Mechanisms of MAdCAM-1 gene expression in human intestinal microvascular endothelial cells

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Ogawa, Hitoshi, David G. Binion, Jan Heidemann, Monica Theriot, Pamela J. Fisher, Nathan A. Johnson, Mary F. Otters, and Parvaneh Rafiee. Mechanisms of MAdCAM-1 gene expression in human intestinal microvascular endothelial cells. Am J Physiol Cell Physiol 288: C272–C281, 2005. First published October 13, 2004; doi:10.1152/ajpcell.00406.2003.—Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is a homing receptor preferentially expressed on gut-associated endothelial cells that plays a central role in leukocyte traffic into the mucosal immune compartment. Although the molecular mechanisms underlying endothelial ICAM-1 or E-selectin expression have been intensively investigated, the mechanisms that regulate human MAdCAM-1 expression have not been defined. We report MAdCAM-1 gene and protein expression in primary cultures of human intestinal microvascular endothelial cells (HIMEC) that was not demonstrated in human umbilical vein endothelial cells. Similar to ICAM-1 and E-selectin expression, MAdCAM-1 gene expression in HIMEC was inducible with TNF-α, IL-1β, or LPS activation. However, in striking contrast to ICAM-1 and E-selectin expression, MAdCAM-1 mRNA and protein expression in HIMEC was heavily dependent on culture duration and/or cellular density, suggesting a prominent role for cell-cell interaction among these endothelial cells in the expression of the mucosal addressin. MAdCAM-1 expression was inhibited by both SN-50 (NF-κB inhibitor) and LY-294002 [phosphatidylinositol 3-kinase (PI3-K) inhibitor], whereas ICAM-1 and E-selectin expression was inhibited by SN-50 but not by LY-294002. The Akt phosphorylation by TNF-α or LPS was greater at higher cell density, demonstrating a pattern similar to that of MAdCAM-1 expression. NF-κB activation was not affected by cellular density in HIMEC. MAdCAM-1 expression in human gut endothelial cells is regulated by distinct signaling mechanisms involving both NF-κB and PI3-K/Akt. These data also suggest that PI3-K/Akt is involved in the gut-specific differentiation of HIMEC, which results in expression of the mucosal addressor MAdCAM-1.

Lymphocyte trafficking into specific organs and tissues is a critical regulatory step for both normal immune homeostasis and the initiation and perpetuation of inflammatory responses (11). Lymphocyte adhesion and migration through the microvessel wall is in large part mediated by the interactions of the endothelial cell adhesion molecules (CAMs) and their integrin and glycoprotein ligands expressed on leukocytes. The current understanding indicates that the process of leukocyte adhesion and emigration involves multiple steps of sequential adhesion and activation (22). The initial rolling interactions between leukocytes and endothelial cells that occur before firm adhesion are mediated by the selectins. This selectin-mediated tethering and rolling is a prerequisite step for the following integrin-mediated interactions and is also considered essential for leukocyte activation by enhancing exposure to chemottractants by prolonging contact with the vessel wall. After rolling adhesion, firm adhesion and diapedesis appear to be mediated by the interaction between leukocyte integrins and the integrin ligands, members of the immunoglobulin gene superfamily such as ICAM-1, expressed on endothelial cells.

Investigation into the mechanisms of selective leukocyte homing into organs and tissues has demonstrated pathways involving distinct glycoproteins expressed on the endothelial lining of specific microvascular beds, known collectively as the addressins. Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is preferentially expressed on endothelial cells in intestinal mucosa, submucosa, and Peyers’ patches and plays a central role in leukocyte homing to the mucosal immune compartment (4). MAdCAM-1 orchestrates both rolling and firm adhesion of lymphocytes to gut endothelial cells through binding of L-selectin or α4β7-integrin, expressed on leukocytes (2). The interaction between MAdCAM-1 and α4β7 integrin has been shown to be the key step in lymphocyte homing to gut mucosa (7), playing an active role in both mucosal immune homeostasis and intestinal inflammation. In the setting of chronic intestinal inflammation, endothelial expression of MAdCAM-1, ICAM-1, and E-selectin is upregulated, and this pattern has been demonstrated in both forms of human inflammatory bowel disease (IBD): Crohn’s disease and ulcerative colitis (1, 4, 21). Our present understanding regarding α4β7-integrin interaction with MAdCAM-1 has provided the scientific rationale for the development of therapeutic compounds inhibiting endothelium-lymphocyte adhesion as novel biological agents for the treatment of IBD (reviewed in Ref. 18; see also Ref. 23).

