Journal of Cellular Biochemistry 53:74–84 (1993)

Modulation of Endothelial Cell Proliferation, Adhesion, and Motility by Recombinant Heparin-Binding Domain and Synthetic Peptides From the Type I Repeats of Thrombospondin

Tikva Vogel, Neng-hua Guo, Henry C. Krutzsch, Diane A. Blake, Jacob Hartman, Simona Mendelovitz, Amos Panet, and David D. Roberts

Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892 (T.V., N.-h.G., H.C.K., D.D.R.); Department of Biochemistry, Meharry Medical College, Nashville, Tennessee 37208 (D.A.B.); and BioTechnology General, Ltd., Rehovot, Israel (T.V., J.H., S.M., A.P.)

Abstract Thrombospondin is an inhibitor of angiogenesis that modulates endothelial cell adhesion, proliferation, and motility. Synthetic peptides from the second type I repeat of human thrombospondin containing the consensus sequence -Trp-Ser-Pro-Trp- and a recombinant heparin binding fragment from the amino-terminus of thrombospondin mimic several of the activities of the intact protein. The peptides and heparin-binding domain promote endothelial cell adhesion, inhibit endothelial cell chemotaxis to basic fibroblast growth factor (bFGF), and inhibit mitogenesis and proliferation of aortic and corneal endothelial cells. The peptides also inhibit heparin-dependent binding of bFGF to corneal endothelial cells. The antiproliferative activities of the peptides correlate with their ability to bind to heparin and to inhibit bFGF binding to heparin. Peptides containing amino acid substitutions that eliminate heparin-binding do not alter chemotaxis or proliferation of endothelial cells. Inhibition of proliferation by the peptide is time-dependent and reversible. Thus, the antiproliferative activities of the thrombospondin peptides and recombinant heparin-binding domain result at least in part from competition with heparin-dependent growth factors for binding to endothelial cell proteoglycans. These results suggest that both the Trp-Ser-Xaa-Trp sequences in the type I repeats and the amino-terminal domain play roles in the antiproliferative activity of thrombospondin. © 1993 Wiley-Liss, Inc.*

Key words: chemotaxis, extracellular matrix, angiogenesis, basic fibroblast growth factor

The requirements for neovascularization during normal development, for reproductive function, wound repair, and tumor growth and metastasis suggest that regulators of this process could be useful therapeutic agents [reviewed in Folkman and Klagsbrun, 1987; Folkman and Shing, 1992]. Several classes of compounds have antiangiogenic activities, including some polysaccharides, proteins, peptides, steroids, and microbial products [Folkman and Klagsbrun, 1987; Folkman and Shing, 1992]. Several of the proteins with these activities are produced by or are associated with endothelium in vivo, and thus are candidates for physiological regulators of endothelial proliferation or migration. These include platelet factor 4 [Maione et al., 1990], osteonectin/SPARC [Funk and Sage, 1991], a proteolytic fragment of fibronectin [Homandberg et al., 1986], interferon α-2a [Ezekowitz et al., 1992], and thrombospondin [Good et al., 1990; Taraboletti et al., 1990; Bagavandoss and Wilks, 1990; Iruela-Arispe et al., 1991].

Thrombospondin is a major component of the α-granules of platelets and is a member of a gene family synthesized by a variety of cultured cell types [reviewed in Frazier, 1991; Mosher, 1990].

Abbreviations used: BAE cells, bovine aortic endothelial cells; bFGF, basic fibroblast growth factor; BCE cells, corneal bovine endothelial cells; P246, synthetic peptide from the second type I repeat of thrombospondin with the sequence Lys-Arg-Phe-Lys-Gln-Asp-Gly-Gly-Trp-Ser-His-Trp-Ser-Pro-Trp-Ser-Ser; P239, Ser-His-Trp-Ser-Pro-Trp-Ser-Ser; P244, Ser-His-Ala-Ser-Pro-Ala-Ser-Ser-Cys-Ser-Val-Thr; P266, Lys-Arg-Phe-Lys-Gln-Asp-Gly-Gly-Ala-Ser-His-Ala-Ser-Pro; TGFβ, transforming growth factor β; TSP18, recombinant heparin-binding fragment of thrombospondin containing residues 1–174.

Received February 25, 1993; accepted May 26, 1993.

Address reprint requests to David D. Roberts, Building 10, Room 2A33, National Institutes of Health, Bethesda, MD 20892.

© 1993 Wiley-Liss, Inc. *This article is a US Government work and, as such, is in the public domain in the United States of America.

Thrombospondin is present at very low levels in plasma, but its concentration is elevated at sites of platelet activation. Thrombospondin is found in intracellular granules of endothelial cells and is enriched in the subendothelial matrix in vivo [Wight et al., 1985; Munjal et al., 1989]. Thus, endothelial cells are probably exposed to variable concentrations of thrombospondin in vivo. Endothelial cell responses to thrombospondin are complex; the magnitude and direction of the responses depend upon the presence of additional matrix components and growth factors. Immobilized thrombospondin promotes endothelial cell adhesion on some substrates [Taraboletti et al., 1990] but inhibits adhesion on others, including substrates coated with fibronectin [Lahav, 1988; Sage and Bornstein, 1991]. Inhibition of adhesion to fibronectin is associated with disruption of focal adhesion contacts [Murphy-Ullrich and Höök, 1989]. Thrombospondin promotes migration of endothelial cells in chemotaxis and haptotaxis assays but inhibits chemotaxis induced by bFGF [Taraboletti et al., 1990]. Thrombospondin inhibits proliferation and spontaneous tube formation by endothelial cells in vitro [Iruela-Arispe et al., 1991] and inhibits angiogenesis in vivo [Good et al., 1990]. A 140 kD fragment of thrombospondin has been identified as the antiangiogenic factor in conditioned medium of hamster kidney cells [Good et al., 1991].

To understand the mechanisms of these diverse and apparently conflicting effects of thrombospondin on endothelial cell behavior, it is necessary to define the domains of thrombospondin that interact with the cells, the identity of the endothelial cell receptors that interact with thrombospondin, and the intracellular responses in transduction and integration of the signals resulting from thrombospondin binding to each receptor. At least three domains of thrombospondin are implicated in interactions with endothelial cells. Adhesion of bovine aortic endothelial cells on thrombospondin is inhibited by peptides containing the Arg-Gly-Asp sequence and may be mediated by a β3 integrin [Lawler et al., 1988; Taraboletti et al., 1990]. Thrombospondin contains an Arg-Gly-Asp sequence that could potentially interact with this receptor [Lawler and Hynes, 1986; Lawler et al., 1988; Sun et al., 1992]. A β3 integrin may also participate in chemotaxis and spreading of endothelial cells on thrombospondin [Taraboletti et al., 1990]. Adhesion and migration of endothelial cells in a thrombospondin gradient is inhibited by an antibody and ligands that bind to the amino terminal heparin-binding domain of thrombospondin [Taraboletti et al., 1990]. The core region of thrombospondin containing the type I repeats has also been shown to contain a cell-binding domain for endothelial cells [Dardik and Lahav, 1991] and other cell types [Asch et al., 1992; Rich et al., 1990; Prater et al., 1991; Guo et al., 1992a].

