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*J Immunol* 2002; 169:5270-5278; ;
doi: 10.4049/jimmunol.169.9.5270
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CD11b/CD18-Dependent Interactions of Neutrophils with Intestinal Epithelium Are Mediated by Fucosylated Proteoglycans

Ke Zen, Yuan Liu, Dana Cairo, and Charles A. Parkos

CD11b/CD18-mediated adhesive interactions play a key role in regulating polymorphonuclear leukocytes (PMN) migration across intestinal epithelium. However, the identity of epithelial ligands for migrating PMN remains obscure. In this study we investigated the role of carbohydrates in mediating adhesive interactions between T84 intestinal epithelial cells and CD11b/CD18 purified from PMN. Fucoidin, heparin/heparin sulfate, N-acetyl-D-glucosamine, mannose-6-phosphate, and laminarin were found to inhibit adhesion of T84 cells to CD11b/CD18. The most potent inhibitory effects were observed with fucoidin (50% inhibition at 1–5 × 10⁻⁸ M). Binding assays demonstrated that fucoidin directly bound to CD11b/CD18 in a divalent cation- and sulfation-dependent fashion that was blocked by anti-CD11b mAbs. Experiments employing CD11b/CD18 as a probe to blot T84 cell fucosylated proteins purified via fucose-specific lectin column revealed several candidate CD11b/CD18 binding proteins with molecular masses of 95, 50, 30, 25, and 20 kDa. Fucosidase treatment of T84 cells resulted in significantly reduced cell adhesion to CD11b/CD18, while no inhibition was observed after neuraminidase treatment. Finally, significant inhibition of T84 cell adhesion to CD11b/CD18 was observed after blocking cell proteoglycan synthesis with p-nitrophenyl-β-D-xylpyranoside. These findings implicate epithelial cell surface proteoglycans decorated with sulfated fucose moieties as ligands for CD11b/CD18 during PMN migration across mucosal surfaces. The Journal of Immunology, 2002, 169: 5270–5278.

Epithelial dysfunction and patient symptoms in inflammatory intestinal diseases such as ulcerative colitis and Crohn’s disease correlate with migration of polymorphonuclear leukocytes (PMN) across epithelial surfaces (1, 2). In studies modeling the process of PMN migration across mucosal surfaces, it has been shown that PMN transepithelial migration is dependent on the leukocyte β₂ integrin CD11b/CD18 (Mac-1, CR3), but not CD11a/CD18 (3). It is generally believed that CD11b/CD18-mediated adhesive interactions play a key role in regulating PMN transendothelial migration, however, have not been defined. Like other β₂ integrins, CD11b/CD18 contains both an I or an A domain and a lectin-like domain that mediate ligand specificity. An ~200-aa region comprising the I domain has been extensively characterized, revealing homology to plasma proteins such as von Willebrand factor, complement factor II, and extracellular matrix proteins, including collagen and cartilage matrix protein (4, 5). It is also regarded as a major recognition site for several ligands of CD11b/CD18 (6). While the precise localization of the lectin-like domain on CD11b is not known, results from a previous study suggested localization between the I domain and C-terminal regions of CD11b (7).

The unique structure of CD11b/CD18 enables it to bind to a wide variety of proteins and carbohydrates. Previously reported ligands for CD11b/CD18 include cell membrane proteins such as ICAM-1 (6, 8) and soluble factors such as iC3b (9, 10), fibronectin (11), LPS (12, 13), elastase (14), oligodeoxynucleotide (15), zymosan (16), β-glucan (17), heparin (heparin sulfate) (18, 19), de-natured proteins (20), and a hookworm-derived neutrophil adhesion inhibitor (21). While ICAM-1 is the only cell membrane protein ligand that has been reported for CD11b/CD18 and has been shown to play an important role in PMN transendothelial migration, it does not appear to play a role in PMN migration across intestinal epithelium. In particular, ICAM-1 expression is induced on the apical membrane of intestinal epithelial cells only under certain inflammatory conditions (3, 22). Under these conditions apically expressed ICAM-1 is not accessible as a ligand for migrating PMN, because transepithelial migration is dependent on interactions of PMN with the basolateral membrane of intestinal epithelial cells. These observations argue against a role for ICAM-1 as an adhesive ligand for PMN during transepithelial migration in the intestine. Previous studies have also suggested that heparin binds to CD11b/CD18 (18, 19). It was proposed that epithelial cell surface proteoglycans decorated with heparin sulfate moieties (HSPGs) might serve as adhesive ligands for CD11b/CD18. However, no such HSPGs have yet been identified. Furthermore, syndecan-1, a heparan sulfate proteoglycan that is expressed primarily on epithelial cells, does not support PMN adhesion (18).

Lectin-like properties of CD11b/CD18 have been borne out by several reports demonstrating carbohydrate binding to CD11b/CD18 (7, 16, 18). However, the role of epithelial cell surface carbohydrates in CD11b/CD18-mediated adhesive interactions has...
not been characterized. Previously, it was shown that certain carbohydrates, such as mannose-6-phosphate, glucose-6-phosphate, heparin, and fucoidin, are effective inhibitors of PMN migration across T84 cell monolayers (23). These results strongly suggested that carbohydrates play a role in regulating PMN transepithelial migration. However, the mechanism by which these carbohydrates regulate PMN transepithelial migration and demonstration of their existence on the epithelial cell surface have not been reported.

