Platelet Thrombospondin Mediates Attachment and Spreading of Human Melanoma Cells

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Abstract. Human platelet thrombospondin adsorbed on plastic promotes attachment and spreading of human G361 melanoma cells. Attachment is rapid, and spreading is maximal by 90 min with 60–90% of the attached cells spread. In contrast, thrombospondin promotes attachment but not spreading of human C32 melanoma cells, which attach and spread only on laminin substrates. The specificity of these interactions and the regions of the thrombospondin molecule involved in attachment and spreading were examined using proteolytic fragments of thrombospondin and by inhibition studies. The sulfated fucan, fucoidan, and monoclonal antibody A2.5, which is directed against the heparin-binding domain of thrombospondin, selectively inhibit spreading but only weakly inhibit attachment. Monoclonal antibodies against some other domains of thrombospondin, however, are potent inhibitors of attachment. The amino-terminal heparin-binding domain of thrombospondin does not promote attachment. Large fragments lacking the heparin-binding domain support attachment but not spreading of G361 cells. Attachment activity is lost following removal of the 18-kD carboxyl-terminal domain. These results suggest that at least two melanoma ligands are involved in cell attachment and spreading on thrombospondin. The carboxyl-terminal region and perhaps other regions of the molecule bind to receptor(s) on the melanoma surface that promote initial attachment but not cell spreading. Interaction of the heparin-binding domain with sulfated glycoconjugates on melanoma surface proteoglycans and/or sulfated glycolipids mediates spreading. Monoclonal antibodies A2.5 and C6.7 also reverse spreading of G361 cells growing on glass culture substrates, suggesting that binding to thrombospondin mediates attachment of these melanoma cells in culture.

Thrombospondin is a glycoprotein that is secreted from platelet α-granules after platelet activation (3, 12). The secreted thrombospondin binds to the surface of activated platelets (20, 32, 47) and may mediate platelet aggregation (9, 13, 26). Thrombospondin also specifically supports adhesion of erythrocytes parasitized with malaria (Plasmodium falciparum) and may mediate adherence of parasitized erythrocytes to melanoma and endothelial cells (42). As thrombospondin is secreted by several cultured cell lines including endothelial (29, 30, 38), melanoma (42), and smooth muscle cells (38), fibroblasts (22, 38), pneumocytes (43), macrophages, and monocytes (21), and is incorporated into the extracellular matrix of some of these cell lines (22, 23, 28), thrombospondin may also mediate additional cell adhesive interactions. Some human melanoma cell lines bind erythrocytes parasitized with falciparum malaria (44). Soluble thrombospondin or antibodies to thrombospondin inhibit adherence of parasitized erythrocytes to thrombospondin or melanoma cells (42), suggesting that these cells have receptors for thrombospondin or a closely related molecule (4). We have examined the interaction of the human melanoma cell lines G361 and C32 with thrombospondin and report here that thrombospondin promotes attachment of both cell lines and spreading of only G361 melanoma cells. Based on ligand and antibody inhibition and adhesive activities of thrombospondin fragments, at least two regions of the molecule are involved in attachment and spreading of G361 cells on surfaces coated with thrombospondin.

Materials and Methods

Materials

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 (17). The purified protein gave a single band (Mr, 180,000) on electrophoresis in the presence of SDS and 2-mercaptoethanol (24) and was stored at −70°C in Tris-buffered saline, pH 7.6, containing 20% sucrose and 0.1 mM CaCl2. Platelet factor 4 was purified from the heparin-bound fraction of the same platelet supernatants by elution from the heparin-agarose column with 2 M NaCl (8, 15). The platelet factor 4 was further purified by gel filtration (15) on Biogel A0.5m. Mouse laminin isolated from the Engelbreth-Holm-Swarm tumor was provided by Dr. Lance Liotta (National Cancer Institute, National Institutes of Health, Bethesda, MD). Synthetic peptides corresponding to the cell attachment site of fibronectin, vitronectin, and monoclonal antibodies 8E6 and 9G2 to vitronectin were provided by Dr. Erkki Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA). Monoclonal antibodies to thrombospondin were provided by Dr. William...
Figure 1. Adsorption of $^{125}$I-thrombospondin onto plastic. $^{125}$I-Thrombospondin (TSP) at 3–200 μg/ml in PBS/Ca was incubated for 18 h at 4°C in bacteriological plastic petri dishes. The supernatant liquid was removed, and the plastic was rinsed with Tris-BSA. Bound radioactivity was used to calculate the amount of thrombospondin adsorbed, and is presented as the mean of triplicate determinations for each concentration.

