

Tenascin Supports Lymphocyte Rolling

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Abstract. Tenascin is a large extracellular matrix molecule expressed at specific sites in the adult, including immune system tissues such as the bone marrow, thymus, spleen, and T cell areas of lymph nodes. Tenascin has been reported to have both adhesive and anti-adhesive effects in static assays. We report here that tenascin supports the tethering and rolling of lymphocytes and lymphoblastic cell lines under flow conditions. Binding was calcium dependent and was not inhibited by treatment of lymphocytes with *O*-glycoprotease or a panel of glycosidases including neuraminidase and heparitinase but was inhibited by treatment of cells with proteinase K. Binding was to the fibrinogen-like terminal domain of tenascin as determined by antibody blocking studies and binding to recombinant tenascin proteins. When compared to rolling of the same cell

type on E-selectin, rolling on tenascin was found to be smoother at all shear stresses tested, suggesting that cells formed a larger number of bonds on the tenascin substrate than on the E-selectin substrate. When protein plating densities were adjusted to give similar profiles of cell detachment under increasing shears, the density of tenascin was 8.5-fold greater than that of E-selectin. Binding to tenascin was not dependent on any molecules previously identified as tenascin receptors and is likely to involve a novel tenascin receptor on lymphocytes. We postulate that the ability of tenascin to support lymphocyte rolling may reflect its ability to support cell migration and that this interaction may be used by lymphocytes migrating through secondary lymphoid organs.

TENASCIN is a large extracellular matrix (ECM)¹ protein expressed in tissues of the immune system, including the bone marrow (37), thymus (32), and the T cell areas of secondary lymphoid organs (11). Tenascin is also expressed in other sites in the adult including tumors (24) and healing wounds (64, 65) and in restricted patterns in the developing embryo (24). Tenascin hexamerizes to form a six-armed structure, or hexabrachion (26). The arms consist of a distinctive pattern of four types of protein motifs that form independently folding globular domains. The NH₂-terminal portion of the tenascin arm contains cysteine residues used in oligomerization, followed by a series of domains homologous to EGF, a series of fibronectin type III (FN-III) domains, and a fibrinogen-like terminal knob (fbg). Two other members of the tenascin family, tenascin-R and tenascin-X, have been described more recently; tenascin-R is expressed mainly in

the central nervous system, and tenascin-X is expressed mainly in muscle, testis, and adrenal gland (10, 29, 45).

The cell adhesion activity of tenascin has produced some controversial results in which the whole molecule and smaller segments promote either adhesion or anti-adhesion for different cell types. Recent studies using bacterial expression proteins have identified two major cell adhesion sites in the tenascin molecule that promote adhesion of a broad range of cell types (see Fig. 3 for the domain structure of tenascin). The third FN-III domain binds three different integrins and mediates adhesion of several cell types (5, 35, 46, 52, 66). The fibrinogen domain mediates adhesion of fibroblasts via a cell surface proteoglycan (5), endothelial cells via an integrin (35), and a variety of other cells (46). In addition to these two sites, neurons can bind to FN-III domains 1–2, 7–8, and the alternatively spliced domains (22, 30, 46).

Peripheral blood leukocytes have not previously been demonstrated to bind tenascin in a cell adhesion assay, but there are indications that lymphocyte interactions with tenascin may be important. Tenascin is prominently expressed in the reticular fibers of secondary lymphoid tissues, including lymph node, spleen, Peyer's patch, tonsil, and appendix (11, and Clark, R.A., and T.A. Springer, unpublished observations). Secondary lymphoid organs experience a constant influx and efflux of migrating lymphocytes that enter via afferent lymph and by transmigration across

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1. *Abbreviations used in this paper:* ECM, extracellular matrix; fbg, fibrinogen-like terminal knob; FN-III, fibronectin type III; HEV, high endothelial venule.

HEV, transit through the lymph node compartments, and finally exit via efferent lymphatics. It has been suggested that lymphocytes may migrate along the reticular fiber network of secondary lymphoid organs using adhesive interactions with the ECM components of these fibers (14, 15, 38). Soluble tenascin has been shown to block T cell activation in two independent studies (33, 50). Bone marrow cells have been shown to adhere to whole tenascin (37), but that study reported no adhesion of peripheral blood leukocytes. In the present study we demonstrate that peripheral blood leukocytes and several cell lines, especially of T cell lineage, can form low-avidity and transient adhesions to tenascin that will produce tethering and rolling under flow. We have mapped the adhesive domain on tenascin and characterized the interaction relative to other rolling adhesions. We discuss the potential utility of this transient interaction for cell migration through the tenascin-rich secondary lymphoid tissues.

Materials and Methods

Proteins

Purified tenascin-C and purified recombinant proteins TNfn1-5, TNfnA-D, TNfn6-8, and TNfnbg were produced as previously described (5). The bacterial recombinant protein TNfn6-8fbg comprises FN-III repeats 6–8 and the fibrinogen domain (16). For use in flow studies, a 25- μ l spot of tenascin (50 μ g/ml) or E-selectin (indicated dilution of column elution fraction) in PBS were coated on 100 \times 15 mm plastic Petri dishes (Nunc, Inc., Naperville, IL) overnight at 4°C. The spot and surrounding area were then blocked with 2% human serum albumin (HSA; Calbiochem, San Diego, CA) in HBSS for 2 h at 37°C, rinsed once with HBSS, and stored in HBSS at 4°C until use. For adsorbing recombinant fragments of tenascin, a 25 μ l spot of control IgG CBRp150/2E1 (100 μ g/ml) in PBS was first preadsorbed to 100 \times 15 mm plastic Petri dishes for 1 h at 37°C. Recombinant protein (50 μ g/ml in 200 μ l) was then incubated on the identical spot overnight at 4°C. The spot and surrounding area were subsequently blocked with 2% HSA in HBSS for 2 h at 37°C. Finally, the plate was rinsed with HBSS and stored at 4°C until use. Substrates produced by coating of recombinant proteins without the preabsorption of a nonspecific protein, e.g., IgG, gave no cell adhesion. No cell adhesion was observed on plates coated only with control IgG and blocked with HSA.

Antibodies

PUJ4 (α_4 integrin mAb) and PUJ5 (α_5 integrin mAb) were both kind gifts of Dr. M. Hemler (Dana-Farber, Boston, MA), DREG 56 was a kind gift of Dr. T.K. Kishimoto (Boehringer Angelheim, Ridgefield, CT), mAb 13 (β_1 integrin mAb) was kindly provided by Dr. K. Yamada (National Institutes of Health, Bethesda, MD). mAbs P1E6 (α_2 integrin mAb) and P4C10 (β_1 integrin mAb) and polyclonal antibody to the α_5 integrin subunit and to tenascin were purchased from GIBCO BRL (Gaithersburg, MD); additional samples of the P1E6 and P4C10 mAbs were obtained from the Fifth International Workshop on Human White Cell Differentiation Antigens. 4B4 (β_1 integrin mAb) was purchased from Coulter Corp. (Hialeah, FL). Rabbit antibodies were prepared to purified recombinant proteins TNfn1-5, TNfnA-D, and a mixture of TNfn6-8 plus TNfnbg (5). TNfn1-5 and TNfnA-D were injected at 50 μ g/rabbit in complete Freund's adjuvant (subcutaneously at six sites), followed by three boosts with 10 μ g. The TNfn6-8 plus TNfnbg mixture contained 100 μ g of each protein for the first injection and 50 μ g each for the boosts, because one attempt at the lower concentration gave no response. mAbs against tenascin, M112 (IgM), M139 (IgG₁), M168 (IgG₁), and M171 (IgM) and control mAb V60 (IgM) recognizing an irrelevant protein were produced by immunizing mice with human tonsillar stromal preparations and screening the resulting mAb by immunohistochemistry on sections of human tonsil. The protocol for fusion and hybridoma maintenance has been described elsewhere in detail (55). M112, M139, M168, and M171 recognize tenascin, as evidenced by their immunohistochemical reactivity patterns in tonsil, mesenteric lymph node, spleen, colon, thymus, appendix, breast, and skin, recognition of immobilized tenascin in dot blots, and immunoprecipitation of

