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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-xyloroynoside. 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.
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 adhesion 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)-carboxy-fluorescein acetoxyethyl ester (BCECF-AM) was purchased from Molecular Probes (Eugene, OR). The glycosidase α-L-fucosidase from bovine kidney (EC 3.2.1.51) and neuraminidase (sialidase) from arthrobacter ureafaciens (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-aminoa- prolic acid N-hydroxysuccinimide ester (biotin-X-NHS), heparin (from porcine intestinal mucosa; M, 13,000–15,000), and heparan sulfate (from bovine intestinal mucosa; M = 7, 500) were purchased from Calbiochem (La Jolla, CA). Other mono- and polysaccharides, including fucoidin, dextran (M, 428,000), dextran sulfate (M, 500,000), mannose-6-phosphate, glucose-6-phosphate, chondroitin sulfate C (from shark cartilage), galactosamine-6-sulfate, N-acetyl-D-glucosamine, and laminarin were purchased from Sigma (St. Louis, MO). FITC-labeled lectin from tetragonalobus purpuratus, tetragonalobus purpuratus 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 as purified IgG. This panel of mAbs included CBRM1/23 (C domain binding, IgG2a), CBRM1/29 (I domain binding, IgG1), CBRM1/31 (I domain binding, IgG1), LM2/1 (I domain binding, purified IgG1), and OKM1 (C domain binding, IgG2b). A rabbit polyclonal Ab against human CD11b R792/8A was raised by immunizing rabbit with a peptide (DOWEX H+ 50 WX8-100) corresponding to the C terminus of CD11b as previously described (26). Hybridoma cells secreting a functionally inhibitory mAb against CD18 (TS1/18, IgG1) 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 (~1011 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 M, of ~150 and 95 kD, characteristic of CD11b and CD18, respectively (not shown). The typical yield of CD11b/CD18 from 1010 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. Nonpecific 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 pretreated 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 dianiminoxylyl. This method has been shown to effectively label polysaccharides while preserving the polysaccharide structure (28). Briefly, 500 μg of polysaccharide was mixed 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 dianiminoxylyl in 15 ml of 0.4 M NaHCO3 solution (pH 9.5). The mixture was gently stirred at 4°C for 1 h and dialyzed against PBS containing 100 mM NaCl. The 200 mg of Aminolkylation-derived dissolved in 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 dia- lyzed. The biotinylated aminolkylation 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 HBSS containing 0.5% BSA and then incubated with HBSS containing 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 alone). 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 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-ethoxycarbonyl-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 hylophilization and storage at 4°C. Further details of the formation of neoproteoglycan were 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 μg/ml) 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 secondary Ab, followed by detection with ECL. All reactions were performed in the presence of 2 mM MgCl2 and CaCl2.

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 × 10⁸) 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 protease 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 8–10 ml/h. After extensive washing with HBSS containing 0.1% Triton X-100, the column was then eluted with a solution of 200 mM Tris, 50 mg/ml α-fucose, and 0.8% SDS, pH 9.5. The protein collection was immediately neutralized with 1.5 M Tris buffer (pH 7.2), dialyzed extensively against HBSS using 10-kDa cutoff dialysis bag, and concentrated before further analysis. Purified proteins were analyzed by SDS-PAGE (10 μg/lane), followed by either direct GelCode Blue staining or CD11b/CD18 probing after transfer onto nitrocellulose filters as described above.

Cell surface deglycosylation experiments

Removal of T84 cell surface fucosides was performed as described by Brennan et al. (24) with slight modifications. Briefly, trypsin/EDTA-elicted T84 cells (5 × 10⁶ cells/ml) were incubated at 37°C for different lengths of time with fucosides (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 HBSS before BICEF-AM labeling with BICEF-AM and cell adhesion assays. To remove T84 cell surface sialic acid residues, trypsin/EDTA-elicted cells were incubated with neuraminidase (0.1, 0.5, and 1.0 U/ml) in PBS (pH 7.0) containing 2.5 mM EDTA, 1 mM benzamidine, and 10 μg/ml each of PMSF and pepstatin A at 37°C for different lengths of time. Cells were then washed with cold HBSS before BICEF-AM labeling and used in adhesion assays. Controls included cells incubated in the same buffers and conditions, but without enzymes. Trypan blue exclusion was assessed to verify cell viability after enzyme treatment.

Cell surface staining with fucose-specific lectin

Cell surface staining with fucosic-specific lectin was performed as previously described (30). Trypsin/EDTA elicted T84 cells were washed three times with cold HBSS and then blocked with HBSS containing 5% FCS for 1 h at room temperature. Cells (5 × 10⁶/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.

Blockage of T84 cell proteoglycan synthesis

Biosynthesis of proteoglycans was blocked in T84 cells as previously described (31). 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% diazole FCS) containing 2 mM n-propenyl-β-D-xylopyranoside (β-xy) overnight (37°C, 5% CO₂). As a control, T84 monolayers were incubated in cell culture medium containing 2 mM n-propenyl-α-D-xylopyranoside (α-xy), which does not inhibit biosynthesis of cell proteoglycans. T84 cell monolayers were washed twice with cell culture medium before harvesting, labeling with BICEF-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 BICEF-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.

