Mutual Regulation of TLR/NLR and CEACAM1 in the Intestinal Microvasculature: Implications for IBD Pathogenesis and Therapy

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Background: Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) displays multiple activities, among which pathogen binding and angiogenesis are particularly prominent. These same functions are also exerted by Toll- and NOD-like receptors (TLRs and NLRs), which are critical mediators of innate immune responses. We investigated whether a functional inter-relationship exists between CEACAM1 and TLRs and NLRs and its potential impact on induction of intestinal angiogenesis.

Methods: This hypothesis was tested using human intestinal microvascular endothelial cells, a unique cell population exposed to microbial products under physiological and pathological conditions.

Results: The results show that activation of TLR2/4, TLR4, NOD1, and NOD2 by specific bacterial ligands selectively and differentially upregulates the levels of cellular and soluble CEACAM1 produced by intestinal microvascular endothelial cells. The results also show that CEACAM1 regulates the migration, transmigration, and tube formation of these endothelial cells and mediates vessel sprouting induced by specific TLR and NLR bacterial ligands. Combined, these results demonstrate a close and reciprocal regulatory interaction between CEACAM1 and bacterial products in mediating multiple functions essential to new vessel formation in the gut mucosa.

Conclusions: A coordinated and reciprocal interaction of CEACAM1 and microbiota-derived factors is necessary to optimize angiogenesis in the gut mucosa. This suggests that a coordination of endogenous and exogenous innate immune responses is necessary to promote intestinal angiogenesis under physiological and inflammatory conditions such as inflammatory bowel disease.

Key Words: CEACAM1, TLR, NLR, innate immunity, intestinal angiogenesis

INTRODUCTION

Angiogenesis is a universal response to changes in tissue homeostasis under physiological and pathological conditions,^{1, 2} such as wound healing,³ malignancies,^{4, 5} infections,⁶ injuries,⁷ and inflammation.^{8, 9} An active process of new vessel formation occurs in organs and tissues affected by autoimmune or chronic inflammatory processes,^{10, 11} including inflammatory bowel disease (IBD).¹² Angiogenesis is actually an integral

component of Crohn's disease (CD) and ulcerative colitis (UC) pathogenesis¹² and contributes to maintenance of gut inflammation, as shown by improvement of experimental colitis with anti-angiogenic therapy.¹³

Angiogenesis is a complex process involving activation of endothelial cells by multiple cellular and soluble factors.^{1, 2} Endothelial cells are also activated in response to Toll- and NOD-like receptor (TLR and NLR) ligands.^{14–16} It is well

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established that the microbiota and its products contribute to physiological intestinal angiogenesis.^{17, 18} In the inflamed gut, this process is exacerbated by an enhanced exposure of the mucosal vasculature to the local microbiota.¹⁹ In fact, luminal microbes represent a rich source of angiogenic stimuli in the form of ligands for pattern recognition receptors (PRRs).^{20–23} We recently reported that microorganisms can activate gut mucosal endothelial cells and induce angiogenesis, and we demonstrated that diverse bacterial ligands can induce proliferation, migration, transmigration, and tube formation of human intestinal microvascular endothelial cells (HIMECs), as well as ex vivo vessel sprouting and in vivo angiogenesis.²⁴ These effects are mediated through TLRs and NLRs, members of a large family of PRRs, and actually represent innate immune responses by endothelial cells resulting in pro-angiogenic effects.²⁴ There is a growing body of evidence indicating that innate immunity receptors are involved in promoting angiogenesis in a variety of in vitro and in vivo systems.^{20–23, 25} Classical ligands for TLR and NLR derive from Gram-negative and Gram-positive bacteria, both commensal and pathogenic, but products from these same microbes also bind to members of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family, particularly CEACAM1.^{26–28}

CEACAM1 is ubiquitously expressed by a large variety of cell types and displays multiple functions involved in physiological and pathological responses, ranging from cell activation, proliferation, migration, and adhesion to angiogenesis, pathogen binding, and tumor growth.²⁸ In endothelial cells, CEACAM1 expression mediates potent pro-angiogenic effects, a property affecting several inflammatory and neoplastic processes.²⁹ CEACAM1 is a potent angiogenic factor in itself and a key mediator of the pro-angiogenic activity of vascular endothelial growth factor (VEGF),³⁰ as shown by CEACAM1 silencing with small interfering RNA (siRNA),³¹ and modulates vascular morphogenesis and remodeling both in vitro and in vivo.³² The pathogen-binding capacity of CEACAM1 has been investigated primarily in epithelial cells,²⁸ but, being also expressed on endothelial cells, microbes also bind to CEACAM1 on endothelial cells, which leads to their activation.³³

