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Altered angiogenic balance in ulcerative colitis: A key to impaired healing? ☆

Zs. Sandor [a,c], X.M. Deng [b,d], T. Khomenko [b,d], A.S. Tarnawski [a,c], S. Szabo [b,d,*]

[a] Medical Health Care Groups, VA Long Beach Healthcare System, Long Beach, CA 90822, USA
[b] Diagnostic and Molecular Medicine Health Care Groups, VA Long Beach Healthcare System, Long Beach, CA 90822, USA
[c] Department of Medicine, Division of Gastroenterology, University of California, Irvine, CA 90822, USA
[d] Departments of Pathology and Pharmacology, University of California, Irvine, CA 90822, USA

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Abstract

Angiogenesis is an essential component of ulcer healing since it assures delivery of oxygen and nutrients to the healing site. Previous studies demonstrated increased serum and tissue levels of vascular endothelial growth factor (VEGF, the most potent angiogenic growth factor) in patients with active ulcerative colitis (UC) and animal models of UC. However, there is no explanation why the healing of UC-related mucosal injury is impaired despite increased expression of VEGF. Expression of angiogenesis inhibitors, angiostatin and/or endostatin, in UC has not been determined before. We examined expression of VEGF, angiostatin, and endostatin in two models of experimental UC. The results revealed that in addition to increased VEGF, both endostatin and angiostatin levels were markedly (2–3-folds) increased in colonic mucosa at early stage of experimental UC. This is the first demonstration that colitis triggers increase in angiostatin and endostatin levels. The results may explain why mucosal lesions heal slowly despite increased VEGF levels, and may provide a novel and mechanistic insight into UC.
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Keywords: Ulcerative colitis; Angiogenic balance; VEGF; Angiostatin; Endostatin; Ulcer healing

Inflammatory bowel diseases (IBD): Ulcerative colitis (UC) and Crohn's disease (CD) are characterized by recurrent, chronic inflammation, and ulcerations of intestinal and/or colonic mucosa and delayed healing [1–3]. Angiogenesis (formation of new blood vessels from pre-existing vessels) is an essential component of ulcer healing and tissue regeneration, since it assures delivery of oxygen and nutrients to the healing site [4–6]. Vascular endothelial growth factor (VEGF) is the most potent and endothelial specific angiogenic growth factor [7,8] and it plays a pivotal role in healing of tissue injury and ulcers [9]. For example, our previous studies demonstrated that recombinant VEGF protein accelerates healing of experimental duodenal ulcers [10] and that gene therapy with VEGF accelerates healing of experimental duodenal and gastric ulcers as well as experimental UC [10–13].

Clinical and experimental studies including our own demonstrated significantly elevated serum and tissue levels of VEGF in patients with active UC, implicating VEGF in the pathogenesis of this disease [14–20]. However, the mechanistic role of VEGF in UC is uncertain and there is no reasonable explanation why the healing of UC-related mucosal injury is impaired despite increased expression of VEGF. This could be explained by a concomitant activation of angiogenesis inhibitors. However, the expression of angiogenesis inhibitors, angiostatin and/or endostatin, in colonic mucosa during UC has not been explored before. We hypothesized that the simultaneous activation of angiogenesis inhibitors, counteracting and/or blocking VEGF angiogenic activity, can explain impaired healing of UC-related mucosal injury and may be a key element in the pathogenesis and chronicity of IBD.

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* Corresponding author. Fax: +1 562 826 5623.
E-mail address: sandor.szabo@med.va.gov (S. Szabo).