radiolabeled tenascin (data not shown). By immunohistochemistry, the V60 mAb reacted only with phagocytic vacuoles in tingible body macrophages in all tissues tested. CBRp150/2E1 (57) to p150,95 was used as a control mAb in flow antibody blocking assays, and TS1/18 was used to the β_2 integrin subunit. All other mAbs were obtained from the Fifth International Workshop on Human White Cell Differentiation Antigens. mAb to α integrin subunits from the Fifth Leukocyte Workshop were IB3.1 (α_1), Gi9 (α_2), J143 (α_3), and LM609 ($\alpha_5\beta_3$). The mAb to CD44 was BRIC235. For inhibition of binding to tenascin in flow, input cells were incubated 40 min on ice with a 1:20 dilution of mAb from the Fifth International Workshop on Leukocyte Differentiation Antigens, 1:1,000 dilution of PUJ4, PUJ5, and P1E6 ascites, 1:500 P4C10 ascites, 1:200 dilution of rabbit antisera, 1:100 dilution of M168, M112, M171, and V60 ascites, 50 μ g/ml of purified mAbs CBRp150/2E1, TS1/18, DREG 56, 4B4, and M139 and 25 μ g/ml of purified mAb 13. For studies using anti-tenascin antibodies, the antibody was added to the flow chamber and incubated for 10 min before the addition of cells.

Isolation of Lymphocytes from Blood and Human Tonsil

Peripheral blood lymphocytes (49) and tonsillar lymphocytes (15) were prepared as described.

Cell Lines

Lymphoblastoid cell lines HuT 78 (American Type Culture Collection, Rockville, MD; TIB 161), Jurkat (American Type Culture Collection; TIB 153), JY 25, SKW3 (originally from Dr. P. Cresswell, Yale University School of Medicine, New Haven, CT), NALM6 (originally from McCaffrey, R.), and Raji (American Type Culture Collection; CCL 86) and myelomonocytic cell lines HL-60 (American Type Culture Collection; CCL 240), KG-1a (American Type Culture Collection; CCL 246.1), HEL 92.1.7 (American Type Culture Collection; TIB 180), THP-1 (American Type Culture Collection; TIB 202), and U937 (American Type Culture Collection; CRL 1593) were maintained in RPMI 1640 medium (JRH Biosciences, Lenexa, KS), 10% fetal bovine serum (JRH Biosciences).

Flow Experiments

Cell tethering and rolling was measured in a parallel-plate flow chamber the lower wall of which was a 100 \times 15 mm plastic Petri dish containing spots of immobilized protein (34, 41). All experiments were in HBSS with 2 mM Ca²⁺ and 1 mM Mg²⁺ unless otherwise described. Video microscopy, analysis of videotape, measurements of cell accumulation in shear flow, and measurement of detachment of cells and rolling velocities were as described (41). For all studies, a total of 2 \times 10⁶ cells at a concentration of 5 \times 10⁶ cells/ml were used per experiment. Unless otherwise noted, cells were accumulated for 40 s each at 0.27 and 0.53 dynes/cm²; adherent cells were counted after the 0.53 dynes/cm² accumulation step.

Enzyme Treatments

Cells (2 \times 10⁶ cells in 200 μ l) were treated with 20 μ g/ml (0.03 U/ml) chondroitinase ABC (from *Proteus vulgaris*; Sigma Chemical Co., St. Louis, MO), 0.01 U/ml of heparitinase I (from *Flavobacterium heparinum*; Sigma Chemical Co.), 1,000 U/ml of hyaluronidase (type IV-S from bovine testes; Sigma Chemical Co.), 0.1 U/ml of neuraminidase (from *Vibrio cholerae*, Sigma Chemical Co.) with or without 0.5 U/ml of exo β -galactosidase (grade VIII from *Escherichia coli*, Sigma Chemical Co.), 60 μ g/ml (0.3 U/ml) *O*-glycoprotease (Accurate Chemical and Scientific Corporation, Westbury, NY), or 100 μ g/ml proteinase K (GIBCO BRL) diluted in RPMI 1640, 10 mM Hepes pH 7.4, 0.1% BSA for 40 min at 37°C. Additional experiments in which cells were incubated with enzymes for 60 min produced identical results. In previous studies, use of chondroitinase ABC and heparitinase I under identical conditions abolished hyaluronan-dependent lymphocyte binding to stromal cell monolayers (15). Neuraminidase and *O*-glycoprotease used under similar reaction conditions prevented sialic acid- and mucin-dependent leukocyte binding (28, 47), and galactose-dependent binding of MOLT-4 cells to thymic epithelial cells via galectin-1 was inhibited using similar buffer conditions and 30-fold less exo- β -galactosidase enzyme than used in these studies (7).

Immunoblots

Intact and recombinant tenascin proteins (1 μ g/spot) were spotted onto nitrocellulose and allowed to dry overnight at room temperature. Nitro-

cellulose strips were then blocked for 1 h with 1% BSA (GIBCO BRL) in tris-buffered saline with 0.05% Tween-20 (TBST) and then incubated with primary antibodies for 1 h at room temperature (1:200 dilution of antisera or monoclonal ascites or 10 $\mu\text{g}/\text{ml}$ of purified mAb M139 in TBST). Strips were rinsed three times for 7 min each in TBST and incubated for 30 min in a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG+A+M or goat anti-rabbit Ig (Zymed Labs, Inc., So. San Francisco, CA) in TBST. Strips were rinsed three times for 7 min each in TBST and developed with Western blue-stabilized AP substrate (Promega Biotech, Madison, WI). Reaction was stopped with distilled water.

Soluble Inhibitors

Chondroitin sulfate B from bovine mucosa (Sigma Chemical Co.), heparin from porcine mucosa (Sigma Chemical Co.), GRGDSP peptide (GIBCO BRL), or fibrinogen γ -chain peptide HHLGGAKQAGDV (Sigma Chemical Co.) were added to the cell suspension and the flow chamber for 10 min before flow experiments and were present throughout the experiments. EDTA (5 mM) or 10 mM EGTA plus 2 mM Mg^{2+} were added to cells immediately before the experiment and were also present in cell-free rinse medium used in the experiment. For experiments using azide and 2-deoxy-D-glucose, cells were pretreated for 10 min at room temperature with 0.1% azide and 50 mM 2-deoxy-D-glucose (Sigma Chemical Co.); these inhibitors were also present throughout the experiments.

Quantitation of Binding Sites

Site densities for plastic-adsorbed E-selectin and tenascin were determined using radiolabeled mAb as previously described (42).

Immunohistochemistry

Human tonsils from tonsillectomies were obtained from Children's Hospital (Boston, MA) or Massachusetts General Hospital (Boston, MA) and snap frozen in liquid nitrogen. Cryostat sections (5 μm) were cut and fixed in room-temperature acetone for 5 min. Sections were incubated for 45 min with a 10 $\mu\text{g}/\text{ml}$ concentration of M168 in PBS, rinsed three times for

5 min each in PBS, 1% BSA (GIBCO BRL) and incubated for 30 min with a 1:40 dilution of FITC-conjugated goat anti-mouse IgG+A+M (Zymed Labs, Inc.). Sections were subsequently rinsed three times for 5 min each in PBS, 1% BSA, and mounted under glass coverslips (Fisher Scientific, Pittsburgh, PA) using Fluoromount-G mounting media (Southern Biotechnology Assoc. Inc., Birmingham, AL). Sections were observed by fluorescence microscopy and photographed immediately after staining.