Based on their commonalities, TLR, NLR, and CEACAM1 can be viewed as broad mediators of a wide range of innate immune responses, but a direct link between CEACAM1 and other bacterial sensors in the induction of angiogenesis has never been reported. In fact, because of their shared pro-angiogenic activity, it is possible that TLR, NLR, and CEACAM1 interact and reciprocally modulate each other's expression and action during endothelial cell activation, proliferation, migration, and new vessel formation. Therefore, we tested the hypothesis of a possible functional inter-relationship of TLR and NLR with CEACAM1 and its potential impact on the induction of angiogenesis. To test this hypothesis, we utilized HIMECs because this unique endothelial cell population is normally exposed to physiological amounts of microbial

products under physiological conditions and increased amounts under inflammatory conditions, such as those found in IBD.^{34, 35} The results show a clear and mutual interdependence of TLRs/NLRs and CEACAM1 in the regulation of responses of gut mucosal microvascular cells to bacterial antigens.

METHODS

Measurement of Colonic Tissue CEACAM1, Serum-Soluble CEACAM1, and HIMEC Culture Supernatants, and Pro-Angiogenic Cytokines Produced by HIMECs

An enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA) was used to measure soluble CEACAM1 in tissue extracts from normal and IBD colonic tissue, serum, and culture supernatants of HIMECs stimulated for 24, 48, and 72 hours with bacterial ligands, tumor necrosis factor- α (TNF- α), or human recombinant CEACAM1 (rCEACAM1) at the concentrations mentioned above. Levels of bFGF and IL-8 levels in culture supernatants of unstimulated or stimulated HIMEC cultures were measured in triplicate using the same ELISA (R&D Systems).

In Vitro Matrigel HIMEC Tube Formation Assay

To assess HIMEC tube formation, growth factor-depleted Matrigel (BD Biosciences, Bedford, MA, USA) was used as described elsewhere.³⁶ Briefly, 24-well plates were coated with 150 μ L of growth factor-depleted Matrigel (BD Biosciences, Bedford, MA, USA) and 150 μ L of medium 199 (Lonza, Walkersville, MD, USA) previously thoroughly mixed on ice. To assess tube formation, 10^5 cells resuspended in media 199 were seeded on Matrigel and left in medium alone or stimulated with bacterial ligands or rCEACAM1 with and without anti-CEACAM1 (clone 4D1/C2). After variable periods of time, the HIMEC monolayers were examined with an inverted phase-contrast microscope (Olympus, IX71, Olympus, Melville, NY, USA) to assess tube formation at different time points up to 24 hours. Five random high-power fields per condition were examined, and experiments were performed in triplicate.

Mouse Aortic Ring Assay

Aortas from 8–12-week-old Balb C mice were harvested and cultured in 3-dimensional collagen gels as previously described.³⁷ Briefly, thoracic aortas were removed from mice killed by CO₂ inhalation and immediately transferred to a culture dish containing ice-cold minimum essential medium (MEM; Sigma-Aldrich, St. Louis, MO, USA). After removing the peri-aortic fibroadipose tissue, 1-mm-long aortic rings were sectioned and extensively rinsed in MEM. Ring-shaped explants of mouse aorta were then embedded in a rat tail interstitial collagen gel (1.5 mg/mL) prepared by mixing 7.5 volumes of 2 mg/mL of collagen (Collagen R, Serva, Heidelberg, Germany),

1 volume of 10× MEM, 1.5 volume of NaHCO_3 (15.6 mg/mL), and approximately 0.1 volume of 1M NaOH to adjust the pH to 7.4. Aortic rings were dropped and positioned into wells of a 48-well plate containing 300 μL of collagen gel, 2.5% mouse serum (Innovative Research, Novi, MI, USA), and 1% pen/strep/amphotericin B mixture (Lonza, Walkersville, MD, USA). After gel polymerization for 20 minutes at 37°C, 500 μL of MCDB131 medium supplemented with 25 mM NaHCO_3 , 2.5% mouse serum, 1% L-Glutamine (Lonza, Walkersville, MD, USA), 1% pen/strep/amphotericin B mixture with and without bacterial ligands, rCEACAM1, or VEGF with and without anti-CEACAM1 antibody (clone 4D1/C2) was added. The cultures were kept at 37°C for 2 weeks, with the medium changed every second day, and gels were examined every other day by phase microscopy.

HIMEC CEACAM1 Isoform Knockdown by siRNA Transfection

HIMECs were transfected with various CEACAM1 isoforms and scrambled control siRNAs using the electroporation Amaxa nucleofector II system (Amaxa Biosystems, Köln, Germany). HIMECs (0.5×10^6) were suspended in 100 μL of nucleofector solution for endothelial cells (Lonza, Walkersville, MD, USA), mixed with different concentrations (0.1, 0.2, and 0.4 nmol) of total CEACAM1, CEACAM1-L (4L), or scrambled siRNA; they were transferred to a cuvette and electroporated according the manufacturer's instructions. Cells were then immediately transferred into 6-well plates containing prewarmed culture medium. As a negative control, some cells were only left in nucleofector solution without applying electroporation. After transfection, cells were incubated overnight, the medium was changed to a serum-reduced (5% FBS), growth factor-free medium, and the cells were incubated an additional 24 hours. At the end of this period, HIMECs were treated with trypsin-EDTA, suspended, counted, and used in the Matrigel in vitro tube formation assay as described above.