The domains of thrombospondin that account for its antiproliferative activity are unclear. Based on inhibition by monoclonal antibodies and sulfated polysaccharides, the heparin-binding domain at the amino-terminus of thrombospondin may be responsible for regulation of endothelial proliferation [Taraboletti et al., 1990]. However, a 140 kD fragment of thrombospondin that lacks the amino-terminal region also suppresses endothelial cell growth [Good et al., 1990]. Thus, multiple sites on the thrombospondin molecule may modulate endothelial cell growth and motility. Moreover, based on recent studies by Murphy-Ullrich et al. [1992], inhibition of bovine endothelial cell growth by thrombospondin is at least partly due to the inhibitory activity of transforming growth factor β, which complexes with thrombospondin and contaminates most thrombospondin preparations.

We have recently shown that synthetic peptides from the type I repeats of thrombospondin containing the consensus sequence -Trp-Ser-Pro-Trp- bind to heparin and sulfatide, inhibit high affinity binding of thrombospondin to melanoma cells, and promote melanoma cell adhesion and chemotaxis [Guo et al., 1992a,b]. We report here that these peptides and a recombinant heparin-binding domain from the amino-terminus of thrombospondin promote endothelial cell adhesion and are potent inhibitors of endothelial cell proliferation and chemotaxis.

MATERIALS AND METHODS

Materials

Thrombospondin was purified from thrombin-stimulated human platelets as previously described [Roberts et al., 1985]. A 140 kD fragment of thrombospondin lacking the amino-terminal heparin-binding domain was prepared by thrombin digestion and purified by heparin affinity chromatography as previously described [Roberts et al., 1987]. Synthetic peptides from the type I repeats of human *THBS1* were prepared and characterized as previously described [Guo et

al., 1992a,b]. Recombinant heparin-binding fragments of thrombospondin were expressed in *Escherichia coli* strain A4255 F- under the control of the thermoinducible λP_L promoter and CII ribosomal binding site. The 28 kD fragment contains amino acids 1–242 of human *THBS1* with a Met residue preceding the first amino acid. The 18 kD fragment contains residues 1–174 with an initiating Met preceding the first residue and the sequence -Arg-Ser-Ala-Ser-Gln added to the carboxyl terminus. The recombinant proteins were purified from inclusion bodies by chromatography on DEAE-Sepharose, CM-Sepharose, and heparin-Sepharose. The 28 kD fragment was oxidized in the presence of 100 μM oxidized glutathione. Both fragments were lyophilized from 1 mM $NaHCO_3$ at 200 μg/ml, pH 8.8, with 1 mM dithiothreitol for the 18 kD fragment, pH 10.5 for the 28 kD fragment. bFGF was obtained from Collaborative Research, Bedford, MA, and Bachem, Torrance, CA. A recombinant fragment of human fibronectin (FN33) contained the cell binding domain of human fibronectin, amino acids 1329–1722, and was prepared as previously described [Werber et al., 1990].

Cell Culture

Bovine aortic endothelial cells (BAE cells) were kindly provided by Dr. E. Gallin (AFRRI, Bethesda, MD), and were used at passages 5–10. BAE cell cultures were routinely maintained in DMEM (low glucose) containing 10% FCS, 4 mM glutamine, 0.05 mg/ml ascorbic acid, and 500 U/ml each of penicillin G potassium and streptomycin sulfate (all media components were from Biofluids Inc., Rockville, MD). BAE cells were grown at 37°C in 5% CO_2. Corneal bovine endothelial cells (BCE cells) were used at passages 2–8 [Munjal et al., 1990]. BCE cell cultures were maintained in the same medium, but without ascorbic acid and including 2.5 μg/ml amphotericin B. BCE cells were grown at 34°C in 5% CO_2. The media were changed every 2–3 days.

Thymidine Incorporation Into DNA

Confluent monolayers of cells were washed once in PBS and then starved in 0.5% FCS-containing medium for 48 h. Cells were trypsinized, washed in 10% FCS-containing medium, resuspended in medium containing 0.1% BSA, and seeded in 24-well plates at 20,000 cells/well in the presence of various concentrations of FCS, growth effectors, and 2.5 μCi/well of thymidine[methyl-^{3}H] (86.1 Ci/mmol, Dupont NEN, Boston, MA). For termination of the assay, cells were washed two times with 1 ml PBS, fixed with 0.3 ml solution of methanol/acetic acid (3:1), washed two times with 0.5 ml of 80% ethanol, and air dried. Cells were extracted from the wells by incubation with 300 μl of trypsin/EDTA (1 h at 37°C and 30 min at room temperature), and by the addition of 100 μl of 1% SDS. The radioactivity of the extracted material was measured in a scintillation counter.

Cell Proliferation Assay

Endothelial cell proliferation was measured using the CellTiter 96™ assay [Promega, Madison, WI]. Cells (5×10^3) were plated into each well of a 96-well culture plate in 0.5 or 5% FCS-containing medium, together with the indicated concentrations of growth effectors. After 72 h, 15 μl of dye solution was added to each well, and the plates were incubated for an additional 4 h. Solubilization solution was added, and absorbance at 570 nm was determined after 24 h as described by the manufacturer.

Chemotaxis

Chemotaxis of endothelial cells was determined in modified Boyden chambers as previously described [Taraboletti et al., 1990]. Trypsinized BCE cells were resuspended in complete medium and allowed to recover in suspension for 2.5 to 3 h. Cells were recovered by centrifugation and suspended in DMEM, 0.1% BSA, at 1.5 to 2×10^6 cells/ml prior to their addition to the upper wells of the chemotaxis chambers. Chemotaxis was measured after incubation at 37°C in 5% CO_2 for 4.5 to 5 h. Trypsinized BAE cells were resuspended in complete medium. The cells were immediately centrifuged, resuspended at 1×10^6 cells/ml in DMEM containing 0.1% BSA, and used without recovery.

Adhesion and Binding Assays

After trypsinization, cells were resuspended in complete medium and allowed to recover for 3 h at 25°C with gentle rocking. Adhesion to immobilized proteins and peptides were determined as previously described [Roberts et al., 1987; Guo et al., 1992a]. Binding of ^{125}I-bFGF to the endothelial cells was determined by a modification of the previously described procedure [Guo et al., 1992a]. Briefly, 10^5 cells in a final volume

of 0.2 ml of Dulbecco's PBS containing 0.2% gelatin were preincubated with the potential inhibitors for 15 min. ^{125}I-bFGF, prepared as described by Neufeld and Gospodarowicz [1985], was added and the cells were incubated on a rotating table for 1 h at 20°C. Bound radioactivity was determined after centrifugation of the cells through oil. Binding of ^{125}I-bFGF to heparin was determined using an immobilized heparin-bovine serum albumin conjugate as previously described [Guo et al., 1992a].

