Thrombospondin 1 and Type I Repeat Peptides of Thrombospondin 1 Specifically Induce Apoptosis of Endothelial Cells

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ABSTRACT

Thrombospondin 1 (TSP1) inhibits angiogenesis and modulates endothelial cell adhesion, motility, and growth. The antiproliferative activity of TSP1 is mimicked by synthetic peptides derived from the type I repeats of TSP1 that antagonize fibroblast growth factor 2 and activate latent transforming growth factor β. These TSP1 analogues induced programmed cell death in bovine aortic endothelial cells based on morphological changes, assessment of DNA fragmentation, and internucleosomal DNA cleavage. Intact TSP1 also induced DNA fragmentation. The endothelial cell response was specific because no DNA fragmentation was induced in MDA-MB-435S breast carcinoma cells, although TSP1 and the peptide conjugates inhibited the growth of both cell types. Apoptosis did not depend on activation of latent transforming growth factor β because peptides lacking the activating sequence RFK were active. Apoptosis was not sensitive to inhibitors of ceramide generation but was inhibited by the phosphatase inhibitor vanadate. Induction of DNA fragmentation by the peptides was decreased when endothelial cell cultures reached confluence. Growth of the cells on a fibronectin substrate also suppressed induction of apoptosis by TSP1 or the peptides. Differential sensitivities to kinase inhibitors suggest that apoptosis and inhibition of proliferation are mediated by distinct signal transduction pathways. These results demonstrate that induction of apoptosis by the TSP1 analogues is not a general cytotoxic effect and is conditional on a lack of strong survival-promoting signals, such as those provided by a fibronectin matrix. The antitumor activity of TSP1 may therefore result from an increased sensitivity to apoptosis in endothelial cells adjacent to a provisional matrix during formation of vascular beds in tumors expressing TSP1.

INTRODUCTION

The extracellular matrix provides both positive and negative signals to regulate endothelial cell growth. Growth factors such as FGF-2 and vascular endothelial cell growth factor promote the growth and survival of nontransformed endothelial cells only when the cells are adherent to an appropriate extracellular matrix. Fibronectin is one of the matrix proteins that provide these signals. Fibronectin is an important matrix component for promoting the survival and growth of many cell types. The binding of fibronectin to the integrin receptor αvβ3 induces activation of signal transduction pathways including the focal adhesion kinase (reviewed in Refs. 1 and 2) and other protein kinases (3–5) and results in signals that maintain viability, such as up-regulation of Bcl-2 in Chinese hamster ovary cells (6) and mitogen-activated protein kinase in fibroblasts (7). The absence of appropriate matrix signals can induce programmed cell death or apoptosis of endothelial cells (8, 9). Inhibitors of a second integrin, αvβ3, also induce apoptosis of angiogenic blood vessels and regression of tumors dependent on this neovascularization (10, 11).

In addition to these positive signals, the extracellular matrix may also provide negative signals to regulate endothelial cell growth. TSP1 (reviewed in Refs. 12 and 13) is one of several matricellular components that, under defined conditions, can inhibit endothelial cell adhesion (14, 15), motility (16), and growth (16–19). TSP1 specifically inhibits endothelial cell adhesion on a fibronectin matrix (14). However, TSP1 can also act as a positive stimulator of endothelial cell adhesion and motility (16, 20), and both positive and negative effects of TSP1 have been reported on angiogenesis in vivo (reviewed in Refs. 21–23). Conflicting signals may therefore arise from the interaction of endothelial cells with TSP1, and further work is needed to define the integration and regulation of these responses. In our experience, however, expression of TSP1 in human breast carcinoma cells suppresses their tumorigenic and angiogenic activity in mouse xenografts (24, 25).

Three domains of TSP1 are implicated to date in the inhibitory activities of TSP1 on endothelial cell growth and motility. The amino-terminal domain of TSP1 mimics the inhibitory activity of intact TSP1 on focal adhesion contacts, and this activity is suppressed by an antibody to this domain (26). The recombinant amino-terminal domain of TSP1 also inhibits proliferation and motility of endothelial cells in response to FGF-2 (27). The TSP1 procollagen domain peptide NGVQYRN inhibits motility of endothelial cells to FGF-2 in vitro and angiogenesis in vivo (28), but its mechanism of action is not known. The type I repeats of TSP1 contain additional inhibitory peptide sequences (27, 28). Peptides from the type I repeats compete with FGF-2 for binding to endothelial cells and inhibit both proliferative and motility responses to this growth factor (27). Because the type I repeat peptides also compete for binding of FGF-2 to heparin or to intact endothelial cells, we proposed that these peptides inhibit endothelial cell responses to FGF-2 by competing with the growth factor for binding to the heparan sulfate proteoglycans that are required for presenting FGF-2 to its high affinity tyrosine kinase receptor (27). On the basis of our recent identification of a TGF-β-activating sequence in this same peptide (29), a second possible mechanism for the observed growth inhibition by TSP1 is by activation of latent TGF-β produced by the endothelial cells (30).