The siRNAs were ON-Target plus smart pool siRNAs from GE Lifesciences/Dharmacon (Lafayette, CO, USA). The siRNA used for inhibition of total CEACAM1 was a commercially available siRNA pool from Dharmacon (L-0009439-01-0005) with a scramble siRNA (D-001810-10). The siRNA for 4L was generated by Thermo-Fisher Scientific (Waltham, MA, USA) using the National Center for Biotechnology Information (NCBI, NIH, Bethesda, MD, USA) reference NM_001712.4 based on the nucleotide 1512–1564 sequence, which appears in the long but not the short form of CEACAM1. An siRNA for 4S could not be generated due to the lack of any nucleotide sequence unique to this isoform (NCBI reference NM_001024912.2).

Reliable antibodies for the protein quantification and differentiation of CEACAM1-L and CEACAM1-S are not readily available, and therefore demonstration of siRNA-mediated

knockdown was performed by assessing levels of their respective mRNAs. Total RNA was harvested from siRNA-electroporated and control HIMECs using Qiagen RNeasy total RNA extraction kit (Valencia, CA, USA), and 600 ng of RNA was used to prepare cDNA using BioRad's iScript cDNA synthesis kit (Hercules, CA, USA). Two μL of cDNA was used in each quantitative polymerase chain reaction (qPCR) using BioRad's iQ SYBR green supermix to determine gene expression. The primer oligonucleotides and conditions used for the real-time PCR to amplify the total (PrimerBank ID 329112546c1), 4L, and 4S isoforms of CEACAM1 were those reported by Singer et al.,³⁸ where the primers for the 4S isoform were designed by targeting the 2 exons flanking the sequence unique to the long form. GAPDH was used as the reference gene. Calculation of the fold changes in gene expression in cells electroporated with CEACAM1-specific siRNAs vs those electroporated with scrambled siRNA was performed according the method of Pfaffl.³⁹ As shown in [Supplementary Figure 1](#), the siRNA for total CEACAM1 induced a dose-dependent inhibition of RNA for the total, 4L, and 4S CEACAM1 isoforms; in contrast, the siRNA for CEACAM1-4L inhibited the total and the 4L isoform mRNA but not the 4S isoform, demonstrating the specificity of the gene knockdown.

Statistical Analysis

Results were analyzed by Graphpad software 4.0 (La Jolla, CA, USA) with expression of mean and standard error. The Student *t* test for paired or unpaired determinations or with analysis of variance was used for all comparisons. Statistical significance was set at $P < 0.05$.

Ethical Considerations

The procurement of intestinal surgical specimens and blood samples for whole-tissue extraction and cell isolation, and serum samples, respectively, was approved by the Institutional Review Board of the Cleveland Clinic.

RESULTS

Microvascular Endothelial Cell CEACAM1 Expression in Human Intestinal Tissue

We initially investigated the expression of CEACAM1 in human colonic mucosa focusing on its expression by local endothelial cells. Co-staining of frozen tissue sections with both vWF and CEACAM1 antibodies demonstrated that mucosal microvascular endothelial cells express CEACAM1, as demonstrated by its co-localization with vWF factor ([Supplementary Fig. 2](#)). Similar to other tissues, CEACAM1 was expressed by small mucosa vessels,⁴⁰ without any obvious differences among normal, CD, and UC tissues.

Next, we measured total levels of CEACAM1 in normal and IBD tissues by ELISA and found that levels of CEACAM1

were significantly higher in both CD and UC compared with normal extracts ($P = 0.01$ and $P = 0.02$, respectively) (Supplementary Fig. 3A). In addition, we investigated whether the differences in CEACAM1 detected at the tissue levels could be reflected in the periphery, as measured by the circulating levels of soluble CEACAM1 in the serum. In fact, the levels of soluble CEACAM1 were significantly higher in the sera of CD and UC patients than in those of control subjects ($P = 0.02$ and $P = 0.03$, respectively) (Supplementary Fig. 3B).