RESULTS

Previous studies from this and other laboratories [Taraboletti et al., 1990; Murphy-Ullrich and Höök, 1989; Good et al., 1990] have demonstrated that thrombospondin modulates various functions of endothelial cells, including adhesion, motility, and proliferation. In order to locate the functional site(s) on thrombospondin, we have used recombinant fragments of thrombospondin containing the heparin-binding domain (TSP18; amino acids 1–174), and synthetic peptides derived from the second type I repeat of human thrombospondin [Guo et al., 1992a,b]. The effect of thrombospondin and thrombospondin-related molecules on endothelial cell growth was determined using mitogenesis assays based both on incorporation of ^{3}H-thymidine in DNA and on cell proliferation. We have tested bovine endothelial cells derived from either the aorta (BAE cells) or the cornea (BCE cells).

P246 and TSP18 Inhibit bFGF-Stimulated DNA Synthesis in Aortic Endothelial Cells

Release of BAE cell cultures from serum starvation by using growth medium containing either 1% or 2.5% FCS resulted in an increase in the incorporation of ^{3}H-thymidine. The addition of bFGF together with the serum resulted in a several-fold stimulation of the serum induced incorporation of the isotope (Fig. 1). The bFGF-stimulated growth was strongly inhibited by either P246 (20 μM), derived from the second type I repeat of human thrombospondin, or by the recombinant heparin-binding domain of thrombospondin TSP18 (0.5 μM) at both high and low serum concentrations. Inhibition of serum-stimulated growth by the two thrombospondin-related molecules in the absence of bFGF, however, was appreciably lower (results not shown).

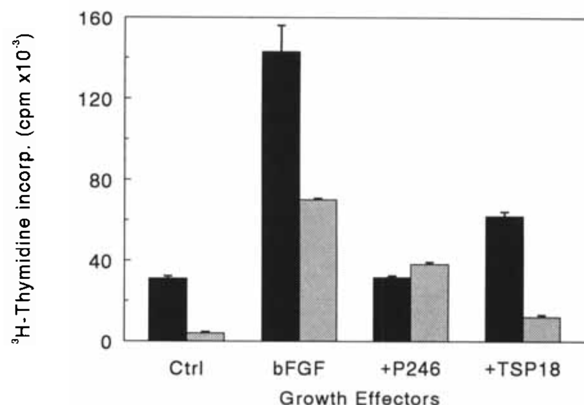

Fig. 1. Inhibition of bFGF-stimulated endothelial cell mitogenesis by recombinant heparin-binding domain or a synthetic peptide from the type I repeats of thrombospondin. Bovine aortic endothelial cells (2 × 10^4/well) were labeled with ^{3}H-thymidine after release from serum starvation in the presence of 1% (solid bars) or 2.5% fetal calf serum (striped bars). Mitogenesis was terminated after 42 h. Control (Ctrl) contained no additions. Other cells were exposed to 10 ng/ml basic fibroblast factor alone (bFGF) or in the presence of 20 μM of the thrombospondin peptide KRFKQDGGWSHWSPWSS (P246) or 0.5 μM recombinant thrombospondin heparin-binding domain (TSP18). Results are presented as mean ± S.D., n = 2.

Endothelial cells were normally serum-starved for 2 days in medium containing 0.5% FCS. When such starved cells were trypsinized and replated at either 1 or 2.5% serum, the incorporation of ^{3}H-thymidine was much higher than in nontrypsinized cells (data not shown). In this situation, the addition of bFGF to a parallel culture did not always result in further stimulation of incorporation. In these assays, however, inhibition of thymidine incorporation by P246 or TSP18 was always greater when bFGF was present. The degree of inhibition by P246 or TSP18 was not affected by modifying cell density from 6,000 to 24,000 cells/well, suggesting that cells were responding to the added bFGF rather than to bFGF produced by the cells.

The time course of ^{3}H-thymidine incorporation in BAE cells after release from serum starvation is presented in Figure 2. When 1% FCS and bFGF were added, cells exhibited a maximum incorporation of isotope at around 40–44 h after replating. In contrast, cells replated under the same conditions with P246 (20 μM) or TSP18 (0.5 μM) exhibited decreased isotope incorporation to approximately 60% of control levels. Treatment of a parallel culture of BAE cells with a recombinant fibronectin molecule containing the cell binding domain, FN33 (0.5 μM), did not affect the kinetics or extent of incorporation of

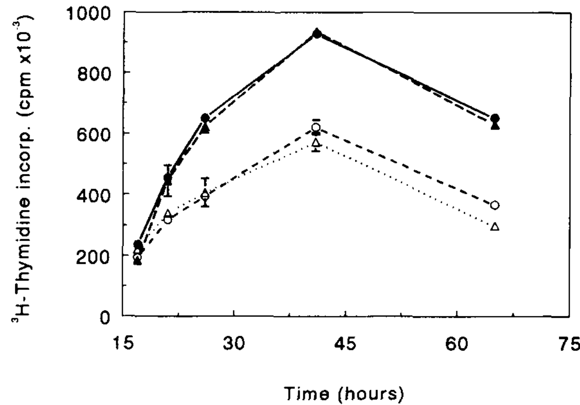

Fig. 2. Time dependence of DNA synthesis in bovine aortic endothelial cells: Effect of thrombospondin or fibronectin fragments. DNA synthesis was tested on newly attached cells. Cells were plated in DMEM containing 1% FCS and 10 ng/ml bFGF *alone (●), or together with 0.5 μM TSP18 (○), FN33 (▲), or 20* μM P246 (△). Results are the mean ± SD of duplicate determinations.

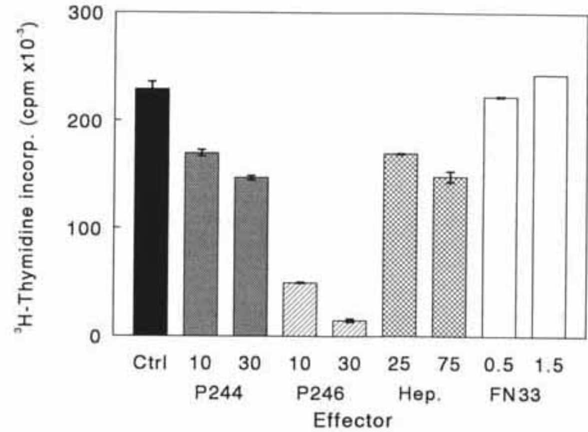

Fig. 3. Effects of heparin, thrombospondin peptides, and fibronectin fragment on DNA synthesis. ^{3}H-Thymidine incorporation into BAE cell DNA was determined 40 h after release from serum starvation, and in the presence of various concentrations of molecules added at time zero: 10 and 30 μM P244 (gray bars) or P246 (striped bars), 25 and 75 μg/ml heparin (hatched bars), or FN 33 at 0.5 and 1.5 μM (open bars).

the isotope, indicating that this inhibition is specific for the thrombospondin-related molecules.

