Thrombospondin Functions as a Cytoadhesion Molecule for Human Hematopoietic Progenitor Cells

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We explored the role that thrombospondin (TSP), a multifunctional extracellular matrix protein, plays in hematopoietic cell-cell and cell-matrix interactions. Thrombospondin synthesis is differentially regulated in human long-term bone marrow cultures. Consistent with this, human hematopoietic progenitor cells of all three lineages (erythrocyte, megakaryocyte, and granulocyte) use TSP as an attachment protein. However, terminally differentiated cells (erythrocytes and neutrophils) show absent or reduced attachment to TSP. The region within the TSP molecule that mediates cell attachment (cell binding domain) was delineated by examining both attachment to proteolytic fragments of TSP and by inhibition of cytoadhesion using monoclonal antibodies directed against TSP domains. The cell binding domain resides toward the C-terminus of a 140 Kd chymotryptic fragment of TSP. We conclude that thrombospondin functions as a hematopoietic cytoadhesion molecule, capable of binding primary hematopoietic progenitor cells, and may, therefore, be important in blood cell development.

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METHODS

Cell attachment assay. Proteins (unless otherwise stated, 20 μg/mL) in serum-free RPMI media were immobilized onto tissue culture plastic by incubation at 37°C for 1 hour. Subsequently, nonspecific plastic binding sites were blocked by incubating (30 minutes at 37°C) with 10 μg/mL bovine serum albumin (BSA) in RPMI 1640 (GIBCO, Grand Island, NY). The immobilized protein was washed twice with fresh RPMI and incubated with washed cells (5 x 10^5/mL suspended in RPMI containing 1% BSA) for 15 minutes at 37°C. Suspension (ie, nonadherent) cells were removed by aspiration, dishes were washed twice with RPMI, adherent cells were removed with the aid of a rubber policeman, and TSP-bound cells were quantitated by hemocytometer. For each of three replicate cultures, two separate hemocytometer counts were done and averaged. HEL cells were cultivated as described elsewhere.10,11

Human bone marrow cell aspirates (≤1.0 mL to prevent peripheral dilution) were obtained after receiving informed consent and were density-separated on Ficoll-Hypaque, and adherent cells were depleted by two rounds of plastic adherence.10,11 Importantly, these nonadherent, low density (NALD) cells do not contain megakaryocytes or platelets. The former are low density in Ficoll and thus not recovered in the mononuclear cell density band. Both residual megakaryocytes and platelets are removed by the subsequent two rounds of plastic adherence. Subsequently, these NALD cells were used in attachment assays as described above.

Hematopoietic progenitor cell attachment is determined as follows. Marrow NALD cells are cultured before (input) and after the cell attachment assay (above), as three to five replicate cultures, under optimal conditions for colony growth.11 For each colony cell...
type (ie, each lineage), replicate colony numbers are averaged. The percent attachment for each colony cell type, for each individual is calculated as the total number of adherent progenitor cells divided by the total number of input progenitor cells times 100. The results are expressed as the mean percent attachment ± 95% confidence interval, based on each individual's percent attachment averaged over the indicated number of experiments (n). Such calculations allow specific attachment to be determined irrespective of the known and large donor-donor variations in progenitor cell number.