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Materials and methods

The animal experiments were approved by the Animal Study Subcommittee of the VALBHS. Sprague–Dawley female rats (170–200 g) had unlimited access to purina food and tap water, and were randomly divided into groups of 3–5 rats. Experimental UC was induced by 6% iodoacetamide, sulfhydryl (SH) alkylator in 1% methylcellulose (0.1 ml/rat, x1), or 6% trinitrobenzene sulfonic acid (TNBS) in 50% ethanol (0.5 ml/rat, ×1) administered intracolonically (i.c.) (about 7 cm from the rectum) using soft plastic catheter. Both, TNBS- and iodoacetaminde-induced colitis, are well-established models of UC [21,22]. Control groups of unfasted rats were given 0.1 ml of vehicle–1% methylcellulose or 0.5 ml of 50% ethanol i.c. Rats were euthanized 0.5, 1, 2, and 6 h after iodoacetamide or TNBS i.c. injection. Colonic mucosal scrapings were homogenized in the presence of protease inhibitors and centrifuged. Isolated proteins were used for Western blotting and enzyme-linked immunosorbent assay (ELISA). All the experiments were repeated twice, and if appropriate, results were pooled.

Western blotting. Aliquots of samples containing 150 μg of total proteins were separated by 12% SDS–PAGE and then transferred onto nitrocellulose membrane (Amersham, MA). The levels of VEGF, endostatin, and angiostatin were detected with antibodies of anti-VEGF (Santa Cruz Biotechnology, CA), anti-endostatin (Lab Vision, Fremont, CA), and anti-angiostatin (Abcam Inc., Cambridge, MA), respectively. The membranes were incubated with Hyper film ECL (Amersham) at room temperature for 1–2 min and exposed to X-ray film. The density of the bands was determined by a scanning densitometer Eagle Eye II (Stratagene).

ELISA. The concentrations of VEGF and endostatin in the colonic mucosa after the administration of iodoacetamide were measured by human VEGF immunoassay kits (R&D Systems, Minneapolis, MN) and mouse endostatin immunoassay kits (CytImmuno, Minneapolis, MN) according to the manufacturer's directions. We calculated the concentrations by a ratio (pg/mg) of endogenous VEGF or endostatin vs. total proteins. The statistical significance of differences among group means was calculated by the non-parametric Mann–Whitney *U*-test. For statistical significance, $p < 0.05$ or smaller values were accepted.

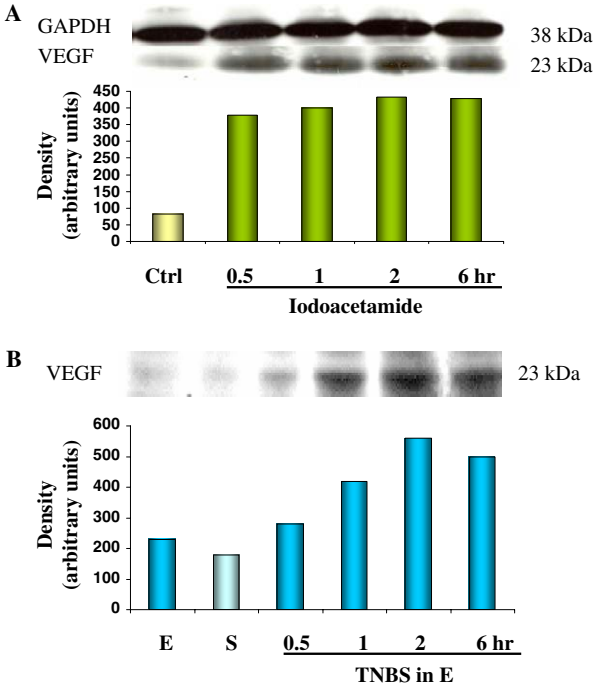

Fig. 1. Western blotting of expression of VEGF in colonic mucosa of rats with ulcerative colitis induced by 6% iodoacetamide or 6% trinitrobenzene sulfonic acid (TNBS). GAPDH was used as a control for loading. Ctrl: control; E: ethanol; S: saline.

Results and discussion

Western blotting revealed that 23 kDa $VEGF_{164}$ levels in colonic mucosa had ~ 4-fold increase at all tested time points in iodoacetamide-induced colitis, and about 2- to 3-fold increase in TNBS-induced colitis when comparing with controls (Fig. 1A and B).