The inhibition of ^{3}H-thymidine incorporation into BAE cells by P246 was mediated at least in part by the heparin-binding motif Trp-Ser-Pro-Trp, as a peptide with Ala residues substituted for the Trp residues (P244) was only weakly active (Fig. 3). Thus, the addition of 10 and 30 μM P244 or 25 and 75 μg/ml heparin caused slight inhibition (20–30%), but addition of 10 and 30 μM P246 resulted in a much stronger inhibition (80–95%). The addition of FN 33 at 0.5 and 1.5 μM had no effect on thymidine incorporation.

Inhibition of DNA Synthesis by the Thrombospondin Peptide 246 Is Time-Dependent and Reversible

To examine the time-dependence of the sensitivity of BAE cells to inhibition of DNA synthesis by P246, serum starved cells were plated into growth medium containing bFGF, FCS (0.5 or 1%) and labeled with ^{3}H-thymidine for 40 h (Fig. 4). When P246 was added at the time of plating, a strong inhibition of incorporation was observed (80–85%). When added after 15 h or 22 h, P246 was considerably less effective and only a minor decrease in incorporation of the isotope was observed (15–20%). This effect was identical at both serum concentrations tested.

The inhibitory effect of peptide 246 on DNA synthesis in BAE cells was reversible, as removal of the peptide after 22 h and readdition of

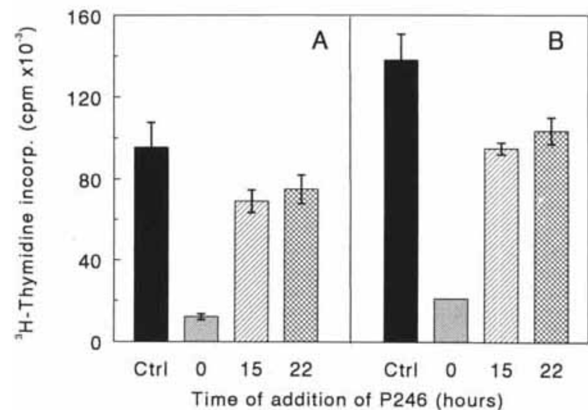

Fig. 4. Effect of time of addition on inhibition of DNA synthesis by thrombospondin peptide 246. Incorporation of [^{3}H]-thymidine into BAE cells was determined in the presence of 10 ng/ml bFGF and 0.5 (**A**) or 1% FCS (**B**) either alone (solid bars) or with 30 μM P246, added to the newly plated cells at time zero (gray bars) or at 15 h (striped bars) or 22 h after plating (hatched bars). [^{3}H]-Thymidine was added at time zero, and the assay was terminated after 40 h. Results are presented (mean ± SD) as a percent of control incorporation determined for cells in 0.5% FCS (95,500 cpm) or 1% FCS (138,000 cpm).

growth medium led to resumption of growth. At 30 μM P246, removal of the peptide after 22 h increased thymidine incorporation at 40 h from 37% of control to 70% of control incorporation, which was determined in duplicate cultures without added peptide.

Thrombospondin-Related Molecules Inhibit DNA Synthesis in Corneal Endothelial Cells

DNA synthesis in bovine corneal endothelial cells (BCE cells) was also sensitive to inhibition

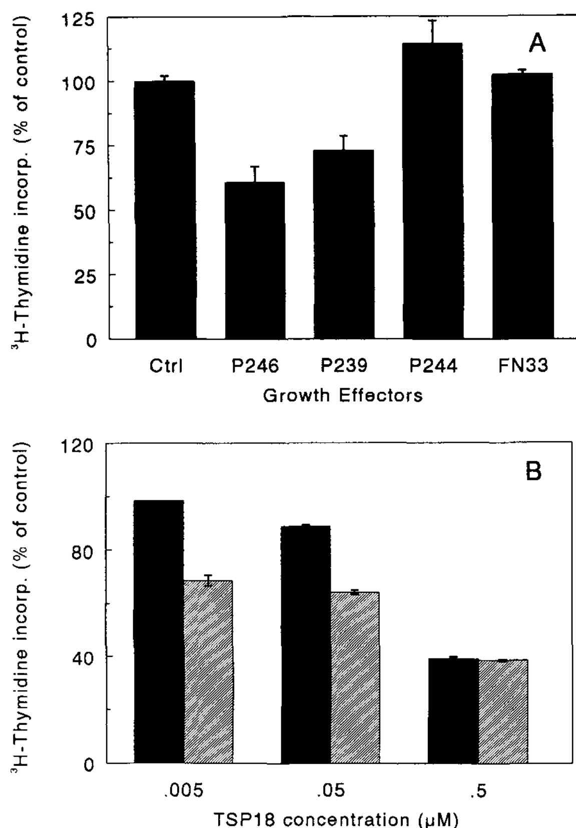

Fig. 5. Inhibition of DNA synthesis in bovine corneal endothelial cells by thrombospondin peptides and recombinant heparin-binding domain. A: Cells were plated in 1% FCS and 10 ng/ml bFGF alone (Ctrl) or together with 30 μM of P246, P239 (SHWSPWSS), or P244 (SHASPASSCSVT). Incorporation was determined after 42 h. B: Thymidine incorporation in BCE cells was determined by protocol 1 in the presence of the indicated concentrations of TSP18 in medium containing 0.5% FCS (solid bars) or 0.5% FCS and 10 ng/ml bFGF (striped bars). Results (mean ± SD) are presented as a percent of control incorporation determined without inhibitor (100% = 215,000 cpm).

by the thrombospondin fragment and peptides (Fig. 5). After release from serum starvation, addition of FCS and bFGF to the culture stimulated incorporation of isotope into DNA. Addition of FN33 or the control peptide P244 at the time of plating had no effect on DNA synthesis. However, addition of P246 or P239 resulted in a 40 or 30% inhibition of isotope incorporation, respectively (Fig. 5A). Inhibition was dose-dependent, although it should be noted that the IC_{50} values for inhibition of growth of BCE cells by the thrombospondin related molecules was usually higher than those observed for BAE cells. The relative inhibitory activities of the peptides suggest that both the consensus heparin-binding sequence at the amino terminus of P246, which is missing in P239, and the tryptophan clusters which are present in both active peptides but not in the inactive analog P244, contribute to the antiproliferative activity of the peptide.

