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Transforming growth factor beta 1 promotes the differentiation of endothelial cells into smooth muscle-like cells in vitro

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Summary

α-Smooth muscle actin is considered a reliable marker for distinguishing between arterial smooth muscle and endothelial cells. Several authors have reported heterogeneity in the expression of this actin isoform in atherosclerotic lesions. Such heterogeneity appears to result from the presence of different smooth muscle cell phenotypes (contractile and synthetic) in these lesions. In the present study, we show that bovine aortic endothelial cells, which are characterised by the presence of Factor VIII-related antigen (FVIII) and by the absence of α-smooth muscle actin (α-SM actin) may be induced to express the latter when exposed to TGF-β1. FVIII was detected by immunofluorescence, α-SM actin was detected by immunofluorescence and immunoblotting. The number of cells expressing α-SM actin increased with time of incubation with TGF-β1, and this increase occurred concomitantly with a decrease in the expression of FVIII. Double immunofluorescence demonstrated the presence of cells that expressed both FVIII and α-SM actin after 5 days of incubation with TGF-β1. With longer incubation times (10-20 days) the loss of FVIII expression was complete and over 90% of the cells expressed α-SM actin.

Ultrastructurally, cells in control cultures showed the typical features of endothelial cells. In the TGF-β1-treated cultures, cells which appeared indistinguishable from contractile and synthetic smooth muscle cells were observed. Withdrawal of TGF-β1 after 10 days incubation resulted in the re-appearance of polygonal cells which were FVIII-positive and α-SM actin-negative. Other cells in the same cultures, however, remained ragged in morphology, FVIII-negative and α-SM actin-positive even after 20 days in control medium. This indicates that the inductive effect of TGF-β1 is partly reversible after 10 days incubation. Such reversibility was no longer apparent after 20 days incubation with TGF-β1.

Our results demonstrate that TGF-β1 induces the differentiation of aortic endothelial cells into a smooth muscle-like phenotype and suggest a novel role for TGF-β1 in atherogenesis.

Key words: endothelial cells, TGF-β1, α-SM actin, smooth muscle cells, differentiation.

Introduction

Six actin isoforms have been described in mammalian cells (Vandekerckhove and Weber, 1978a,b). Of these, alpha-smooth muscle actin (α-SM actin) is considered the major form in arterial smooth muscle cells (Gabbiani et al., 1981; Owens et al., 1986; Skalli et al., 1987), whilst beta and gamma actins (β- and γ-actins) are the predominant forms present in fibroblasts (Leavit et al., 1985; Skalli et al., 1987) and endothelial cells (Ketis et al., 1986; Otey et al., 1986; Gottlieb et al., 1991). α-SM actin has never been detected in aortic endothelial cells (Kocher and Madri, 1989) and it is therefore considered a reliable marker for distinguishing between arterial smooth muscle and endothelial cells.

Several authors have reported changes in the expression of α-SM actin during atherosclerosis. These changes appear to be related to the presence of different smooth muscle cell (SMC) populations in atherosclerotic lesions (Ross et al., 1984; Gabbiani et al., 1984; Babaev et al., 1990; Kocher et al., 1991). Smooth muscle cell differentiation has been observed both in vivo and in vitro (Chamley-Campbell et al., 1979; Schwartz et al., 1986). These cells may express two distinct phenotypes: synthetic and contractile. Since these phenotypes are reversible, some authors describe this process as modulation rather than "differentiation and de-differentiation". The synthetic phenotype is characterised ultrastructurally by an extensive endoplasmic reticulum, rich Golgi complex and sparsity of microfilaments. This phenotype is metabolically active regarding the synthesis and deposition of extracellular matrix and is able to proliferate. By contrast, the contractile phenotype does not proliferate, is rich in α-SM actin-containing microfilaments (that coalesce into focal densities) and contains very few organelles dedicated to matrix biosynthesis (Chamley-

Campbell et al., 1979; Schwartz et al., 1986). During development, synthetic SMC proliferate in the arterial media and secrete extracellular matrix (ECM) components. Maturation of the vessel is accompanied by differentiation of the SMC from a synthetic to a contractile phenotype. These cells stop proliferating and acquire their contractile ability. A modulation from a contractile to a synthetic phenotype occurs in atherosclerosis, where intimal hyperplasia is the result of SMC migration, proliferation and extracellular matrix deposition. Various reports have described heterogeneity of the SMC population present in atherosclerotic plaques, with reference to the structural characteristics and the expression of myosin and α-SM actin. These studies suggest the presence of contractile, synthetic and luminal SMC (Babaev et al., 1990; Kocher et al., 1991). The latter are able to form focal tight junctions amongst themselves, a feature normally associated with endothelial rather than SMC (Kocher et al., 1991). A similar modulation of SMC phenotype occurs in tissue culture (Chamley-Campbell et al., 1979).

The key events in the pathogenesis of atherosclerosis are believed to involve initial damage to the arterial endothelium, followed by the release of vascular cell regulatory factors such as platelet-derived growth factor (PDGF) and transforming growth factor beta 1 (TGF-β1) from macrophages, platelets and damaged endothelial cells (Ross et al., 1986; Schwartz et al., 1990; Ferns et al., 1992). Many of the cellular changes that occur during atherogenesis can be attributed to the effects of PDGF and TGF-β1 upon endothelial cells and SMC at the site of the lesion. PDGF stimulates migration, proliferation and matrix biosynthesis by cultured SMC (Ross et al., 1986; Ross et al., 1990); TGF-β1 inhibits the migration and proliferation of endothelial cells in vitro (Baird and Durkin, 1986; Frater-Schroder et al., 1986; Heimark et al., 1986; Sutton et al., 1991) and it has been reported to both stimulate and inhibit SMC proliferation, depending on tissue culture conditions (Assoian and Sporn, 1986; Majack, 1987; Morisaki et al., 1991; Merwin et al., 1991). The synthesis of α-SM actin by arterial smooth muscle cells in culture is enhanced by TGF-β1 (Bjorkerud, 1991) and is inhibited by PDGF at both protein and mRNA levels (Blank et al., 1988; Corjay et al., 1989).

On the basis of in vitro data it is believed that during atherogenesis PDGF released at the site of injury attracts SMC from the media into the intima and also induces them to proliferate, deposit extracellular matrix and reduce the synthesis of α-SM actin. The SMC phenotype, therefore changes from contractile to synthetic. At the same time, TGF-β1 slows down re-endothelialisation of the luminal surface and stimulates the synthesis of α-SM actin by SMC (Ross et al., 1986; Schwartz et al., 1990; Ferns et al., 1992).