Frazier (Washington University School of Medicine, St. Louis, MO). Human von Willebrand factor was provided by Dr. Harvey Gralnick (Clinical Pathology Department, NIH). Human plasma fibronectin was obtained from Collaborative Research, Inc., Waltham, MA. Human fibrinogen and fucoidan were obtained from Sigma Chemical Co., St. Louis, MO. Heparin was from the Upjohn Co. (Kalamazoo, MI).

Cell Culture

Human melanoma cell lines C32 (ATCC CRL 1585) and G361 (ATCC CRL 1424) were maintained by monolayer culture on 75-cm$^2$ tissue culture flasks (Costar, Cambridge, MA) in RPMI 1640 containing 10% FBS (Gibco, Grand Island, NY). For attachment assays, cells were passaged at 10$^4$ cells/cm$^2$ and used between days 5 and 8.

Attachment Assays

Cells were detached by replacing the medium with PBS, pH 7.2, containing 2.5 mM EDTA and incubating for 60 min at 37°C. The cells were collected by centrifugation and resuspended immediately before use in RPMI 1640 without NaHCO$_3$ and containing 30 mM Hepes, pH 7.2.

Adhesive proteins were adsorbed onto 13-mm diameter plastic disks cut from bacteriological plastic dishes (Falcon 3007) (14) by incubation with the proteins in PBS, pH 7.2, containing 1 mM CaCl$_2$ for 16–24 h at 4°C. The supernatant was removed and the disks immersed in Tris–BSA (50 mM Tris–HCl, 110 mM NaCl, 5 mM CaCl$_2$, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1% BSA 0.02% NaN$_3$, pH 7.8) for 30 min to minimize nonspecific adhesion. The disks were washed twice with RPMI and overlaid with melanoma cells at a density of 4–6 × 10$^4$/cm$^2$, 0.5 ml/well in 24-well plates. After incubation for 90 min at 37°C, the disks were washed by dipping six times in RPMI, fixed with 1% glutaraldehyde in PBS, pH 7.2, and stained with 1.5% Giemsa. Attached and spread cells were counted microscopically.

For inhibition studies, disks coated with thrombospondin (50 μg/ml) were preincubated for 30 min at 25°C in 0.4 ml of RPMI containing the antibodies or ligands to be tested before addition of melanoma cells. Melanoma cells were added in 0.1 ml RPMI and incubated for 90 min at 37°C. The disks were fixed with 1% glutaraldehyde and stained for counting.

Thrombospondin Adsorption to Plastic

Thrombospondin was labeled with Na $^{125}$I (ICN Radiochemicals, Irvine, CA) using Iodogen (Pierce Chemical Co., Rockford, IL) as previously described (41). Immediately before use, 40 μCi of $^{125}$I-thrombospondin (10 μCi/μg) was applied to a 0.2-ml column of heparin–agarose (Sigma Chemical Co.) in 20 mM Tris-buffered saline, pH 7.6, containing 1 mM CaCl$_2$ and eluted with the same buffer containing 0.6 M NaCl. The repurified $^{125}$I-thrombospondin was diluted with unlabeled thrombospondin to final concentrations of 3 to 200 μg/ml in PBS, pH 7.2, 1 mM CaCl$_2$. Incubation on plastic disks was done as described for cell attachment assays. After washing, adsorbed thrombospondin was measured by counting the bound radioactivity.

Limited Proteolysis of Thrombospondin

Thrombospondin was digested with 4 U/ml reagent thrombin (provided by Dr. Jules Gladner, NIH), 0.05%–5% wt/wt L-1-tosylamido-2-phenylethylchloromethylketone-treated trypsin (CooperBiomedical Inc., Malvern, PA), or 0.5% wt/wt chymotrypsin (67 U/mg, CooperBiomedical) in 20 mM Tris-buffered saline, pH 7.6, containing 1 mM CaCl$_2$ and 0.02% NaN$_3$. Digestions were stopped by addition of 2 mM PMSF and the digests were fractionated by chromatography on heparin–agarose (8). The fragments were analyzed by electrophoresis in the presence of SDS and 2-mercapto-
Results

Thrombospondin Adsorption

Adsorption of thrombospondin to bacteriological plastic was quantified using $^{125}$I-thrombospondin (Fig. 1). At low concentrations, ~70% of the added thrombospondin remains bound following washing with Tris-BSA buffer. At saturation, 15 ng of thrombospondin is bound per mm$^2$ of plastic. Adsorption of thrombospondin is similar to that reported previously for fibronectin and other proteins (14, 40).