Results

Plastic-adsorbed Tenascin Supports Lymphocyte Rolling

Freshly isolated peripheral blood lymphocytes and tonsillar lymphocytes introduced under flow adhered to plastic-adsorbed tenascin at wall shear stresses of 0.27 and 0.53 dynes/cm² (Fig. 1 *a*). In contrast, neutrophils from peripheral blood did not bind tenascin under flow. Adherent lymphocytes began rolling immediately after binding to the substrate and continued to roll as shear was increased. Binding to tenascin was threefold higher for tonsillar than for peripheral blood lymphocytes. Lymphocytes harvested from tonsil tissue contain activated cells that express the activation antigens CD26, CD30, CD39, CD69, CD70, and CD71 (8, 21, 58). Because cell lines are useful models for activated leukocytes, several cell lines were also evaluated for their ability to form rolling attachments to tenascin (Fig. 1 *a*). Most cell lines of T cell, B cell, and myeloid lineage bound tenascin, but the efficiency of binding varied widely. The SKW3 T lymphoblastoid cell line was chosen for subsequent studies because of its robust binding to tenascin under flow.

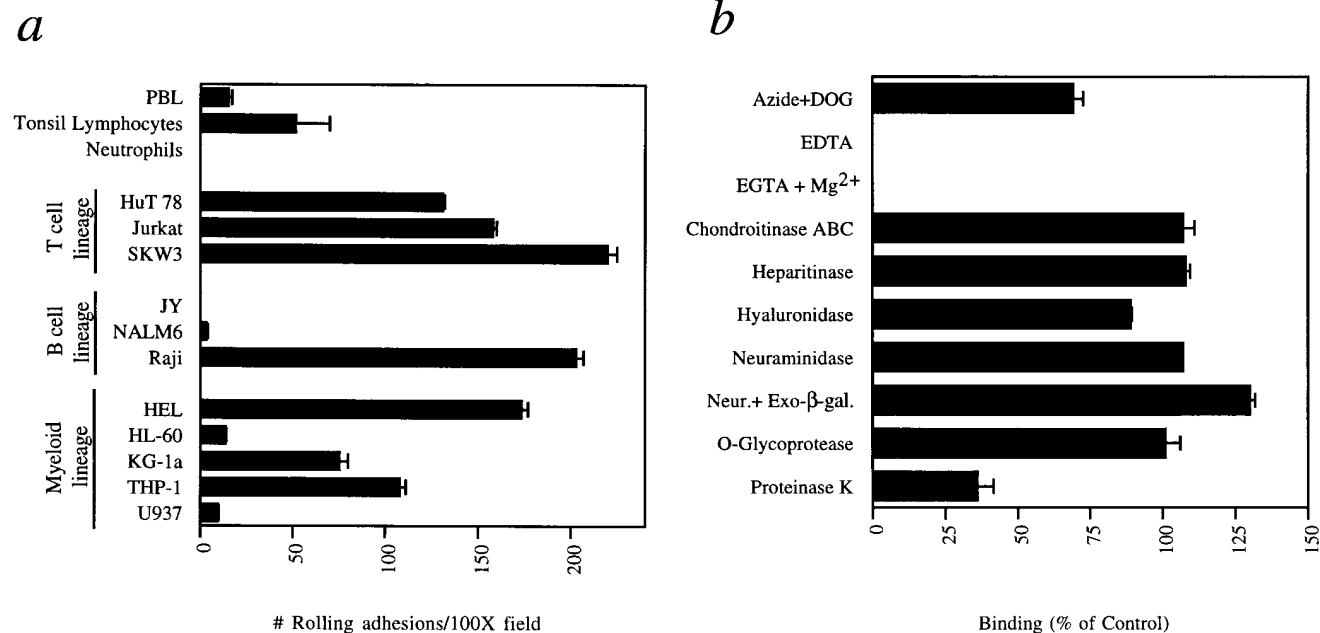


Figure 1. Cell accumulation on plastic-adsorbed tenascin. (*a*) Ability of various cell types to accumulate on tenascin under flow. Cells were accumulated for 40 s each at 0.27 and 0.53 dynes/cm². Cells were counted after the 0.53 dynes/cm² accumulation step; all adherent cells were rolled. One of two representative experiments is shown with the range indicated by bars. (*b*) Effects of inhibitors and enzyme treatments on SKW3 cell accumulation on tenascin. SKW3 cells were treated for 10 min at 25°C with either 0.1% azide and 50 mM 2-deoxyglucose (*Azide+DOG*), inclusion of 5 mM EDTA or 10 mM EGTA and 1 mM Mg^{2+} in the assay, or treatment for 40 min at 37°C with chondroitinase ABC, heparitinase, hyaluronidase, neuraminidase, neuraminidase and exo- β -galactosidase, O-glycoprotease, or proteinase K. Control binding represents binding of control-treated cells to the same tenascin substrate immediately before binding of treated cells. Mean values for two experiments are shown with the range indicated by bars.

The ability of SKW3 cells to form rolling adhesions was completely blocked by inclusion of EDTA or EGTA plus Mg^{2+} in the rolling assay and was therefore calcium dependent (Fig. 1 *b*). Likewise, the binding of all other cell lines shown to tether and roll on tenascin was completely abolished by EGTA plus Mg^{2+} (data not shown). Treatment of SKW3 cells with a panel of glycosidases and with *O*-glycoprotease failed to inhibit the ability of these cells to bind, suggesting that the SKW3 cell ligand activity is not dependent upon chondroitin sulfates, heparin, hyaluronan, sialic acid residues, galactose, or mucin-like molecules (Fig. 1 *b*). However, treatment of SKW3 cells with proteinase K decreased binding to tenascin by 64%; identically treated cells analyzed by immunofluorescence flow cytometry showed a 92% decrease in CD44 staining, a 31% decrease in β_2 , and a 22% decrease in α_4 integrin subunit staining. The reduction in binding after proteinase K treatment suggests that the tenascin ligand on SKW3 cells is either a protein or a carbohydrate dependent on protein scaffolding for presentation. Pretreatment of SKW3 cells with 0.1% sodium azide and 50 mM 2-deoxy-D-glucose inhibited binding by 30%.

The ability of rolling SKW3 cells to accumulate on tenascin decreased with increasing shear stresses (Fig. 2 *a*) with a profile similar to that seen for SKW3 cells on purified hyaluronan (15). Accumulation was less efficient than was seen with neutrophils (PMN) on E-selectin, where accumulation was still evident at 1.6 dynes/cm² and declined to zero between 3 to 4.5 dynes/cm² (48). Rolling velocities of cells on tenascin increased with increasing wall shear stress and were on the order of 10 μ m/second (Fig. 2 *b*), similar to the rolling velocities of PMN on E-selectin (48) but faster than the rolling velocities of SKW3 cells on hyaluronan, which were on the order of 1 μ m/s (15). SKW3 cells rolling

on tenascin detached with increasing shear stress (Fig. 2 *c*). SKW3 cells detached from tenascin at lower wall shear stresses than seen with PMN on E-selectin or SKW3 cells on hyaluronan; at 35 dynes/cm², 96% of adherent SKW3 cells on tenascin had detached, whereas only 60% of PMN on E-selectin and 20% of SKW3 cells on hyaluronan had detached, respectively (15, 48). The ability of cells to accumulate in shear flow, rolling velocity, and resistance to detachment depend not only on intrinsic properties of the receptor–ligand interaction (48) but also to some extent upon the number of binding sites on the substrate, which is a function of the concentration of proteins used during adsorption to plastic. Therefore, the above observations depend on the plating concentrations used in these studies and are not necessarily general properties of the interaction.

