

Thrombospondin-induced Tumor Cell Migration: Haptotaxis and Chemotaxis Are Mediated by Different Molecular Domains

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Abstract. Thrombospondin induces the migration of human melanoma and carcinoma cells. Using a modified Boyden chamber assay, tumor cells migrated to a gradient of soluble thrombospondin (chemotaxis). Checkerboard analysis indicated that directional migration was induced 27-fold greater than stimulation of random motility. Tumor cells also migrated in a dose-dependent manner to a gradient of substratum-bound thrombospondin (haptotaxis). A series of human melanoma and carcinoma cells were compared for their relative motility stimulation by thrombospondin haptotaxis vs. chemotaxis. Some cell lines exhibited a stronger haptotactic response compared to their chemotactic response while other lines exhibited little or no migration response to thrombospondin. Human A2058 melanoma cells which exhibit a strong haptotactic and chemotactic response to thrombospondin were used to study the structural domains of thrombospondin required for the response. Monoclonal antibody C6.7, which binds to the COOH-terminal region of thrombospondin, inhibited haptotaxis in a dose-dependent optimal manner. C6.7 had no significant

effect on thrombospondin chemotaxis. In contrast, monoclonal antibody A2.5, heparin, and fucoidan, which bind to the NH₂-terminal heparin-binding domain of thrombospondin, inhibited thrombospondin chemotaxis but not haptotaxis. Monoclonal antibody A6.1 directed against the internal core region of thrombospondin had no significant effect on haptotaxis or chemotaxis. Synthetic peptides GRGDS (50 µg/ml), but not GRGES, blocked tumor cell haptotaxis on fibronectin, but had minimal effect on thrombospondin or laminin haptotaxis. The 140-kD fragment of thrombospondin lacking the heparin-binding amino-terminal region retained the property to fully mediate haptotaxis but not chemotaxis. When the COOH region of the 140-kD fragment, containing the C6.7-binding site, was cleaved off, the resulting 120-kD fragment (which retains the RGDA sequence) failed to induce haptotaxis. Separate structural domains of thrombospondin are therefore required for tumor cell haptotaxis vs. chemotaxis. This may have implications during hematogenous cancer metastases formation.

THROMBOSPONDIN is a multifunctional glycoprotein first described as a secretion product of thrombin-stimulated platelets (2). Thrombospondin is a component of the extracellular matrix in a wide variety of tissues. By immunohistology, thrombospondin is localized in basement membranes, vessel walls, and interstitium (32). Thrombospondin is also secreted by several cultured cell lines including endothelial, smooth muscle cells, fibroblasts, pneumocytes, macrophages, monocytes, and tumor cells (11, 12, 14, 17, 24, 26).

Like fibronectin and laminin, thrombospondin binds to a diverse array of macromolecules and mediates cell adhesion in vitro (5, 6, 14, 15, 21–24, 31). Based on activities of proteolytic fragments and using ligands and antibodies to thrombospondin to inhibit adherence, at least two regions of the molecule are involved in attachment and spreading of human

G361 melanoma cells on surfaces coated with thrombospondin (23). The monoclonal antibody A2.5 directed against the heparin-binding domain of thrombospondin (8, 9) selectively inhibits melanoma spreading but only weakly inhibits attachment. Large fragments of thrombospondin lacking the heparin-binding domain support attachment but not spreading of G361 cells, and this activity is lost after removal of the 18-kD carboxyl-terminal domain. The heparin-binding domain of thrombospondin recognizes cell surface sulfatides and other sulfated glycoconjugates which may serve as receptors distinct from those which recognize the carboxyl terminus of thrombospondin (15, 23, 26–28, 33).

Tumor invasion and metastases is a multistep process which requires active tumor cell migration through extracellular matrix barriers (16). Circulating tumor cells can potentially interact with thrombospondin during the process of

extravasation. In this process, tumor cells interact with platelets, migrate underneath endothelial cells, and traverse the subendothelial basement membrane (16). All of these surfaces may be rich in thrombospondin. Although thrombospondin is known to play a role in cell attachment and growth (17, 23, 31), little is known concerning its role in tumor cell locomotion. The present study was performed to compare the promotion of tumor cell migration by substrate-bound (haptotaxis) vs. soluble thrombospondin (chemotaxis). The results indicate that separate domains of thrombospondin are involved in haptotaxis vs. chemotaxis of human tumor cells.

