

VEGF-A Links Angiogenesis and Inflammation in Inflammatory Bowel Disease Pathogenesis

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Background & Aims: Vascular endothelial growth factor A (VEGF-A) mediates angiogenesis and might also have a role in inflammation and immunity. We examined whether VEGF-A signaling has a role in inflammatory bowel disease (IBD).

Methods: Expression levels of VEGF-A, and its receptors VEGFR-1 and VEGFR-2, were examined in samples from patients with IBD and compared with those of controls. The capacity of VEGF-A to induce angiogenesis was tested in human intestinal microvascular endothelial cells using cell-migration and matrigel tubule-formation assays. Levels of vascular cellular adhesion molecule-1 and intercellular adhesion molecule were measured by flow cytometry to determine induction of inflammation; neutrophil adhesion was also assayed. Expression patterns were determined in tissues from mice with dextran sulfate sodium (DSS)-induced colitis; the effects of VEGF-A overexpression and blockade were assessed in these mice by adenoviral transfer of VEGF-A and soluble VEGFR-1. Intestinal angiogenesis was measured by quantitative CD31 staining and leukocyte adhesion in vivo by intravital microscopy. **Results:** Levels of VEGF-A and VEGFR-2 increased in samples from patients with IBD and colitic mice. VEGF-A induced angiogenesis of human intestinal microvascular endothelial cells in vitro as well as an inflammatory phenotype and adherence of neutrophils to intestinal endothelium. Overexpression of VEGF-A in mice with DSS-induced colitis worsened their condition, whereas overexpression of soluble VEGFR-1 had the opposite effect. Furthermore, overexpression of VEGF-A increased mucosal angiogenesis and stimulated leukocyte adhesion in vivo. **Conclusions:** VEGF-A appears to be a novel mediator of IBD by promoting intestinal angiogenesis and inflammation. Agents that block

VEGF-A signaling might reduce intestinal inflammation in patients with IBD.

Inflammatory bowel disease (IBD) pathogenesis involves the interplay of multiple biologic components, among which nonimmune cells play a crucial role.¹⁻³ In particular, endothelial cells play a key role in multiple aspects of chronic intestinal inflammation, including expression of cell adhesion molecules (CAM) and chemokine secretion, recruitment of leukocytes and platelets, acquisition of a prothrombotic phenotype, and through immune-driven angiogenesis.^{4,5} Angiogenesis is therefore a complex process mediated by multiple cell types and mediators^{6,7} and is fundamental to many biologic processes, including growth, development, and repair.

Besides its well-known role in cancer, it has become clear that angiogenesis is also an integral component of a diverse range of nonneoplastic chronic inflammatory and autoimmune diseases, including atherosclerosis, rheumatoid arthritis, diabetic retinopathy, psoriasis, airway inflammation, peptic ulcers, and Alzheimer's disease.^{6,8,9} Indeed, angiogenesis is intrinsic to chronic inflammation and is associated with structural changes, including activation and proliferation of endothelial cells, and capillary and venule remodeling, all of which result in expansion of the tissue microvascular bed.¹⁰⁻¹² A potential functional consequence of this expansion is the promotion of inflammation through various correlated mechanisms. First, influx of inflammatory cells may increase; second, there is an increased nutrient supply to the metabolically active immune process; and, third, the activated endothelium contributes to the local production of cytokines, chemokines, and matrix metallopro-

Abbreviations used in this paper: CD, Crohn's disease; CAM, cell adhesion molecules; HIMEC, human intestinal microvascular endothelial cell; ICAM, intercellular adhesion molecule; UC, ulcerative colitis; VCAM, vascular cellular adhesion molecule; VEGF-A, vascular endothelial growth factor A; VEGFR, VEGF receptor.

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teinases.^{13,14} The anatomic expansion of the microvascular bed combined with its increased functional activation can therefore foster further recruitment of inflammatory cells, and angiogenesis and inflammation become chronically codependent processes.^{10,12,14,15} In addition, many of the mediators that are fundamental players in angiogenesis are also inflammatory molecules.^{16,17}

The angiogenic role played by the pathways involving the vascular endothelial growth factors (VEGFs) and their receptors is well characterized. There are 7 members of the VEGF family, ie, VEGF-A, -B, -C, -D, -E, -F, and placental growth factor, and these each interact with specific receptors, such as VEGFR-1 (flt-1), VEGFR-2 (KDR), and VEGFR-3.^{18,19} VEGF-A is the best characterized^{7,20,21} and is a fundamental mediator of pathologic angiogenesis, such as in neoplasia and chronic inflammation. Indeed, targeted blockade of VEGF-A is currently being used as a therapeutic approach to block angiogenesis in malignant tumors.^{22,23}

VEGF-A is crucially involved in several chronic inflammatory disorders,²⁴⁻²⁸ in which VEGF-A not only promotes pathologic angiogenesis but directly fosters inflammation.^{7,18,25,26} It is now well established that, in diseases such as rheumatoid arthritis, psoriasis, atherosclerosis, and chronic lung inflammation, VEGF-A is intimately involved in disease pathogenesis, and targeting VEGF-A is a promising new therapeutic strategy to dampen inflammation.^{7,9,18,27-32}

Studies from our laboratory and others have shown that angiogenesis is a novel component of both ulcerative colitis (UC) and Crohn's disease (CD) and that targeting angiogenesis by integrin $\alpha\beta3$ blockade is an effective and entirely novel approach to block experimental colitis.³³⁻³⁶ However, the specific mediators involved in immune-driven angiogenesis associated with IBD are still poorly defined.³⁷

A few reports have described overexpression of VEGF-A in humans with IBD,^{4,37} but the functional significance of such up-regulation is not yet understood. In addition, the messenger RNA (mRNA) for VEGF-A is strongly up-regulated in animals with chronic experimental colitis.³⁵ In murine colonic-derived endothelial cells, VEGF-A triggers an inflammatory phenotype by up-regulating CAMs and inducing adhesion of neutrophils and T cells, thus supporting an inflammatory role for this cytokine in the intestine.³⁸ However, thus far, VEGF-A and its receptors have not been fully characterized in patients with IBD nor has the functional role of VEGF-A been studied in these patients.

We have therefore evaluated the role of the VEGF-A pathway³⁹ in the pathogenesis of IBD. Here, we show that VEGF-A is up-regulated in involved tissues in humans with IBD and colitic mice, as is its receptor VEGFR-2, but not VEGFR-1. In vitro, VEGF-A induces both angiogenic activity and an inflammatory phenotype in human intestinal microvascular endothelial cells (HIMEC), whereas

overexpression in vivo increases disease severity and blockade decreases disease severity in colitic mice. This in vivo effect correlated with increased or decreased angiogenesis, respectively. In addition, VEGF-A induced recruitment of leukocytes to the inflamed intestine in vivo, thus fostering inflammation. These results strongly support the important role played by the VEGF pathway in both inflammation and the angiogenesis that underlies disease pathogenesis in IBD.

Materials and Methods

For additional information on materials and methods, see supplementary materials and methods section (see supplementary materials and methods online at www.gastrojournal.org).

Patient Population

Patients with active and inactive CD and UC were studied, and healthy individuals were enrolled as controls. Patients and controls were recruited at the Division of Gastroenterology, Istituto Clinico Humanitas, Milan, Italy, and the study was approved by the Institutional Review Board. Ethical guidelines were followed by the investigator in studies on humans or animals and described in the paper. Clinical disease activity was assessed by the Harvey-Bradshaw Activity Index and the Colitis Activity Index, as previously reported.³³ All diagnoses were confirmed by clinical, radiologic, endoscopic, and histologic criteria.

Immunostaining of Mucosal Expression of VEGFR-1 and -2 in Human and Murine Colonic Tissues and CD31 in Murine Colonic Tissues

Immunostaining was performed as previously described⁴⁰ (see supplementary materials and methods online at www.gastrojournal.org).

Isolation and Culture of HIMEC

HIMEC were isolated as previously described⁴¹ (see supplementary materials and methods online at www.gastrojournal.org).

Western Blotting Analysis

Immunoblotting was performed as previously described⁴² (see supplementary materials and methods online at www.gastrojournal.org).

Tubule Formation and Migration Assay

Endothelial cell tube formation was assessed using Matrigel (BD Biosciences, San Jose, CA), as previously described³⁶ (see supplementary materials and methods online at www.gastrojournal.org). Chemotaxis was assessed as previously reported^{40,43} (see supplementary materials and methods online at www.gastrojournal.org).

Analysis of HIMEC by Flow Cytometry

Detection of expression of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on HIMEC was performed by flow cytometry as previously described⁴⁴ (see supplementary materials and methods online at www.gastrojournal.org).