Data presented in this paper suggest that endothelial cells may differentiate into smooth muscle-like cells when exposed to TGF-β1. On the basis of these observations, we propose a new model of the cellular events contributing to atherogenesis.

Materials and methods

Cell culture

Bovine aortic endothelial cells were isolated from adult animals and cultured as previously described (Schor et al., 1983; Schor and Schor, 1986). Cells were identified as endothelial on the basis of their morphology, positive staining with factor VIII-related antibody (FVIII) and negative staining for α-SM actin. The endothelial stock cultures were routinely maintained on gelatin-coated Petri 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 medium is referred to as 20% DCS-MEM). Cultures were incubated at 37°C in a humidified atmosphere consisting of 5% CO_2, 95% air. Four lines of endothelial cells were used between passages 11 and 30. Of these lines two were cloned and two uncloned. Identical results were obtained with these four lines. Using published criteria (Schor and Schor, 1986; Schor et al., 1992), extensive investigations were carried out to confirm that the uncloned lines were free from contaminating smooth muscle cells and fibroblasts. For comparative studies we used two lines of SMC (passages 5 and 12) and two lines of bovine retinal pericytes (passages 3 and 5). These lines were isolated and cultured as described previously (Schor and Schor, 1986). Cell numbers were determined with a Coulter counter (Schor, 1980; Schor and Schor, 1986).

Culture conditions

The endothelial cells were plated sparsely (0.6×10^3 to 1.2×10^3 cells cm^{-2}) on gelatin-coated dishes and allowed to attach and spread for 24 hours in 20% DCS-MEM. At this point, the medium was replaced by either control medium or medium containing 1 ng ml^{-1} of human platelet TGF-β1 (from British Biotechnology Ltd, Cowley, Oxford, UK). The cultures were incubated for up to 20 days, changing the medium every 2-3 days during this period. Every experiment reported in this communication has been repeated at least 7 times with consistent results.

Immunofluorescence staining

Fixed and permeabilised cells were stained by indirect immunofluorescence according to standard techniques (Schor et al., 1991). The following specific antisera were used: rabbit anti-human factor VIII-related antigen from Dako Ltd (Bucks, England), mouse ascites monoclonal anti-α-SM actin from Sigma (Dorset, England) and mouse monoclonal anti-desmin from Dako Ltd (Bucks, England). The second antisera used were rabbit anti-mouse, either rhodamine or fluorescein isothiocyanate (FITC)-conjugated (Dako Ltd) and swine anti-rabbit, either rhodamine or FITC-conjugated (Dako Ltd).

The cultures were observed on an immunofluorescence microscope at ×10 and ×100 magnification. In order to estimate the percentage of cells that were positively stained, triplicate cultures were examined and between 200 and 600 cells were counted per experiment.

Identification of α-SM actin by immunoblotting

Cultures were washed twice with Hanks' balanced salt solution, and the cell layer-matrix proteins were extracted in 4 M guanidinium chloride, 50 mM Tris-HCl, pH 7.4, for 48 hours at 4°C. Between 90 and 95% of the total proteins deposited into the cell layer/matrix were extracted by this procedure. Insoluble material was removed by centrifugation (14,000 ***g*** for 20 min) and the solubilised proteins were dialysed extensively against 0.5 M acetic acid at 4°C. The protein content of each sample was determined using Coomassie protein assay reagent from Pierce (Rockford, Illinois, USA). These proteins were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions on 6.5% resolving gels (Laemmli, 1970) and transferred to nitrocellulose (Schleicher and Schuell, Germany) by semi-dry blotting.

An equal amount of protein (5 μg) was loaded onto each track and prestained relative molecular mass markers (M_r 14,300 to

200,000; Gibco, BRL) were electrophoresed at the same time. Active sites on the nitrocellulose were blocked by incubation in phosphate buffered saline (PBS) containing bovine serum albumin (BSA) (5%, w/v) and Tween 20 (0.05%, v/v) for 1 hour at room temperature, followed by 3×10-minute washes in PBS, Tween 20 (0.05%, v/v). The nitrocellulose filter was then incubated with mouse anti-α-SM actin antibody (Sigma) diluted 1:2000 in PBS containing BSA (0.1%, w/v) and 10 mM sodium azide for 2 hours at room temperature. The nitrocellulose was subsequently washed in PBS, Tween 20 (0.05%, v/v) (3×10 min) and incubated with peroxidase-conjugated rabbit anti-mouse IgG (Dako) diluted 1:1000 in PBS, BSA (0.1%, w/v) for $1^1/_2$ hours at room temperture. Finally, the nitrocellulose was washed extensively in PBS, Tween 20 (0.05%, v/v) for 1 hour and the immobilised proteins were detected by enhanced chemiluminescence using a kit from Amersham PLC (Amersham, UK).

Electron microscopy

Transmission electron microscopy (TEM) was performed as previously reported (Schor et al., 1983). Briefly, cultures were fixed in 2.5% glutaraldehyde in 0.15 M Sorensen's buffer (pH 7.3), washed three times with this buffer, and post-fixed for 1 hour in 1% OsO_4 in the same buffer. Samples were dehydrated, infiltrated and embedded in situ, using Luft's Epon. Thin sections were stained with uranyl acetate and lead citrate and viewed at 80 kV in a Philips 400-TEM.

Results

Presence of FVIII and α-SM actin in control and TGF-β1-treated cultures

Aortic endothelial cells were plated on gelatin-coated dishes as described in the Materials and methods. One day after plating, the cells covered approximately 20% of the substratum. At this point the medium was changed to either control or TGF-β1 (1 $ng.ml^{-1}$)-containing medium and then replaced every 2-3 days throughout the experiment.

Control cultures

The cells proliferated in control medium, reaching confluence 8-10 days after plating. At confluence the cells formed a typical monolayer of cobblestone appearance. A second layer of elongated sprouting cells appeared underneath the cobblestone monolayer in post-confluent cultures as previously reported (Schor et al., 1983). Control cultures were maintained for up to 20 days after plating and stained at various stages with antibodies to FVIII and α-SM actin; FVIII was present in all endothelial cells at all stages, including sprouting cells (Fig. 1A), whereas α-SM actin was never detected by either immunofluorescence (not shown) or immunoblotting (Fig. 2, tracks 1 and 2).

