VEGF-A stimulation of leukocyte adhesion to colonic microvascular endothelium: implications for inflammatory bowel disease

Stephen Goebel,1 Meng Huang,1 William C. Davis,1 Merilyn Jennings,2 Teruna J. Siahaan,3 J. Steven Alexander,2 and Christopher G. Kevil1,2

1Department of Pathology and 2Department of Molecular and Cellular Physiology, Louisiana State University Health Sciences Center, Shreveport, Louisiana; and 3Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas

Submitted 6 October 2005; accepted in final form 9 November 2005

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder characterized by increased leukocyte recruitment and subsequent tissue damage. An increase in the density of the microvasculature of the colon during IBD has been suggested, leading to the concept that angiogenesis may play a pathological role in IBD. Increased tissue and serum levels of the angiogenic cytokine VEGF-A have been reported in cases of active IBD. In this study, we examined the hypothesis that VEGF-A exerts a proinflammatory effect on colonic microvascular endothelium that contributes to colonic inflammation. Leukocyte adhesion to VEGF-A-stimulated colon microvascular endothelial cells was examined using a parallel-plate hydrodynamic flow chamber. ICAM-1 adhesion molecule expression on colonic microvascular endothelium also was determined in response to VEGF-A stimulation, along with characterization of leukocyte adhesion molecule expression. High-dose VEGF-A (50 ng/ml) stimulation increased neutrophil and T cell adhesion to and decreased rolling velocities on activated endothelium, whereas low-dose VEGF-A (10 ng/ml) was without effect. Colonic endothelium constitutively expressed ICAM-1, which was significantly increased by treatment with 50 ng/ml VEGF-A or 10 ng/ml TNF-α but not 10 ng/ml VEGF-A. T cells expressed CD18 and CD11a with no expression of CD11b, whereas neutrophils expressed CD18, CD11a, and CD11b. Finally, VEGF-A-dependent leukocyte adhesion was found to occur in a CD18-dependent manner. These results demonstrate that VEGF-A levels found in IBD exert a proinflammatory effect similar to other inflammatory agents and suggest that this cytokine may serve as an intermediary between angiogenic stimulation and cell-mediated immune responses.

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder that involves immune dysfunction, genetic susceptibility, and bacterial flora in the intestinal environment (14, 36). Although the specific causes of IBD are poorly understood, the pathological nature of this disorder entails leukocyte recruitment and infiltration, tissue ulceration, and subsequent regeneration of intestinal mucosa (2, 4, 15). A critical feature of IBD is chronic immune dysregulation, which is the primary mediator of tissue damage associated with colitis (32, 35, 38, 41). However, recent clinical evidence suggests that stimulation of angiogenesis may play an important pathological role during colitis. Spalinger et al. (39) reported increased vessel density with Doppler ultrasonography during active Crohn’s disease that is not enhanced in unaffected tissue or disease remission. Moreover, a report by Fishman et al. (16) and others have established long-term remission of active Crohn’s disease with the antiangiogenic agent thalidomide. Together, these reports suggest that increased neovascularization may play an important role in IBD pathogenesis.

Vascular endothelial cell growth factor-A (VEGF-A) is a potent angiogenic cytokine that increases endothelial solute permeability, cell motility, and proliferation (28, 33). Several reports have documented increased serum and tissue concentrations of VEGF-A in IBD patients that appear to follow periods of disease activity versus quiescence (8, 22, 23). Moreover, VEGF-A was recently reported to increase endothelial cell adhesion molecule expression, such as ICAM-1, on human umbilical vein endothelial cells in vitro and to stimulate chronic inflammatory states upon transgenic or ectopic overexpression in vivo (13, 29, 37, 42). Thus VEGF-A may serve as an intermediary between angiogenic and inflammatory processes in IBD that could perpetuate a state of chronic inflammation. Moreover, leukocyte-endothelial cell adhesion molecules have been linked to angiogenesis in chronic inflammatory disorders such as diabetic retinopathy and rheumatoid arthritis, further suggesting that increased angiogenesis may enhance the chronic inflammatory response associated with IBD (19, 21, 31).

