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Association of thrombospondin-1 with osteogenic differentiation of retinal pericytes in vitro

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SUMMARY

Vascular pericytes can differentiate into osteoblast-like cells in vitro, suggesting that these cells may represent a potential source of osteoprogenitor cells in the adult. Pericyte differentiation is associated with a characteristic pattern of nodule formation and mineralisation. Nodules are formed in post-confluent cultures by the retraction of multilayered areas. Crystals of hydroxyapatite are deposited on the extracellular matrix of these nodules which then becomes mineralised. We now demonstrate that thrombospondin-1 (TSP-1) gene expression is modulated during pericyte differentiation in vitro. That is, the relative levels of TSP-1 (protein and mRNA) increased markedly during nodule formation and then decreased when mineralisation of the nodules had taken place. TSP-1 was localised throughout non-mineralised nodules but it was largely excluded from the inner mass of mineralised nodules. The production of a mineralised matrix by vascular pericytes was promoted by the presence of antibodies to TSP-1 in the culture medium and was inhibited by exogenous TSP-1. These effects did not appear to be mediated through the activation of latent TGF-β, since neither exogenous TGF-β nor neutralising antibodies to TGF-β had any effect on the rate or extent of mineralisation seen in the pericyte cultures. Taken together these results suggest that high levels of TSP-1 inhibit pericyte mineralisation, supporting the view that this protein plays a role in pericyte differentiation and bone formation.

Key words: Pericyte, Thrombospondin, Osteogenesis, Differentiation

INTRODUCTION

It is still not clear how mineralisation and bone formation are controlled, although a number of molecules have been implicated. These include soluble growth factors (e.g. TGF-β, IGF-1 and bone morphogenetic proteins), hormones (e.g. parathyroid hormone and calcitonin), matrix macromolecules (e.g. type I collagen, decorin and biglycan) and bone-related proteins (e.g. osteocalcin, osteonectin, bone sialoprotein and osteopontin) (Raisz, 1988; Gowen, 1992; Price and Russell, 1992; Denhardt and Guo, 1993; Kingsley, 1994; Bonewald and Dallas, 1994). Recent evidence has suggested that the matrix macromolecule thrombospondin-1 (TSP-1) is also involved in bone formation, although the precise role played by this protein remains to be determined (Gehron-Robey et al., 1989; Sherbina and Bornstein, 1992; Grzesik and Gehron-Robey, 1994).

TSP-1 is a large (M_r 420,000) trimeric modular protein released from platelet α-granules and also secreted by a wide variety of cell types including fibroblasts, endothelial cells, pericytes and osteoblasts (Clezardin et al., 1989; Canfield et al., 1990a; Frazier, 1991). This protein can bind to other matrix proteins and to the cell surface and may therefore play a crucial role in modulating cell-matrix interactions during development (Sage and Bornstein, 1991; Frazier, 1991; Adams and Lawler, 1993). TSP-1 is found mainly in the periosteum and in the osteoid layer on the surface of the mineralised bone, whereas little TSP-1 is detected within the mature bone matrix (Gehron-Robey et al., 1989; Grzesik and Gehron-Robey, 1994). Sherbina and Bornstein (1992) found that changes in the level of alkaline phosphatase activity in a mouse osteogenic cell line (MC3T3-E1) were closely paralleled by changes in the level of TSP-1 mRNA; based on these results, they suggested that TSP-1 may be a marker for osteoblast differentiation.

Osteogenesis is normally preceded by the ingrowth of new blood vessels (Sevitt, 1981). Pericytes form an integral part of the microvasculature, being embedded within the basement membrane of both mature and newly-formed microvessels (Sims, 1991). Recent evidence has shown that microvascular pericytes can differentiate along the osteogenic pathway in vitro and it has therefore been suggested that these cells may represent a source of osteoprogenitor cells in the adult (Schor et al., 1990, 1995; Canfield et al., 1990a; Brighton et al., 1992). The differentiation of pericytes into osteoblast-like cells was also found to be associated with the expression of alkaline

phosphatase, osteonectin and osteocalcin and the synthesis of several matrix macromolecules including: collagen types I and IV, BRP collagen (which is related to collagen type X), laminin, tenascin and TSP-1 (Schor et al., 1990, 1991, 1995; Canfield et al., 1990a; Canfield and Schor, 1991; Brighton et al., 1992).

The objective of the present study has been to investigate the possible role of TSP-1 in the differentiation of pericytes into osteoblast-like cells. This has involved determining the expression and localisation of TSP-1 at specific stages of pericyte differentiation by SDS-PAGE, northern analysis, immunolocalisation and in situ hybridisation. Functional assays have also been used to demonstrate the involvement of TSP-1 in pericyte mineralisation.

MATERIALS AND METHODS

Cells and culture conditions

Pericytes were isolated from the retinal microvasculature of adult cows as previously described (Schor and Schor, 1986). The cells were routinely grown on plastic tissue culture dishes in Eagle's minimum essential medium (MEM) supplemented with 20% donor calf serum, 50 μg/ml ascorbic acid, 2 mM glutamine, 1 mM sodium pyruvate and non-essential amino acids (Gibco-Biocult Ltd, Uxbridge, UK). This complete growth medium will be referred to hereafter as 20% DCS-MEM. Medium was changed on stock cultures three times a week. When nearly confluent, stock cultures were subcultured in a 1:2 split ratio using 0.05% crystalline trypsin (Gibco) and 2 mM EGTA in Dulbecco's phosphate buffered saline (trypsin-EGTA). Cultures were incubated at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air. The cells were free from mycoplasmal contamination as assessed by fluorescent Hoechst 33256 stain (Chen, 1977).

The cells used in this study were identified as pericytes according to the following criteria: (i) morphology; (ii) lack of Factor-VIII related antigen and glial fibrillary acidic protein; (iii) presence of α-smooth muscle actin and high molecular weight-melanoma associated antigen; (iv) ability to contract three-dimensional collagen gels; and (v) migratory behaviour on these gels as previously reported (Schor et al., 1990, 1991, 1995). The results presented in this communication were obtained using 8 different lines of pericytes between passages 1 and 4.

Experimental protocols

Pericytes were plated at sparse densities (between 1.1 and 1.25×10^4 cells/cm^2) on plastic tissue culture dishes and maintained in the presence of 20% DCS-MEM for up to 35 days. The cultures were examined when they reached the following stages: (i) when the cells were semiconfluent, occupying approximately 40-50% of the area of the dish (days 2 to 3); (ii) at confluence (between days 4 and 7); (iii) when large multicellular nodules were present (between days 12 and 20); and (iv) when the nodules had mineralised (between days 22 and 35). For most experiments, the cultures were analysed at the last 3 stages described above.

Cell numbers were determined with a Coulter electronic particle counter (Schor and Schor, 1986). The DNA content of the cultures was determined as described by Leyva and Kelley (1974). Duplicate cultures were monitored daily for nodule formation and mineralisation using phase contrast microscopy. Mineralised nodules appear opaque under phase contrast (Schor et al., 1990). To quantitate the number of nodules, the cultures were placed on a grid and both the total number of nodules present and the number of mineralised nodules per culture were counted.