Attachment and Spreading of Melanoma Cells on Thrombospondin and Other Proteins

Surfaces coated with laminin, thrombospondin, platelet factor 4, or albumin were tested for promotion of cell attachment using C32 and G361 human melanoma cell lines. Both cell lines attach on all of the proteins except albumin (Fig. 2). The cell lines differ, however, in their spreading behavior. G361 cells spread well on thrombospondin and laminin but not on platelet factor 4 substrates. C32 cells spread on laminin substrate but remain rounded on thrombospondin or platelet factor 4 even after 2 h at 37°C. G361 cells were also tested for attachment on surfaces coated with von Willebrand factor or vitronectin (data not shown). von Willebrand factor did not promote attachment. Surfaces coated with 50 μg/ml of vitronectin promoted attachment of G361 cells at levels similar to laminin or thrombospondin. Approximately 30–40% of the cells spread on vitronectin after 90 min.

Kinetics for G361 melanoma cell attachment on substrates coated with thrombospondin or laminin are identical (Fig. 3). Maximal attachment is attained at 30 min. In three experiments, 60–80% of the added cells attached to both substrates. The attached cells, however, spread faster on laminin than on thrombospondin substrates (Fig. 3 B).

The dependence on adsorbed protein concentration for the attachment and spreading of G361 melanoma cell is presented in Fig. 4. Cell attachment on thrombospondin closely parallels the adsorption isotherm for thrombospondin in Fig. 1. Spreading is maximal at 50 μg/ml thrombospondin and falls slightly at lower concentrations.

Attachment is similar on laminin-coated substrates (Fig. 4 C) compared to thrombospondin-coated substrates (Fig. 4 A) except that lower concentrations of laminin are required for comparable attachment activity. G361 cells also spread well on laminin (Fig. 4 D). Whereas platelet factor 4 also promotes efficient attachment (Fig. 4 C), only 20% of the cells spread on this substrate at even high concentrations (Fig. 4 D). Furthermore, the cells that do spread are much less flattened than on thrombospondin or laminin. Thus attachment and spreading on thrombospondin is not due to contamination with platelet factor 4.

Cell Attachment to Thrombospondin Fragments

Thrombospondin fragments generated by limited proteolysis in the presence of calcium (6, 8, 25, 39) were tested for promotion of cell attachment and spreading on plastic to define those regions of the molecule which interact with the melanoma cells. Thrombin, trypsin, and chymotrypsin digests of thrombospondin were analyzed by SDS gel electrophoresis (Fig. 5) and adsorbed on plastic to quantify G361 melanoma cell attachment (Table I). Digestion with thrombin in the presence of calcium selectively removes the 25-kD amino-terminal heparin-binding fragment (25) to give a 140-kD fragment (Fig. 5, lane a). The heparin-binding fragment, undegraded thrombospondin, and 140-kD fragment presumably disulfide bonded to intact chains are bound when the digest is applied to the heparin–agarose column and eluted with high salt. The attachment and spreading of cells on this fraction is probably attributable to undegraded thrombospondin. The unbound fraction contains only the 140-kD fragment lacking the amino terminus (Fig. 5, lane d). This fraction supports cell attachment but only 6% of the cells spread on the 140-kD fragment. Similar results are obtained by digestion with 0.5% wt/wt chymotrypsin at 25°C which also produces primarily the 140-kD fragment (9). The unbound fraction contains 140-kD and some 120-kD fragments (Fig. 5, lane f) and supports attachment but not spreading. The bound fraction contains the 25-kD heparin-binding fragment but lacks detectable intact thrombospondin and has no attachment activity. Heparin-binding fragment prepared by trypsin digestion is also inactive. Digestion with chymotrypsin at 37°C results in the further cleavage of the 140-kD fragment to a 120-kD fragment (Fig. 5, lane e) and loss of attachment activity. Digestion with 0.5% wt/wt trypsin also produces primarily the 120-kD fragment (Fig. 5, lane b).
This preparation has some attachment activity, probably due to 140-kD fragment, but no spreading activity. Further digestion using 5% wt/wt trypsin yields 90-, 105-, and 110-kD fragments (Fig. 5, lane c) which are also inactive.