TLR- and NLR-Mediated Upregulation of CEACAM1 Expression by HIMECs

The upregulation of endothelial CEACAM1 by infectious agents is well known,³³ but it is less certain whether products from nonpathogenic microbes, like those of the commensal gut microbiota, would induce a similar response. To investigate this, we initially examined whether activation of TLRs or NLRs would modulate CEACAM1 expression or distribution in HIMECs. Immunoblot analysis revealed that ligands for TLR2/6, TLR4, NOD1, and NOD2 robustly increased CEACAM1 expression to a level comparable to that induced by VEGF, whereas the pro-inflammatory cytokine TNF- α only had a slight enhancing effect (Fig. 1). Confocal microscopic analysis of the HIMECs used in these experiments confirmed the upregulation of CEACAM1 expression by the TLR and NLR ligands (Fig. 2). Interestingly, each ligand induced a distinctive CEACAM1 distribution pattern compared with the diffuse enhancement promoted by VEGF: TLR2/6L primarily increased the transmembrane expression whereas TLR4L augmented only the cytoplasmic form; NOD1L upregulated the

cytoplasmic more than the transmembrane expression, whereas NOD2L enhanced the transmembrane more than the cytoplasmic CEACAM1 (Fig. 2). Of note, TNF- α failed to modulate the distribution of either form of CEACAM1.

In view of the above results, we investigated whether TLR and NLR ligands, in addition to modulating CEACAM1 expression and distribution in HIMEC, would also affect the production of the soluble form of CEACAM1. Soluble CEACAM1 release was significantly increased in a time-dependent manner by VEGF, TNF- α , and binding of TLR4 and NOD2 ($P = 0.01$ – 0.002), but not TLR2/6 or NOD1 (Fig. 3A). Interestingly, the increase in soluble CEACAM1 induced by TLR4L and NOD2L was greater than that induced by VEGF or TNF- α ($P < 0.05$ – 0.01). No significant differences between normal and IBD or between CD and UC HIMECs were noted.

Induction of HIMEC Proliferation, Migration, and Transmigration

Having shown that HIMECs express and release CEACAM1 and that this response is modulated by TLR and NLR activation, we next performed a series of experiments to investigate whether CEACAM1 and bacterial ligands could induce an angiogenic response by HIMECs. As no significant differences were noted between normal and IBD HIMECs in the previous experiments, the results for normal, CD, and UC HIMECs were combined in all subsequent experiments.

To learn whether CEACAM1 could mediate HIMEC proliferation, cells were exposed to a previously determined concentration of rCEACAM1³⁰ and proliferation was measured by [³H]thymidine uptake. When compared with unstimulated

FIGURE 1. Upregulation of HIMEC CEACAM1 by TLR and NLR ligands. HIMEC monolayers were exposed to medium alone or optimal stimulatory doses of VEGF, TLR2/6, TLR4, NOD1, or NOD2 ligands for 48 hours and were washed, harvested, and lysed; protein was extracted for immunoblotting using an anti-CEACAM1 antibody. The same blot was stripped and immunoblotted using an anti-GAPDH antibody. The figure is representative of 10 HIMEC lines (6 normal and 4 IBD lines). Abbreviation: L, ligand.

FIGURE 2. Differential modulation of HIMEC cytoplasmic and transmembrane CEACAM1 by TLR and NLR ligands. HIMEC monolayers were exposed to medium alone or optimal stimulatory doses of VEGF, TNF- α , and TLR2/6, TLR4, NOD1, and NOD2 ligands for 48 hours; they were washed and then stained with an anti-CEACAM1 antibody. Confocal microscopy shows the relative cytoplasmic and transmembrane distribution and expression level of CEACAM1 in HIMECs. All panels are from the same HIMEC line concomitantly treated with the indicated stimuli. The panel is representative of 6 HIMEC lines (2 normal and 4 IBD lines). Abbreviation: L, ligand.

cells, rCEACAM1 induced a significant increase in proliferation of HIMECs in a dose-dependent manner in the range of 100–300 ng/mL ($P < 0.02$) (Fig. 3B). Among bacterial ligands, only NOD1L significantly enhanced HIMEC proliferation ($P = 0.02$) (Fig. 3B).

Cell migration is essential to new vessel formation during angiogenesis, and therefore we tested whether rCEACAM1 could stimulate HIMEC migration. Using a wounded cell monolayer assay system, rCEACAM1 induced a dose-dependent migration of HIMECs comparable to that of VEGF (Supplementary Fig. 4). In contrast to the results of the proliferation assay, all bacterial ligands significantly enhanced HIMEC migration, with the strongest effect by TLR2/6L and NOD2L (Fig. 4A) and a lesser but still significant effect by TLR4L and NOD1L (not shown). In contrast, when TNF- α was used, no modulation of cell migration was observed. After showing that both rCEACAM1 and bacterial ligands were able to

upregulate HIMEC migration, we investigated a possible interaction between them by performing the same experiments in the presence of CEACAM1-blocking antibodies. Surprisingly, the blockade of CEACAM1 not only abrogated the migration of HIMECs in response to rCEACAM1, but also to TLR2/6L and NOD2L (Fig. 4A).