To test the effects of TSP-1 and TGF-β1 (TGF-β) on pericyte mineralisation, cultures were incubated in the presence of: (i) specific antibodies against these proteins; and (ii) exogenous TSP-1 or TGF-β. Briefly, pericytes were plated at 1.2×10^4 cells/cm^2 in either 2 cm^2 or 8 cm^2 dishes in 20% DCS-MEM. The following day, the medium was changed and either antibodies to TSP-1, antibodies to TGF-β, or exogenous human TGF-β (R&D Systems; 0.05 to 5 ng/ml) were added and the incubations continued for up to 30 days. Normal rabbit serum was added to the control dishes. The antibody to TGF-β used was a polyclonal anti-porcine TGF-β1 neutralizing antibody (Catalogue code BDA19, R & D Systems, Oxford, England). It was used at 20 μg/ml. According to the manufacturer's data, 2-3 μg/ml of this antibody will neutralise 50% of the biological activity due to 250 pg of TGF-β1. We have confirmed this data using the inhibition of Mv1Lu epithelial cells as a bioassay (not shown). In addition, using the induction of α-smooth muscle actin expression by endothelial cells as a bioassay (Arciniegas et al., 1992), we have shown that 20 μg/ml of this antibody neutralises the activity of 5 ng/ml of TGF-β1 (unpublished data). This antibody has been previously shown to neutralise the activity of porcine, human and bovine TGF-β1 (Keski-Oja et al., 1987; Sato and Rifkin, 1989). The anti-TSP-1 antibodies used were: (i) a polyclonal antibody raised against human platelet TSP-1 (20 μl/ml medium; antibody kindly provided by Dr N. R. Hunter, SNBT National Science Laboratory, Edinburgh, UK); and (ii) a monoclonal antibody which recognises the 70 kDa core fragment of TSP-1 (3 μl/ml medium; Sigma Chemical Co., Poole, Dorset, UK; Dardik and Lahav, 1991). In pilot experiments, these antibodies were tested at various dilutions; the final dilutions used, shown above within brackets, were those which produced maximum effect on mineralisation without affecting cell proliferation or viability. For example, the monoclonal antibody to TSP-1 used at concentrations above 7 μl/ml significantly reduced the number of pericytes present in semiconfluent cultures after 3 days incubation (results not shown). The polyclonal antibody did not affect cell numbers at the concentrations tested. Fresh antisera or protein were added every time the medium was changed (three times per week). Exogenous TSP-1 (20 μg/ml; Calbiochem Novabiochem Ltd, Beeston, Nottingham, UK) was added to post-confluent cultures containing non-mineralised nodules. Higher concentrations of exogenous TSP-1 (50 μg/ml) have previously been shown to inhibit mitogenesis induced by 10% FCS (Taraboletti et al., 1990). After 24 hours, disodium β-glycerophosphate (10 mM; Sigma) was added to the cultures and these were maintained for a further 11 day period. Fresh TSP-1 and β-glycerophosphate were added to the cultures every time the medium was changed. Control cultures were maintained in the presence of β-glycerophosphate alone.

The formation of nodules and mineralisation of the pericyte nodules was monitored by microscopic examination as described above. All experiments were conducted a minimum of three times with duplicate dishes being used for each determination. The two-tailed Student's *t*-test was used to determine the significance of differences between means.

Immunohistochemical staining

The presence of TSP-1 in pericyte cultures was assessed by indirect immunofluorescence according to standard techniques (Schor et al., 1991). The monoclonal antibody to TSP-1 (MA-IV; kindly provided by Dr J. Lawler, Dept of Pathology, Harvard Medical School, Boston, USA) and normal mouse serum were used at a 1:50 dilution in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin and incubated for 1 hour at room temperature. The second antiserum (fluorescein isothiocyanate-conjugated anti-mouse IgG; Dako Ltd, High Wycombe, Bucks., UK) was applied for 1 hour at room temperature. Photographs were taken with an automatic camera; the exposure time was generally between 30 and 90 seconds, negative controls were stopped manually after 5 minutes exposure.

In situ hybridisation

Pericytes were grown on tissue culture slides (Gibco) in 20% DCS-MEM. When large, mineralised nodules were present in the cultures,

the cells were fixed for 1 hour in 4% (w/v) paraformaldehyde in PBS and then washed in PBS. The prehybridisation treatments used were essentially as described previously (Hoyland et al., 1991). Briefly, the slides were immersed sequentially in 0.2 M HCl (20 minutes), 2× SSC (1× SSC (standard saline citrate) = 0.15 M sodium chloride and 0.015 M sodium citrate; 10 minutes), and PBS (10 minutes). Control slides were then treated with 10 mg/ml RNase A (Boehringer Mannheim, Lewes, East Sussex, BN7 1LG, UK) in 0.5× SSC for 1 hour at 37°C and rinsed in PBS. All slides were post-fixed for 20 minutes in 0.4% (w/v) paraformaldehyde in PBS and then immersed for 10 minutes in freshly prepared 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine, pH 8.0. Slides were prehybridised for 1 hour at 37°C in 50% formamide, 1 mg/ml of bovine serum albumin, 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinyl pyrrolidone, 0.6 M NaCl, 0.2 mg/ml of sheared salmon sperm DNA, 10 mM Tris (pH 7.4), 0.5 mM EDTA, 10 mM dithiothreitol (DTT) and 10% (w/v) dextran sulphate. Hybridisation with aliquots (50 μl) of heat-denatured ^{35}S-labelled probe (100 ng/ml prehybridisation mixture) was carried out overnight in prehybridisation solution. Hybridisation probes were prepared by random oligolabelling with [α-^{35}S]dCTP (Amersham International, Bucks., UK) to specific radioactivities of approximately 1×10^8 cpm/μg of DNA. The cDNA probe used in this study was a 1,100 bp *Eco*RI insert in pGEM-2 coding for amino acids at the N terminus of human endothelial thrombospondin-1 (Lawler and Hynes, 1986; kindly provided by Dr J. Lawler). After hybridisation, the slides were washed with a series of high stringency washes: twice for 5 minutes in 0.5× SSC containing 1 mM EDTA and 10 mM DTT; twice for 5 minutes in 0.5× SSC with 1 mM NaCl; 15 minutes in 50% formamide, 0.15 M NaCl, 5 mM Tris-HCl, pH 7.5, and 0.5 mM EDTA; four times 5 minutes in 0.5× SSC at 55°C, followed by 5 minutes at room temperature in 0.5× SSC. Slides were then dehydrated in 70% and 95% ethanol with 0.3 M ammonium acetate and air dried. Autoradiography was performed with Ilford K5 emulsion melted at 40°C and diluted 1:1 with distilled water. The slides were exposed at 4°C for 14 days and then developed in Kodak D-19 developer for 5 minutes, rinsed, fixed for 5 minutes, and counterstained with hematoxylin and eosin.

Radiolabelling of cell cultures and analysis of newly synthesised proteins

At specific time points (i.e. confluent, non-mineralised nodule and mineralised nodule stages), pericyte cultures were incubated with 20% DCS-MEM containing 20 μCi/ml of [^{3}H]proline (Amersham) for 24 hours. At the end of the incubation period, the medium was collected and the attached cell layer/matrix washed twice with cold Eagle's MEM. The medium and the washings were combined and phenylmethanesulphonyl fluoride (2 mM; Sigma) and *N*-ethylmaleimide (10 mM; Sigma) added to the final concentrations indicated. Proteins present in the cell layer/matrix were extracted with 4 M guanidinium chloride/50 mM Tris-HCl, pH 7.4, for 24 hours at 4°C. Guanidinium chloride-insoluble material was removed by centrifugation (15,000 ***g*** for 20 minutes at 4°C) and the supernatant dialysed extensively against 0.5 M acetic acid at 4°C (Canfield et al., 1994). The incorporation of radioactivity into newly synthesised proteins secreted into the medium and present in the cell layer/matrix was determined as previously described (Canfield et al., 1994). Samples were either analysed immediately or stored at −20°C before analysis.