Induction of Colitis in Mice and Overexpression of VEGF-A and sVEGFR-1 Using Adenovirus

Colitis was induced in C57BL/6N mice by administration of 2.5% dextran sulfate sodium (DSS) (molecular mass, 40 kilodaltons; MP Biomedicals, Cleveland, OH) in filter-purified (Millipore Corporate, Billerica, MA) drinking water for 10 days. The severity of colitis was assessed on a daily measurement of weight loss^{34,45} (see supplementary materials and methods online at www.gastrojournal.org).

Engineered adenovirus encoding VEGF-A (phVEGF₁₆₅), soluble VEGFR-1 (sVEGFR-1), or vector alone (LacZ) were the generous gift of S. Yla-Herttuala and R. Pola and were generated as previously reported⁴⁶⁻⁵¹ (see supplementary materials and methods online at www.gastrojournal.org).

HIMEC-Neutrophil Adhesion Assay and Intravital Microscopy Studies

Adhesion assays were performed as previously reported^{44,45} (see supplementary materials and methods online at www.gastrojournal.org). Intravital microscopy experiments were performed as previously described⁴⁵ (see supplementary materials and methods online at www.gastrojournal.org).

Statistical Analysis

For statistical analysis, see supplementary materials and methods (see supplementary materials and methods online at www.gastrojournal.org).

Results

Mucosal and Plasma Levels of VEGF-A Are Up-Regulated in Patients With IBD

To compare the expression of VEGF-A under physiologic conditions and chronic inflammation, we

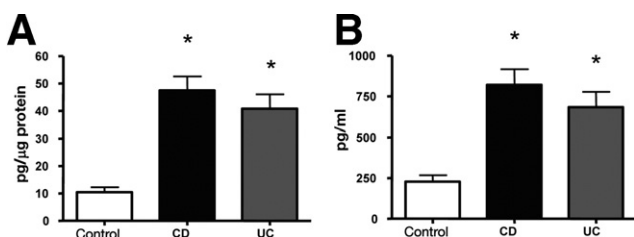


Figure 1. Expression of the VEGF-A protein is up-regulated in the mucosa and plasma of patients with IBD. Control (n = 16) or actively inflamed CD (n = 15) and UC (n = 16) patients were investigated for VEGF mucosal content in mucosal extracts (A) or in plasma (B). VEGF was measured by ELISA. * $P < .001$ for UC and CD compared with control.

first measured the levels of the VEGF-A protein in mucosal extracts from patients with active IBD and control individuals, using quantitative enzyme-linked immunosorbent assays (ELISA) of homogenized tissue samples (Figure 1A). The levels of VEGF-A in the mucosa of patients with either CD or UC were markedly ($P < .05$) enhanced compared with control individuals.

In addition, we also measured the levels of VEGF-A in the plasma of control individuals and compared those with that of patients with active CD or UC (Figure 1B). Again, there was a significant increase ($P < .05$) in the levels of VEGF-A in the plasma of patients with either form of IBD. VEGF-A was therefore up-regulated at both the systemic and intestinal levels in patients with IBD.

VEGFR-2 Is Up-Regulated, Whereas Levels of VEGFR-1 Remain Unchanged in the Mucosa of Patients With IBD

Having demonstrated that VEGF-A is up-regulated in the mucosa of patients with IBD, we next investigated the expression of its 2 receptors: VEGFR-1 and -2. First, we performed immunohistochemical staining of mucosal tissues from healthy control individuals, and the inflamed mucosa of patients with CD and UC. Endothelial cells in the colonic mucosa of both control individuals and actively inflamed tissues from patients with IBD were positive for VEGFR-1 immunostaining (Figure 2A), with no apparent differences in expression levels between the control (2.4 ± 0.2) and inflamed CD (2.3 ± 0.2) and UC (2.4 ± 0.2) mucosa.

To define the cell types on which VEGFR-1 was expressed, serial sections were immunostained for the following markers: CD31 (endothelial cells), CD68 (macrophages), CD3 (T cells), CD11C (dendritic cells), and MPO (neutrophils). As shown in Figure 2, VEGFR-1 immunolocalized with CD31, CD68, and epithelial cells, indicating that expression of VEGFR-1 is mainly found in the endothelium, macrophages, and epithelial cells.¹⁸ No difference in expression level was found between control and IBD tissues. CD3-, CD11C-, and myeloperoxidase-positive cells were negative for colocalization with VEGFR-1 (data not shown). Next, we investigated VEGFR-2, the inducible receptor for VEGF-A.¹⁸ Using the same serial sections used for VEGFR-1 immunostaining, we found that VEGFR-2 was also expressed by the endothelial microvasculature (CD31), macrophages (CD68), and epithelial cells, as reported in Figure 2B. VEGFR-2 was expressed at low levels in the microvasculature of control mucosa (0.7 ± 0.2), but its expression was strongly up-regulated in actively inflamed CD (2.0 ± 0.2 , $P < .01$) and UC (1.9 ± 0.3 , $P < .01$) mucosa (Figure 2B). The number of positive macrophages was higher in IBD tissues, but no differences in the expression level were observed between control and IBD tissues. No difference was found also in the expression of VEGFR-2 by epithe-

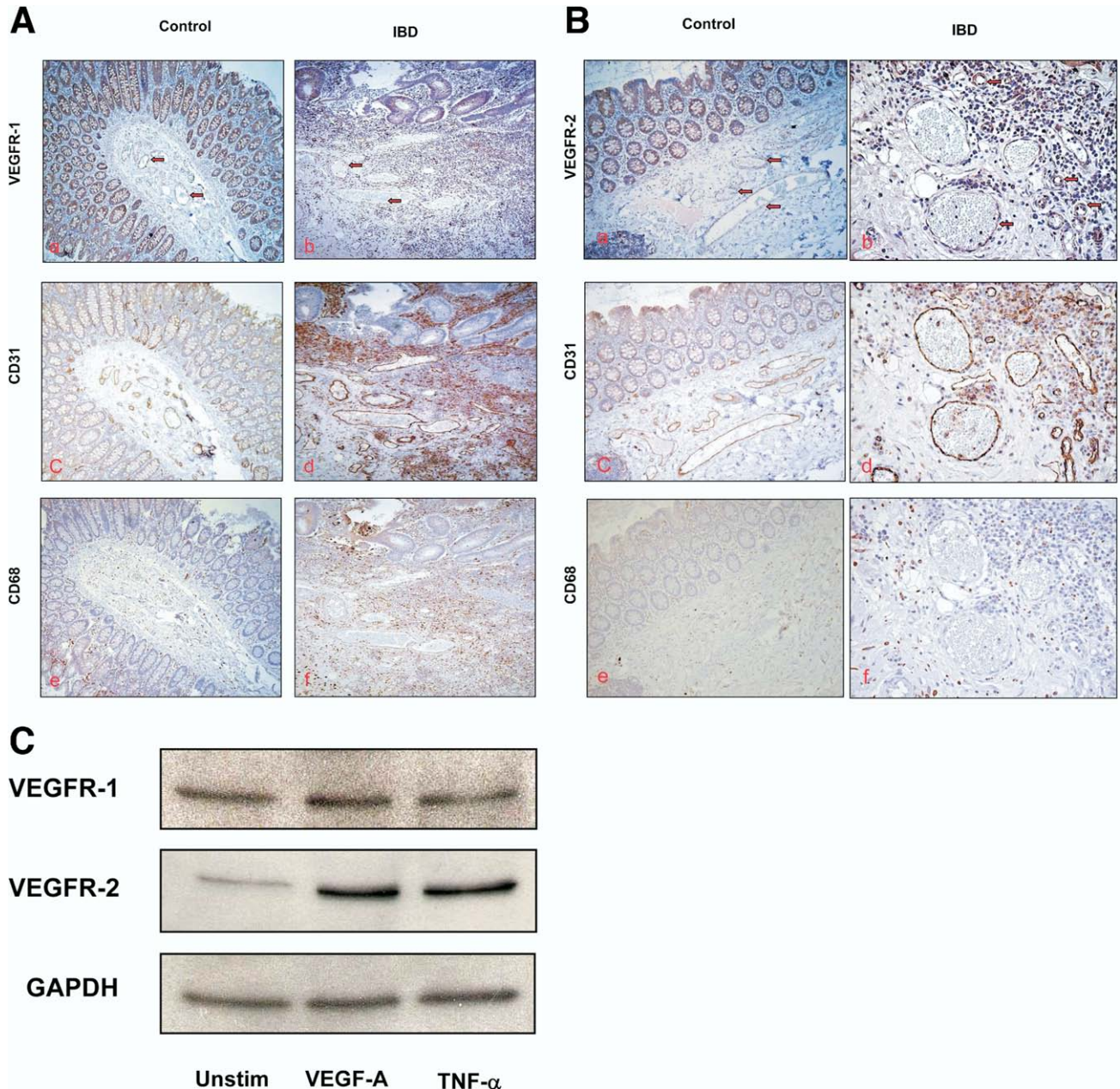


Figure 2. VEGFR-2 but not VEGFR-1 is up-regulated in human IBD. (A) The panels show brown immunohistochemical staining for VEGFR-1, CD31, and CD68 in serial sections of the colonic mucosa and submucosa from histologically normal control (a, c, e), active IBD (b, d, f). Original magnification, $\times 10$. (B) The panels show brown immunohistochemical staining for VEGFR-2, CD31, and CD68 in serial sections of the colonic mucosa and submucosa from histologically normal control (a, c, e; original magnification, $\times 10$), active UC, and active CD tissue (b, d, f; magnification, $\times 40$). The panels are representative of 10 control, 9 UC, and 12 CD samples, respectively. Red arrows indicate intestinal microvasculature. (C) HIMEC were left unstimulated or stimulated with VEGF-A or TNF- α then lysed and their expression of VEGFR-1 and -2 assessed by Western blotting.

lial cells between controls and IBD. No differences were found between uninflamed IBD and control mucosa (data not shown).

