Thrombospondin 2 Inhibits Microvascular Endothelial Cell Proliferation by a Caspase-independent Mechanism

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The matricellular protein thrombospondin 2 (TSP2) regulates a variety of cell–matrix interactions. A prominent feature of TSP2-null mice is increased microvascular density, particularly in connective tissues synthesized after injury. We investigated the cellular basis for the regulation of angiogenesis by TSP2 in cultures of murine and human fibroblasts and endothelial cells. Fibroblasts isolated from murine and human dermis synthesize TSP2 mRNA and secrete significant amounts of immunoreactive TSP2, whereas endothelial cells from mouse lung and human dermis did not synthesize TSP2 mRNA or protein. Recombinant mouse TSP2 inhibited growth of human microvascular endothelial cells (HMVECs) mediated by basic fibroblast growth factor, insulin-like growth factor-1, epidermal growth factor, and vascular endothelial growth factor (VEGF). HMVECs exposed to TSP2 in the presence of these growth factors had a decreased proportion of cells in S and G2/M phases. HMVECs cultured with a combination of basic fibroblast growth factor, insulin-like growth factor-1, and epidermal growth factor displayed an increased proportion of nonviable cells in the presence of TSP2, but the addition of VEGF blocked this TSP2-mediated impairment of cell viability. TSP2-mediated inhibition of DNA synthesis by HMVECs in the presence of VEGF was not affected by the broad-spectrum caspase inhibitor zVAD-fmk. Similar findings were obtained with TSP1. Taken together, these observations indicate that either TSP2 or TSP1 can inhibit HMVEC proliferation by inhibition of cell cycle progression and induction of cell death, but the mechanisms responsible for TSP2-mediated inhibition of cell cycle progression are independent from those leading to cell death.

INTRODUCTION

The process of wound healing is highly dependent on angiogenesis to provide a vascular network for the regenerating tissue. The study of mechanisms governing vascularization of healing connective tissues has primarily focused on proangiogenic factors occurring early in the wound-healing process. Degranulating platelets and a fibrin clot provide growth and chemotactic factors and an adhesive substrate for initial influx of endothelial cells (ECs) (Singer and Clark, 1999; Tonnesen et al., 2000). Infiltrating inflammatory cells, such as macrophages, appear at the wound site shortly after the wounding event and are also potent sources of proangiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Sunderkötter et al., 1994). A number of these factors also induce migration of fibroblasts into the wound area and stimulate fibroblasts to deposit extracellular matrix (Kalluri and Sukhatme, 2000). The direct influence of fibroblasts on ECs in determining the vascularity of the healing wound is less clear. Regression of blood vessels seen in the resolution phase of a wound, in which fibroblasts become the predominant cell type, suggests an inhibitory role for fibroblasts in the angiogenic process (Kyriakides et al., 1999b).

A large number of factors have been found to antagonize one or more stages of the angiogenic process and are thus
candidates for mediators of vascular regression during wound healing (Carmeliet and Jain, 2000). In particular, the ability of thrombospondin 1 (TSP1) to inhibit angiogenesis in cultured cells and in vivo has been well documented (reviewed in Armstrong, et al., 1998; Dawson and Bousk, 1999; Chen et al., 2000). Studies with cultured cells indicate that TSP2 also contains antiangiogenic activity (Volpert et al., 1995; Panetti et al., 1997; Bornstein et al., 2000a). By examination of mice with a targeted deletion of the gene encoding TSP2 (Kyriakides et al., 1998b), our laboratory has shown that TSP2 is a physiological inhibitor of angiogenesis. In particular, TSP2-null mice display increased vascularity in healing excisional wounds (Kyriakides et al., 1999b; Bornstein et al., 2000b), in the capsules surrounding subcutaneously implanted silicone discs (Kyriakides et al., 1999a), and in granulation tissue invading subcutaneously implanted polyvinyl alcohol sponges (Kyriakides et al., 2001). These observations indicate that TSP2 preferentially inhibits vascularization of fibroblast-rich connective tissues in healing wounds, the foreign body response, and tumors.

Increasing evidence supports the idea that TSP1 and TSP2 influence angiogenesis by similar mechanisms, but in distinct locations and points in time by virtue of their different patterns and regulation of expression (Bornstein, 1995, 2001). In this study, we sought to identify the cellular sources of TSP2 by examination of production of TSP2 by fibroblasts and ECs, and to determine the cellular basis and mechanisms by which TSP2 inhibits vascular density. We find that TSP2 is largely absent from cultured ECs, but is expressed at high levels by fibroblasts. By quantifying TSP2 produced by fibroblasts, we find that fibroblasts secrete TSP2 at levels sufficient to inhibit proliferation of ECs. Both TSP1 and TSP2 increase caspase activity and impair viability in HMVECs in the absence of VEGF, but inhibit VEGF-stimulated cell cycle progression in a caspase-independent manner. Thus, we conclude that TSP1 and TSP2 can inhibit EC proliferation without causing cell death.

