MAdCAM mediates lymphocyte-endothelial cell adhesion in a murine model of chronic colitis

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The discovery of adhesion molecules that mediate the recruitment of leukocytes to sites of inflammation represents a major advancement in our understanding of the events associated with an inflammatory response. Different families of adhesion glycoproteins have been identified and characterized for both leukocytes and endothelial cells. It is well recognized that these adhesion molecules normally either exist in an inactive state or remain localized within the cell interior. However, on activation of the cell (leukocyte and/or endothelial cell), the avidity of the adhesion molecule for its ligand is increased (e.g., due to conformation changes in the glycoprotein), the intracellular pool of preformed glycoprotein is mobilized to the cell surface, and/or transcription-dependent glycoprotein synthesis leads to increased cell surface expression. When tissues are exposed to an intense inflammatory stimulus (e.g., cytokines), the expression of several cell adhesion molecules (CAMs), including P-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), is increased on the surface of vascular endothelial cells. The high level of expression of several CAMs serves many useful purposes, including 1) the coordinated and sequential mediation of leukocyte rolling, firm adhesion, and transendothelial migration and 2) the selective recruitment of specific leukocyte populations into certain tissues (e.g., lymphocyte recruitment into lymphoid tissues) (4, 34, 39).

Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is an endothelial CAM of the immunoglobulin superfamily (along with ICAM-1 and VCAM-1) that has been implicated in the selective recruitment of lymphocytes to sites of inflammation in the gut (3, 6, 27, 38). MAdCAM-1 is expressed on the specialized microvascular endothelial cells lining venules (high endothelial venules) of Peyer’s patches, and it is found on endothelial cells in the lamina propria of small and large intestine (2, 28, 36). Increased MAdCAM-1 expression has been described in the colonic microvasculature of different animal models of colitis (15, 21). Similarly, immunohistochemical methods have revealed that MAdCAM-1 is upregulated in the inflamed colonic mucosa of humans with ulcerative colitis or...
Crohn’s disease (3, 32). Although the expression of other endothelial CAMs (e.g., VCAM-1 and ICAM-1) is also increased in the inflamed colon of humans and experimental animals, the relative functional importance of the elevated MadCAM-1 expression is evidenced by reports demonstrating reduced inflammation and mucosal damage in different animal models of colitis after immunoneutralization of MadCAM-1 (7, 14). Nonetheless, neutralizing monoclonal antibodies (mAbs) directed against other endothelial CAMs (e.g., VCAM-1) have also shown efficacy in some animal models of colitis (1, 9, 31, 37).

Because endothelial CAMs, such as MadCAM-1 and VCAM-1, are upregulated and neutralizing mAbs against these CAMs afford protection in different lymphocyte-dependent models of colitis (1, 9, 14, 31, 37), it is generally assumed that MadCAM-1, VCAM-1, and/or other endothelial CAMs mediate lymphocyte recruitment into the inflamed gut by promoting adhesive interactions between lymphocytes and vascular endothelial cells. Although immunohistochemical and flow cytometric determinations of lymphocyte infiltration in tissue samples of inflamed bowel tend to support this assumption, there are very few reports in the literature that directly assess lymphocyte-endothelial cell interactions in the microvasculature of control or inflamed intestines. Miura and co-workers (11, 22, 23) have employed the technique of intravital videomicroscopy to quantify the homing of T lymphocytes in venules of lymphoid (Peyer’s patches) and nonlymphoid (villus) regions of small bowel, under basal conditions or following stimulation with endotoxin (lipopolysaccharide) or concanavalin A. These studies also implicated a variety of different leukocyte CAMs, including L-selectin, α4-integrins, and β2-integrins, as mediators of the lymphocyte-endothelial cell adhesion observed in the unstimulated and challenged intestinal vasculature. It remains unclear, however, whether and how these observations relate to the lymphocyte recruitment that occurs in colonic microvasculature during experimentally induced colitis.