Immunoprecipitation

Thrombospondin was identified by immunoprecipitation using a polyclonal antibody raised against human platelet thrombospondin (kindly provided by Dr N. R. Hunter) as previously described (Canfield et al., 1990a,b).

Electrophoretic analysis

Newly synthesised proteins secreted into the medium by retinal pericytes were examined by discontinuous SDS-PAGE under reducing conditions using 6% polyacrylamide gels and were visualised by fluorography as described elsewhere (Canfield et al., 1990a). An equal number of cpm were loaded onto each track to permit comparison of the relative levels of specific proteins; ^{14}C methylated M_r standard proteins were run in parallel; these 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) and carbonic anhydrase (M_r 30,000) (Amersham). Measurements of the relative intensities of specific bands of interest was carried out with a TV monochrome camera (Bosch TYK) linked to a microcomputer-based image analysis system (an Olivetti M28 computer with a Matrox PIP 1024 frame-grabber; Matrox Electronics Systems Ltd, Quebec, Canada) as described by Thornton et al. (1989).

RNA isolation and northern blot analyses

Total RNA was prepared from pericyte cultures using RNAzol (Biogenesis Ltd, Bournemouth, UK) according to the manufacturers' instructions. 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 (Sambrook et al., 1989).

Samples of RNA (10 μg) obtained from pericyte cultures were separated in 1% agarose gels containing 2.2 M formaldehyde, transferred to nylon membranes (Hybond-N; Amersham) by capillary action and fixed by UV irradiation. Hybridisation probes were prepared by random oligolabelling with [α-^{32}P]dCTP using a Pharmacia kit to specific radioactivities of 1×10^9 cpm/μg of DNA. The cDNA probe used in this study was a 1,100 bp *Eco*RI insert in pGEM-2 coding for amino acids at the N terminus of human endothelial thrombospondin-1 (Lawler and Hynes, 1986). Filters were prehybridised and hybridised at 65°C in phosphate buffer containing 1% BSA and 7% SDS (Church and Gilbert, 1984). Filters were washed in 2× SSC, 1% SDS at 65°C for 15 minutes, and in several washes of 0.2× SSC, 0.1% SDS at 65°C for 1 hour. Filters were then exposed to Kodak X-Omat AR-film at −70°C. Measurements of the relative intensities of specific bands of interest were carried out as described above.

RESULTS

Differentiation of pericytes: DNA and total protein synthesis

Bovine retinal pericytes reproducibly differentiate into an osteogenic phenotype in vitro (Schor et al., 1990, 1991, 1995). After reaching confluence ($4\text{-}6\times10^4$ cells/cm^2) on 2-D substrata (Fig. 1A), pericytes do not become contact-inhibited, but continue to proliferate forming multicellular areas which retract, giving rise to nodules (Fig. 1B). These nodules are composed of viable cells and extracellular matrix; the latter becomes gradually mineralised, giving an opaque appearance under phase microscopy (Fig. 1C). The presence of bone-like mineral deposits in the extracellular matrix of these nodules has been confirmed by electron microscopy, X-ray microprobe analysis and histochemical staining (Schor et al., 1990).

Pericyte cultures were examined at the three stages of their differentiation shown in Fig. 1, namely: (a) when the cells were confluent; (b) when large non-mineralised nodules were present in the cultures (between 12 and 20 days after plating); and (c) when these nodules were heavily mineralised (between 10 and 15 days after nodule formation).

Table 1 shows the DNA contents of pericyte cultures at these

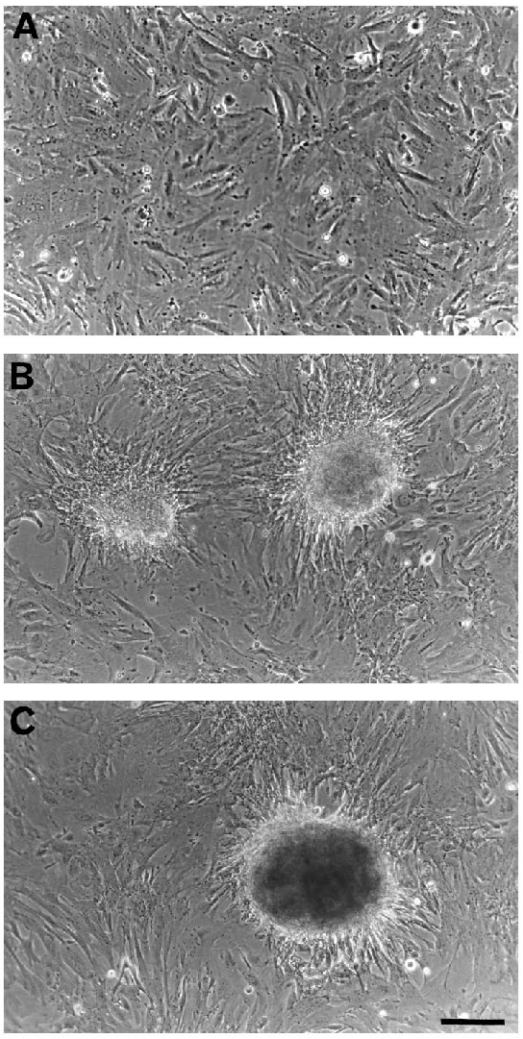

Fig. 1. Pericyte differentiation in vitro. Phase contrast micrographs of pericyte cultures. (A) Pericytes display a characteristic stellate morphology at confluence. (B) Multicellular nodules are formed in post-confluent cultures. (C) The matrix within these nodules becomes mineralised a few days later, giving an opaque appearance to the nodules. Bar, 150 μm.

three stages. The data demonstrate that pericyte cultures containing mineralised nodules have a significantly higher DNA content (indicating a higher cell number) than either confluent cultures or cultures containing non-mineralised nodules. Total protein synthesis was also measured in pericyte cultures at the three stages described above. In these experiments, the cells were incubated with [^{3}H]proline for 24 hours and the amount of radioactivity incorporated into newly synthesised proteins secreted into the medium and deposited into the cell layer/matrix was determined. Between 30% and 50% of the total [^{3}H]proline incorporated into newly synthesised proteins were secreted into the medium, the remaining 50% to 70% were associated with the cell layer/matrix. The medium and cell layer/matrix values were pooled to give total protein synthesis (in cpm) per culture shown in Table 1; these values have also been expressed per μg DNA (Table 1). The results show that pericytes actively synthesise proteins at the three stages tested; protein synthesis per μg DNA is similar at confluence and nodule stages but decreases significantly once the nodules have mineralised.

Table 1. DNA contents and total protein synthesis by pericytes at different stages of differentiation

Stage	Total cpm/culture ($\times10^4$)	DNA (μg/dish)	cpm/μgDNA
C	81±5	12±2	6.9±1.2
N	115±9	15±2	7.8±1.2
M	48±4	24±1.5*	2.0±0.3*

BRP were plated at 1.2×10^4 cells/cm^2 and maintained in 20% DCS-MEM for 30 days. Duplicate cultures were labelled with [^{3}H]proline (20 μCi/ml) for 24 hours when the cells were confluent (C), when non-mineralised nodules were present (N) and when the nodules had mineralised (M). The total incorporation of radioactivity into newly synthesised proteins was determined as described in Materials and Methods. The proteins secreted into the medium represented 30-50% of the total proteins synthesised. DNA was also analysed at the three stages shown. Results represent the mean ± s.d. of duplicate cultures. DNA contents and cpm/μg DNA at the mineralised stage (M), marked with an asterisk, are significantly different from the corresponding values at the confluent (C) and non-mineralised nodule (N) stages (P<0.05).