In the present study we tested a wide range of mono- and polysaccharides for inhibition of epithelial T84 cell adhesion to purified CD11b/CD18. We demonstrate that several carbohydrates, including fucoidin, heparin/heparin sulfate, N-acetyl-D-glucosamine, mannose-6-phosphate, and laminarin, can significantly inhibit the adhesion of T84 cells to purified CD11b/CD18. We have determined that fucoidin is the most potent inhibitor of cell adhesion and does so by binding to CD11b/CD18 in a divalent cation-dependent fashion. We also determined the role of cell surface fucose moieties and fucosylated proteoglycans in regulating T84 cell adherence to CD11b/CD18. We show that fucosylated proteins derived from solubilized intestinal epithelial cells via the fucose-specific lectin column contain several candidate CD11b/CD18 binding proteins. Based on these results we conclude that epithelial T84 cell surface proteoglycans decorated with sulfated fucose moieties serve as adhesive ligands for CD11b/CD18 during the transmigration response.

Materials and Methods

Cell lines and reagents

T84 intestinal epithelial cells were grown in a 1/1 mixture of DMEM and Ham’s F-12 medium supplemented with 15 mM HEPES buffer (pH 7.5), 14 mM NaHCO₃, 40 μg/ml penicillin, 8 μg/ml ampicillin, 90 μg/ml streptomycin, and 5% newborn calf serum (Life Technologies, Gaithersburg, MD). The fluorescent probe 2',7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester (BCFEF-AM) was purchased from Molecular Probes (Eugene, OR). The glycosidase α-1-fucosidase from bovine kidney (EC 3.2.1.51) and neuraminidase (sialidase) from arthrobacter ureaficiens (EC 3.2.1.18), which cleaves both α-2,3- and α-2,6-linked sialic acid (24), were obtained from Roche (Indianapolis, IN). Biotin-ε-aminocaproic acid N-hydroxysuccinimide ester (biotin-X-NHS), heparin (from porcine intestinal mucosa; M₀ = 13,000–15,000), and heparan sulfate (from bovine intestinal mucosa; M₀ = 7, 500) were purchased from Calbiochem (La Jolla, CA). Other mono- and polysaccharides, including fucoidin, dextran (M₀ = 42,000), dextran sulfate (M₀ = 500,000), mannose-6-phosphate, glucose-6-phosphate, chondroitin sulfate C (from shark cartilage), galactosyl-fucose, and laminarin were purchased from Sigma (St. Louis, MO). FITC-labeled lectin from tetragonolobus purpureas, tetragonolobus purpureas immobilized on 4% beaded agarose, and an anion exchanger (Dowex H⁺ 50 WX8-100) were also obtained from Sigma. A panel of functionally characterized mAbs that had been previously shown to bind to the defined regions of the CD11b extracellular domain (18, 25) was used to purify IgG. This panel of mAbs included CBRM1/23 (C domain binding, IgG2a), CBRM1/29 (1 domain binding, IgG1), CBRM1/31 (1 domain binding, IgG1), LM2/1 (1 domain binding, purified IgG1), and OKM1 (C domain binding, IgG2b). A rabbit polyclonal Ab against human CD11b R7928A was raised by immunizing rabbit with a peptide (DMMSEGPP- PGQPLQ) corresponding to the C terminus of CD11b as previously described (26). Hybridoma cells secreting a functionally inhibitory mAb against CD18 (TS1/18, IgGl) were purchased from American Type Culture Collection (Manassas, VA). A protease inhibitor mixture consisting of soybean trypsin inhibitor, benzamidine, leupeptin, pepstatin A, bestatin, and aprotinin was obtained from Calbiochem. GelCode Blue stain Reagent was obtained from Pierce (no. 24590; Rockford, IL). Nitrocellulose membrane and pre-stained m.w. markers were purchased from Bio-Rad (Richmond, CA). All other reagents, unless stated, were obtained from Sigma.

Purification of CD11b/CD18

Functionally active CD11b/CD18 was purified to homogeneity from large quantities of human PMN (~10⁶ cells) by immunoaffinity chromatography using LM2/1-Sepharose, as previously described by Diamond et al. (8). SDS-PAGE of the purified integrin, followed by Coomassie blue staining, revealed two prominent protein bands with M₀ of ~150 and 92 kDa, characteristic of CD11b and CD18, respectively (not shown). The typical yield of CD11b/CD18 from 10⁶ PMN ranged from 1 to 2 mg.