Expression of the cell adhesion molecules ICAM-1 and E-selectin on endothelial cells, including human intestinal microvascular endothelial cells (HIMEC), have been well characterized (5, 17). In marked contrast, the mechanisms underlying human MAdCAM-1 expression have not been well defined, in large part because of the lack of a cell culture system in which endothelial cells express this organ-specific adhesion molecule in vitro.

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Our laboratory has focused investigation on HIMEC, developing protocols for the routine isolation and culture of this gut-specific microvascular population. Previous investigation has demonstrated that HIMEC possess classic features of endothelial cells, including Weibel-Palade bodies and factor VIII-associated antigen, but simultaneously demonstrate unique features, including patterns of cell adhesion molecule expression distinct from that of large-vessel endothelium [i.e., human umbilical vein endothelial cells (HUVEC)] (3). In the present study, we have demonstrated that primary cultures of HIMEC express MadCAM-1 gene in response to inflammatory cytokines and bacterial endotoxin (LPS). In contrast to ICAM-1 and E-selectin, MadCAM-1 shows unique expression patterns associated with cell-cell interaction, and this expression is regulated through distinct intracellular signaling pathways involving phosphatidylinositol 3-kinase (PI3-K)/Akt.

**Material and Methods**

**Reagents.** Human recombinant TNF-α and IL-1β were purchased from R&D Systems (Minneapolis, MN) and used at 100 U/ml. Bacterial LPS (from *Escherichia coli* 0111:B4) was purchased from Sigma Chemical (St. Louis, MO) and used at 1 μg/ml. SN-50 (specific inhibitor of NF-κB) and LY-294002 (specific inhibitor of PI3-K) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA) and used at the concentrations indicated.

**Isolation and culture of HIMEC.** HIMEC isolation was performed as described previously (3). In brief, surgical specimens obtained from normal disappearing small intestine or colon were rinsed, and mucosal strips were dissected, washed, and digested in a type II collagenase solution (Worthington Biochemical, Freehold, NJ). Microvascular endothelial cells were extracted by mechanical compression and plated onto fibronectin-coated tissue culture dishes in MCDB 131 medium (Sigma) containing 20% fetal bovine serum (FBS) and endothelial cell growth supplement (ECGS; Upstate Biotechnology, Lake Placid, NY). After 7–10 days of culture, endothelial cell clusters were physically isolated, and a pure culture was obtained and characterized as described previously. All experiments were carried out on cultures between passages 8 and 14. HUVEC were freshly isolated from placenta and used during passages 3–5 as previously described (3). HUVEC were cultured using the same protocols described above for HIMEC.

The isolated HIMEC monolayers were then cultured under specific conditions for different experiments as follows. To determine the effect of culture duration on CAM expression, HIMEC were seeded at 1.5 × 10⁴ cells/cm² and then cultured until confluent (~2 days). Thereafter, confluent HIMEC monolayers were cultured for another 1, 3, or 5 days, followed by stimulation with TNF-α or LPS for 4 h. To determine the effect of cellular densities on CAM expression or intracellular signaling events, HIMEC were seeded at 0.75, 1.5, or 3.0 × 10⁴ cells/cm² and then cultured for 2–3 days before stimulation with TNF-α or LPS. In other experiments, unless specifically indicated, the endothelial monolayer (HIMEC or HUVEC) was seeded at 1.5 × 10⁴ cells/cm² and cultured for 3–5 days after they reached confluence. The culture media were changed every 2 days.

