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Expression of Thrombospondins by Endothelial Cells

Injury Is Correlated with TSP-1

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The thrombospondins (TSP-1, -2, and -3) comprise a family of proteins that are homologous at the carboxy terminus but have unique sequences at the amino terminus that might be correlated with the regulation of cell behavior. To investigate the expression of TSP-1, -2, and -3 in endothelial cells, we examined developing murine blood vessels and human atherosclerotic plaques by* in situ *hybridization. The expression of TSP-1 was also characterized in cultured bovine aortic endothelial cells. Expression of TSP-2 was seen in the dorsal aorta as early as embryonic day 10; TSP-1 was not detected in endothelial cells until later stages, and TSP-3 was not apparent in the vasculature. In atherosclerotic specimens, TSP-1 mRNA was detected in many intraplaque microvessels and in the endothelium lining the atheromatous plaque; TSP-2 was absent from these regions. Cultured bovine aortic endothelial cells did not transcribe TSP-2 mRNA at detectable levels. There were high steady-state levels of TSP-1 mRNA in subconfluent bovine aortic endothelial cells before confluence and at the wound edge after injury of the cell monolayer, with maximal expression of TSP-1 in cultures at a time during which approximately 35% of the cells were in S phase. As the majority of these cells subsequently undergo mitosis, these data are consistent with TSP-1 as an inhibitor of endothelial cell proliferation that functions in G1. These results support the conclusion that, despite sequence homology, the TSPs have distinct functions in vascular biology. (Am J Pathol 1995, 147:1068–1080)

Thrombospondins (TSPs)-1, -2, and -3 are members of a family of at least 5 proteins (other members include TSP-4 and COMP/TSP-5) with related sequences but diverse tissue distributions.[1,2] TSP-1 was initially described as a major component of platelet α-granules that functions in aggregation and clot formation.[3] TSP-1, which is secreted by numerous cell types that include endothelial cells, fibroblasts, macrophages, and smooth muscle cells[4–7] enhances the growth of smooth muscle cells and fibroblasts,[8–12] whereas it inhibits endothelial cell proliferation and angiogenesis *in vitro* and *in vivo*.[13–16] Although TSP-1 appears to promote substrate adhesion of a variety of cells (keratinocytes, melanoma cells, platelets, and myoblasts),[17–20] it reduces focal adhesions in endothelial cells and fibroblasts.[18,21] TSP-1 binds to and activates transforming growth factor (TGF)-β1[22,23] and exhibits increased expression in response to serum, platelet-derived growth factor, and basic fibroblast growth factor.[8,24,25] Moreover, the kinetics of induction of TSP-1 by platelet-derived growth factor are similar to those of other inducible, immediate early response genes, a feature consistent with a role for TSP-1 in the regulation of cell cycle progression.[24,26]

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TSP-2 is also expressed by many different cells including fibroblasts, smooth muscle cells, and osteosarcoma cells; however, it has not been detected in cultured endothelial cells.[27] In murine and avian embryos, TSP-2 has been found in the central nervous system, liver, lung, gut, perichondrium, and blood vessels[1,28] in a pattern different from that of TSP-1.[1,29,30] Expression of TSP-2 is enhanced by stimulation of fibroblasts with growth factors.[30] However, unlike TSP-1, TSP-2 lacks a serum-response element in its promoter and is not expressed as an immediate early gene in response to mitogens.[31]

Little is known about the expression of TSP-3 *in vitro*.[32] In murine embryos, a transcript for TSP-3 has been detected in the gut, cartilage, lung, and central nervous system.[1,33] Immunohistochemical studies of TSP-3 in the mouse have revealed restricted expression during early embryogenesis, increased levels during organogenesis, and maximal expression at days 17 to 19 of development.[33]

Homology among the TSPs is greatest at the carboxy terminus, which contains peptide sequences that mediate binding to cell surface receptors.[34,35] Near the amino terminus, TSP-3 is significantly different from TSP-1 or TSP-2. For example, TSP-3 contains four rather than three type II (epidermal growth factor-like) repeats and lacks the procollagen and type I (properdin-like) repeats that, in TSP-1, have been shown to inhibit the angiogenic response mediated by basic fibroblast growth factor.[36] We would therefore predict that differences in the functions of the TSPs in the regulation of endothelial cells are mediated by this region. Thus, TSP-1 and -2, which exhibit significant sequence homology within the procollagen and type I repeats, might have similar functions as anti-angiogenic proteins that would not be shared by TSP-3.

Differential expression of TSPs in endothelial cells during development, injury, and *in vitro* would be consistent with separate functions for these structurally related proteins. Accordingly, we have studied TSP-1, -2, and -3 during blood vessel development in the mouse embryo as well as in atherosclerotic human arterial tissue that has undergone neovascularization. We have also examined TSP-1 and -2 in endothelial cells *in vitro* and have further characterized the expression of TSP-1 in a wound model *in vitro* and by cells plated at different densities. Our results are consistent with disparate expression of TSPs in endothelial cells, *in vivo* and *in vitro*. Transcripts for TSP-3 were not detected during development. TSP-2 mRNA was expressed in endothelial cells during early development but was not found in atheromas or in cultured cells. In contrast, TSP-1 was present in endothelial cells at later stages of blood vessel development and in bovine aortic endothelial cells (BAECs) after injury, both *in vivo* and *in vitro*. The maximal expression of TSP-1 in cultures was noted just before confluence after both disruption of the monolayer and plating at subconfluent density. This expression correlated with the transition of BAECs between S phase and G2 and was consistent with the role of this protein as an inhibitor of endothelial cell proliferation that acted principally in G1. Despite the sequence homology between TSP-1 and -2, our results support the conclusion that these proteins exert distinct functions in vascular biology.

Materials and Methods

cDNA and Riboprobes

For Northern blots, the TSP-1 probe was a 1.3-kb *Eco*RI/*Eco*RI fragment from human TSP-1 cDNA, and the TSP-2 probe was a 500-bp (nucleotides 330 to 830) insert subcloned from a 1.3-kb *Bam*HI/*Eco*RI fragment of human TSP-2 cDNA. All cDNA probes were labeled with α-[^{32}P]dCTP by random priming (Amersham, Arlington Heights, IL) and were purified on a Sephacryl S-500 minicolumn (Pharmacia, Piscataway, NJ).