TGF-β1-treated cultures

The addition of TGF-β1 to endothelial cells inhibited cell proliferation and induced morphological changes as previously reported (Sutton et al., 1991). The expression of FVIII and α-SM actin changed in these cultures with time, as described below and summarised in Table 1.

After 3 days of incubation with TGF-β1 the cells were clearly enlarged by comparison to the controls, displaying a characteristic "ragged" morphology. At this stage only 50-60% of the cells stained positively with FVIII antibody, and this staining was weaker than in the control cultures, where 100% of the cells were positive for FVIII (Fig. 1A). α-SM actin was not detected in these cultures (not shown). After 5 days of incubation the percentage of FVIII-positive cells had decreased to between 30 and 40% (Fig. 1B) and the observed staining was very weak. In the same cultures, between 40 and 60% of the cells were found to stain positively for α-SM actin (Fig. 1C). At this stage the intensity of the staining for α-SM actin varied; approximately 20% of the positively stained cells showed strong fluorescence, with the remaining cells more weakly fluorescent. Double immunofluorescence revealed that these latter cells were usually positive for both FVIII and α-SM actin (Fig. 1D,E,F); the cells which were strongly positive for α-SM actin were generally negative for FVIII. Some of the cells were positive for FVIII and negative for α-SM actin and some were negative for both.

After 10 days in culture the ragged cells were very spread out and appeared to cover most of the surface of the dish. These cells were 100% negative for FVIII (Fig. 1G, H) whereas the number of cells staining strongly with α-SM actin antibody had increased to approximately 80-90% (Fig. 1I). Under phase-contrast microscopy, these cells appeared to display a large number of typical actin microfilaments.

Cultures incubated with TGF-β1 for longer periods (20 days) contained overlapping ragged cells which were negative for FVIII. Approximately 90-95% of the cells were strongly positive for α-SM actin (not shown).

Identical results were obtained with 4 lines of cells (2 cloned, 2 uncloned) used in this study. The presence of α-SM actin was confirmed by immunoblotting (Fig. 2) as described in Materials and methods. Control endothelial cultures were examined when semiconfluent and post-confluent. Semi-confluent cultures contained a similar number of cells as the TGF-β1-treated cultures, post-confluent cultures had been maintained in parallel with those treated with TGF-β1. α-SM actin was not detected in either of these control cultures (Fig. 2, tracks 1 and 2). By contrast, α-SM actin was present in cultures treated with TGF-β1 (Fig. 2, track 3), as well as in control, confluent, bovine aortic smooth muscle cells (Fig. 2, track 4).

Comparative studies with aortic SMC and retinal pericytes

Aortic SMC (two lines, passages 5 and 12) and retinal pericytes (two lines, passages 3 and 5) were plated under the

Table 1. *Presence of FVIII and α-SM actin in cultures incubated with TGF-β1*

Incubation time (days)	FVIII	α-SM actin
3	50-60 (++)	0
5	30-40 (+)	20 (+++) 40 (+)
10	0	80-90 (+++)
20	0	90-95 (+++)

The percentage of cells stained and the intensity are indicated.
Control cultures were always 100% positive for FVIII (+++) and negative for α-SM actin. Intensity of staining: +, weak; ++, moderate; +++, strong.