The expression of TSP-1 during pericyte differentiation in vitro

The synthesis of TSP-1 by bovine retinal pericytes was examined at the three stages of differentiation described above, namely: (a) at confluence, (b) when non-mineralised nodules were present and (c) when the nodules had mineralised. Pericytes were incubated with [^{3}H]proline at these three stages and the newly synthesised proteins secreted into the medium were analysed as described in Materials and Methods. An equal number of cpm were loaded onto each track of the polyacrylamide gel or were immunoprecipitated with antibodies to TSP-1. This protocol allowed us to compare the amount of TSP-1 synthesised by the cells, relative to total protein synthesis at each stage. Fig. 2 shows a fluorogram of total

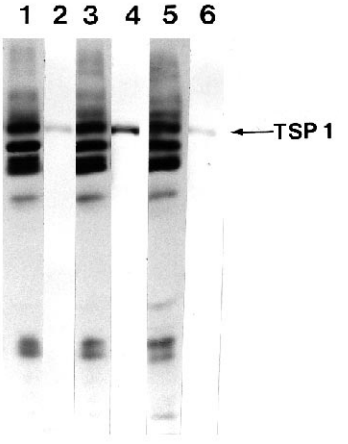

Fig. 2. Thrombospondin-1 synthesis by cultured pericytes. Pericytes were incubated with [^{3}H]proline at: (a) confluence (tracks 1 and 2); (b) when nodules were present in the cultures (tracks 3 and 4); and (c) when these nodules had mineralised (tracks 5 and 6). The newly synthesised proteins secreted into the medium were then analysed by SDS-PAGE and fluorography before (tracks 1, 3 and 5) and after (tracks 2, 4 and 6) precipitation with antibodies to TSP-1, as described in Materials and Methods. The position of migration of TSP-1 is shown.

proteins secreted into the medium (tracks 1, 3 and 5; representing stages a, b and c, respectively) and the proteins immunoprecipitated with antibodies to TSP-1 (tracks 2, 4 and 6). This figure shows that the relative synthesis of TSP-1 was highest in cultures containing non-mineralised nodules compared to confluent cultures (Fig. 2, compare tracks 2 and 4) or cultures containing mineralised nodules (Fig. 2, compare tracks 4 and 6). The ratios of TSP-1 in confluent cultures, cultures containing non-mineralised nodules and cultures containing mineralised nodules was 1:4:1 (respectively) in the fluorogram shown in Fig. 2. In other experiments, such ratios varied between 1:2:1 and 1:8:1 (not shown).

In order to determine whether the regulation in TSP-1 expression occurred at the level of transcription or translation, total RNA was prepared from pericyte cultures at the same three stages of differentiation and subjected to northern analysis. Fig. 3A shows the results obtained when the filter was hybridised with an α-^{32}P labelled cDNA probe for TSP-1; Fig. 3B shows the ethidium bromide-stained gel which confirms that equal amounts of RNA were loaded onto the gel. The cDNA probe for TSP-1 hybridised with 2 bands of approximately 6.1 kb and 7.5 kb (Fig. 3A). The principal TSP-1 message has previously been shown to be 6.1 kb; however, an extra band at 7.5 kb has also been described in other cell lines and tissues (Gehron-Robey et al., 1989; Labell et al., 1992). Preliminary experiments indicated that the 7.5 kb message in pericyte RNA was not related to TSP-2. Thus, when the filters were hybridised with a cDNA probe to the 3′ untranslated region of TSP-2 (Labell et al., 1992) no message was detected (results not shown). Fig. 3 shows that cultures with non-mineralised nodules expressed significantly more 6.1 kb message for TSP-1 than those cultures which were either confluent (arrowheads; compare tracks 1 and 2) or contained mineralised nodules (arrowheads; compare tracks 2 and 3). The level of the initial increase in TSP-1 mRNA was found to vary from experiment to experiment (between 2- and 10-fold), suggesting that the levels of TSP-1 gene expression may be dependent on the precise stage of pericyte differentiation at which the RNA was collected. By comparison, the levels of the 7.5 kb message remained relatively constant during pericyte differentiation.

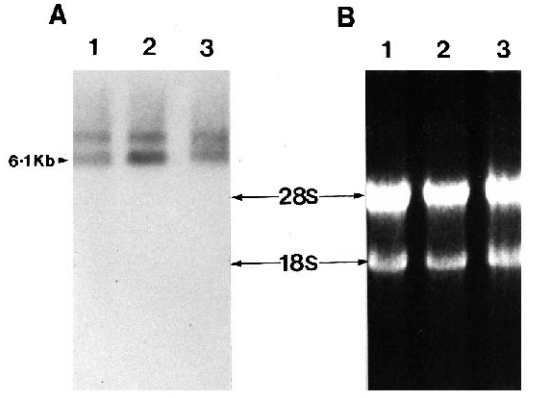

Fig. 3. Northern blot analysis of mRNA for TSP-1 in cultured pericytes. Total RNA was isolated from cultures of retinal pericytes at the same three stages of their differentiation as shown in Fig. 2. That is: at confluence (track 1); when nodules were present (track 2); and when these nodules had mineralised (track 3). Samples (10 μg) were subjected to northern analysis using α-^{32}P labelled cDNA probe for TSP-1, as described in Materials and Methods. (A) Hybridisation with the cDNA probe for TSP-1. (B) Ethidium bromide staining, confirming that equal amounts of RNA were loaded onto each track of the gel.

Localisation of TSP-1 in pericyte cultures

Immunofluorescence microscopy revealed that TSP-1 was present in pericyte cultures at every stage of their growth and differentiation (Figs 4, 5). When the cells were semi-confluent, TSP-1 showed a cytoplasmic, perinuclear location (Fig. 4A,B). In confluent and post-confluent cultures a dense, fibrillary, extracellular staining was also observed (Fig. 4C,D). Multilayered areas and nodules stained strongly with antibodies to TSP-1. Frequently, the periphery of the nodule stained more intensely than the centre mass (Fig. 4E,F). Controls, including the use of normal rabbit serum (Fig. 4G,H) or normal mouse serum (not shown) instead of the TSP-1 antiserum were negative. As the nodules mineralised, the difference in the intensity of staining between the periphery and the centre of the nodules became more apparent, so that the staining often appeared as a 'halo' surrounding a weakly positive or negative nodule (Fig. 5A,B). Focusing within such a nodule revealed discrete positive areas (Fig. 5C-F), amongst weakly positive or negative areas. Interestingly, we have previously shown that cell density is highest in the surface of the nodules (Canfield et al., 1990a); however, mineralised pericyte nodules stain uniformly with antibodies to laminin, type IV collagen and type X collagen (Schor et al., 1991).

After nodule formation, pericyte cultures contain cells at various stages of growth and differentiation (Schor et al., 1990). Therefore, we used in situ hybridisation to determine whether mRNA for TSP-1 was expressed uniformly by all cells in pericyte cultures or only by cells at specific stages of differentiation. We found an intense hybridisation signal with TSP-1 cDNA throughout the pericyte cultures. That is, TSP-1 message was expressed by cells in sparse (proliferating), confluent and multilayered areas, as well as in non-mineralised and mineralised nodules. A mineralised nodule surrounded by a monolayer area is shown in Fig. 6A. Due to the high cell density at the surface of the nodule (Schor et al., 1990; Canfield et al., 1990a), and to the particular plane of focus used, the message appears to be concentrated in the periphery of the nodule in this figure. RNase digestion eliminated the reaction (Fig. 6B).