To determine if conventional static binding assays would detect the interaction of SKW3 cells with tenascin, plastic-adsorbed tenascin substrates identical to those used in the flow assays were used in static experiments. Using a Petri dish adhesion assay, with very mild washing conditions, that detects shear-sensitive, i.e., low avidity, interactions of integrins (18), SKW3 cells incubated with tenascin substrates showed no binding at 30 min and <1 bound cell/100 \times field at 1 h and at 90 min (data not shown). Thus, while binding to tenascin is easily demonstrable using the flow assay, it is not surprising that previous studies using static assays have failed to observe adhesive interactions (37).

Lymphocyte Rolling Is Mediated by the Fibrinogen-like Terminal Knob of Tenascin

To determine the region of the tenascin molecule that binds to lymphocytes under flow, we used a panel of anti-

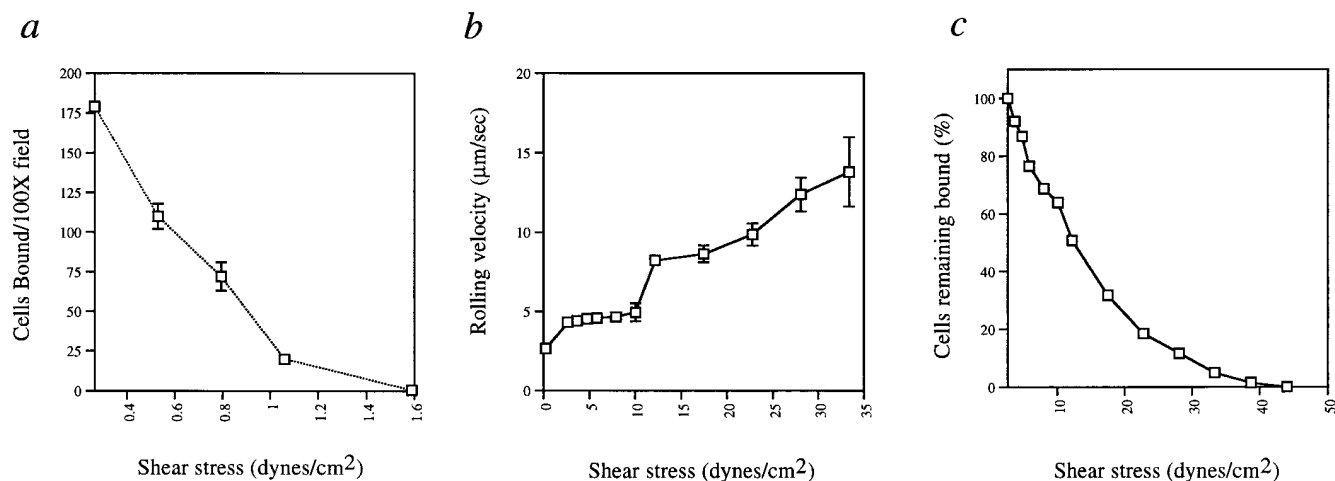


Figure 2. Accumulation, rolling velocity, and resistance to detachment of SKW3 cells on tenascin. (*a*) Accumulation of SKW3 cells on plastic-adsorbed tenascin at various shears. Cells introduced at a concentration of 5×10^6 cells/ml were accumulated for 40 s at the indicated shear stresses in separate experiments. The mean values of two experiments are shown with the range indicated by bars. (*b*) Rolling velocities of SKW3 cells initially accumulated on plastic-adsorbed tenascin for 40 s at 0.27 dynes/cm². Velocity measurements were done on all cells in the field; bars represent SEM for each measurement. (*c*) Detachment of SKW3 cells bound to plastic-immobilized tenascin under increasing shear. Cells were initially accumulated for 40 s at 0.27 dynes/cm² and then perfused with cell-free medium for 10 s at each of the indicated shears. Cell accumulation at the end of the 0.27 dynes/cm² accumulation step was considered 100% binding; the numbers of cells remaining at the end of subsequent steps were compared to this value to determine percent detachment. The mean values of two experiments are shown; bars representing the range of measurements are present but are too small to be seen.

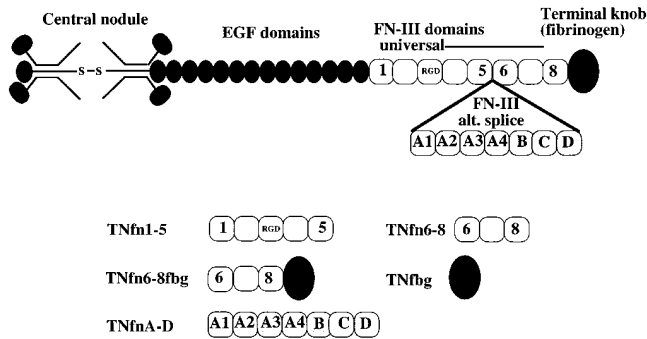


Figure 3. The domain structure of the hexabrachion arm of human tenascin (above) and the bacterial expression proteins used in this study (below), modified from reference 5.

bodies against tenascin. Rabbit polyclonal antibodies were prepared against recombinant tenascin fragments TNfn1-5, TNfnA-D, and TNfn6-8+TNfbg (Fig. 3). Monoclonal antibodies recognizing tenascin were produced by immunizing mice with preparations of human tonsillar stroma. mAbs were screened by immunohistochemistry, and those that recognized high endothelial venule (HEV) or ECM components of secondary lymphoid organs were further analyzed. A significant proportion of mAbs made in this way was found to recognize tenascin. The sites of antibody binding to tenascin were mapped by immunoblotting of recombinant tenascin proteins (Fig 4 a). Rabbit antibodies showed the expected specificity, and additionally, antibodies to TNfn6-8+TNfbg crossreacted weakly with TNfnA-D and

TNfn1-5. mAbs M139 and M171 were specific for TNfn1-5, and mAb M112 reacted with TNfn1-5 and more weakly with TNfn6-8fbg and TNfbg. mAb M168 reacted with TNfn6-8fbg and TNfbg and not with TNfn6-8 and therefore appears specific for the fibrinogen-like terminal domain of tenascin. Antisera produced by immunizing rabbits with the TNfn1-5 and TNfnA-D tenascin proteins had little effect on the binding of SKW3 cells to intact tenascin under flow, while antisera raised against the TNfn6-8fbg recombinant tenascin protein completely blocked binding (Fig. 4 b). Antibodies M112, M139, and M171 to TNfn1-5 domains of tenascin did not significantly inhibit SKW3 cell binding. By contrast, mAb M168 to the terminal fb domain of tenascin reduced binding by 99%. These studies suggest that the terminal fibrinogen-like domain of tenascin is responsible for binding to SKW3 cells.