Riboprobes for *in situ* hybridization were derived from regions of low homology to generate transcript-specific probes. Riboprobe vectors were constructed by standard recombinant techniques as follows. TSP-1 riboprobes were transcribed from a 294-bp *Bgl*II/*Pst*I mouse TSP-1 fragment generated by the polymerase chain reaction and cloned in an antisense orientation to the T3 promoter into the vector pBSM13$^+$ and from a 1.3-kb *Eco*RI/*Eco*RI human TSP-1 cDNA fragment cloned in an antisense orientation to the SP6 promoter into the vector pGEM2. For TSP-2 riboprobes, a 514-bp *Pst*I/*Pst*I mouse TSP-2 cDNA, generated by the polymerase chain reaction, was cloned into the vector pBSM13$^+$ in an antisense orientation relative to the T7 promoter. For TSP-3 riboprobes, a 950-bp *Eco*RI/*Eco*RI mouse TSP-3 cDNA was cloned into a pBSK$^-$ vector in an antisense orientation relative to the T7 promoter. The corresponding riboprobes, transcribed in a sense orientation, were used for control experiments. Riboprobes were transcribed with an RNA transcription kit (Promega, Madison, WI) in the presence of α-[^{35}S]dUTP (Amersham). The specific activity of probes was approximately 3×10^7 to 4×10^7 cpm/μg.

Animals and Preparation of Tissue

Swiss-Webster female mice were mated and were checked daily for a vaginal plug, the appearance of which represented day 0. Females were sacrificed daily between 10 and 18 days after conception. Embryos were fixed by immersion for 12 to 16 hours in 3% paraformaldehyde in 0.1 mol/L sodium phosphate (pH 7.6) containing 1% sucrose at 4°C. The age of the embryos was confirmed by morphological characteristics according to Rafferty.[37] Embryos were embedded in paraffin blocks, from which sagittal sections of 5 μm were prepared and mounted on Probe-On slides (Fisher, Pittsburgh, PA). At least five sections per animal and three different animals per stage of development were analyzed in separate *in situ* hybridization experiments.

Atherectomy Specimens

The expression of TSP-1 mRNA was examined in human atherosclerotic tissue after ethical approval for the secondary use of the tissue was granted. Twelve directional peripheral atherectomy specimens from ten superficial femoral and two iliac artery lesions were collected from nine patients (eight males, one female; ages 54 to 80 years old, with an average age of 69) undergoing treatment of peripheral ischemic syndromes at Sequoia Hospital, Redwood City, CA. Samples were obtained from two different stenoses in three of the patients. Six of the arterial specimens were from primary lesions, and the remainder were from restenotic lesions treated 7 months to 9 years previously by an initial interventional procedure. The approximate weight of the specimens was 100 to 150 mg. Each specimen was fixed in 10% neutral buffered formalin overnight and was subsequently embedded in paraffin. Five-micron sections were placed on Superfrost Plus glass slides (Fisher).

Cell Culture

Three different strains of BAECs were isolated as described.[38] All cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) with 10% (vol/vol) heat-inactivated fetal calf serum (Hyclone, Logan, UT), 250 μg/ml amphotericin B (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin G, and 100 U/ml streptomycin sulfate.

Cell Density Experiments

BAECs were plated on 100-mm^2 dishes and were grown to confluence. Two days after cells attained confluence and were growth-arrested, as determined by flow cytometric analysis and incorporation of [^{3}H]thymidine, the cells were dispersed with trypsin and were replated at different densities to attain 25, 50, 75, 90, or 100% confluence after 24 to 34 hours. Cells were plated simultaneously on 100-mm^2 dishes for RNA extraction, 12-well plates for flow cytometry, and 24-well plates for analysis of incorporation of [^{3}H]thymidine. Cell counts were performed with a hemocytometer at the end of the 24-hour period for measurement of percent confluence; 25% confluence represents approximately 2×10^4 cells/cm^2 and 100% confluence represents approximately 20×10^4 cells/cm^2. Correlation between degree of confluence and cell count is not exact because of changes in cell shape and volume that occur as the cells approach confluence.

Wounding of the Monolayer

BAECs were plated on 24-well plates (for incorporation of [^{3}H]thymidine), 100-mm^2 dishes (for RNA extraction), or Probe-On slides (for *in situ* hybridization) and were grown to confluence. Two days after the cells became confluent, the monolayers were wounded with a rake made from nylon bristles that were 0.5 mm in diameter and were separated by a distance of 1 mm.[39] BAECs were prepared for measurements of incorporation of [^{3}H]thymidine, *in situ* hybridization, and RNA levels at subconfluent and confluent densities and at 1, 4, 12, 18, 24, and 44 hours after injury of the cell monolayer.

Incorporation of [^{3}H]Thymidine

BAECs were plated at various densities in 24-well plates in sextuplicate. At the end of 24 hours, three wells of each density were supplemented with 1 μCi/ml [methyl-^{3}H]thymidine (New United Kingdom Nuclear, Boston, MA). After 4 hours, the labeled cells were washed twice with serum-free DMEM, fixed with ice-cold 10% trichloroacetic acid for 25 minutes, washed with cold ethanol, and air dried. Material insoluble in trichloracetic acid was hydrolyzed in 200 μl of 0.4 mol/L NaOH at 80°C for 20 minutes, neutralized with an equal volume of glacial acetic acid, combined with 3 ml of scintillation fluid, and analyzed for radioactivity in a scintillation counter. Cells were removed from the three unlabeled wells with trypsin and were counted by means of a hemocy-

tometer. Cell counts from unlabeled wells were used to normalize incorporation of [^{3}H]thymidine to cell number.

Flow Cytometric Analysis

Synchronized cells were plated in 12- or 24-well plates (Corning, Corning, NY) at 25, 50, 75, 90, and 100% confluence. At various time points, duplicate wells were washed with versene, exposed to trypsin, recovered in DMEM/10% fetal calf serum, and centrifuged. The pellet was resuspended in a buffer containing 10% dimethylsulfoxide and 4,6-diamidino-2-phenylindole. Samples were frozen and stored at −20°C until all wells had been processed. Flow cytometry and data analysis were performed by the laboratory of Dr. P. Rabinovitch (University of Washington).

RNA Extraction and Northern Blot Analysis

Total RNA was isolated from BAECs according to Chomczynski and Sacchi,[40] resolved by electrophoresis in a denaturing 1.2% agarose gel, and stained with 0.5 μg/ml ethidium bromide. Ten micrograms of total RNA was applied to each lane of the gel. RNA was transferred to Duralose-UV (Stratagene, La Jolla, CA) and was crosslinked by ultraviolet radiation.