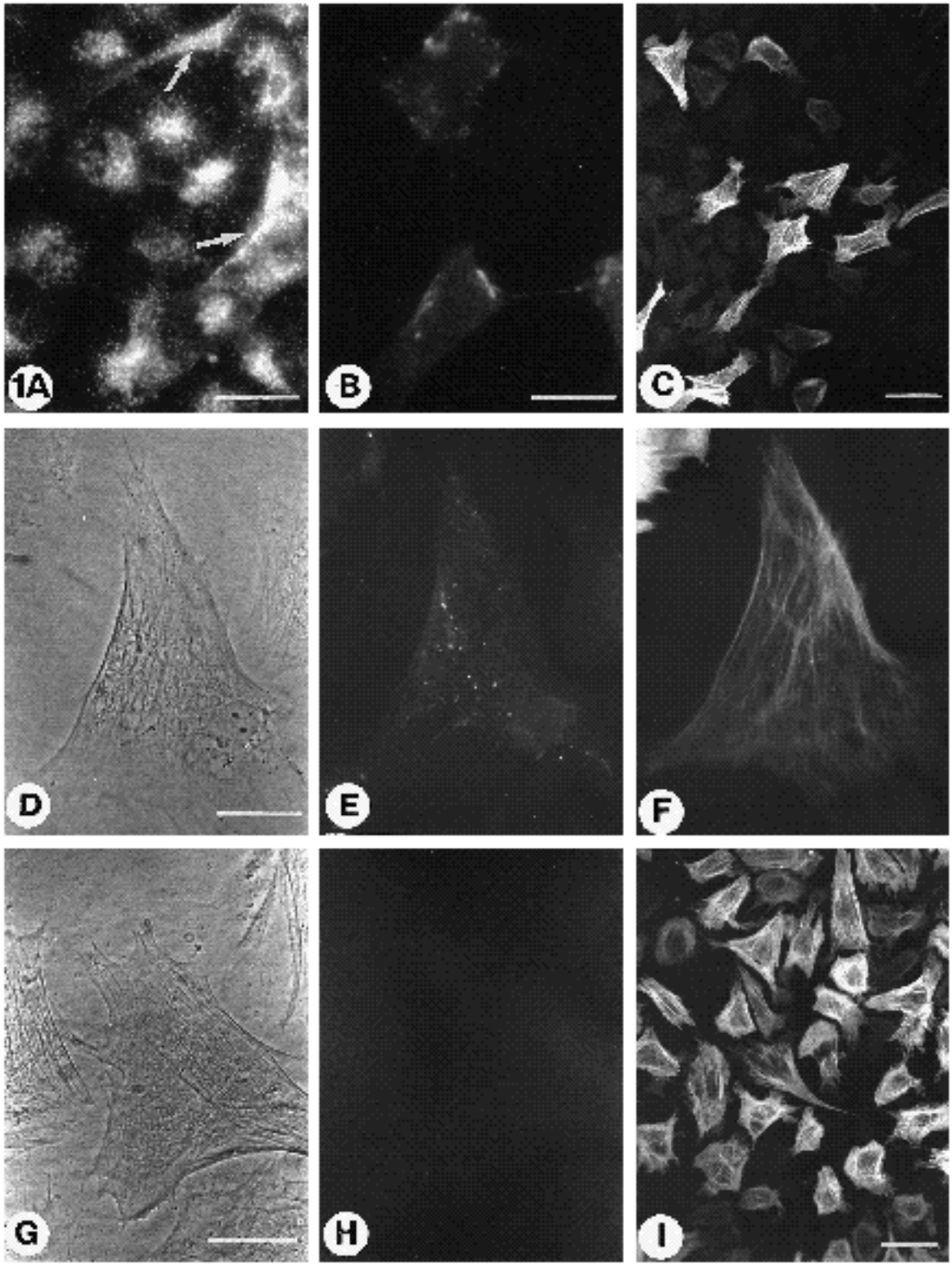


Fig. 1. Immunolocalisation of Factor VIII-related antigen and alpha-smooth muscle actin (α-SM actin) in control and TGF-β1-treated endothelial cultures. (A) Post-confluent control culture stained with FVIII antibody. Strong staining is observed in all cobblestone and sprouting (arrowed) cells. (B,C,D,E,F) Cultures incubated with TGF-β1 for 5 days. (B) FVIII was weakly expressed by approximately 40% of the cells, the rest were negative. (C) Localisation of α-SM actin. Strongly positive, weakly positive and negative cells are present. (D,E,F) Phase-contrast (D) and immunofluorescence micrographs of the same field stained using double immunofluorescence with FVIII (E) and α-SM actin (F) antibodies. Both FVIII and α-SM actin are expressed by the same cell. (G,H,I) Cultures incubated with TGF-β1 for 10 days. (G,H) Phase-contrast (G) and immunofluorescence (H) micrographs of the same field showing lack of staining with FVIII antibody. (I) Presence of α-SM actin in the same culture shown in H. Bar: 150 μm for C and I; 50 μm for A,B,D,E,F,G and H.

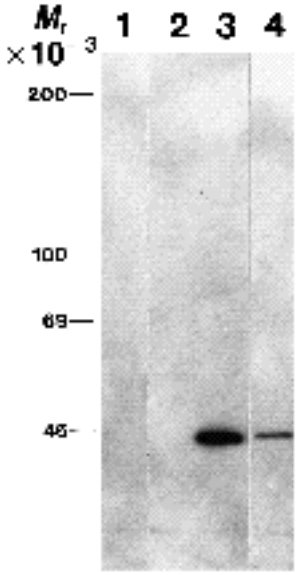


Fig. 2. Identification of α-SM actin by immunoblotting. Cultures of uncloned aortic endothelial and smooth muscle cells were extracted with 4 M guanidinium chloride, 50 mM Tris-HCl, pH 7.4, and the cell layer/matrix proteins were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose sheets were then incubated with anti-α-SM actin serum as described in Materials and methods. Control endothelial cultures were examined when semiconfluent (track 1) and postconfluent (track 2). Semiconfluent cultures contained similar cell numbers to the TGF-β1-treated cultures (track 3). Postconfluent control cultures (track 2) had been maintained in culture for the same length of time as the TGF-β1-treated cells (13 days). Neither of the control endothelial cultures contained α-SM actin. In contrast, cultures treated with TGF-β1 expressed α-SM actin (track 3). This actin isoform was also expressed by control smooth muscle cell cultures (track 4). Migration positions of prestained M_r markers are indicated.

same conditions used for the control endothelial cells and stained with anti-α-SM actin and anti-desmin antibodies when sparse and at confluence. Control endothelial cells were examined when sparse, confluent and post-confluent. The latter contained a cobblestone monolayer as well as sprouting cells (Schor et al., 1983). Ragged endothelial cells were examined after 20 days of incubation with TGF-β1. The results (not shown) demonstrated that control endothelial cells were always negative for α-SM actin and desmin. Approximately 90-95% of the ragged (TGF-β1-treated) endothelial cells were positive for α-actin; desmin was not detected. Both SMC and pericytes were 100% positive for α-SM actin. Desmin was not detected in the SMC, whereas approximately 35% of the pericytes stained strongly with anti-desmin antibody. These results agree with previously published work regarding the presence of α-SM actin and desmin in these vascular cells (Schor et al., 1992).

Reversibility of the effects of TGF-β1

The possible reversibility of the effects of TGF-β1 on endothelial cell morphology and expression of FVIII and α-SM actin was examined in cultures that had been incubated with TGF-β1 for 10 and 20 days. In both cases the cultures were washed 3 times and incubated with 20% DCS-MEM in the absence of TGF-β1 for an additional 10- to 20-day period.

In the cultures that had been incubated with TGF-β1 for 10 days the presence of polygonal cells was observed after 5 days in control medium. These polygonal cells increased in numbers and formed typical cobblestone areas which were surrounded by ragged cells. After 20 days in control medium a large number of ragged cells still remained in the cultures, apparently separating cobblestone areas (Fig. 3A). Double immunofluorescence staining demonstrated that the polygonal, cobblestone, cells were positive for FVIII (Fig. 3B) and negative for α-SM actin (Fig. 3C),