**Antibody and Ligand Inhibition of Melanoma Cell Attachment**

Four monoclonal antibodies to thrombospondin were examined for their effects on melanoma attachment to thrombospondin (Table II). Antibody C6.7 is a potent inhibitor of attachment, even at the lowest concentrations tested. This antibody is also a potent inhibitor of thrombin-induced platelet aggregation (9) and binds to the 18-kD carboxyl-terminal region which is present on the 140-kD but not the 120-kD fragment of thrombospondin (9, 11). Antibody A6.1, which binds to the protease-resistant core of thrombospondin (6, 11), also inhibits melanoma cell attachment, but higher concentrations are required. Antibody A2.5, which binds to

**Table I. G361 Melanoma Attachment to Thrombospondin Fragments**

| Proteolytic digestion          | Major* unbound fragment | Not bound by heparin | Heparin bound |
|-------------------------------|-------------------------|----------------------|--------------|
|                               | KD                      | Attached†            | Spread       | Attached‡ | Spread  |
| Trypsin 0.05% wt/wt 37°C, 60 min | 180                     | 0                    | –            | 67 ± 31    | 73 ± 9   |
| Trypsin 0.5% wt/wt 37°C, 60 min | 120, (140)              | 35 ± 25             | 3 ± 2        | 0          | –        |
| Trypsin 5% wt/wt 37°C, 60 min  | 90, (105,110)           | 4 ± 2               | 0            | 0          | –        |
| Thrombin 4 U/ml 37°C, 16 h     | 140                     | 88 ± 27             | 6 ± 2        | 77 ± 24    | 49 ± 15  |
| Chymotrypsin 0.5% wt/wt 25°C, 30 min | 140, (120)          | 80 ± 13             | 5 ± 2        | 1 ± 1      | 0        |
| Chymotrypsin 0.5% wt/wt 37°C, 60 min | 120                 | 3 ± 2               | 0            | 0          | –        |

* Sizes of minor fragments are indicated in parenthesis.
†Control: 182 ± 12 cells attached/mm², 78 ± 1% spread.
Table II. Inhibition of G361 Melanoma Cell Attachment to Thrombospondin by Monoclonal Antibodies

| Antibody | Concentration µg/ml | Attached cells/mm² | % of control | Spread % of control |
|----------|---------------------|---------------------|--------------|--------------------|
| Control 1 |                     |                     |              |                    |
| A2.5     | 50                  | 41                  | 98 ± 19      | 102 ± 17           |
|          | 20                  | 55                  | 110 ± 14     | 111 ± 18           |
|          | 10                  | 110                 | 111 ± 11     | 113 ± 21           |
|          | 5                   | 111                 | 113 ± 11     | 115 ± 21           |
|          | 2.5                 | 183                 | 115 ± 11     | 117 ± 21           |
| A6.1     | 50                  | 1                   | 100 ± 18     | 113 ± 27           |
|          | 20                  | 17                  | 110 ± 18     | 112 ± 27           |
|          | 10                  | 167                 | 112 ± 18     | 114 ± 27           |
|          | 5                   | 175                 | 114 ± 18     | 116 ± 27           |
|          | 2.5                 | 203                 | 116 ± 18     | 118 ± 27           |
| D4.6     | 50                  | 186                 | 118 ± 18     | 120 ± 27           |
| Control II |                   |                     |              |                    |
| C6.7     | 50                  | 1                   | 100 ± 18     | 113 ± 27           |
|          | 20                  | 6                   | 110 ± 18     | 112 ± 27           |
|          | 10                  | 51                  | 112 ± 18     | 114 ± 27           |
|          | 5                   | 86                  | 114 ± 18     | 116 ± 27           |
|          | 2                   | 90                  | 116 ± 18     | 118 ± 27           |

Two monoclonal antibodies to vitronectin were also examined as inhibitors of G361 cell attachment and spreading on thrombospondin and vitronectin. Antibody 8E6 (1:50 dilution of ascites) completely inhibited G361 cell attachment to vitronectin (50 µg/ml) but had no effect on attachment or spreading on thrombospondin (50 µg/ml). Attachment and spreading on thrombospondin were 80% and 86% of control levels, respectively. Antibody 9G2 did not inhibit attachment or spreading on either substrate.