The recombinant heparin-binding fragment TSP18 also inhibited incorporation of ^{3}H-thymidine in BCE cell cultures (Fig. 5B). In this experiment, plating of BCE cell cultures either in the presence of 1% FCS alone or together with bFGF resulted in approximately equal levels of isotope incorporation. TSP18 was a dose dependent inhibitor of thymidine incorporation. At lower doses of TSP18 (0.005 and 0.05 μM), inhibition was greater in the cultures containing bFGF than those treated with FCS alone. At 0.5 μM TSP18, however, the extent of inhibition was equal under both conditions (45%).

Thrombospondin Peptide 246 and Recombinant Heparin Binding Domain, TSP18, Inhibit Proliferation of Corneal and Aortic Endothelial Cells

Addition of either soluble native thrombospondin or recombinant TSP18 to BCE cell cultures resulted in a dramatic and dose-dependent inhibition of cell proliferation in response to both bFGF and FCS (Fig. 6). The IC_{50} values for the inhibition were approximately 70 nM for native thrombospondin and 2–3 μM for TSP18. FN33, a recombinant polypeptide containing the cell-binding domain of human fibronectin, did not significantly inhibit the proliferative response at

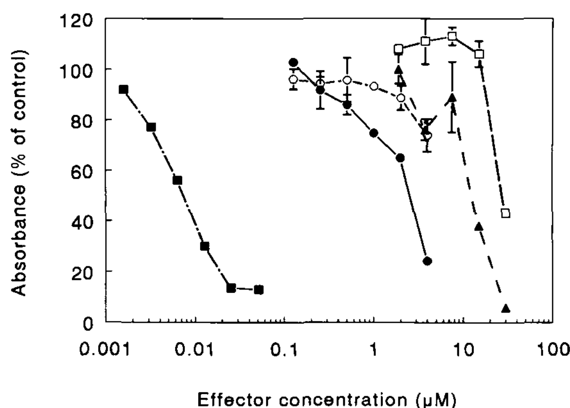

Fig. 6. Inhibition of BCE cell proliferation by thrombospondin, recombinant TSP18, and synthetic peptides. The proliferation of BCE cells was measured after 3 days in medium containing 10 ng/ml bFGF and 0.5% FCS in the presence of the indicated concentrations of native thrombospondin (■), TSP18 (●), FN33 (○), or P246 (□), or 5% FCS in the presence of P246 (▲). Cell number was determined by the CellTiter assay using Absorbance = 0.960(A_{570nm} − A_{630nm}) and is expressed as a percent of net stimulated proliferation determined in the absence of inhibitors, mean ± SD, n = 2.

concentrations where TSP was strongly inhibitory. Addition of P246 to the culture resulted in a dose-dependent inhibition of proliferation at both the high and low concentrations of serum used. However, at the higher concentration of serum, inhibition of proliferation occurred at lower concentrations of P246.

TSP-18 (0.4 μM) and P246 (15 μM) also inhibited proliferation of BAE cells to 72 ± 14% and 65 ± 5% of control levels, respectively. In combination, the two molecules inhibited proliferation to 56 ± 3% of control. However, in additional experiments, additivity of the effects of TSP18m and P246 was not consistently observed (results not shown).

Thrombospondin Fragments and Peptides Mimic the Effects of the Intact Molecule on Endothelial Cell Motility

As was observed for thrombospondin as a modulator of bFGF-stimulated endothelial cell motility [Taraboletti et al., 1990], peptide 246 had a biphasic effect on BCE cell motility. In the absence of bFGF, peptide 246 stimulated the chemotactic response of BCE cells (Fig. 7A). The positive chemotactic response required higher concentrations of P246 than needed to stimulate chemotaxis of other cell lines tested [Guo et al., 1992b]. Addition of bFGF to the lower well of the chemotaxis chamber stimulated motility of BCE cells approximately 2-fold. Addition of low concentrations of P246 produced a dose-dependent inhibition of the bFGF-stimulated motility. However, at concentrations greater than 12.5 μM, P246 also produced a positive chemotactic response by BCE cells in the presence of bFGF. The effects of P246 on BCE cell migration were specific in that P266, which contains the basic amino acid motif of P246 but has Ala substituted for both Trp residues, did not stimulate BCE cell motility at concentrations up to 50 μM and did not significantly inhibit bFGF-stimulated motility (results not shown). TSP18 did not stimulate motility of BCE cells, and inhibited their basal motility when added to the lower wells (data not shown).

Peptide 246 inhibited basal and bFGF stimulated chemotaxis of BAE cells (Fig. 7B). Addition of 25 μM P246 to the lower well eliminated the bFGF stimulated motility at all concentrations of bFGF. Addition of P246 to the upper well inhibited migration of BAE cells to 50 ng/ml bFGF, but the inhibition was overcome by increasing the concentration of bFGF to 200 ng/ml. The latter result indicates that the inhibition is not due to a toxic effect of the peptide on the cells and is consistent with a competitive mode of inhibition.

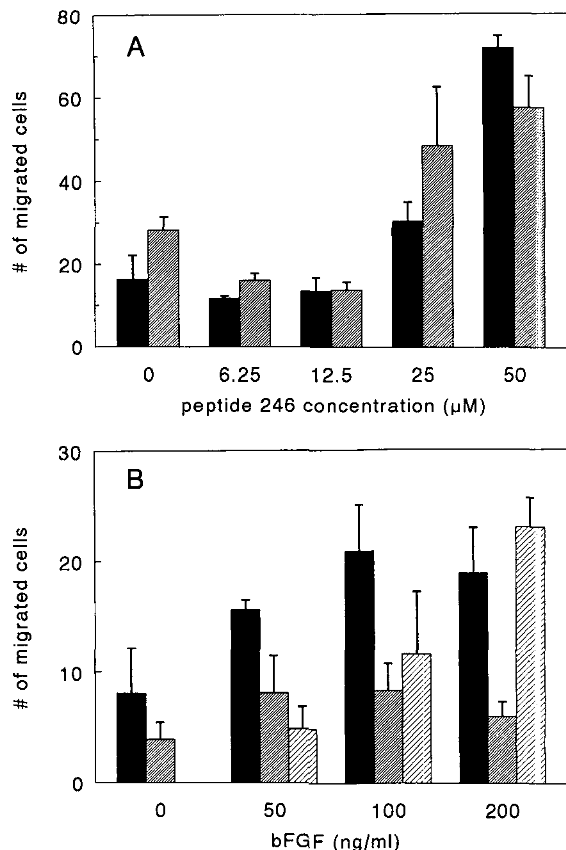

Fig. 7. Modulation of endothelial cell chemotaxis by thrombospondin peptide 246. Chemotaxis was measured in modified Boyden chambers as described in Materials and Methods. **A:** BCE cell migration to the indicated concentrations of P246 in the lower chamber was determined in the absence (solid bars) or in the presence of 100 ng/ml bFGF. Migrated cells/field are presented as mean ± SD, n = 3. **B:** BAE cell migration to the indicated concentrations of bFGF added to the lower chamber was determined alone (solid bars) or in the presence of 25 μM P246 added to the lower (gray bars) or upper chamber (striped bars).