Northern blots were prehybridized for 2 hours and were subsequently hybridized with DNA probes as previously described.[16] Relative levels of mRNA for each TSP were measured by hybridization to their corresponding α-^{32}P-labeled cDNA probes. After hybridization, blots were washed at a final stringency of 0.1X standard saline citrate (1X SSC = 0.15 mol/L NaCl/0.015 mol/L sodium citrate, pH 6.8) containing 0.1% sodium dodecyl sulfate at 55°C. Radioactive bands were visualized by autoradiography and were subjected to quantitative densitometry by the PhosphorImager Facility at the University of Washington. Variations in the amount of total RNA applied to the gels were normalized by hybridization of the blots with a bovine cDNA probe for 28S rRNA.[16]

In Situ *Hybridization*

The *in situ* hybridization protocol was a modification of a method described by Holland et al.[41] Slides with subconfluent, confluent, and wounded BAECs were rinsed in serum-free DMEM, postfixed with 4% paraformaldehyde for 20 minutes, rinsed in phosphate-buffered saline, and digested for 12 minutes with 20 μg/ml proteinase K. All slides (mouse sections and BAECs) were subsequently prepared and exposed to prehybridization solution as previously described.[42] The samples were hybridized at 50°C overnight in prehybridization solution that contained 10% dextran sulfate and 0.1 μg/ml of the TSP-1, TSP-2, or TSP-3 riboprobes. Samples were washed, dehydrated, air dried, and exposed to emulsion (Kodak, Rochester, NY) as described previously. Slides were exposed for 10 to 14 days and subsequently developed in Kodak D-19 developer and fixed in GBX fixer (Kodak). Samples were counterstained with methyl green/pyronin, dehydrated with ethanol (solutions of 70 to 100%), cleared in xylene, and mounted with Permount.

Results

Expression of TSP-1, -2, and -3 in Blood Vessels of Murine Embryos

To discern potential functions of the TSPs in angiogenesis, we studied the expression of TSP-1, -2, and -3 mRNA in blood vessels during murine development from embryonic day 10 to young adult. Serial sections of all specimens were examined by hematoxylin and eosin stain to determine the size and type of blood vessels that were present. Figure 1 shows dark-field images of *in situ* hybridization with antisense riboprobes corresponding to TSP-1 (Figure 1, A–C) and TSP-2 (Figure 1, D–F). Transcripts for TSP-2 were present in the intima of large blood vessels, such as aorta, as early as day 10 (Figure 1D). Later, TSP-2 mRNA diminished significantly and was not detected in aortas of day 14 embryos (data not shown). Nevertheless, hybridization for TSP-2 mRNA persisted in many medium-sized blood vessels (Figure 1E, arrow) throughout development, as well as in mesenchyme and perichondrium. In Figure 1F, transcripts are noted in postnatal, medium-sized vessels (arrow). Transcripts for TSP-1 were not apparent in blood vessels at day 10 (Figure 1A) and were present only in blood vessels of later fetal stages and in the adult. By day 17, hybridization for TSP-1 was observed in several blood vessels. In venules, TSP-1 transcripts were detected in the individual endothelial cells that comprise the intima (Figure 1B, arrow). In arterioles, however, expression was also detected in the smooth muscle cells of the media (Figure 1B, vessel, upper left). Hybridization of TSP-1 to individual endothelial cells was noted in some postnatal, medium-sized vessels (Figure 1C, arrow). No signal was detected for TSP-3 in the intima of blood vessels of the embryonic or postnatal mouse (data not

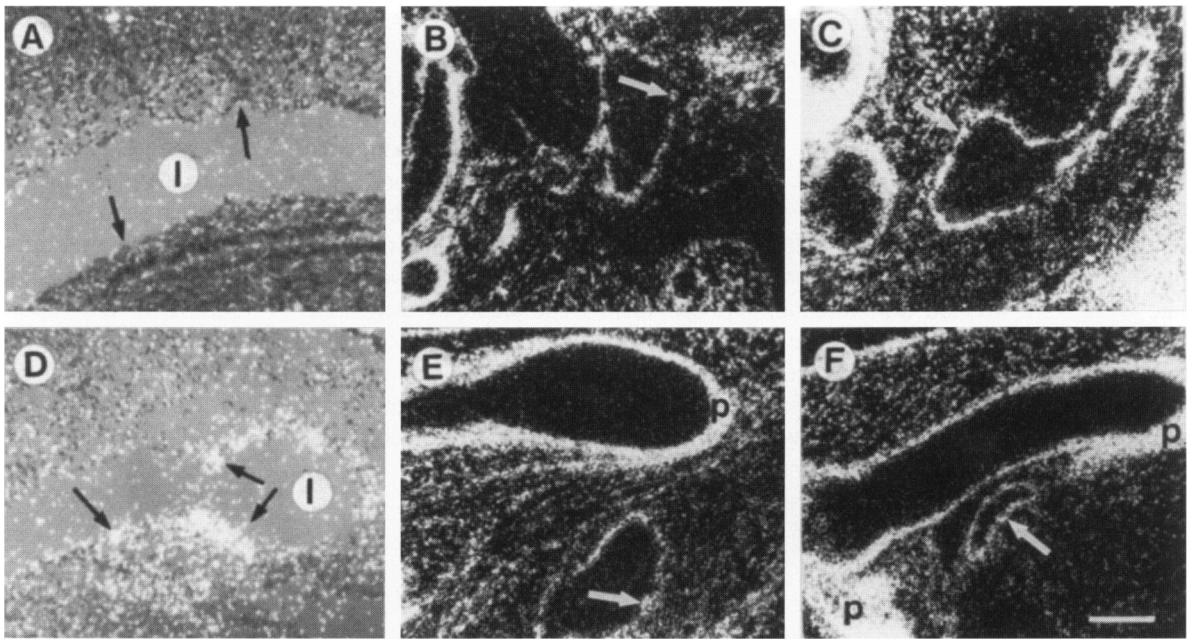

Figure 1. *Expression of TSP-1 and TSP-2 mRNA in blood vessels of embryonic mice.* **A–F:** *Dark-field images of* in situ *hybridization with murine antisense TSP-1* (**A**, **B**, *and* **C**) *and TSP-2* (**D**, **E**, *and* **F**) *riboprobes.* **A** *and* **D:** *10-day mouse embryonic tissues.* **B** *and* **E:** *17-day mouse embryonic tissues.* **C** *and* **F:** *2-day postnatal mouse tissues. TSP-1 transcripts are detected in the endothelial cells that comprise the intima* (**B**, **arrow**) *at 17 days but not at 10 days* (**A**, **arrows**). *Hybridization of TSP-1 antisense RNA to individual endothelial cells is detected in some postnatal medium-sized vessels* (**C**, **arrow**). *Transcripts for TSP-2 are present in the intima of blood vessels at 10 days* (**D**, **arrows**) *and 17 days* (**E**, **arrow**) *as well as in the perichondrium* (*p*; **E–F**). **F:** *Transcripts in postnatal medium-sized vessels* (**arrow**). *l, lumen of blood vessels; bar, 100 μm.*

shown). In summary, comparison of the TSPs during these developmental time points showed disparate expression; TSP-2 appeared early in gestation, whereas TSP-1 appeared later in development and TSP-3 was not detected.