Monoclonal antibody OKM5 binds to C32 but not G361 melanoma cells and inhibits adherence of erythrocytes parasitized with falciparum malaria to C32 cells (4). Antibody OKM5 at 10 µg/ml does not inhibit attachment or spreading of either melanoma cell line on thrombospondin (Table III).

Several ligands of thrombospondin were also examined as inhibitors (Table III). Fibrinogen binds to a site near the carboxyl terminus of thrombospondin (7). Fibrinogen at 1 mg/ml has no effect on attachment or spreading. Fucoidan and heparin are potent inhibitors of thrombospondin binding to sulfatides and erythrocyte agglutination (41). Fucoidan and

Table III. Inhibition of G361 Melanoma Cell Attachment to Thrombospondin

| Inhibitor          | Concentration µg/ml | Attachment % of control | Spreading % of control |
|--------------------|---------------------|-------------------------|------------------------|
| Fibrinogen         | 0.1 mg/ml           | 98 ± 19                 | 102 ± 17               |
| Fibrinogen         | 1 mg/ml             | 110 ± 26                | 111 ± 18               |
| Gly-Arg-Gly-Asp-Pro | 0.68 mM            | 105 ± 14                | 94 ± 12                |
| Gly-Arg-Gly-Glu-Pro | 0.83 mM            | 107 ± 15                | 105 ± 19               |
| Fucoidan           | 10 µg/ml            | 107 ± 32                | 47 ± 21                |
| Fucoidan           | 100 µg/ml           | 41 ± 18                 | 31 ± 18                |
| Fucoidan           | 200 µg/ml           | 65 ± 7                  | 22 ± 2                 |
| Heparin            | 10 µg/ml            | 101 ± 4                 | 76 ± 10                |
| Heparin            | 100 µg/ml           | 94 ± 17                 | 63 ± 16                |
| Antibody OKM5      | 10 µg/ml            | 100 ± 18                | 113 ± 27               |

* Mean ± SD, n = 4, with n = 8 for control binding in the absence of inhibitor.

the amino-terminal heparin-binding fragment (10, 11), only weakly inhibits attachment. Spreading, however, is inhibited 70% at 2.5 µg/ml and completely inhibited at 50 µg/ml. This result is consistent with the observation that spreading activity is lost after proteolytic removal of the heparin-binding fragment from thrombospondin. Antibody D4.6 (6) has no effect on attachment or spreading at 50 µg/ml.

In control experiments, the specificity of the antibody effects were examined using laminin or platelet factor 4 substrates. Neither A2.5 or C6.7 at 50 µg/ml inhibit attachment of G361 cells to platelet factor 4 (20 µg/ml), and C6.7 did not inhibit attachment or spreading of G361 cells on vitronectin (50 µg/ml, data not shown). The same antibodies at 50 µg/ml also do not inhibit attachment to laminin (20 µg/ml), although the cells spread on laminin extended fewer processes in the presence of both antibodies (results not shown).
Figure 6. Reversal of G361 melanoma cell spreading by monoclonal antibodies to thrombospondin. G361 melanoma cells were plated in eight chamber Lab-Tek glass tissue culture slides at $1 \times 10^4$ cells per well in RPMI medium containing 10% FCS and grown for 48 h at 37°C. The medium was removed and replaced with RPMI (a and f) or RPMI containing 10 μg/ml of monoclonal antibodies to thrombospondin: A2.5 (b), A6.1 (c), C6.7 (d), or D4.6 (e). The cells were incubated at 37°C for 90 min and fixed with 1% glutaraldehyde. Photomicrographs were taken using Hoffman modulation contrast optics. Bar, 70 μm.

heparin also selectively inhibit spreading of G361 cells on thrombospondin. Inhibition of spreading by heparin is less complete than by fucoidan. Peptides comprising the cell attachment site of fibronectin (33) were also examined as inhibitors (Table III). Neither the active sequence Gly-Arg-Gly-Asp-Ser-Pro or the inactive analog Gly-Arg-Gly-Glu-Ser-Pro at 0.7 to 0.8 mM had any effect on attachment or spreading on thrombospondin substrates.