Taken together, our results suggest that the majority of cells within pericyte cultures synthesise TSP-1, irrespective of their growth or differentiation status. The level of synthesis, however, depends on the stage of differentiation. Furthermore, TSP-1 is largely excluded from the mineralised matrix of the nodules.

The role of TSP-1 in pericyte mineralisation

The results presented above demonstrated that the synthesis of TSP-1 by pericytes was dependent upon the stage of differentiation of the cells. We next investigated whether TSP-1 played a role in the mineralisation of these cells by incubating cultures of pericytes with: (i) antibodies to TSP-1 and (ii) exogenous TSP-1. The rate and extent of mineralisation of the nodules was then assessed by microscopic examination of the cultures as described in Materials and Methods.

For these experiments, two different lines of bovine retinal pericytes and two different antibodies were used. The antibodies were: (i) a polyclonal antibody raised against human

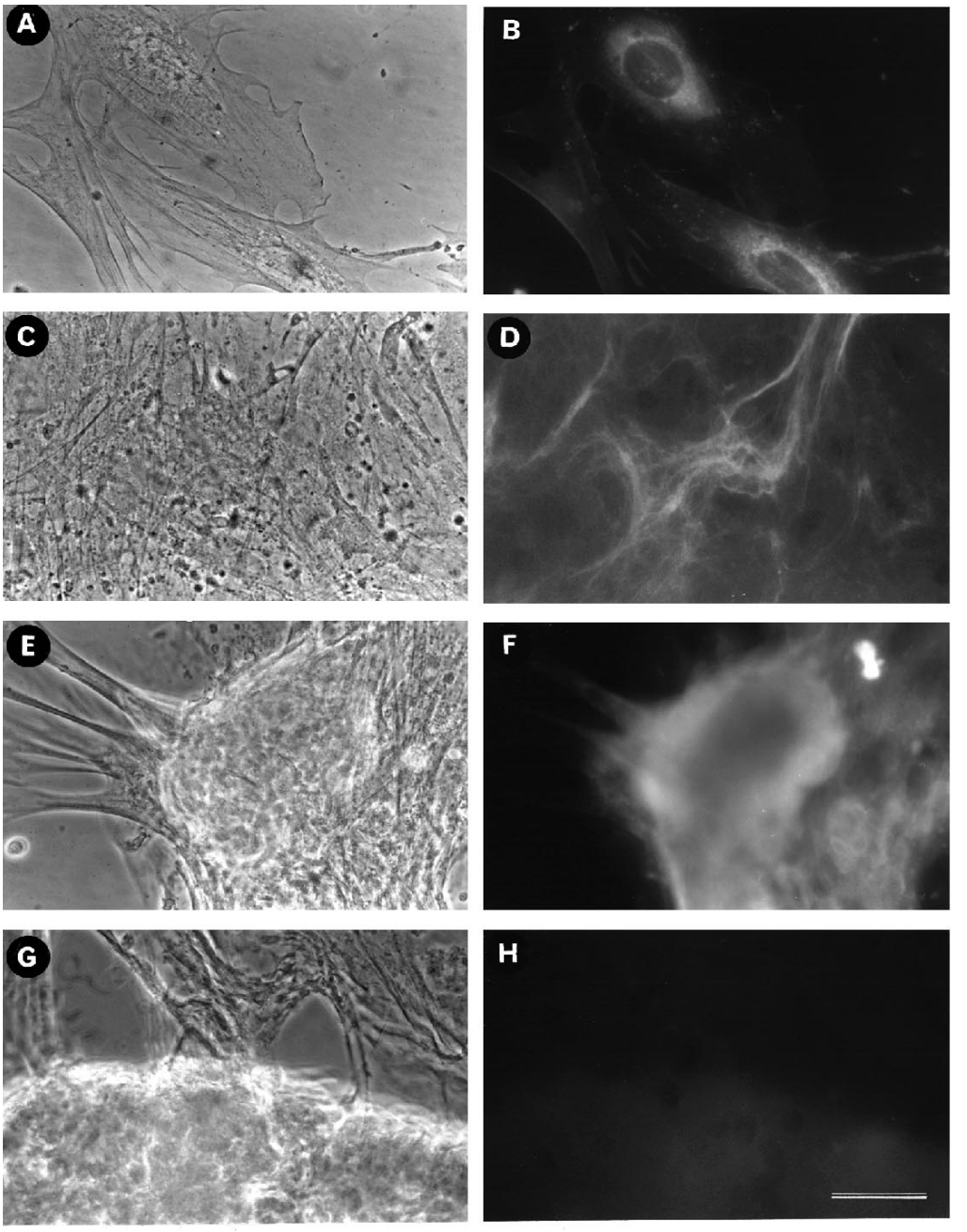

Fig. 4. Immunolocalisation of TSP-1 in pericyte cultures before mineralisation. Pericytes were grown in 20% DCS-MEM until the cells were semi-confluent (A and B), 1-2 days post-confluent (C and D) or nodules were present (E-H). At these times, cultures were stained either with monoclonal antibodies to TSP-1 (MA-IV; A-F) or normal rabbit serum (G and H), as described in Materials and Methods. (A,B) Semi-confluent culture of pericytes. Phase (A) and immunofluorescence (B) micrograph of the same field showing a cytoplasmic, perinuclear staining for TSP-1. (C,D) Early post-confluent culture of pericytes. Phase (C) and immunofluorescence (D) micrographs of the same field showing a dense, fibrillary staining of the matrix. (E,F) Culture of pericytes containing non-mineralised nodules. Phase (E) and immunofluorescence (F) micrographs of the same field showing that the nodules stain strongly with anti-TSP sera. (G,H) Culture of pericytes containing non-mineralised nodules. No staining was seen with normal rabbit serum. Bar, 150 μm.

platelet TSP-1; and (ii) a monoclonal antibody which recognises the 70 kDa core fragment of TSP-1. Retinal pericytes were plated at sparse densities and allowed to attach for 24 hours in 20% DCS-MEM. At this time medium containing either antibodies to TSP-1 or normal rabbit serum was added to duplicate cultures and the incubations continued for a further 20 to 32 days. Fresh antisera were added every time the medium was changed. At the doses used in these experiments, the antisera to TSP-1 did not affect the rate of cell proliferation (not shown) or nodule formation (Table 2). However, in the presence of antibodies to TSP-1, mineralisation began immediately after the nodules had formed and proceeded rapidly so that within 5 days the nodules had become heavily mineralised. At this time (up to 17-20 days after plating) no mineralisation was seen in the control cultures (Table 2 and Fig. 7). The control cultures proceeded to mineralise at the normal (i.e. slower) rate, with nodules reaching the extent of mineralisation shown in Fig. 7B, 15 days later (Table 2). The increased rate of mineralisation of pericyte nodules in the presence of antibodies to TSP-1 was observed in four separate experiments with both antibodies.