The levels of expression of the 2 receptors were also investigated in cultured HIMEC by Western blot analysis and quantified by densitometry. HIMEC constitutively expressed VEGFR-1 in unstimulated cultures

(0.91 ± 0.05), with no increase when cultures were stimulated with VEGF-A (0.99 ± 0.02) or tumor necrosis factor (TNF)- α (0.88 ± 0.01) (Figure 2C). In contrast to VEGFR-1, both VEGF-A (1.053 ± 0.02) and TNF- α (1.027 ± 0.02) induced significant ($P < .05$) up-regulation of the expression of VEGFR-2 on HIMEC (0.61 ± 0.02) (Figure 2B). There was therefore

no evidence for a change in the levels of VEGFR-1 in response to proinflammatory stimuli, or in the inflamed mucosa, whereas VEGFR-2 was clearly overexpressed in the inflamed mucosa, and its expression was increased in response to proinflammatory stimuli.

VEGF-A Induces Angiogenic Activity of HIMEC In Vitro

To assess whether VEGFR-1 and -2 expressed on HIMEC are functional, we investigated the capacity of VEGF-A to induce angiogenesis. We first determined the ability of VEGF-A to induce angiogenesis in vitro using a Matrigel tubule formation assay (Figure 3A). Unstimulated HIMEC failed to form tubules, whereas VEGF-A readily promoted tubule formation. The specificity of this response was confirmed by the application of an anti-VEGF antibody simultaneously with VEGF-A, which reduced tubule formation.

Next, we investigated the functional capacity of VEGF-A in mucosal extracts from control individuals and patients with IBD to induce migration of HIMEC in vitro (Figure 3B and C). No significant difference was observed between unstimulated HIMEC and HIMEC stimulated with mucosal extracts from control individuals. However,

mucosal extracts from patients with IBD potently induced migration of HIMEC ($P < .01$). This induction was significant ($P < .05$), although not completely dependent on the presence of VEGF-A in the mucosal extracts from patients with IBD because application of an anti-VEGF antibody at the same time as the mucosal extracts reduced the migration of the HIMEC.

VEGF-A Induces an Inflammatory Phenotype in HIMEC

We also investigated whether VEGF-A has the capacity to induce a proinflammatory phenotype in HIMEC. We first measured the expression of vascular ICAM-1 and VCAM-1. Unstimulated HIMEC constitutively expressed ICAM-1, but expression was strongly up-regulated (3- to 4-fold increase) by exposure to VEGF-A (Figure 4A). By contrast, unstimulated HIMEC expressed very low levels of VCAM-1, and no up-regulation in expression was observed after stimulation with VEGF-A (data not shown). On the other hand, when a similar concentration of TNF- α was used as positive control, the expression of ICAM-1 and VCAM-1 was increased by up to 5- to 6- and 50- to 60-folds, respectively, over baseline expression levels (data not shown). Next, we quantified the adhesion of neutrophils to VEGF-A-stimulated HIMEC. Unstimulated HIMEC bound few neutrophils (54 ± 11 cells/field), but this number significantly (622 ± 56 cells/field, $P < .001$) increased after stimulation with VEGF-A (Figure 4B). TNF- α (50 ng/mL) induced a further increase in neutrophil adhesion (918 ± 40 cells/field, not shown). Addition of an antibody that blocked endothelial ICAM-1 significantly (217 ± 16 cells/field, $P < .05$) decreased neutrophil adhesion, demonstrating a functional role for ICAM-1 in VEGF-A-dependent neutrophil adhesion (Figure 4B). Control antibodies failed to inhibit the induction of adhesion of neutrophils to HIMEC by VEGF-A or TNF- α (data not shown).

The VEGF Pathway Is Also Activated in Mice With DSS-Induced Colitis, With Up-Regulation of VEGF-A and VEGFR-2 But Not VEGFR-1

To investigate the mucosal expression of VEGF-A during the induction of experimental colitis, we measured the levels of the VEGF-A protein at different time points in mucosal extracts of mice administered 2.5% DSS. As measured by Western blot, VEGF-A was expressed in healthy mice, but its expression was markedly enhanced during induction of colitis (Figure 5A). We next investigated the expression levels of VEGFR-1 and -2 by immunohistochemical staining of mucosal tissues from control and DSS colitic mice. Colonic mucosa from the control mice showed a physiologic (2.5 ± 0.2) vascular immunostaining for VEGFR-1 (Figure 5B), with a similar degree of immunoreactivity detected in the colonic mucosa of DSS-treated mice (2.4 ± 0.3). On the contrary, VEGFR-2 was expressed at very low levels in the microvasculature of normal mice (0.4 ± 0.2), but its

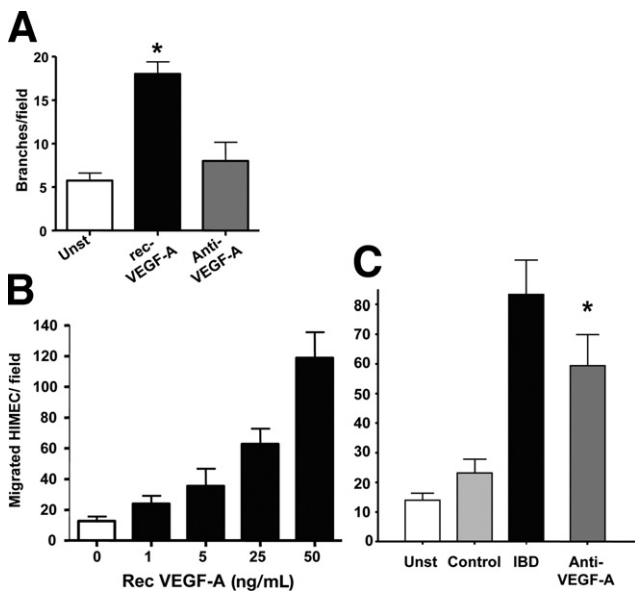


Figure 3. VEGF-A mediates angiogenesis in mucosal endothelial cells in vitro, causing both formation of tubules and migration of cultured HIMEC. (A) Tubule formation assays were performed with Matrigel. HIMEC were left untreated, or stimulated with VEGF, in the presence or absence of anti-VEGF-A blocking antibodies, stained with calcein. Results are representative of 4 independent experiments. Five high-power fields per culture condition were examined at magnification $\times 40$. (B and C) HIMEC were seeded on a transwell insert and left untreated, exposed to several doses of recombinant VEGF-A, or to a 1:10 concentration of extracts from normal or IBD mucosa (3 CD and 3 UC). Experiments were carried out in the absence and presence of blocking antibodies against VEGF-A. Migrated cells were labeled with calcein and counted. Data are derived from 6 separate experiments. * $P < .05$ for anti-VEGF-treated compared with untreated IBD mucosal extracts.