The recombinant heparin-binding fragment TSP18 also caused dose-dependent inhibition of BAE cell chemotaxis to bFGF (Fig. 8). In addition, TSP18 inhibited the basal motility of BAE cells in the absence of bFGF, a result similar to that observed with P246. Fibronectin also stimulated chemotaxis of BAE cells, but TSP18 did not inhibit fibronectin-stimulated chemotaxis (results not shown), demonstrating the specificity of TSP18 for inhibiting bFGF-stimulated motility.

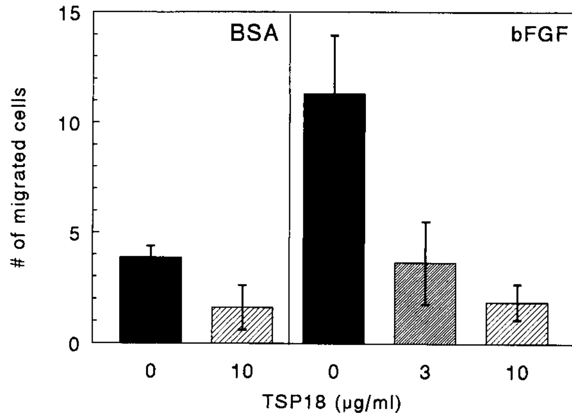

Fig. 8. Inhibition of BAE cell migration by recombinant TSP18. BAE cell migration to DMEM/0.1% BSA medium (BSA) or medium containing 33 ng/ml bFGF added to the lower chamber was determined alone (solid bars) or in the presence of 3 μg/ml (gray bars) or 10 μg/ml TSP18 added to the lower chamber (striped bars).

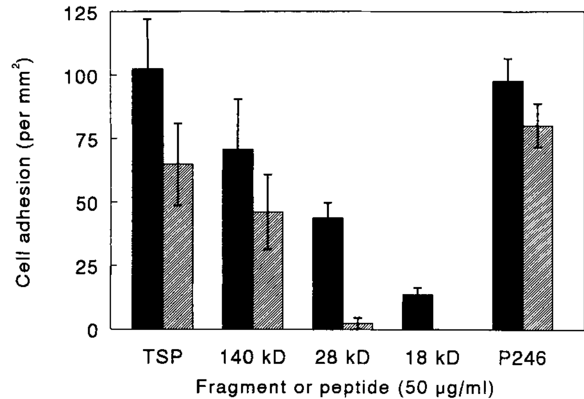

Fig. 9. Adhesion of BCE cells on substrates coated with thrombospondin or thrombospondin fragments. Bacteriological plastic disks were coated with 50 μg/ml of TSP or the indicated fragments: 140 kD thrombin digested fragment (140 kD), recombinant 28 kD fragment (28 kD), recombinant 18 kD fragment (18 kD), or the synthetic peptide P246. Attachment (solid bars) and spreading (gray bars) are presented as mean ± SD, n = 6.

Adhesion of Corneal Endothelial Cells to Thrombospondin Fragments

Thrombospondin immobilized on plastic supported attachment and spreading of BCE cells (Fig. 9). The activities of the intact molecule for promoting attachment and spreading were mimicked by a 140 kD fragment containing all but the amino-terminal heparin-binding domains. Two recombinant fragments of thrombospondin containing the heparin-binding domain promoted attachment but not spreading of the cells, and P246 from the second type I repeat strongly promoted attachment and spreading.

To further define the domains of thrombospondin involved in adhesion, heparin and monoclonal antibodies to thrombospondin were tested as inhibitors of adhesion (Fig. 10). Approximately 90% of BCE cell adhesion to thrombospondin was inhibited by heparin. Antibody A2.5, which binds to the amino-terminal heparin-binding domain, and A4.1, which binds to a 70 kD fragment of thrombospondin containing the type I repeats, were dose-dependent inhibitors of BCE cell adhesion. Two additional antibodies to other domains of thrombospondin, A6.1 and D4.6, were inactive (results not shown).

Inhibition of bFGF Binding to Intact Cells or Heparin by Thrombospondin Fragments or Peptides

Binding of ^{125}I-bFGF to BCE cells was inhibited by the thrombospondin fragments (Fig. 11). Typically, 90% of bFGF binding to BCE cells

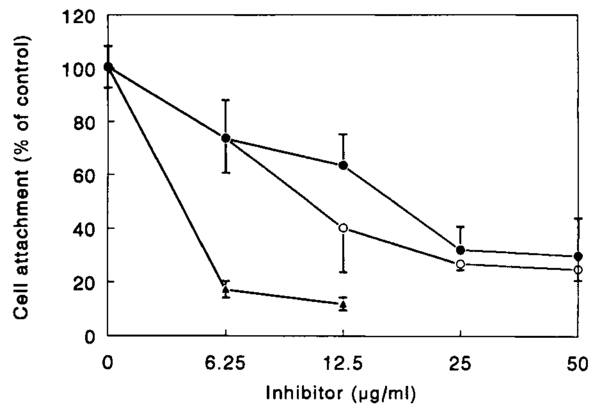

Fig. 10. Inhibition of endothelial cell adhesion on thrombospondin. BCE cell adhesion to disks coated with 50 μg/ml TSP was determined in the presence of the indicated concentrations of heparin (▲) or thrombospondin antibodies A4.1 (●) or A2.5 (○). Results are presented as a percent of control adhesion, mean ± SD, n = 6.

was inhibitable by heparin (IC_{50} = 0.56 μg/ml). Binding was strongly inhibited by P246 (IC_{50} = 3.5 μM) and partially inhibited by 6 μM TSP18. Remarkably, although binding of ^{125}I-bFGF to BAE cells was consistently inhibited more than 90% by heparin (IC_{50} = 0.61 μg/ml), only 10 to 45% of bFGF binding to BAE cells could be inhibited by 50 μM P246 (Fig. 11 and data not shown). Inhibition was minimal in cells grown in complete medium but was enhanced by serum starvation (data not shown).

These results suggest that the thrombospondin fragments act in part by competing for binding of bFGF to proteoglycans on the cell surface.