**Purification of TSP proteolytic fragments.** Human TSP was purified from human platelets by a combination of heparin-Sepharose and gel filtration chromatography, as described previously. Proteolytic fragments were purified as follows: The 140 Kd fragment (Fig 1) was generated by subjecting TSP to digestion with 0.1% (wt/wt) chymotrypsin (60 U/mg; Sigma Chemical Co, St Louis, MO) in 20 mmol/L Tris, pH 7.6, 0.15 mol/L NaCl and 1 mmol/L CaCl₂, for 20 minutes at 37°C. The digestion was stopped by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 1 mmol/L. The resulting digest was fractionated by heparin-Sepharose chromatography. The nonheparin-binding 140 Kd fragment was present in the column runthrough. The 25 Kd heparin binding fragment was eluted with high salt-containing buffer (20 mmol/L Tris, pH 7.6, 0.6 mol/L NaCl). The 120 Kd/18 Kd fragment was formed by letting the chymotrypsin digestion proceed longer (40 minutes) using the same enzyme:substrate ratio, temperature, and buffer as described above for the generation of the 140 Kd fragment. The nonheparin-binding 120 Kd/18 Kd fragment was similarly resolved from the 25 Kd heparin-binding fragment by heparin-Sepharose chromatography (proteolytic fragment resolution on SDS-PAGE is shown in Fig 7, see below). As with intact TSP, 20 µg/ml of proteolytic TSP fragments were immobilized onto tissue culture plastic, and cell attachment was done as described above. To quantitatively assess the binding of the various TSP fragments to tissue culture plastic, we used radioiodinated fragments of known specific activity. Both the intact 140 Kd and 120/18 Kd fragments bound in equimolar amounts. The isolated 25 Kd heparin-binding domain bound in 20-fold molar excess, probably reflecting the hydrophobic character of this domain.

**Anti-thrombospondin monoclonal antibodies.** Murine monoclonal anti-TSP antibodies were prepared and purified by protein A affinity chromatography as described previously. These antibodies and their TSP binding domains are as follows: C6.7, which reacts with the 18 Kd fragment; A6.1, A4.1, and D4.6, which react with the region proximal to the carboxy terminus of the 120 Kd fragment; and A2.5, which reacts with the 25 Kd heparin-binding domain. These antibodies are specific for their stated epitopes and do not crossreact with other matrix proteins such as fibronectin.

**Metabolic labeling.** Human erythroleukemia cells (1 x 10⁶) were metabolically labeled with ³⁵S methionine (100 µCi per dish for 6 hours) in methionine-free minimal essential medium (GIBCO) containing 10% dialyzed fetal calf serum, which had been depleted of exogenous TSP by affinity chromatography on an anti-TSP monoclonal antibody (MoAb) column. After the labeling period, the media were aspirated off the cell layer, and both detergents (1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) and protease inhibitor (1 mmol/L PMSF) were added to give the indicated final concentrations. Immunoreactive TSP was precipitated with a 1:100 dilution of polyclonal rabbit anti-TSP antisera and protein A sepharose as described by Varani et al. Pre-immune rabbit serum served as control antibody. The washed immunoprecipitates were eluted by boiling in Laemmli SDS-PAGE sample buffer in the presence of 2% 2-mercaptoethanol and fractionated on a 5% Laemmli polyacrylamide gel. Radioactive bands were visualized by fluorography with En'Hance (NEN Research Products, Boston, MA) and subsequent autoradiography. Human marrow NALD cells were labeled identically, except that the cells were cultivated serum-free with 1% BSA replacing the 10% dialyzed fetal calf serum.

![Diagram of TSP subunit structure showing the location of functional domains and antibody binding sites in relation to cell adhesion.](image)

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Hemopoietic progenitor cell assays. Human hemopoietic progenitor cell assays were done using accessory cell-depleted marrow as target cells and optimal concentrations of hemopoietic growth factors. For progenitor cell attachment to TSP, human marrow NALD cells are attached and removed as described above, and cultured to determine the actual frequency of TSP-bound progenitors. Granulocyte and megakaryocyte progenitor cell cultures are cultivated as described by Long et al. Erythroid and mixedcellularity progenitors were grown as described by Lim et al. Progenitor cells are cultivated for 14 days at 37°C, 100% humidity, 7% CO₂ in room air, in the presence of hemopoietic growth factors at limiting cell dilutions of 50,000 cells/mL.