To confirm that CEACAM1 and bacterial ligands could induce an angiogenic response in HIMECs, and that this response was dependent on the interaction between CEACAM1 and bacterial ligands, additional experiments were carried out to study HIMEC transmigration using the Boyden chamber system. rCEACAM1 significantly upregulated HIMEC transmigration in a dose-dependent manner (Fig. 4B), but, in contrast to the migration assay, ligands for TLR4 and NOD1, but not for TLR2/6 or NOD2, significantly enhanced HIMEC transmigration (Fig. 4B). As observed in the migration assays, blockade of CEACAM1 not only inhibited the transmigration

FIGURE 3. A, Differential upregulation of HIMEC-soluble CEACAM1 production by TLR and NLR ligands. HIMEC monolayers were exposed to medium alone or optimal stimulatory doses of VEGF, TNF- α , and TLR2/6, TLR4, NOD1, and NOD2 ligands for 24, 48, and 72 hours; they were washed, and levels of soluble CEACAM1 were measured by ELISA in the culture supernatants. *Significantly increased amounts, as shown by the P values at each respective time point, compared with medium alone ($n = 10$, including 4 normal, 3 CD, and 3 UC HIMEC lines). B, Differential upregulation of HIMEC proliferation by rCEACAM1 and TLR and NLR ligands. HIMEC monolayers were exposed to medium alone or optimal stimulatory doses of bFGF, TNF- α , TLR2/6, TLR4, NOD1, and NOD2 ligands or increasing concentrations of rCEACAM1 for 24 hours; they were washed, and proliferation was assessed by ^3H thymidine uptake. *Significantly increased proliferation, as shown by the respective P values, compared with medium alone ($n = 6$, including 3 normal and 3 IBD HIMEC lines). Abbreviation: L, ligand.

of HIMECs in response to rCEACAM1, but also to TLR4L and NOD1L (Fig. 4B). Once again, TNF- α failed to modulate HIMEC transmigration (Fig. 4B).

Mucosal Microvascular Cell Tube Formation

To complement the above angiogenic assays, we also carried out experiments with HIMECs on Matrigel, a system promoting tube formation that mimics microvessel formation.²⁴ In preliminary experiments, we determined that the ligand for NOD1 was the most potent inducer of tube formation, and therefore all subsequent experiments were carried out using this ligand in addition to rCEACAM1. In the absence of any exogenous stimuli, HIMECs only showed a trend toward spontaneous tube formation. When rCEACAM1 was added, a borderline increase in tube formation was observed, whereas the addition of anti-CEACAM1 antibodies eliminated even the spontaneous trend toward tube formation (Fig. 5). Ligation of NOD1 was particularly effective in inducing HIMEC tube formation, and this angiogenic response was inhibited by the blockade of CEACAM1 (Fig. 5).

Murine Aortic Ring Endothelial Cell Sprouting

To further corroborate the above in vitro results, we explored the pro-angiogenic activity of rCEACAM1, TLR4,

and NOD1L by assessing the sprouting of vessels from murine aortic rings, an ex vivo angiogenesis model that mimics in vivo events.³⁷ In preliminary experiments, we determined that TLR4L and NOD1L were the strongest inducers of vessel sprouting, and therefore all subsequent experiments were carried out using these ligands in addition to rCEACAM1. rCEACAM1 induced a remarkably strong sprouting of vessels, whereas TLR4L and NOD1L induced sprouting to a similar or slightly smaller degree (Fig. 6). In view of the results of the tube formation assays showing the apparent dependence on CEACAM1 for induction of angiogenesis by NOD1L (Fig. 5), we performed additional experiments blocking endogenous CEACAM1 while stimulating the aortic rings with TLR4L and NOD1L. This resulted in essentially complete abrogation of the vessel sprouting induced by both bacterial ligands (Fig. 6).

CEACAM1 Isoforms and Mucosal Microvascular Angiogenesis

To determine which CEACAM1 isoform was mainly responsible for the pro-angiogenic effects of bacterial ligands, the Matrigel in vitro tube formation assay was performed using HIMECs transfected or not with siRNA specific to total and the 4L isoform of CEACAM1. HIMECs submitted