**MATERIALS AND METHODS**

**Preparation of Recombinant Full-Length TSP2 and Fragments of TSP2**

Recombinant murine TSP1 and TSP2 were expressed by infection of insect cells with baculoviruses containing TSP1 or TSP2 cDNA (a gift from D. Mosher, University of Wisconsin, Madison, WI), and purified by heparin affinity chromatography as described previously (Chen et al., 1994, 1996). A fragment of murine TSP2 encompassing the procollagen domain and type I repeats (PC/1, amino acids 320–549 with the initiator methionine as 1) was prepared by cloning the corresponding cDNA into pcAcGP67.coco (Misenheimer et al., 2000; a gift from D. Mosher) in frame with the upstream signal sequence and downstream polyhistidine tag. A fragment of murine TSP2 encompassing the C-terminal half of TSP2 (amino acids 377–1172) was also prepared by cloning into pAcGP67.coco. The resulting plasmids were cotransfected with linearized BaculoGold baculovirus genome into Sf9 cells, as recommended by the manufacturer (BD PharMingen, San Diego, CA). The His-tagged TSP2 fragments were purified from conditioned media of Hi5 cells by Ni-chelate affinity chromatography. Concentrations of the proteins were determined by A280 with an extinction coefficient as described previously (Chen et al., 1994) and by Bradford assay with bovine serum albumin as a standard; equivalent results were obtained. Preparations of recombinant proteins were tested for endotoxin by the LAL method (Associates of Cape Cod, Falmouth, MA) and found to contain <1 EU/μg protein. In addition, transforming growth factor-β activity in purified recombinant proteins was found to be undetectable, as determined by activation of a PAI-1 promoter/luciferase construct in transformed mink lung epithelial cells (Abe et al., 1994). Other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

**Preparation of Polyclonal and Monoclonal Antibodies against Murine TSP2**

Polyclonal antibodies against the N terminus of murine TSP2 were prepared as described previously (Kyriakides et al., 1998b). To prepare monoclonal antibodies against murine TSP2, TSP2-null mice were each injected i.p. with a mixture of full-length TSP2 and N-terminal, PC/1, and C-terminal fragments of TSP2. One hundred wells were selected based on differential reactivity patterns from the fragments. Possible precipitating antibodies were identified by plating supernatant from each well into 96-well plates and incubating with biotinylated TSP2, which was detected with horseradish peroxidase-streptavidin. Selected hybridomas (monoclonal antibodies [mAbs] 61, 93, and 156; described in Results) were cloned by limiting dilution. Monoclonal antibodies were purified from hybridoma-conditioned media by chromatography on a HiTrap Protein G column (Amersham Biosciences, Piscataway, NJ). Monoclonal antibodies produced by each of the primary clones were evaluated for IgG subclass and light chain composition by using the Mouse Monoclonal Antibody Isotyping kit (Roche Applied Science, Indianapolis, IN). Cross-reactivity of the antibodies with insect cell-derived recombinant mouse TSP1 was determined by ELISA.

**Preparation and Culture of Primary Human and Mouse ECs and Fibroblasts**

Wild-type mice or Immortomice (Jat et al., 1991) wild type or null for TSP2 (Yang et al., 2000a) were used as sources of murine cells. Immortomice carry an interferon-γ-inducible promoter driving the conditionally immortalizing simian virus 40 large T-antigen H-2Kk-tsAS8 as a transgene. Dermal cells were prepared by collagenase digestion as described previously (Yang et al., 2000b), except that the cells were initially plated in EC growth medium (EGM2-MV; BioWhittaker, Walkersville, MD) and were used as a starting material for preparation of the dermal ECs described below. To prepare lung cells, lungs from three wild-type or TSP2-null Immortomice were perfused with phosphate-buffered saline (PBS) and digested with 0.2% collagenase in Hanks’ balanced salt solution. After shearing through a 14-gauge cannula to generate a single-cell suspension, cells were dispensed onto a 75-cm2 tissue culture-treated flask preincubated with EGM2-MV and allowed to adhere for 24 h. ECs from lung and dermal cell populations were prepared as follows. For a first round of negative selection to remove macrophages and other immune cells, antimouse cell beads were prepared by incubating sheep anti-rat IgG-conjugated Dynabeads (Dynal, Lake Success, NY) with anti-mouse FcγRIII/II (BD PharMingen) in PBS containing 2% fetal calf serum (FCS) at 4°C overnight, and washing three times.
with PBS-FCS. Cells in monolayer were incubated with beads in growth medium for 1 h at 4°C, washed twice with PBS, and de- 
tached with 0.05% trypsin/1 mM EDTA. Bead-bound immune cells were removed with a magnet, and the unbound fraction, containing fibroblasts and ECs, was plated in a 75-cm² flask. After 2 d, positive selection of EC was performed with Dynabeads coated with anti- 
mouse intercellular adhesion molecule-2 (BD PharMingen) pre- 
pared as described above. The unbound fraction, consisting mainly of fibroblasts, was plated on tissue culture-treated plastic in DMEM containing 10% FCS and 20 U/ml interferon-γ (Invitrogen, Carls- 
bad, CA) and grown at 33°C. The bead-bound fraction was sus- 
pended in EGM2-MV containing 20 U/ml interferon-γ and dispensed into tissue culture-treated flasks precoated with EGM2-MV. 
ECs were propagated at 33°C and subjected to two subsequent rounds of positive selection when cultures neared confluence. Pu- 
ri ty of EC preparations was determined by plating 4 × 10⁴ cells/ 
well of eight-well chamber slides preincubated with media, and after 48 h, incubating with 1 µg/ml acetylated low-density lipoprotein 
leaving with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbo- 
cyanine perchlorate (DiI-Ac-RL; Biomedical Technologies, Stough- 
ton, MA) for 16 h. Cells were then fixed with 10% formalin buffered 
with zinc (Z-Fix; Anatech, Battle Creek, MI), stained with 5 µg/ml 
4',6-diamidino-2-phenylindole dihydrochloride (DAPI), and 
mounted with Vectashield (Vector Laboratories, Burlington, CA).