**RNA extraction and RT-PCR.** Total RNA was extracted using TRIzol (Life Technologies, Rockville, MD) before treatment with deoxyribonuclease I, amplification grade (Life Technologies) according to the manufacturer’s instruction. The complementary DNA (cDNA) was generated from 1 μg of total RNA with oligo(dT) primer by using the Superscript First-Strand synthesis system for RT-PCR (Life Technologies), filled to a volume of 80 μl. cDNA solution (4 μl) was used for polymerase chain reaction (PCR) in a total volume of 40 μl containing 0.5 μM of sense and antisense primers for either human MadCAM-1 (5′-AGG TTT ATT TGC CAA AGC CTC-3′ and 5′-CCC CTG TGA AAG CAA AAT AGC-3′), ICAM-1 (5′-TAT GCC AAC GAC TTC TTC T-3′ and 5′-CAT TCA GGC TCA CCT TGG-3′), E-selectin (5′-CCA GTG CCT ATT GTG AGC-3′ and 5′-CACC ATT GCA GGC TGG AAT-3′), or β-actin (5′-CCA GAG CAA GAG AGG CAT CC-3′ and 5′-CTG TGG TGA AGC TGA AG-3′). PCR amplifications were performed at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 35 (MadCAM-1 and ICAM-1), 28 (E-selectin), or 25 cycles (β-actin), followed by final extension for 5 min. PCR product (20 μl) was visualized on 1.2% agarose gels stained with ethidium bromide. The MadCAM-1 sequence of the PCR products was confirmed using automated DNA sequence analysis.

**Western blotting for MadCAM-1 and Akt.** HIMEC were stimulated with TNF-α or LPS for 5–60 min and were lysed in buffer containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1% (vol/vol) Nonidet P-40 (NP-40), 0.25% (wt/vol) SDS, 1 mM EDTA (pH 8.0), 10 mM sodium orthovanadate (pH 10), 40 mM β-glycerolphosphate, 20 mM sodium fluoride, and 0.5% (vol/vol) protease inhibitor cocktail (protease inhibitor cocktail III; Calbiochem, San Diego, CA) and incubated on ice for 15 min. Protein (30 μg/lane) was separated using SDS-12% PAGE and transferred onto nitrocellulose membranes by using a semi-dry blotter (Buchler Instruments, Kansas City, MO). Nitrocellulose membranes were blocked by incubation for 1 h in Tris-buffered saline with Tween 20 [TBS-T: 20 mM Tris·Cl, 137 mM NaCl, 0.1% (wt/vol) Tween 20, pH 7.6] containing 5% (wt/vol) nonfat dry milk and probed overnight at 4°C with either anti-MadCAM-1 (Oncogene Research Products, San Diego, CA) or anti-phospho-Akt (Ser473) polyclonal antibody (1:1,000 in TBS-T; Cell Signaling Technology, Beverly, MA). After three washes in TBS-T, membranes were incubated for 1 h at room temperature with secondary horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and washed three times in TBS-T. Bands were detected using enhanced chemiluminescence (ECL) reagents (Amersham Bioscience). After detection, the bound antibody was stripped by incubation in a stripping buffer containing 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris·HCl (pH 6.7) for 30 min at 50°C. After stripping, the membrane was washed, blocked, and reprobed with anti-total Akt antibody (Cell Signaling Technology) as described above. The bands were detected using ECL.

**EMSA for NF-κB translocation.** HIMEC grown on 60-mm plates were stimulated with either TNF-α or LPS for 1 h and washed twice with ice-cold PBS, and nuclear protein extracts were prepared as described previously (15). For the DNA binding assay, a double-stranded NF-κB oligonucleotide (5′-AGTTGAGGGAGCTTCCAGGC-3′; Promega, Madison, WI) was end-labeled with [γ-32P]ATP (3,000 Ci/mmol at 10 mCi/ml; Amersham Bioscience). Binding reactions (20-μl volume) consisted of 3 μg of poly(dI-dC) (Amersham Bioscience), 3 μg of nuclear protein extract, and 35 fmol of oligonucleotide and were incubated at room temperature for 30 min. Reaction products were separated through a 4% polyacrylamide/Tris-borate-EDTA gel, transferred to Whatman paper, dried, and autoradiographed at ~80°C.