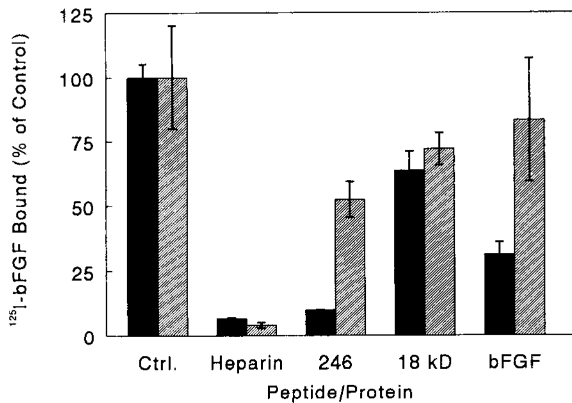

Fig. 11. Inhibition of ^{125}I-bFGF binding to endothelial cells by heparin and thrombospondin fragments. Binding of 0.16 μg/ml ^{125}I-bFGF to 1 × 10^5 BCE cells (solid bars) or BAE cells starved by growing for 2 days in medium containing 0.5% FCS (gray bars) was determined as described in Materials and Methods. Binding in the presence of 100 μg/ml heparin, 50 μM P246, 5.6 μM *TSP18, or* 2 μg/ml bFGF are presented as a percent of net control binding determined in the absence of inhibitors, mean ± SD, n = 3.

This hypothesis was supported by the observation that intact thrombospondin, TSP18, and P246 were dose-dependent inhibitors of bFGF binding to immobilized heparin-BSA, with IC_{50} values of 0.14 μM, 1.8 μM, and 1.6 μM, respectively (data not shown). The control proteins laminin and a 31 kD recombinant fibrin-binding domain of fibronectin and the control peptides P244 and 245 (VTCGGGVQKRSRL) did not inhibit bFGF binding to heparin.

DISCUSSION

Using both a recombinant fragment and synthetic peptides from thrombospondin, we have identified two regions of the molecule that mimic the activities of platelet thrombospondin on endothelial cells using in vitro mitogenesis, proliferation, and migration assays. The recombinant amino-terminal heparin-binding domain specifically inhibits mitogenic and proliferative responses of two endothelial cells from two sources, and inhibits chemotactic responses to bFGF. Synthetic peptides from the type I repeats of thrombospondin also inhibit the mitogenic and proliferative responses of endothelial cells to bFGF. They also have a bimodal inhibitory/stimulatory effect on bFGF stimulated endothelial cell chemotaxis that mimics the bimodal response of endothelial cells to intact thrombospondin [Taraboletti et al., 1990]. Because these molecules are synthetic or derived from bacterial sources, their biological activities are not due to contamination with TGFβ. Although native thrombospondin is antiadhesive in some settings [Lahav, 1988; Murphy-Ullrich and Höök, 1989], the intact molecule, the recombinant heparin-binding fragment, and the synthetic peptides from the type I repeats promote adhesion of endothelial cells when adsorbed on plastic.

The simplest model to explain the inhibitory activity of the peptides and recombinant fragments of thrombospondin on bFGF-dependent proliferation and motility is based on their shared heparin-binding activities. These heparin-binding molecules could act by competing for bFGF binding to a low affinity HSPG receptor on the endothelial cells, which is essential for bFGF action [reviewed in Klagsbrun and Baird, 1991]. This model would account for the fact that most of the reported antiangiogenic proteins bind to heparin. The ability of thrombospondin, TSP18, and P246 to compete for bFGF binding to heparin and of P246 to inhibit bFGF binding to BCE cells are consistent with this model. Dose responses for the biological effects of the peptide and fragments are generally consistent with their IC_{50} values for binding to heparin [Guo et al., 1992a,b] and the IC_{50} value for inhibiting bFGF binding to BCE cells by P246 matches that for inhibiting bFGF binding to heparin. P246 is less able to inhibit the binding of bFGF to BAE cells; however, the cells are sensitive to inhibition by P246 of bFGF-stimulated growth and chemotaxis. In some cases, therefore, the thrombospondin peptide P246 may be acting through some other mechanism.

Since the active amino acid sequence in P246 is conserved in various growth factors and cytokine receptors and may be essential for their function [Bazan, 1990; Guo et al., 1992b; Miyazaki et al., 1991; Yoshimura et al., 1992], additional effects of the peptide on growth factor responses should be considered. Our data suggests that serum-derived growth factors other than bFGF are also sensitive to inhibition by the thrombospondin fragments and peptides. It remains to be determined whether these growth factors require heparin-dependent binding for their growth-promoting activities.

Another possible mechanism for inhibition of cell proliferation by thrombospondin and its fragments is based on their antiadhesive activities. Ingber and Folkman [1989] proposed that

growth or differentiation of endothelial cells is regulated by extracellular matrix components through a mechanochemical coupling that induces cell spreading or retraction. Retraction was associated with cessation of cell proliferation. We have observed that TSP18 and P246 induce partial rounding of endothelial cells in proliferation assays. Both the rounding response and inhibition of proliferation were partially reversed by addition of FN33 which contains the cell-binding domain of fibronectin (T. Vogel and D.D. Roberts, unpublished results). Thus, a second possible mechanism for inhibition of endothelial cell growth by the thrombospondin fragments is by disruption of specific interactions with extracellular matrix components required for endothelial cell spreading.

Although there is evidence that at least two domains of intact thrombospondin function in its interactions with endothelial cells, their relative roles remain to be determined. Monoclonal antibodies to the amino-terminal heparin-binding domain inhibited thrombospondin-induced chemotaxis [Taraboletti et al., 1990]. The present data demonstrate that this domain, when expressed as a recombinant protein, inhibits bFGF stimulated chemotaxis and growth. The antiproliferative and antiangiogenic activities of a 140 kD fragment of thrombospondin lacking the amino-terminal heparin-binding domains [Good et al., 1990], indicate that an additional domain of the molecule has activity for inhibiting endothelial cell growth. This fragment of thrombospondin contains the integrin ligand Arg-Gly-Asp-Ala [Lawler and Hynes, 1986; Lawler et al., 1988; Sun et al., 1992]. However, this sequence does not appear to participate in the antiproliferative activity of thrombospondin, since RGD peptides did not block the inhibition [Taraboletti et al., 1990]. We have now identified a peptide sequence from the type I repeats of the 140 kD fragment that mimics many of the activities of the intact protein on endothelial cells. Because TGFβ associated with platelet thrombospondin also inhibits proliferation and can only be removed using harsh denaturing conditions [Murphy-Ullrich et al., 1992], it will be difficult to define the role of each site in the intrinsic antiangiogenic activities of platelet-derived thrombospondin. The further use of recombinant proteins and synthetic peptides, therefore, remains a promising alternative approach to this problem.

ACKNOWLEDGMENTS

We thank Dr. William Frazier for providing monoclonal antibodies to thrombospondin, Dr. Elaine Gallin for providing aortic endothelial cells, and Mr. Russel DeCamp, who was supported by an ASBMB High School Teacher Summer Fellowship, for assisting with the proliferation and migration assays. This work was supported in part by National Institutes of Health Grant R01 EY09092.