T84 cell adhesion to purified CD11b/CD18

T84 cell adhesion to immobilized, purified CD11b/CD18 was performed as previously described (25). Briefly, purified CD11b/CD18 (~100 μg/ml in 150 mM NaCl, 2 mM MgCl₂, 2 mM CaCl₂, 100 mM Tris, and 1% N-octyl-β-D-glucopyranoside, pH 7.4) was diluted 20-fold with HBSS and immediately added to 96-well, flat-bottom, microtiter plates (ICN Biomedical, Aurora, OH; 50 μl/well). Microtiter plates were kept at 4°C overnight to allow for protein binding. Non specific protein binding was blocked by adding 1% BSA in HBSS solution for 1 h at room temperature. Trypsin/EDTA-elicted T84 cells were then washed twice with HBSS and incubated with 5 μg/ml BCECF-AM (Molecular Probe) in HBSS for 15 min at 37°C. After washing by centrifugation, fluorescence-labeled T84 cells were added to CD11b/CD18-coated plates (~2.5×10⁵ cells/well in a total volume of 150 μl), followed by stationary incubation at 37°C for 1 h to allow for cell adhesion. To quantify adhesion, plates were gently washed three times, and the fluorescence of each well was determined using a fluorescence microtiter plate reader at excitation/emission wavelengths of 485/535 nm (Millipore, Milford, MA). Cell adherence was calculated as the percentage of total applied cells: 100 × (postwash fluorescence/pre-wash fluorescence). In some experiments, T84 cells were pre-treated with test compounds/mAbs as indicated before addition to CD11b/CD18-coated wells.

Biochemical modification of fucoidin

Desulfation of fucoidin was achieved by solvolysis in dimethylsulfoxide as previously described (27). Briefly, the sodium salt of fucoidin (250 mg) was converted into free acid using Dowex 50 W-X8 (H⁺) (Sigma), neutralized with pyridine, and lyophilized. The pyridinium salt was then dissolved in 10 ml of 10% aqueous Me₂SO. The solution was heated at 80°C for 90 min and cooled in an ice bath. The reaction mixture was diluted to 20 ml with water, and the pH was adjusted to 9.0 using 0.1 M NaOH. The mixture was then dialyzed and lyophilized.

Fucoidin (normal and desulfated) was biotinylated using biotin-X-NHS after cyanogen bromide activation and aminolkylation with dianinomethane. This method has been shown to effectively label polysaccharides while preserving the polysaccharide structure (28). Briefly, 500 μg of fucoidin was activated with 875 mg of cyanogen bromide in 28 ml of 0.5 M NaHCO₃ solution (pH 11) with vigorous stirring for 30 min. Activated carbohydrates were de-salted over a Sephadex G-50 column and mixed with 750 mg of dianinomethane in 15 ml of 0.4 M NaHCO₃ solution (pH 8.5). The mixture was gently stirred at 4°C for 1 h and dialyzed against PBS containing 0.2% Triton X-100 for 24 h. One mg of Aminolkyld derivative dissolved in 100 ml of 0.1 M NaHCO₃ (pH 8.0) was reacted with 27 mg of biotin-X-NHS (Calbiochem). After 6 h at 25°C the solution was extensively dialyzed. The biotinylated aminomethyl derivatives were recovered by lyophilization.

Fucoidin-CD11b/CD18 binding assay

The binding of fucoidin to CD11b/CD18 was studied using two reciprocal methods. First, to assay binding of biotinylated fucoidin to immobilized CD11b/CD18, purified CD11b/CD18 was diluted (~20-fold) with HBSS and bound to 96-well, flat-bottom, microtiter plates as described above. After blocking with 2% BSA in HBSS, biotinylated fucoidin (10 μg/ml) was added (37°C, 30 min). Plates were washed three times with PBS containing 0.5% BSA and then incubated with HRP-conjugated streptavidin in HBSS containing 2% BSA for 30 min at room temperature. Polysaccharide/CD11b complexes were detected colorimetrically using ABTS. Controls included BSA-coated wells or wells without fucoidin (streptavidin) instead of fucoidin. As a reciprocal method, CD11b/CD18 was assayed for binding to immobilized fucoidin. Here, 100 μl of fucoidin (5 mg/ml) in HBSS was added to microtiter wells, followed by incubation for 2 h at 37°C. After washing off unbound fucoidin, fucoidin-coated wells were blocked with 2% BSA in HBSS for 1 h at room temperature. Purified CD11b/CD18 (5 μg/ml) in HBSS containing 0.1% Triton X-100 was added to the fucoidin-coated microtiter wells. After 1-h incubation at 37°C, the plates were washed three times with HBSS containing 0.1% Triton X-100, followed by incubation with anti-CD18 mAb TS1/18 (1/500 dilution) or polyclonal Ab R7928A (1/200 dilution) in HBSS containing 2% BSA. After washing away unbound Ab, wells were incubated with HRP-conjugated secondary Ab, followed by addition of substrate (ABTS) for color development and OD measurement. Wells coated with BSA (no fucoidin) or chondroitin sulfate C served as controls.
Neoproteoglycan preparation and CD11b/CD18 binding experiments