Conversely, the addition of exogenous TSP-1 to cultures of pericytes was found to inhibit mineralisation of the nodules. These experiments were done three times, using two different lines of bovine retinal pericytes. The cells were grown in 20% DCS-MEM until large (non-mineralised) nodules were present. TSP-1 (20 μg/ml) was then added to half of the cultures; 24 hours later, sodium-β-glycerophosphate was added to every culture, as this compound has previously been shown to accelerate pericyte mineralisation (Schor et al.,

1990). Cells were then grown in the continual presence of either TSP-1 and β-glycerophosphate (test cultures) or β-glycerophosphate alone (control cultures); mineralisation of the nodules was estimated by microscopic observation as described in Materials and Methods. The rate of nodule formation, as well as the rate and extent of mineralisation of the nodules, was found to vary between the two cell lines used in these studies. Nodule formation and mineralisation was therefore assessed 4 and 11 days after the addition of TSP-1 as indicated in Table 3. The results (Table 3) show that exogenous TSP-1 inhibited mineralisation. Thus, the total number of nodules present on day 11 (two experiments) was reduced in the presence of TSP-1 by comparison to the control cultures; this inhibition was statistically significant ($P<0.05$) in experiment C (asterisk in Table 3). This decrease was not, however, apparent on day 4 (experiment B). The total number of mineralised nodules per culture was reduced by the presence of exogenous TSP-1 in all three experiments; this reduction was significant ($P<0.05$) in experiments B and C.

The role of TGF-β in pericyte mineralisation

TGF-β1 (TGF-β) has previously been shown to inhibit nodule formation and mineralisation in a number of different cell lines in vitro (Bonewald and Dallas, 1994). As latent TGF-β can be activated by TSP-1 (Schultz-Cherry and Murphy-Ullrich, 1993; Schultz-Cherry et al., 1994), we investigated whether the effects of TSP-1 on pericyte mineralisation were mediated through an indirect effect on TGF-β activity. Sparse cultures of pericytes were therefore incubated with either exogenous TGF-β or antibodies to TGF-β and the effects on nodule formation and mineralisation were determined. The results shown in Table 4 demonstrate that the addition of TGF-β (1 ng/ml) or antisera to TGF-β (20 μg/ml) to pericyte cultures had no effect on nodule formation or the production of a mineralised matrix by these cells. Thus, on day 16 approximately 45 nodules were present in all of the cultures and none of these were mineralised. By day 30, the number of nodules in these cultures had increased and the majority of these were mineralised (Table 4). In other experiments, TGF-β was added at a range of doses (from 0.05 to 5 ng/ml) and similar results were obtained. These results therefore suggest that the effects of TSP-1 are not mediated through activation of latent TGF-β.

DISCUSSION

It has previously been shown that vascular pericytes can differentiate into osteoblast-like cells in vitro and may therefore represent a source of osteoprogenitor cells in the adult (Schor et al., 1990, 1995; Brighton et al., 1992). The results presented in this paper demonstrate that the matrix glycoprotein TSP-1 plays a role in regulating pericyte differentiation and in the production of a mineralised matrix by these cells and that these effects are not due to activation of latent TGF-β.

Pericyte differentiation is associated with a characteristic

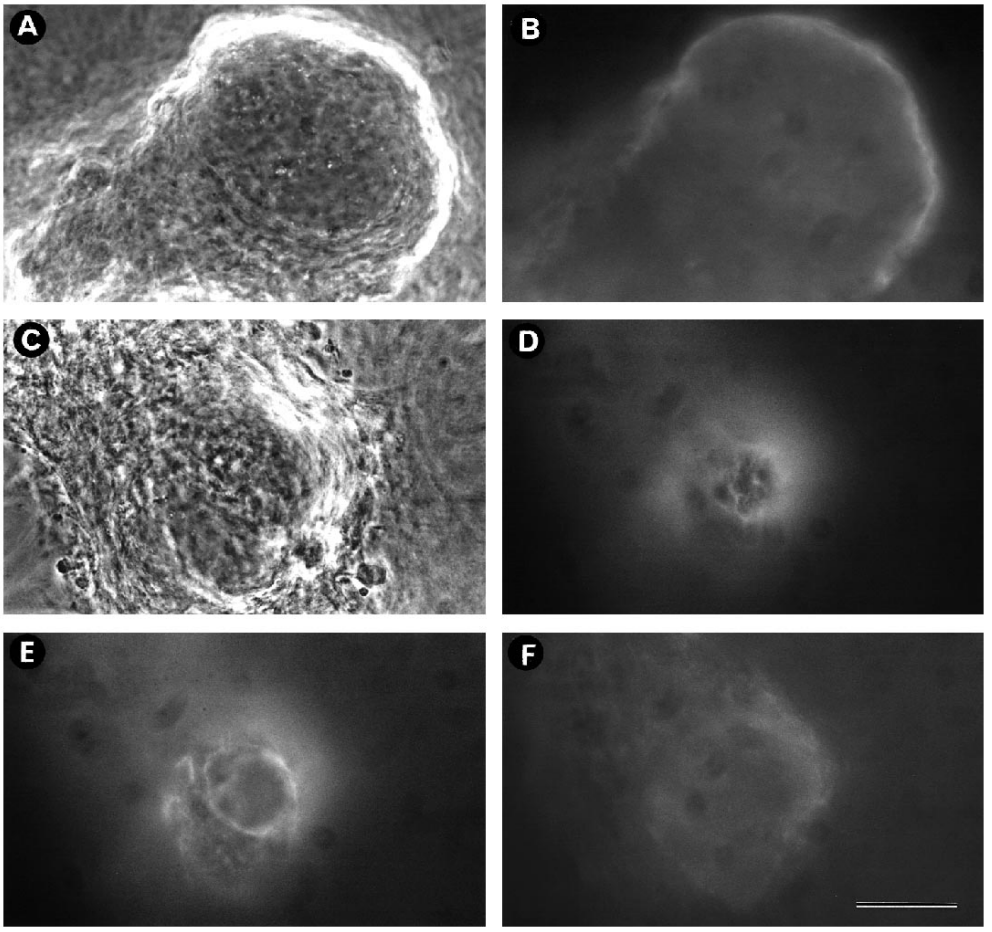

Fig. 5. Immunolocalisation of TSP-1 in mineralised pericyte nodules. Pericyte cultures containing mineralised nodules were stained with antibodies to TSP-1 as described in the legend to Fig. 4. (A,B) Phase (A) and immunofluorescence (B) micrographs of the same field showing that the periphery of the mineralised nodule stains more intensely than the centre mass. (C-F) Phase (C) and immunofluorescence (D-F) micrographs obtained by focusing at different planes through a mineralised nodule. These micrographs show the presence of discrete, positively stained areas within the nodule adjacent to weakly positive and negative areas. Bar, 150 μm.