REFERENCES

Asch AS, Silbiger S, Heimer E, Nachman RL (1992): Thrombospondin sequence motif (CSVTCG) is responsible for CD 36 binding. Biochem Biophys Res Commun 182:1208–1217.

Bagavandoss P, Wilks JW (1990): Specific inhibition of endothelial cell proliferation by thrombospondin. Biochem Biophys Res Commun 170:867–872.

Bazan JF (1990): Structural design and molecular evolution of a cytokine receptor superfamily. Proc Natl Acad Sci USA 87:6934–6938.

Dardik R, Lahav J (1991): Cell-binding domain of endothelial thrombospondin: Localization to the 70 kDa core fragment and determination of binding characteristics. Biochemistry 30:9378–9386.

Ezekowitz RAB, Mulliken JB, Folkman J (1992): Interferon alfa-2a therapy for life-threatening hemangiomas of infancy. New Engl J Med 326:1456–1463.

Folkman J, Klagsbrun M (1987): Angiogenic factors. Science 235:442–447.

Folkman J, Shing Y (1992): Angiogenesis. J Biol Chem 267:10931–10934.

Frazier WA (1991): Thrombospondins. Curr Opin Cell Biol 3:792–799.

Funk SE, Sage H (1991): The Ca2+-binding glycoprotein SPARC modulates cell cycle progression in bovine aortic endothelial cells. Proc Natl Acad Sci USA 88:2648–2652.

Good DJ, Polverine PJ, Rastinejad F, LeBeau MM, Lemons RS, Frazier WA, Bouck NP (1990): A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci USA 87:6624–6628.

Guo N, Krutzsch HC, Nègre E, Vogel T, Blake DA, Roberts DD (1992a): Heparin- and sulfatide-binding peptides from the type I repeats of thrombospondin promote melanoma cell adhesion. Proc Natl Acad Sci USA 89:3040–3044.

Guo N, Krutzsch HC, Nègre E, Zabrenetzky VS, Roberts DD (1992b): Heparin-binding peptides from the type I repeats of thrombospondin: Structural requirements for heparin binding and promotion of melanoma cell adhesion and chemotaxis. J Biol Chem 267:19349–19355.

Homandberg GA, Kramer-Bjerke J, Grant D, Christianson G, Eisenstein R (1986): Heparin-binding fragments of fibronectin are potent inhibitors of endothelial cell growth: Structure-function correlations. Biochim Biophys Acta 874:61–71.

Ingber DE, Folkman J (1989): Mechanochemical switching between growth and differentiation during fibroblast

growth factor-stimulated angiogenesis in vitro: Role of extracellular matrix. J Cell Biol 109:317–330.

Iruela-Arispe ML, Bornstein P, Sage H (1991): Thrombospondin exerts an antiangiogenic effect on cord formation by endothelial cells in vitro. Proc Natl Acad Sci USA 88:5026–5030.

Klagsbrun M, Baird A (1991): A dual receptor system is required for basic fibroblast growth factor activity. Cell 67:229–231.

Lahav J (1988): Thrombospondin inhibits adhesion of endothelial cells. Exp Cell Res 177:199–204.

Lawler J, Hynes RO (1986): The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins. J Cell Biol 103:1635–1648.

Lawler J, Weinstein R, Hynes RO (1988): Cell attachment to thrombospondin: The role of Arg-Gly-Asp, calcium, and integrin receptors. J Cell Biol 107:2351–2361.

Maione TE, Gray GS, Petro J, Hunt AJ, Donner AL, Bauer SI, *Carson* HF, Sharpe RJ (1990): Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. Science 247:77–79.

Miyazaki T, Maruyama M, Yamada G, Hatakeyama M, Taniguchi T (1991): The integrity of the conserved "WS motif" common to IL-2 and other cytokine receptors is essential for ligand binding and signal transduction. EMBO J 10:3191–3197.

Mosher DF (1990): Physiology of thrombospondin. Annu Rev Med 41:85–97.

Munjal ID, Blake DA, Sabet MD, Gordon SR (1990): Thrombospondin: Biosynthesis, distribution, and changes associated with wound repair in corneal endothelium. Eur J Cell Biol 52:252–263.

Murphy-Ullrich JE, Höök M (1989): Thrombospondin modulates focal adhesions in endothelial cells. J Cell Biol 109:1309–1319.

Murphy-Ullrich JE, Schultz-Cherry S, Höök M (1992): Transforming growth factor-β complexes with thrombospondin. Mol Biol Cell 3:181–188.

Neufeld G, Gospodarowicz D (1985): The identification and partial characterization of the fibroblast growth factor receptor of baby hamster kidney cells. J Biol Chem 260: 13860–13868.

Prater CA, Plotkin J, Jaye D, Frazier WA (1991): The properdin-like type I repeats of human thrombospondin contain a cell attachment site. J Cell Biol 112:1031–1040.

Rich KA, George FW, Law JL, Martin WJ (1990): Cell-adhesive motif in region II of malarial circumsporozoite protein. Science 249:1574–1577.

Roberts DD, Haverstick DM, Dixit VM, Frazier WA, Santoro SA, Ginsburg V (1985): The platelet glycoprotein thrombospondin binds specifically to sulfatides. J Biol Chem 260: 9405–9411.

Roberts DD, Sherwood JA, Ginsburg V (1987): Platelet thrombospondin mediates attachment and spreading of human melanoma cells. J Cell Biol 104:131–139.

Sage EH, Bornstein P (1991): Extracellular proteins that modulate cell matrix interactions. J Biol Chem 266:14831–14834.

Sun X, Skorstengaard K, Mosher DF (1992): Disulfides modulate RGD-inhibitable cell adhesive activity of thrombospondin. J Cell Biol 118:693–701.

Taraboletti G, Roberts DD, Liotta LA, Giavazzi R (1990): Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: A potential angiogenesis regulatory factor. J Cell Biol 111:765–772.

Werber M, Vogel T, Kook M, Greenstein LA, Levanon A, Zelig Y, Havron A, Gorecki M, Panet A (1990): Large scale purification of recombinant proteins derived from fibronectin domains. In White MD, Reuvery S, Shafferman A (eds): "Biologicals From Recombinant Microorganisms and Animal Cells Production and Recovery." Weinheim: VCH, pp 369–382.

Wight TN, Raugi GJ, Mumby SM, Bornstein P (1985): Light microscopic immunolocation of thrombospondin in human tissues. J Histochem Cytochem 33:295–302.

Yoshimura A, Zimmers T, Neumann D, Longmore G, Yoshimura Y, Lodish HF (1992): Mutations in the Trp-Ser-X-Trp-Ser motif of the erythropoietin receptor abolish processing, ligand binding, and activation of the receptor. J Biol Chem 267:11619–11625.