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# A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin

**(neovascularization/human chromosome 15/adhesive glycoproteins)**

DEBORAH J. GOOD*, PETER J. POLVERINI†, FARZAN RASTINEJAD*‡, MICHELLE M. LE BEAU§, RICHARD S. LEMONS¶, WILLIAM A. FRAZIER‖, AND NOEL P. BOUCK*,**

*Department of Microbiology-Immunology and Cancer Center, and †Department of Pathology, Northwestern University, Chicago, IL 60611; §Section of Hematology/Oncology, Department of Medicine, The University of Chicago, Chicago, IL 60637; ¶Department of Pediatrics, University of Utah School of Medicine, Salt Lake City, UT 84132; and ‖Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Saint Louis, MO 63110

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ABSTRACT **A secreted inhibitor of angiogenesis that is controlled by a tumor suppressor gene in hamster cells has been found to be similar to a fragment of the platelet and matrix protein thrombospondin. The two proteins were biochemically similar and immunologically crossreactive and could substitute for one another in two functional assays. Human thrombospondin inhibited neovascularization *in vivo* and endothelial cell migration *in vitro*, as does the hamster protein, gp140. gp140 sensitized smooth muscle cells to stimulation by epidermal growth factor, as does human thrombospondin. The thrombospondin gene has been localized on human chromosome 15. These results demonstrate a function for the ubiquitous adhesive glycoprotein thrombospondin that is likely to be important in the normal physiological down-regulation of neovascularization. In addition, they raise the possibility that thrombospondin may be one of a number of target molecules through which a tumor suppressor gene could act to restrain tumor growth.**

As a normal cell develops into a solid tumor it undergoes a series of changes. At the genetic level, oncogenes may be activated and multiple tumor suppressor genes may be inactivated (1–5). At the physiological level, growth is enhanced, immunity is evaded, and neovascularization is induced. For solid tumors, neovascularization appears to be a prerequisite. Experimental solid tumors are unable to grow beyond a few millimeters in thickness without a new blood supply (6–8) and most naturally occurring solid tumors elaborate angiogenic factors that attract the new vessels on which they depend (9). In the immortal hamster cell line BHK21/cl13 it has been possible to link the loss of a tumor suppressor gene with the physiological acquisition of angiogenic activity (10). These nontumorigenic cells produce angiogenic factors and a 140-kDa inhibitor that blocks the action of these factors. The production of the inhibitor, and thus the inability of the cells and their conditioned media to induce neovascularization, is strictly dependent upon the presence in the cells of an active suppressor gene.

Here this 140-kDa suppressor-dependent inhibitor of angiogenesis is shown to be indistinguishable from a portion of the well-characterized adhesive glycoprotein thrombospondin (TSP) (11–14). In addition, native human TSP inhibits angiogenesis efficiently *in vivo* and suppresses endothelial cell migration *in vitro*. TSP is the most abundant protein in the platelet $\alpha$ granule and is found in blood clots and wounds where it initially serves to stabilize platelet aggregates. It is also produced and incorporated into the matrix by a variety of other cell types *in vitro* and presumably *in vivo* where anti-TSP antibodies react with basement membranes, vessel walls, and some interstitial material (11–14). The ability of exogenous TSP to block angiogenesis suggests a function for this ubiquitous protein in curtailing the physiological neovascularization that occurs briefly during wound healing and accompanies normal embryogenesis. It also identifies TSP as a protein whose loss could contribute to the ability of a cell to grow successfully as a solid tumor.

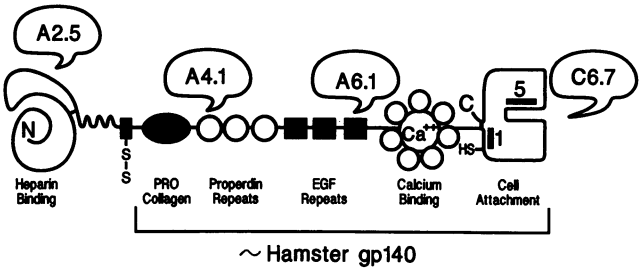


FIG. 1. Cartoon of the 180-kDa subunit of the homotrimeric human TSP molecule. Binding sites for individual monoclonal antibodies raised to TSP are indicated above the diagram; specific domains are below. The portion of the TSP molecule containing homology to the N-terminal amino acids of gp140 and shared antigenic sites is indicated by the bar. Numbers 1 and 5 mark the positions of the peptides used to make TSP anti-peptide serum. EGF, epidermal growth factor.