Leukocyte integrins and endothelial cell adhesion molecules work in concert to facilitate leukocyte rolling, firm adhesion, and transmigration across the endothelium (24, 25). Cytokine stimulation of either leukocytes or endothelial cells results in increased adhesion molecule expression and activation, thereby facilitating the process of leukocyte recruitment through adhesion molecule interactions. Members of the leukocyte β2-integrins LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), p150/90 (CD11c/CD18), and CD11d/CD18 are involved in mediating immune cell adhesion to vascular endothelium through interaction with ICAM-1 and other counterligands (17). In this study we examined whether the angiogenic cytokine VEGF-A could stimulate increased leukocyte adhesion to colonic microvascular endothelial cells and
Fig. 1. Neutrophil adhesion under hydrodynamic flow conditions. A: number of firmly adherent neutrophils on TNF-α- or VEGF-A-stimulated colon endothelium. B: average rolling velocities. C: frequency of total neutrophil rolling at various velocity intervals. *P < 0.05, treatments vs. nonstimulated.

Fig. 2. T cell adhesion under hydrodynamic flow conditions. A: T cell firm adhesion to TNF-α- or VEGF-A-stimulated colon endothelium. B: average T cell rolling velocities on activated endothelial monolayers. C: frequency of T cell rolling at various velocity intervals. *P < 0.05, treatments vs. nonstimulated.
determined the adhesion molecule requirements for VEGF-A-dependent leukocyte recruitment.

MATERIALS AND METHODS

Reagents. All cell culture material and supplies were purchased from Sigma (St. Louis, MO). Recombinant murine VEGF-A164 and TNF-α were purchased from Calbiochem (San Diego, CA). All antibodies for flow cytometry analysis were purchased from BD Laboratories (Franklin Lakes, NJ).

Animals. Mice used in this study were bred and housed at the Association for Assessment and Accreditation of Laboratory Animal Care, International-accredited Louisiana State University Health Sciences Center-Shreveport animal resource facility and maintained according to the National Research Council’s Guide for Care and Use of Laboratory Animals, and protocols were approved by the Institutional Animal Care and Use Committee. Male CD18−/− null (Igβ2tm2Bay) C57BL/6 mice were used for leukocyte isolations, whereas wild-type mice were used for colonic microvascular endothelial cell isolations.

In vitro cell culture. Mouse colonic endothelial cells were isolated from mucosal scrapings and cultured in Eagle’s minimum essential medium containing d-valine supplemented with fetal bovine serum (FBS), l-glutamine, nonessential amino acids, and antibiotic-antimycotic as previously reported (3). The colonic endothelial cells were stimulated with 10 ng/ml murine TNF-α or 10 or 50 ng/ml murine VEGF-A164 4 h before use in flow cytometric analysis and hydrodynamic flow chamber adhesion assays. The mouse T-lymphocyte cell line WEHI 7.1 was purchased from American Type Culture Collection and kept in Dulbecco’s modified Eagle’s medium supplemented with FBS, l-glutamine, and antibiotic-antimycotic as previously reported (18).

Isolation of mouse bone marrow neutrophils. Mouse neutrophils were collected from isolated bone marrow as previously reported (43). Briefly, marrow was flushed from femurs using RPMI culture medium. The resulting cell suspension was passed over a 100-µm sterile nylon filter screen to remove cell aggregates and other debris. The resulting filtrate was layered onto an equal volume of Histopaque 1077 density centrifugation medium and spun at 700 x g for 30 min. The resulting pellet was resuspended in red blood cell lysis buffer, spun, and washed using phosphate-buffered saline (PBS).