To determine if recombinant tenascin proteins could support rolling, we immobilized the proteins on plastic precoated with nonspecific IgG and HSA (as described in Materials and Methods) and used them as substrates in the rolling assay. Rolling of SKW3 cells was observed on TNfbg and TNfn6-8fbg but not on the other recombinant fragments (Fig. 5). Overall levels of accumulation on recombinant TNfbg was ~10% of that observed with the intact protein and was therefore considerably less efficient. Binding to TNfn6-8fbg was even less efficient, perhaps reflecting reduced binding to IgG-coated plates. Transient tethering, defined as cells that bound under flow and remained adherent to the substrate for at least 1 s, was observed at low levels for TNfnA-D, TNfn1-5, and TNfn6-8 and not for control substrates. Transient tethering of cells to TNfn6-8fbg and TNfbg was two- and fivefold higher, re-

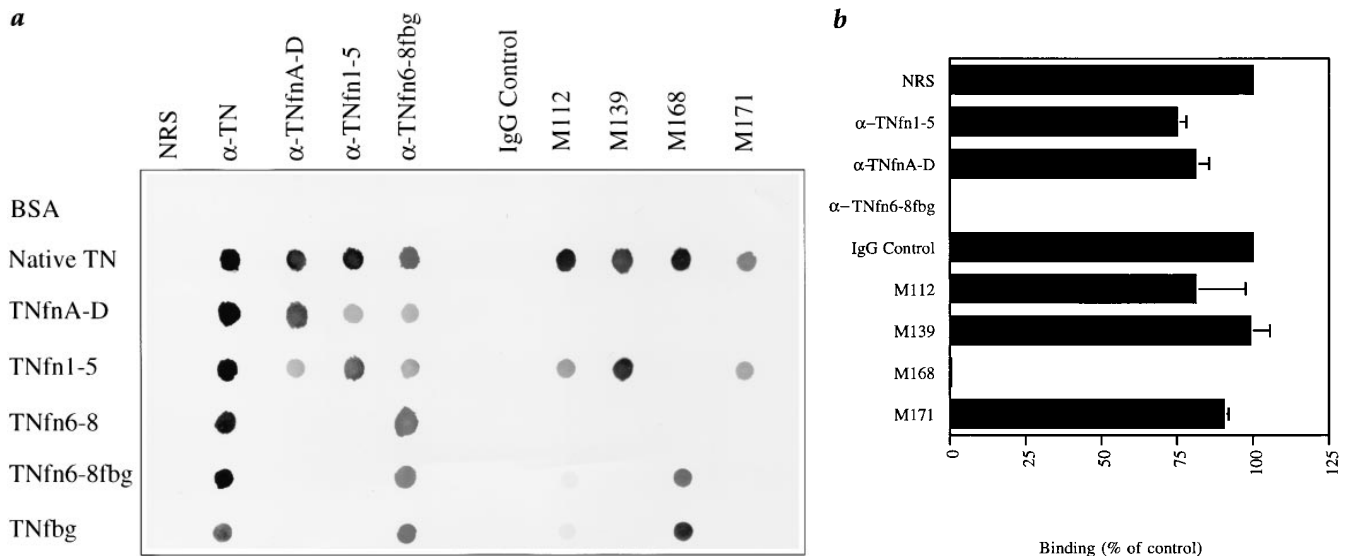


Figure 4. Localization of antibody binding sites with immunoblotting and inhibition of cell binding to tenascin. (a) Recognition of tenascin and recombinant tenascin proteins by antibodies. Proteins were spotted on nitrocellulose and detected with the indicated antibodies and alkaline phosphatase-anti-Ig. Antibodies included normal rabbit serum (NRS); rabbit antibodies against intact tenascin (α -TN), recombinant TNfnA-D (α -TNfnA-D), recombinant TNfn1-5 (α -TNfn1-5), and a mixture of recombinant TNfn6-8 and TNfbg (TNfn6-8fbg); a negative IgG control mAb; and mAb M112, M139, M168, and M171 against tenascin. (b) Inhibition of SKW3 cell binding to plastic-adsorbed tenascin by tenascin antibodies. Binding of SKW3 cells was measured before and after treatment of the spots of plastic-immobilized tenascin with polyclonal antisera (1:50), ascites (1:100), or purified mAb (50 μ g/ml) for 10 min. Binding to control NRS-treated spot was 250 cells and binding to IgG control-treated spot was 224 cells. Mean values of two experiments are shown, with the range indicated by bars.

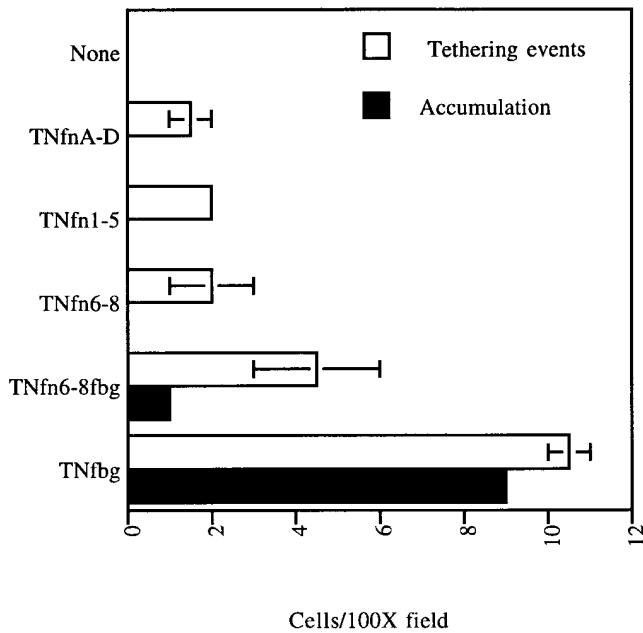


Figure 5. Ability of recombinant tenascin proteins to support SKW3 cell rolling. Cells were accumulated for 40 s at 0.27 dynes/cm² shear stress; adhesion events were counted throughout this 40 s period. “Accumulation” represents cells that rolled a distance of at least four cell diameters. “Tethering events” were defined as cells that tethered under flow and remained bound to the substrate for at least one second. Mean values of two experiments are shown, and the range is indicated by bars.

spectively, than that seen with the other proteins. The recombinant tenascin fragments expressed in bacteria are not glycosylated, and thus carbohydrate on tenascin is not required for rolling adhesion. The observation that recombinant proteins containing the fibrinogen-like domain of tenascin can support cell rolling, and the ability of antibodies to this region to block rolling suggests that this domain supports rolling of SKW3 cells.

Lymphocyte Rolling on Tenascin is Smoother Than Rolling on Selectins

The characteristics of tenascin-mediated rolling were compared to rolling on E-selectin using KG1a cells, which bind to both tenascin and E-selectin (28). To directly compare the two adhesion systems, plating concentrations of E-selectin were varied until the detachment profile of KG1a cells on E-selectin was similar to that observed using a 50 μg/ml plating concentration of tenascin (Fig. 6 *a*). This required plating of purified E-selectin at concentrations 5- to 10-fold lower than the amount used routinely for experiments in our laboratory (42). At an E-selectin plating dilution of 1:1,000, a level at which detachment from the two substrates was most similar, the E-selectin substrate was found to contain 34 sites/μm², as measured by binding of radiolabeled mAb CL3. In contrast, the tenascin substrate contained 290 sites/μm², as measured by binding of the function-blocking mAb M168. The tenascin substrate therefore had 8.5-fold more binding sites than the E-selectin substrate under conditions in which cells detached at roughly the same shear stresses.