We examined also the distribution of TSP-1 (Figure 2, A and B) and TSP-2 (Figure 2, C and D) in blood vessels of the 4-week-old adult mouse. The size and type of blood vessels present were assessed by examination of serial sections stained with hematoxylin and eosin. TSP-1 mRNA was apparent in the endothelium of venules (Figure 2, A and B, transverse section) and arterioles (Figure 2, A and B, longitudinal section). In addition, the subendothelium of some arteries (arrow in Figure 2B), as well as smooth muscle cells of the media (arrowhead in Figure 2B), also contained TSP-1 transcripts. Signal in the lumen of blood vessels is generally artifactual, although this could also represent TSP-1 mRNA in platelets.[43] TSP-2 mRNA was detected in medium-sized venules, although the hybridization signal was confined largely to the intima (Figure 2, C and D). Thus, the expression of TSP-2 in endothelial cells was detected in blood vessels of embryos as well as of adults. Transcripts for TSP-1 appeared in endothelial cells later in development and were also present in adult animals. Within a single animal, transcripts for both TSP-1 and TSP-2 were not found consistently in all vessels of an equivalent size. In comparison with TSP-2, TSP-1 was expressed in a greater number of vessels and was more abundant during the late stages of development and in the adult.

Expression of TSP-1 and TSP-2 in the Endothelium of Atherosclerotic Tissue

Transcripts for both TSP-1 and TSP-2 mRNA were detected in quiescent vessels of the young adult. However, different patterns were observed in activated or injured endothelium such as that of atherosclerotic vessels (Figure 3) and atherectomy specimens (Figure 4). For example, TSP-1 mRNA was present in the neointima of atherosclerotic coronary arteries, as demonstrated by bright-field and dark-field images of a coronary artery with an eccentric atherosclerotic lesion (Figure 3). TSP-1 mRNA was detected in endothelial cells lining the vessel on the stenotic side but not on the normal side of the artery (Figure 3, A–C). There was no TSP-2 mRNA on either surface of the stenotic coronary artery (Figure 3D). Transcripts for TSP-2 were present in fibroblasts and smooth muscle cells in the intima, media, and adventitia. The lack of an intact endothelial lining in the other stenotic coronary arteries that were examined

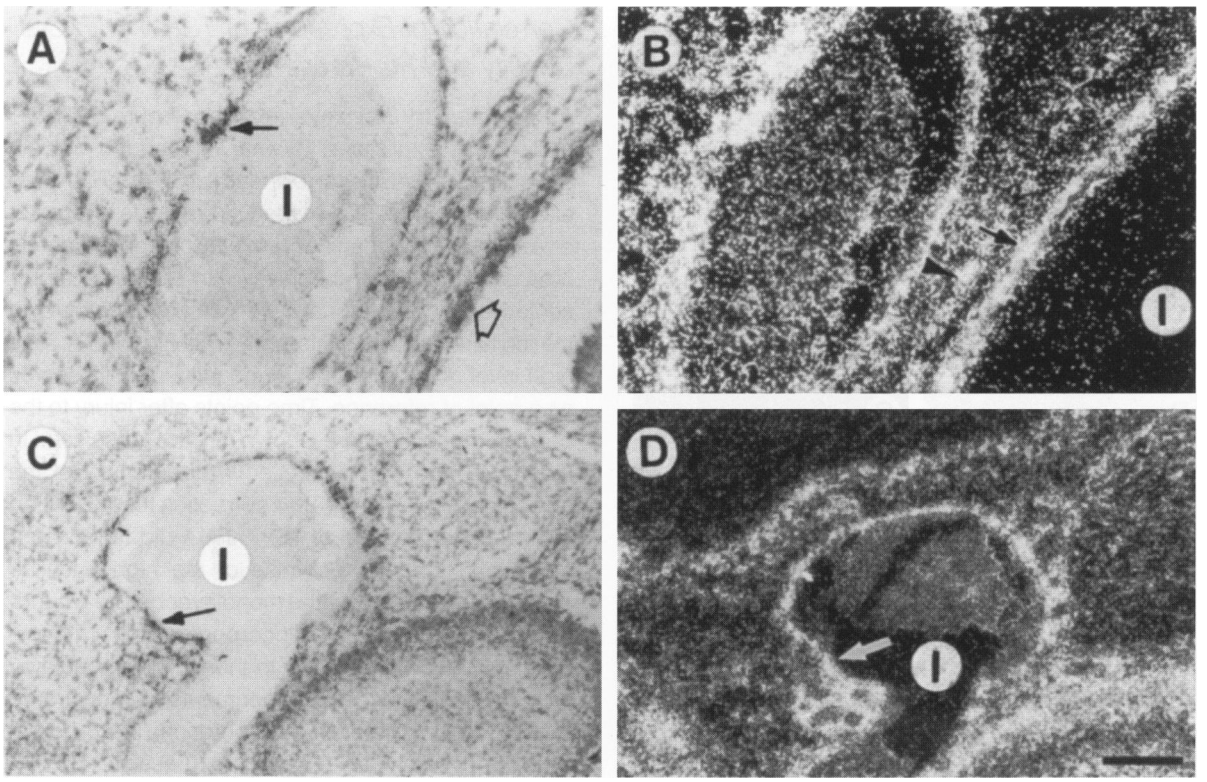

Figure 2. *Distribution of TSP-1 and TSP-2 mRNA in blood vessels of the adult mouse. Hybridization of TSP-1* (A *and* B) *and TSP-2* (C *and* D) *antisense riboprobes to 4-week-old murine blood vessels. Bright-field images* (A *and* C) *are compared with the dark-field counterparts* (B *and* D). A *and* B: *TSP-1 mRNA is detected in the endothelium of venules* (A, arrow) *and arterioles* (A, arrowhead). *In addition, the subendothelium of some arteries* (B, arrow) *and smooth muscle cells of the media* (B, arrowhead) *also contain TSP-1 transcripts.* C *and* D: *TSP-2 mRNA is apparent in this oblique section of a medium-sized venule, although the hybridization signal is largely confined to the intima* (C *and* D, arrow). *l, lumen of blood vessels; bar, 100 μm, with all panels shown at equal magnification.*

precluded additional study. Therefore, we focused on specimens that had been obtained from directional atherectomies of stenotic iliac arteries. Several areas of these specimens exhibited neovascularization in hypercellular regions as well as in areas composed primarily of extracellular matrix. As shown in Figure 4, TSP-1 mRNA was apparent in the endothelium of microvessels within the atherosclerotic plaque. Bright-field (Figure 4, A and D) and dark-field (Figure 4, B and E) images of a representative microvessel (Figure 4, A–D, arrowheads) in an atherectomy specimen from the iliac artery exhibited transcripts for TSP-1 in the endothelial cells lining the microvessel. Transcripts were confirmed to be in the endothelial lining of blood vessels with bright-field images of the lumen (Figure 4C, arrowheads) as well as by immunohistochemistry for CD34, a marker of endothelial cells, in serial sections of the same specimen (data not shown). In contrast, there was no expression of TSP-2 mRNA in the endothelial cells of these vessels (Figure 4F, arrowheads).