**Immunofluorescent staining of HIMEC.** For immunofluorescent staining to demonstrate translocation of p65 NF-κB subunit, cells were grown on fibronectin-coated glass coverslips as previously described (16). Monolayers were assessed directly or after activation with TNF-α (100 U/ml) for 30 min. Cells were then fixed with 3% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature and washed three times (5 min each) with washing buffer [PBS containing 0.01% (vol/vol) NP-40]. After being blocked for 30 min as described above, the coverslips were incubated with the anti-p65 NF-κB subunit antibody (Santa Cruz Biotechnology) for 60 min at room temperature. Unbound antibody was removed by rinsing three times with washing buffer, and the coverslips were incubated for 30 min with a fluorescein-conjugated secondary antibody (Santa Cruz Biotechnology), washed three times, mounted, and visualized using an Olympus BX-40 fluorescence microscope, and...
photographed with an Olympus PM20 camera with fixed shutter speed.

Immunofluorescent staining for occludin and ZO-1 in HIMEC monolayers was performed as previously described (16).

Inhibition of PI3-K/Akt pathway or NF-κB in HIMEC. To define the intracellular signaling pathways regulating MAdCAM-1, ICAM-1 or E-selectin gene expression, we pretreated HIMEC monolayers with LY-294002 (10 μM) or SN-50 (10 or 50 μg/ml) for 2 h before TNF-α or LPS stimulation. Each CAM gene expression in HIMEC was determined using RT-PCR as described above.

RESULTS

MAdCAM-1 mRNA expression is induced by TNF-α, IL-1β, or LPS. Previous investigation of murine MAdCAM-1 expression demonstrated induction of this molecule in immortalized mouse endothelial cell lines after activation with TNF-α (14). As shown in Fig. 1A, human MAdCAM-1 mRNA expression was strongly induced by TNF-α, IL-1β, or LPS stimulation. The enhanced expression was observed 4–24 h after TNF-α stimulation of HIMEC (Fig. 1B). HUVEC failed to express MAdCAM-1 constitutively or after induction with TNF-α or IL-1β (Fig. 1C). These data demonstrate that the specialized gut microvascular endothelial cells express the mucosal homing receptor MAdCAM-1, which is not seen in the large-vessel, nonmucosal umbilical vein endothelial cultures.

MAdCAM-1 mRNA and protein expression in response to TNF-α or LPS is increased after longer culture duration or higher cellular densities. During the repeated RT-PCR analysis using several different HIMEC cultures, we noticed that MAdCAM-1 mRNA expression in response to cytokines or LPS was greater after longer culture duration. In Fig. 2A, HIMEC were seeded at 1.5 × 10^4 cells/cm^2 (confluent HIMEC monolayers contain ~3 × 10^4 cells/cm^2) and cultured for 3–7 days in MCDB 131 medium containing 20% FBS. Under these culture conditions, most HIMEC monolayers reached confluence on the second day after seeding. As shown in Fig. 2A, MAdCAM-1 expression was enhanced only slightly by LPS stimulation after 3 days of culture (1 day after having reached confluence) but was greater at later time points. In marked contrast, ICAM-1 or E-selectin induction was not affected by...
HIMEC culture duration. Similar results were observed when HIMEC were stimulated with TNF-α (data not shown). In the next experiments, we seeded HIMEC at different cellular densities (0.75, 1.5, or $3 \times 10^4$ cells/cm$^2$) and cultured them for 2 days followed by stimulation with LPS or TNF-α. Under these culture conditions, the monolayers showed 70–90% confluence, <100% confluence, or tight confluence, respectively. As shown in Fig. 2B, MAdCAM-1 expression in response to LPS was greater at higher cellular densities, whereas ICAM-1 or E-selectin expression was not affected by the number of cells. Similar results were observed when cells were stimulated with TNF-α. These data suggest that cell-cell interactions among HIMEC are critically important for MAdCAM-1 expression in response to cytokine or LPS.
We next confirmed that MAdCAM-1 gene expression also resulted in protein expression in HIMEC by using monoclonal antibody against human MAdCAM-1 and performing Western blot analysis (Fig. 2C). Low levels of unstimulated MAdCAM-1 protein expression in HIMEC were detected, and MAdCAM-1 expression was induced after TNF-α stimulation. We next analyzed the effect of prolonged culture on MAdCAM-1 protein expression in HIMEC. Again, we found that increased TNF-α-activated MAdCAM-1 protein expression was detected in HIMEC monolayers that were cultured for longer time periods (6–10 days), which parallels our findings with MAdCAM-1 gene expression (Fig. 2A).