Materials and Methods

Calcium-replete thrombospondin was purified from the supernatant of thrombin-stimulated human platelets by affinity chromatography on gelatin-agarose and heparin-agarose and gel filtration on Bio-gel A0.5m (10). The purified protein migrated as a single band (M_r 180,000) on electrophoresis in the presence of SDS and 2-mercaptoethanol (13) and was stored at -70°C in Tris-buffered saline, pH 7.6, containing 20% sucrose and 0.1 mM CaCl_2 . Synthetic peptides corresponding to the cell-attachment site for fibronectin (GRGDS and the control GRGES) were provided by Dr. Kenneth M. Yamada (National Cancer Institute, National Institutes of Health, Bethesda, MD). Monoclonal antibodies to thrombospondin were provided by Dr. William Frazier (Washington University School of Medicine, St. Louis, MO). Laminin was purified from Engelbreth-Holm swarm (EHS) tumor as described (29). Fibronectin and type IV collagen were purchased from Collaborative Research (Bedford, MA) or Gibco (Grand Island, NY).

Cell Lines

A2058 human melanoma (30), MDA-MB-361 human breast adenocarcinoma (ATCC HTB 27), MDA-MB-435S human breast ductal carcinoma (ATCC HTB 129), and Hs578 Bst human breast-immortalized myoepithelial cells (ATCC HTB 125) were maintained in culture in DME supplemented with 10% FCS. Human melanoma cell lines C32 (ATCC CRL 1585) and G361 (ATCC CRL 1424) were maintained in vitro in RPMI 1640 containing 10% FCS. Media and serum were provided by Meloy Laboratories, Inc. (Springfield, VA) or Gibco.

Limited Proteolysis of Thrombospondin

Thrombospondin was digested with 4 U/ml reagent thrombin (provided by Dr. Jules Gladner, NIH) in 20 mM Tris-buffered saline, pH 7.6, containing 0.1 mM or 2 mM CaCl_2 , for 16 h at 37°C or with trypsin, chymotrypsin (0.5% wt/wt), or thermolysin (1:100 wt/wt) as described previously (10, 23). The digestions were stopped by addition of 2 mM phenylmethylsulfonyl fluoride (PMSF) and the cleavage products were fractionated by chromatography on heparin-agarose (8, 9, 23). The fragments were judged pure by electrophoresis in the presence of SDS and 2-mercaptoethanol as shown previously (23, 26–28) and tested for chemotactic and haptotactic activities.

Cell Motility Assays

Chemotaxis and haptotaxis were assayed using modified Boyden chambers, with 8- μm pore size polycarbonate Nucleopore filters.

For haptotaxis, filters were coated on one side by floating them overnight at 37°C on a solution of thrombospondin (20 $\mu\text{g}/\text{ml}$), in Dulbecco's PBS or laminin or fibronectin (10 $\mu\text{g}/\text{ml}$ in 50 mM carbonate buffer, pH 9.6). The coating method used was identical to that described by McCarthy et al. (20). Chambers were then assembled with serum-free medium containing 0.1% BSA. The side of the filter with the highest concentration of protein faced the lower compartment.

For chemotaxis, filters were soaked overnight at 37°C in Dulbecco's PBS. To test thrombospondin-mediated chemotaxis on different substrates, filters were soaked overnight in solutions of laminin (10 $\mu\text{g}/\text{ml}$ in 50 mM carbonate buffer, pH 9.6), type IV collagen (40 $\mu\text{g}/\text{ml}$ in 0.1 M acetic acid), or gelatin (100 $\mu\text{g}/\text{ml}$ in 0.1% acetic acid). All filters were washed five times in Dulbecco's PBS before use. Chemotaxis was conducted as described previously (19, 25).

Cells in logarithmic phase of growth were detached by brief exposure to 0.05% trypsin/0.02% EDTA and kept 1 h in serum-containing medium be-

fore the assay. The upper compartment of the chamber was then filled with 4×10^5 cells in serum-free medium supplemented with 0.1% BSA. After a 4–5-h incubation at 37°C filters were stained with Diff Quick (American Scientific Products, MacGraw Park, IL) and the amount of the migrated cells was measured with a 2202 Ultrascan Laser Densitometer (LKB Instruments, Gaithersburg, MD). We found a linear correlation ($r = 0.99$) between the number of migrated cells counted per high power field (50 \times) and the optical density area units.

The effect of antibodies and thrombospondin-binding molecules, heparin, and fucoidan on motility was assayed by adding them in the lower compartment of the chamber with the thrombospondin for chemotaxis. For haptotaxis the antibodies or polysaccharides were added to either the top or the bottom of the modified Boyden chamber.