Expression of TSP-1 and -2 by Endothelial Cells in Vitro

To characterize further the expression of TSPs by the endothelium, we examined endothelial cells derived from the bovine aorta. We used three different strains of BAECs that exhibited a contact-inhibited monolayer of polygonal cells. It has been confirmed by several investigators that there are high levels of TSP-1 mRNA in subconfluent endothelial cells, with decreased expression in cells at confluence and during angiogenesis.[16,44] In contrast, TSP-2 was not expressed by subconfluent or confluent BAECs (Figure 5, lanes 1 and 2). BAECs that spontaneously formed cord-containing cultures exhibited a low to nondetectable signal (Figure 5, lane 3). Endothelial cells from fetal bovine aorta and bovine capillaries were also negative for TSP-2 mRNA (Figure 5, lanes 5 and 6), although a clear signal was obtained from bovine smooth muscle cells (lane 4). These data concur with previous reports that endothelial cells do

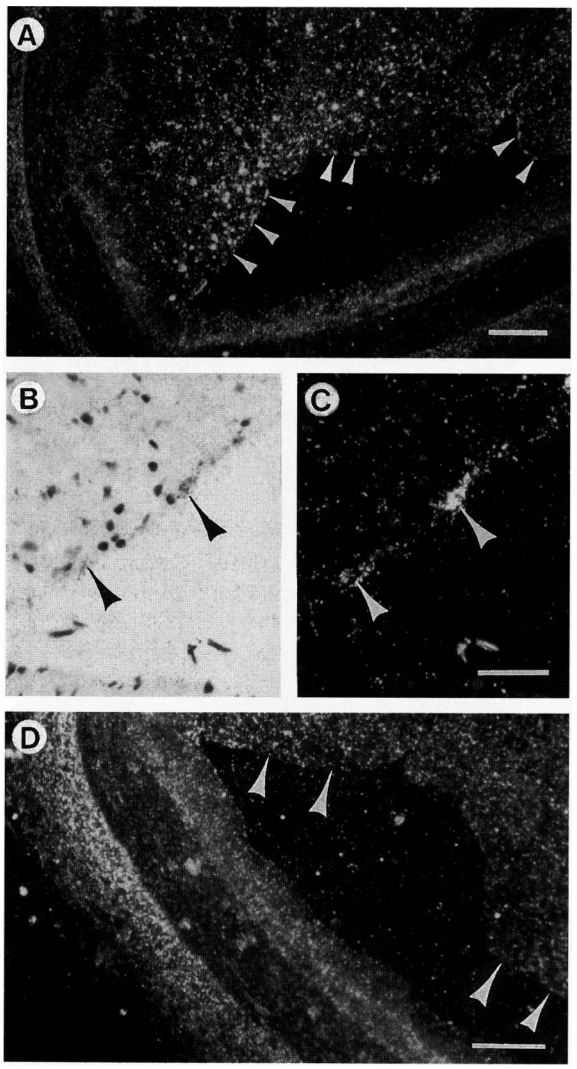

Figure 3. *TSP-1 mRNA is expressed in the neointima on the stenotic surface of an atherosclerotic coronary artery. Hybridization of a TSP-1 antisense riboprobe to the endothelial lining of a human coronary artery containing an eccentric atherosclerotic plaque. Shown are dark-field* (**A**, **C**, *and* **D**) *and bright-field* (**B**) *images of a representative atherosclerotic coronary artery.* **A** *and* **D**: *Low power views of the same vessel shown in* **B** *and* **C**. *Transcripts for TSP-1 are detected in endothelial cells that overlie the atherosclerotic intima* (**A–C**, **arrowheads**) *but not on the opposite arterial wall, which has very minimal intimal thickening.* **D**: *Serial section of the same specimen shown in* **A** *shows a lack of signal for TSP-2 mRNA in the endothelial cells lining the vessel* (**arrowheads**). *Bar, 100 μm* (**A**); *50 μm* (**B** *and* **C**); *and 100 μm* (**D**)

not express TSP-2 in culture.[27] Human omental endothelial cells did contain TSP-2 mRNA (data not shown); however, these cells were cultured in the presence of epidermal growth factor, the effect of which on the expression of TSP-2 is not known. These data show that the expression of TSP-2 in endothelial cells is limited. In contrast, transcript for TSP-1 is found in many (if not all) endothelial cells.[45] Expression of TSP-1 was therefore examined also *in vitro* as a function of cell density, proliferation, and response to injury.

Correlation of Expression of TSP-1 with Cell Density and Proliferation

As illustrated by *in situ* hybridization (Figure 6), TSP-1 mRNA was expressed by many (although not all) subconfluent BAECs (arrowhead). TSP-1 mRNA was also present in BAECs at the wound edge (Figure 6, arrows) after mechanical removal of approximately 50% of the cellular monolayer with a rake. A representative Northern blot of TSP-1 mRNA after injury to the cellular monolayer is shown in Figure 7A. The high level of expression in subconfluent cells serves as a point of reference. Time points after injury to the cellular monolayer are shown on the abscissa. In Figure 7B, the ordinate reflects the intensity of the signal for TSP-1 mRNA after correction for 28S rRNA. Increases in the level of TSP mRNA did not exceed more than 2.5-fold, because of the high baseline expression of TSP-1 by the monolayer before wounding. Measurements of the incorporation of [^{3}H]thymidine by BAECs after injury to the cellular monolayer in a duplicate series of experiments demonstrated that the highest level of incorporation (at 18 hours) correlated with the maximal expression of TSP-1 mRNA at 12 to 18 hours after injury to the monolayer (Figure 7C).