HIMEC demonstrate cell-cell interaction in vitro. Immunofluorescent staining of confluent HIMEC monolayers with antibodies against occludin and ZO-1 demonstrated cell-cell interaction and specific patterns of molecular expression, whereas isolated HIMEC did not show this phenomenon (Fig. 3). These data support the concept that cell-cell interaction occurs in cultured HIMEC monolayers and could play a role in enhanced MAdCAM-1 expression.

NF-κB activation is not affected by cellular densities in HIMEC. The results described above suggested that cell density and, potentially, endothelial cell-cell interaction played a critical role in the capacity of HIMEC to express MAdCAM-1. In the next series of experiments, we sought to define the intracellular signaling pathways that regulate MAdCAM-1 expression and might also be affected by HIMEC cell density and cell-cell interaction. First, we examined NF-κB activation in HIMEC in response to TNF-α or LPS at different cellular densities, because previous data have demonstrated that NF-κB is an important transcription factor regulating ICAM-1 or E-selectin expression in HUVEC as well as playing a central role in murine MAdCAM-1 expression. As shown in Fig. 4A, NF-κB translocation at 1 h after TNF-α stimulation was not significantly affected by cellular density. This result corresponded with the ICAM-1 and E-selectin expression patterns described above but could not explain the MAdCAM-1 expression pattern that was clearly linked to cell-cell contact.

We next used immunofluorescent staining to confirm that NF-κB translocation in HIMEC monolayers followed activation with either TNF-α or LPS. Figure 4B demonstrates translocation of the p65 subunit of NF-κB in response to TNF-α activation, which was inhibited by pretreatment of the cells with SN-50.

PI3-K/Akt activation in response to TNF-α or LPS is increased at higher cellular densities. The next experiments focused on the PI3-K/Akt signaling pathway because this signaling mechanism is widely involved in cellular differentiation, and we hypothesized that it could be associated with MAdCAM-1 expression. Because MAdCAM-1 is exclusively expressed in a tissue-specific and differentiated microvascular distribution, PI3-K/Akt represented a promising signaling pathway that may underlie this pattern of regulated expression. First, we examined whether Akt is activated in response to TNF-α or LPS stimulation in HIMEC cultures. As shown in Fig. 5A, Akt phosphorylation was observed 15–60 min after TNF-α or LPS stimulation. Next, experiments were performed to determine whether cell density would influence activation of PI3-K/Akt activation in HIMEC. HIMEC were seeded at 0.75, 1.5, or 3 × 10^5 cells/cm² and cultured for 2 days (Fig. 5B), which were the same culture conditions used for the RT-PCR or EMSA experiments. Thirty minutes after TNF-α or LPS stimulation, Akt phosphorylation was slightly increased in HIMEC seeded at 0.75 × 10^5 cells/cm², which were 70–90% confluent at the time of stimulation. The HIMEC cultures at 1.5 or 3 × 10^5 cells/cm² (which showed <100% or tight confluence at the time of stimulation, respectively) demonstrated that Akt phosphorylation in response to TNF-α or LPS was greater at higher cellular densities. Figure 5C demonstrates that pre-
treatment with the PI3-K inhibitor LY-294002 also inhibited Akt phosphorylation in HIMEC stimulated with TNF-α. These results parallel the MAdCAM-1 expression patterns as shown in the RT-PCR experiments and also indicate that the degree of PI3-K/Akt activation by TNF-α or LPS is influenced in part by endothelial cellular densities.