## MATERIALS AND METHODS

The gp140 inhibitor was purified from the serum-free conditioned medium of normal BHK21/cl13 cells, subclone SN-10J6S1, by column chromatography as described (10). Its N-terminal amino acid sequence was determined by Al Smith (Protein Structure Laboratory, University of California, Davis, CA). Human TSP was purified from platelet releasates (15).

Western blots were prepared from gels containing 0.5 $\mu$g of pure protein per lane or 10 $\mu$g of conditioned medium per lane as described (10) and developed using polyclonal rabbit antiserum to gp140 (10) or monoclonal antibodies to human

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Abbreviations: TSP, thrombospondin; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor.

‡Present address: Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.
**To whom reprint requests should be addressed at: Department of Microbiology-Immunology, Northwestern University, 303 East Chicago Avenue, Chicago, IL 60611.

Table 1. Amino acid sequence similarity between hamster gp140 and human TSP

| | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | | | | | | | | | | | | | | | | | | 20 |
| gp140 | Leu | – | Arg | – | Arg | – | Pro | – | * | – | Leu | – | * | – | Phe | – | His | – | Asn | – | Gly | – | Val | – | Gln | – | Tyr | – | Arg | – | Asn | – | Asn | – | Glu | – | Glu | – | Trp |
| TSP | Glu | – | Leu | – | Arg | – | Arg | – | Pro | – | Pro | – | Leu | – | Cys | – | Tyr | – | His | – | Asn | – | Gly | – | Val | – | Gln | – | Tyr | – | Arg | – | Asn | – | Asn | – | Glu | – | Glu | – | Trp |
| | 294 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 313 |

Residue numbers are indicated.
*Residue not recovered; presumed to be a cysteine or modified amino acid.

TSP (11). Anti-TSP peptide serum is a polyclonal rabbit serum raised to two peptides containing residues 941–970 and 1041–1070 of human TSP.

Southern blot analysis of a somatic cell hybrid panel was performed as described (16), using as a probe the human cDNA plasmid p6SXE (17). Filters were stripped and rehybridized with a v-*fes* probe that identifies the *FES* protooncogene previously mapped to chromosome 15 (18). *In situ* hybridization was performed as described (19), also using TSP plasmid p6SXE.

*In vitro* migration of endothelial cells was measured in triplicate in a modified Boyden chamber (10) using basic fibroblast growth factor (bFGF) at 20 ng per well and gp140 or TSP at 0.125 μg per well. *In vivo* angiogenesis was assessed by implanting Hydron pellets of test material into the cornea of the rat eye and recording responses at 7 days (10). Pellets were formulated where indicated with 50 ng of bFGF, 3 μg of antibody protein, and, unless indicated otherwise, 0.125 μg of gp140 or TSP. Anti-bFGF was from R & D Systems (Minneapolis).

Mitogenesis assays on rat smooth muscle cells followed precisely the scheme devised by Majack *et al.* (20), using cells cultured from rat aortic explants (21).

## RESULTS

**Biochemical and Antigenic Similarities.** Biochemically the gp140 inhibitor purified from nontumorigenic hamster BHK cells closely resembled human TSP. Seventeen of the 20 N-terminal amino acids matched a sequence corresponding to residues 294–313 in the 1152-amino acid human TSP peptide chain (17, 22) (Table 1). Of the three possible mismatches, one is due to the conservative substitution of phenylalanine for tyrosine 301 in TSP and the other two result from the inability of the gas-phase sequencer to recover residues 5 and 7 in gp140, indicating they are either cysteines, as is expected if residue 7 corresponds to cysteine 300 in TSP, or amino acids that have been modified, for example by O-linked glycosylation or phosphorylation. Of the 20 residues sequenced, only residue 5, corresponding to proline 298 in TSP, represents a clear significant deviation in primary sequence between the hamster and human proteins. TSP and gp140 also have similar amino acid compositions and similar proportions of carbohydrate, 6% for TSP (23, 24) and 6.4% for gp140 (data not shown).