Immunofluorescence staining of cell adhesion molecules and flow cytometric analysis. The following antibodies were used to characterize mouse WEHI 7.1 T cells and bone marrow neutrophils: phycoerythrin-conjugated anti-mouse CD18, CD11a, and CD11b. The fluorescein isothiocyanate-conjugated anti-mouse CD54 (ICAM-1) was used to detect expression of this molecule on nonstimulated, 10 ng/ml of TNF-α, 10 and 50 ng/ml VEGF-stimulated mouse colonic endothelial cells. PE-conjugated rat IgG2a κ and rat IgG2b κ were used as isotype controls for CD11a, CD11b, and CD18, whereas FITC-conjugated IgG1 κ was used as an isotype control for CD54.

In vitro hydrodynamic flow chamber adhesion assay. Hydrodynamic parallel-plate flow chamber studies were performed as previously reported (18, 26). Briefly, mouse leukocytes were labeled with a fluorescent dye by 30-min incubation at 37°C with 200 nM CMPTX Cell Tracker red (Molecular Probes). The labeled cells were resuspended in HBSS at 2 x 10⁷ cells/ml in a 200-ml beaker kept at 37°C and stirred at 60 rpm. A Glycotech flow chamber insert and gasket...
were used to form a laminar plate flow chamber that could be viewed on a microscope. The labeled cells were drawn from the beaker into the flow chamber across a monolayer of colonic endothelial cells at a physiological shear rate of 1.5 dyn/cm² with a programmable digital syringe pump. The fluorescent cells were then viewed using a Nikon Eclipse TE-2000 epifluorescent microscope equipped with a Hamamatsu digital camera, and digital video was captured at 29 images/s using SIMPLE PCI software from Compix. The software’s motion tracking analysis feature enabled calculation of individual cell rolling velocity. Firmly adherent cells were defined as a cell that did not move one cell diameter over a 5-s period as determined by automated tracking and manual review of individual cells in each experimental field of view.

Statistical analyses. Data were statistically compared using Prism 4.0 software (GraphPad). All experiments were repeated at least four times. The number of firmly adherent cells was compared using a standard ANOVA with Bonferroni’s posttest to determine statistical differences between experimental groups. Firm adhesion data is reported as the mean and standard error. Rolling velocity data from 1,200 cells per treatment group were compared using a Kruskal-Wallis nonparametric ANOVA with a Dunn’s posttest to determine statistical differences between experimental groups. Rolling velocity data are presented as a bar graph illustrating the mean rolling velocity and standard error, as well as a relative frequency histogram distribution identifying cell populations rolling at various velocity intervals.

RESULTS

VEGF stimulates neutrophil and T cell adhesion under hydrodynamic flow conditions. Mouse T cells or neutrophils were drawn across mouse colonic endothelium in a hydrodynamic parallel-plate flow chamber at 1.5 dyn/cm² to measure leukocyte-endothelial cell biophysical interactions as previously reported (18, 26). Figure 1 shows biophysical data of neutrophil interactions under hydrodynamic flow conditions with monolayers of nonstimulated, TNF-α-stimulated, or VEGF-A-stimulated mouse colonic endothelium. TNF-α (10 ng/ml) stimulation significantly increased neutrophil adhesion to colonic microvascular endothelial cell monolayers, whereas high-dose (50 ng/ml) VEGF-A treatment also significantly increased neutrophil adhesion to a similar extent (Fig. 1A). Importantly, low-dose (10 ng/ml) VEGF-A did not increase neutrophil adhesion to colonic endothelium. Efficient leukocyte adhesion typically requires increased sampling of adhesion molecules on the endothelial cell surface by interacting immune cells (26). To increase immune cell sampling of the endothelial surface, leukocyte rolling velocities must decrease in order for adhesion molecules to engage counterligands, thus forming multiple strong adhesion bonds. Figure 1B shows that both TNF-α and VEGF-A (50 ng/ml) significantly decreased the average rolling velocity of neutrophils on colonic endothelium. Importantly, the frequency of neutrophils rolling between 0 and 49 μm/s was significantly elevated by either agent compared with nonstimulated conditions (Fig. 1C).