Results

Thrombospondin-induced Chemotaxis

Thrombospondin stimulated the migration of human melanoma cells in the modified Boyden chamber assay (Fig. 1). When a series of thrombospondin concentrations was introduced into the lower chamber, migration was stimulated in a dose-dependent manner. An increase in migration occurred within a range of 1.0–150 nM and reached a plateau at thrombospondin concentrations higher than this. Checkerboard analysis was performed to investigate the random or directed nature of the tumor cell migration response to thrombospondin (Fig. 2). In this assay, the migration of tumor cells was studied using different ratios of thrombospondin above and below the filter. The squares below the diagonal reflect a positive gradient, while those above the diagonal reflect a negative gradient. The values along the diagonal indicate random motility to a uniform concentration of soluble thrombospondin added to both sides of the chamber. The checkerboard analysis indicated that migration was directional in nature occurring to the highest level in a positive gradient (maximum of 27-fold increase) and greatly inhibited in a negative gradient (squares above diagonal). Ran-

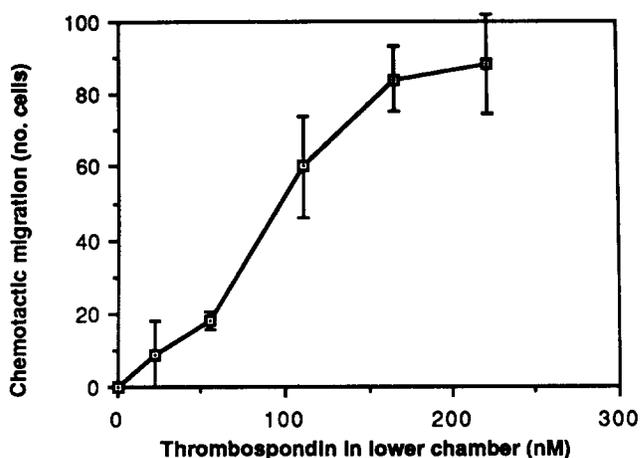


Figure 1. Dose-response curve for chemotactic response of A2058 melanoma cells to thrombospondin. Increasing concentrations of thrombospondin were added to the lower chamber, diluted in DME, supplemented with 0.1% BSA, in a volume of 42 μl . The chambers were then assembled with filters (preincubated overnight in Dulbecco's PBS and washed); 4×10^5 cells in DME, supplemented with 0.1% BSA were added, and the incubation time was 4 h. Data are expressed as mean number \pm SEM of migrated cells per high power field, as measured with the Ultrascan Densitometer.

		TSP (µg / ml) IN THE UPPER CHAMBER			
		0	25	50	100
TSP (µg / ml) IN THE LOWER CHAMBER	0	2.6	0.7	0	0
	25	22.2	2.5	0.6	0
	50	70.6	15.7	5.3	1.4
	100	47.8	37.4	19.1	4.1

Figure 2. Checkerboard analysis of the chemotactic activity of thrombospondin. A2058 melanoma cells were tested for chemotactic response to soluble thrombospondin in presence of different gradients (positive, negative, or null). The data shown are the mean number of migrated cells as in Fig. 1.

dom migration was slightly increased (maximum of twofold increase; lower right along the diagonal).

The effect of substratum composition on thrombospondin-mediated chemotaxis was studied by coating the polycarbonate filters with a uniform concentration of laminin or proteins that do not significantly bind thrombospondin. Uncoated filters, laminin, type IV collagen, and gelatin all mediated cell adhesion and served as a migration surface which allowed the cells to sense and respond to the gradient of soluble thrombospondin in the fluid phase (Table I). When the filters were coated with BSA which failed to mediate tumor cell adhesion, no chemotaxis was observed.

Thrombospondin-induced Haptotaxis

Tumor cells migrated over substratum-bound thrombospondin in the absence of soluble thrombospondin. The migration of cells to a substratum-bound attractant, termed haptotaxis, was pronounced in the presence of a step density gradient of substratum-bound thrombospondin. When the magnitude of the gradient was increased from 0:0 to 0:100 nM, the level of haptotaxis increased and began to reach a plateau above 60 nM (Fig. 3). Only a very low level of random migration was observed for filters precoated on both sides with uniform concentrations of thrombospondin.

Table I. Thrombospondin-induced Chemotaxis on Different Substrates

	Migrated cells*	Attachment
Laminin	89.0 ± 46.2	+
Type IV collagen	29.5 ± 8.1	+
Gelatin	79.0 ± 7.1	+
Gelatin	45.3 ± 8.4	+
BSA	0	-

Filters were coated with substrates as described in Materials and Methods. Chemotactic test was performed using a solution of 50 µg/ml thrombospondin in DME supplemented with 0.1% BSA. A2058 melanoma cells were used in this assay. Data are expressed as directed motility, obtained by subtracting nonstimulated motility from total migration.