FIGURE 4. A, Differential upregulation of HIMEC migration by rCEACAM1 and TLR and NLR ligands. Confluent HIMEC monolayers were scraped with a sterile razor blade, washed to remove cell debris, and cultured in medium alone, optimal stimulatory doses of bFGF, TNF- α , TLR2/6, TLR4 (not shown), NOD1 (not shown), and NOD2 ligands or increasing doses of rCEACAM1 and the number of migrated cells were counted after 24 hours. Some experiments were performed in the presence of anti-CEACAM1 antibody. Migration was assessed by counting the total number of cells below and above the wound in 4 random microscopic high-power fields at 100X magnification using an image analysis system. *Significantly increased migration, as shown by the respective P values, compared with medium alone or stimuli ($n = 24$, including 8 normal and 16 IBD HIMEC lines). B, Differential upregulation of HIMEC transmigration by rCEACAM1 and TLR and NLR ligands. HIMECs were placed in the upper compartment of a Boyden chamber, and optimal stimulatory doses of bFGF, TNF- α , TLR2/6 (not shown), TLR4, NOD1, and NOD2 (not shown) ligands or increasing doses of rCEACAM1 were placed in the lower compartment, and the number of transmigrated cells was counted after 8 hours. Some experiments were performed in the presence of anti-CEACAM1 antibody. Transmigrated cells on the lower side of the filter were fixed, stained, and counted in 4 random high-power fields at 200X magnification using an image analysis system. *Significantly increased migration, as shown by the respective P values, compared with medium alone or stimuli ($n = 21$, including 9 normal and 12 IBD HIMEC lines). Abbreviations: hpf, high-power field; L, ligand.

to electroporation with scramble siRNA and then cultured in medium alone or with NOD1L or TLR4L showed comparable degrees of tube formation, which was visibly less pronounced in cells pretreated with total CEACAM1 or CEACAM1-4L siRNAs (Supplementary Fig. 5). An siRNA for the 4S isoforms could not be developed because of a lack of any nucleotide sequence unique to this isoform (see M&M).

Pro-angiogenic Factors Production by Mucosal Microvascular Cells

To complete the investigation of the role of CEACAM1 and bacterial ligands as mediators of HIMEC angiogenesis, we examined whether both classes of stimuli could induce the release of typical pro-angiogenic factors such as IL-8 and

bFGF. rCEACAM1 failed to stimulate IL-8 and bFGF production by HIMECs, regardless of the concentration used (Fig. 7A and B); in contrast, bacterial ligands, with the exception for TLR2/6L, significantly enhanced IL-8 and bFGF production by HIMECs (Fig. 7A and B). VEGF failed to upregulate both angiogenic factors, whereas TNF- α significantly induced IL-8 and bFGF production by HIMECs.

DISCUSSION

This study shows that bacterial products selectively upregulate the levels of cellular and soluble CEACAM1 produced by intestinal microvascular cells while, in turn, CEACAM1 regulates endothelial cell migration, transmigration, tube formation, and vessel sprouting induced by bacterial products. Together,

FIGURE 5. Induction of HIMEC tube formation by rCEACAM1 and NOD1 ligand. HIMECs were seeded on Matrigel in the presence and absence of optimal stimulatory doses of rCEACAM1, NOD1L, or mouse IgG, and tube formation was visually assessed at up to 24 hours. Parallel experiments were performed in the presence of anti-CEACAM1 antibody. The figure is representative of 2 and 3 experiments for normal and IBD HIMEC lines, respectively, at 4 hours.

these results point to a close and reciprocal regulatory interaction between the microbiota and CEACAM1 in the process of new vessel formation in the gut. This interaction is directly relevant to IBD given the established role of the microbiota and angiogenesis in disease pathogenesis^{12,41} and the novel biological therapies targeting the trafficking of immune cells to the gut microvasculature.⁴²

Some evidence for an interaction between CEACAM1 and bacterial receptors comes from studies of CEACAM1 and TLR2 in human pulmonary epithelial cells,⁴³ the involvement of TLR4 in the upregulation of endothelial cell CEACAM1 by Gram-negative bacteria,³³ and the promotion of CEACAM1 mRNA expression in the murine small bowel epithelium by the gut microbiota.⁴⁴ However, the extent and effects of the interaction between CEACAM1 and PRRs in the induction of intestinal angiogenesis are unknown. Our results show that upregulation of HIMEC CEACAM1 levels is induced by ligands for TLR2/6, TLR4, NOD1, and NOD2 and that this effect was quantitatively similar to that of VEGF, a strong inducer of CEACAM1,²⁹ whereas TNF- α was less effective. This suggests that when bacterial products translocate into an inflamed organ,^{45,46} such as the intestine in CD and UC,⁴⁷ they can upregulate endothelial CEACAM1 expression and do so even more efficiently than products generated from the ensuing immune response.