To prepare conditioned media from mouse fibroblasts and ECs, confluent cells in T25 flasks were fed with 6 ml of fresh growth 
media, and 300-µl aliquots of conditioned media were collected 
daily for 3 d. Cells were subsequently detached with trypsin/EDTA and 
counted with a hemocytometer. Human foreskin fibroblasts (a gift from 
David Morris, University of Washington, Seattle, WA) and human gingival and periodontal ligament fibroblasts (gifts from Dr. 
A.S. Narayanan, University of Washington) were propagated in 
DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 
µg/ml streptomycin, and 0.25 µg/ml amphotericin B. HMVECs (BioWhittaker) and human umbilical vein endothelial cells (HUVECs) (provided by the cell culture core group of the Biology of 
the Artery Wall Program Project, University of Washington) were 
propagated in EGM2-MV in a 37°C incubator under 5% CO₂ and 
used between passages 4 and 10. Conditioned media from human 
cells were prepared by incubating confluent cells with their respec- 
tive media lacking serum for 48 h.

Detection of TSP2 by Immunofluorescence
Murine fibroblasts and ECs were plated on glass eight-well chamber 
slides at 1 × 10⁵ cells/ml and incubated for 48 h. Cells were fixed 
with Z-Fix and permeabilized in 100% ethanol. After blocking with 
2% normal goat serum, mAb 93 was added at 25 
µg/ml for 1 h. Fluorescein isothiocyanate-conjugated anti-mouse IgG, diluted in 
blocking solution containing 5 µg/ml DAPI, was added for 1 h, and 
the slide was mounted with Vectashield. Images were obtained with a 
photomicroscope (Nikon, Tokyo, Japan) and processed with Meta- 
Morph software (Universal Imaging, Downingtown, PA).

Analysis of mRNA Encoding TSP2 and S6 
Ribosomal Protein by Reverse Transcription-
Polymerase Chain Reaction (RT-PCR)
RNA was isolated from cells by acid guanidinium/phenol/chloro- 
form extraction or with RNeasy kit (QIAGEN, Valencia, CA). Com- 
plementary DNA was synthesized with OmniScript reverse trans- 
scriptase (QIAGEN). Polymerase chain reaction amplification of 
cDNA encoding murine TSP2 and ribosomal protein S6 was pre- 
fomed for 30 cycles of 95°C for 1 min, 55°C for 2 min, and 72°C for 
3 min. Polymerase chain reaction amplification of human TSP2 and 
S6 cDNAs was performed for 30 cycles of 95°C for 1 min, 55°C for 
1 min, and 72°C for 2 min. Primer sequences were as follows: murine TSP2, forward 5'-CTGGTACACGCTCAAGAGCACACT- 
TCGAT-3', reverse 5'-ATGCACTTTTGGCCAGCTACCTCG-3'; 
murine S6, forward 5'-AACCGTCTGGCACCTTCTATGAGA-3', 
reverse 5'-TGAAGTCACGCTGAAATAGTGAAAG-3'; human TSP2, forward 5'-TTTGTTTTTGCACTGAGGTACG-3', reverse 5'-GAGCCCTTTGGTACGCTACGT-3'; and human S6, forward 5'- 
CGTACTTCTATGAAAGCCTGATGCG-3', reverse 5'-AAAGTC- 
TGGCACTTCTTGGCAATT-3'.

Detection and Quantification of TSP2 by Western 
Blotting and Enzyme-linked Immunosorbent 
Assay (ELISA)
Samples were resolved by SDS-PAGE under reducing or nonreduc- 
ting conditions, transferred to nitrocellulose membranes, incubated overnight with monoclonal anti-mouse TSP2 antibody 93, rabbit 
polyclonal antiserum against murine N terminus of TSP2, or mono- 
clonal anti-human TSP2 (BD PharMingen; Transduction Laborato- 
ries, Lexington, KY). Blots were then incubated with anti-mouse or 
anti-rabbit IgG-alkaline phosphatase conjugates, followed by color 
development with 5-bromo-4-chloro-3-indoly phosphate/nitro 
blue tetrazolium. For the sandwich ELISA, antibody 156 was dia- 
yzed against PBS and biotinylated with Sulfo-NHS-LC-biotin 
(Pierce Chemical, Rockford, IL). ProBond 96-well plates (Fisher 
Scientific, Pittsburgh, PA) were coated with antibody 61 at 20 
µg/ml in PBS and blocked with 5% FCS and 100 µg/ml amphotericin B. HMVECs (BioWhittaker) and human umbilical vein endothelial cells (HUVECs) (provided by the cell culture core group of the Biology of 
the Artery Wall Program Project, University of Washington) were 
propagated in EGM2-MV in a 37°C incubator under 5% CO₂ and 
used between passages 4 and 10. Conditioned media from human 
cells were prepared by incubating confluent cells with their respec- 
tive media lacking serum for 48 h.