MAdCAM-1, but not ICAM-1 or E-selectin, gene expression is inhibited by PI3-K inhibitors in HIMEC. On the basis of the above-described data, we examined whether NF-κB activation or the PI3-K/Akt pathway is involved in expression of MAdCAM-1, ICAM-1, and/or E-selectin in HIMEC. HIMEC monolayers were cultured 3–5 days after confluence and then stimulated with TNF-α or LPS, with or without specific inhibitors of NF-κB (SN-50) or PI3-K (LY-294002). As shown in Fig. 6A, SN-50 inhibited all MAdCAM-1, ICAM-1, and E-selectin mRNA expression, indicating that these genes all require NF-κB activation to be induced by TNF-α or LPS stimulation. In marked contrast, MAdCAM-1 expression was
inhibited by LY-294002, whereas ICAM-1 or E-selectin was not affected (Fig. 6B). MAdCAM-1 protein expression in response to TNF-α was also inhibited by SN-50 and LY-294002 (Fig. 6C). Together, these results indicate that ICAM-1 and E-selectin expression are regulated by the transcription factor NF-κB but not by the PI3-K/Akt pathway, whereas MAdCAM-1 requires both NF-κB and PI3-K/Akt activation for the induction of mRNA and protein expression.

DISCUSSION

In the present study, we report MAdCAM-1 gene expression in primary cultures of human gut-derived microvascular endothelial cells as well as the molecular mechanisms underlying expression. Our data demonstrate that similar to expression of the cell adhesion molecules ICAM-1 and E-selectin (3), MAdCAM-1 expression was strongly induced by TNF-α, LPS, or IL-1β stimulation in HIMEC. To our knowledge, this is the first report demonstrating MAdCAM-1 gene expression in primary cultures of human intestinal endothelial cells. More importantly, our data demonstrate that in addition to activation with inflammatory cytokines and LPS, endothelial cell-cell interaction plays a critical role in the expression of MAdCAM-1, a phenomenon that is not linked to the expression of ICAM-1 or E-selectin. However, it is important to consider that the apparent correlation between cell density and MAdCAM-1 expression could also be explained by increased exposure to paracrine/autocrine factors secreted by HIMEC themselves. Because expression of MAdCAM-1 is highly regulated with a restricted distribution in the lining of organ-specific microvascular beds, our data further demonstrate that the PI3-K/Akt signaling pathway is integral in gut-specific expression of this addressin glycoprotein as well as in mucosal immune homeostasis.

To date, there are extremely limited data regarding the expression and function of MAdCAM-1 in human microvessels, with a majority of information generated from histological analysis (4). MAdCAM-1 distribution in human intestinal microvessels appears to be localized to the postcapillary venule, which supports the role for this molecule in selective leukocyte recruitment through interaction with α4-integrin-expressing leukocytes. In the setting of IBD, there is increased