The overall objectives of this study were 1) to characterize the kinetics and localization of T lymphocyte-endothelial cell adhesion in venules of the chronically inflamed colon and 2) to define the endothelial cell adhesion molecules (ICAM-1, VCAM-1, MadCAM-1) that mediate these lymphocyte-endothelial cell interactions. Whereas a variety of animal models of inflammatory bowel disease (IBD) have been introduced over the past decade, the severe combined immunodeficient (SCID) mouse that is reconstituted with congeneric CD4+, CD45RB(high) T cells was selected for this study because it mimics several characteristic features of the human disease and the kinetics of endothelial CAM expression, including ICAM-1, VCAM-1, and MadCAM-1, have been determined in a systematic, quantitative fashion (16). Our findings indicate that the onset of clinically evident colitis is associated with profound lymphocyte-endothelial cell adhesion in different-sized colonic venules and that MadCAM-1 expression is a major determinant of this lymphocyte recruitment process.

MATERIALS AND METHODS

Cell purification and flow cytometry. Female donor CB-17 and male CB-17 SCID mice were obtained from Taconic Laboratories (Germantown, NY) and subsequently housed under specific pathogen-free conditions. The donor mice were anesthetized with ketamine (150 mg/kg body wt im) and xylazine (7.5 mg/kg body wt im), and the spleens were removed and then teased into single-cell suspensions in PBS containing 1% fetal calf serum. Erythrocytes were removed by hypotonic lysis. Enrichment of CD4+ T cells was achieved with a MACS system (Milteny Biotech, Auburn, CA) for negative selection by magnetic cell sorting using a modification of the method described by Mackay et al. (19). Briefly, cells were incubated with anti-B220 mAb-FITC, anti-CD8 mAb-FITC, and anti-Mac-1 mAb-FITC (all from PharMingen; San Diego, CA) and thereafter labeled with anti-FITC microbeads (Milteny Biotech). Unlabeled cells were separated from labeled cells on a deletion column (column type CS; Milteny Biotech) assembled into the magnetic separator (VarioMACS; Milteny Biotech). Enriched CD4+ T cells were labeled with FITC-conjugated anti-CD4 mAb GK1.5 as well as phycoerythrin (PE)-conjugated anti-CD45RB mAb (PharMingen) and then fractionated into CD4+ CD45RB(high) and CD4+ CD45RB(low) fractions by two-color sorting on a FACS Vantage (Becton-Dickinson, San Jose, CA). The CD45RB(high) lymphocytes were defined as the brightest, staining 40% of the CD4+ T cells, and were >98% pure on reanalysis.

Animals and induction of colitis. Male CB-17 SCID mice, 6–8 wk of age, were injected (intraperitoneal) with 5×10^6 CD4+, CD45RB(high) T cells suspended in 500 μl of PBS isolated from spleens of female CB-17 donor mice described above. Body weights and fecal status were followed and recorded weekly from the time of the cell injection. At 4–7 wk following reconstitution, animals lost 10–15% of their initial body weight. Intravital microscopic studies were performed on both precolitic (3 wk after reconstitution with CD4+, CD45RB(high) T cells) and colitic (7 wk after reconstitution) mice as well as normal SCID (no T cell reconstitution) and wild-type mice.

Lymphocyte labeling with carboxyfluorescein diacetate, succinimidyl ester. T lymphocytes were separated from spleens of wild-type BALB/C mice using negative selection (Immulan, Biotex Laboratories, Houston, TX). The purity of the isolated cells was >95%. Carboxyfluorescein diacetate, succinimidyl ester (CFDASE; Molecular Probes, Eugene, OR) was dissolved in DMSO to a concentration of 15.6 mM and was stored at −20°C until use. Before injection into recipient mice, lymphocytes (2×10^7) in 20 ml Hanks’ balanced salt solution were incubated with 100 μl CFDASE solution for 10 min at 37°C. These manipulations had no significant effect on the activity or viability of the T lymphocytes as assessed by flow cytometry and trypan blue exclusion.