* Mean number of cells ± SEM.

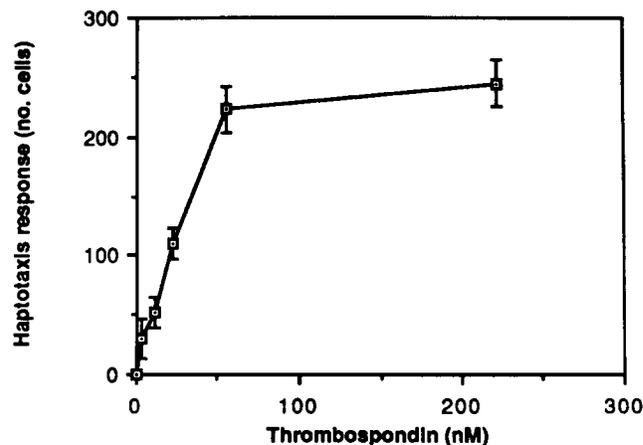


Figure 3. Dose-response relationship in the haptotactic response of G361 melanoma cells to thrombospondin. Filters were coated with a step gradient of increasing concentrations of thrombospondin. Data are expressed as mean ± SEM of migrated cells per high power field.

Heterogeneity of Thrombospondin-mediated Chemotaxis and Haptotaxis by Different Human Tumor Cell Lines

A series of human melanoma and breast carcinoma cell lines were compared for their migration response to a soluble or solid phase gradient of thrombospondin. As shown in Table II, six separate human tumor cell lines exhibited wide differences in their relative migration induced by thrombospondin. Two of three melanoma lines (A2058 and G361) exhibited both a chemotactic and a haptotactic response to thrombospondin. The A2058 melanoma cells showed a similar rate of migration by both haptotaxis and chemotaxis. The G361 melanoma line demonstrated a greater mean haptotactic response compared to chemotaxis. The melanoma line C32 was devoid of any migration response to soluble or solid-phase thrombospondin. The three human breast cell lines also differed significantly in their response to thrombospondin-induced migration. MDA-MB-435S tumor cells showed almost no thrombospondin chemotactic response, but did respond by haptotaxis. MDA-MB-361 cells failed to

Table II. Heterogeneity in Thrombospondin-mediated Chemotactic and Haptotactic Response by Different Tumor Cell Lines

Tumor cell	Spontaneous migration	Chemotaxis*	Haptotaxis*
A2058	1.75 ± 1.2	93.4 ± 21.0	107.4 ± 27.6
G361	0	80.7 ± 18.7	153.9 ± 38.2
C32	0	0	0
MDA435	0	2.4 ± 2.4	30.0 ± 23.7
MDA361	0	2.7 ± 0.2	0
HS578	0	62.5 ± 22.3	91.5 ± 25.6

The different cell lines were maintained in culture as described in Materials and Methods. The test was performed using their usual culture medium, without serum and containing 0.1% BSA. All lines were tested at the same cell concentration and for the same incubation time (4.5 h). For haptotaxis, filters were coated using a solution of 20 µg/ml of thrombospondin in Dulbecco's PBS. For chemotaxis, 42 µl of a solution 50 µg/ml thrombospondin in DME was added to the lower chamber.

* Mean number of cells ± SEM.

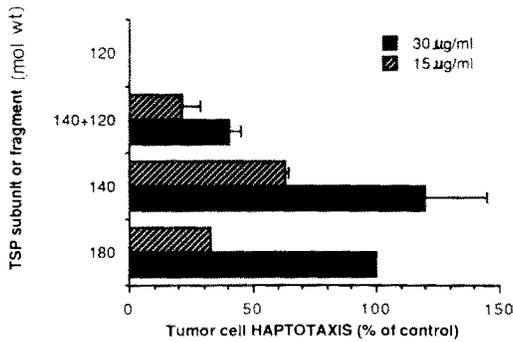


Figure 4. Haptotactic activity of enzymatic fragments of thrombospondin. Filters were coated overnight on one side with a solution of 30 or 15 µg/ml of fragments in Dulbecco's PBS. Data are expressed as percentage of control (undigested thrombospondin). (180) Mock-digested control thrombospondin. (140) 140-kD thrombin generated fragment. (140 + 120) Equal molar (1:1) ratio of 140- and 120-kD fragments. (120) Purified 120-kD chymotrypsin fragment (devoid of haptotaxis activity). Coating concentrations were adjusted for molarity equal to whole thrombospondin.

respond by either haptotaxis or chemotaxis, whereas Hs578 Bst cells migrated toward both types of gradients. While these cell lines differed significantly from one to another, they were highly consistent in their response to thrombospondin when the same cell line was repeatedly tested. These data indicate that different tumor cells regardless of their histologic origin may respond to thrombospondin haptotaxis and chemotaxis through different mechanisms and to different magnitudes.

