988) have shown that TGF/β can cause the chemotaxis and activation of macrophages, which may then release other angiogenic factors, e.g. TNFα. However, despite being angiogenic in vivo TNFα like TGF/β inhibits endothelial cell proliferation in vitro (Leibovich et al. 1987). Furthermore, Yang and Moses (1990) did not find any association between endothelial cell proliferation and the presence of macrophages in the chick chorioallantoic membrane. Nevertheless, it is still feasible that TGF/β acts indirectly on endothelial cells in vivo to induce angiogenesis, either through modification of the surrounding matrix, or by stimulation of a secondary ‘peri-endothelial’ cell type.

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References