We next asked whether the proliferative status of the cell or the degree of confluence correlated with the increased expression of TSP-1 mRNA. BAECs were grown to confluence, made quiescent, and were subsequently plated at different densities (approximate ranges of confluence are shown in the legend to Figure 8). High levels of TSP-1 are generally found in subconfluent cultures of BAECs[43,16] changes in this expression as a function of different degrees of subconfluence in several different strains of BAECs had not been examined previously. Duplicate plates of synchronized cells were analyzed by flow cytometry for a more precise determination of the percentage of cells that were in S phase, and a concurrent set of triplicate plates underwent RNA extraction to determine the levels of expression of TSP-1 steady-state mRNA. Flow cytometry was performed on cultures plated at different densities at multiple time points after plating to ensure that the cells had adequate time to recover from passage before analysis. As shown in Figure 8, the highest percentage of cells in S phase occurred just before confluence (Figure 8C). The maximal expression of TSP-1 mRNA (Figure 8, A and B) was also noted at 90% confluence. We also performed concurrent experiments to measure TSP-1 protein and found an increase in levels, similar to the mRNA, as the cells approached confluence (data not shown). These

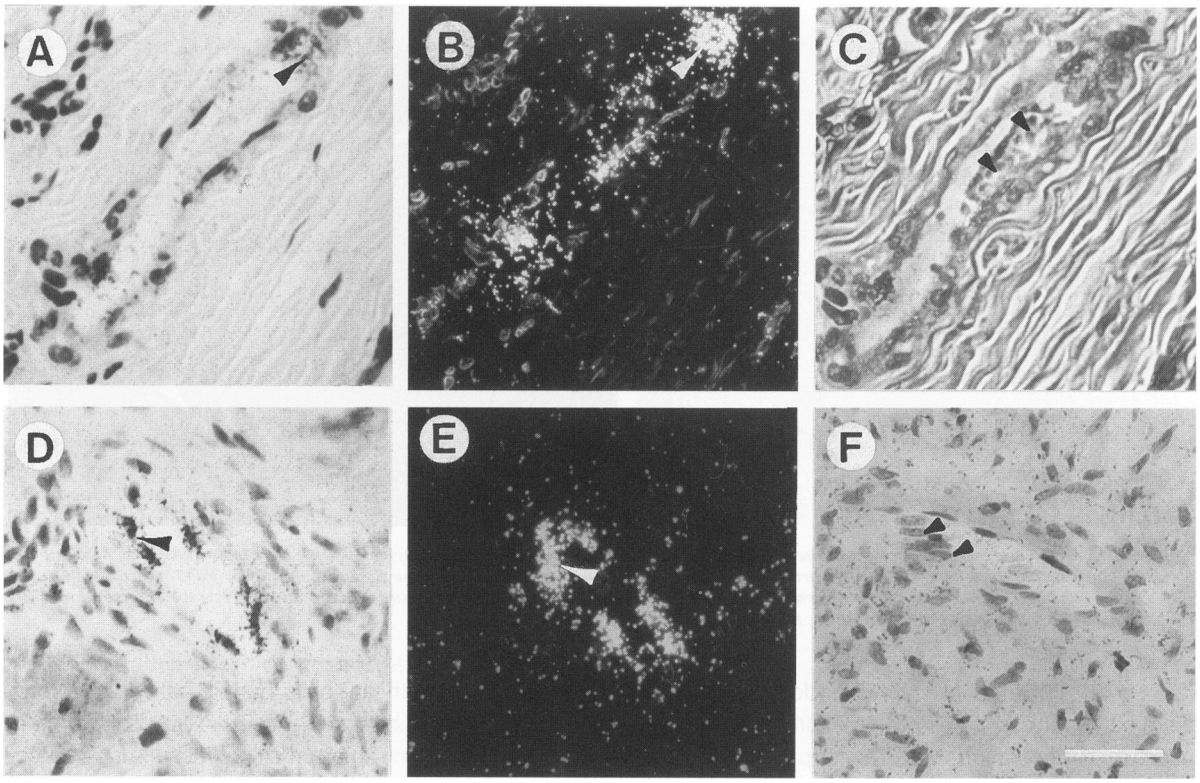

Figure 4. *TSP-1 mRNA is expressed in the endothelium of blood vessels within atherectomy specimens. Hybridization of a TSP-1 antisense riboprobe to blood vessels in human atherectomy specimens derived from an atherosclerotic iliac artery. Bright-field images* (A *and* D) *are compared with the dark-field* (B *and* E) *counterparts.* A, B, D, *and* E: *Transcripts for TSP-1 are detected in the endothelial cells lining the blood vessels* (arrowheads). C: *Phase contrast photomicrograph of the vessel* (arrowheads) *in a serial section of the same specimen shown in* A *and* B. F: *Serial section of the same specimen shown in* D *and* E *shows a lack of signal for TSP-2 mRNA in the endothelium* (arrowheads) *of the vessel. Bar, 50 μm*

data differ from the studies by Mumby et al,[44] which showed that levels of TSP-1 protein decreased in one strain of BAEC as the culture approached confluence. This disparity could reflect differences in cell types and culture conditions (see Discussion). Increases in the level of TSP-1 mRNA did not exceed threefold, because of the high baseline expression of TSP-1. Calculations of the percentage and absolute number of cells that were in the various stages of the cell cycle showed that the highest levels of TSP-1 expression occurred in cultures with the greatest percentage of cells in S phase. TSP-1 is therefore likely to exert its described inhibitory effect on proliferation of BAECs on cells in G1, when maximal amounts of TSP-1 have accumulated in the media and after the remaining cells in S phase have completed the cell cycle.

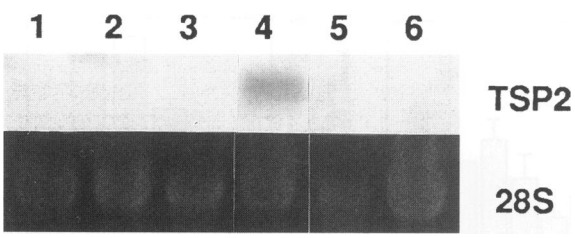

Figure 5. *Bovine endothelial cells do not express TSP-2 mRNA. A Northern blot of total RNA shows the approximately 6-kb RNA corresponding to TSP-2 expressed by bovine smooth muscle cells* (lane 4) *after hybridization with the corresponding ^{32}P-labeled cDNA. In contrast, there was no detectable expression of TSP-2 mRNA by subconfluent BAECs, confluent BAECs, or BAECs that spontaneously form cords* (lanes 1–3). *Moreover, there was no detectable expression by microvascular endothelial cells isolated from the fetal adrenal gland* (lane 5) *or by large vessel endothelial cells derived from the fetal bovine aorta* (lane 6).