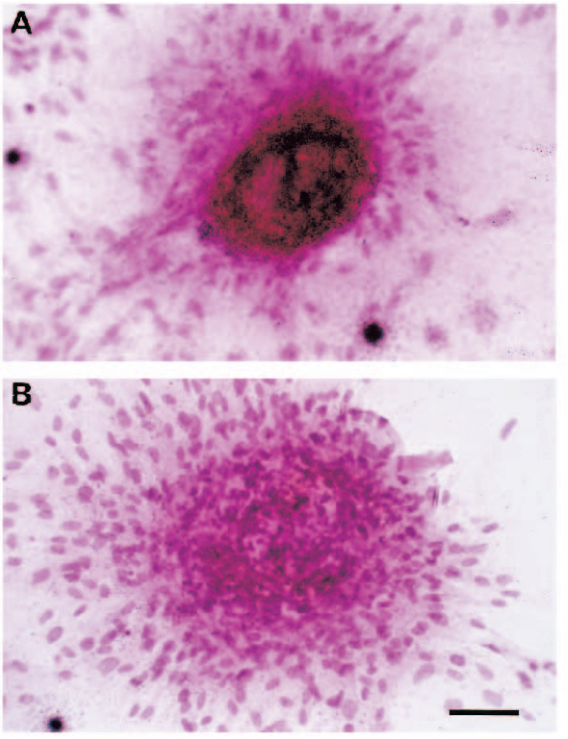

Fig. 6. Expression of thrombospondin-1 mRNA in mineralised pericyte nodules. Pericyte cultures containing mineralised nodules were subjected to in situ hybridisation using a cDNA probe for TSP-1 as described in Materials and Methods. (A) A strong hybridisation signal for TSP-1 mRNA is seen over a mineralised nodule and the surrounding area. (B) RNase-treated control, resulting in the loss of hybridisation signal. Bar, 200 μm.

pattern of cell proliferation, nodule formation and protein synthesis. Pericytes continue to proliferate once confluency has been reached forming multilayered areas which eventually retract to form nodules. Crystals of hydroxyapatite are deposited on the extracellular matrix of these nodules which then become mineralised. Identification of mineral deposits within the nodules has been confirmed by electron microscopy, histochemical staining and X-ray microprobe analysis (Schor et al., 1990). Nodule formation and mineralisation is a reproducible event in pericyte cultures. However, the time taken for mineralisation to occur, and the extent of mineralisation, are dependent on a number of factors, including: (i) the plating density of the cells; (ii) the passage number of the cells; (iii) the substratum on which the cells are plated (i.e. mineralisation occurs more quickly on a native collagen substratum than on plastic tissue culture dishes); and (iv) the presence of exogenous factors in the growth medium (i.e. β-glycerophosphate increases the rate of mineralisation whereas levamisole inhibits mineralisation) (Schor et al., 1990, 1995).

Both the nature and the total amount of proteins synthesised by pericytes varies according to the state of differentiation of the pericyte cultures. Our results demonstrate that pericytes are most active in protein synthesis at confluence and when nodules are present in the cultures. Once these nodules have mineralised, the incorporation of radioactivity into newly synthesised proteins decreases markedly. Cultured pericytes synthesise a number of extracellular matrix components,

Table 2. The effects of antibodies to TSP on nodule formation and mineralisation

	Nodules in early cultures		Nodules in late cultures	
Additive	Number	% Mineralised	Number	% Mineralised
nrs(20 μl/ml)	93±7	0	120±15	95±3
Ab to TSP (monoclonal, 3 μl/ml)	88±11	66±5*	131±12	99±1
Ab to TSP (polyclonal, 20 μl/ml)	85±8	88±4*	125±11	98±2

BRP were plated in 20% DCS-MEM at 1.2×10^4 cells/cm^2 in 2 cm^2 dishes. The following day the medium was changed and either normal rabbit serum (nrs) or antisera to TSP-1 were added. Medium and additives were replaced every 2-3 days for up to 32 days. The number of nodules and the percentage of mineralised nodules were determined on day 17 (early cultures) and day 32 (late cultures). An asterisk denotes values significantly different (P<0.005) from the nrs controls.

including: collagens I, III and IV, BRP collagen, laminin, fibronectin, tenascin (Canfield and Schor, 1991; Canfield et al., 1990a; Schor et al., 1991) and TSP-1 (this paper, Figs 2-6). The results presented in this paper demonstrate that TSP-1 gene expression is modulated during pericyte differentiation in vitro, with the highest levels of TSP-1 being expressed at the

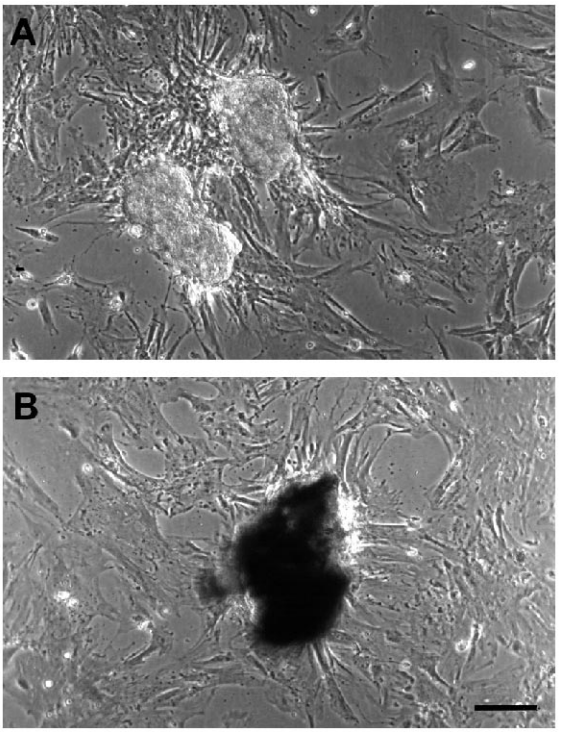

Fig. 7. The effects of antibodies to TSP-1 on the mineralisation of pericyte nodules. Pericytes were plated at semi-confluent density and allowed to attach for 24 hours in standard growth medium. At this time, medium containing either antibodies to TSP-1 or normal rabbit serum was added to duplicate cultures and the incubations continued for a further 20 to 30 days, as described in Materials and Methods. (A) A 20 day culture incubated with normal rabbit serum. No mineralisation of the nodules is seen. (B) Corresponding 20 day culture incubated with antibodies to TSP-1. These nodules have become heavily mineralised. Bar, 150 μm

Table 3. The effects of exogenous TSP-1 on nodule formation and mineralisation in pericyte cultures

		Number of nodules per culture			
		Total		Mineralised	
Expt	Day	C	TSP-1	C	TSP-1
A	11	90±29	47±7	38±15	10±6
B	4	53±11	51±4	16±1	1.5±0.5*
C	11	312±46	143±24*	71±5	35±8*

Pericytes were grown in normal growth medium until large, non-mineralised nodules were present in the cultures. At this time exogenous TSP-1 (20 μg/ml) was added. 24 hours later, disodium-β-glycerophosphate (10 mM) was added to the cultures and the incubations continued for a further 11 day period. Controls (C) were incubated in the presence of disodium-β-glycerophosphate alone. Fresh TSP-1 and disodium-β-glycerophosphate were added every time the medium was changed. Experiment A was conducted with one cell line; experiments B and C were conducted with a different cell line. Duplicate dishes were used in each experiment. The total number of nodules present and the total number of mineralised nodules increased with time in culture and was determined microscopically as described in the Materials and Methods. An asterisk (*) denotes a statistically significant ($P<0.05$) difference between control and TSP-1-treated cultures.

nodule stage (Figs 2, 3); once mineralisation has taken place, TSP-1 synthesis decreases to baseline levels (Figs 2, 3). In the MC3T3-E1 cell line, which also undergoes osteogenic differentiation in vitro, the levels of TSP-1 mRNA have also been found to increase initially, and then decrease markedly (Sherbina and Bornstein, 1992); mineralisation or nodule formation were not reported in this study.