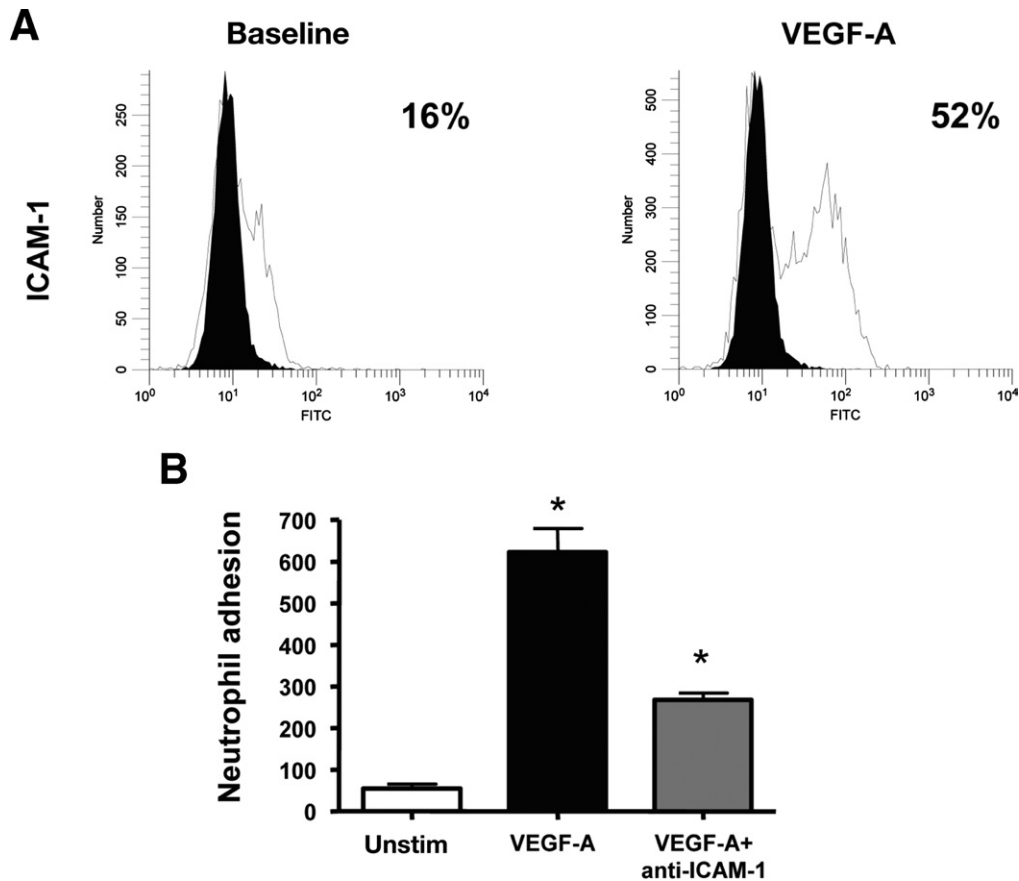


Figure 4. VEGF-A triggers an inflammatory phenotype in HIMEC in vitro by inducing expression of ICAM-1, as well as ICAM-1-dependent neutrophil adhesion. (A) HIMEC were left untreated or stimulated with VEGF-A. After 24 hours, expression of ICAM-1 was measured by flow cytometry. The black curve represents the background signal from the isotype control. The Figure is representative of 5 separate experiments. Numbers represent the net percentage of positive cells. (B) HIMEC were left untreated (baseline) or stimulated with VEGF-A, with or without monoclonal antibodies against ICAM-1. Calcein-labeled neutrophils were added to the HIMEC monolayers. The number of adherent cells/mm² in each experimental condition was expressed as mean \pm SEM of 5 separate experiments. * $P < .05$ for VEGF stimulated HIMEC vs untreated HIMEC or vs anti-ICAM-1 treated HIMEC.

expression was strongly up-regulated (2.3 ± 0.3 , $P < .001$) in the actively inflamed mucosa of DSS colitic mice (Figure 5B). These findings mirror those observed in humans, as described above.

In Vivo Manipulation of the Expression of VEGF-A Affects the Course of Experimental Colitis

To investigate whether VEGF-A plays a key role in the pathogenesis of experimental colitis, we undertook a series of experiments using adenovirus constructs to overexpress VEGF-A or a soluble form of its receptor VEGFR-1 (sVEGFR-1). This experimental approach has been successfully used in several disease models but not in experimental IBD.^{50,52-54} Initially, we transfected healthy mice with the adenovirus encoding for VEGF-A or a control adenovirus, both of which have previously been described.⁴⁶⁻⁵¹ The mice were killed every other day and compared with control adenovirus-infected mice. We found that the plasma of animals that received the VEGF-A-encoding adenovirus contained high and sus-

tained levels of VEGF-A and sVEGFR-1 (see supplementary Figure 1A and B online at www.gastrojournal.org). To determine whether the virus localizes to the intestine, we transfected mice with adenovirus-LacZ and immunostained the gut with X-gal. Mice transfected with the VEGF-A adenovirus displayed intense X-gal staining in their mucosa, indicative of LacZ expression, whereas no X-gal staining was observed in the mice transfected with the control adenovirus (data not shown). In addition, to verify whether the VEGF-A expressed by the transfected virus caused a significant increase in levels of VEGF-A at the tissue level, we analyzed mucosal protein extracts derived from mice transfected with control or VEGF-A adenovirus by Western blot. VEGF-A was also more abundantly expressed in the intestine of the healthy VEGF-A adenovirus-transfected mice compared with control adenovirus-transfected mice (data not shown). Similar overexpression of VEGF-A was found in the lung, kidney, and liver, although no resulting pathologic features were observed in the transfected tissues (data not shown).

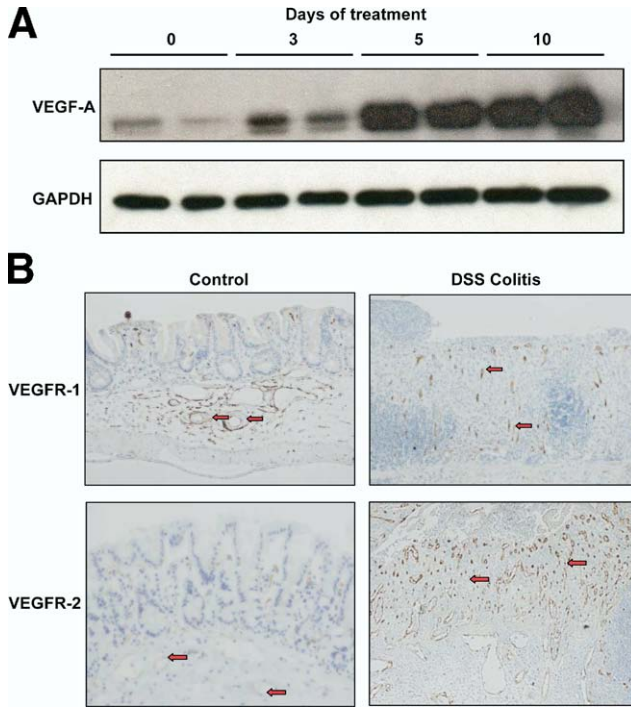


Figure 5. VEGF-A is up-regulated in mice with DSS-induced colitis, as is its receptor VEGFR-2, but not VEGFR-1. (A) Mucosal extracts were obtained from mice undergoing DSS treatment, and their VEGF-A content was assessed by Western blotting. (B) The panels show brown immunohistochemical staining for VEGFR-1 and -2 in the microvasculature of colonic mucosa and submucosa from histologically normal control mice (A and C) and DSS-colitic mice (B and D). The panels are representative of 6 control and 7 DSS colitic mice.

The VEGF-A adenovirus construct was then used to overexpress VEGF-A in mice with DSS-induced colitis to determine the effects on colitis. In addition, the effect of blocking VEGF-A by administering an adenovirus that encodes the soluble receptor VEGFR-1 (sVEGFR-1) was determined (Figure 6). Compared with mice administered control adenovirus, mice administered the VEGF-A adenovirus developed significantly more severe colitis ($P < .05$), whereas mice administered the sVEGFR-1 adenovirus displayed a significantly ($P < .05$) less severe colitis, as assessed by weight loss (Figure 6A) and histologic scores (Figure 6B). In particular, there was 100% mortality in the VEGF-A adenovirus group by day 9, whereas only 20% of the mice that received the control adenovirus and none that received the sVEGFR-1 adenovirus died (data not shown).

Intestinal inflammation is associated with a local increase in the production of cytokines and chemokines. To test whether the severe colitis we observed in the mice overexpressing VEGF-A mice was associated with an increase in cytokine and chemokine production in the intestinal mucosa, the levels of TNF- α , the mouse homolog of human interleukin (IL)-8 (KC) in the colonic mucosa were measured in an organ culture system. After colitis was established, mice that overexpressed VEGF-A

produced more TNF- α ($0.8 \pm 0.1 \text{ pg}/\mu\text{g}$) and KC ($45 \pm 3 \text{ pg}/\mu\text{g}$) than control mice ($0.4 \pm 0.03 \text{ pg}/\mu\text{g}$ and $20 \pm 2 \text{ pg}/\mu\text{g}$, respectively, both $P < .05$). Notably, mice in which VEGF-A was blocked produced significantly (both, $P < .05$) less TNF- α ($0.2 \pm 0.04 \text{ pg}/\mu\text{g}$) and KC ($7 \pm 1 \text{ pg}/\mu\text{g}$).