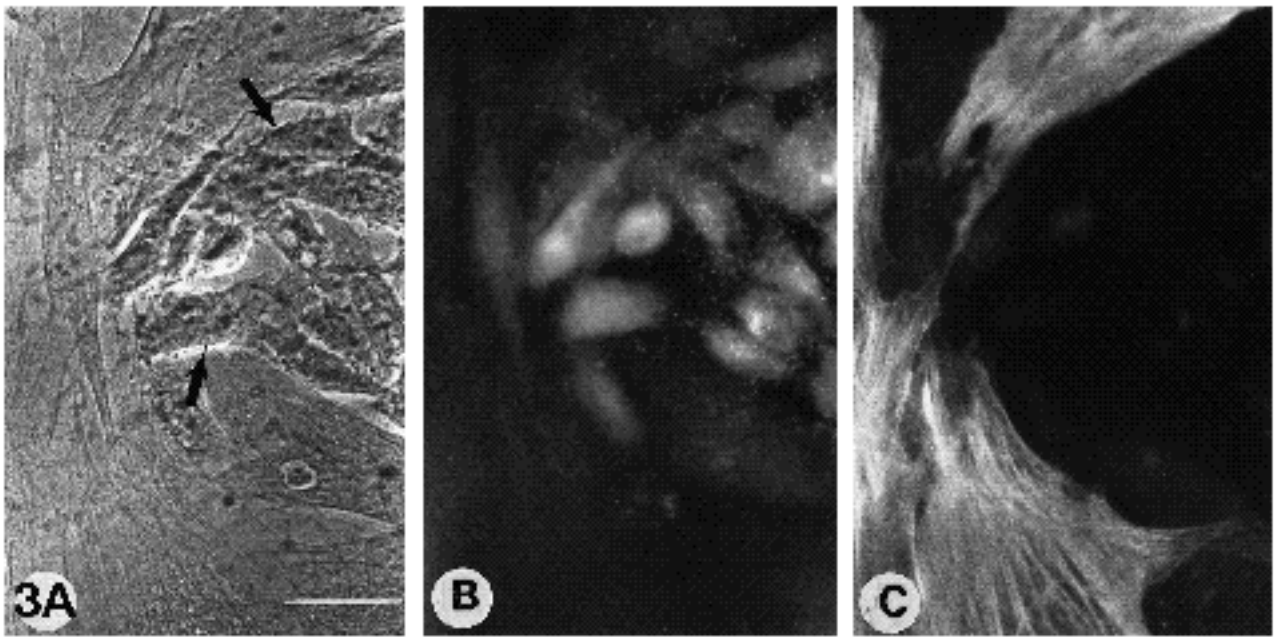


Fig. 3. Reversibility of the effects of TGF-β1. Phase-contrast (A) and immunofluorescence micrographs of the same field stained using double immunofluorescence for FVIII (B) and α-SM actin (C). This culture was incubated for 10 days with TGF-β1 and then for 10 more days in the absence of this factor. After the initial 10 days with TGF-β1, all the cells showed a ragged morphology and FVIII was not present. 10 days after the addition of control medium some cells reversed to a polygonal morphology (A, arrows). The polygonal cells expressed FVIII (B) and were surrounded by ragged cells which were negative for FVIII (B) and positive for α-SM actin (C). Bar, 50 μm.