Several properties of gp140 are consistent with its lacking the first 293 residues found in TSP. As purified from platelet releasates, native TSP is a homotrimer in which three identical subunits are linked by disulfide bridges thought to involve cysteine residues 252 and 256 (ref. 11; see Fig. 1). As expected for a protein missing these cysteines, gp140 behaved as a monomer, running on sizing columns and on both native and SDS nonreducing gels at 140 kDa (data not shown). It is thus distinct from the *ca.* 140-kDa peptide released from TSP by chymotrypsin digestion, which remains disulfide linked in a trimer (25). The 25-kDa N-terminal portion of human TSP contains the high-affinity heparin-binding domain (26–28). gp140 inhibitor apparently lacks this domain for it was unable to bind to a heparin-Sepharose column (data not shown).

A strong crossreaction on Western blots was seen between TSP and gp140. Polyclonal rabbit antiserum raised to gp140 and affinity purified from a Western blot of a 140-kDa band of pure gp140 recognized TSP (data not shown), and polyclonal antisera raised to TSP peptides recognized gp140 (Fig. 2). No crossreaction was detected in similar assays between gp140 and either of the transformation-sensitive proteins, fibronectin or collagen VI (data not shown).

A panel of monoclonal antibodies has been developed against human platelet TSP and the epitope recognized by each assigned to a specific domain of the linear TSP subunit (ref. 11; see Fig. 1). Those monoclonal antibodies whose epitopes lie C-terminal to the S—S link in the TSP molecule were able to recognize purified hamster gp140 and to detect the large amount of gp140 in a complex mix of conditioned medium of normal BHK cells and the much smaller amount present in conditioned medium from DMN4A (10), a BHK cell line grown under conditions where its temperature-sensitive suppressor gene was inactive (Fig. 2). Monoclonal antibody A2.5, specific for the N-terminal heparin-binding domain of TSP (29), did not recognize hamster gp140, as expected if it indeed lacks TSP residues 1–293 (Fig. 2).

**Reciprocal Functional Similarities.** gp140 was identified and purified from hamster fibroblasts on the basis of its ability to inhibit migration of endothelial cells toward angiogenic factors *in vitro* and to block neovascularization in the rat cornea *in vivo* (10). Native trimeric TSP purified from a human platelet releasate also performed these functions. Although TSP and gp140 were purified from different sources in different laboratories using different procedures, similar concentrations of either protein inhibited migration of cultured

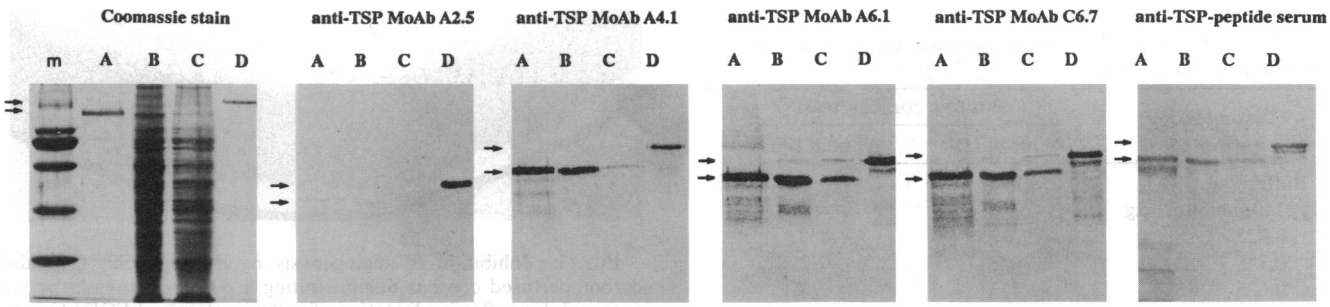


FIG. 2. Western blots developed with the antibodies indicated demonstrate crossreactivity between the hamster gp140 and human TSP. MoAb, monoclonal antibody. Lanes: A, gp140; B, conditioned medium from normal BHK cells; C, conditioned medium from transformed BHK cells; D, TSP. The gel on the left was stained with Coomassie blue R-250. m, Molecular mass markers of 150, 116, 97, 68, 43, and 29 kDa. Small arrows indicate the positions of the 180-kDa subunit of TSP (top arrow) and the 140-kDa gp140 (lower arrow).