A neoproteoglycan derivative of fucoidin was prepared according to the method described by Matsumoto et al. (29). Briefly, 30 mg of fucoidin was dissolved in 4 ml of distilled water and mixed with 30 mg of N-ethylcarbonyl-2-ethoxy-1,2-dihydroquinoline in 6 ml of ethanol. After preincubation at room temperature for 2 h, 60 mg of BSA was added, and the mixture was incubated at 4°C for 2 days with gentle shaking. The mixture was then dialyzed against PBS, followed by dialyization and storage at 4°C. The formation of neoproteoglycan was confirmed by SDS-PAGE, demonstrating a shift in the m.w. of BSA under nonreducing conditions. To test the binding of fucoidin neoproteoglycan to CD11b/CD18, fucoidin neoproteoglycan (fucoidin-BSA conjugate) and BSA (control) were subjected to SDS-PAGE analysis on a 4–15% gradient gel (5 μg/lane). Proteins were directly visualized with silver staining or were electrophoretically transferred onto nitrocellulose filter (Bio-Rad, Richmond, CA), followed by probing with CD11b/CD18. To probe with CD11b/CD18, nitrocellulose filters were first incubated with blocking solution (HBSS containing 0.2% Triton X-100 and 1% BSA) for 1 h at room temperature. The filters were then incubated with purified CD11b/CD18 (final concentration, 5–10 μg/ml) in blocking solution for 2 h at 37°C. After washing filters with HBSS containing 0.1% Triton X-100 (three times for 5–10 min each time), nitrocellulose filters were incubated sequentially with anti-CD18 mAb TS1/18 (1/1000 dilution) and HRP-conjugated goat antimouse antibodies (1/1000 dilution), followed by detection with ECL. All reactions were performed in the presence of 2 mM Mg2+ and Ca2+.

Probing T84 cell fucose residue-containing proteins with CD11b/CD18

Isolation of fucosylated proteins from detergent lysate of T84 cells was performed using a fucose-specific lectin column. Briefly, T84 cells (~4 x 106) were harvested with trypsin/EDTA and lysed with 30–35 ml of lysis buffer containing 100 mM Tris, 150 mM NaCl, 2.5 mM CaCl2, 2.5 mM MgCl2, 1% Triton X-100, and a mixture of proteinase inhibitors, pH 7.3. After a 1-h high speed (45,000 g) centrifugation, the cell lysate supernatant was pumped through a fucose-specific lectin column (tetragonolobus purpureas) at 8°C. FITC-conjugated fucose-specific lectin was performed as previously described (30). Trypsin/EDTA elicited T84 cells were washed three times with cold HBBS before B7ECF-AM labeling and use in adhesion assays. To remove T84 cell surface sialic acid residues, trypsin/EDTA-elicited cells were incubated with neuraminidase (0.1, 0.5, and 1.0 U/ml) in PBS containing 2.5 mM EDTA, 1 mM benzamidine, and 10 μg/ml each of PMSF and pepstatin A, pH 7.0. Cells were then washed with cold HBBS before B7ECF-AM labeling and cell adhesion assays. To remove T84 cell surface fucose residues, trypsin/EDTA-elicited cells were incubated with fucose-specific lectin column as previously described (30). Trypsin/EDTA elicted T84 cells were washed three times with cold HBBS and then blocked with HBSS containing 5% FCS for 1 h at room temperature. Cells (5 x 105/ml) were then incubated with FITC-conjugated fucose-specific lectin from tetragonolobus purpureas (final concentration, 20 μg/ml) in HBSS containing 5% FCS for 30 min at room temperature. Cells were washed three times with HBSS, shortly fixed with 1% paraformaldehyde (1–5 min), washed twice with HBSS, mounted on slides, and observed under a fluorescence microscope. In this experiment T84 cells were also treated with 1.0 U/ml fucosidase at 37°C for 1 h before labeling with FITC-lectin.

Results

Certain carbohydrates inhibit T84 cell adhesion to immobilized CD11b/CD18

We tested a wide variety of carbohydrates for inhibition of epithelial cell adhesion to purified CD11b/CD18. We previously demonstrated that purified CD11b/CD18 immobilized in microtiter wells supported binding of 30–50% of the total applied T84 cell load after 1 h of incubation at 37°C, while <5% of applied cells adhered to wells coated with BSA alone (25). Complete inhibition of T84 cell adhesion to CD11b/CD18 by mAb CBRM1/29 demonstrates the specificity of this interaction. Table I represents a summary of the effects of a panel of carbohydrate moieties on T84 cell adhesion to CD11b/CD18. As shown, among all carbohydrates tested, fucoidin potently inhibited adhesion in a concentration-dependent fashion. At a low concentration of 0.1 mg/ml, fucoidin significantly inhibited cell-integrin adhesion (79.3 ± 6.8% inhibition; p < 0.001). Adhesion of T84 cells to CD11b/CD18 was also significantly reduced by heparin (46.5 ± 8.1% inhibition; p < 0.03), heparin sulfate (43.2 ± 9.4% inhibition; p < 0.03), N-acetyl-D-glucosamine (40.8 ± 10.2% inhibition; p < 0.03), mannose-6-phosphate (38.5 ± 6.4% inhibition; p < 0.03), and

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*Data are presented as the percent inhibition by each carbohydrate ± SD described in Materials and Methods. Results are from three separate experiments.

p < 0.03.

p < 0.001.