Effect of Antibodies to Thrombospondin on Chemotaxis vs. Haptotaxis

Affinity-purified monoclonal antibodies recognizing specific domains of thrombospondin were studied for their ability to inhibit thrombospondin-mediated haptotaxis or chemotaxis. A striking difference in the antibody specificity was noted (Table III). Monoclonal antibody C6.7, which recognizes the globular carboxyl terminus of thrombospondin (7, 9), at a concentration of 10 µg/ml inhibited haptotaxis by 90% (Table III). The inhibition was equally effective if the antibodies

Table III. Inhibition of A2.5 and C6.7 Monoclonal Antibody on Thrombospondin-induced Cell Motility

Antibody	Concentration µg/ml	Chemotaxis*	Haptotaxis*
C6.7	10	80.8 ± 17.6	10.0 ± 5.0
	1	92.7 ± 12.5	63.0 ± 11.0
	0.1	85.0 ± 18.1	91.5 ± 8.5
A2.5	10	38.0 ± 7.2	90.7 ± 21.9
	1	68.7 ± 8.2	100.0 ± 21.3
	0.1	59.0 ± 12.2	95.0 ± 70.0
A6.1	10	90.6 ± 36.4	72.0 ± 7.0

Chemotactic test was performed using a solution of 50 µg/ml thrombospondin in DME/0.1% BSA. Different dilutions of monoclonal antibodies were preincubated with TSP for 45 min, then both were placed in the lower chamber. Haptotaxis was assayed as described; antibodies were added to the lower compartment. Data are expressed as mean (3-6 experiments) percentage of control ± SEM.

* % control ± SEM.

were added to the top or bottom chamber. The level of haptotactic inhibition decreased proportionally as the C6.7 antibodies were diluted. In contrast to its inhibition of haptotaxis, antibody C6.7 had no significant dose-dependent effect on thrombospondin-induced chemotaxis. Monoclonal antibody A2.5, which recognizes the amino terminus of thrombospondin, exhibited the opposite pattern for haptotactic vs. chemotactic inhibition when compared to antibody C6.7. Antibody A2.5 inhibited thrombospondin chemotaxis in a dose-dependent manner, after a 100-fold difference in concentration, but had no significant dose-dependent effect on haptotaxis. Antibody A6.1, which recognizes the core 70-kD internal domain fragment of thrombospondin (4), did not significantly inhibit chemotaxis and only slightly inhibited haptotaxis.

Effect of Fucoidan, Heparin, and RGD Peptides

Antibody A2.5 which inhibits chemotaxis but not haptotaxis (Table III) recognizes the heparin-binding amino-terminal domain of thrombospondin. This region of thrombospondin binds both to sulfated polysaccharides (e.g., heparin), and to sulfated glycolipids. Roberts et al. (23) have reported that the sulfated fucan, fucoidan, and the monoclonal antibody A2.5 selectively inhibit melanoma cell spreading, but only weakly inhibit attachment. Therefore, the inhibitory effects of heparin and fucoidan were studied for thrombospondin-mediated tumor cell migration. Heparin and fucoidan both showed an inhibition of thrombospondin chemotaxis but had no effect on, or in some cases slightly stimulated, haptotaxis (Table IV). Considering the very large standard errors, the statistical significance of any potential dose-dependent inhibition cannot be tested by comparing different doses. Nevertheless, in the aggregate these data show some inhibition at all concentrations, most of which are statistically significant. This finding is in keeping with a major role for the amino-terminal domain in the mediation of chemotaxis but not haptotaxis. Thrombospondin contains an RGD sequence located 241 amino acid residues from the carboxyl-terminal end of the molecule (15). The glycoprotein IIb/IIIa complex of platelets has been identified as a receptor which recognizes the RGD sequence of fibronectin, fibrinogen, von Willibrand factor, and vitronectin (27). Parallel studies with thrombospondin indicate that this receptor may also bind thrombospondin weakly (14, 26), although platelets lacking IIb/IIIa retain normal thrombospondin binding (1). Consequently, the effect of purified RGD synthetic peptides were studied for their role in thrombospondin-mediated haptotaxis. Fibronectin