Table 2. Inhibition of *in vitro* migration of capillary endothelial cells

| Addition | % of control migration |
|---|---|
| bFGF | 100* |
| bFGF + gp140 | <4 |
| bFGF + gp140 + A4.1† | 46 |
| bFGF + TSP | <4 |
| bFGF + TSP + A4.1† | 108 |

*43 ± 4.9 cells migrated per 10 fields toward bFGF alone.
†Anti-TSP monoclonal antibody.

endothelial cells toward bFGF in a Boyden chamber *in vitro* (Table 2) and blocked neovascularization toward bFGF in the rat cornea *in vivo* (Table 3; Fig. 3).

In addition, antibodies directed against either protein were able to block the inhibitory function of the other. Monoclonal antibody A4.1 recognizes the central stalk of the TSP subunit (ref. 11; Fig. 1) and blocked the ability of TSP and gp140 to inhibit endothelial cell migration *in vitro* (Table 2) and angiogenesis *in vivo* (Table 4). Polyclonal rabbit serum raised to gp140 blocked the inhibition of bFGF-induced angiogenesis by gp140 and TSP. The rabbit serum and the monoclonal antibodies were not angiogenic when implanted alone nor were they inhibitory when implanted with bFGF. Immune complexes themselves were not angiogenic for there was no neovascularization in response to pellets containing bFGF with anti-bFGF.

Monoclonal antibody A6.1, known to recognize human TSP with a higher affinity in the absence of calcium (30), blocked gp140 activity efficiently only after the gp140 had been treated with EDTA (zero of three positive cornea responses to bFGF in the presence of untreated gp140 plus A6.1; three of four positive responses and one ± cornea response to bFGF in the presence of EDTA-treated gp140 plus A6.1). The EDTA requirement for A6.1 effects on gp140 suggests that the hamster protein may have a calcium-sensitive domain analogous to that in human TSP.

gp140 is also able to perform one unusual function attributed to TSP—namely, the ability to augment synergistically the mitogenic response of vascular smooth muscle cells to EGF (20). Purified hamster gp140 also performs this function (Fig. 4). In experiments run in parallel, gp140 is more effective than trimeric TSP, perhaps due to an increased effective concentration resulting from its monomer structure.

**Localization of TSP Gene on Human Chromosome 15.** Southern blot analyses of a somatic cell hybrid panel showed perfect concordance between TSP coding sequences and human chromosome 15 (data not shown). All other chromosomes showed significant discordance (18–50%) with TSP. No new bands or segregation of bands indicative of a pseudogene on a chromosome other than number 15 were seen. After stripping and rehybridization of filters, perfect concordance was seen between TSP and *FES*, previously mapped to chromosome 15 (18). To sublocalize TSP, we hybridized the TSP cDNA plasmid to normal human metaphase chromosomes. This hybridization resulted in specific labeling only of chromosome 15 (Fig. 5). The largest number of grains was observed at 15q21. Similar results were obtained in a second hybridization experiment. *In situ* hybridization together with somatic cell hybrid analysis localized the TSP gene to human chromosome 15 at bands q15-22.