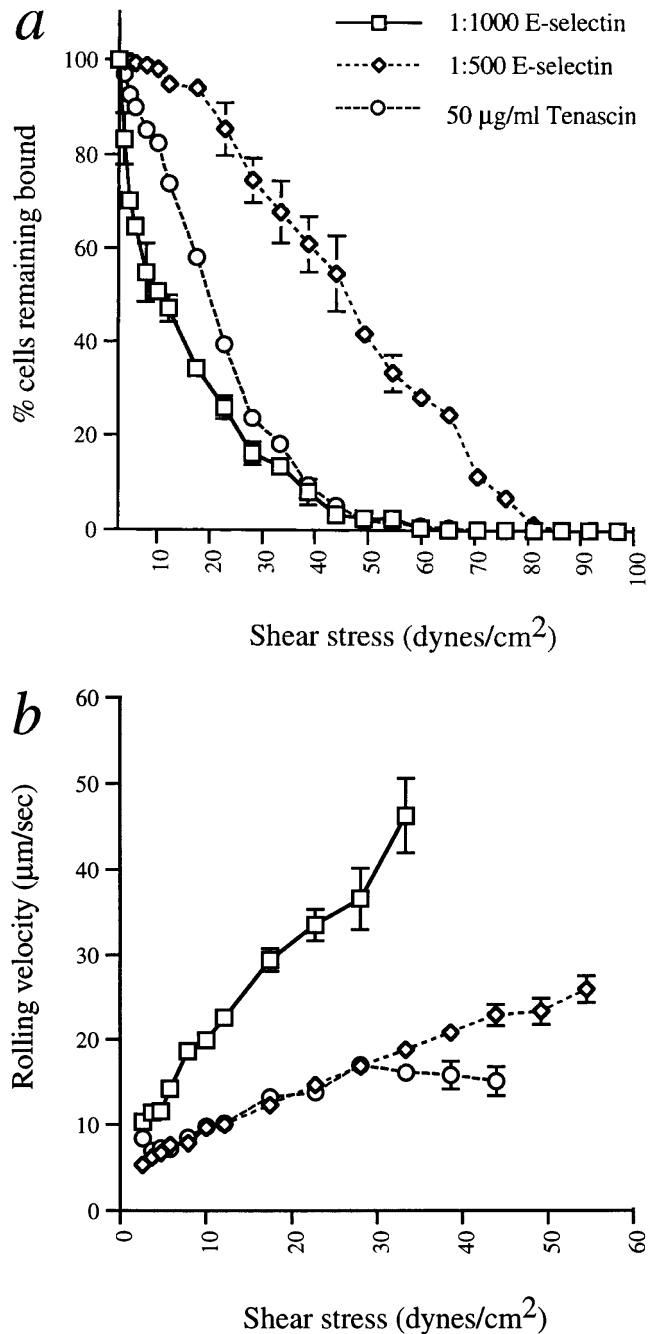


Figure 6. Comparison of KG1a cells rolling on tenascin and E-selectin. (*a*) Detachment profiles of KG1a cells on tenascin and two plating dilutions of E-selectin. (*b*) Rolling velocities of KG1a cells on tenascin and E-selectin. The mean values of two experiments are shown; ranges are indicated by bars.

KG1a cells on tenascin at 290 sites/μm² rolled at similar velocities to KG1a cells on a plating dilution of 1:500 E-selectin (Fig. 6 *b*). During these experiments it was noted that KG1a cells rolling on E-selectin appeared to move in a series of jerks, as previously noted for rolling on selectins (3), whereas cells attached to tenascin rolled more smoothly. We quantitated variation in rolling velocity using separate conditions under which either the wall shear stress or the average rolling velocity were identical on the three sub-

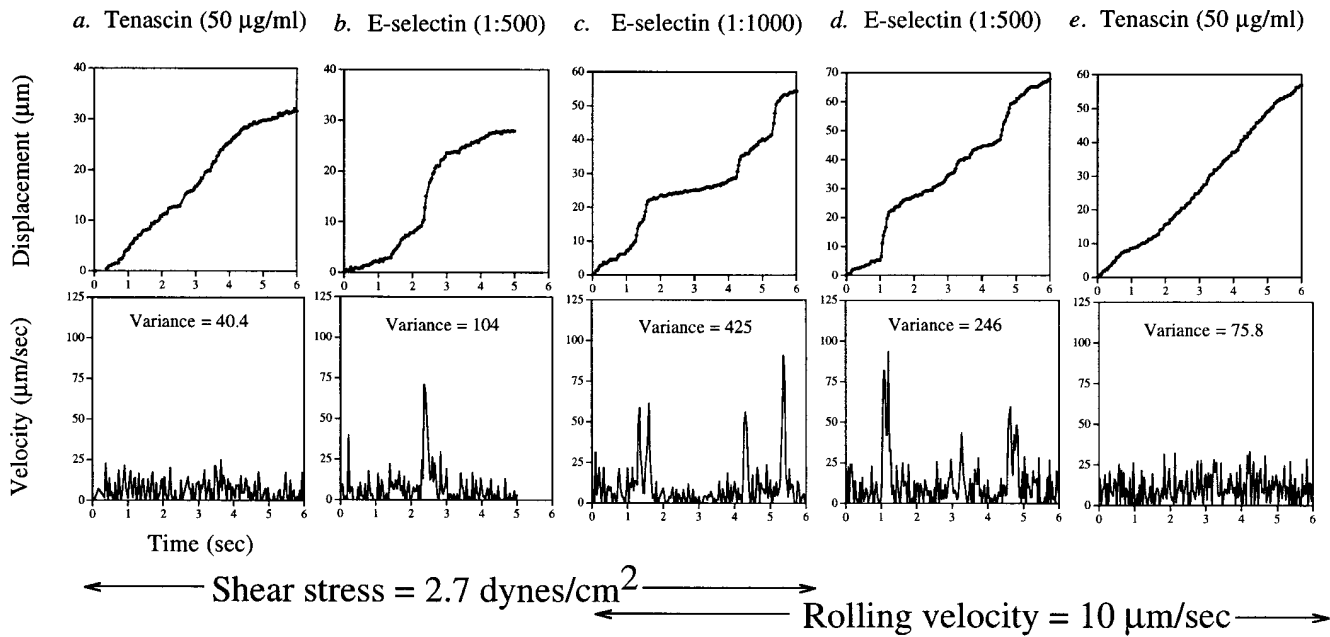


Figure 7. Rolling on tenascin is smoother than rolling on E-selectin at all shear stresses examined. (a–c) Displacement and velocity profiles of cells rolling on tenascin and E-selectin at 2.7 dynes/cm² shear stress. (c–e) Change in velocity profiles of cells rolling on tenascin and E-selectin at 10 µm/s. The variance of velocity measurements is as shown.

strates. The position of representative rolling cells was measured every 0.033 s. For each condition, both the displacement (Fig. 7, top graph) and the velocity (Fig. 7, bottom graph) are shown. A relatively straight tracing on the displacement versus time graph indicates a cell that rolled smoothly at a constant velocity, while a tracing with changes in slope indicates a cell that rolled in a more jerky manner. Similarly, the range of velocities for a smoothly rolling cell is small, while the intermittent motion of a jerky cell is reflected in a larger range of rolling velocities. This was quantified by calculating the variance in cell velocities for cells rolling on the three substrates. Cells rolling on tenascin had a variance in velocities of 40.4, whereas cells rolling on 1:1,000 and 1:500 dilutions of E-selectin had larger variances, 425 and 104, respectively. Therefore, cells rolled more smoothly on tenascin at 2.7 dynes/cm² shear stress than cells rolling on either site density of E-selectin (Fig. 7, a–c). To compare cells rolling at a similar velocity of 10 µm/s, cells rolling at 2.7 dynes/cm² shear stress for E-selectin (1:1,000) and at 10 dynes/cm² for E-selectin (1:500) and tenascin were compared (Figs. 6 b and 7, d–f). Cells bound to tenascin rolled more smoothly than cells bound to E-selectin when rolling at similar velocities. The variance in cell velocities was 75.8 for cells rolling on tenascin and 425 and 246 for cells rolling on 1:1,000 and 1:500 dilutions of E-selectin, respectively. These direct comparisons suggest that the smoother rolling observed with tenascin is an inherent characteristic of the molecular interaction of tenascin with its cellular receptor.