Figure 2 shows biophysical data of T cell interactions under hydrodynamic flow conditions with monolayers of nonstimulated, TNF-α-stimulated, or VEGF-A-stimulated mouse colonic endothelium. Figure 2A indicates the number of firmly adherent T cells to TNF-α- or VEGF-A-treated colon microvascular endothelial cells. High-dose (50 ng/ml) VEGF-A, but not low-dose (10 ng/ml) VEGF-A, significantly increased T cell adhesion as shown with neutrophils. Figure 2B demonstrates that both TNF-α and VEGF-A significantly decreased the average rolling velocity of T cells on the endothelial cell surface. Moreover, Figure 2C shows a large increase in the frequency of T cells rolling at average velocities between 0 and 49 μm/s. These data clearly demonstrate increased leukocyte-endothelial cell interactions under physiological hydrodynamic flow conditions in response to high-dose VEGF-A.

Fig. 4. Leukocyte surface adhesion molecule expression. A and D: CD18 expression on T cells and neutrophils, respectively. B and E: CD11a expression on T cells and neutrophils, respectively. C and F: CD11b expression on T cells and neutrophils, respectively. PE, phycoerythrin.
Characterization of surface adhesion molecule expression. Flow cytometric analysis was performed on nonstimulated, TNF-α/H9251-stimulated, and VEGF-A-stimulated mouse colonic endothelial cells to detect expression of the endothelial cell adhesion molecule ICAM-1 (Fig. 3). A low basal level of constitutive ICAM-1 expression was observed on nonstimulated colonic endothelium. Expression of ICAM-1 increased upon stimulation with 10 ng/ml TNF-α/H9251 (Fig. 3A). Low-dose (10 ng/ml) VEGF-A stimulation of colonic microvascular endothelial cells did not significantly alter ICAM-1 expression (Fig. 3C). However, high-dose (50 ng/ml) VEGF-A treatment significantly increased ICAM-1 expression to levels similar to those observed upon TNF-α stimulation. These data demonstrate a dose-dependent threshold of VEGF-A-mediated ICAM-1 expression.

The expression of CD18, CD11a, and CD11b was examined on T cells and neutrophils by using flow cytometric analysis (Fig. 4). Positive staining for CD18, the β2-subunit of the β2-integrins, was observed in both T cells and neutrophils (Fig. 4, A and D, respectively). Similarly, the αL integrin, CD11a, was expressed in both T cells and neutrophils (Fig. 4, B and E, respectively). However, the αM integrin, CD11b, was largely expressed on neutrophils and minimally expressed on T cells (Fig. 4, C and F, respectively). Thus a clear distinguishing difference between T cells and neutrophils was the lack of CD11b in T cells.

VEGF-A-mediated leukocyte adhesion is CD18 dependent. Numerous different leukocyte adhesion molecules mediate adhesion to endothelial cells; however, members of the β2-integrin (CD18) family serve as primary ligands for ICAM-1 (17, 24). Therefore, we determined the importance of leukocyte CD18 in mediating VEGF-A-dependent adhesion. Figure 5A shows that gene-targeted null deletion of CD18 completely abolished VEGF-A-mediated neutrophil adhesion compared with wild-type neutrophils. Moreover, loss of CD18 prevented any significant VEGF-A-induced neutrophil adhesion compared with basal CD18 null neutrophil adhesion to nonstimulated endothelium, whereas TNF-α stimulation still increased CD18 null neutrophil adhesion over basal CD18 null neutrophil adhesion, but this was significantly diminished compared with TNF-α-induced wild-type neutrophil adhesion. Figure 5B shows that the average neutrophil rolling velocities were still significantly slower in response to either VEGF-A or TNF-α, independent of CD18 expression. Figure 6 shows the importance of CD18 for VEGF-A-mediated T cell adhesion. Blockade of CD18 with a competitive cyclic peptide antagonist derived from the I-like domain CD18 sequence cLBE [cyclo(1,12)PenDLRNVKKLGCG-OH] significantly attenuated T cell adhesion on both TNF-α- and VEGF-A-stimulated colonic microvascular endothelium (Fig. 6A). Likewise, blockade of CD18 did not significantly affect T cell slow rolling on TNF-α- or VEGF-A-stimulated monolayers. Together, these data clearly demonstrate that VEGF-A-mediated neutrophil and T cell adhesion is primarily dependent on CD18.