Table 3. TSP and gp140 inhibit neovascularization in the rat cornea at equivalent doses

| Test material implanted | Positive corneas/total | |
|---|---|---|
| | With bFGF | Without bFGF |
| Buffer | 4/4 | 0/3 |
| gp140 inhibitor, μg | | |
| 0.025 | 3/3 | 0/3 |
| 0.125 | 0/3 | 0/2 |
| 0.625 | 1/3 | 0/3 |
| TSP, μg | | |
| 0.025 | 3/3 | 0/2 |
| 0.125 | 0/4 | 0/2 |
| 0.625 | 0/3 | 0/2 |

## DISCUSSION

The biochemical, immunological, and functional similarities between hamster gp140 and human TSP indicate that the suppressor-controlled gp140 inhibitor of angiogenesis is an isoform of hamster TSP. The observations that the function of gp140 is blocked by more than one anti-TSP monoclonal antibody and that TSP itself has an activity similar to that of gp140 seem to eliminate the possibility that the gp140 preparation consists of two proteins of 140 kDa, one a TSP fragment unable to inhibit angiogenesis and the other an active inhibitor. Nor is it likely that the inhibition of angiogenesis is due to a very active inhibitor that does not run with a molecular mass of 140 kDa and is an unseen contaminant in purified preparations of TSP and gp140. When gp140 was incubated 10 min at 37°C with 1% SDS, run on a polyacrylamide gel, electroeluted from the 140-kDa region, and extensively dialyzed, it retained its inhibitory activity (only one of five positive cornea responses to bFGF in the presence of eluted gp140). An equal amount of material electroeluted in

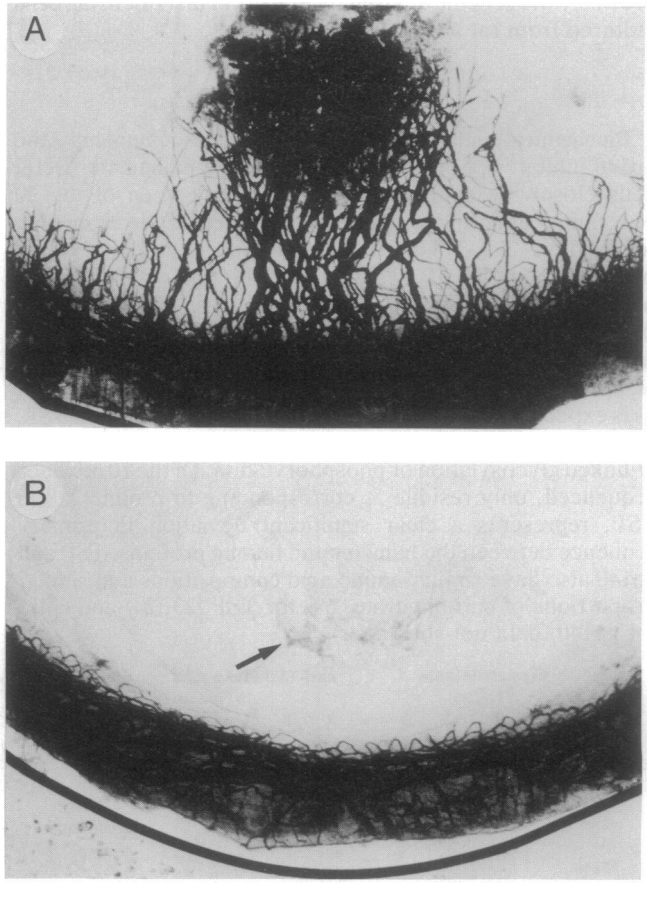


FIG. 3. Inhibition of angiogenesis *in vivo* by TSP: Colloidal carbon-perfused corneas demonstrating a positive neovascular response 7 days after implantation of pellet containing bFGF (*A*; note brush-like network of capillary sprouts surrounding the implant) and a negative response 7 days after implantation of pellet containing bFGF and 125 ng of human TSP [*B*; note complete absence of new capillary growth toward the pellet (arrow)].