Quantification of EC Proliferation, DNA Synthesis, 
and Caspase Activity
HMVECs or HUVECs were suspended in EBM2 (BioWhittaker) supplemented with 5% FCS (basal medium) and plated in gelati- 
nized 48-well plates at 2000 cells/well. After 5 h, the medium was 
replaced with basal medium supplemented with various combina- 	ions of VEGF (10 ng/ml; Pierce Chemical), bFGF (20 ng/ml, a gift from 
Scios Nova, Mountain View, CA), insulin-like growth factor-1 
(IGF-1, 20 ng/ml; R & D Systems, Minneapolis, MN), epidermal 
growth factor (EGF, 20 ng/ml; Invitrogen), and recombinant TSP2. 
Cells were incubated for 5 d, with one change of medium. Cell 
number was estimated indirectly by incubation in EGM2-MV sup- 
plemented with a metabolic dye (Cell Titer96; Promega, Madison, 
WI) for 1 h and measurement of the medium at 490 nm. To quantify 
DNA synthesis, HMVECs were plated at 1.5 × 10⁴ cells/well in 
48-well plates and incubated overnight in EBM2 containing 5% FCS. 
Media were replaced with media containing growth factors and/or 
TSP2 or TSP1 as indicated (0.2 ml/well). In experiments examining 
effects of inhibition of caspases on DNA synthesis, caspase inhibitor 
I (zVAD-fmk; Calbiochem, San Diego, CA) was added to 100 
µM from a stock of 50 mM in dimethyl sulfoxide (DMSO), and control 
cultures contained an equivalent volume of DMSO. Cells were preincubated with the caspase inhibitor or DMSO for 1 h before 
addition of growth factors and TSP2. After incubation for 22 h, 
[³H]thymidine (1 µCi/well; Amersham Biosciences) was added and 
icubation continued for 2 h. Cells were washed with cold 5% 
trichloroacetic acid and incubated on ice in 5% trichloroacetic acid 
for 20 min. The cell layer was washed twice with cold PBS, and 
DNA was solubilized with 0.25 N NaOH. Extracts were mixed with 
Biosafe II scintillation cocktail (Research Products International, Mt. 
Prospect, IL) and counted. To measure caspase activity, HMVECs 
were cultured and treated with test substances in the same manner, 
except that the incubation time was 4 h. Total cellular caspase 
activity was determined with the Homogeneous Caspase Assay kit

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To determine whether human cells resembled mouse cells in expression of TSP2, RT-PCR for TSP2 was performed on HMVECs and fibroblasts from foreskin, periodontal ligament, and gingiva. TSP2 mRNA was faintly detectable in HMVECs but was present in much larger amounts in fibroblasts (lanes 2, 3, 6, and 7), and HMVECs (lanes 4 and 8). Arrows, as described in C.

**RESULTS**

**TSP2 Is Expressed at High Levels by Fibroblasts, but Is Absent from ECs**

To clarify the issue of cellular sources of TSP2, we purified ECs and fibroblasts from lung and dermis and compared expression of TSP2 mRNA and secretion of TSP2. We were able to obtain essentially pure populations of ECs, containing <2% non-Dil-Ac-LDL–staining cells, from mouse lungs. Separation of cells from dermis by the same procedure yielded mixtures of cell types, of which ~70% were ECs as determined by uptake of Dil-Ac-LDL (Figure 3D).

RNA was isolated from murine endothelial and fibroblast populations, and mRNA encoding TSP2 was detected by RT-PCR. ECs from murine lung contained no detectable TSP2 mRNA, whereas fibroblasts from murine lung contained abundant TSP2 mRNA (Figure 1A). Impure preparations of ECs from dermis contained TSP2 mRNA; however, the amount of TSP2 mRNA decreased with increasing purity of EC, indicating that contaminating fibroblasts secreted large amounts of TSP2 and might be responsible for all of the TSP2 produced by the population (our unpublished data).

To determine whether human cells resembled mouse cells in expression of TSP2, RT-PCR for TSP2 was performed on HMVECs and fibroblasts from foreskin, periodontal ligament, and gingiva. TSP2 mRNA was faintly detectable in HMVECs but was present in much larger amounts in fibroblasts from all sources (Figure 1B). The mRNA encoding S6 ribosomal protein was detected in essentially equal amounts in all of the cell types, indicating that the RNA from HMVECs is intact. Thus, mRNA encoding TSP2 is absent or present at very low levels in ECs but is abundant in fibroblasts of both murine and human origin.

To obtain antibodies for detection and quantification of murine TSP2, we prepared monoclonal antibodies against recombinant murine TSP2. Immunization of TSP2-null mice with recombinant TSP2 resulted in a strong immune response, culminating in the identification of >300 positive clones in the primary screen after fusion of splenocytes with myeloma cells. In contrast, a fusion of similar scale after immunization of wild-type rats with TSP2 yielded only 16
positive wells (our unpublished data). Thus, absence of endogenous TSP2 rendered exogenous TSP2 highly immunogenic. A secondary screen against three recombinant fragments of TSP2 permitted the distinction of antibodies against various regions of the TSP2 molecule. Further screening for binding to TSP2 in solution and specific staining of wild-type, but not TSP2-null, fibroblasts led to the selection of wells 61 (IgG2a; C terminus specific), 93 (IgG1; N-terminus specific), and 156 (IgG2b; type I repeats specific) for further analysis. All antibodies possessed a κ light chain. Binding of each antibody to purified recombinant mouse TSP1 or mouse fibroblast-derived TSP1 was undetectable by ELISA or Western blotting analysis (our unpublished data).

Conditioned media were collected from fibroblasts isolated from wild-type and TSP2-null Immortomice and ECs from lungs of Immortal mice wild type for TSP2, and resolved by SDS-PAGE under reducing and nonreducing conditions. Subsequently, Western blotting analysis with monoclonal anti-mouse TSP2 antibody 93 was performed. Under nonreducing conditions, a 450-kDa band was detected at high levels in wild-type fibroblast-conditioned medium, whereas under reducing conditions, a single band at 195 kDa was detected (Figure 1C). Neither of these bands was evident in conditioned media from TSP2-null fibroblasts or wild-type lung ECs. Similar results were obtained with a polyclonal antibody against the N-terminal fragment of TPS2 (our unpublished data).