Immunocytochemistry. Bone marrow adherent stromal cell populations were grown in long-term bone marrow cultures for 1 to 4 weeks according to the procedure of Gartner and Kaplan. As described previously, cell-laden glass coverslips were methanol fixed, the endogenous peroxidase activity blocked with 0.03% H₂O₂, and Fc receptors blocked with 3% goat sera. Next, the cells were incubated with polyclonal anti-TSP IgG and antibody-localized using a biotinylated anti-IgG avidin-peroxidase procedure. Controls done each week of culture were as follows: coverslips not exposed to anti-TSP (ie, secondary antibody control), to determine nonspecific binding; and cells exposed to blocking concentrations of H₂O₂, to test for residual endogenous peroxidase activity.

RESULTS

Thrombospondin localization in human long-term bone marrow cultures. In order to examine TSP expression by human bone marrow stromal cells, adherent cell populations were grown in long-term bone marrow cultures for 1 to 4 weeks. Many cultivated marrow cells show intracellular TSP (Fig 2), with megakaryocytes having the highest intensity (++)+, mononuclear cells intermediate in reactivity (3-4+), fibroblast-like cells staining weakly (1+-/trace), and macrophage-like cells negative. Developmentally, long-term bone marrow cultures first evolve hemopoietic foci that consist of hemopoietic cells intimately attached to underlying (plastic-adherent) stromal cells. During the first week of culture, these foci show intermediate (1+/3+) TSP reactivity in the attached hemopoietic cells and little to no reactivity in the stromal cells (Fig 2A). At week 2, the stromal cells are more confluent, and the attached hemopoietic cells are strongly (3-4+) positive (Fig 2B). By week 4, TSP-positive strands of extracellular matrix are observed that are closely associated with the developing hemopoietic cells (Fig 2C). At this time, fibroblast-like cells (which were negative early in culture) are strongly positive (data not shown). No peroxidase activity is seen in secondary antibody controls (ie, in the absence of primary antibody; Fig 2D) or in cells in which endogenous activity is blocked by MeOH/H₂O₂ (data not shown).

Cytoadhesin properties of thrombospondin. To assess the cell attachment capacity to TSP, an in vitro assay was developed using plastic-immobilized proteins (see Methods). Thrombospondin was observed to function as a cytoadhesion molecule for human erythroleukemia (HEL) cells, a leukemic cell line expressing erythroid, macrophage, and megakaryocytic characteristics. Cellular attachment studies indicate that a significant proportion of leukemic HEL cells (34% ± 10%) attach to TSP-coated dishes, as compared with low attachment (6.0% ± 4%) to BSA-coated control dishes (P < 0.05 by Student’s t test; n = 36) (Fig 3). The extent of adherence reaches a plateau at 10 μg/dish (Fig 4), and equilibrium binding occurs within a 15-minute time period (Fig 5). Human erythroleukemia cells do not attach to laminin, an extracellular matrix protein that does not show cytoadhesive properties for hemopoietic cells. Erythroleukemia cells also bind to fibronectin (total binding approximately 40%; laminin and fibronectin data not shown), which is consistent with previous observations indicating that erythroid cells bind to this molecule.

In order to determine if the TSP attachment characteristic of HEL cells is a function of leukemic transformation, we examined TSP attachment of both human bone marrow cells and the promegakaryocytic cell line, HL60. HL60 cells did not attach to varying concentrations of TSP (data not shown). In contrast, nonadherent (to plastic) bone marrow cells attached to TSP, showing a fivefold (5.2 ± 2, n = 4) greater attachment than the control protein (BSA). Both of these observations suggested that TSP attachment is not due to leukemic transformation per se.

We further explored the role of TSP as a hemopoietic cytoadhesion molecule by examining the attachment of committed hemopoietic progenitor cells to this molecule. Hemopoietic progenitor cells of each lineage (granulocytic, megakaryocytic, and erythroidic) specifically attach to TSP (Fig 6). Importantly, pluripotent progenitor cells (CFU-GEMM) adhere to TSP. Control protein (BSA-coated) cultures show only low numbers (if any) of granulocyte progenitor cells (Fig 6) consistently attaching to this immobilized protein, whereas other progenitor cell phenotypes (data not shown) fail to attach to BSA. Finally, circulating human erythrocytes fail to attach to TSP (<0.1%), whereas a low percentage of circulating neutrophils retain this capacity (14.4% and 7.1% neutrophil binding to TSP and BSA, respectively).