Table IV. Inhibition of Thrombospondin-induced Cell Motility

Inhibitor	Dose µg/ml	Chemotaxis	Haptotaxis
Fucoidan	100	35.0 ± 5.8	146.0 ± 9.8
	50	47.0 ± 17.1	87.7 ± 16.2
	10	53.0 ± 10.7	105.0 ± 63.9
Heparin	100	56.3 ± 7.6	143.0 ± 21
	50	66.0 ± 7.0	133.3 ± 59.3
	10	59.3 ± 31.3	115.0 ± 19.4

Data are expressed as mean (3-6 experiments) percentage compared to control ± SEM.

and laminin both elicited significant haptotaxis using A2058 melanoma cells. GRGDS at a concentration of 50 $\mu\text{g/ml}$ abolished fibronectin haptotaxis but GRGES had no effect (Table VI). GRGES at 50 $\mu\text{g/ml}$ had no effect on laminin haptotaxis, compared to GRGDS (which slightly stimulated laminin haptotaxis) (Table VII). GRGDS and GRGES at a concentration of 62.5 $\mu\text{g/ml}$ both partially inhibited thrombospondin haptotaxis. At 250 $\mu\text{g/ml}$, GRGDS inhibited 30% more than GRGES, a fivefold higher concentration of GRGDS compared with that which inhibited haptotaxis on fibronectin.

Effect of Thrombospondin Fragments

Thrombin digestion of thrombospondin in the presence of different concentrations of Ca^{++} followed by further separation on a heparin affinity column yields two classes of fragments. The 140-kD fragment contains the COOH-terminal end of thrombospondin but lacks the heparin-binding domain residing in a region located at the NH_2 -terminal domain of intact thrombospondin (10). The 140-kD fragment can be further cleaved to a 120-kD fragment which lacks the COOH-binding region for mAb C6.7. Both the 120- and the 140-kD fragments have identical amino-terminal sequences (9). The thrombospondin fragments were studied for the ability to promote tumor cell migration (Fig. 4). The 140- and the 120-kD fragments were both devoid of chemotactic activity. In contrast, the 140-kD fragment retained full ability to elicit haptotaxis with a response equal to or greater than the control undigested thrombospondin. When the 140-kD fragment was partially cleaved to the 120-kD form (equal molar amounts of 120- and 140-kD fragments), significant haptotaxis was lost. Furthermore, the fully cleaved 120-kD fragment was unable to generate a haptotactic response. The 120-kD fragment lacks the C6.7-binding site which is retained on the 140-kD fragment. We also studied the induction of A2058 cell motility by the isolated heparin-binding domain, obtained by digestion of thrombospondin with chymotrypsin or thermolysin. All the thrombospondin fragments which retained the heparin-binding domain induced chemotaxis when tested at the same molarity corresponding to 50 $\mu\text{g/ml}$ of whole thrombospondin. All the fragments failed to induce chemotaxis on plain filters, however, using gelatin-coated filters, the heparin-binding fragments obtained by digestion with thermolysin induced the same degree of chemotactic response as did whole thrombospondin ($106.6\% \pm 45$), while the heparin-binding thermolysin-generated fragments in-

Table V. Effect of GRGDS and GRGES on Thrombospondin-induced Haptotaxis on A2058 Melanoma Cells

	Dose	Haptotaxis
	$\mu\text{g/ml}$	
GRGDS	250	48.5 \pm 15.5
	125	71.5 \pm 6.5
	62.5	66.0 \pm 29.0
GRGES	250	80.0 \pm 15.0
	125	81.5 \pm 6.5
	62.5	65.0 \pm 13.0

Data are expressed as percentage of control \pm SEM. (Mean of 3-6 experiments).

Table VI. Effect of GRGDS and GRGES on Fibronectin-mediated Haptotaxis

	Dose	Haptotaxis
	$\mu\text{g/ml}$	
GRGDS	50	0 (complete inhibition)
GRGES	50	103.2 \pm 23.6

Data are expressed as percentage of control \pm SEM. (Mean of 3-6 experiments).

duced a twofold chemotactic response ($195\% \pm 12.4$) compared to equal molar quantities of whole thrombospondin. All the above heparin-binding fragments were unable to induce any significant haptotaxis. The thrombospondin fragment results are consistent with the antibody inhibition data, further demonstrating that the heparin-binding amino-terminal region of thrombospondin is required for chemotaxis, whereas the C6.7-binding COOH-terminal region of thrombospondin is required for haptotaxis.