Rolling of Lymphocytes on Tenascin May Use a Novel Receptor

More than a dozen molecules have been identified as tenascin receptors (Table I). Among them are a number of members of the integrin family. Most notably, binding via $\alpha_2\beta_1$ may involve the fbg region of the tenascin molecule (35, 56). We therefore investigated whether treatment of SKW3 cells with blocking mAbs to these integrins would inhibit tethering and rolling on tenascin. Two different blocking mAbs to the β_1 integrin had little or no effect on attachment of SKW3 cells to tenascin (Fig. 8), as did a third β_1 -blocking mAb, 4B4 (data not shown). Most significantly, the β_1 mAb P4C10 obtained from two different sources did not inhibit binding of SKW3 cells in the shear flow assay despite reports that it blocks the binding of the $\alpha_2\beta_1$ integrin to tenascin (56). A panel of blocking antibodies to the α_1 , α_2 , α_3 , α_4 , α_5 , α_6 , and α_v integrin subunits also had no effect (Fig. 8); these included two different samples of P1E6, the α_2 mAb reported to inhibit $\alpha_2\beta_1$ interaction with tenascin (56). Furthermore, expression of the α_2 integrin subunit did not correlate with the ability of cell lines to bind to tenascin. Hut 78 and Raji cell lines do not express the α_2 integrin subunit (53) but bind efficiently to tenascin. The blocking $\alpha_v\beta_3$ mAb LM609 did not inhibit SKW3 cell binding, despite its ability to inhibit tenascin binding via $\alpha_v\beta_3$ (56), and a polyclonal antibody to the human α_v subunit did not inhibit binding despite its ability to block α_v -mediated binding of human cell lines to chicken tenascin (46). In addition, the RGD peptide GRGDSP, reported to block $\alpha_2\beta_1$ and $\alpha_v\beta_3$ binding to tenascin (35, 56), also had no effect (Fig. 8). $\alpha_2\beta_1$ is the only integrin thought to interact with the fbg region of tenascin; other integrins recognize tenascin's FN-III repeats (Table I). These integrins contain either α_v or β_1 subunits; however, multiple antibodies to these subunits failed to inhibit binding to tenascin in the flow assay.

Heparin binding to tenascin also involves tenascin's fbg

Table I. Tenascin Receptors

Receptor	Tenascin binding site	Characteristics	Reference
$\alpha_2\beta_1$	Unknown, possibly TNfbg	Blocked by β_1 mAb P4C10 and α_2 mAb P1E6. Divalent cation dependent, RGD sensitive	(35, 56)
$\alpha_8\beta_1$	TNfn3		(52, 61)
$\alpha_9\beta_1$	TNfn3	Not inhibited by RGD peptide or by mutating RGD site	(66)
$\alpha_v\beta_3$	TNfn3	Inhibited by mAb LM609 and RGD. Divalent cation dependent	(35, 56)
$\alpha_v\beta_6$	TNfn3	Blocked by α_v polyclonal antibody	(46)
Annexin II	TNfnA-D		(12)
Contactin	TNfn5 and TNfn6	Requires TNfn5 and TNfn6 to be adjacent	(62)
Fibronectin	TNfn3		(13)
Glypican	TNfn5	Blocked by heparin	(62)
Heparin	TNfbg, TNfn5	Cation and RGD insensitive	(5, 27, 63)
Neurocan	Unknown		(31)
Perlecan	TNfn3-5		(26a)
Phosphacan	Unknown		(6, 44)
Syndecan	Unknown	Abolished by heparitinase treatment by syndecan	(51)
Versican (lectin domain)	Unknown	Calcium dependent, blocked by chemical deglycosylation of TN	(4, 32)

region (5, 27). In addition to the ability of tenascin to bind purified heparin (5, 27, 63), the binding of tenascin to both syndecan (51), a cell surface proteoglycan on fibroblasts (5), and the proteoglycan glypican (62) is also heparin dependent, as shown by the ability of heparitinase treatment and/or soluble heparin to inhibit binding to these ligands. Heparitinase treatment of SKW3 cells did not inhibit binding to tenascin at the recommended enzyme level of 0.01 U/ml (Fig. 1 *b*), nor did it when used at the much higher doses of 0.05 and 0.1 U/ml (data not shown). Inclusion of 100 μ g/ml heparin in the rolling assay also failed to inhibit binding of SKW3 cells, and inclusion of 200 and 500 μ g/ml was not inhibitory relative to the inclusion of similar amounts of a control, chondroitin sulfate B (data not shown). In contrast, adhesion of fibroblasts to TNfbg was inhibited by as little as 10 μ g/ml heparin (5). In light of these results we believe that the interactions we observe with tenascin are not mediated by tenascin binding to heparin or heparin-like structures on SKW3 cells.

The remaining identified tenascin ligands include annexin II (12), a protein that binds tenascin via TNfnA-D; fibronectin (13), which binds tenascin via TNfn3; neurocan and phosphacan (6, 31, 44), two soluble chondroitin sulfate proteoglycans specific to nervous tissue; contactin (62), a neural cell adhesion molecule and a member of the immunoglobulin superfamily; and perlecan, a proteoglycan component of basement membranes (Table I). None of these molecules are known to be expressed on lymphocytes or leukocyte cell lines, nor have any been reported to bind via the fbg region of tenascin. To rule out contributions by L-selectin and CD44, two molecules known to mediate

lymphocyte rolling (15, 17), we treated SKW3 cells with mAbs known to block rolling via these molecules (15, 36); these mAbs also had no effect (Fig. 8). The fbg region of tenascin bears a 40% amino acid sequence homology to fibrinogen (20), raising the question of whether known fibrinogen-binding molecules may be involved in lymphocyte binding to tenascin. Fibrinogen γ -chain peptide HHLGGAKQAGDV has been reported to block the binding of fibrinogen to platelets and endothelial cells (59), but it had no effect on SKW3 cell binding to tenascin (data not shown). ICAM-1 has also been reported to mediate binding of leukocytes and endothelial cells to fibrinogen (39). However, ICAM-1 is present on both the cell lines that bind tenascin, e.g., Raji and SKW3, and the cell lines that do not bind tenascin, e.g., JY (53), suggesting that the presence of ICAM-1 does not correlate with tenascin binding. Adhesion in shear flow of lymphocytes and leukocyte cell lines to tenascin may therefore involve a novel receptor.

Discussion

Previous flow studies have demonstrated that two well-known classes of molecules are capable of mediating rolling: the selectins, E-selectin, L-selectin, and P-selectin (54); and specific members of the integrin family, such as VLA-4 that binds to VCAM-1 (2, 9), $\alpha_4\beta_7$ that binds to MADCAM-1 (9), and $\alpha_6\beta_4$ that binds to laminin (60). Recently, it was found that hyaluronan, a component of the ECM network of lymph nodes, mediated lymphocyte rolling via CD44 (15, 17). We therefore investigated whether

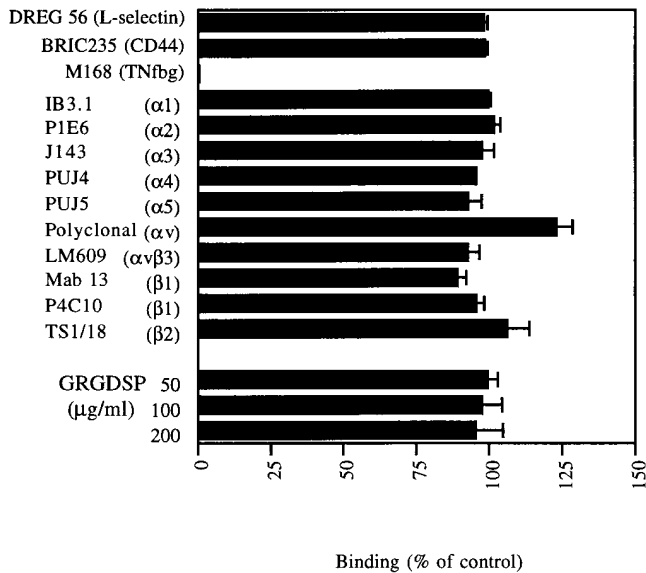


Figure 8. Binding of SKW3 cells to tenascin is not inhibited by mAbs to known tenascin ligands or RGD peptide. SKW3 cells were treated with the indicated mAbs or GRGDSP peptide before flow assays; M168 and GRGDSP were also added to the flow chamber before the addition of cells. For GRGDSP peptide experiments, cells were maintained in the continued presence of the peptide. Observed binding was compared to binding of cells treated with control IgG CBRp150/2E1 (mAb experiments; control binding was 162 cells), normal rabbit serum (rabbit Ab; control binding was 171 cells), or untreated cells (GRGDSP peptide experiments; control binding was 293 cells) to obtain percent control binding. The mean values of two experiments are shown; ranges are indicated by bars.