We also observed that endothelial cells lose their normal morphology when treated with peptides from the type I repeats of TSP1 and that cell numbers decreased after incubation with the peptides. Although the peptides also inhibited the growth of a human breast carcinoma cell line, we did not observe a decrease in cell number. This suggested that the peptides may either have a specific cytotoxic activity toward endothelial cells or trigger programmed death of these cells. We have further examined the effects of TSP1 and the peptides on endothelial and breast carcinoma cells, and we report here that TSP1 and the thrombospondin peptides specifically induce apoptosis.

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3 The abbreviations used are: FGF-2, fibroblast growth factor 2; BAE, bovine aortic endothelial; TPA, 12-0-tetradecanoylphorbol-13-acetate; TGF-β, transforming growth factor β1; TSP1, thrombospondin 1; BrdUrd, bromodeoxyuridine.

in endothelial cells and that this activity is independent of their ability to activate latent TGF-β.

MATERIALS AND METHODS

TSP1 was purified from thrombin-stimulated human platelets as described previously (31). Fibronectin was purified from human plasma (NIH Blood Bank) as described (32). Recombinant human TGF-β1 was obtained from R&D Systems, Inc. Synthetic peptides from the type I repeats of human TSP1 were prepared and characterized as described previously (33, 34). Analogues of the TSP1 sequence (KRAKAGGWSHPSWSSC, KRKFQDGDHASPASSC) were prepared with appropriate Ala substitutions to eliminate the essential Phe residue for TGF-β activation or the Trp residues required for heparin binding and contain a carboxyl-terminal Cys residue to allow conjugation to polysucrose. Structures of the peptides used are summarized in Table 1. Peptides with Ala substitutions for Phe were unable to activate a mixture of latent TGF-β1 and TGF-β2 in BAE cell conditioned medium as assessed by NRK fibroblast colony formation in soft agar. Conjugation of the peptides to polysucrose was performed as described previously. In all cases, the peptides were used as polysucrose conjugates, which lack the adhesive activity of the free peptides but retain their other biological activities to regulate cell proliferation.

Cell Culture. BAE cells were kindly provided by Dr. E. Gallin (Armed Forces Radiobiology Research Institute, Bethesda, MD) and were used at passages 4–10. BAE cells were maintained at 37°C in 5% CO₂ in DMEM (low glucose) containing 10% FCS, 4 μg/ml transferrin, 25 μg/ml ascorbic acid, and 500 units/ml each of penicillin G, potassium, and streptomycin sulfate. Media components were obtained from Biofluids Inc. (Rockville, MD). MDA-MB-435S and MCF7 breast carcinoma cells (American Type Culture Collection) were grown in RPMI 1640 containing 10% FCS, 450 μM glucose, and 500 units/ml each of penicillin G, potassium, and streptomycin. Media components were obtained from Biofluids Inc. (Rockville, MD). MDA-MB-435S and MCF7 breast carcinoma cells (American Type Culture Collection) were grown in RPMI 1640 containing 10% FCS. Okadaic acid, TPA, fumonisin B1, herbimycin A, and sodium vanadate were purchased from Sigma.

TGF-β Assays. NRK fibroblast bioassays for TGF-β activity were conducted as described previously (29, 35). Serum-free conditioned medium (35) prepared from BAE cells at 70% confluence was used as a source of latent TGF-β for detecting activation by synthetic peptides in the NRK colony-forming assay. NRK colonies in soft agar were quantified microscopically. TGF-β1 was also quantified using an immunoassay specific for this isoform (Genzyme Corp.). BAE cells secrete latent TGF-β1 and TGF-β2 (36), and MDA-MB-435S cells produce TGF-β1, TGF-β2, and TGF-β3 (37). Using the TGF-β1-specific ELISA, conditioned media from BAE, MCF7, and MDA-MB-435S cells contained 2.05, 2.53, and 1.68 ng/ml acid-activatable TGF-β1. More than 90% of total TGF-β activity was latent in media from each cell line as assessed by the NRK bioassay.