Manipulation of VEGF-A Expression In Vivo Affects Both Angiogenesis and Inflammation In Vivo

Finally, we investigated the effects of VEGF-A on angiogenesis and intestinal inflammation during the course of colitis. First, we measured the effects of overexpression of VEGF-A and sVEGFR-1 on intestinal angiogenesis by investigating the expression of CD31, an established marker of angiogenesis, and by quantifying microvascular density. Mice that overexpressed VEGF-A had a significantly increased ($P < .05$) number of microvessels ($112 \pm 8 \text{ vessel}/\text{field}$) compared with mice administered the control adenovirus ($79 \pm 12 \text{ vessel}/\text{field}$) or healthy control mice ($38 \pm 3 \text{ vessel}/\text{field}$). Im-

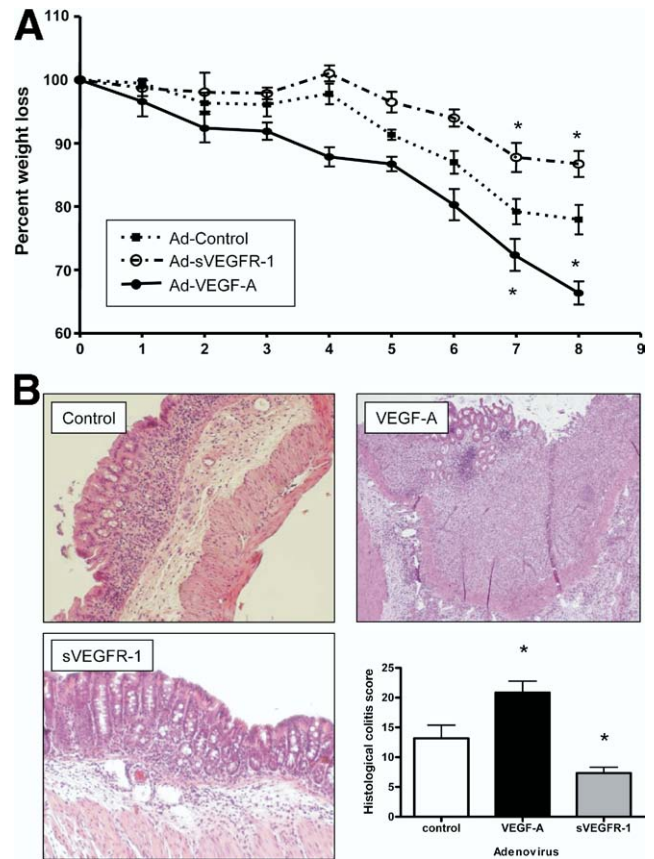


Figure 6. Overexpression of VEGF-A increases the severity of colitis in DSS treated mice, whereas VEGFR-1 decreases the severity. (A) Mice undergoing DSS treatment were injected with adenoviruses encoding VEGF-A ($n = 11$), sVEGFR-1 ($n = 9$), or a control virus ($n = 8$) and monitored daily for weight loss. (B) After 8 days, mice were killed and their colons assessed for histologic colitis. * $P < .05$ for mice overexpressing VEGF ($n = 6$) compared with control mice ($n = 6$) and for mice overexpressing sVEGFR-1 ($n = 6$) vs control mice.

BASIC-ALIMENTARY TRACT

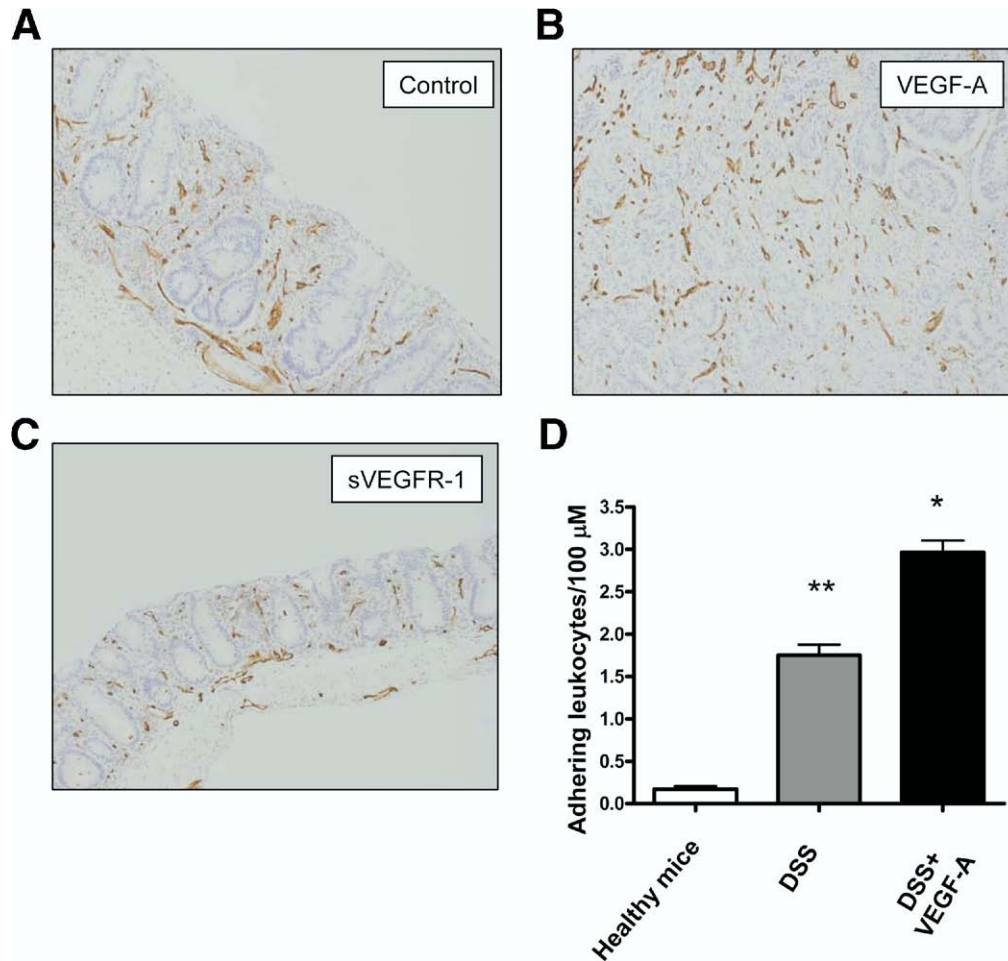


Figure 7. Modulation of the VEGF pathway affects angiogenesis and leukocyte adhesion in mice with DSS-induced colitis. Mice undergoing DSS treatment were injected with adenoviruses encoding a control virus (A, $n = 8$), VEGF-A (B, $n = 11$), or sVEGFR-1 (C, $n = 9$) and were killed after 8 days of treatment. Their colons were immunostained for CD31 and vascular density was measured. (D) Leukocyte-endothelial cell interactions were assessed by intravital microscopy in 3 groups of animals: healthy mice (open bar; $n = 6$), DSS colitic mice (shaded bar; $n = 6$), and DSS colitic mice, treated with daily injections of VEGF (solid bar; $n = 6$). Leukocyte adhesion is expressed as the number of firmly adherent leukocytes to the endothelium per 100 μm of venule. ** $P < .001$ between healthy and colitic mice; * $P < .05$ between colitic/placebo and colitic/VEGF treated mice.

portantly, mice administered the adenovirus encoding for sVEGR-1 had a significantly decreased ($P < .05$) number of mucosal vessels (44 ± 11 vessel/field) (Figure 7).

Second, we investigated whether VEGF-A could trigger leukocyte adhesion to the intestinal endothelium *in vivo*, thus triggering and promoting inflammation. We quantified the number of adhering leukocytes in the colonic microcirculation by intravital microscopy, as previously reported.⁴⁵ In healthy mice, very few leukocytes adhered to colonic venules (Figure 7D). By contrast, a large number of leukocytes adhered to the colonic microvascular endothelium in mice with DSS-induced colitis ($P < .001$). In addition, colitic mice that were also administered 1 $\mu\text{g/g}$ recombinant VEGF had a further significant increase in leukocyte adhesion to the intestine (Figure 7D), an effect that was abrogated if recombinant VEGF was blocked by monoclonal antibodies (data not shown).

Healthy mice treated with recombinant VEGF alone had no significant increase of leukocyte adhesion to the intestinal microvascular endothelium.