Surgical procedure. The wild-type and SCID mice (nonreconstituted controls, precolitic, and colitic) were anesthetized with ketamine (150 mg/kg body wt im) and xylazine (7.5 mg/kg body wt im). The right carotid artery was cannulated with a Statham P23A pressure transducer (Gould, Oxnard, CA) connected to the carotid artery cannula. Systemic blood pressure and heart rate were continuously recorded with a phys-
ological recorder (Grass Instruments, Quincy, MA). The left jugular vein was also cannulated for administration of fluoroscopically labeled T cells and drugs. The experimental procedures described herein were performed according to the criteria outlined in the National Institutes of Health and were approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee.

**Intravital microscopic assessment of T lymphocyte-endothelial cell adhesion in submucosal venules.** The colonic microcirculation was observed with an inverted intravital microscope (TNMD-2S, Diaphot, Nikon) assisted by a silicon intensified target camera (C-2400–08, Hamamatsu Photonics). The proximal (ascending) colon was carefully placed on an adjustable Plexiglas microscope stage to minimize the influence of respiratory movement. The tissue surface was moistened with PBS and covered with saline-soaked cotton gauze. Images of submucosal venules that were parallel with the serosal surface were observed through a \( \times 10 \) objective lens (Nikon) and recorded on videotape using a videocassette recorder (NV8950, Panasonic). A video time-date generator (WJ835, Panasonic) projected the stopwatch function onto the monitor. CFDASE-labeled T lymphocytes (2.0 \( \times 10^7 \)) suspended in 100–150 \( \mu l \) saline were injected intravenously, and CFDASE-associated fluorescence was visualized by epillumination at 420–490 nm using a 520-nm emission filter. The number of adherent lymphocytes was determined in different-sized submucosal venules during playback of videotaped images. A lymphocyte was considered stationary within the microcirculation if it remained stationary for \( \geq 30 \) s.

**Experimental protocols.** The fluorescently labeled T lymphocytes were injected into mice, and their interactions with different sized venules in the submucosa were monitored and quantified for up to 120 min after T cell injection. Lymphocyte-endothelial cell adhesion was studied in first (1V; >50 \( \mu m \) diameter), second (2V; 25–50 \( \mu m \) diameter), and third-order (3V; <25 \( \mu m \) diameter) submucosal venules. In some experiments, an anti-murine mAb directed against MadCAM-1 (MECA-367, Pharmingen), VCAM-1 (M/K-2, R&D Systems, Minneapolis, MN), or ICAM-1 (YN-1, a gift from Dr. M. Gerritsen, Genentech, San Francesco, CA) was administered via the jugular vein, each at a dosage of 2 mg/kg, 15 min before the injection of lymphocytes.

**Immunohistochemical localization of MadCAM-1 in inflamed colon.** In control, precolitic, and colitic mice, the MadCAM-1-directed mAb (2 mg/kg) was administered (as the primary antibody) 30 min after injection of the CFDASE-labeled T lymphocytes. Thirty minutes thereafter, the mice were killed and samples (~5mm) of the large intestine were obtained for immunohistochemical localization of MadCAM-1. The tissue samples were immersed in ice-cold Zamboni’s fixative (35), coarsely chopped, and fixed for at least 24 h. The tissue was then removed from the fixative, dipped in PBS, trimmed, and stored in PBS at 4°C. The samples were washed in 80% ethanol (3 times at 20 min) and then permeabilized using 100% DMSO (3 times at 10 min) and rinsed in PBS (3 times at 15 min) and cytprotected in 30% sucrose before freezing. Frozen sections (5–10 \( \mu m \)) were cut on a cryostat and stored at –20°C until immunohistochemistry was performed. Nonspecific staining was minimized by incubating the samples in normal donkey serum (10%, Sigma Chemicals, St. Louis) diluted in antibody diluent (Biogenex, San Ramon, CA) for 1 h at room temperature and rinsed in PBS (3 times at 10 min). The samples were then washed in PBS, and the secondary antibody (diluted 1:100 in antibody diluent; Biogenex) was applied. Secondary antibodies were conjugated to Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA). After the tissues were incubated with the secondary antibody, they were washed in glycerol for 2 h at room temperature and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) to minimize photobleaching. Negative controls were prepared by substituting a nonbinding antibody (diluted 1:100 in antibody diluent; Biogenex) for the primary antibody. Samples were imaged with a SySys digital camera (Photometrics), and the digital images were analyzed using MetaMorph (Universal Imaging).