Discussion

Differences in protein sequence among the TSPs have been correlated with unique functions of the members of this family.[2] Moreover, domains that are similar among these proteins should mediate analogous roles in the regulation of cell behavior. One would therefore predict, as has been found *in vitro*,[36] that the procollagen homology and the type I repeats that are found in TSP-1 and TSP-2, but not TSP-3, would confer anti-angiogenic properties on TSP-1 and TSP-2. We sought to define further the differential expression of TSPs in endothelial cells *in vivo* during development and injury. The association of

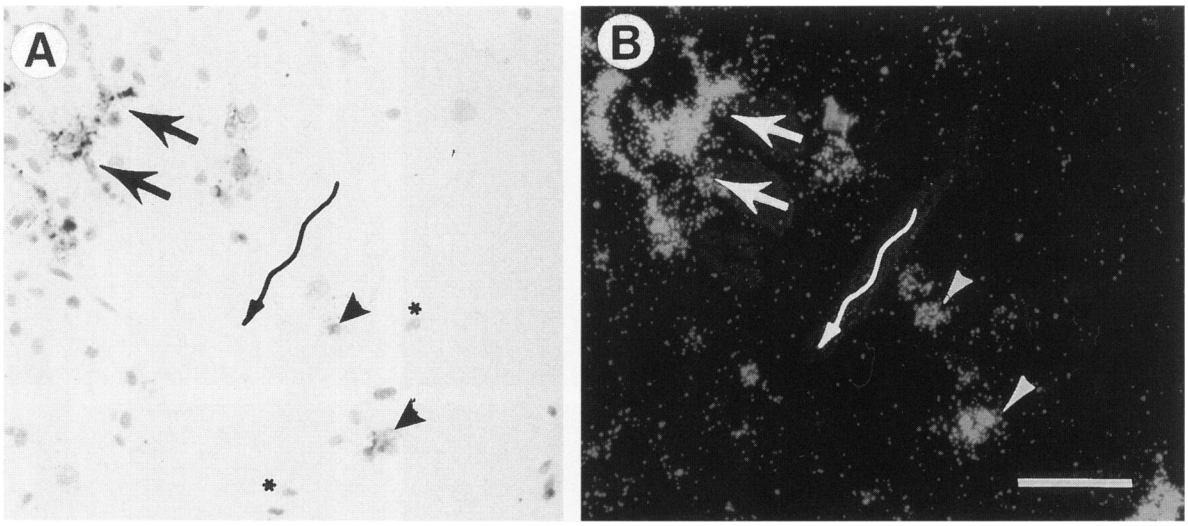

Figure 6. *TSP-1 mRNA is expressed by subconfluent BAECs and by cells at the wound edge after injury of the cellular monolayer. Bright-field image* (A) *is compared with a dark-field* (B) *counterpart after* in situ *hybridization with a TSP-1 antisense riboprobe.* A *and* B*: TSP-1 mRNA is detected in BAECs at the edge of a cellular monolayer* (arrows) *that has been mechanically disrupted by a rake* (curved arrow). *Note the expression of TSP-1 mRNA in many* (B, arrowheads) *but not all* (A, asterisks) *subconfluent BAECs. Bar, 50 μm*

TSP-1 with activated or injured endothelium led to examination of correlates of this expression *in vitro*.

In situ hybridization with transcript-specific riboprobes demonstrated that TSP-3 was not expressed in endothelial cells during the time points of development that we examined. The absence of TSP-3 mRNA in blood vessels was not unexpected and concurs with previous immunohistochemical studies of murine development.[33] In contrast, TSP-2 transcripts were present in endothelial cells of small, medium, and large vessels as early as embryonic day 10 and were also present in the adult. The expression of TSP-2 in blood vessels early in development has also been reported in avian embryos.[28] TSP-2 might indirectly serve as an angiogenic protein by acting as a structural homologue that blocks the function of TSP-1. We did not detect TSP-2 transcripts in endothelial cells during pathological processes that exhibit angiogenesis *in vivo*, for example, newly formed intraplaque microvessels in atherectomy specimens or in the neointima of atherosclerotic arteries.[46] The activation of endothelial cells in these diseases occurs early and the detection of transcript for TSP-2 could have been missed. In quiescent vessels of the murine adult, transcripts for both TSP-1 and TSP-2 were present. However, the expression of TSP-1 was more prevalent than TSP-2, with transcript for the latter detected in only some of the vessels. Additional characterization of the divergent as well as similar roles of TSP-2 and TSP-1 in endothelial cells would benefit from studies *in vitro*. Further studies of TSP-2 were not pursued because of the absence of TSP-2 mRNA in endothelial cells derived from both fetal and adult bovine aortas as

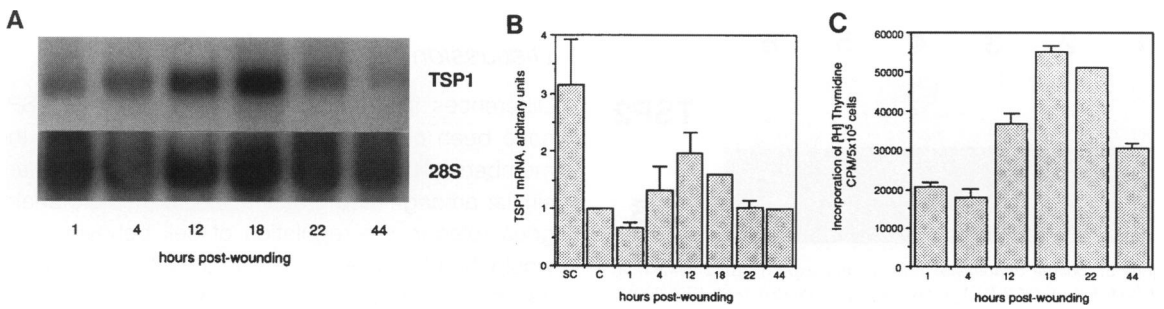

Figure 7. *Expression of TSP-1 mRNA and incorporation of [^{3}H]thymidine by endothelial cells after mechanical injury of the cellular monolayer.* A: *Representative Northern blot of total RNA derived from BAECs at 1, 4, 12, 18, 22, and 44 hours after mechanical injury to the cellular monolayer. The blot revealed the 6-kb mRNA for TSP-1 after hybridization with the corresponding α-^{32}P-labeled cDNA.* B: *Relative levels of expression of TSP-1 mRNA (in arbitrary units) at the same time points after injury, derived from phosphorimage analyses of the Northern blots. Values were normalized to the corresponding signal for 28S rRNA. The expression of TSP-1 mRNA was maximal at 12 hours (determined from the average of four separate experiments).* C: *Incorporation of [^{3}H]thymidine by the cellular monolayer. Values were normalized to 5 × 10^5 cells. The incorporation of thymidine was maximal at 18 hours (determined by two separate experiments performed in triplicate). SC, subconfluent; C, confluent.*