Blockage of T84 cell proteoglycan synthesis

Biosynthesis of proteoglycans was blocked in T84 cells as previously described (30). T84 cells in 12-well tissue culture plates (~75% confluence) were grown in cell culture medium (1:1 mixture of DMEM and Ham’s F-12 medium in 5% dialyzed FCS) containing 2 mM N-(p-nitrophenyl)-β-D-xlylopyranoside (β-xy) overnight (37°C, 5% CO2). As a control, T84 monolayers were incubated in cell culture medium containing 2 mM N-(p-nitrophenyl-α-D-xlylopyranoside (α-xy), which does not inhibit biosynthesis of cell proteoglycans. T84 cell monolayers were washed twice with cell culture medium before harvesting, labeling with B7ECF-AM, and used in cell adhesion assays. In some experiments cells were also treated with a combination of fucosidase and 2 mM β-xy. The cells that were grown in the presence of 2 mM β-xy were harvested with trypsin/EDTA and further incubated with fucosidase (1.0 U/ml in PBS containing 2.5 mM EDTA, 1 mM benzamidine, and 10 μg/ml each of PMSF and pepstatin A for 60 min at 37°C. After washing with cold HBSS, cells were labeled with B7ECF-AM in HBSS and assayed for adhesion to immobilized CD11b/CD18. Trypan blue exclusion was assessed to ensure cell viability.

Statistics

Data are presented as the mean ± SD and were compared by Student’s t-test.
laminarin (39.7 ± 11.3%; p < 0.03), but only at high concentration (10 mg/ml). However, no significant inhibition was found in the presence of glucose-6-phosphate, dextran, dextran sulfate, chondroitin sulfate C, l-fucose, or galactose. Unlike fucoidin, which is a polymer of sulfated l-fucose, the monosaccharide l-fucose had no effect on T84 cell adhesion to CD11b/CD18. As shown in Fig. 1, 50% inhibition of T84 cell binding to CD11b/CD18 was observed at fucoidin concentrations of 1–5 × 10−8 M.

To determine whether the observed inhibitory effects on adhesion were the result of fucoidin binding to CD11b/CD18, trypsin/EDTA-elicted T84 cells or microtiter wells containing immobilized CD11b/CD18 were preincubated with fucoidin as detailed in Materials and Methods. After washing away unbound fucoidin with HBSS, cell adhesion assays were performed in the absence of fucoidin. As shown in Fig. 2, preincubation of T84 cells with fucoidin did not affect cell adhesion to CD11b/CD18 whereas preincubation of immobilized CD11b/CD18 with fucoidin strongly reduced the T84 cell adhesion. These results suggest that fucoidin inhibits T84 cell adhesion to CD11b/CD18 through direct interactions with CD11b/CD18.

**Fucoidin binds directly to CD11b/CD18**

The cell adhesion results suggested the possibility of binding interactions between fucoidin and CD11b/CD18, and additional experiments were performed to examine this possibility. The binding of fucoidin to CD11b/CD18 was investigated using two reciprocal binding assays: biotinylated fucoidin binding to immobilized CD11b/CD18 and CD11b/CD18 binding to immobilized fucoidin. For the first assay fucoidin was biotinylated (28) and added to CD11b/CD18-coated microtiter wells. After incubation at 37°C for 1 h, bound fucoidin was detected with HRP-streptavidin and color development. We observed that biotinylated fucoidin bound to CD11b/CD18, but not to BSA (Fig. 3A). In the reciprocal assay microtiter wells were first coated with unlabeled fucoidin, followed by addition of purified CD11b/CD18 in buffer containing Mg²⁺ and detergent. CD11b/CD18 binding was then detected with anti-CD11b Ab (TS1/18), HRP-conjugated goat anti-mouse secondary Ab, and color development. As shown in Fig. 3B, CD11b/CD18 bound to fucoidin-coated wells; in contrast, there was little binding of CD11b/CD18 to either BSA-coated wells or chondroitin sulfate C-coated wells.

To further characterize the binding properties of fucoidin to CD11b/CD18, binding assays were performed using a panel of well-characterized anti-CD11b/CD18 mAbs that had previously been mapped to various domains on the extracellular portion of CD11b (18, 25) and in the absence of divalent cations. As shown in Fig. 4, the binding of biotinylated fucoidin to CD11b/CD18 was largely blocked by anti-CD11b Abs with epitopes on the membrane-proximal, C-terminal region of CD11b (CBRM1/23 and OKM1) as well as anti-CD11b Abs with epitopes residing within the I domain and amino-terminal region (CBRM1/29, CBRM1/31, CBRM1/32, and CBRM1/40).

**FIGURE 1.** Concentration-dependent inhibition of T84 cell adhesion to CD11b/CD18 by fucoidin. As detailed in Materials and Methods, BCECF-labeled T84 cells were incubated with CD11b/CD18-coated wells for 1 h at 37°C in the presence of polysaccharides. T84 cell adhesion is expressed as a percentage of the total applied cells. Values are the mean ± SD of three separate experiments, each performed in triplicate.

**FIGURE 2.** Fucoidin-mediated inhibition of T84 cell adhesion to CD11b/CD18 occurs through direct interactions with CD11b/CD18. Before cell adhesion assays, T84 cells (F+T84 cells) or CD11b/CD18-coated microtiter wells (F+CD11b/CD18) were preincubated with 10 mg/ml of fucoidin for 30 min at 37°C, followed by washing. Cell adhesion assays were then performed in the absence of fucoidin as detailed in Materials and Methods. Control wells (no treatment) represent T84 cell binding to CD11b/CD18 in the absence of fucoidin pretreatment, whereas blank represents binding of T84 cells to wells coated with BSA only. Values are the mean ± SD of three separate experiments, each performed in triplicate.