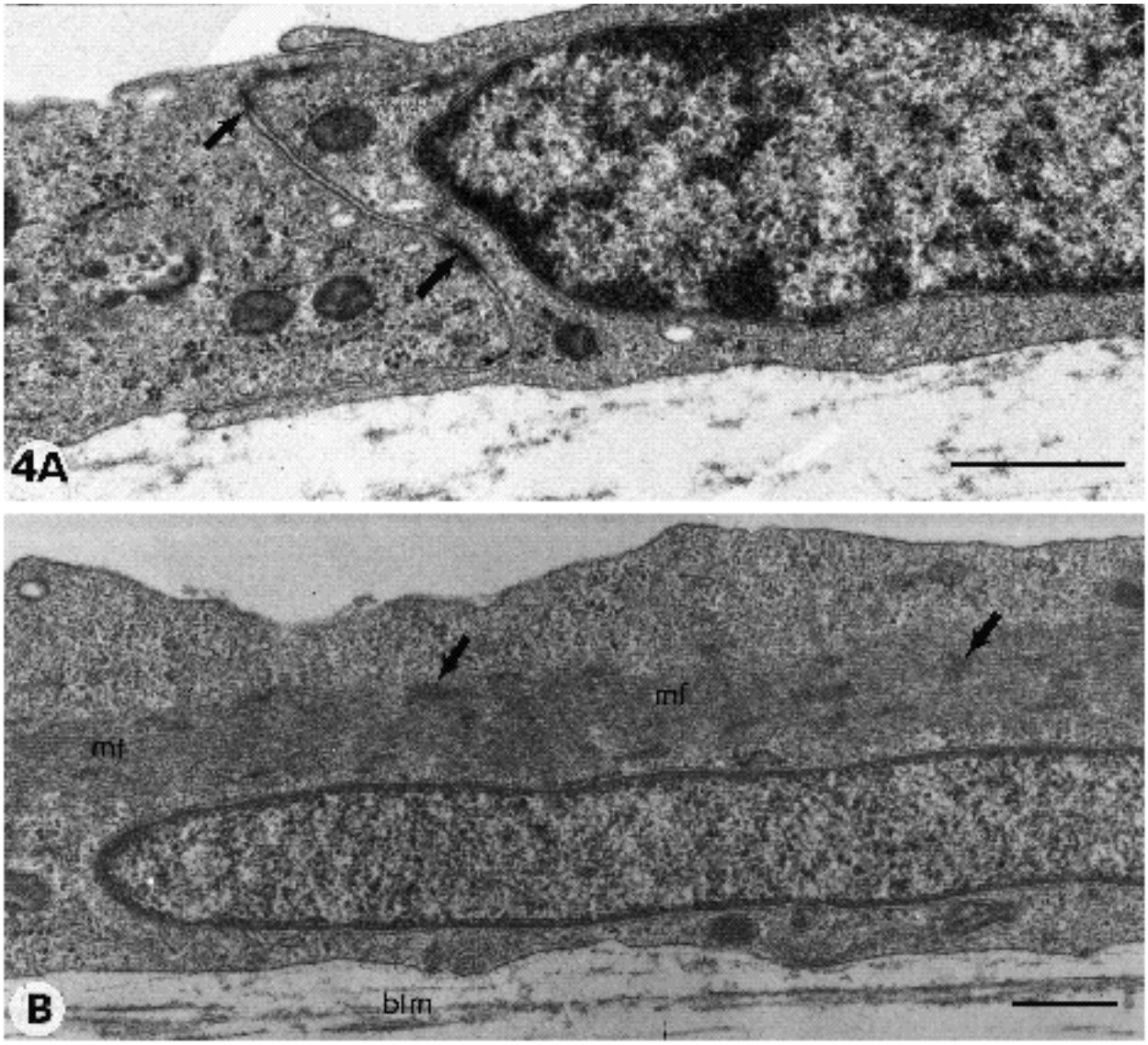


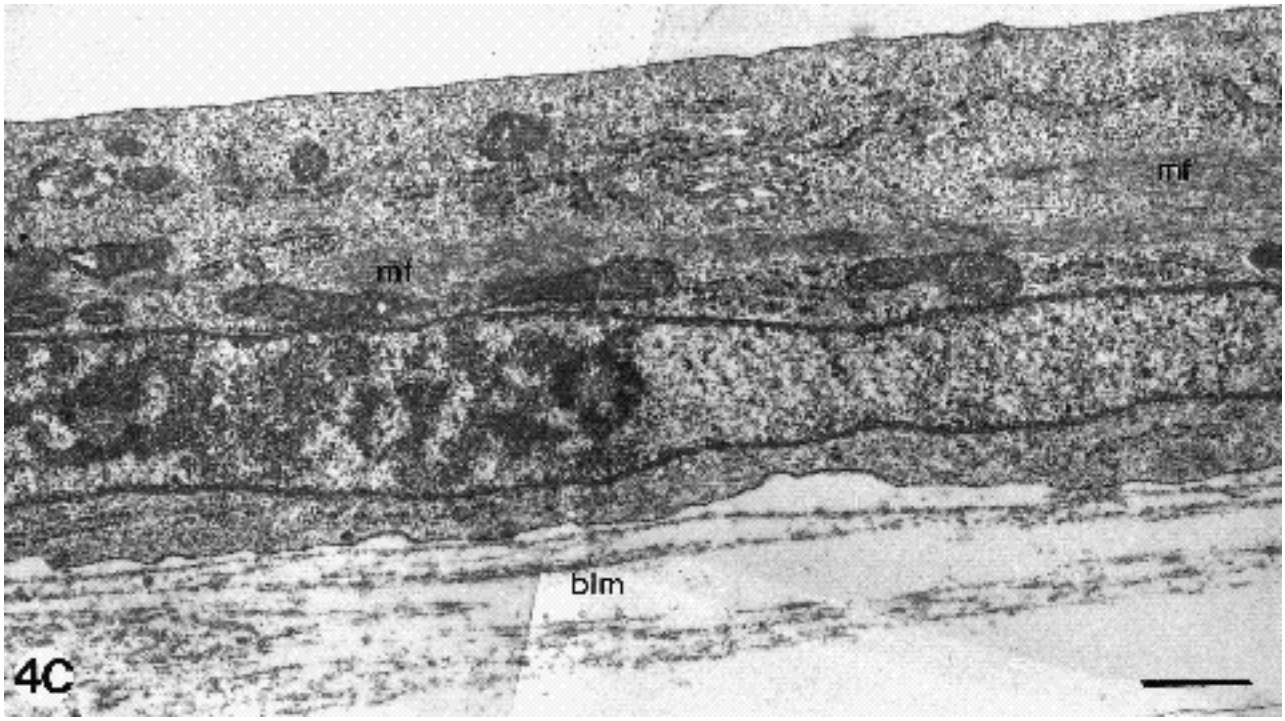
Fig. 4. Transmission electron micrographs of control and TGF-β1-treated cultures. (A) Control endothelial cells are attached to one another by junctional complexes (arrows) and contain a variety of cytoplasmic organelles, including smooth and rough endoplasmic reticulum, polysomes and Golgi complex. Bar, 1 μm. (B) Contractile smooth muscle-like cell present in TGF-β1-treated cultures. The cytoplasm is rich in microfilaments (mf) coalescing into dense bodies (arrows). The cells contain numerous mitochondria and few, poorly developed, organelles involved in the synthesis and secretion of proteins. Bar, 0.5 μm. (C) TGF-β1-treated cultures also contain cells resembling synthetic smooth muscle cells. These exhibit few microfilaments (mf), a prominent Golgi complex and extensive rough endoplasmic reticulum. Bar, 0.5 μm. Note, in (A), (B) and (C), the presence of an amorphous electron-dense basal lamina-like material (blm) adjacent to the plasma membrane.

whereas the ragged cells were negative for FVIII (Fig. 3B) and positive for α-SM actin (Fig. 3C).

Cultures that had been incubated with TGF-β1 for 20 days were also maintained in control medium for up to 10 days. During this time polygonal cells were not observed and the ragged cells remained negative for FVIII (not shown). These observations suggest that in long-term cultures the effects of TGF-β1 may become irreversible.

Transmission electron microscopy (TEM)

TEM was performed on cultures maintained in the presence and absence of TGF-β1 for 20 days. Ultrathin sections cut perpendicularly to the surface of the dish showed that the cells in the control cultures (Fig. 4A) were attached to one another by junctional complexes and contained a variety of cytoplasmic organelles, including smooth and rough endoplasmic reticulum, polysomes and prominent Golgi complex. The basal lamina was thin and continuous. Weibel-Palade bodies were absent. Two types of cells were observed in the TGF-β1-treated cultures. The majority of the cells had the appearance of contractile smooth muscle cells (Chamley-Campbell et al., 1979; Schwartz et al., 1986) as they contained numerous microfilaments coalesc-



ing into dense bodies (Fig. 4B). These cells were also rich in mitochondria but they contained few and poorly developed organelles involved in the synthesis and secretion of proteins. The second cell type (Fig. 4C) could be identified as synthetic smooth muscle cells (Chamley-Campbell et al., 1979; Schwartz et al., 1986) as they exhibited few microfilaments, a prominent Golgi complex and extensive rough endoplasmic reticulum. Both contractile and synthetic smooth muscle cells had an electron-dense basal lamina material adjacent to the plasma membrane and did not appear to form complex junctions.

Discussion

In the present study we show that bovine aortic endothelial cells, which are characterised by the presence of FVIII-related antigen and by the absence of α-SM actin (Ketis et al., 1986; Kocher and Madri, 1989; Gottlieb et al., 1991), are able to express this actin isoform when exposed to TGF-β1. TGF-β1 has been shown previously to increase the synthesis of α-SM actin by cells which already express this actin isoform (Bjorkerud, 1991; Kocher and Madri, 1989). To our knowledge this is the first time that expression of α-SM actin has been induced in a cell type which does not normally express it. Interestingly, cardiac fibroblasts treated with TGF-β1 have been found to differentiate into cells that express sarcomeric actin, a feature of cardiac myocytes (Eghbali et al., 1991).

α-SM actin is considered a reliable marker for distinguishing between arterial smooth muscle and endothelial cells. This actin isoform has never been found in aortic endothelial cells (see Introduction). It has been found, however, in capillary endothelial cells derived from the rat epididymal fat pad (Kocher and Madri, 1989). These cells are apparently unique amongst endothelial cells in that they express both FVIII and α-SM actin.

Our results demonstrate that exposure to TGF-β1 first reduced the number of endothelial cells positive for FVIII as well as the intensity of the staining. The expression of α-SM actin by these cells occurred 1-2 days later. Double immunofluorescence demonstrated the presence of cells that expressed both FVIII and α-SM actin after 5 days incubation with TGF-β1. These cells may be considered as an intermediary phenotype between endothelial and smooth muscle cells. With longer incubation times (10-20 days) the loss of FVIII expression was complete and over 90% of the cells expressed α-SM actin. A small number of cells that did not express α-SM actin after 20 days incubation were morphologically identical to the α-SM actin-positive cells. Aortic SMC have been shown to be positive for α-SM actin and negative for desmin (Gabbiani et al., 1981; Lazarides, 1982; this communication), while pericytes may be positive for both antibodies (Sims, 1991; Schor et al., 1992; this communication). The ragged (TGF-β1-treated) endothelial cells did not express desmin and were positive for α-SM actin, suggesting that they may be more akin to SMC than to pericytes.