Identification of the thrombospondin cellular binding domain. Identification of the hemopoietic cell binding domain of TSP was done with two complementary approaches: attachment to proteolytic fragments of TSP and inhibition of cellular attachment by TSP-specific MoAbs. Proteolytic fragments of TSP (as shown in Fig 1) were prepared by chymotrypsin digestion and heparin-Sepharose affinity chromatography. Digestion of intact TSP by chymotrypsin for 30 minutes results in the generation of a 25 Kd heparin-binding domain and a large 140 Kd fragment. Upon further exposure to chymotrypsin, the 140 Kd fragment is cleaved to a 120 Kd and an 18 Kd fragment. However, the 18 Kd fragment remains in disulfide linkage to the 120 Kd species.

The purified 140 Kd fragment contains small amounts of the 120 Kd fragment (Fig 7 A, lane 2), and the purified 120 Kd fragment contains residual (undigested) 140 Kd fragment (Fig 7 A, lane 3). These minor residual proteolytic fragments are not of significance in cellular attachment as complete cell attachment is observed only when the 140 Kd fragment is the predominant species (Fig 7 C). When the combined 120/18 Kd fragment is the predominant species, cells fail to bind the immobilized peptide (Fig 7 C). HEL cells...
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Fig 3. Cellular attachment to TSP. HEL cells were allowed to attach to plastic tissue culture dishes coated with the indicated concentrations of (plastic-immobilized) TSP or BSA. For each experiment, all conditions are cultured as three replicates per condition. Values are expressed as mean ± SD of the mean replicate culture values averaged over the indicated number (n) of experiments. Immobilized protein indicates HEL cell binding to intact TSP immobilized on tissue culture plastic (n = 36); monoclonal antibodies, HEL cell attachment to immobilized TSP in the presence of monoclonal murine anti-TSP antibodies. Attachment of TSP-nonadherent cells in secondary attachment assays shows no further attachment on TSP-coated dishes (data not shown). The antibodies (50 μg/mL) used and their specificities are described in Methods. lnapp, inappropriate antibody (murine IgG; 50 μg/mL).

also fail to bind to the 25 Kd heparin-binding domain (Fig 7C). Consistent with this observation, cell attachment is not inhibited by heparin (data not shown). The 140 Kd fragment contains an Arg-Gly-Asp-Ser cell recognition sequence.26 However, this particular sequence is not involved in the attachment of hematopoietic progenitor cells as a vast molar excess (1 mmol/L) of the synthetic peptide Arg-Gly-Asp-Ser fails to inhibit attachment (data not shown).

Inhibition of cellular attachment by monoclonal anti-TSP antibodies not only confirms the conclusions drawn from the work with proteolytic fragments, but also serves to establish the specificity of cell attachment to TSP. Treatment with MoAbs that bind proximal to the carboxy-terminal 18 Kd fragment (A 4.1, A 6.1, and D 4.6); anti-TSP antibody specificity shown in Fig 1) completely abrogates the attachment of HEL cells to TSP (Fig 3). However, attachment is unaffected by the presence of MoAbs (A 2.5) that bind the 25 Kd heparin-binding domain,18 the 18 Kd (C 6.7) platelet-binding fragment,19 or inappropriate antibody controls (Fig 3).