Conditioned media were also obtained from HMVECs, human neonatal dermal fibroblasts, and human periodontal ligament fibroblasts, and subjected to Western blotting with an antibody against human TSP2. Similar to murine cells, an immunoreactive band was found at 195 kDa under reducing conditions in fibroblast-conditioned media, but was absent from EC-conditioned media (Figure 1D).

Preliminary experiments revealed that the monoclonal anti-mouse TSP2 antibodies, in any combination, were highly sensitive in detecting murine TSP2 when used in a sandwich ELISA. Further experiments using unlabeled antibody 61 in the solid phase and biotinylated antibody 156 for detection of bound TSP2 yielded a linear signal from 1.25 to 20 ng of murine TSP2. Quantification of TSP2 by sandwich ELISA indicated that confluent monolayers (~0.5 × 10⁶ cells) of wild-type untransformed and Immortal mouse dermal fibroblasts in a 25-cm² flask accumulated TSP2 in the medium at concentrations up to 0.5 and 5 μg/ml, respectively (Figure 2). Murine lung ECs isolated from Immortal mice and grown under permissive conditions were found to accumulate TSP2 to 12 ng/ml (Figure 2). This amount of TSP2 could be produced by the small number of fibroblasts remaining in the EC preparations. Specificity of the assay for TSP2 was confirmed by the lack of a signal in conditioned media from TSP2-null fibroblasts (our unpublished data). Thus, dermal fibroblasts synthesize at least 40-fold more TSP2 than do lung EC.

To further characterize the dermal cell population synthesizing TSP2, fibroblasts and partially purified ECs from murine dermis were immunostained for TSP2 with mAb 93. Wild-type fibroblasts stained strongly for TSP2 (Figure 3A), whereas the TSP2-null fibroblasts were negative for TSP2 (Figure 3B). In partially purified preparations of dermal EC, the majority of cells in the EC-enriched fraction did not stain for TSP2 (Figure 3C). The small number of cells staining for TSP2 seemed to form clusters and multicellular strands amid the TSP2-negative monolayer. In parallel cultures stained with Dil-Ac-LDL and DAPI, the clustered cells did not incorporate Dil-Ac-LDL, whereas the cells in the monolayer displayed robust incorporation of Dil-Ac-LDL (Figure 3D). The Dil-Ac-LDL-negative, TSP2-positive clusters most likely represent fibroblasts, and the Dil-Ac-LDL-positive, TSP2-negative monolayer cells are most likely ECs. Thus, we conclude that cultured murine ECs synthesize largely undetectable TSP2 mRNA and protein, whereas fibroblasts synthesize considerable TSP2.

**Inhibition of EC Proliferation and Cell Cycle Progression by TSP2**

To determine whether TSP2 preferentially inhibits growth of ECs mediated by specific growth factors, purified recombinant TSP2 was incubated with ECs in the presence of bFGF, IGF-1, EGF, or VEGF for 4 d, and cell number was estimated by a colorimetric assay. TSP2 inhibited growth mediated by all of the growth factors in this assay (our unpublished data). Proliferation of sparsely plated HMVECs, stimulated by growth factors in the absence or presence of TSP2 (2 μg/ml), was also quantified by direct cell counting after 3 d. In basal medium lacking added growth factors, HMVECs did not proliferate, and TSP2 had no effect on cell number (Figure 4A). However, increases in cell number mediated by VEGF or a combination of bFGF, IGF-1, and EGF were inhibited by TSP2 and TSP1 to a similar degree (Figure 4A). To determine whether TSP2 slowed or arrested proliferation of HMVECs, cells were incubated in medium containing bFGF, IGF-1, and EGF in the presence or absence of TSP2, and cell number was determined over time. In contrast to the increase in cell number in the presence of growth factors, cell number remained constant in the presence of TSP2 (Figure 4B), indicating that TSP2 causes arrest of proliferation. To determine the range of concentrations at which TSP2 inhibits EC proliferation, HMVECs were plated at defined density and incubated in basal medium supplemented with bFGF and...
IGF-1 with increasing concentrations of recombinant murine TSP2. Concentrations of recombinant murine TSP2 as low as 0.25 μg/ml (0.6 nM) were effective in inhibiting proliferation of ECs (Figure 4C). Addition of polymyxin B, an inhibitor of endotoxin activity, had no effect on TSP2-mediated inhibition of proliferation, indicating that the very low levels of endotoxin in our preparations of TSP2 were not contributing to the antiproliferative effect of TSP2 (our unpublished data).

To determine whether different types of ECs were susceptible to inhibition by TSP2, growth of HUVECs incubated with bFGF in the presence or absence of TSP2 was determined. In contrast to HMVECs, proliferation of HUVECs was resistant to inhibition by TSP2 (Figure 4D).

To determine whether the reduction in cell number in the presence of TSP2 might be a consequence of inhibition of cell cycle progression, HMVECs were incubated with bFGF, EGF, and IGF-1 in the presence or absence of VEGF and TSP2 for 24 h. The adherent and detached cells were pooled, labeled with propidium iodide, and subjected to FACS analysis. After 24 h, TSP2-treated cells, in the presence of all combinations of growth factors, were found to have a reduced proportion of cells in the S and G2/M fractions (Figure 5). Direct comparison of cell cycle distribution in response to TSP1 and TSP2 indicated that TSP1 and TSP2 have similar abilities to cause arrest at the G0/G1 phase (Figure 6). Thus, both TSP1 and TSP2 can cause impairment of cell cycle progression in HMVECs in the presence of all growth factors tested.