Endothelial Cell Proliferation. Cell proliferation was measured using the Cell-Titer colorimetric assay (Promega) as described previously (27). In brief, cells were trypsinized and suspended in complete medium (BAE cells in DMEM; MDA-MB-435S cells in RPMI 1640) containing 10% FCS at 1–1.5 × 10⁵ cells/ml. Inhibitors were added to a 96-well plate (Costar Corp.) in 50 μl of medium without FCS followed by 50 μl of the endothelial cell suspension. The plates were incubated at 37°C in a 5% CO₂ atmosphere for 72 h. For determining the effect of okadaic acid, phorbol, herbinycin, fumonisin B1, or TPA on proliferation, the reagents were added to a 96-well plate (Costar) precoated with either medium or culture medium only (100 μl/well) to yield a final volume of 200 μl/well. The cells were incubated for 24–72 h as indicated at 37°C in a humidified atmosphere with 5% CO₂. For determining the effect of inhibitors on confluent endothelial cells, cells were cultured for 24–48 h at 37°C until the cells reached confluence, and inhibitors were added into the wells and incubated as described above.

After incubation, the cells in the plate were lysed by adding 20 μl of washing buffer (10X) for 30 min at room temperature. The microtiter plate was centrifuged at 250 × g for 3 min, and 100 μl of supernatant was transferred to replicate wells of a microtiter plate precoated with anti-DNA antibody. The samples were incubated for 90 min at room temperature. After washing, the samples were denatured and fixed by microwave irradiation of the plate for 5 min. After cooling the plate for 10 min at −20°C, anti-BrdUrd peroxidase conjugate solution was added and incubated for an additional 90 min at room temperature. After washing, immune-complexed anti-BrdUrd peroxidase was detected by 3,3',5,5'-tetramethylbenzidine substrate. After incubation for 10–20 min at room temperature in the dark, absorbance was detected by monitoring at 450 nm.

Gel Analysis of DNA Fragmentation. Cells (5 × 10⁶/well in 1.5 ml) were cultured on 6-well Nunc tissue culture plates in 10% FCS complete medium for 24 h. The medium was replaced with medium containing 5% FCS and the inhibitors to be tested. After incubating for 24 h at 37°C, the cells were removed by trypsinization and collected by centrifugation at 1,000 rpm for 3 min in complete medium. Lysis buffer [0.5 ml; containing 5 mM guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 100 mM 2-mercaptoethanol, and 0.5 mg/ml proteinase K] was added to the cell pellet and incubated at 37°C for 30 min (38). The lysate was vortexed for 15 s and precipitated with an equal volume of isopropanol at −70°C for 1 h. The samples were centrifuged for 30 min at 12,000 × g at 4°C, and the DNA pellets were washed in 70% ethanol at room temperature. After drying in a Speedvac concentrator for 15 min, the samples were dissolved in 25 μl of Tris-EDTA buffer containing 0.6 mg/ml RNase A and incubated overnight at 37°C. The samples were reextracted, washed, and dried as described above. The pellets were dissolved in 30 μl of Tris-EDTA buffer, and the DNA was subjected to electrophoresis on a horizontal 2% agarose gel in Tris-borate EDTA buffer. The DNA was stained with SYBR green solution (Molecular Probes, Inc., Eugene, OR) diluted 1:5,000 in running buffer.

RESULTS

We have previously shown that peptides derived from the second type I repeat of TSP1 inhibit proliferation of endothelial cells and a breast carcinoma cell line (27). Some of these peptides also promote cell adhesion (34). Because these two activities may elicit opposing signals in cells, polysucrose conjugates of the peptides, which do not promote cell adhesion, were used instead of free peptides in the current studies. The peptide conjugates arrested growth of both endothelial and breast carcinoma cells, but endothelial cell numbers also decreased after this treatment. The decrease in endothelial cell number was preceded by morphological changes in the treated endothelial cells that are characteristic of programmed cell death (Fig. 1c), including membrane blebbing, nuclear condensation, and loss of adhe-