**Reversal of Spreading**

The potential role in substrate attachment of thrombospondin either derived from serum in the growth medium or secreted by growing melanoma cells was examined by addition of the monoclonal antibodies to live G361 melanoma cells on glass slides (Fig. 6). Antibodies A2.5 and C6.7 (10 μg/ml) both caused retraction of cell processes. The rounded cells remained attached to substrate. Antibody C6.7 induced retraction equally in both sparse and confluent cultures, whereas the effect of A2.5 was greater in sparse cultures. Antibodies A6.1 and D4.6 had no effect on G361 melanoma cell cultures. The cell attachment peptide of fibronectin, Gly-Arg-Gly-Asp-Ser-Pro, causes similar rounding and detachment of several cell lines from culture substrates (18). This peptide at 0.5 mM but not the analog Gly-Arg-Gly-Glu-Ser-Pro also
cause G361 melanoma cells to round up on the glass substrate (results not shown). Some cells were detached after incubation with the peptide.

Discussion

Although thrombospondin is secreted by many cell lines in culture and is localized by antibodies (22, 23) and by uptake of radiolabeled protein (28) in the extracellular matrix, evidence for its function in cell adhesion is limited to aggregation of thrombin- or ionophore-activated platelets (9, 13, 26) and adhesion of erythrocytes parasitized with falciparum malaria (42). The present results demonstrate that thrombospondin also mediates attachment and spreading of some human melanoma cell lines. Attachment and spreading of G361 melanoma cells on thrombospondin is similar to that obtained with the well-characterized cell adhesion protein, laminin (27). Attachment to both proteins is rapid and is followed by extensive spreading on the protein substrates (Fig. 3). Although C32 and G361 melanoma cells (Fig. 1) and some other cell lines (unpublished results) attach to thrombospondin, of those tested only the G361 melanoma cells spread on thrombospondin.

The activity of thrombospondin is not due to contamination with other proteins. Several adhesive proteins are in platelet supernatants including fibronectin, platelet factor 4, and von Willebrand factor. von Willebrand factor does not promote attachment of G361 cells. During purification of thrombospondin, fibronectin is removed by chromatography on gelatin–agarose and platelet factor 4 by gel filtration. G361 melanoma cells spread on thrombospondin but not on platelet factor 4 substrates. Fibronectin peptides do not inhibit adhesion to thrombospondin (Table III) but do inhibit cell attachment on fibronectin (33) or vitronectin (18) and inhibit adhesion to thrombospondin (Table III) but do inhibit cell attachment on fibronectin (33) or vitronectin (18) and binding of fibronectin, fibrinogen, and von Willebrand factor to platelets (16, 35, 37). Finally, monoclonal antibodies to thrombospondin specifically block attachment and spreading on thrombospondin but not on other proteins, whereas an antibody to vitronectin blocks attachment to vitronectin but not to thrombospondin.

At least two regions of thrombospondin interact with G361 melanoma cells. A site present in the 140-kD but not in the 120-kD proteolytic fragments of thrombospondin is required for attachment. This region, which is near the carboxyl terminus (9) and is contained in the large globular domain seen by electron microscopy (11), is also implicated in binding to fibrinogen and thrombin-activated platelets. Antibody C6.7, which binds to this region and inhibits platelet aggregation (9), strongly inhibits attachment of G361 cells to thrombospondin (Table II). However, fibrinogen has no effect (Table III). The second important region is the amino-terminal heparin-binding site. This site is not required for attachment, as the 140-kD fragment lacks the heparin-binding site but strongly promotes attachment. Partial inhibition of attachment by high concentrations of antibody A2.5 could be steric or result from a change in the conformation of thrombospondin induced by this antibody (II). Proteolytic fragments lacking the heparin-binding domain do not promote spreading of G361 cells. Spreading is also inhibited by fucoidan and antibody A2.5 which binds to this fragment. A third region may also interact with melanoma cells since antibody A6.1 inhibits attachment. This antibody binds to the core region of thrombospondin (6) which contains a binding site for type V collagen (31). Fragments containing this region such as the 120-kD fragment, however, do not support attachment. Interaction with this region may be weak or inhibition by A6.1 could be due to steric effects.