Table 4. Antibody blockades of TSP and gp140 inhibition of corneal neovascularization

| Test material implanted along with bFGF | Corneal response to bFGF | | |
|---|---|---|---|
| | + | ± | − |
| Buffer | 11 | 1 | 0 |
| gp140 (0.125 μg) | 0 | 0 | 9 |
| + anti-gp140 rabbit serum | 4 | 0 | 0 |
| + preimmune rabbit serum | 0 | 1 | 3 |
| + A4.1* | 4 | 1 | 0 |
| + control monoclonal antibody | 0 | 0 | 2 |
| TSP (0.125 μg) | 0 | 1 | 5 |
| + anti-gp140 rabbit serum | 3 | 1 | 0 |
| + preimmune rabbit serum | 0 | 1 | 3 |
| + A4.1* | 6 | 0 | 0 |
| Anti-bFGF | 0 | 0 | 2 |
| A4.1* | 3 | 0 | 0 |

+, Number of corneas showing a vigorous, sustained ingrowth of capillaries toward implant; ±, a few sprouts but no sustained growth; −, no visible ingrowth.
*Anti-TSP monoclonal antibody.

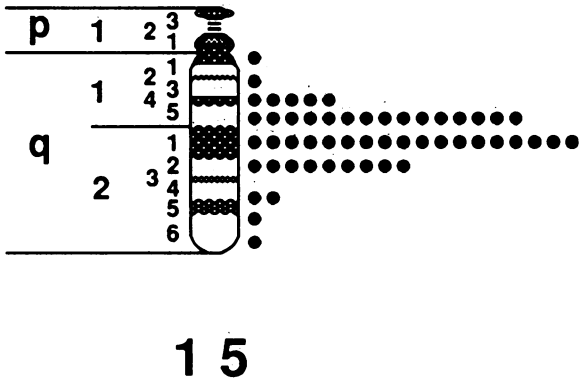


FIG. 5. Localization of TSP on human chromosome 15. Distribution of labeled sites on chromosome 15 in 100 normal human metaphase cells from phytohemagglutinin-stimulated peripheral blood lymphocytes that were hybridized with a TSP cDNA probe. Each dot indicates one labeled site observed in the corresponding band. Eighty-nine percent (47/53) of the labeled sites on chromosome 15 were located at q14-22; this cluster represented 20.6% of all labeled sites (47/228). Cumulative probability for the Poisson distribution is << 0.0005.

parallel from an adjacent empty lane of the gel was not inhibitory.

The fact that gp140 begins at a site corresponding to human TSP residue 294 suggests that (*i*) the hamster TSP gene is shortened relative to the human TSP gene; (*ii*) gp140 is a proteolytic fragment of a "full-length" hamster TSP homolog; (*iii*) gp140 is an alternatively spliced product of a full-length hamster gene; or (*iv*) gp140 is coded for by a second gene exceedingly similar to that coding for TSP. The occasional detection of minor amounts of a 450-kDa protein in addition to gp140 on native gels and of a 180-kDa protein on Western blots of conditioned medium indicates that BHK cells can make TSP of a size comparable to full-length human TSP. It is doubtful that gp140 is processed from a 180-kDa precursor since the N-terminal sequence of gp140 does not correspond to a known proteolytic cleavage site of human TSP. Assuming that hamsters, like humans, have only a single TSP gene, gp140 seems most likely to be produced by an alternatively spliced message. Such messages code for different isoforms of other extracellular glycoproteins (see, for example, ref. 31), and alternatively spliced TSP mRNA can be detected in human cells using polymerase chain reaction (W.A.F. and L. Paul, unpublished results), although none detected so far predicts a 140-kDa product analogous to gp140.

gp140 is reduced in level but not eliminated in tumorigenic cells that have lost the suppressor gene controlling it (ref. 10; Fig. 2). This reduction in concentration of the inhibitor is sufficient to allow angiogenesis to proceed *in vivo*, as a reduction of similar magnitude in the amount of pure protein gp140 or TSP incorporated into a Hydron pellet resulted in loss of the inhibitory effect in the cornea assay.