**Growth Factors and Caspase Inhibitors Block TSP2-mediated Cell Death and Caspase Activation but Do Not Block TSP2-mediated Inhibition of Cell Cycle Progression**

To determine whether impairment of viability contributes to the ability of TSP2 to inhibit proliferation of microvascular ECs, HMVECs were incubated with different combinations of growth factors in the presence or absence of TSP2, and cell death was assessed by flow cytometry using an annexin V-FITC/propidium iodide double staining kit.

**Figure 3.** Detection of TSP2 by immunofluorescence in fibroblasts but not in EC isolated from mouse dermis. Dermal fibroblasts from wild-type (A) and TSP2-null (B) mice and partially purified dermal ECs from wild-type mice (C) were stained for TSP2 (green) with mAb 93 and counterstained with DAPI (violet). The arrow in C indicates a cluster of cells positive for TSP2 amid the monolayer of TSP2-negative cells. In a parallel slide (D), the partially purified dermal ECs from wild-type mice were incubated overnight with DiI-Ac-LDL (red) and subsequently fixed and stained for DAPI (violet). Arrow, a cluster of fibroblasts negative for uptake of DiI-Ac-LDL.
viability was determined by staining with trypan blue. After 72 h, HMVECs exposed to TSP2 or TSP1 in basal medium alone exhibited no change in viability (Figure 7A). Cells treated with a combination of bFGF, IGF-1, and EGF contained a higher proportion of nonviable cells in the presence of TSP2 or TSP1 (Figure 7A). However, the presence of TSP2 or TSP1 did not affect the proportion of nonviable cells in HMVECs incubated in the presence of VEGF alone or with the above-mentioned growth factors (Figure 7A). To determine whether caspase activation was responsible for the selective impairment of viability by TSP2 in the presence of bFGF, IGF-1, and EGF, caspase activity was measured after a 7-h incubation with these factors. Caspase activation in HMVECs in basal medium was induced by TSP2 (Figure 7B). Interestingly, both VEGF alone and the combination of bFGF, EGF, and IGF-1 blocked TSP2-mediated caspase activation (Figure 7B), suggesting that a caspase-independent pathway might be responsible for TSP2-mediated impairment of viability of HMVECs in the presence of bFGF, IGF-1, and EGF. Comparison of the relative abilities of TSP1 and TSP2 to activate caspases in the presence or absence of VEGF indicated that TSP1 and TSP2 activate caspase activity under basal conditions to similar degrees, and this activation was blocked by VEGF (Figure 7C).

To assess whether caspase-dependent proapoptotic pathways were responsible for TSP2-mediated inhibition of proliferation in the presence of growth factors, [3H]thymidine incorporation by HMVECs in EBM2/5% FCS alone or containing a combination of VEGF, bFGF, IGF-1, and EGF, in the presence or absence of TSP2 and a broad-spectrum caspase inhibitor, zVAD-fmk, was determined. TSP2 inhibited DNA synthesis in the presence or absence of growth factors, and inhibition of caspases by zVAD-fmk had no effect on this TSP2-mediated inhibition of [3H]thymidine incorporation (Figure 8, top). Inhibition of DNA synthesis by TSP1 was also not affected by the presence of zVAD-fmk (our unpublished data). Total caspase activity was assayed in cells subjected to the identical treatment described above for 4 h. TSP2 increased caspase activity in HMVECs in basal medium, but had no effect in the presence of growth factors (Figures 7 and 8, bottom). Furthermore, zVAD-fmk blocked the TSP2-mediated increase in caspase activity in the absence of growth factors (Figure 8, bottom). The observation that both growth factors and a broad-spectrum caspase inhibitor block TSP2-mediated caspase activation, but have no effect on TSP2-mediated inhibition of DNA synthesis, indicates that TSP2-mediated inhibition of cell-cycle progression is caspase independent (Figure 9).

DISCUSSION

In this study, we have explored the mechanisms by which TSP2 inhibits proliferation of ECs. Because proliferation represents a balance between cell division and cell death, we examined both cell death and inhibition of cell cycle progression of HMVECs mediated by TSP2 in the presence of various growth factors. We found that TSP2 promotes cell death, because it decreased cell viability in the presence of bFGF, IGF-1, and EGF, and increased caspase activity under
basal conditions. However, we also determined that VEGF protects HMVECs from TSP2-mediated cell death-promoting pathways, as determined both by viability assays and caspase activity (Figures 7 and 8). In contrast, we observed that TSP2 inhibits cell cycle progression of HMVECs mediated by both bFGF and VEGF (Figure 5) and that caspase inhibitors failed to block TSP2-mediated inhibition of cell cycle progression (Figure 8). Thus, TSP2 inhibits cell cycle progression by a signaling pathway that is independent from those that promote caspase activation or caspase-independent cell death (Figure 9). The same results in these experiments were obtained with TSP1.

We have determined the cellular source of TSP2, and documented that ECs from several sources do not produce significant amounts of TSP2, thus ruling out an autocrine mechanism for TSP2-mediated inhibition of vascular density. We found that fibroblasts in culture produce sufficient TSP2 to inhibit proliferation of ECs. Thus, we conclude that fibroblasts, and possibly other mesenchymal cells, secrete TSP2 as a paracrine mediator of EC proliferation (Figure 9).