Multiple interactions between adhesive proteins and cells may be a general phenomenon. The attachment and spreading activities of laminin can be separated using proteolytic fragments (45). Multiple sites may also be involved in interaction of fibronectin with cells (1, 5).

Inhibition of thrombospondin-mediated melanoma cell attachment and platelet aggregation by antibody C6.7 suggests that a common receptor for thrombospondin is on the two cell types. Based on antibody inhibition, fibrinogen, fibrinectin, von Willebrand factor, and thrombospondin may bind to the glycoprotein IIb/IIIa complex on activated platelets (34, 47). Thrombospondin may also bind to a second class of receptor on resting platelets (47). Binding of fibrinogen, fibrinectin, and von Willebrand factor to platelets is inhibited by Arg-Gly-Asp-containing peptides (16, 35), and glycoprotein IIb/IIIa incorporated in liposomes binds to this sequence (37). However, this receptor in liposomes does not bind to thrombospondin (37), and thrombospondin binds to thrombusthetic platelets lacking glycoprotein IIb/IIIa (36).

The present results demonstrate that attachment to thrombospondin mediated by the receptor on G361 melanoma cells is not inhibited by an Arg-Gly-Asp-containing peptide. As parasitized erythrocytes adhere to C32 but not to G361 melanoma cells (44), thrombospondin may have different receptors on the two cell lines. The antigen defined by antibody OKM5 may be a receptor for thrombospondin-mediated parasite adhesion on C32 cells (4) and for thrombospondin binding to C32 cells (2). However, OKM5 does not inhibit attachment or spreading of C32 or G361 cells (Table III and our unpublished results). Thus, additional receptors for thrombospondin are probably present on both cell lines. Further work is required to identify these receptors and to determine their relationship to the platelet receptor.

Sulfated glycoconjugates are a second class of receptors on melanoma cells for thrombospondin. The isolated heparin-binding domain does not support attachment but is required for spreading of G361 cells (Table I). As platelet factor 4 does not promote spreading of G361 cells, however, binding to heparin is not sufficient for promoting spreading. Thrombospondin binds both to sulfated polysaccharides (e.g., heparin, [8]) and to sulfated glycolipids (41). The relative importance of sulfated glycolipids and heparan sulfate proteoglycans in mediating spreading remains to be determined. Different distributions of sulfated glycolipids and proteoglycans in C32 and G361 cells may account for their different spreading responses to thrombospondin.

Because some antibodies to thrombospondin disrupt spreading of G361 melanoma cells growing on glass substrates, thrombospondin derived from serum in the medium or synthesized by these cells (42; Sherwood, J. A., D. D. Roberts, S. L. Spitalnik, K. Marsh, E. B. Harvey, J. W. Lawler, L. H. Miller, and R. J. Howard, manuscript in preparation) may function in substrate attachment during growth in culture. Deposition of labeled thrombospondin in extracellular matrix (28) and localization in endothelial cell matrices (19, 23) support this hypothesis. It is unlikely that the reversal of spreading is an indirect effect of antibody binding to thrombospondin on the apical surface of the cells because not all antibodies to thrombospondin disrupt spreading. It is more
likely that the antibodies directly inhibit interactions between thrombospondin in the extracellular matrix and melanoma surface receptors. As the fibronectin peptide Gly-Arg-Gly-Asp-Ser-Pro specifically induces rounding of G361 cells on glass but does not inhibit attachment or spreading on thrombospondin, other serum proteins including fibronectin and vitronectin probably contribute to spreading (18). The finding that antibodies to thrombospondin round up but do not detach G361 cells may be due to residual interaction or the melanoma cells with fibronectin and vitronectin on the glass surface.

Malignant melanoma can metastasize locally through epidermal basement membrane or distantly through blood or lymphatic vessels. Melanoma cell interaction with adhesion molecules may play a role in metastasis (27). Because thrombospondin is associated with endothelium in vivo (46), adhesion of melanoma cells to thrombospondin in subendothelial matrix may be important for passage of melanoma cells into circulation. If thrombospondin is on the luminal surface of endothelial cells, binding to thrombospondin could be an early event in metastatic arrest.

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