Western blotting also showed increased expression of both angiostatin and endostatin in colonic mucosa after iodoacetamide or TNBS administration (Fig. 2). 19 kDa endostatin and 50 kDa angiostatin were increased in a time-dependent manner during the 0.5–2 h period after iodoacetamide administration (Figs. 2A and B), while endostatin levels in TNBS-induced colitis had increase of more than 2-fold from 1 to 6 h after TNBS administration comparing to the control (Fig. 2C).

ELISA confirmed the above changes of VEGF and endostatin in colonic mucosa after iodoacetamide administration (Fig. 3). The concentrations of VEGF were significantly increased in all time points after iodoacetamide (Fig. 3A), and the concentrations of endostatin were significantly increased 2 h after iodoacetamide (Fig. 3B).

Gastrointestinal (GI) tissue injury is usually followed by healing that requires production of granulation tissue, i.e., proliferations of fibroblasts, deposition of connective tissue matrix, and most important angiogenesis for reconstruction of mucosal microvessel critical for delivery of oxygen and nutrients to the healing site [9–13,23]. In the final stage of healing, re-epithelialization and reconstruction of epithelial structures take place. Angiogenesis in injured tissue is dependent on activation and increased expression of pro-angiogenic growth factors from which VEGF plays a critical, rate limiting-role [7,8]. In both clinical and experimental UC there is increased activation of VEGF gene and increased expression of VEGF protein in colonic mucosa [14–20]. Despite this, the healing of colonic ulcerations is very slow. This indicates a lack of response of endothelial cells to VEGF stimulation, perhaps due to presence of angiogenic inhibitors. Surprisingly, expression of angiostatin and/or endostatin in clinical or experimental UC has not been examined before. Several literature searches revealed no information regarding expression of angiostatin and/or endostatin in colonic mucosa during ulcerative colitis. Our study showed for the first time that experimental colitis triggers not only increased expression and levels of VEGF, but also significantly enhanced levels of angiostatin and endostatin in the early stages of UC. These events which preceded development of colonic mucosal lesions shift the angiogenic balance toward anti-angiogenesis.

The anti-angiogenesis factors such as angiostatin and endostatin are generated through activated proteinases by cleaving extracellular matrix (ECM) and/or extravasated

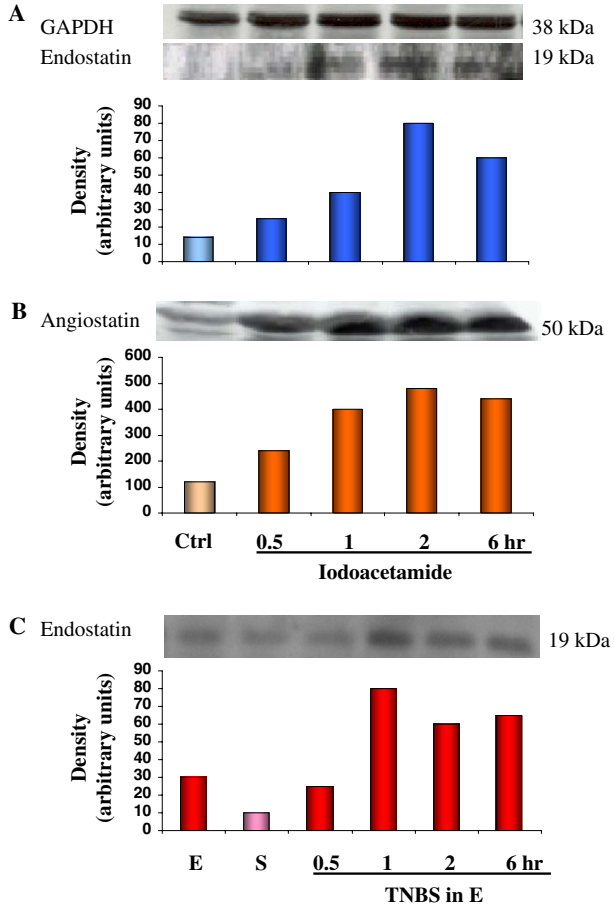

Fig. 2. Western blotting of expression of endostatin and angiostatin in colonic mucosa of rats with ulcerative colitis induced by 6% iodoacetamide or 6% trinitrobenzene sulfonic acid (TNBS). GAPDH was used as a control for loading. Crtl: control; E: ethanol; S: saline.