The differential upregulation of gut endothelial CEACAM1 expression by the microbiota became evident when

the cellular distribution of CEACAM1 was analyzed in HIMEC monolayers. All bacterial ligands increased CEACAM1 levels and did so in a selective fashion: transmembrane CEACAM1 was upregulated strongly by the TLR2/6 ligand and moderately by the ligand for NOD2, whereas ligands for TLR4 and NOD1 increased almost exclusively cytoplasmic CEACAM1. These differential effects are intriguing and have functional implications, as the transmembrane and cytoplasmic forms of CEACAM1 mediate distinct cellular functions.^{48–50} In the intestine, where numerous and diverse microbial niches exist all along the gastrointestinal tract,⁵¹ distinct bacteria may differentially regulate the CEACAM1 expression level, its cellular distribution, and the subsequent CEACAM1-dependent effects. The same is likely to be true in the IBD intestine, whose dysbiosis is associated with both quantitative and qualitative changes of microbial composition.⁵² Of note, bacterial ligands upregulated cytoplasmic and transmembrane CEACAM1 as strongly as VEGF, whereas TNF- α barely increased the levels of intracellular CEACAM1.

Bacterial products also enhanced the production of soluble CEACAM1 in HIMEC culture, and this occurred in a discriminating manner. Ligands for TLR4 and NOD2 were particularly potent, even to a higher degree than VEGF or TNF- α , whereas ligands for TLR2/6 and NOD1 failed to significantly increase secretion of soluble CEACAM1 by HIMECs. The ligand for TLR4 is LPS, which is found primarily in Gram-negative bacteria, and the ligand for NOD2 is MDP, which is

FIGURE 6. Vessel sprouting induced by CEACAM1 and TLR and NLR ligands in the aortic ring assay. Murine aortic rings were placed in Matrigel in the presence and absence of optimal stimulatory doses of rCEACAM1 and TLR4 and NOD2 ligands, and vessel sprouting was visually assessed throughout 6 days. Parallel experiments were performed in the presence of anti-CEACAM1 antibody. The figure is representative of 4 experiments for normal and IBD HIMECs, respectively.

found in a large variety of bacteria.⁵³ These diverse ligand–receptor pairs would allow a wide range of microorganisms to promote soluble CEACAM1 secretion and make it locally available to further induce pro-angiogenic functions in vivo,⁵⁴ as suggested by our HIMEC-based in vitro systems.

We measured total CEACAM1 in tissues of non-IBD histologically, in CD- and UC-involved intestine, and in the serum of the respective subjects and found significantly increased levels in both forms of IBD. This is in agreement with the reported increase of CEACAM1 RNA and protein levels in IBD tissues, where CEACAM1 is expressed not only in the epithelium but also in the blood and lymphatic vessels, a finding consistent with a role in local angiogenesis.⁵⁵ CEACAM1 has multiple origins, and our results do not allow for determination of specific cellular sources. However, one could assume

that, in addition to inflammatory products, microbes contribute to the increased levels of CEACAM1 given the fact that the gut microbiota is an essential component of IBD pathogenesis and translocates into the inflamed tissues.^{41, 47} The increased level of CEACAM1 found in serum and intestinal tissues can be reasonably assumed to be functional in vivo and justify the investigation of whether CEACAM1 modulates bacterial product–induced effects directly relevant to angiogenesis, as we recently reported.²⁴ In addition, it is also possible that enhanced expression of CEACAM1 in the gut contributes to the increased angiogenesis found in both the CD intestine and the UC intestine.¹² In this regard, it is interesting that blood flow and oxygen tension can be reduced in the inflamed mucosa, whereas increased angiogenesis in IBD mucosa would suggest that inflammation increases blood

FIGURE 7. Differential production of HIMEC angiogenic factors by rCEACAM1 and TLR and NLR ligands. HIMEC monolayers were exposed to medium alone, optimal stimulatory doses of VEGF or TNF- α , increasing concentrations of rCEACAM1, and optimal stimulatory doses of TLR2/6, TLR4, NOD1, and NOD2 ligands for 24 hours; they were washed, and levels of IL-8 and bFGF were measured by ELISA in the culture supernatants. *Significantly increased amounts, as shown by the P values at the respective stimuli, compared with medium alone ($n = 9$, including 4 normal and 5 UC HIMEC lines). Abbreviation: L, ligand.

flow.⁵⁶ This apparent conflict was addressed in a study of structural adaptations of the murine colonic microvascular bed in a model of acute trinitrobenzene sulfonic acid induced colitis.⁵⁷ The results showed that structural changes in the colonic microcirculation are functionally associated with mononuclear cell transmigration and that vascular prominence, increased volumetric flow, and decreased blood flow coexist in the gut inflammatory response.⁵⁷

Apart from promoting HIMEC proliferation, when recombinant CEACAM1 was added to the cultures, we also observed induction of HIMEC migration, transmigration, and tube formation. When these CEACAM1-dependent responses were assessed in the presence of anti-CEACAM1-blocking antibodies, not only was the recombinant CEACAM1-dependent response abrogated, but also those induced by the bacterial ligands. The increased migration of HIMECs induced by TLR2/6 and NOD2 ligands was completely eliminated, as was the increased transmigration of HIMECs induced by TLR4 and NOD1 ligands. In the tube formation assay performed on Matrigel, the ligand for NOD1 failed to form thin endothelial cell bridges when CEACAM1 was blocked with specific antibodies.