**Statistics.** Standard statistical analyses, i.e., one-way ANOVA and Scheffe’s (post hoc) test were applied to the data. All values are reported as means \( \pm \) SE, with at least 5 mice per group. Statistical significance was set at \( P < 0.05 \).

**RESULTS**

The body weight of SCID mice reconstituted with CD45Rb\(^{high} \) T lymphocytes increased by \( \sim 10\% \) at 3 wk after reconstitution. The mice also appeared in good health. However, beginning 4 wk after lymphocyte reconstitution, the mice began to lose weight (to \( \sim 90\% \) of original weight) with further weight loss (to 85% of original weight) noted at 7 wk after reconstitution with CD4\(^+\), CD45Rb\(^{high} \) T cells. At 7 wk, the mice appeared ill, as evidenced by piloerection and a hunched-over appearance, diarrhea, and occult blood in the stool, as described previously (16). Only mice that exhibited clinical signs of colitis were included in the “colitis” group and examined by intravital microscopy.

Table 1 summarizes the values of microvessel diameter that were obtained for 1V and 3V venules and first-order arterioles in the different experimental groups. Compared with SCID mice, vessel diameter increased by 47%, 12%, and 16% in colitic mice for 1V and 2V venules and first-order arterioles, respectively. No changes in vessel diameter were noted in precolitic mice. Although negligible lymphocyte-endothelial cell adhesion was noted in control (wild type) and SCID mice (Fig. 1), two patterns of lymphocyte adhesion were detected in SCID mice reconstituted with CD45Rb\(^{high} \) T lymphocytes. The first pattern was a more intense, focal accumulation of T lymphocytes in colonic venules, whereas the second pattern was characterized by a uniform distribution of adherent T lymphocytes in submucosal venules, whereas the second pattern was a more intense, focal accumulation of adherent T cells in discrete regions of colonic venules (see Fig. 2). The latter pattern appeared to occur in

**Table 1. Microvascular diameters in different experimental groups**

<table>
<thead>
<tr>
<th>Mice</th>
<th>Diameter, ( \mu m )</th>
<th>1V</th>
<th>2V</th>
<th>1A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CB17)</td>
<td>16.98 ( \pm ) 0.08</td>
<td>8.06 ( \pm ) 0.44</td>
<td>8.57 ( \pm ) 0.64</td>
<td></td>
</tr>
<tr>
<td>SCID</td>
<td>16.00 ( \pm ) 0.57</td>
<td>8.62 ( \pm ) 0.46</td>
<td>9.00 ( \pm ) 0.61</td>
<td></td>
</tr>
<tr>
<td>Precolitic</td>
<td>17.59 ( \pm ) 0.76</td>
<td>8.58 ( \pm ) 0.31</td>
<td>8.87 ( \pm ) 0.52</td>
<td></td>
</tr>
<tr>
<td>Colitic</td>
<td>23.48 ( \pm ) 1.31†‡</td>
<td>10.02 ( \pm ) 0.39†‡</td>
<td>10.48 ( \pm ) 0.61</td>
<td></td>
</tr>
</tbody>
</table>

*Values are means \( \pm \) SE. 1V, first-order venules; 2V, second-order venules; 1A, first-order arterioles. SCID, severe combined immunodeficient. *\( P < 0.05 \) relative to wild-type controls (CB17); †\( P < 0.05 \) relative to SCID; ‡\( P < 0.05 \) relative to precolitic.*
smaller venules, whose diameter was difficult to quantify because of the intense fluorescence. Figure 1 also illustrates the differences in T cell adhesion (uniform pattern of adhesion) between different-sized colonic venules at 60 min after T cell injection in normal, precolitic (3 wk after reconstitution) and colitic (7 wk after reconstitution) SCID mice. No significant T cell adhesion was noted throughout the 2-h observation period in all venules of normal SCID mice. Although there was a tendency for increased lymphocyte-endothelial cell adhesion in SCID mice reconstituted with CD4<sup>+</sup>, CD45R<sup>b</sup>high T cells for 3 wk (precolitic mice), this uniform pattern of adhesion did not achieve statistical significance. However, after 7 wk of reconstitution (colitic mice), a large number of adherent T lymphocytes was detected in all sized colonic venules. The lymphocyte-endothelial cell adhesion noted in venules of colitic mice was significantly greater than that observed in control and precolitic SCID mice.