other ECM components of secondary lymphoid organs might also support binding of lymphocytes under flow. We report here that tenascin mediates tethering and rolling of lymphocytes and of lymphoid and myeloid cell lines. Although tenascin has been shown to bind to fibroblasts and other cell types (Table I), this is the first report of lymphocyte adhesion to tenascin. Using static binding assays, tenascin has been shown to be both adhesive and anti-adhesive, depending on the experimental conditions (24). Our findings that tenascin mediates rolling may have implications for the mechanical nature of these particular tenascin–ligand bonds and may explain why earlier studies of lymphocyte binding using static assays did not detect these interactions. Fast off rates have been shown for selectin-mediated rolling, and most likely fast on-rates are also involved (1). The receptor–ligand bonds involved in rolling adhesions must be highly labile, with rapid formation and dissolution of bonds, so that adhesions formed in the trailing edge of the adhesive contact zone do not retard the cell but instead release and allow the cell to roll forward and form new adhesions at the leading edge. In our studies the same plastic-adsorbed tenascin substrates used in the rolling assays failed to support the binding of SKW3 cells in conventional static binding assays. Thus static assays would have produced a null result in this case. The sort of transient binding known to support cell rolling, and exemplified best by the selectins, is not detected well by traditional

static assay systems and yet can play major roles in cell behavior *in vivo*.

Our studies showed that the fibrinogen-like terminal knob of the tenascin molecule was responsible for tenascin’s ability to support rolling of SKW3 cells. Polyclonal and monoclonal antibodies to this terminal domain completely blocked binding to native tenascin. Furthermore, recombinant proteins containing the fibrinogen-like terminal domain supported tethering and rolling of SKW3 cells. Tenascin has been found in all vertebrates studied and has remained remarkably conserved throughout evolution (25). The fb_g region of tenascin is perched on the COOH terminus of the tenascin arm, while the NH₂ terminus of the arm contains domains that bind to the other monomers in the hexabrachion. This is analogous to the positioning of the binding domains of selectins and IgSF members that mediate rolling at the membrane-distal ends of these molecules and may facilitate accessibility for interactions in shear flow.

Tenascin is expressed in several hematopoietic and lymphoid tissues including bone marrow (37), thymus (32), spleen (43), lymph node, tonsil, and Peyer’s patches (11, and Clark, R.A., unpublished observations). In the lymph node and tonsil, tenascin is expressed in the reticular fibers of the T cell areas as well as the basal surfaces of HEVs, suggesting a role in cell migration as opposed to lymphocyte binding to the luminal surface of HEVs (Fig. 9). Previous studies have suggested that during lymphocyte migration through secondary lymphoid organs, lymphocytes may migrate along the reticular fiber network via adhesive interactions with the ECM components of these fibers (14, 15, 38). Although the rolling adhesions that we have demonstrated *in vitro* are not likely to occur *in vivo* in secondary lymphoid organs, the dynamic nature of this interaction may be well suited to support cellular migration. The dynamic reversibility of bonds formed between a cell and the substrate is a key part of cellular migration (23). Lymphocytes form bonds with tenascin that are sufficiently strong to tether the cell to the substrate but not

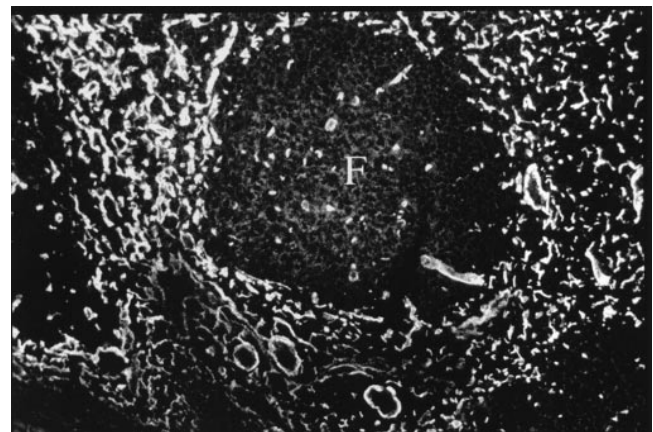


Figure 9. Immunofluorescent staining of human tonsil with tenascin mAb M168. Tenascin is heavily expressed by HEV and the reticular fiber network of the interfollicular T cell areas, while B cell follicles show sparse expression. “F” denotes a B cell follicle; three prominent HEV are visible below the follicle.

strong enough to induce cell arrest and sticking. Migration speed has been suggested to be biphasic with respect to attachment strengths; low attachment strengths do not permit the transmission of the motile force within the cell to the substrate, intermediate attachment strengths produce the fastest migration, and higher attachment strengths produce cell arrest and spreading (19, 40). Indeed, the speed of smooth muscle cell migration on fibronectin and type IV collagen substrates was found to be fastest at intermediate attachment strengths (19). Thus, we predict that the ability of tenascin to support lymphocyte rolling may also reflect the ability of this protein to support cellular migration. Alternatively, cells may simultaneously bind to tenascin through a labile interaction that does not impede but may help guide the direction of cell migration, and to other extracellular matrix components through firmer adhesive interactions that may provide traction for migration but not directionality cues.

Quite aside from this possibility, the fact that tenascin supports rolling provides interesting information about the mechanics of this protein's interaction with its receptor. Rolling of cells on tenascin is smoother than rolling of the same cell type on E-selectin at comparable rolling velocity, wall shear stress, and resistance to detachment. This suggests that a greater number of bonds is present between the cell and the substrate on tenascin than on E-selectin; the smoothness of rolling is thought to reflect small movements of the cell upon dissociation of each bond, requiring closer spacing between bonds and a greater number of bond dissociation events per unit time. This is consistent with the finding that a tenascin substrate that produced cell binding with similar shear resistance to an E-selectin substrate was found to have an 8.5-fold higher site density. However, because the density and affinity of the receptors on KG1a cells for E-selectin and tenascin are not known, the number of receptor-ligand bonds may not be proportional to the ligand density on the substrate.

The cellular receptor mediating the attachment of lymphocytes and leukocytic cell lines to tenascin under shear flow remains unidentified. Because we have ruled out all known tenascin receptors that interact with the fbg region of tenascin and that may be expressed on lymphocytes, we believe these interactions are mediated by a novel tenascin receptor. Other receptors for tenascin have been characterized with nonhematopoietic cell types; because nonhematopoietic cells can also be used in rolling assays in shear flow, it will be interesting to compare the different receptors that bind tenascin for rolling in shear flow.

In addition to the interaction with tenascin described here, laminin supports rolling via the integrin $\alpha\beta_4$ (60), and hyaluronan supports rolling via CD44 (15, 17). In addition to the molecules on cell surfaces known to support rolling, our report brings to three the number of ECM components that mediate leukocyte rolling.

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