Ultrastructurally, cells in control cultures showed the typical features of endothelial cells, whereas the TGF-β1-treated cells appeared indistinguishable from smooth muscle cells. Two types of smooth muscle cell phenotypes, contractile and synthetic, have been described in the literature (Chamley-Campbell et al., 1979; Schwartz et al., 1986). In vivo these phenotypes are defined by their ultrastructure; both cell types were observed in our cultures. The majority of the cells appeared to be contractile smooth muscle cells, with typical microfilaments forming focal

densities, and few cytoplasmic organelles. The synthetic phenotype is characterised by the presence of numerous cytoplasmic organelles and the sparsity of microfilaments. This phenotype is ultrastructurally different from the endothelial cells, which contain some microfilaments (although not α-SM actin), are rich in organelles and establish complex junctions amongst themselves. It is possible that the cells resembling synthetic smooth muscle cells may be the same cells found to be negative when stained for α-SM actin, although this has not been investigated.

After 10 days of incubation with TGF-β1 the effects of this factor were partly reversible. FVIII-positive cells were observed 5 days after TGF-β1 withdrawal but a large number of cells remained FVIII-negative and α-SM actin-positive 20 days after withdrawal. Cells incubated with TGF-β1 for 20 days were examined for 10 days after removal of TGF-β1. During this period polygonal FVIII-positive cells did not appear in the cultures. These results suggest that TGF-β1 induces the differentiation of endothelial cells into SMC-like cells in a gradual fashion, involving several overlapping stages. In the first stage there is a reduction in the synthesis of FVIII-related antigen, in the last stage examined so far the endothelial cells appear committed to express a phenotype that we describe as SMC-like on the basis of their ultrastructural morphology, the presence of α-SM actin and the absence of desmin and FVIII.

The expression of α-SM actin has been considered as an indicator of the degree of differentiation in smooth muscle cells. Changes in the expression of α-SM actin have been found during the development of the aorta (Kocher et al., 1985), in wound healing (Darbi et al., 1990), in atherosclerotic lesions (Gabbiani et al., 1984; Kocher et al., 1991) and in cultured smooth muscle cells obtained from aortic explants (Chamley-Cambell et al., 1979). During the development of atherosclerotic lesions, both TGF-β1 and PDGF are released from platelets and macrophages (see Introduction). In such a situation current theories suggest that TGF-β1 would slow down re-endothelialisation, since it inhibits endothelial migration and proliferation. At the same time, PDGF would recruit smooth muscle cells into the site of injury, as this factor has been shown to stimulate smooth muscle cell proliferation and migration. PDGF would also stimulate matrix biosynthesis and inhibit α-actin synthesis by SMC. Once the smooth muscle cells have been recruited, TGF-β1 would increase the expression of α-SM actin by these cells (see Introduction).

Our results suggest a parallel role for TGF-β1, whereby this factor would induce the differentiation of endothelial cells into contractile, synthetic and possibly luminal smooth muscle cells. Since smooth muscle cells in atherosclerotic lesions are heterogeneous regarding their structure and the expression of α-SM actin (Babaev et al., 1990; Kocher et al., 1991), our hypothesis is consistent with the experimental and clinical findings.

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References

Assoian, R.K. and Sporn, M.B. (1986). Type β transforming growth factor in human platelets: Release during platelet degranulation and action on vascular smooth muscle cells. *J. Cell Biol.* **102**, 1217-1223.

Babaev, V.R., Bobryshev, Y.V., Stenina, O.V., Tararak, E.M. and Gabbiani, G. (1990). Heterogeneity of smooth muscle cells in atheromatous plaque of human aorta. *Am. J. Pathol.* **136**, 1031-1042.

Baird, A. and Durkin, T. (1986). Inhibition of endothelial cell proliferation by type β-transforming growth factor: Interactions with acidic and basic fibroblast growth factors. *Biochem. Biophys. Res. Commun.* **138**, 476-482.

Bjorkerud, S. (1991). Effects of transforming growth factor-β1 on human arterial smooth muscle cells in vitro. *Arterioscler. Thromb.* **11**, 892-902.

Blank, R., Thompson, M.M. and Owens, G.K. (1988). Cell cycle versus density dependence of smooth muscle alpha actin expression in cultured rat aortic smooth muscle cells. *J. Cell Biol.* **107**, 299-306.

Corjay, M.H., Thompson, M.M., Lynchs, KR. and Owens, G.K. (1989). Differential effect of platelet-derived growth factor-versus serum induced growth on smooth muscle α-actin and non-muscle β-actin mRNA expression in cultured rat aortic smooth muscle cells. *J. Biol. Chem.* **264**, 10,501-10,506.

Chamley-Campbell, J., Campbell, G. and Ross, R. (1979). The smooth muscle cell in culture. *Physiol. Rev.* **59**, 1-61.

Darbi I., Skalli, O. and Gabbiani, G. (1990). α-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab. Invest.* **63**, 21-29.

Eghbali, M., Tomek, R., Woods, C.H. and Bhambi, B. (1991). Cardiac fibroblasts are predisposed to convert into myocyte phenotype: Specific effect of transforming growth factor β1. *Proc. Nat. Acad. Sci. USA* **88**, 795-799.

Ferns, G.A., Stewart-Lee, A.L. and Anggard, E.E. (1992). Arterial response to mechanical injury: balloon catheter de-endothelialisation. *Atherosclerosis* **92**, 89-104.

Frater-Schroder, M., Muller, G., Birchmeier, W. and Bohlen, P. (1986). Transforming growth factor-beta inhibits endothelial cell proliferation. *Biochem. Biophys. Res. Commun.* **137**, 295-302.

Gabbiani, G., Kocher, O., Bloom, W.S., Vandekerckhove, J. and Weber, K. (1984). Actin expression in smooth muscle cells of rat aortic intimal thickening, human atheromatous plaque and cultured rat aortic media. *J. Clin. Invest.* **73**, 148-152.

Gabbiani, G., Schmid, E., Winter, S., Chaponnier, Chj. De Chastonay, C., Vandekerkhove, J., Weber, K. and Franke, W. (1981). Vascular smooth muscle cells differ from other smooth muscle cells: Predominance of vimentin filaments and a specific α-type actin. *Proc. Nat. Acad. Sci. USA* **78**, 298-302.