Figure 2 illustrates the patterns of lymphocyte-endothelial cell adhesion that were detected (at 60 min after T cell injection) in SCID mice reconstituted for 7 wk with CD4<sup>+</sup>, CD45R<sup>b</sup>high T cells and compares these patterns with control SCID mice as well as colitic mice treated with a MAdCAM-1-specific mAb. Although negligible lymphocyte adhesion was detected in venules of control SCID mice (Fig. 2A), a significant number of adherent lymphocytes was noted in venules of colitic mice, with both a uniform (Fig. 2B) and focal (Fig. 2C) pattern of adhesion. Figure 2D illustrates the profound attenuation of lymphocyte adhesion observed in colitic mice receiving a MAdCAM-1-specific mAb. This mAb was equally effective in blunting both uniform and focal patterns of lymphocyte-endothelial cell adhesion.

Some venules in precolitic mice exhibited the intense focal pattern of lymphocyte adhesion. Figure 3 shows the time course (Fig. 3A) of lymphocyte-endothelial cell adhesion in venular (2V) regions exhibiting a focal, intense adhesion response in untreated precolitic mice and precolitic mice receiving a blocking monoclonal antibody directed against MAdCAM-1. A: time course of T cell adhesion in these groups, controls (wild-type) and SCID mice. B: mean data 60 min after T lymphocyte administration. †P < 0.05 vs. control; *P < 0.05 vs. SCID; #P < 0.05 vs. precolitic mice.
adhesion in these venules and compares these adhesion values with corresponding values obtained in control mice, SCID mice, and in precolitic SCID mice treated with an anti-MAdCAM-1 mAb. Figure 3B illustrates the profound inhibitory effect of the anti-MAdCAM-1 mAb on the focal T cell adhesion observed at 60 min after lymphocyte administration in the precolitic SCID mice. Treatment with either an anti-ICAM-1 or -VCAM-1 mAb had no significant effect on the focal lymphocyte adhesion response in precolitic mice.

Figure 4 compares the efficacy of different endothelial CAM-directed mAbs in reducing the lymphocyte-endothelial cell adhesion observed in 2V venules of colitic mice at 60 min after T cell administration. Although the anti-ICAM-1 and -VCAM-1 mAb, either alone or in combination, had no significant effect on the lymphocyte adhesion response, it was virtually prevented by the anti-MAdCAM-1 mAb. The same results were obtained for both the uniform and focal patterns of lymphocyte-endothelial cell adhesion.

Figure 5 illustrates that the T lymphocyte adhesion observed in colitic mice occurred in MAdCAM-1-expressing colonic venules, which were found in both the lamina propria and the submucosa.

DISCUSSION

IBD can be induced in experimental animals either through targeted gene deletion (17, 30), transgenic overexpression (5, 10, 12), immune manipulation (25, 29), or chemical injury to the gut mucosa (20, 24, 26). Several important insights into the pathogenesis of IBD have emerged from studies employing these animal models, including the rate-determining contribution of colonic vascular endothelial cells to the recruitment of different populations of inflammatory cells into the inflamed bowel. This regulatory property of colonic endothelial cells has been attributed to the ability of these cells to express different adhesion glycoproteins that can facilitate the rolling, firm adhesion and emigration of leukocytes. Immunohistochemical staining methods have been used to demonstrate an increased expression of ICAM-1, MAdCAM-1, VCAM-1, and E-selectin in biopsy specimens from patients with ulcerative colitis or Crohn’s disease. Similar immunohistochemical methods as well as the dual radiolabeled mAb technique have revealed profound upregulation of these endothelial CAMs in animal models of colitis. For example, the SCID/CD45Rbhigh T cell model of colitis, which results from a dysregulated immune response to components of the normal gut flora, exhibits threefold increases in the expression of ICAM-1 and VCAM-1 and a ninefold increase in MAdCAM-1 expression within the inflamed colon at 6–8 wk after reconstitution with CD4+ , CD45Rbhigh T lymphocytes (16). In the present study, we extend the previously reported observations on endothelial CAM expression in the SCID/CD45Rbhigh T cell model of colitis by defining the relative quantitative significance of these CAMs in mediating the resultant lymphocyte-endothelial cell adhesion.