Table 1 Structures of TSP1 peptides and mimetics

<table>
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<tr>
<th>Peptide Origin</th>
<th>Sequence</th>
<th>Table</th>
<th>407</th>
<th>TSP1 type 1 (residues 429–447)</th>
<th>KRKFQDGDHASPASSC</th>
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<td>407</td>
<td>TSP1 type 1 (residues 429–447)</td>
<td>KRKFQDGDHASPASSC</td>
<td></td>
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<tr>
<td>389</td>
<td>peptide 407 (Try1 → Ala)</td>
<td>KRKFQDGDHASPASSC</td>
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<tr>
<td>450</td>
<td>peptide 407 (Phe, GlnAsp → Ala)</td>
<td>acKRKFQKGDHASPASSC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>416</td>
<td>peptide 407 (retro-inverso)</td>
<td>all ⇒ acKDFWPSWHSWGQDKFRKam</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>493</td>
<td>TSP1 procollagen (residues 321–327)</td>
<td>NOQVYRNC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>493</td>
<td>TSP1 residues 436–444 (retro-inverso)</td>
<td>all ⇒ tpAAKPSWHSWGQam</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>521</td>
<td>TSP1 residues 429–446 (retro-inverso)</td>
<td>all ⇒ tpSDWPSWHSWGQDKFRKam</td>
<td></td>
<td></td>
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</tbody>
</table>

Sequences are depicted using single-letter codes and are t-amino acids except where indicated; try, thryopyrrolion; am, amide; ac, acetyl. |
Thrombospondin I induces endothelial cell apoptosis. The morphological changes were specifically induced by the type I repeat peptides because a control conjugate containing an antiangiogenic peptide from the procollagen domain of TSP1 was inactive (Fig. 1g).

BAE cells secrete latent TGF-β1 and TGF-β2 (36), and the TSP1 sequence KRFK activates latent TGF-β (29). Conditioned medium from the BAE cells used for this assay contained 0.01 ng/ml TGF-β1 immunoreactivity and 2.05 ng/ml acid-activatable TGF-β1. Because similar morphology changes were induced by a modified TSP1 sequence in which the TGF-β-activating sequence KRFK was altered to the inactive sequence KRAK (Fig. 1e), the effect of the peptide did not require activation of latent TGF-β produced by the endothelial cells.

Induction of DNA Fragmentation. Analysis of low molecular weight DNA extracted from endothelial cells treated with the active TSP1 peptides demonstrated a characteristic ladder pattern resulting from internucleosomal cleavage of the genomic DNA (Fig. 2). Polysucrose conjugates containing 0.4 μM of either the native TSP1 sequence KRFKQDGGWSHWSPWSSC (Lane b) or the modified sequence KRAKAAGGWSHWSPWSSC (Lane e), which lacks the TGF-β-activating sequence, equally stimulated DNA fragmentation. The basic residues and the WSXW motif were both required for optimal activity of these peptide conjugates, based on the weak activities of conjugates containing KRFKQDGGASHASPASSC (Lane a) or GGWSHWSPWSSC (Lane d), which lack either the Trp residues or the basic motif. The appearance of cleaved DNA fragments was specific for the active type I repeat peptides because a polysucrose conjugate containing the TSP1 procollagen peptide NGVQYRNC was inactive (Lane f). Two conjugated retro-inverso mimetics of the type I sequence were also active (Lanes c and g). Exposure of the cells to 1 μg/ml TSP1 did not result in detectable DNA fragmentation by this method (Lane h).

Apoptosis Is Blocked on a Fibronectin Matrix. Because loss of matrix adhesion is a known inducer of apoptosis in endothelial cells (8, 9), an antiadhesive activity was considered as a mechanism for the activity of the peptides. Precoating the tissue culture plastic with fibronectin did not alter the morphology of untreated cells (Fig. 1, a and b) but prevented the morphology changes induced by the TSP1 peptides (Fig. 1, d and f). The antiproliferative activities of the native TSP1 sequence (KRFKQDGGWSHWSPWSSC-polysucrose, 4071) and an analogue without the TGF-β-activating sequence RGK (KRAKAAGGWSHWSPWSSC-polysucrose, 450f) were also decreased by growth of the endothelial cells on a fibronectin matrix (Fig. 3). In contrast, the antiproliferative activities of intact TSP1 or TGF-β were not significantly decreased by attachment of the endothelial cells on fibronectin (Fig. 3).