TSP-1 protein, though still synthesised and secreted in cultures containing mineralised nodules, was largely excluded from the mineralised matrix deposited by the cells (Figs 4, 5, 6). In comparison, we have previously shown that mineralised pericyte nodules stained uniformly with antibodies to laminin, type IV collagen and type X collagen (Schor et al., 1991). The apparent absence of TSP-1 from the mineralised matrix deposited by retinal pericytes parallels the localisation of TSP-1 seen in bone in vivo. That is, TSP-1 has been detected mainly in the osteoid and periosteum of human bone whereas little TSP-1 was found in the mature bone matrix (Gehron-Robey et al., 1989; Grzesik and Gehron-Robey, 1994).

The cDNA probe for TSP-1 consistently hybridised with 2 messages of 6.1 kb and 7.5 kb in the pericyte RNA (Fig. 3); by comparison we have previously found that this probe hybridises with only one message (6.1 kb) in endothelial cells (Canfield et al., 1990b). Interestingly, only the 6.1 kb message was modulated during pericyte differentiation (Fig. 3). The identity of the protein encoded by the 7.5 kb message in pericytes is not known, but preliminary data suggest that it is not TSP-2. Two messages of 6.1 kb and 7.5 kb have also been observed in other cell lines and tissue samples using a cDNA probe to TSP-1 (Gehron-Robey et al., 1989; Labell et al., 1992) and it has been suggested that the 7.5 kb message encodes either a protein related to TSP-1 or an additional form of TSP-1 (Labell et al., 1992).

The results presented in this paper also suggest a functional role for TSP-1 in pericyte differentiation and mineralisation since the production of a mineralised matrix by vascular pericytes was promoted by the presence of antibodies to TSP-1 in the culture medium and was inhibited by high levels of exogenous TSP-1 (Fig. 7; Tables 2 and 3). The addition of antibodies to sub-confluent pericyte cultures did not affect the proliferation of the cells or the formation of non-mineralised nodules. These data suggest that TSP-1 is not required for nodule formation and that the high levels of TSP-1 synthesis found at the nodule stage occur either as a consequence of nodule formation or concomitantly with it. The mineralisation of pericyte nodules was promoted by the presence of antibodies to TSP-1 in the culture medium (Table 2). Conversely, the addition of exogenous TSP-1 (20 μg/ml) after nodule formation inhibited mineralisation of the nodules and slowed down the formation of new nodules (Table 3). The possible effects of exogenous TSP-1 on pericyte proliferation have not been determined in this study; however, antibodies to TSP-1 did not affect pericyte proliferation or viability at the concentrations used (not shown). It should be noted that exogenous TSP-1 at higher concentrations (50 μg/ml) than those used in our study have previously been shown to inhibit endothelial cell proliferation induced by 10% foetal calf serum (Taraboletti et al., 1990). Taken together, these results suggest that the high levels of TSP-1 synthesised by cells within the non-mineralised nodules prevents their mineralisation. A marked decrease in TSP-1 synthesis (such as that found from the non-mineralised to the mineralised nodule stages; Figs 2, 3) would therefore be a pre-requisite for mineralisation to occur.

It is now well established that the extracellular matrix (ECM) can regulate cellular differentiation and development in vivo. In addition to providing a structural support for the cells, interactions between cells and the ECM through cell surface receptors can induce transcriptional activation of specific sets of genes by the cells (reviewed by Adams and Watt, 1993). Cell behaviour can also be modulated through the interactions of specific cytokines with certain matrix macromolecules (Nathan and Sporn, 1991; Schubert, 1992). The composition of the ECM in different tissues and at different stages of development is extremely diverse. In this regard, the expression of TSP-1 is particularly high in tissues in which cells are actively undergoing proliferation, migration and adhesion (O'Shea and Dixit, 1988; O'Shea et al., 1990). It has therefore been suggested that this glycoprotein may regulate cell-cell and cell-matrix interactions in areas of tissue morphogenesis (Sage and Bornstein, 1991). Interestingly, TSP-1 has previously been reported to affect cell differentiation. For example, TSP-1 is required for trophoblast outgrowth (O'Shea et al., 1990), the differentiation of embryonal carcinoma cells

Table 4. Effects of TGF-β1 and antibodies to TGF-β1 on nodule formation and mineralisation

	Nodules in early cultures		Nodules in late cultures	
Additive	Number	% Mineralised	Number	% Mineralised
None	45±6	0	89±10	94±5
TGF-β (1 ng/ml)	47±8	0	92±5	95±4
Ab to TGF-β (20 μg/ml)	43±9	0	90±12	93±6

BRP were plated at 1.2×10^4 cells/cm^2 in 2 cm^2 dishes in 20% DCS-MEM. The following day the medium was changed and TGF-β or antibodies to TGF-β were added. Medium and additives were replaced every 2-3 days for up to 30 days. The number of nodules and the percentage of mineralised nodules were determined on day 16 (early cultures) and day 30 (late cultures).

into neurons, glial cells and fibroblasts (Liska et al., 1994) and the early stages of retinoic acid induced neuroblastoma cell differentiation (Castle et al., 1992).

The mechanism whereby TSP-1 exerts its observed effects on mineralisation are not known but may involve: (a) the induction of signal transduction cascades by ligation of its cell surface receptors; (b) interactions with other matrix macromolecules for example, fibronectin, collagen and osteonectin (Frazier, 1991; Clezardin et al., 1988); and/or (c) activation or interactions with cytokines for example TGF-β (Schultz-Cherry and Murphy-Ullrich, 1993; Schultz-Cherry et al., 1994). In this regard, TGF-β has recently been shown to inhibit nodule formation and mineralisation in a number of different cell lines in vitro (reviewed by Bonewald and Dallas, 1994). On the basis of the data presented in this communication, TSP-1 does not appear to exert its effects on pericyte mineralisation through the activation of latent TGF-β. Thus, neither exogenous TGF-β nor neutralising antibodies to TGF-β had any effect on the rate or extent of mineralisation seen in the pericyte cultures (Table 4).

Several proteins, such as osteonectin, osteocalcin, osteopontin and bone sialoprotein are believed to play a role in the control of mineralisation. Of these proteins, osteopontin and bone sialoprotein, like TSP-1, have been localised at the periphery of mineralising new bone matrix suggesting that they may play a role in the early stages of mineralisation (Denhardt and Guo, 1993; Roach, 1994). Interestingly, osteopontin expression is also induced just before the onset of mineralisation and, furthermore, mineralisation is increased if the synthesis of osteopontin is inhibited (Ibaraki et al., 1992; Sodek et al., 1992). It has therefore been suggested that osteopontin may act either to prevent inappropriate crystal formation (Roach, 1994) or to influence the rate of mineralisation (Denhardt and Guo, 1993). Recent data showing that bone sialoprotein increases the amount of hydroxyapatite formed in a cell-free system suggests that this protein may act as a nucleator for crystal formation (Hunter and Goldberg, 1993). By comparison, osteocalcin and osteonectin have been identified in the fully mineralised matrix (Carlson et al., 1993; Roach, 1994) and recent evidence suggests that these proteins may serve to prevent excessive mineralisation by controlling the growth and size of hydroxyapatite crystals (Doi et al., 1992).

The results presented in this communication, taken together with the low levels of TSP-1 present in mature bone (Gehron-Robey et al., 1989; Grzesik and Gehron-Robey, 1994), indicate that TSP-1 may be an inhibitor of mineralisation. Mineralisation is likely to be regulated by the co-ordinated interactions between components of the ECM, cytokines and hormones (Raisz, 1988; Gowen, 1992; Price and Russell, 1992; Denhardt and Guo, 1993; Bonewald and Dallas, 1994). Further studies are therefore required to ascertain the precise function of each of these components, including TSP-1, during mineralisation and bone formation in vivo.

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