Results of the analysis of somatic cell hybrids and *in situ* hybridization indicate that the human TSP gene (symbol *THBS1*) is located on chromosome 15, at bands q15-22. This location distinguishes it from the tumor suppressor activity previously identified on chromosome 1 that is able to reverse transformation and angiogenesis in transformed hamster cells (32). The region of chromosome 15 to which TSP maps also contains the breakpoint of a recurring chromosomal abnormality in a human tumor—namely, the t(15;17)(q22;q11.2-12) that is seen in virtually all cases of acute promyelocytic leukemia (APL-M3) (33, 34). APL is characterized by an infiltration of the bone marrow by promyelocytes in association with a disseminated intravascular coagulation. The unregulated production of TSP, which is an active participant

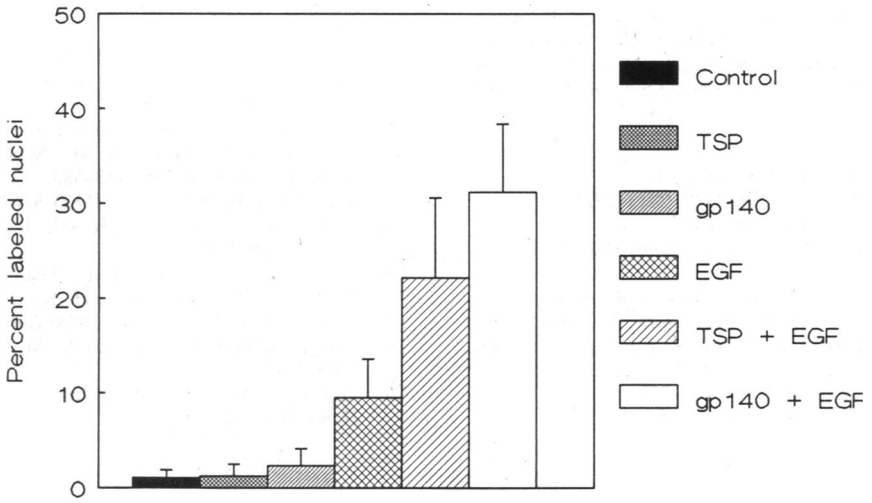


FIG. 4. gp140 or TSP can act synergistically with EGF to induce vascular smooth muscle cells to enter mitosis. Serum-deprived cells were exposed to the compounds indicated for 30 hr along with [$^3$H]thymidine, and labeled nuclei were counted after autoradiography.

in normal thrombosis, could contribute to the hemorrhagic diathesis of APL.

Inhibition of angiogenesis is a newly identified function for TSP, likely to be relevant in a number of normal and pathological situations. It is consistent with the observed absence of this protein from areas where new vessels are forming in mouse embryos (35) and in early wounds (36) and with its presence where neovascularization is ending, adjacent to vessels in late wounds. TSP produced by macrophages may also play a role in regulating the angiogenic activity that is central to the neovascular response that accompanies the formation of inflammatory granulation tissue during wound repair (37). Mouse and human macrophages produce TSP when resting, responsive, or primed but stop producing it upon final activation, and purified gp140 is able to decrease the formation of granulation tissue in polyvinyl sponges implanted subcutaneously in hamsters (J. Holland and P.J.P., unpublished). Endothelial cells themselves appear to stop producing TSP when cultured on collagen gels permissive for *in vitro* angiogenesis (38) and upon spontaneous transformation to hemangioendothelioma cells (39). Polyoma virus, which induces these tumors in transgenic mice (40), can prevent the production of gp140 when it transforms hamster fibroblasts (N.P.B., unpublished).

Soluble TSP, or an active fragment, seems to block neovascularization by affecting the endothelial cells themselves as it blocked their migration *in vitro*. TSP may act indirectly, for example, by altering contacts between endothelial cells and appropriate matrix components that are vital for successful angiogenesis (41, 42), as exogenous TSP can decrease cell affinity for substratum (43). Alternatively, TSP may act more directly. Preliminary experiments suggest that it does not sequester bFGF, but it can bind to endothelial cells in suspension (44) and may negatively modulate the activity of the endothelial cell, as it is known to positively modulate the growth of smooth muscle cells (20).

The identification of suppressor-controlled gp140 as a TSP isoform opens up to direct analysis the mechanism by which a tumor suppressor gene controls a molecule potentially able to influence tumorigenicity and the means by which ubiquitous, multifaceted TSP may contribute to the regression of physiological neovascularization.

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