Fibronectin also inhibited the appearance of the DNA ladder in endothelial cells treated with a TSP1 peptide analogue from the type I repeat (Fig. 4). DNA fragmentation induced by the peptide KRFKQDGGWSHWSPWSSC conjugate was reduced by 64% in cells attached on wells coated with 10 μg/ml fibronectin. A conjugate containing the TSP1 procollagen peptide NGVQYRNC was used as a negative control in this experiment and did not significantly induce DNA fragmentation. Similar reductions in DNA fragmentation were observed for the other active TSP1 peptide analogues when cells were attached on fibronectin (data not shown).

Quantitative Analysis of DNA Fragmentation. An ELISA assay for detecting DNA fragmentation was used to quantify the activity of
Because the same peptides inhibited the growth of MDA-MB-435S cells, the induction of apoptosis can be independent of the antiproliferative effects of the TSP1 peptides. A second breast carcinoma cell line, MCF7, showed DNA fragmentation in response to the peptide 407 conjugate but not to intact TSP1 (Fig. 5B). The magnitude of the peptide response was similar to the DNA fragmentation induced in the same cells by camptothecin.

On the basis of the protective activity of fibronectin observed in Figs. 1 and 4, the effect of endothelial cell adhesion on the induction of DNA fragmentation was further examined using the quantitative DNA fragment ELISA. The adhesion of endothelial cells on a fibronectin matrix inhibited DNA fragmentation induced by TSP1 (Fig. 6A) or by a TSP1 peptide analogue from the type I repeats (Fig. 6B). Consistent with the report that vanadate suppresses the induction of endothelial cell death induced by removal of extracellular matrix signals (8), the addition of 50 μM vanadate decreased the fragmentation induced by the active TSP1 peptide KRKFQDGGWSHWSPWSSC (407F) or by the analogue lacking the TGF-β-activating sequence (450F; Fig. 7). The serine/threonine phosphatase inhibitor okadaic acid, at 5 nM, also inhibited DNA fragmentation induced by these peptides, whereas the ceramide

the peptides. On the basis of this sensitive and quantitative assay for DNA fragmentation, both TSP1 and the type I repeat peptides induced significant DNA fragmentation in endothelial cells (Fig. 5A). The activity of TSP1 was weaker than that of the synthetic peptide conjugates to elicit DNA fragmentation but was consistently observed in several independent experiments. The TSP1 procollagen domain peptide, however, was inactive. Treatment of BAE cells with TGF-β induced DNA fragmentation to a similar extent as TSP1. The stimulation of DNA fragmentation by TSP1 and the peptide conjugates was specific because no DNA fragmentation was induced in MDA-MB-435S breast carcinoma cells by the peptide conjugates (Fig. 5B). DNA fragmentation was induced in MDA-MB-435S cells by the topoisomerase I inhibitor camptothecin, indicating that these cells can initiate programmed cell death.

Fig. 3. Attachment of endothelial cells on fibronectin partially reverses the inhibition of proliferation by type 1 repeat peptides but not by TSP1 or TGF-β. Proliferation of BAE cells was determined on untreated tissue culture plastic (●) or on plastic coated with 10 μg/ml fibronectin (◻) in DMEM medium containing 1% FBS and the indicated concentrations of the TSP1 peptide KRKFQDGGWSHWSPWSSC (407F), an analogue without the TGF-β-activating sequence acKRAKAAGGWSHWSPWSSCam (450F), TGF-β (TGF-β), TSP1, or the TSP1 procollagen domain peptide NGVQYRNC (500F). The cell number was quantified after 72 h using the Cell-Titer assay and is presented as a percentage of that determined in the same medium without additions, mean ± SD, n = 3.

Fig. 4. Fibronectin inhibits TSP1 peptide-induced DNA fragmentation. DNA fragmentation was determined as described in the legend to Fig. 2. Band intensity was determined by image analysis and is plotted with background subtraction using control cells as a reference for endothelial cells treated with a conjugate of peptide KRKFQDGGWSHWSPWSSC (●), cells treated with the same peptide in a well coated with 10 μg/ml fibronectin (◻), or cells treated with a conjugate of the TSP1 procollagen peptide NGVQYRNC (○). The migration of DNA size standards is as indicated.