Discussion

Tumor cells encounter a variety of soluble and substratum-bound factors which may influence their directed migration at different stages in the process of tumor invasion and metastases (3, 16, 18). Such factors can promote the directed movement of tumor cells by at least two mechanisms, termed chemotaxis and haptotaxis. Chemotaxis refers to the directed migration of cells toward a soluble gradient of the attractant. The chemoattractant gradient is sensed by the migrating cell and influences the direction of locomotion. Haptotaxis, on the other hand, refers to the directed migration of cells along a gradient of substratum-bound insolubilized attractant. As shown in the present report, thrombospondin can induce tumor cell migration by both chemotaxis and haptotaxis (Figs. 1-3, and Table I), and this requires separate domains of thrombospondin for each type of migration response. In the case of chemotaxis, the substratum adhesion factors used by the migrating cells may be unrelated to the chemoattractant which exists in a soluble form in the fluid phase (25). This is true for leukocytes which respond to the chemoattractant FMLP (25). In this example, attachment to the substratum is required for leukocytes to migrate toward FMLP, but FMLP does not directly mediate adhesion (25). Thrombospondin can also mediate a true chemotactic response which is dependent of the mechanism used for cell-substratum attachment. As shown in Table I, thrombospondin mediated chemotaxis of melanoma cells when the filters were coated with a variety of proteins which mediate adher-

Table VII. Effect of GRGDS and GRGES on Laminin-mediated Haptotaxis

	Dose	Haptotaxis
	$\mu\text{g/ml}$	
GRGDS	50	157.9 \pm 4.1
GRGES	50	100.9 \pm 44.7

Data are expressed as percentage of control \pm SEM. (Mean of 3-6 experiments).

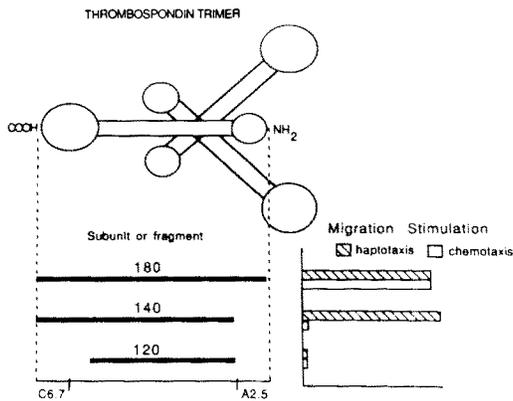


Figure 5. Thrombospondin subunit structure related to chemotaxis- and haptotaxis-promoting domains. The thrombospondin trimer is composed of three subunits that are linked by interchain disulfide bonds forming a protease-resistant core near the amino-terminal globular region. The alignment of proteolytic fragments and antibody-binding sites are shown and correlated with a differential haptotactic or chemotactic response.

ence. In particular, when the filters were coated with gelatin, which mediates tumor cell attachment but does not bind thrombospondin, a chemotactic response was observed. In contrast, when the filters were coated with BSA, which did not mediate A2058 melanoma cell attachment and did not bind thrombospondin, the tumor cells failed to attach and therefore failed to exhibit a chemotactic response.

Thrombospondin is a trimer composed of subunits that contain a small (8 nm) globular domain, a connecting rod-shaped (~20 nm) region, and a larger (17 nm) globular domain (Fig. 5). The small globular domain represents the NH₂ terminus of the peptide chain. Three subunits are joined along the rod-shaped region at a point ~16 nm from the carboxy terminus which represents the large globular domain. The two monoclonal antibodies A2.5 and C6.7, which bind to two epitopes at essentially the maximum possible distance on the thrombospondin molecule, exhibit a remarkable difference in their inhibition of thrombospondin-induced tumor cell migration. C6.7, which binds to the large globular COOH domain, blocks thrombospondin-mediated haptotaxis, but not chemotaxis. A2.5, which binds to the small globular NH₂ end, inhibits chemotaxis, but not haptotaxis. The differential effect strongly suggests that opposite domains of thrombospondin are involved in separate types of motility responses.

McCarthy et al. have reported that adhesion and motility functions can be separated using fragments of fibronectin (19). In the case of thrombospondin, adhesion and motility can also be separated. Furthermore, chemotaxis can be mediated by thrombospondin when the cells are migrating on a surface coated with adhesion proteins different from thrombospondin (Table I).

Well-defined proteolytic fragments of thrombospondin have been prepared which retain one or more of its multiple-binding domains (Fig. 5). When native thrombospondin (180-kD) is digested with thrombin, 25- and 140-kD fragments are generated. The 25-kD fragment is derived from the amino terminus (6), binds to heparin-Sepharose, and contains the binding site for mAb A2.5. Rotary shadowing of thrombospondin bound to A2.5 demonstrated that this site

is in the small globular end region of the thrombospondin subunits (9). Monoclonal antibody C6.7 binds to the large globular domain on the opposite end of the thrombospondin molecule compared to the binding location of A2.5 (9). Amino acid sequence data confirms that C6.7 binds near the extreme COOH terminus of the peptide chain, which is retained on the 140-kD fragment. The 140-kD fragment can be cleaved to form a 120-kD fragment which lacks the C6.7-binding domain but retains the fibrinogen-binding domain. The 120-kD fragment can be further cleaved to generate a 70-kD fragment which retains the ability to bind type V collagen and fibrinogen (5).