Finally, compared with the intestinal vascular permeability of healthy mice administered the control adenovirus (06 ± 0.2 mg/g), permeability was not significantly increased in healthy mice given the adeno-VEGF-A virus (0.7 ± 0.3 mg/g, not significant). In addition, permeability increased when colitis was established at day 7 compared with in healthy mice (5 ± 0.5 mg/g, $P < .05$). Notably, adeno-VEGF-A transfected colitic mice displayed a further abnormal and significant increase (9.3 ± 0.9 mg/g, $P < .05$) compared with untransfected DSS colitic mice, whereas transfection with adeno-sVEGFR1 attenuated the increase to levels below those in uninfected DSS colitic mice (2.6 ± 0.3 mg/g), suggesting that VEGF-A regulates permeability.

Discussion

It has now been clearly established that the microvascular changes associated with angiogenesis are key contributors to the tissue injury and remodeling process that inevitably accompanies chronic inflammation.^{10,12,14,15} However, the important role played by angiogenesis in several chronic inflammatory diseases is still being elucidated.¹⁵ We and others have shown that intense angiogenesis occurs in humans with IBD in animals with experimental colitis.³³⁻³⁵

Increasing evidence suggests that VEGF-A is one of the major proangiogenic factors involved in pathologic angiogenesis. The expression of VEGF-A and its receptors is elevated in patients with inflammatory skin diseases that are associated with enhanced vascularity such as psoriasis.^{55,56} Similarly in human and experimental rheumatoid arthritis, the VEGF-A pathway is strongly overexpressed and activated, and its blockade is clinically beneficial.⁵⁷

In this study, we demonstrate activation of the VEGF pathway in the actively inflamed mucosa of patients with IBD. Expression of both VEGF-A and its receptor VEGFR-2 are enhanced in tissue biopsy specimens from inflamed bowel segments. To test whether the proinflammatory milieu of the IBD mucosa can directly affect the expression of VEGFR-1 and -2 on the endothelium, we investigated whether TNF- α or the receptor ligand VEGF-A could enhance receptor expression levels. Unstimulated HIMEC express both VEGFR-1 and VEGFR-2, but only VEGFR-2 was up-regulated in response to VEGF-A or the proinflammatory cytokine TNF- α , suggesting that the overexpression observed in vivo in patients with IBD may be due to a mucosal milieu rich in inflammatory mediators. We next investigated whether the enhanced expression of VEGF-A observed in patients with IBD can affect the angiogenic activity of endothelial cells using microtubule formation and migration assays. When HIMEC were stimulated with VEGF-A, there was rapid formation of microtubules, an effect inhibited by an anti-VEGF antibody, which completely inhibited microtubule formation. In addition, mucosal extracts from patients with IBD potentially induced migration of HIMEC, which was again VEGF-A dependant because migration was reduced by the anti-VEGF-A antibody.

Interestingly, besides its classical angiogenic activities, we also found that VEGF-A can exert proinflammatory effects on intestinal endothelium, both in vitro and in vivo. When the endothelium becomes inflamed, it expresses enhanced levels of cell adhesion molecules. This was also true for HIMEC that had been stimulated with VEGF-A, which caused enhanced expression of ICAM-1. The functional consequences of expression of ICAM-1 by HIMEC were confirmed by the demonstration that they were able to mediate neutrophil adhesion. Taken together, these data suggest that, besides acting as an angiogenic mediator, VEGF-A is also an inflammatory mol-

ecule acting on mucosal endothelial cells during the course of IBD.

Next, we investigated the expression of VEGF-A and its receptors in the DSS model of colitis. We found that the effects on the VEGF pathway in this experimental model of colitis mirrored that found in humans. VEGF-A, and its receptors VEGFR-1 and VEGFR-2, were all expressed under physiologic conditions. However, after the induction of colitis, the expression of both VEGF-A and VEGFR-2 were markedly enhanced, whereas no increase in the expression of VEGFR-1 was observed. These findings indicate that this model of colitis offers a good platform to manipulate the VEGF pathway and thereby affect the course of colitis.

Adenoviruses are frequently used for the in vivo overexpression of proteins, and their safety for use in humans is well established.⁴⁶ Adenoviruses for overexpression of VEGF-A have previously been used to investigate VEGF-A in several murine models of chronic inflammation, such as atherosclerosis, arthritis, diabetes, sepsis, and vascular inflammation.⁴⁶⁻⁵⁰ In such models, systemic overexpression of VEGF-A induces generalized up-regulation of VEGFR-2 by endothelial cells in several organs.^{23,58,59} In addition, we also used an adenovirus for overexpression of a soluble form of VEGFR-1 to block the activity of VEGF-A in vivo. Adenoviral transfer of VEGF-A overexpresses plasma concentration nearly to 6 ng/mL, a value comparable with the in vitro experiments we performed. In line with our observations that expression of VEGF-A is enhanced in humans with IBD and mice with experimental colitis and that VEGF-A induces an inflammatory phenotype in HIMEC, overexpression of VEGF-A in the intestinal mucosa of mice with DSS-induced colitis caused a significantly more severe disease, including increased colonic cytokine and chemokine production, with 100% mortality by day 9. On the other hand, blockade of VEGF-A with sVEGFR-1 significantly ameliorated the severity of disease and decreased mucosal production of inflammatory cytokines and eliminated mortality.

To address directly the changes that are occurring during manipulation of the VEGF pathway, we investigated angiogenic changes in these animals. The levels of the angiogenic marker CD31 were markedly increased in mice overexpressing VEGF-A, whereas its blockade inhibited angiogenesis. In addition, the increased leukocyte adhesion observed by intravital microscopy during overexpression of VEGF-A indicates that the increased severity of inflammation results from a direct effect on the inflammatory phenotype of the endothelial cells. Finally, because VEGF-A is also a crucial gatekeeper of vascular permeability, we measured its effect on the regulation of intestinal microvascular permeability. Adeno-VEGF-A-treated mice had an increase of Evans blue leakage, both under normal and colitic conditions. This effect was reduced by blockade of VEGF-A, suggesting that VEGF-A is responsible for exacerbation of the tissue edema that

accompanies the colitis. However, VEGF-A induction provoked Evans blue leakage specifically in the gut and not in other tissues, leading to the premise that VEGF-A is necessary, but not sufficient, for disease activity in experimental IBD.

Even though the overexpression of VEGF-A in experimental colitis might induce levels that are high compared with those observed in humans with IBD, it provides a very useful tool to study the contribution of VEGF-A to the pathogenesis of intestinal inflammation. In addition, combining these data with the beneficial effects of blockade of VEGF-A observed both *in vitro* and *in vivo*, it compellingly supports the proinflammatory role of VEGF-A in intestinal inflammation.

In conclusion, our results identify VEGF-A as a molecule intimately involved in IBD pathogenesis and one that acts at the crossroads between inflammatory-driven angiogenesis and mucosal inflammation. This suggests that blockade of VEGF-A may represent a new strategy to dampen intestinal inflammation.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: [10.1053/j.gastro.2008.09.064](https://doi.org/10.1053/j.gastro.2008.09.064).

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Supplementary Materials and Methods

Isolation and Culture of HIMEC

HIMEC were isolated as previously described.¹ Briefly, HIMEC were obtained from surgical specimens of patients with CD and UC and from normal areas of the intestine of patients admitted for bowel resection because of colon cancer, polyps, or diverticulosis. HIMEC were isolated by enzymatic digestion of intestinal mucosal strips followed by gentle compression to extrude endothelial cell clumps, which adhere to fibronectin-coated plates and were subsequently cultured in MCDB131 medium (Sigma, St. Louis, MO) supplemented with 20% fetal bovine serum (FBS), antibiotics, heparin, and endothelial cell growth factor. Cultures of HIMEC were maintained at 37°C in 5% CO₂, fed twice a week, and split at confluence. HIMEC were used between passages 3 and 12.

Tubule Formation and Migration Assay

Endothelial cell tube formation was assessed using Matrigel (BD Biosciences, San Jose, CA), as previously described.² Briefly, multiwell dishes were coated with 250 μ L of complete medium containing 5 mg/mL Matrigel, and HIMEC were resuspended in complete growth medium containing only VEGF-A or a combination of VEGF-A and anti-VEGF-A (25 μ g/mL; both from R&D Systems, Minneapolis, MN) then were seeded at a density of 5×10^4 . Cells were cultured on Matrigel for 16 hours, and inverted phase-contrast microscopy was used to assess formation of endothelial tube-like structures. Five high-power fields per condition were examined, and experiments were performed in duplicate.