**FIGURE 3.** Binding of fucoidin to CD11b/CD18. A, Binding of biotinylated fucoidin (B-fucoidin) to immobilized CD11b. As detailed in Materials and Methods, microtiter wells coated with CD11b/CD18 were blocked with BSA, followed by addition of 100 µg/ml B-fucoidin for 1 h at 37°C. B-fucoidin binding was assessed after addition of HRP-streptavidin, followed by color development and OD measurement (405 nm). B, Binding of CD11b/CD18 to immobilized fucoidin and chondroitin sulfate C (control). Polysaccharides (5 mg/ml in HBSS) were added to wells (50 µl/well, 37°C, 2 h), followed by blocking with BSA and addition of CD11b/CD18 (2.5 µg/ml, 37°C, 1 h). CD11b/CD18 binding to fucoidin was assessed after addition of mAb TS1/18, followed by incubation with HRP-conjugated secondary mAb, color development, and OD measurement (405 nm). Binding assays were performed in the presence of 2 mM Ca²⁺ and Mg²⁺. BSA-coated wells served as controls in both experiments. Values are the mean ± SD of three separate experiments, each performed in triplicate.
Sulfation of fucoidin determines binding to CD11b/CD18

Sulfation of polysaccharides has been shown to be important for a number of glycosylated proteins (proteoglycans) interacting with integrins (32–34). Thus, experiments were performed to examine whether sulfation of fucoidin plays a role in binding to CD11b. For these experiments fucoidin was chemically desulfated, followed by biotinylation before use in binding assays with CD11b/CD18. The desulfation method we used in the experiments did not destroy the polymer structure of fucoidin and resulted in 75%–90% desulfation of fucoidin polysaccharide. As shown in Fig. 6A, the binding of desulfated fucoidin to immobilized CD11b/CD18 was diminished by 65% compared with that observed with untreated fucoidin. In Fig. 6B, desulfated fucoidin was also tested for inhibition of T84 cell adhesion to purified CD11b/CD18. As shown, binding of T84 cells to CD11b/CD18 in the presence of 10 mg/ml of desulfated fucoidin was only mildly inhibited (∼25% decrease), whereas binding in the presence of untreated fucoidin was reduced by 95%.

Fucosidase treatment of T84 cells diminishes adhesion to CD11b/CD18

To determine whether epithelial cell surface fucosylation plays a role in adhesion to CD11b/CD18, T84 cells were treated with fucosidase and assayed for adhesion. For these experiments trypsin/EDTA-elicited T84 cells were labeled with BCECF, followed by incubation with different amounts of fucosidase at 37°C. As a control, parallel experiments were performed in which cells were incubated with neuraminidase to remove cell surface sialic acid residues. The enzyme-treated cells were still 95% viable based on trypan blue exclusion 2 h after enzymatic treatments. Fucosidase treatment significantly reduced T84 cell adhesion to CD11b/CD18.
in a concentration- and time-dependent fashion (Fig. 7). In contrast, no significant inhibition was observed after pretreatment of cells with neuraminidase. These results suggest that T84 cells contain cell surface fucose-like structures that partially mediate binding to CD11b/CD18.

Labeling experiments were also performed to examine the effect of fucosidase treatment on binding of fucose-specific lectin (tetragonolobus purpureas) to T84 cells. Suspensions of T84 cells were treated with fucosidase (1.0 U/ml, 60 min), followed by fixation and staining with FITC-labeled lectin (Fig. 8). Untreated T84 cells were strongly labeled with the lectin (Fig. 8B). In contrast, fucosidase treatment demonstrated a dramatic reduction in labeling of T84 cells with FITC-lectin (Fig. 8D). As in controls, there was no decrease in labeling with FITC-wheat germ agglutinin after fucosidase treatment, nor was tetragonolobus purpureas labeling altered by neuraminidase treatment (data not shown).

Inhibition of proteoglycan synthesis reduces T84 cell adhesion to CD11b/CD18

Since the above results and those reported previously (35, 36) confirm the existence of fucose residues on the epithelial cell surface, we reasoned that inhibition of the synthesis of cell surface proteins decorated with such residues might influence adhesive interactions with CD11b/CD18. Candidate cell surface molecules containing fucose moieties include glycolipids and proteoglycans that are decorated with sulfated fucose polymers. To inhibit proteoglycan synthesis, T84 cell monolayers were cultured overnight in the presence of 2 mM β-xyl, followed by labeling and use in cell adhesion assays. As a control, parallel incubations were performed in the presence of 2 mM α-xyl, which does not inhibit proteoglycan synthesis. In both conditions cell viability was >95% after treatment as assessed by trypan blue exclusion. As shown in Fig. 9, treatment of T84 cells with α-xyl had no significant effect on cell adhesion to CD11b/CD18 compared with untreated cells (52.5 ± 6.7 vs 51.7 ± 5.9% of total applied cells adhering to CD11b/CD18 for α-xyl vs

FIGURE 7. Effect of fucosidase on epithelial cell adherence to CD11b/CD18. As detailed in Materials and Methods, trypsin/EDTA-elicited T84 cells were pretreated with fucosidase or neuraminidase (0, 0.1, 0.5, and 1.0 U/ml, respectively) for 30 min at 37°C, washed twice with HBSS and used in adhesion assays (A). B, Cells were pretreated with fucosidase (0.5 U/ml) or neuraminidase (0.5 U/ml) at 37°C for different lengths of time as indicated, followed by washing with HBSS, and were used in CD11b/CD18 adhesion assays. Values are the mean ± SD of three separate experiments, each performed in triplicate.