Unfortunately, purification of primary hematopoietic progenitor cells in quantities sufficient for biochemical determinations is not technically feasible. However, radioimmunoassays (RIAs) of whole marrow (NALD) cell lysates are possible. Thrombospondin content was determined by RIA as described elsewhere.7 Marrow NALD cells were washed three times in PBS and cultivated for 2 days in the presence of TSP-depleted fetal calf serum. Thrombospondin depletion was done using an affinity column containing covalently coupled MoAb directed against TSP. Determination of TSP content in whole cell lysates of marrow NALD cells shows that large amounts of TSP (1,500 μg/105 cells) are produced. These RIA data were confirmed by metabolic labeling and immunoprecipitation, done as described previously,7 of NALD human marrow cells. Metabolic labeling studies show that significant amounts of TSP are synthesized by this nonadherent population of marrow cells, as well as by HEL cells (Fig 7D). Moreover, metabolically labeled TSP is detectable in bone marrow (ie, NALD cell) conditioned media, showing that TSP is also secreted by this cell population (data not shown).

FIGURE 3.ellular attachment to varying concentrations of TSP.

DISCUSSION

We conclude that thrombospondin, a well-characterized extracellular matrix (ECM) protein is synthesized by bone marrow cells and deposited within the ECM, and it functions within the hematopoietic microenvironment as a cytoadhesion protein for hematopoietic cells. Both pluripotent and committed progenitor cells bind to this molecule. The ability to use TSP as an attachment molecule is lost in mature erythroid cells but is retained in a proportion of mature neutrophils, although the variability in neutrophil binding makes the degree of reduction difficult to interpret. Meta-

Fig 2. Immunolocalization of TSP in human long-term bone marrow stromal cell populations. Long-term marrow cultures were established as described in Methods. At weekly intervals, cell-laden coverslips were removed, and TSP was localized by immunoperoxidase labeling.16-17 (A) Hematopoietic foci at 1 week of culture. Underlying stromal cells stain weakly for TSP, whereas attached hematopoietic cells show intense staining. (B) At 2 weeks, cultures show further development of hematopoietic foci with many TSP-positive cells. (C) At 4 weeks of culture, TSP-positive strands of ECM are observed surrounded by developing hematopoietic cells. (D) Hematopoietic foci at 2 weeks in which endogenous peroxidase and Fc receptor binding are blocked (see Methods) and then stained in the absence of primary antibody. Original magnification in A, B, and D is 40×; C, 25×.
Hematopoietic cells synthesize and release TSP, suggesting that perhaps these cells synthesize and attach to their own cytoadhesion molecule. Importantly, differential thrombospondin attachment is seen in leukemic cell lines. Human erythroleukemia cells attach in an equivalent fashion to normal bone marrow progenitor cells, whereas progranulocytic leukemia cells (HL60) fail to adhere to this molecule, suggesting that the characteristic attachment of HEL cells to TSP is not due to leukemic transformation per se.

The data indicating that thrombospondin functions as a cytoadhesion molecule for human hematopoietic cells shows the specific attachment of primary human pluripotent progenitor cells (CFC-GEMM) to this molecule, as well as progenitor cells committed to the erythroid, granulocytic, and megakaryocytic lineages. Furthermore, granulocytes and erythrocytes reduce or lose (respectively) the capacity to use thrombospondin as an attachment protein. Similar observations exist concerning the ability of fibronectin to function as a cytoadhesion protein. Recently, Tsai et al reported the adherence of early erythroid precursors to this protein. Coulombel et al recently demonstrated the attachment of human progenitor cells to narrow fibroblast-derived ECM. Their observations of reduced erythroid progenitor cell attachment to purified fibronectin and only partial inhibition of attachment with anti-fibronectin led to the conclusion that another matrix molecule is involved in erythroid progenitor...
cell attachment. Confirming this suggestion, we show that human erythroid progenitor cells attach to thrombospondin.