Chemotaxis was assessed using previously reported methods. Briefly, fluorescence-blocked polycarbonate filters (8- μ m pore size; BD Bioscience, Franklin Lakes, NJ) were coated with human fibronectin (10 μ g/mL) for 1 hour at room temperature. Using a transwell system, 35×10^4 HIMEC were plated in the upper chamber in MCDB-131 medium, while the lower chamber contained chemotaxis buffer with growing concentration of VEGF (1–50 ng/mL) as positive controls or control or IBD-derived mucosal extracts. In some experiments, 25 μ g/mL of blocking antibodies against VEGF, or control antibody, were added to the chemotaxis buffer. After 4 hours, buffer was removed from both chambers, and HIMEC migrated onto the lower surface of the porous membrane were washed twice in phosphate-buffered saline (PBS) and stained with calcein for 15 minutes at 37°C. Duplicates of migrated cells were observed with an inverted fluorescence microscope and counted in 6 random high-power ($\times 200$) fields. Quantitative analysis of data was performed using the Image Pro Plus software (Media Cybernetics, Inc, Bethesda, MD).

Intravital Microscopy Study of Leukocyte-Endothelium Interactions in the Bowel Microvasculature

Mice were anesthetized with subcutaneous ketamine (150 mg/kg) and xylazine (7.5 mg/kg), and a tail vein was cannulated. Throughout the experiments, rectal temperature was monitored using an electrothermometer and was maintained between 36.5°C and 37.5°C with an infrared heat lamp. The abdomen was opened via a mid-line incision, and a segment of the distal colon was chosen for microscopic examination, exteriorized, and covered with a cotton gauze soaked with bicarbonate buffer. Mice were then placed on an adjustable microscope stage, and the colon was extended over a nonautofluorescent coverslip that allowed observation of a 2-cm² segment of tissue. An inverted microscope (Diaphot 300; Nikon, Tokyo, Japan) with a CF Fluor 403 objective lens (Nikon) was used. A charge-coupled device camera (model XC-77; Hamamatsu Photonics, Hamamatsu, Japan) with a C2400 charge-coupled device camera control unit and a C2400-68 intensifier head (Hamamatsu Photonics), mounted on the microscope, projected the image onto a monitor (Trinitron KX-14CP1; Sony, Tokyo, Japan), and the images were recorded using a videocassette recorder (SR-S368E; JVC, Tokyo, Japan) for off-line analysis. Leukocytes were labeled *in vivo* by subcutaneous injection of rhodamine-6G (Molecular Probes, Leiden, The Netherlands). Rhodamine-6G-associated fluorescence was visualized by epi-illumination at 510–560 nm, using a 590-nm emission filter. Single unbranched submucosal and lamina propria venules with internal diameters of 25–40 μ m were selected for observation. The flux of rolling leukocytes, leukocyte rolling velocity, number of adherent leukocytes, venular blood flow, and venular wall shear rate were determined off-line after playback of the videotapes, as previously described.³ Rolling leukocytes were defined as those white blood cells that moved at a velocity less than that of free-flowing leukocytes in the same vessel. The flux of rolling leukocytes was measured as the number of rolling leukocytes that passed a fixed point within a small (10 mm) viewing area of the vessel in a 1-minute period. Leukocytes were considered adherent to venular endothelium when stationary for 30 seconds or longer and expressed as the number per 100- μ m length of venule. In each animal, 3 to 6 random venules were examined, and results were calculated as the mean of each parameter in all venules examined.

Western Blotting Analysis

Confluent HIMEC monolayers were left unstimulated or stimulated with VEGF-A (50 ng/mL) or TNF- α (50 ng/mL) both from R&D Systems) for 48 hours in regular culture medium supplemented with 5% FBS. The cells were lysed with extraction buffer containing 50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 10% glycerol, 1% Triton

X-100, and 50 mmol/L protease plus 50 mmol/L phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The protein concentration of lysates was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Immunoblotting was performed as previously described.⁴ Proteins (20 μ g) were separated on a 10% Tris-glycine gel and electrotransferred to a nitrocellulose membrane (Novex, San Diego, CA). Nonspecific binding was blocked by incubation with 5% milk in 0.1% Tween 20/tris-buffered saline (Fisher Scientific, Hanover Park, IL), followed by overnight incubation at 4°C with the primary antibody: either mouse anti-human VEGF-R1 or rabbit anti-human VEGF-R2, both diluted at 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA).

Membranes were washed for 20 minutes in 0.1% Tween 20/tris-buffered saline and then incubated for 1 hour with the appropriate horseradish peroxidase-conjugated secondary antibody, goat anti-mouse antibody, or goat anti-rabbit antibody (1:2000, Santa Cruz Biotechnology). The membranes were incubated with chemiluminescent substrate (Super Signal; Pierce, Rockford, IL) for 5 minutes, after which they were exposed to film (Amersham, Arlington Heights, IL).

Colonic samples from colitic mice were frozen in liquid nitrogen at the time of removal. Following mechanical homogenization in liquid nitrogen, specimens were processed in a lysing buffer for protein extraction as described above. Samples were then sonicated (twice for 5 seconds), and insoluble material was removed by centrifugation for 15 minutes at 16,000g at 4°C. The concentration of proteins in each lysate was measured using the Bio-Rad protein assay (Bio-Rad Laboratories). Immunoblotting was performed as described above, using the rabbit anti-mouse VEGF-A (R&D Systems).

Migration Assay

Chemotaxis was assessed as previously reported,^{5,6} with some modifications. Briefly, fluorescence-blocked polycarbonate filters (8- μ m pore size; BD Bioscience, Franklin Lakes, NJ) were coated with human fibronectin (10 μ g/mL) for 1 hour at room temperature. By using a transwell system, HIMEC were plated in the upper chamber in MCDB-131 medium, whereas the lower chamber contained chemotaxis buffer with VEGF-A (50 ng/mL) as positive controls or control or IBD-derived mucosal extracts. In some wells, 25 μ g/mL of blocking antibodies against VEGF-A or control antibody were added to the chemotaxis buffer. After 4 hours, buffer was removed from both chambers, and the HIMEC that had migrated onto the lower surface of the porous membrane were washed twice in PBS and stained with calcein for 15 minutes at 37°C. Duplicates of migrated cells were observed with an inverted fluorescence microscope and counted in 6 random high-power (\times 200) fields. Quantitative analysis of data was performed using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

Mucosal extracts from control patients or from patients with IBD were also applied, then the same experimental conditions were used. Mucosal extracts were obtained as described above.

Induction of Colitis in Mice

Animal studies were approved by the Ethical Committee of the Instituto Clinico Humanitas and Hospital Clinic y Provincial, Barcelona, Spain. Colitis was induced in C57BL/6N mice by administration of 2.5% DSS (molecular mass, 40 kilodaltons; MP Biomedicals, Cleveland, OH) in filter-purified (Millipore Corporate, Billerica, MA) drinking water for 10 days. The severity of colitis was assessed on a daily measurement of weight loss.^{3,7}

For each animal, histologic examination was performed on 3 samples from the distal colon; samples were fixed in 10% formalin before staining with H&E. All histologic quantification was performed blinded using a scoring system that has previously been described.^{3,7} Three independent parameters were measured: severity of inflammation (0–3: none, slight, moderate, severe), extent of injury (0–3: none, mucosal, mucosal and submucosal, transmural), and crypt damage (0–4: none, basal one-third damaged, basal two-thirds damaged, only surface epithelium intact, entire crypt and epithelium lost). The score of each parameter was multiplied by a factor reflecting the percentage of tissue involvement (\times 1, 0%–25%; \times 2, 26%–50%; \times 3, 51%–75%; \times 4, 76%–100%), and all numbers were summed. The maximum possible histologic score was 40. Mice were killed every other day for assessment of the expression of VEGF-A in mucosal extracts.

In Vivo Overexpression of VEGF-A and sVEGFR-1 Using Adenovirus

Mice were injected with the adenovirus (1×10^9 plaque-forming units) into their tail vein 2 days prior to the administration of DSS, at the following doses: 250 μ g of the VEGF-A adenovirus, 250 μ g of the VEGFR-1 adenovirus, and 250 μ g of the LacZ adenovirus. To test whether the virus localizes in the intestine, healthy mice were injected with adenovirus-LacZ or adenovirus-vector. After 8 days, mice were killed then the colon was fixed in 4% paraformaldehyde for 3 hours at room temperature and incubated in X-gal solution overnight at 37°C. The target tissue samples were then placed in PBS and examined under a dissecting microscope to detect lacZ-expressing cells macroscopically. In addition, histologic sections were counterstained with nuclear fast red under \times 40 magnification, and X-gal-positive cells (blue-stained cells) per sample were counted in a blinded manner. In some experiments, the pathogenic effect of VEGF was tested by daily administration of intraperitoneal injections of recombinant VEGF-A (1 μ g/g). Bulk quantities of VEGF

are provided free of charge by the BRB Preclinical Repository of the NCI (web.ncifcrf.gov/research/brb/preclin/).