FIGURE 8. Fluorescence staining of T84 cell surface for fucosylated structures. As detailed in Materials and Methods, trypsin/EDTA-elicited T84 cells were incubated with (C and D) or without (A and B) fucosidase (1.0 U/ml). Cells were then washed three times with cold blocking buffer (HBSS with 1% BSA) and incubated with FITC-labeled lectin from tetragonolobus purpureas (25 μg/ml) on ice for 30 min. After a short fixation with paraformaldehyde, cells were then mounted on glass slides and visualized under a fluorescence microscope. A and C. Phase contrast images of the fluorescence photomicrographs of cells in B and D, respectively. Bars = 50 μm.

FIGURE 9. Inhibition of T84 cell proteoglycan synthesis reduces cell adhesion to CD11b/CD18. As detailed in Materials and Methods, T84 cells were cultured in the presence of 2 mM β-xyl or 2 mM α-xyl in DMEM overnight, followed by harvest and BCECF labeling for cell adhesion assays. In a subset of experiments T84 cells pretreated with β-xyl were further incubated with fucosidase (1.0 U/ml) for 30 min at 37°C before adhesion assays. Values are the mean ± SD of three separate experiments, each performed in triplicate. *, p < 0.01.
no drug treatment, respectively). In contrast, adhesion of β-xy-treated T84 cells to purified CD11b/CD18 was significantly reduced compared with that of α-xy-treated cells (33.4 ± 4.8 vs 52.5 ± 6.7% of applied cells adhering to CD11b/CD18 for β-xy vs α-xy, respectively). Interestingly, incubation of β-xy-treated T84 cells with fucosidase (1.0 U/ml, 60 min, 37°C) did not result in further significant inhibition of adhesion to CD11b/CD18.

Identification of fucose-containing proteins from T84 cells that bind to CD11b/CD18

Experiments were performed to identify fucosylated protein(s) in T84 cells that bind to CD11b/CD18. As detailed in Materials and Methods, fucose-containing proteins were first purified from T84 cells using a fucose-specific lectin column (tetragonolobus purpureas immobilized on 4% beaded agarose). The mixture of fucosylated proteins was dialyzed, concentrated, and separated by SDS-PAGE (12% gel concentration). Proteins were also transferred to nitrocellulose filters for our modified Western blot analysis (probing filters with purified CD11b/CD18 protein in the presence of Mg2+ and detergent). In our experiment, ~800 μg of total fucosylated proteins were isolated from ~4 × 10^7 T84 cells. As shown in Fig. 10, CD11b/CD18 recognized several major bands at Mr of 95, 50, 30, 25, and 20 kDa (lane 3, arrowheads). Those protein bands were absent in the control experiment in which no CD11b/CD18, but only anti-CD18 mAb and HRP-conjugated secondary mAb, was used (Fig. 10, lane 2). The same amount of all fucosylated proteins from T84 cells was displayed by staining with GelCode Blue (Fig. 10, lane 1).

Discussion

Transepithelial migration of PMN is a pathological hallmark of disease states such as ulcerative colitis, Crohn’s disease, and infectious enterocolitis. It has been well established that the β2 integrin CD11b/CD18 plays a key role in mediating the initial stage of transepithelial migration consisting of PMN adhesion to the basolateral epithelial surface (25, 37–39). In the present study we have further characterized the interaction of carbohydrates with CD11b/CD18 and demonstrated a role of fucosylated proteoglycans in CD11b/CD18-mediated epithelial cell adhesion.

In a previous study we reported that several sulfated polysaccharides, including fucoidin, inhibited PMN transmigrational motion (23). Since these experiments consisted of cell-cell adhesion and transmigration assays, the molecular mechanisms of the carbohydrate-mediated inhibition observed were not clear. The present study was undertaken to better define the mechanism of carbohydrate-mediated inhibition of transepithelial migration and the nature of epithelial ligands for migrating PMN. Here, using assays of T84 cell adhesion to highly purified CD11b/CD18, we screened a wide range of mono- and polysaccharides and identified several carbohydrates that significantly inhibit such adhesive interactions. We found that the sulfated, l-fucose-rich polysaccharide fucoidin is the most potent inhibitor (>90% inhibition) and produces 50% inhibition at a concentration of 1–5.0 × 10^{-8} M. Using a number of binding assays, we determined that fucoidin binds directly and specifically to CD11b/CD18.