Our observation that recombiant TSP2 strongly inhibits proliferation of HMVECs mediated by VEGF, IGF-1, EGF, and bFGF suggests that TSP2 modulates the response of ECs to a variety of mitogenic stimuli, and is consistent with previous studies demonstrating that migration of microvascular ECs stimulated by a variety of chemotactic factors, and proliferation of bovine aortic ECs stimulated by lysophosphatidic acid, are inhibited by TSP2 (Volpert et al., 1995; Panetti et al., 1997). Our findings that TSP2 can increase caspase activity in, and decrease cell viability of, HMVECs is consistent with the observation that TSP1 induces apoptosis of ECs (Guo et al., 1997; Jimenez et al., 2000; Nor et al., 2000).

However, our results indicate a more complex role for TSP2 in causing cell death. Although both TSP1 and 2 increased caspase activity in HMVECs in basal medium, neither protein had an effect on cell viability under these conditions (Figure 7). This result might be caused by further stimulation of caspasases by TSP1 and TSP2 in cells already undergoing cell death induced by growth factor withdrawal. Alternatively, the modest increases in caspase activation induced by TSP1 and 2 in basal medium might not be sufficient to cause frank apoptosis. TSP2 did impair viability in the presence of a combination of bFGF, IGF-1, and EGF, but these growth factors blocked TSP2-mediated activation of caspase activity (Figure 7). This observation reveals the ability of TSP2 to induce a caspase-independent form of cell death under some circumstances. On the other hand, the presence of VEGF blocked both TSP2-mediated caspase activation and impairment of viability (Figure 7). The greater effectiveness of VEGF in protecting cells from the cell death-promoting effects of TSP2 might result from the greater ability of VEGF to activate the phosphotidylinositol 3-kinase/Akt pathway, which induces expression of cytoprotective proteins of the IAP and bcl-2 families (Gerber et al., 1998a,b; Tran et al., 1999; Mesri et al., 2001).

Differences have also been observed in the ability of TSP1 to inhibit angiogenesis mediated by VEGF and bFGF. A previous study found that, in the chick chorioallantoic membrane assay, peptides from the tryptophan-rich region of the type I repeats of TSP1 inhibited angiogenesis mediated by
bFGF, but not VEGF, whereas peptides from the CD36-binding region inhibited angiogenesis mediated by both growth factors (Iruela-Arispe et al., 1999). Binding of bFGF by TSP1 has been demonstrated previously (Taraboletti et al., 1997), and TSP2 could also inhibit bFGF-mediated cell cycle progression and cytoprotection by directly binding bFGF (our unpublished observations). It should be noted that the magnitude of caspase activation by TSP2 and TSP1 (1.3-fold) was modest in comparison with the 3.2-fold increase in caspase activation in HMVECs treated with staurosporine (our unpublished observations). Taken together, these observations characterize TSP2 and TSP1 as weak, contextual inducers of cell death, in contrast to broad-spectrum chemical inducers of apoptosis.

TSP2 impairs cell cycle progression, as indicated by a decrease in the proportion of cells in the S and G2/M phases of the cell cycle in HMVECs stimulated by all combinations of growth factors that we tested. Our observations that 1) the presence of VEGF renders HMVECs resistant to TSP2-mediated cell death but does not protect these cells from TSP2-mediated impairment of cell cycle progression; and 2) TSP2-mediated inhibition of cell cycle progression could not be inhibited by a broad-spectrum inhibitor of caspases, indicate that caspase activation does not play a role in TSP2-mediated inhibition of cell cycle progression in HMVECs. The independence of TSP2-mediated inhibition of cell cycle progression from cell death pathways stands in contrast to the observation that TSP1-mediated inhibition of microvascular EC migration is dependent on caspase activation (Jimenez et al., 2000). Further experimentation is necessary to characterize the pathways by which TSP2 influences cell death and cell cycle progression and to determine the relative importance of cell death and cell cycle progression in TSP2-mediated inhibition of angiogenesis in vivo. The possibility exists that cell death and inhibition of cell cycle progression might

Figure 6. TSP2 and TSP1 inhibit cell cycle progression equally in HMVECs. HMVECs were incubated in basal medium alone or supplemented with bFGF, EGF, IGF-1, and VEGF (GF), in the absence or presence of TSP1 or TSP2. Cells were analyzed for cell cycle distribution as described above. TSP1 and TSP2 have a similar ability to arrest HMVECs in the G0/G1 phase of the cell cycle. *, p < 0.0005, compared with cell-cycle phase in the presence of growth factors alone, by a two-tailed t test (n = 3). Error bars represent SD of the mean. Data are representative of two independent experiments.

Figure 7. VEGF blocks TSP2-mediated impairment of HMVEC viability. (A) Sparsely plated HMVECs were incubated for 72 h in medium containing 5% serum alone, or supplemented with a mixture of bFGF, IGF-1, and EGF (FIE), or with VEGF alone (V), in the presence or absence of 2 μg/ml TSP2 or TSP1. Adherent and detached cells were collected and stained with trypan blue. The proportion of nonviable, trypan blue-positive cells was determined. Error bars represent SD of the mean (n = 3), * , p < 0.005 by two-tailed t test. TSP2 and TSP1 impair viability of HMVECs in the presence of bFGF, IGF-1, and EGF, but not in the presence of VEGF. (B) HMVECs plated and incubated with growth factors and TSP2 as described above for 7 h were analyzed for caspase activity. TSP2 induced caspase activity in basal media, but both VEGF and the combination of bFGF, IGF-1, and EGF blocked TSP2-mediated caspase activation. *, p < 0.05 by two-tailed t test (n = 3). (C) HMVECs plated and incubated in the presence or absence of TSP1, TSP2, or VEGF were analyzed for caspase activity. TSP1 and TSP2 induce activation of caspases to similar degrees, but VEGF blocks TSP1- and TSP2-mediated caspase activation. *, p < 0.05 by two-tailed t test (n = 3).
both be necessary for TSP2-dependent vascular regression, such as occurs during the late stages of healing of an excisional wound (Kyriakides et al., 1999b). The observation that macrophage-dependent capillary regression in the pupillary membrane of the eye occurs specifically when the ECs are in G1 phase (Diez-Roux et al., 1999) suggests a potential mechanism by which TSP2 might initiate vascular regression by inhibiting cell cycle progression in microvascular ECs.