Fig. 5. Detection of TSP1- and peptide-induced apoptosis in BAE and human breast carcinoma cells by a DNA fragment ELISA. DNA fragmentation in BAE cells (A), MCF7 breast carcinoma cells (B, ■), or MDA-MB-435S breast carcinoma cells (B, □) was quantified by an ELISA assay using BrdUrd-labeled cells. Target cells (10 ml; 2 × 10⁶ cells/ml) were labeled overnight using 10 μM BrdUrd. After labeling, a cell suspension containing 1 × 10⁵ cells/ml was transferred to replicate wells of a microtiter plate (100 μl/well) containing 100 μl of culture medium containing inhibitors (25 μg/ml TSP1, 4 μM KRKFQDGGWSHWSPWSSC (407F), NGVQYRNC (500F), 400 ng/ml camptothecin, or 10 ng/ml TGF-β) or medium only (Control). After incubation for 24 h at 37°C, the cells in the wells were lysed and centrifuged. Released DNA fragments in 100 μl of supernatant were quantified using a sandwich ELISA using anti-DNA capture antibody and anti-BrdUrd peroxidase conjugate for detection. After washing, immune-complexed anti-BrdUrd peroxidase was detected using 3,3′,5,5′-tetramethylbenzidine substrate. Absorbance was measured at 450 nm and is presented as mean ± SD, n = 3.

Fig. 6. Effect of extracellular matrix signals on the induction of apoptosis. Migration of BAE cells (A) and human breast carcinoma cells (B) was determined on untreated tissue culture plastic or plastic coated with 10 μg/ml fibronectin (+), or cells treated with a conjugate of the TSP1 procollagen peptide NGVQYRNC (500F), TGF-β, or the TSP1 procollagen domain peptide NGVQYRNC (500F). The cell number was quantified after 72 h using the Cell-Titer assay and is presented as a percentage of that determined in the same medium without additions, mean ± SD, n = 3.
cells produced a similar suppression of the DNA fragmentation response to the peptide (Fig. 8).

Phosphorylation Differentially Modulates Antiproliferative Responses to Peptides and Thrombospondin. On the basis of the observation that phosphatase inhibition differentially affected apoptotic responses to TSP1 and the peptides, we further examined the mechanism of endothelial growth inhibition by the TSP1 type I repeat peptides. Sodium vanadate significantly inhibited the antiproliferative activity of TSP1 and TGF-β but did not inhibit the antiproliferative activity of the TSP1 peptides (Table 2). Blocking of the TSP1 and TGF-β activities by vanadate was specific in that the serine/threonine phosphatase inhibitor okadaic acid had no effect at a concentration sufficient to inhibit protein phosphatase 2A (39). However, at concentrations sufficient to inhibit protein phosphatase 1 (25 nm), okadaic acid alone inhibited endothelial growth and strongly induced DNA fragmentation (results not shown).

Although the activity of the phosphatase inhibitor vanadate to antagonize the antiproliferative effect of TSP1 suggests that hyperphosphorylation prevents the antiproliferative activity of TSP1, a tyrosine kinase may also mediate the growth-suppressive activity of TSP1 and the peptides. The tyrosine kinase inhibitor herbimycin A, used at concentrations below those that directly blocked endothelial

Table 2 Effect of signal transduction modulators on inhibition of endothelial cell proliferation by TSP1, TGF-β, and TSP1 peptides

Inhibition of BAE cell proliferation by TSP1, TGF-β, or the indicated thrombospondin peptide analogues were determined in the presence of inhibits of tyrosine kinase (herbimycin), ceramide synthase (fumonisin), phosphatases (vanadate or okadaic acid), or a stimulator of protein kinase C (TPA). Net inhibition of proliferation, expressed as mean ± SD, n = 3.
proliferation, strongly suppressed the antiproliferative activities of the TSP1 peptides and completely blocked the antiproliferative activities of TSP1 and TGF-β (Table 2). Because herbimycin also blocked the antiproliferative activity of the TSP1 peptide analogue 450, which lacks a latent TGF-β-activating sequence, herbimycin can prevent the activity of the TSP1 peptides independently of blocking TGF-β-mediated signaling. In contrast, fumonisin B1, an inhibitor of ceramide synthase and ceramide-mediated apoptosis (40), had no effect on the activity of the peptides at 15 μM (Kₐ = 0.2 μM for ceramide synthase; Ref. 40). At higher doses, fumonisin B1 also inhibited endothelial cell proliferation and directly induced DNA fragmentation (results not shown).