Gottlieb, A.I., Langille, L.B., Wong, M.K. and Kim, D.W. (1991). Structure and function of the endothelial cytoskeleton. *Lab. Invest.* **65**, 123-137.

Heimark, R.L., Twardzik, D.T. and Schwarzt, S.M. (1986). Inhibition of endothelial regeneration by type-Beta transforming growth factor from platelets. *Science* **233**, 1078-1080.

Ketis, N.V., Hoover, R.L. and Karnovsky, M.J. (1986). Isolation of bovine aortic endothelial cell plasma membranes: Identification of membrane-associated cytoskeletal proteins. *J. Cell. Physiol.* **128**, 162-170.

Kocher, O., Gabbiani, F., Gabbiani, G., Reidy, M.A., Cokay, M.S., Peters, H. and Huttner,. I. (1991). Phenotypic features of smooth muscle cells during the evolution of experimental carotid arterty intimal thickening. *Lab. Invest.* **65**, 459-470.

Kocher, O. and Madri, J. (1989). Modulation of actin mRNAs in cultured vascular cells by matrix components and TGF-β1. *In Vitro* **25**, 424-434.

Kocher, O., Skalli, O., Cerutti, D. and Gabbiani, G. (1985). Cytoskeletal features of rat aortic cells during development. An electron microscopic, immunohistochemical and biochemical study. *Circ. Res.* **56**, 829-838.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lazarides, E. (1982). Intermediate filaments: A chemically heterogeneous,

developmentally regulated class of proteins. *Annu. Rev. Biochem.* **51**, 219-250.

Leavit, J., Gunning, P., Kedes, L. and Jariwalla, R. (1985). Smooth muscle α-actin is a transformation sensitive marker for mouse WIH3T3 and Rat-2 cells. *Nature* **316**, 840-842.

Majack, R.A. (1987). Beta-type transforming growth factor specifies organisational behaviour in vascular smooth muscle cell cultures. *J. Cell Biol.* **105**, 465-471.

Merwin, J.R., Newman, U., Dawsoin, L., Tucker, A. and Madri, J. (1991). Vascular cells respond differentially to transforming growth factors beta 1 and beta 2 in vitro. *Am. J. Pathol.* **138**, 37-51.

Morisaki, N., Kawano, M., Koyama, N., Koshikawa, T., Umemiya, K., Saito, Y. and Yoshida, S. (1991). Effects of transforming growth factor β1 on growth of aortic smooth muscle cells. Influences of interactions with growth factors, cell state, cell phenotype and cell cycle. *Atherosclerosis* **88**, 227-234.

Otey, C.A., Kalnoski, M.H., Lessard, J.L. and Bulinski, J. Ch. (1986). Immunolocalization of the gamma isoform of non-muscle actin in cultured cells. *J. Cell Biol.* **102**, 1726-1737.

Owens, G.K., Loeb, A., Gordon, D. and Thompson, M.M. (1986). Expression of smooth muscle-specific α-isoactin in cultured vascular smooth muscle cells: Relationship between growth and cytodifferentiation. *J. Cell Biol.* **102**, 343-352.

Ross, R., Bowen-Pope, D.F. and Raines, E.W. (1990). Platelet-derived growth factor and its role in health and disease. *Phil. Trans. R. Soc. Lond.* **B327**, 155-169.

Ross, R., Raines, E.W. and Bowen-Pope, D.F. (1986). The biology of platelet-derived growth factor. *Cell* **46**, 155-169.

Ross, R., Wight, T.N., Strandness, E. and Thiele, B. (1984). Human atherosclerosis. Cell constitution and characteristics of advanced lesions of the superficial femoral artery. *Am. J. Pathol.* **144**, 79-93.

Schor, A.M., Canfield, A.E., Sutton, A.B., Allen, T.D., Sloan, P. and Schor, S.L. (1992). The behaviour of pericytes in vitro: Relevance to angiogenesis and differentiation. In *Angiogenesis: Key Principles-Science-Technology-Medicine* (ed. R. Steiner, P.B. Weiz and R. Langer), pp. 167-178. Birkhauser-Verlag, Basel, Switzerland.

Schor, A.M., Canfield, A.E., Sloan, P. and Schor, S.L. (1991). Differentiation of pericytes in culture is accompanied by changes in the extracellular matrix. *In Vitro* **27A**, 651-659.

Schor, A.M. and Schor, S.L. (1986). The isolation and culture of endothelial cells and pericytes from bovine retinal microvasculature: A comparative study with large vessel vascular cells. *Microvasc. Res.* **32**, 21-38.

Schor, A.M., Schor, S.L. and Allen, T.D. (1983). Effects of culture conditions on the proliferation, morphology and migration of bovine aortic endothelial cells. *J. Cell Sci.* **62**, 267-285.

Schor, S.L. (1980). Cell proliferation and migration on collagen substrata in vitro. *J. Cell Sci.* **41**, 159-175.

Schwartz, S.M., Campbell, G.R. and Campbell, J.H. (1986). Replication of smooth muscle cells in vascular disease. *Circ. Res.* **58**, 427-444.

Schwartz, S.M., Heimark, R.L. and Majesky, M.W. (1990). Developmental mechanism underlying pathology of arteries. *Physiol. Rev.* **70**, 1177-1209.

Sims, D.E. (1991). Recent advances in pericyte biology. Implications for health and disease. *Can. J. Cardiol.* **7**, 431-443.

Skalli, O., Vandekerckhove, J. and Gabbiani, G. (1987). Actin-isoform pattern as a marker of normal or pathological smooth-muscle and fibroblastic tissues. *Differentiation* **33**, 232-238.

Sutton, A.B., Canfield, A.E., Schor, S.L., Grant, M.E. and Schor, A.M. (1991). The response of endothelial cells to TGF-β1 is dependent upon cell shape, proliferative state and the nature of the substratum. *J. Cell Sci.* **99**, 777-787.

Vandekerckhove, J. and Weber, K. (1978a). At least six different actins are expressed in a higher mammal: An analysis based on the amino acid sequence of the amino-terminal tryptic peptide. *J. Mol. Biol.* **126**, 783-802.

Vandekerckhove, J. and Weber, K. (1978b). Mammalian cytoplasmic actins are the products of at least two genes and differ in primary structure in at least 25 identified positions from skeletal muscle actins. *Proc. Nat. Acad. Sci. USA* **75**, 1106-1110.

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