Purified recombinant TSP2 inhibits growth of HMVECs, but not HUVECs, indicating that only certain subsets of ECs are sensitive to inhibition of growth by TSP2. Similar findings have been reported for TSP1, and reflect the fact that CD36, a receptor for TSP1, is present on HMVECs but not HUVECs (Dawson et al., 1997). The extent to which CD36 participates in TSP2-mediated inhibition of proliferation is currently under investigation.

Immunohistochemical and in situ hybridization studies have yielded conflicting information regarding the cellular source of TSP2. An in situ hybridization study performed on mouse embryos found TSP2 mRNA to be associated with both endothelium and mesenchyme in several tissues (Iruela-Arispe et al., 1993). Immunohistochemical analyses located TSP2 predominantly in mesenchymal cells of connective tissues and occasionally in endothelium (Kyriakides et al., 1998b; Tooney et al., 1998). In healing excisional wounds and fibrotic capsules surrounding subcutaneously implanted silicone discs, TSP2 was detected by immunohistochemistry throughout the fibrillar collagen-rich extracellular matrix and was not detected in association with the endothelium (Kyriakides et al., 1999b). In previous studies from our laboratory, we detected TSP2 by Western blotting in conditioned media from primary cultures of murine dermal fibroblasts and marrow-derived stromal cells (Kyriakides et al., 1998b; Hankenson et al., 2000; Yang et al., 2000b). The
quantitative experiments presented herein indicate that TSP2 accumulates in conditioned media from murine dermal fibroblasts to concentrations at which recombinant TSP2 can inhibit proliferation of ECs. In contrast, we determined that ECs from several sources express very low or undetectable TSP2 mRNA and protein. The small amounts of TSP2 mRNA and protein that we detected in HMVECs and mural lung ECs, respectively, are likely to arise from the small number (<2%) of contaminating fibroblasts in each preparation. Therefore, we conclude that TSP2 is absent from cultured ECs and that TSP2 can serve as a highly sensitive marker for the presence of contaminating fibroblasts in preparations of murine ECs. However, we cannot rule out the possibility that subsets of ECs not examined in this study express TSP2, or that ECs can be induced to express TSP2 with specific stimuli. In this respect, TSP2 differs from TSP1 in that TSP1 is more prominently expressed in endothelial cells during embryogenesis (Iruela-Arispe et al., 1993) and is synthesized in significant amounts by cultured ECs (McPherson et al., 1981; Mosher et al., 1982). Thus, unlike TSP2, TSP1 seems to be an autocrine inhibitor of EC function (Iruela-Arispe et al., 1991; DiPietro et al., 1994; Tolasma et al., 1997). Our findings that TSP2 and TSP1 have similar effects on proliferation and cell death in endothelial cells support the concept that the different distributions, rather than intrinsic biochemical properties, of TSP2 and TSP1 determine the differences in antiangiogenic functions of the proteins in vivo.

The influence of fibroblasts on the process of angiogenesis seems to be complex. Several studies have identified a role for fibroblasts in stabilizing ECs in three-dimensional networks (Montesano et al., 1993; Kuzuya and Kinsella, 1994; Villaschi and Nicosa, 1994; Xin et al., 2001). Conditioned medium from fibroblasts was also found to have a neutral (Kuzuya and Kinsella, 1994) or somewhat stimulatory effect (Zhao and Eghbal/Webb, 2001) on proliferation of ECs, but strongly inhibited migration of ECs (Volpert et al., 1997). Although the production of physiologically significant amounts of a cell death-inducing protein by fibroblasts is apparently contradictory to the proposed role of fibroblasts in stabilizing the microvasculature, the cell death-promoting effects of TSP2 might be counteracted by protective factors secreted by fibroblasts. Indeed, our observation that HMVECs exhibit reduced transition from G0/G1 to S phase, but maintain high viability in the presence of TSP2 and VEGF, provides a potential mechanism for maintaining the capillary endothelium in a state of quiescence. Thus, it is conceivable that fibroblasts inhibit proliferation and migration of ECs to prevent new vessel growth, but also promote the stability of preexisting vessels. A further point of uncertainty is the extent to which fibroblasts contact and thus exert influence on ECs in vivo. ECs that have synthesized a basement membrane and become invested with pericytes might be refractory to the inhibitory effects of fibroblast-derived TSP2. Interestingly, smooth muscle cells, which are related phenotypically to pericytes, seem to synthesize TSP2 (Armstrong, Yang, and Bornstein, unpublished observations). Quantification of TSP2 by cultured pericytes and smooth muscle cells will be necessary to determine whether such mural cells might also be a significant source of TSP2 that can inhibit EC proliferation in vivo.

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