Fig. 5. Phosphatidylinositol 3-kinase (PI3-K)/Akt activation in response to TNF-α or LPS is increased at higher cellular densities. A: confluent HIMEC monolayers were stimulated with TNF-α for 5–60 min, and Akt phosphorylation was examined using Western blot and densitometric analyses. Phosphorylated Akt was increased from 15 to 60 min after TNF-α stimulation (top blot). The membrane was stripped and reprobed with anti-Akt antibody as control (bottom blot). Similar results were observed when cells were stimulated with LPS. Representative results are from 2 experiments performed on distinct HIMEC lines. B: HIMEC were seeded at 1, 2, or 3 × 10⁴ cells/cm² and cultured for 2 days, and Akt phosphorylation after TNF-α (100 U/ml) stimulation for 30 min was examined using Western blot and densitometric analyses. The degree of Akt phosphorylation was increased at higher cellular densities. Representative results are from 3 experiments performed on distinct HIMEC lines. C: confluent HIMEC monolayers were pretreated with LY-294002 (PI3-K inhibitor) and SN-50 (NF-κB inhibitor) and then stimulated with TNF-α for 60 min, and Akt phosphorylation was examined using Western blot and densitometric analyses. LY-294002 abolished Akt phosphorylation (top blot). The membrane was stripped and reprobed with anti-Akt antibody as control (bottom blot). Similar results were observed when cells were stimulated with LPS. Representative results are from 3 experiments performed on distinct HIMEC lines.
MAdCAM-1 expression in both inflamed Crohn’s disease and ulcerative colitis intestine, with increased expression in deeper layers of the bowel wall in surgically resected Crohn’s tissues. This histological analysis suggests that MAdCAM-1 expression is substantially increased in the setting of human gut inflammation (1).

The majority of information characterizing the expression of MAdCAM-1 has been limited to experiments in rodents and murine models of IBD. MAdCAM-1 is present in the mouse intestine, cecum, and colon, and after the development of spontaneous enterocolitis in animals with genetically deleted production of IL-10 (i.e., IL-10−/− mice), there is a four- to fivefold induction of this molecule as indicated by dual radio-labeled antibody detection (6). Further investigation of the induction of MAdCAM-1 mRNA after the development of enterocolitis in IL-10−/− mice demonstrated a 23-fold induction in gene expression, compared with a 10-fold induction of ICAM-1 and a 5-fold induction of VCAM-1 mRNA (9). Work by Oshima et al. (14) characterized the regulation of MAdCAM-1 expression by using an immortalized murine endothelial cell line, demonstrating TNF-α induction that is linked to NF-κB, tyrosine kinase, and MAPK activation. The ability of IL-10 to influence the expression and function of MAdCAM-1 was investigated by Sasaki et al. (19), who demonstrated that transfection of an IL-10 expression vector resulted in a significant diminution of MAdCAM-1-mediated leukocyte binding in an immortalized mouse gut endothelial cell line.

The demonstration of MAdCAM-1 in isolated human microvascular endothelial cells, as well as information regarding the regulated expression of this molecule, has been extremely limited to date. A recent report demonstrated MAdCAM-1 expression in immortalized human gut microvascular endothelial cells (10). Although multiple groups have utilized HIMEC for characterization of gut-specific microvascular function, characterization of MAdCAM-1 has been problematic. An early report (24) suggested that MAdCAM-1 expression using immunohistochemical detection diminished after the in vitro isolation of gut-specific endothelial cells. We suspect that the requirement for prolonged culture duration and the increased cell density in the HIMEC monolayers that is necessary for demonstration of MAdCAM-1 gene and protein expression may be partly responsible for past difficulties in detection of this molecule.