DISCUSSION

The exact role of angiogenesis in the pathogenesis of IBD is unknown, but the increase in the colonic microvasculature in active IBD suggests that angiogenesis may relate somehow
with the inflammation and tissue damage characteristic of the disease. Tissue and serum levels of angiogenic agents, namely, VEGF-A, have been reported to be higher in active IBD than during remission with serum concentrations between 30 and 200 pg/ml and tissue levels between 50 and 150 ng/ml (8, 11, 22, 23). Thus increased VEGF-A expression and concomitant angiogenesis could facilitate tissue healing and ulcer repair. However, recent reports have shown that high expression levels of VEGF-A stimulate pathological angiogenesis and increase inflammatory responses (12, 30, 37, 40). The question then is not only whether VEGF-A acts as a proangiogenic agent but whether it also exerts proinflammatory responses in colonic endothelium, as well. In a previous study, VEGF-A stimulation of human umbilical vein endothelial cells increased expression of the inflammatory adhesion molecule ICAM-1, which has been reported to be important for leukocyte adhesion and recruitment in IBD (7, 29). Data presented in our study demonstrate that VEGF-A treatment of colonic microvascular endothelial cells significantly increases endothelial cell ICAM-1 surface expression that can facilitate leukocyte adhesion to activated endothelium (Figs. 1–3). These proinflammatory effects of VEGF-A are evident by the increased adhesion of both T cells and neutrophils to colonic endothelium under physiological hydrodynamic flow conditions and the dependency of CD18 for these interactions (Figs. 4–6).

Leukocyte β2-integrins have been reported to play an important role in chronic inflammatory disorders, including systemic lupus erythematosus (SLE), psoriasis, diabetes, and IBD (1, 5, 6, 20, 27). Increased angiogenic activity has been reported in these chronic diseases, suggesting that β2-integrins may modulate immune cell recruitment under such pathological conditions (10, 40). Consistent with this hypothesis, our data demonstrate a primary role for the β2-integrin CD18 in mediating both neutrophil and T cell adhesion to VEGF-A-activated colonic endothelium (Figs. 5 and 6). Our findings are interesting, because a previous report has shown that VEGF-A overexpression in the skin results in increased leukocyte adhesion to skin microvessels in a VCAM-1/VLA-4-dependent pathway (13). Together, our findings suggest that VEGF-A-dependent leukocyte recruitment may be differentially regulated in various tissues, possibly because of differences in the tissue microvascular endothelium.

Increased angiogenic activity and blood vessel density has long been observed in several chronic inflammatory disorders (9, 34). The exact pathological or physiological function of this relationship still remains largely unknown. However, VEGF-A stimulation of the angiogenic process could contribute to the sustenance of chronic inflammation through changes in microvascular permeability, provisional matrix deposition and remodeling, and altered vascular tone and increased blood flow, as well as increased vascular surface area available for leukocyte-endothelial cell interactions. In this study, we clearly demonstrated that VEGF-A also can increase endothelial cell adhesion molecule expression and CD18-dependent leukocyte adhesion under physiological flow conditions. Future studies are necessary to determine which members of the leukocyte β2-integrins (e.g., LFA-1 and Mac-1, among others) are required for VEGF-A-dependent leukocyte adhesion and whether in vivo inhibition of VEGF-A could alter immune cell recruitment during experimental colitis. Thus VEGF-A inhibition may represent a novel target for therapeutic intervention in IBD.

GRANTS
This work was supported by the Louisiana State University Health Sciences Center-Shreveport Center for Excellence in Arthritis and Rheumatology and National Institutes of Health (NIH) Grant DK-43785. Financial support also was received from a Self Faculty Scholar from the University of Kansas and NIH Grant AI-65302 (to T. J. Siahaan).

REFERENCES