Lastly, to gain further insights into the dependence on CEACAM1 for bacterial product-induced angiogenic responses, we carried out additional experiments using the vessel sprouting aortic ring assay.³⁷ Both the ligands for TLR4

and NOD1 were highly effective in stimulating microvessel sprouting, and this occurred to a degree virtually comparable to that of recombinant CEACAM1. Remarkably, this response was totally prevented by the blockade of CEACAM1 with specific neutralizing antibodies.

To identify the most effective isoform of CEACAM1 involved in TLR4 and NOD1 ligand-induced HIMEC angiogenesis, we performed the Matrigel tube formation assay in the presence of siRNAs for the total and 4L isoform of CEACAM1.⁵⁸ When compared with the effect of scrambled siRNA, both siRNAs reduced tube formation at generally comparable degrees, suggesting that the 4L isoform is the main molecular moiety involved in bacterial product-mediated angiogenesis. This is interesting because the L form of CEACAM1 is known to activate angiogenesis by affecting integrin-mediated signaling,⁴⁸ with potential implications for gut homing and anti-adhesion therapies in IBD.⁴² No studies have been performed to determine whether any specific CEACAM1 isoform is specifically activated in IBD. Because multiple isoforms are expressed by various cell types, like the short form in intestinal T cells⁵⁹ and the long form in the vasculature,⁴⁸ multiple isoforms are probably involved and together contribute to the mucosal inflammatory process.

Finally, to test whether CEACAM1 can stimulate the production of angiogenic factors by endothelial cells, HIMECs

were exposed to various amounts of recombinant CEACAM1 and optimal stimulatory doses of TLR2/6, TLR4, NOD1, and NOD ligands, which we have previously shown to induce angiogenesis.²⁴ The bacterial ligands were broadly effective in inducing production of IL-8 and bFGF, but neither of the 2 angiogenic factors was induced by CEACAM1. This suggests that although CEACAM1 has obvious pro-angiogenic properties, its capacity does not include the ability to promote the secretion of endogenous pro-angiogenic soluble factors that could act in an autocrine fashion and further strengthen or accelerate angiogenesis. We did not formally examine whether increased CEACAM1 expression would alter HIMEC barrier function, but the role of CEACAM1 in vascular homeostasis and the endothelial barrier is well recognized,⁶⁰ regulating the leukocyte–endothelium interaction and endothelial barrier leakiness.⁶¹ However, it is highly likely that in the IBD mucosa multiple other cell surface receptors and soluble factors contribute to disruption of the endothelial barrier in addition to CEACAM1.

Taking all of the above results together, it is obvious that different bacterial products have distinct capacities to modulate CEACAM1 expression and CEACAM1-dependent functions, probably due to the intrinsic properties of each ligand and the specific activation pathways triggered by each ligand.⁶² More importantly, however, is the discovery that the multiple pro-angiogenic effects induced by microbial products cannot optimally take place unless functional CEACAM1 is co-expressed in the endothelial cells. This conclusion indicates that TLRs/NLRs and CEACAM1 act concomitantly and reciprocally in the induction of intestinal angiogenesis. In fact, both TLRs and CEACAM1 upregulate VEGF and VEGF-R2 expression levels in vascular cells^{24, 31, 63} and activate signaling pathways involved in angiogenesis, such as those leading to cellular and integrin activation, like NF- κ B, MAPKs, p38, ERK, and FAK.^{64–68} These shared functions could underlie, at least in part, the reported association between gut microbiome composition and response to integrin blockade therapy in IBD patients.⁶⁹

The convergence of TLR-, NLR-, and CEACAM1-mediated signals during angiogenesis can be viewed as a harmonizing form of innate immunity. TLR- and NLR-initiated effects are typical responses of classical innate immune cells,⁵³ but it is now recognized that similar responses are also mediated by nonimmune cells, such as those of the vascular system under physiological and pathological circumstances.^{22, 70–72} The same is true for CEACAM1-induced effects. Based on its T-cell modulatory function, the role of CEACAM1 in adaptive immunity is well established,^{59, 73} but its role is being expanded to innate immunity by a growing body of complementary evidence,^{43, 74–76} including the results of this study. A recent report found that the clinical response to anti-integrin therapy in IBD patients is associated with substantial downregulation of mucosal innate immune responses, such as microbial sensing and chemoattraction.⁷⁷ TLR/NLR/CEACAM1 interactions mediate these

events in the gut endothelium. It is intriguing to speculate that a concomitant decrease of the gut microbial load might suppress angiogenesis and further enhance the clinical response to anti-integrin therapy in IBD.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

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