Attachment studies using TSP proteolytic fragments indicate that the 140 Kd proteolytic fragment contains the intact cell binding domain, whereas the 120/18 Kd disulfide-linked fragment fails to support cell adhesion. Since both the 140 Kd and 120 Kd fragments share a common amino terminal sequence, it follows that proteolysis must occur at the carboxy terminus of the 140 Kd fragment, resulting in both the formation of the disulfide linked 120/18 Kd fragment and loss of the ability to support cell adhesion. Consistent with this observation, antibodies that bind close to the site of proteolytic cleavage (ie, the site that results in generation of the 120/18 Kd fragment; Fig 1, asterisk) are capable of inhibiting cell attachment to TSP. Mechanistically, the anti-TSP antibodies specifically bind to an area of the TSP molecule that would span at least 65 to 70 Kd, thus sterically inhibiting the binding of cells in an area that is much larger than the actual hematopoietic cell binding site. However, the proteolytic cleavage data further localizes the binding region to a region spanning approximately 20 to 40 amino acids just adjacent to the carboxy terminus. Whether this cleavage site represents the actual location of the cell-binding domain is presently being investigated.

Thrombospondin contains an RGDS sequence that resides within the 140 Kd fragment, near the carboxy terminus. However, excess concentrations of a tetrapeptide containing this sequence fail to abrogate cell attachment. Similar results are seen in other cell systems, indicating that this sequence does not play a role in cellular attachment for this molecule. In addition to hematopoietic cell attachment, the 140 Kd fragment also supports the attachment and spreading of squamous carcinoma cells and keratinocytes. In contrast, the 140 Kd fragment supports only the attachment of melanoma cells to TSP; spreading requires the additional presence of the 25 Kd heparin-binding domain. Thus, cellular responsiveness to TSP binding is highly dependent on cell phenotype.

The functional role of cytoadhesion molecules in the hematopoietic microenvironment is not well understood. To date, hematopoietic cells are known to specifically attach to three cytoadhesion proteins. Fibronectin, a ubiquitous ECM protein, mediates the attachment of many hematopoietic cell lines. The capacity to attach to fibronectin is developmentally regulated during erythropoiesis and also occurs in human erythroid progenitor cells, and attachment is required for murine erythroleukemia cell differentiation. Recently, a novel cytoadhesion molecule, hemonectin, was described. Hemonectin is restricted to the bone marrow in its organ distribution and shows a developmentally regulated, lineage-specific attachment of granulocytic cells. Finally, thrombospondin must be added to this category of hematopoietic cytoadhesion molecules and is the first molecule shown to attach pluripotent hematopoietic progenitor cells, as well as committed progenitor cells of each lineage.

Given the ubiquitous distribution of both fibronectin and thrombospondin, the question arises as to how specificity of either progenitor cell attachment or stem cell homing occurs. For cellular attachment, specificity may be regulated either at the level of receptor expression or receptor avidity. Stem cell homing, however, requires a highly specific recognition system. A number of recent studies demonstrate that lymphocyte homing involves specific interactions with vascular endothelial cell molecules (addressins) that import positional information to recirculating lymphocytes (reviewed in reference 39). The receptor for these addressins appears to be a lectin that recognizes phosphomannose. Less is understood concerning hematopoietic stem cell trafficking (homing). Interestingly, work by Aizawa and Tavassoli demonstrates that the homing receptor is a mannose/glucosamine specific lectin receptor. Unfortunately, hematopoietic addressins remain unidentified. Surely, these will be different from tissue nonspecific molecules, such as thrombospondin or fibronectin. Perhaps the role for TSP and other hematopoietic cytoadhesion molecules is to strengthen the eventual localization (bond) to the appropriate microenvironmental niche. Clearly, further studies are required to understand the role of thrombospondin and other cytoadhesion molecules in hematopoiesis and bone marrow transplantation.

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REFERENCES

1. Dexter TM, Wright EG, Kriza F, Lajtha LG: Regulation of haemopoietic stem cell proliferation in long term bone marrow cultures. Biomedicine 27:344, 1977
10. Long MW, Smolen JE, Szczepanski P, Boxer LA: Role of
40. Stoolman LM, Tenford TS, Rosen SD: Phosphomannosyl receptors may participate in the adhesive interaction between lymphocytes and high endothelial venules. J Cell Biol 99:1535, 1984
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