The 140-kD thrombospondin fragment, which lacks the heparin-binding site, retains full haptotactic activity but is devoid of chemotactic activity (Figs. 4 and 5). When this fragment is further cleaved to yield a 120-kD fragment, the haptotactic activity is totally lost. Since the binding site for C6.7, which blocks haptotaxis, is cleaved off the 140-kD fragment to yield the 120-kD fragment, it can be concluded that the region of thrombospondin essential for haptotactic promotion is located on the COOH domain distal to the chymotrypsin cleavage site. This COOH-terminal region comprises 122 amino acid residues starting with the sequence DTNPTRAQGY, but lacks the RGDA sequence which is located 241 amino acid residues from the COOH end of thrombospondin. Synthetic RGD-containing peptides failed to significantly inhibit haptotaxis in a dose-dependent manner (Table V). The same RGD peptides at 50 µg/ml exhibited a remarkable ability to block tumor cell haptotaxis on fibronectin but not laminin (Table V). As previously reported (23), RGD peptides do not inhibit tumor cell attachment or spreading on thrombospondin. Thus, the RGD recognition sequence does not seem to be directly involved in the adhesive and haptotactic interactions of these human tumor cells with thrombospondin, although an ancillary, indirect role for a cell surface RGD integrin family receptor cannot be ruled out. The putative tumor cell surface receptor which recognizes the COOH-terminal region of thrombospondin during haptotaxis remains to be identified. Nevertheless, it is likely to be similar to the receptor used for tumor cell attachment to thrombospondin and thrombospondin-mediated platelet aggregation, since C6.7 (but not A2.5) inhibits both activities (7, 23).

Thrombospondin binds specifically to purified sulfatides (galactosylceramide-[³S]sulfate), and the binding was inhibited strongly by antibody A2.5 (22). Thrombospondin failed to bind to other purified lipids including cholesterol 3-sulfate, phospholipids, neutral glycolipids, and gangliosides. Fucoidan, a sulfated fucan, and heparin strongly inhibited binding of thrombospondin to sulfatides. Thus, the NH₂-terminal A2.5-binding domain of thrombospondin contains an important binding site for sulfated glycoconjugates. Thrombospondin-mediated tumor cell spreading is inhibited by A2.5, heparin, and fucoidan (23) suggesting that tumor cell surface glycoconjugates serve as receptors for spreading. Autocrine growth-stimulating activity of thrombospondin is also inhibited by heparin and may involve the same receptor (17). The same interaction appears to play an important role in tumor cell chemotaxis. A2.5, heparin, and fucoidan all inhibit chemotaxis but not haptotaxis (Tables III and IV). The 140- and 120-kD fragments of thrombospondin produced by thrombin, trypsin, or chymotrypsin, which lack

the heparin-binding domain, fail to induce chemotaxis. All these results are in keeping with the location of a specific site on the NH₂-terminus of thrombospondin which mediates chemotaxis. This site must be exposed on the soluble thrombospondin molecule.

In the thrombospondin chemotactic assay, binding of the soluble thrombospondin to the filter to indirectly generate a haptotactic gradient was negligible as determined by labeled thrombospondin binding to filters. Furthermore, if thrombospondin chemotaxis was, in fact, indirectly mediated haptotaxis, C6.7, A2.5, heparin, fucoidan, and protease fragments retaining or lacking the heparin-binding domain would not exhibit the markedly differential inhibition of chemotaxis vs. haptotaxis shown in Tables III and IV, and in Fig. 5.

Roberts et al. (23) and Varani et al. (31) demonstrated that thrombospondin can mediate tumor cell adhesion. Tumor cells encounter thrombospondin at many stages of tumor invasion, extravasation, and metastasis. The present data suggest that the role played by thrombospondin is more than just as an adhesion protein. Thrombospondin is present in plasma in a low concentration (20 ng/ml). However, in areas of platelet stimulation, such as in the proximity of a vessel injury, it can reach chemotactically active concentrations (>15 µg/ml) (15). Thus, thrombospondin can potentially attract circulating tumor cells toward a site of endothelial injury. Once they arrive at this site, the tumor cells can adhere to the insolubilized thrombospondin present on the endothelial cell surface or imbedded in the exposed extracellular matrix. Here the directional migration can be stimulated by a haptotactic mechanism.

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