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), mannos-6-phosphate (38.5 ± 6.4% inhibition; p < 0.03), and

Table I. Summary of carbohydrate effects on T84 cell-CD11b/CD18 adhesion

<table>
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<tr>
<th>Carbohydrate</th>
<th>0.1 mg/ml</th>
<th>1.0 mg/ml</th>
<th>10 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-fucose</td>
<td>4.1 ± 2.1</td>
<td>3.9 ± 1.7</td>
<td>4.5 ± 1.7</td>
</tr>
<tr>
<td>Galactose</td>
<td>3.5 ± 1.9</td>
<td>4.6 ± 3.6</td>
<td>6.4 ± 4.2</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>1.9 ± 1.2</td>
<td>4.5 ± 2.1</td>
<td>7.6 ± 3.6</td>
</tr>
<tr>
<td>Dextran</td>
<td>3.9 ± 2.8</td>
<td>5.4 ± 3.8</td>
<td>8.6 ± 5.2</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>2.8 ± 2.1</td>
<td>6.7 ± 4.3</td>
<td>7.3 ± 6.1</td>
</tr>
<tr>
<td>Chondroitin C</td>
<td>1.8 ± 1.1</td>
<td>4.2 ± 2.2</td>
<td>5.8 ± 2.6</td>
</tr>
<tr>
<td>Heparin</td>
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<td>10.6 ± 3.9</td>
<td>46.5 ± 8.1a</td>
</tr>
<tr>
<td>Heparin sulfate</td>
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<td>9.7 ± 2.6</td>
<td>43.2 ± 9.4a</td>
</tr>
<tr>
<td>N-acetyl-d-glucosamine</td>
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<td>11.3 ± 3.6</td>
<td>40.8 ± 10.2a</td>
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<tr>
<td>Laminarin</td>
<td>7.6 ± 3.1</td>
<td>13.2 ± 4.7</td>
<td>39.7 ± 11.3a</td>
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<tr>
<td>Mannos-6-phosphate</td>
<td>9.1 ± 3.6</td>
<td>14.6 ± 6.2</td>
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</tr>
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<td>Fucoidin</td>
<td>79.3 ± 6.8</td>
<td>94.6 ± 8.4</td>
<td>97.1 ± 7.1a</td>
</tr>
</tbody>
</table>

a 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.
CD18 was observed at fucoidin concentrations of 1 M shown in Fig. 1, 50% inhibition of T84 cell binding to CD11b/fucose had no effect on T84 cell adhesion to CD11b/CD18. As microtiter wells were A CD11b/CD18, but not to BSA (Fig. 3 for development. We observed that biotinylated fucoidin bound to ° CD11b/CD18-coated microtiter wells. After incubation at 37 C for 1 h, bound fucoidin was detected with HRP-streptavidin and color development. 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 Mg 2+ 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, and CBRM1/39).

**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.
and LM2/1). Interestingly, mAb TS1/18, which binds to CD18, did not inhibit fucoidin-CD11b/CD18 binding. These results suggest that the fucoidin binding site is on CD11b, but is not localized to a specific domain, as defined by previously characterized mAbs. We also observed that the binding of fucoidin to CD11b/CD18 was abolished by 5 mM EDTA, indicating that such binding is dependent on divalent cation (Fig. 4).

**Fucosylated neoproteoglycans bind to CD11b/CD18**

A modified Western blotting protocol was employed using purified CD11b/CD18 as a probe to evaluate binding to synthetic fucoidin-containing neoproteoglycans. For these experiments a BSA-fucoidin conjugate (BSA-F) was prepared as previously described (29) with minor modifications. SDS-PAGE of BSA-F demonstrated a large shift in the molecular mass of the complex to a higher apparent molecular mass on a 4–15% gradient gel (Fig. 5A, lane 1) compared with normal BSA alone (Fig. 5A, lane 2). Fig. 5B represents a modified Western blot in which samples of BSA-F (lane 1) and BSA (lane 2) were subjected to SDS-PAGE and transferred to nitrocellulose filters, followed by incubation with CD11b/CD18 in the presence of 2 mM Mg$^{2+}$ and detergent at room temperature. Bound CD11b/CD18 was detected by probing washed blots with biotinylated fucoidin (Fig. 5B, lane 2), but was completely absent in the BSA control (lane 1).

**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 β-xyl).
no drug treatment, respectively). In contrast, adhesion of β-xylopyranosidase-treated T84 cells to purified CD11b/CD18 was significantly reduced compared with that of α-xylopyranosidase-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-xylopyranosidase-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 purpureus) and then incu...
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 α-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 purifying CD11b/CD18, it is possible that such interactions might be exploited therapeutically. For example, in an inflammatory bowel disease. Gastroenterology. 120:1031.

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References