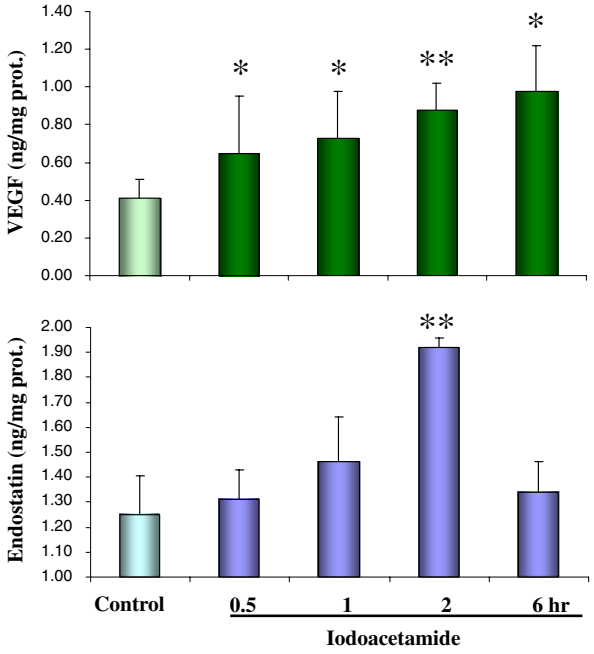

Fig. 3. Concentrations of VEGF and endostatin measured by ELISA in colonic mucosa of rats with ulcerative colitis induced by 6% iodoacetamide. $^{*}p < 0.05$; $^{**}p < 0.01$.

plasma proteins. In addition, elevated levels of proteinases can degrade VEGF [24]. Angiostatin—a 38 kDa fragment of the first four kringle domain of plasminogen inhibits angiogenesis *in vitro* and *in vivo* [24–28]. Detailed analysis demonstrated that angiostatin antagonizes the action of VEGF in human umbilical vascular endothelial cells via two distinct pathways: One, intrinsic mediated by p53 and other, extrinsic, involving Fas ligand and mitochondrial dysfunction [29]. Other studies showed that angiostatin inhibits bovine aortic endothelial cell proliferation by downregulation of cell cycle regulatory protein cdk5 [30]. A number of proteinases, such as elastase and various matrix metalloproteinases, can generate angiostatin-related fragments *in vitro* and *in vivo*. *In vitro*, these fragments inhibit endothelial cell proliferation and migration, and induce endothelial cell apoptosis. However, the anti-angiogenic activity of these fragments *in vivo* is unclear. Another anti-angiogenic molecule generated by cleavage of the ECM is endostatin. Endostatin is a 20 kDa fragment of collagen XVIII, a basement membrane heparin sulfate proteoglycan which is present in abundance in blood vessels in the skin [31–33]. Various forms of endostatin are generated *in vitro* by proteinases. *In vitro*, endostatin induces endothelial cell apoptosis and inhibits the proliferation and migration of some types of endothelial cells, and *in vivo* it has potent anti-angiogenic activity [31–33].

Our present study is in agreement with previous studies relevant to chronic dermal ulcers, which showed that fluid from chronic venous leg ulcers, particularly those that heal slowly, inhibits in *vitro* angiogenesis despite elevated level of VEGF [34–37]. Since angiogenesis outcome depends on a balance between pro- and anti-angiogenic factors, it is very likely that a net excess of anti-angiogenic factors in the ulcer environment will inhibit ulcer healing and cause its chronicity.

In summary, our present study revealed a novel, previously unrecognized mechanism that might explain why the healing of mucosal ulcerations in UC takes a long time despite activation of VEGF gene and increased expression of VEGF in colonic mucosa.

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