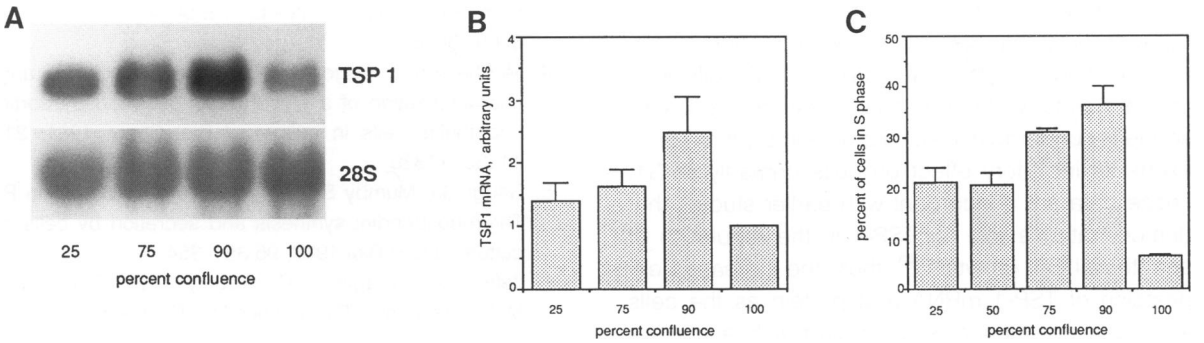

Figure 8. *Expression of TSP-1 mRNA as a function of cell cycle and density.* A: *Representative Northern blot of total RNA derived from BAECs at 25% (range, 25 to 40), 75% (range, 75 to 85), 90% (range, 90 to 95), and 100% confluence. Various confluencies were achieved by the plating of cells at different densities. All samples were plated (time 0) and harvested simultaneously (34 hours later), and the degree of confluence at the time of harvest was verified by cell counts. The blot shows the 6-kb mRNA for TSP-1 after hybridization with the corresponding* α-^{32}*P-labeled cDNA.* B: *Levels of expression of TSP-1 mRNA, derived from phosphorimage analyses of Northern blots at different degrees of confluence. Values were normalized to the corresponding signal for 28S rRNA.* C: *Percentage of BAECs in S phase, determined by flow cytometry, at 25, 50, 75, 90, and 100% confluence. The expression of TSP-1 mRNA and the percentage of cells in S phase were maximal at 90% cell density, just before confluence (determined by the average of three separate experiments).*

well as in endothelial cells derived from microvessels of the bovine adrenal gland.

In contrast to TSP-2, TSP-1 mRNA was not detected until day 17 of gestation, when it was noted in small, medium, and large vessels. The demonstration of TSP-1 transcripts in the late stages of blood vessel development is consistent with its role as an inhibitor of angiogenesis.[13–16] As TSP protein has been identified in the basement membrane of blood vessels,[47] it is possible that TSP-1 transcripts are expressed as vessels conclude morphogenesis and that the protein becomes incorporated into the basement membrane. The late appearance of TSP-1 mRNA in endothelium during development might provide a matrix that stabilizes blood vessels and inhibits the additional proliferation of endothelial cells and angiogenesis.

In human atherosclerotic tissue, TSP-1 mRNA was present in endothelium on the stenotic surface of the neointima of a coronary artery and in many microvessels in atherectomy specimens. In atherosclerotic human coronary plaques, cells that exhibit immunostain for the proliferating cell nuclear antigen are more commonly found in specimens that also contain intraplaque microvessels.[46] During atherogenesis, the expression of TSP-1 transcripts by activated endothelial cells could be consistent with its proposed role in the enhancement of proliferation of the vascular smooth muscle cells and fibroblasts that constitute a large portion of the stenosis.[10]

The proliferation of cells in culture, especially at low density or after a rake wound, also results in injury or activation of endothelial cells.[39] Our experiments *in vitro* demonstrated the expression of TSP-1 in activated or injured BAECs. Levels of TSP-1 mRNA and protein were maximal as the cells approached confluence, a temporal pattern consistent with the role of TSP-1 as an inhibitor of endothelial proliferation. This pattern differs from early work by Mumby et al,[44] which showed that levels of secreted TSP-1 protein decreased, on a per cell basis, as cells approached confluence. This disparity is likely a result of several differences in the methods of cell culture. In contrast to the earlier work, our experiments utilized three different strains of BAECs, the cells were synchronized before plating, and experiments were performed in low serum, as well as in DMEM containing 10% fetal calf serum, with similar results.

We also examined the expression of TSP-1 mRNA in BAECs after wounding of the cellular monolayer, at various degrees of confluence and as a function of cell cycle. These cells at low plating densities have been shown to express high levels of TSP-1, which are diminished at confluence.[16,44] After the cells were rake wounded, the monolayer expressed TSP-1 mRNA in cells at the wound edge and in many (although not all) subconfluent cells at the periphery of the monolayer. When we measured [^{3}H]thymidine incorporation in duplicate plates of cells that were injured in an identical fashion, we found a high percentage of cells in S phase, immediately before confluence, which was correlated with maximal expression of TSP-1 mRNA. The expression of TSP-1 was also assessed in cells that had been plated simultaneously at various densities, and determination of the percentage of cells in S phase was made by flow cytometry. As we saw in the wound model, a high percentage of cells in S phase, immediately before confluence, was correlated with maximal expression of TSP-1 mRNA. High levels of expression were coincident with high density but not with confluence. Calculations of the percentage and absolute number

of cells that were in the various stages of the cell cycle showed that TSP-1 levels were highest in cultures having the greatest proportion of cells in S phase. As many (if not most) of these cells will subsequently undergo mitosis, our data imply that TSP-1 exerts its inhibitory effect on cells primarily in G1. These data are concordant with earlier studies that demonstrated a role for TSP-1 in the regulation of endothelial cell growth[48,49]; thus, the increased expression of TSP-1 mRNA and protein as the cells approach confluence is consistent with a role for TSP-1 in the inhibition of additional proliferation. This effect could result directly from TSP-1, or by an indirect mechanism whereby TSP-1 binds and activates latent TGF-β1,[22,50] a known inhibitor of endothelial cell proliferation in culture.[51] The interaction between TSP-1 and TGF-β1 is mediated by the type I repeats of TSP-1 and appears to protect TGF-β1 from degradation.[23] Thus, the presence of TSP-1 could potentiate the ability of TGF-β1 to inhibit the proliferation of endothelial cells and to augment the migration or proliferation of smooth muscle cells and fibroblasts.

In conclusion, our studies demonstrate disparate expression of the TSPs in endothelial cells. TSP-3 was not expressed in blood vessels during the developmental stages we examined. Moreover, despite the sequence homology between TSP-1 and -2, these proteins exhibited different patterns of expression during development. Finally, our studies *in vitro* corroborate the role of TSP-1 as an inhibitor of endothelial cell proliferation, and the presence of this TSP in the endothelium of atherosclerotic specimens is consistent with its proposed function in the modulation of proliferation of other cell types.

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