The CD11b/CD18 binding properties of fucoidin are similar to those of L-selectin, which has also been shown to bind fucoidin (40, 41). In particular, fucoidin binds to the lectin-like domain of L-selectin with properties characteristic of C-type lectin-carbohydrate binding interactions. CD11b also has a lectin-like domain that is most likely present between the I domain and C-terminal regions of the extracellular domain (7, 16). Our results indicate that the binding of fucoidin to CD11b/CD18 is divalent cation dependent, which is a major binding property of C-type lectins. The similarity of carbohydrate binding patterns between CD11b/CD18 and selectin have also been demonstrated in other cases. For example, heparin and complement factor H have been shown as ligands for both integrins (13, 18, 19, 42, 43). We have attempted to map the fucoidin binding site on CD11b/CD18 using anti-CD11b mAbs with defined epitopes. Since fucoidin binding to CD11b/CD18 was strongly inhibited by I domain and C-terminal mAbs (Fig. 4), it is likely that fucoidin binds to a range of sites on CD11b/CD18. Interestingly, in assays of PMN transmigrational migration and T84 cell adhesion to purified CD11b/CD18, it is clear that the I domain is the most critical functional binding domain (25). Furthermore, mAb TS1/18, which binds to CD18, strongly inhibits T84 cell binding to CD11b/CD18, but has no effect on fucoidin binding to CD11b/CD18, suggesting that the fucoidin binding site is localized on CD11b. Despite these differences, fucoidin is a potent inhibitor of PMN transmigrational migration (23).

The binding of fucoidin to CD11b/CD18 was determined to be strongly dependent on the degree of sulfation and polymer structure. As shown in Table I, the monosaccharide l-fucose does not affect cell-CD11b/CD18 adhesion, nor does D-fucose even at concentrations as high as 20 mM (data not shown). The functional activity of fucoidin in our CD11b/CD18 binding assays was significantly reduced by desulfation, suggesting a critical role of sulfation in fucoidin-CD11b/CD18 interactions. One explanation for this observation would be that basic amino acid residues in CD11b play a role in binding to sulfated fucans, as reported for other adhesion proteins (44). However, other sulfated polysaccharides, such as chondroitin sulfate C and dextran sulfate, with comparable charge densities and m.w. did not inhibit T84 cell binding to CD11b/CD18. These observations suggest that sulfation of fucoidin may play an important role in modifying or maintaining the structure necessary for binding to CD11b/CD18 instead of simple charge contributions. This hypothesis is further supported by our finding that neuraminidase treatment of T84 cells, which results in removal of negatively charged sialic acid residues, did not reduce adhesion to CD11b/CD18. Interestingly, CD18 contains a basic amino acid domain that has been implicated in carbohydrate binding interactions (45). While we only tested one CD18-reactive mAb TS1/18, we failed to detect inhibition with this function blocking mAb.
As a ligand for L-selectin (40, 41) or the scavenger receptor (46, 47), fucoidin has been implicated in a wide range of physiological events, including lymphocyte tethering and rolling (48) and phagocytosis (47, 49). A role for fucoidin in cell adhesion has also been reported. In particular, bovine sperm binding to oviductal epithelium has been shown to be dependent on fucose residues (50) and can be abolished by treatment of the epithelium with fucosidase (36). Fucoidin and other sulfated polysaccharides have also been shown to be involved in sperm-egg interactions in birds (30). In vitro studies of human cells have shown that sulfated polysaccharides, including fucoidin, can inhibit lymphocyte-to-epithelial transmission of HIV-1 (51, 52). Furthermore, others have reported that adhesion of both T and B lymphocytes to cultured fibroblasts was strongly inhibited by fucoidin, whereas fucose and mannan had no effect (53). These results are in agreement with our observations and support the functional relevance of fucoidin in epithelial cell interactions with CD11b/CD18. It is possible that such interactions might be exploited therapeutically. For example in animal models, cellular infiltrates associated with meningitis can be reduced by infusion of fucoidin (22). Whether fucoidin could be used to inhibit neutrophil transepithelial migration in ulcerative colitis or Crohn’s disease remains to be determined.

While the nature of fucoidin-like epithelial counter-receptors for CD11b/CD18 remains undefined, candidate structures have been reported on epithelial cell surface (35, 54). In particular, there are a number of structures on the epithelial cell surface that contain fucose residues, including glycolipids, membrane glycoproteins with terminal fucosylation, and cell surface proteoglycans decorated with fucose or sulfated fucose sugar chains. Our data suggest that intestinal epithelial cell surface proteoglycans decorated with sulfated fucose polymers play an important role in CD11b/CD18-mediated adhesive interactions. This is consistent with our finding that blockage of biosynthesis of epithelial cell proteoglycans by β-xy reduced T84 cell adhesion to CD11b/CD18 by the same amount observed after treatment with a fucosidase (Fig. 9). While the exact identities of such proteoglycans are not known, we have identified several candidate fucosylated proteins that can bind to CD11b/CD18. Due to the material (whole cell lysates from epithelial cells) we used in purification, it is possible that some of these fucosylated proteins recognized by CD11b/CD18 are not expressed on the cell surface. Further characterization, including use of purified epithelial cell plasma membranes or selective cell surface labeling with tagged lectin as starting material, will facilitate identification of the subset of epithelial cell surface proteoglycans that serves as adhesive ligand for CD11b/CD18.

Acknowledgments

We thank Susan Voss for her help with the T84 and H29 cell culture.

References