The results of this study provide the first direct evidence for lymphocyte-endothelial cell adhesion in a clinically relevant model of colitis. Our findings indicate that the adhesion of lymphocytes in colonic venules occurs in two distinct patterns, a uniform, diffuse adhesion of T cells in broad regions of the colonic microcirculation (1V, 2V, 3V; Fig. 3B) and a more focal, intense accumulation of lymphocytes in specific regions (in small-diameter venules) of the colonic vasculature (Fig. 3C). We also found that the intensity of the lymphocyte-endothelial cell adhesion did not vary significantly between different-sized venules (when normalized to 150-µm venule length) and that the magnitude of the adhesion response was related to the clinical signs of disease severity (e.g., weight loss, diarrhea). These observations support the view that the SCID/CD45Rbhigh T cell model of colitis is lymphocyte dependent and that lymphocyte-directed interventions can reduce the inflammatory response and blunt mucosal injury (27).

The structural and/or functional basis for the two patterns of lymphocyte-endothelial cell adhesion observed in colonic venules of precolitic and colitic mice is
LYMPHOCYTE ADHESION IN CHRONIC COLITIS

not readily apparent from our data. The two vessel populations may exhibit unique endothelial cell structures and/or phenotypes that explain the observed patterns. For example, the focal intense accumulation of lymphocytes may occur in the morphologically distinct “high endothelial venules,” which are known to sustain intense lymphocyte traffic in lymphoid tissues (8, 13). Alternatively, the two populations of venules may not differ morphologically; however, those vessels exhibiting the focal intense accumulation of lymphocytes may respond to the inflammatory stimulus with a more pronounced expression of adhesion molecules on the surface of endothelial cells.

Our data suggest that the endothelial cell adhesion molecule that is most likely to mediate the lymphocyte-endothelial cell adhesion that is observed in both colonic vessel populations of precolitic and colitic mice is MAdCAM-1. Two lines of evidence support this contention: 1) a neutralizing mAb directed against MAdCAM-1, but not mAbs against ICAM-1 or VCAM-1, virtually abolished the lymphocyte-endothelial cell adhesion associated with colitis, and 2) adherent T cells were detected on colonic endothelial cells that are immunohistochemically positive for MAdCAM-1 (Fig. 5). Although alternate adhesion molecules (e.g., VCAM-1) have been implicated in other models of colitis (33), this variation likely reflects differences in the underlying mechanism that drives the inflammatory response. For example, the model of dextran sulphate sodium colitis is likely to be initiated by chemical injury to the colonic mucosa, whereas the SCID/CD45Rbhigh T cell model appears to be entirely immunologically driven.

Our conclusion that MAdCAM-1 is the major endothelial CAM that mediates lymphocyte-endothelial cell adhesion in the SCID/CD45Rbhigh T cell model of colitis is consistent with previously reported quantitative estimates of an increased MAdCAM-1 (mRNA and protein) expression in colonic venules after 6–8 wk of lymphocyte reconstitution (16) as well as the reduced severity of colonic inflammation in SCID/CD45Rbhigh T cell reconstituted mice treated with a MAdCAM-1-blocking mAb (27). Our findings coupled with the previous reports dealing with MAdCAM-1 in experimental colitis (7, 14) lend strong support for the proposal that MAdCAM-1 may be a relevant tissue-specific target for therapeutic modulation of disease activity in patients with ulcerative colitis or Crohn’s disease.

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