The protein kinase C stimulator TPA, which blocks ionizing radiation-induced ceramide generation and apoptosis of BAE cells (41, 42), stimulated proliferation of the cells but had no effect on the antiproliferative activities of the TSP1 peptides or TSP1 (Table 2). The inactive analogue 4α-phorbol did not stimulate proliferation, verifying the specificity of the proliferative response to TPA. TPA also had no effect on the generation of DNA fragmentation induced by the TSP1 peptides as assessed by the DNA ladder assay (results not shown).

**DISCUSSION**

Previous studies have demonstrated the positive effects of extracellular matrix components on endothelial cell survival (8, 9, 11). Recently, however, TSP1 and several other matricellular components have been found to negatively modulate cell adhesion (12, 43). Because adhesion provides signals essential for survival of nontransformed cells, these observations suggested that TSP1 may also regulate cell survival. The present results demonstrate a negative effect of TSP1 on endothelial cell survival. The ability of TSP1 or the TSP1 peptide analogues to inhibit growth and induce apoptosis, however, is dependent on other external signals. Confluent quiescent cells were resistant to the induction of cell death, as were subconfluent cells attached to a pure fibronectin matrix or cells treated with vanadate. As was demonstrated for the interaction of cells with fibronectin (4), the signals resulting from the interaction of endothelial cells with TSP1 may be complex and involve multiple signal transduction pathways. TSP1 and the TSP1 peptides elicit changes in both endothelial cell proliferation and survival. On the basis of their differential sensitivities to fibronectin matrix signals and agents that modulate several signal transduction pathways, these responses probably involve different signaling pathways.

Several results indicate that induction of apoptosis is independent of the growth-inhibitory activities of TSP1 and the TSP1 type I repeat peptides. Proliferation of breast carcinoma and endothelial cells are both inhibited by TSP1 and the peptides, but only the latter cells exhibited an apoptosis response. Differential sensitivity of the endothelial cell apoptosis and proliferative responses to vanadate inhibition for the TSP1 peptides also suggest that distinct mechanisms may mediate growth inhibition and apoptosis. Likewise, the proliferative and survival responses to intact TSP1 differ in that fibronectin reverses the apoptotic response but did not reverse the antiproliferative activity of TSP1.

The parallel proliferative and survival responses of TSP1 and TGF-β-treated endothelial cells to many of the inhibitors tested suggest that TGF-β may mediate the activity of intact TSP1, although it is not required for activity of the TSP1 peptides. Part of the observed response to TSP1 could result from active TGF-β contaminating the platelet TSP1, but the measured concentration of TGF-β was insufficient to account for most of the activity observed. The TSP1 may also activate latent TGF-β1 produced by the BAE cells (30). As was observed with the peptides, however, sensitivities to signal transduction inhibitors differ for TSP1-mediated growth inhibition and induction of DNA fragmentation. Vanadate completely reversed the antiproliferative activity of TSP1 but augmented DNA fragmentation. Likewise, fumonisin B1 had no effect on the antiproliferative activity of TSP1 but also augmented DNA fragmentation. This pattern parallels previous reports that apoptotic and growth-inhibitory responses to TGF-β may also involve distinct signaling pathways (38).

Peptides from the type I repeats of TSP1 elicit a strong apoptotic response in endothelial cells. To date, no other region of TSP1 has been found to induce apoptosis, and the present data exclude this as a mechanism for the antiangiogenic activity of the procollagen domain peptide (28). Mutagenesis of the type I repeat sequences will be required to confirm the role of the type I repeats in the activity of the intact protein and to determine whether other regions of TSP1 participate in the cell death response to intact TSP1.

The mechanism of action of the TSP1 peptides is clearly not from direct cytotoxicity, based on the resistance of endothelial cells plated on fibronectin or at confluence to apoptosis in the presence of active concentrations of the peptides. The peptides may act outside of the cell to block FGF-2 presentation to and activation of its tyrosine kinase receptor (27). This hypothesis is consistent with protection by vanadate from peptide-mediated apoptosis. However, some other results question this hypothesis. Partial reversal of the peptide antiproliferative activities by herbimycin and the lack of vanadate sensitivity are not consistent with their acting by antagonizing a tyrosine kinase-dependent receptor. Furthermore, FGF-2 is known to suppress ceramide-mediated apoptosis of BAE cells, and this activity is mediated by protein kinase C (42). Because TPA stimulation of PKC protects BAE cells from ceramide-mediated apoptosis but did not protect our BAE cells from an inhibition of growth by the TSP1 peptides, the antiproliferative activity of the peptides cannot arise exclusively from inhibition of an essential FGF-2 survival signal. Although ceramide has recently been shown to mediate apoptosis of many cell types in response to various stimuli (reviewed in Ref. 44) and participates in radiation-induced apoptosis of BAE cells (41, 42), the lack of effect of fumonisin B1 and TPA on the activities of the peptides suggests that the pathway for inducing cell death by the TSP1 peptides is distinct from that of ionizing radiation and does not require ceramide generation. On the basis of the apparent synergism of fumonisin B1 with TSP1 to induce DNA fragmentation, however, the apoptotic response to the intact protein may be regulated by ceramide generation.