Fig. 6. MAdCAM-1 expression is regulated through distinct signaling pathways. A: semi-quantitative RT-PCR demonstrates the increased level of MAdCAM-1, ICAM-1, and E-selectin mRNA expression in HIMEC stimulated with TNF-α (100 U/ml, 4 h) with or without SN-50 pretreatment (10 or 50 μg/ml, 2 h). All 3 CAM gene expressions were inhibited by SN-50. β-Actin served as internal control. Representative results are from 3 experiments performed on distinct HIMEC lines. B: semi-quantitative RT-PCR demonstrates the increased level of MAdCAM-1, ICAM-1, and E-selectin mRNA expression in HIMEC stimulated with TNF-α (100 U/ml, 4 h) with or without LY-294002 pretreatment (10 μM, 2 h). MAdCAM-1 expression, but not ICAM-1 or E-selectin expression, was inhibited by LY-294002. β-Actin served as internal control. Representative results are from 3 experiments performed on distinct HIMEC lines. C: confluent HIMEC monolayers were pretreated with LY-294002 (10 μM, 2 h) and SN-50 (50 μg/ml, 2 h) and then stimulated with TNF-α (100 U/ml, 4 h), and MAdCAM-1 protein expression was examined using Western blot and densitometric analyses. Both LY-294002 and SN-50 abolished MAdCAM-1 protein expression. Representative results are from 3 experiments performed on distinct HIMEC lines.
In the present study we have demonstrated that MadCAM-1 expression in response to TNF-α or LPS requires both NF-κB and PI3-K/Akt activation, whereas E-selectin or ICAM-1 mRNA expression is also regulated by NF-κB but not by the PI3-K/Akt pathway. These results are in part consistent with a previous report (13) demonstrating that LY-294002, a selective PI3-K inhibitor, did not prevent E-selectin or ICAM-1 gene transcription and protein expression in response to TNF-α or IL-1β in large-vessel endothelial cells. NF-κB is a well-established transcription factor regulating ICAM-1 and E-selectin expression in human endothelial cells as well as MadCAM-1 in mouse endothelial cells (14). The human MadCAM-1 promoter also contains an NF-κB binding site (12), although direct evidence has not been previously obtained demonstrating that NF-κB activation underlies human MadCAM-1 gene expression. Our present report provides the first direct evidence that human MadCAM-1 mRNA expression in HIMEC is dependent in part on NF-κB activation. Moreover, our results also demonstrate that the PI3-K/Akt pathway is also integrally involved in MadCAM-1 expression.

PI3-K is a ubiquitous heterodimeric lipid-modifying enzyme consisting of p85 regulatory and p110 catalytic subunits. Upon stimulation, activated p110 catalyzes the phosphorylation of membrane phosphatidylinositol 4,5-bisphosphate to generate phosphatidylinositol 3,4,5-trisphosphate, leading to downstream Akt activation by its phosphorylation on both Thr308 and Ser473 (20). These events constitute the start of a complex signaling cascade, which may result in proliferation, differentiation, chemotaxis, survival, glucose metabolism, or nitric oxide production, thus demonstrating that the PI3-K/Akt pathway plays a central role in cell physiology and homeostasis (8, 19). In the present study, we found that human MadCAM-1 is one of the molecules regulated by the PI3-K/Akt pathway. In addition, we determined that PI3-K/Akt activation in response to TNF-α or LPS is greater at higher cell densities, paralleling the pattern of MadCAM-1 expression. Together, these results strongly suggest that MadCAM-1 mRNA expression is regulated in part by the PI3-K/Akt pathway, whose degree of activation is further dependent on endothelial cell-cell interaction. These distinct signaling pathways governing MadCAM-1 expression further suggest that the gut-specific differentiation of endothelial cells into HIMEC is dependent in part on PI3-K/Akt activity. Because PI3-K/Akt is known to undergo activation in large-vessel endothelial populations as well (13), we suspect that additional signaling pathways will ultimately be shown to be involved in the differentiation of gut-specific microvascular endothelial populations.

In summary, we provide the initial report that primary cultures of human intestinal microvascular endothelial cells express mRNA for the mucosal leukocyte-homing molecule MadCAM-1 after prolonged culture duration, which is not seen in large-vessel endothelial cells. The regulation of MadCAM-1 gene expression in human gut microvascular endothelial cells is dependent on NF-κB activation, which is similar to the regulated expression of ICAM-1 and E-selectin. However, unlike expression of these ubiquitous endothelial cell adhesion molecules, MadCAM-1 expression is also dependent on PI3-K/Akt signaling, and the degree of activation of this pathway is directly linked to endothelial cell-cell contact. Finally, our data suggest that the differentiation of gut-specific endothelial cell lines, as assessed by the expression of the mucosa-specific leukocyte-homing receptor MadCAM-1, is dependent in part on PI3-K/Akt signaling.

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