Enzyme-Linked Immunosorbent Assay

Circulating VEGF-A was measured in blood and mucosal biopsy extracts using an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (R&D Systems). To determine the circulating levels of VEGF-A, blood was collected from control individuals and patients with IBD and diluted 1:10 in the anticoagulant sodium citrate (0.13 mol/L). The concentration of VEGF-A was then assessed and expressed as pg/mL. Mucosal VEGF-A was assessed in endoscopic biopsy specimens collected from the actively inflamed mucosa of patients with UC and CD and from normal areas of the colons of control individuals undergoing colonoscopy for non-IBD- or inflammatory-related bowel diseases. Biopsy samples were homogenized and sonicated on ice in extraction buffer (10 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% Triton X-100) supplemented with a cocktail of protease inhibitors. Samples were centrifuged at 900g for 15 minutes, then the supernatants were collected and stored at -80°C . The protein concentration was measured using the Bio-Rad protein assay as per manufacturer's instructions (Bio-Rad Laboratories)⁸, then the concentration of VEGF-A was expressed as pg/ μg of protein.

Measurement of VEGF-A and sVEGFR-1 in the plasma of adenoviral transfected mice was performed by ELISA, according to the manufacturer's instructions (R&D Systems). For measurement of colonic cytokines, colons from all mice were excised, opened, and cut longitudinally into 3 parts. One part was washed in cold PBS supplemented with penicillin, streptomycin, and amphotericin B (BioWhittaker, Cambrex, East Rutherford, NJ) and incubated in serum-free RPMI 1640 medium with 0.1% FBS, penicillin, streptomycin, and amphotericin B, at 37°C in 5% CO_2 . After 24 hours, the supernatant was collected, centrifuged, and stored at -20°C . Supernatants were analyzed for TNF- α and KC content in duplicate using commercially available ELISA kits, as previously reported (R&D Systems).³

Immunostaining of Mucosal Expression of VEGFR-1 and -2 in Human and Murine Colonic Tissues and CD31 in Murine Colonic Tissues

Immunostaining was performed as previously described.⁵ Briefly, paraffin-embedded intestinal sections of histologically normal control and IBD-involved and -uninvolved mucosa and of mice were cut into 3- μm slices, deparaffinized then hydrated, blocked for endogenous peroxidase using 3% $\text{H}_2\text{O}_2/\text{H}_2\text{O}$, then subjected to microwave epitope enhancement using a Dako Target retrieval solution (Dako, Carpinteria, CA). Incubation with a primary antibody; mouse anti-human VEGFR-1 or rabbit anti-human VEGFR-2 (both from Santa Cruz Biotech-

nology); and rabbit anti-mouse VEGF-R1 (Abcam, Cambridge, MA), rabbit anti-mouse VEGFR-2 (Gene Tex, San Antonio, TX), or anti-mouse CD31 (Santa Cruz Biotechnology) was performed at a 1:50 dilution for 1 hour at room temperature. Detection was achieved with a standard streptavidin-biotin system (Vector Laboratories, Burlingame, CA), and antigen localization was visualized with 3',3'-diaminobenzidine (Vector Laboratories). Mucosal vascularization was quantified as reported.⁹ Stained colonic sections were scanned at low power ($\times 40$) to detect the most vascularized area, after which at least 5 microphotographs at high magnification ($\times 200$) were taken.

Quantification of VEGFR-1 and -2 expression was performed on immunostained sections by a semiquantitative method (scores from 0 to 3), as previously described.³ For microvessel density analysis, counts were performed as previously reported.⁷

Analysis of HIMEC by Flow Cytometry

Detection of expression of ICAM-1 and VCAM-1 on HIMEC was performed by flow cytometry as previously described.¹⁰ Briefly, HIMEC were plated onto plastic in unsupplemented media for 48 hours then exposed to 50 ng/mL of VEGF or TNF- α . After 24 hours, HIMEC were rapidly trypsinized, washed twice, and incubated with PE mouse anti-human ICAM-1 and phycoerythrin mouse anti-human (both from BD Pharmingen, San Diego, CA) or isotype control antibody for 30 minutes on ice, washed again, and incubated with a mouse secondary-fluorescein isothiocyanate conjugated antibody. After additional washing, HIMEC were analyzed by quantitative flow cytometry using a Coulter Epics XL Flow Cytometer (Beckman Coulter, Inc, Fullerton, CA). Each analysis was performed on at least 10,000 events. ICAM-1 expression was quantified using the Winlist software program (Verity Software House, Topsham, ME).

HIMEC-Neutrophil Adhesion Assay

Adhesion assays were performed as previously reported.^{3,10} Neutrophils were isolated from healthy controls from peripheral blood and utilized for the adhesion assay.¹ Confluent HIMEC monolayers were left alone or stimulated with 50 ng/mL TNF- α . After 1 hour, 10^6 calcein (Molecular Probes, Eugene, OR)-labeled neutrophils were overlaid and allowed to adhere at 37°C in a 5% CO_2 incubator. In some experiments, HIMEC were preincubated for 2 hours at 37°C with 5 $\mu\text{g}/\text{mL}$ of polyclonal anti-ICAM-1 neutralizing or control antibodies (R&D Systems). Following 1 hour of coculture, nonadherent neutrophils cells were removed by 3 sequential rinsings and washing with cold PBS. Fluorescent adherent cells were quantified in 10 randomly selected fields by an imaging system (Image Pro Plus; Media Cybernetics, Silver Spring, MD) connected to an Optronics Color digital camera (Olympus, Tokyo, Japan) on an inverted fluores-

cence microscope. All experiments were performed in duplicate wells and results expressed as adherent cells/mm².

Measurement of Intestinal Microvascular Permeability

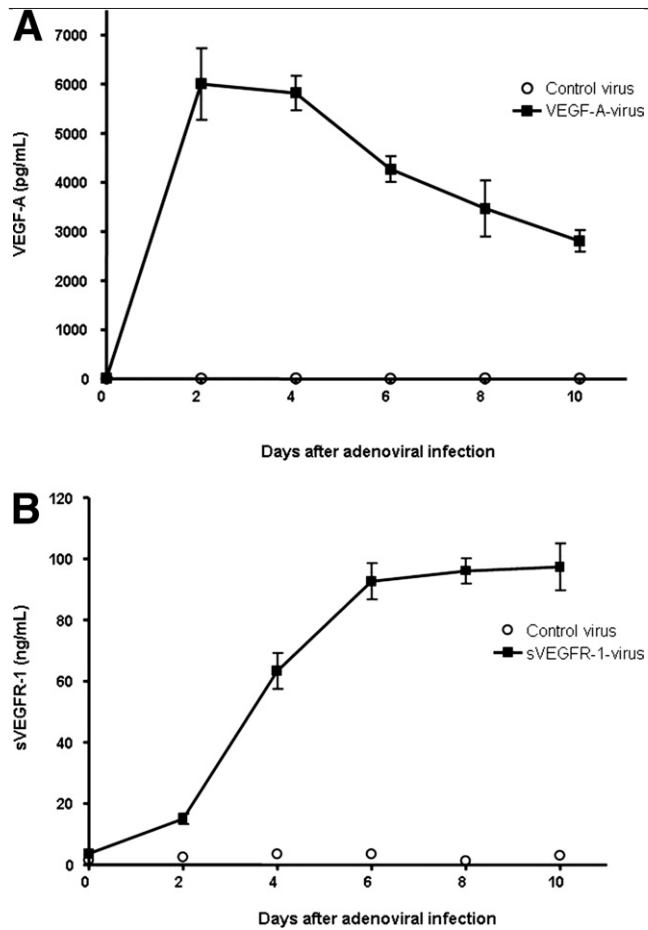
For quantification of intestinal vascular permeability, we used Evans blue that binds to albumin. Its leakage reflects increased vascular permeability of macromolecules. Adeno-VEGF-A- and adeno-sVEGFR-1-treated mice, as well as control mice, were anesthetized, and Evans blue (0.4 mg/100 g in PBS) was injected intravenously 15 minutes before death. Colons were then removed and rinsed, Evans blue was extracted from the tissue using chloroform and measured by spectrophotometry at 520 nm, and results were expressed as milligrams dye/grams wet weight colon.

Statistical Analysis

Data were analyzed by Graphpad software (San Diego, CA) and expressed as mean \pm SEM. Student *t* test or analysis of variance followed by the appropriate post hoc test was used when appropriate. Statistical significance was set at $P < .05$.

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Supplementary Figure 1. Adenoviral transfection induced sustained plasmatic levels of VEGF-A and sVEGFR-1. Mice were transfected with an adenovirus encoding for VEGF-A, sVEGFR-1, or a control adenovirus. Mice were killed every other day, and the plasmatic concentrations of VEGF-A (A) and sVEGFR-1 (B) were measured by ELISA. Data represent mean \pm SEM (mice = 3 per each time point).