TSP1 inhibits focal adhesion contacts in endothelial cells attached on fibronectin (15). This mechanism could participate in the activity of TSP1 but not that of the peptides because the amino-terminal domain of TSP1 is responsible for the former activity. Treatment with the peptide conjugates, however, also results in the loss of endothelial cell adhesion. It remains to be determined whether this loss of adhesion causes programmed cell death or is an indirect effect of other signals induced in the cells by the peptides.

Attachment of the endothelial cells to fibronectin or being at confluence generates a signal that reverses the apoptotic and antiproliferative responses to the peptides. Because fibronectin binding to the integrin α5β1 promotes endothelial cell survival (8), this signal may involve activation of focal adhesion kinase or other adhesion-dependent tyrosine kinases. Fibronectin or antibody engagement of β1 or β3 integrins on endothelial cells results in tyrosine kinase-dependent phosphorylation of focal adhesion kinase and a 70-kDa protein (45). Vanadate can replace the fibronectin signal to prevent endothelial cell death (8) and presumably maintains the targets of these kinases in a phosphorylated state by inhibiting the corresponding phosphatases. This model is consistent with the ability of vanadate to suppress DNA fragmentation induced by the TSP1 peptide KRFKQDGWHSWP- WSSC and to reverse growth inhibition by intact TSP1. However, it
does not account for the ability of vanadate to stimulate apoptosis induced by intact TSP1 or the ability of herbimycin A to prevent growth inhibition by TSP1 or the peptides. The latter result was also unexpected because herbimycin is reported to inhibit angiogenesis (46), most integrin signaling (4, 45), and apoptotic responses in several cell types (47). The data can be rationalized by proposing that the peptides elicit a second inhibitory tyrosine kinase pathway that is sensitive to herbimycin.

The role of programmed cell death in the biological activities of TSP1 in vivo remains to be examined. TSP1 overexpression in MDA-MB-435S breast carcinoma cells reduced tumor growth in vivo but had no effect on the growth of these cells or the formation of colonies in soft agar (24). These observations are consistent with the inability of TSP1 to induce apoptosis of MDA-MB-435S cells. The resistance of MDA-MB-435S breast carcinoma cells to induction of apoptosis by the TSP1 peptides may result from mutation of p53 in this cell line (48), whereas the MCF7 cells have wild-type p53 and are sensitive to the induction of apoptosis. Normal p53 function may therefore be required for the apoptotic response to TSP1 peptides.

Reduction of angiogenesis in tumors formed by TSP1-transfected MDA-MB-435S cells (24) could result from the induction of apoptosis in endothelial cells during vascularization of the tumor. A similar mechanism has been proposed for the antitumor activity of antibodies to the avβ3 integrin, which induce apoptosis in developing tumor blood vessels (10, 11). Thus, extracellular matrix signals may be absent in newly formed tumor blood vessels and sensitize this endothelium to the effects of TSP1 secreted by the transfected MDA-MB-435S cells. Although TSP1 is a ligand for avβ3 (20), the active TSP1 peptides do not contain the Arg-Gly-Asp sequence recognized by the avβ3 integrin.

The TSP1 peptides are potent inducers of DNA fragmentation in BAE cells in vitro. This activity may account for the differential effects of these peptides on endothelial and breast carcinoma cell proliferation in vitro. We recently found that stable analogues of the TSP1 peptides inhibit tumor growth in vivo in MDA-MB-435S xenografts in nude mice. The present data suggest that the selective induction of apoptosis of tumor endothelium may explain the activity of the peptides in vivo. The resistance of confluent endothelial cells to the induction of apoptosis by the peptides in vitro is consistent with their lack of toxicity in vivo.

REFERENCES

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