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Current Pharmaceutical Design, **2013,** *19,* **17-28** **17**

Novel Pharmacologic Approaches to the Prevention and Treatment of Ulcerative Colitis

Xiaoming Deng[a,c], Sandor Szabo[a,c,d], Tetyana Khomenko[a,c], Ganna Tolstanova[a,c,g], Brankica Paunovic[a], Samuel W. French[f] and Zsuzsanna Sandor[b,e,*]

[a]Pathology & Laboratory Medicine Service, and [b]Primary Care & Specialty HCG, VA Long Beach Healthcare System; and Departments of [c]Pathology, [d]Pharmacology & [e]Medicine, University of California-Irvine, Long Beach, CA; [f]Harbor-UCLA Medical Center, Los Angeles, CA, USA; [g]Department of Pharmaco-Physiology, Kiev National Shevchenko University, Kiev, Ukraine

Abstract: Ulcerative colitis (UC) is a chronic, relapsing inflammatory disorder of the colonic mucosa followed by poor quality of healing and recurring lesions. Recent studies demonstrated that the poor healing and chronic inflammation in colon of UC could be the result of microvascular dysfunction and endothelial barrier defect, resulting in sustained tissue hypoperfusion and ischemia in the colon. Long before angiogenesis became a popular research topic, our laboratory was the first to postulate that stimulation of angiogenesis alone might be sufficient to accelerate ulcer healing in the gastrointestinal tract. Our earlier studies demonstrated that therapy with genes or peptides of angiogenic growth factors, e.g., bFGF, PDGF and VEGF significantly accelerated healing of experimental duodenal ulcers (DU), while blockade of these angiogenic factors resulted in impaired healing of DU. However, unlike the angiogenesis in DU, increasing evidences from us and others indicate that angiogenesis plays a pathogenic role in UC, e.g., VEGF induces an abnormal "pathologic" angiogenesis which interferes with UC healing. Recently, another angiogenic factor, placental growth factor (PlGF), has also been suggested to be a marker of pathologic angiogenesis and may play a critical role in pathogenesis of UC. Although inhibition of pathologic angiogenesis by, e.g., anti-VEGF or –PlGF, was demonstrated to be a new approach to attenuate UC development, additional data of our and others showed that stimulating angiogenesis by administration of PDGF or bFGF significantly accelerated healing of UC. Also, activation of Rac1, a small GTPase, markedly improved VEGF-induced neovessel architecture defect and reduced vascular permeability (VP) in an angiogenic model. Thus, it seems that both angiogenic and anti-angiogenic therapies may be used in various stages of UC. More recently, we demonstrated that increased VP in colonic mucosa is an early and essential element in the initiation and progression of UC. The increased VP is initiated by early release of histamine and maintained/aggravated by VEGF, leading to perivascular edema, vascular stasis, hypoxia, inflammatory cell infiltration, and colonic erosions/ulcers. Inhibition of increased VP prevents or reduces development and progression of UC.

In this review, we discuss novel pharmacologic approaches to prevent UC, differential actions of angiogenic growth factors in UC pathogenesis and blocking the early increase in VP in UC development, these new findings may provide new insights into the regulation of angiogenesis in UC and may lead to development of VP-related drugs to accelerate the healing of UC.

Keywords: Angiogenesis, angiogenic growth factors, bFGF, PDGF, VEGF, PlGF, Rac-1, pathologic angiogenesis, histamine, Src kinase, VE-cadherin, vascular permeability, ulcer healing, ulcerative colitis.

INTRODUCTION

Ulcerative colitis (UC) is a chronic and recurring disease with a major clinical and economic impact and its incidence is growing. In UC, the ulcers are surrounded by acute (e.g., neutrophils) and/or chronic (e.g., lymphocytes, plasma cells, macrophages) inflammatory cells, hence often leading to the mistaken pathogenic target of treating UC by anti-inflammatory drugs to suppress the secondary inflammatory response to the previously induced ulcerative lesions. For unknown reasons, nevertheless, UC often leads to carcinomas [1,2].

Although recent studies on mechanisms of chronic inflammatory components of UC have brought progress in UC therapy, current anti-TNFα antibody (infliximab) and other anti-inflammatory treatment (e.g., mesalamine) induced sustained clinical long term remission at 52 weeks in fewer than 34% of patients [3-5]. This means that in the majority of patients these treatments do not induce sustained remission and complete healing or prevent recurrence. The healing aspects of UC and the mechanisms governing the healing component of UC have not been explored in depth and are not well understood. Thus, research is needed to address our current lack of understanding of how UC are triggered, why it easily recurs, and what the precise cellular and molecular targets are for complete and permanent healing.

*Address correspondence to this author at the VA Medical Center (05/113), 5901 East 7th Street, Long Beach, CA 90822, USA; Tel: 562-826-5513; Fax: 562-826-5768; E-mail: zsuzsanna.sandor@va.gov

The main focus of our research laboratory has been the vascular elements of gastrointestinal (GI) mucosal injury and healing. Long before angiogenesis became a popular term and research topic, we proposed that growth of new capillaries from existing blood vessels surrounding the ulcer crater is a rate-limiting step in ulcer healing. Stimulation of angiogenesis leads to development of granulation tissue that is essential for the healing process. Our earlier studies demonstrated that therapy with genes or peptides of angiogenic factors such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), significantly accelerated healing of experimental duodenal ulcers (DU), while blockade of these angiogenic factors resulted in impaired healing of DU [6-9]. Since histologically and pathologically gastroduodenal ulcers look similar to ulcers in the lower GI tract, we also predicted that the healing of UC might be also improved by stimulating angiogenesis. However, recent data of ours and others showed that angiogenic therapy targeting VEGF aggravated healing of experimental UC but anti-VEGF treatment improved the healing [10,11]. Increasing evidences indicate that VEGF induces pathologic "abnormal" angiogenesis that links inflammation and plays a pathogenic role in experimental UC [12,13]. However, it has been reported that anti-VEGF therapy often results in gastric ulcer perforation and skin ulcers in patients [14,15] as anti-VEGF may also affect physiologic angiogenesis needed in tissue repair and organ regeneration. Thus, it is important to recognize the inducers and modulators of pathologic angiogenesis. Recently, placental growth factor (PlGF) has been identified as a marker of pathologic angiogenesis and may play a specific role in

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abnormal angiogenesis only, e.g., inhibition of PlGF did not affect quiescent vessels in healthy organs [16-18]. In addition, our and other's data also showed that treatment with other angiogenic growth factors, e.g., PDGF or bFGF significantly improved healing of experimental UC [19-23], indicating that therapeutic angiogenesis is still needed in healing of UC. Recent publications also showed that activation of Rac1, a small GTPase involved in regulating neovascularization, markedly improved VEGF-induced neovessel architecture defect and reduced vascular permeability (VP) in an established angiogenic model [24,25].

In addition to investigations on the therapeutic approaches to angiogenesis and anti-angiogenesis in UC, we recently demonstrated that abnormal VP is also one of the major pathogenic factors of UC. Our studies showed that vascular injury and increased VP in colonic mucosa preceded the dysfunction of colonic epithelial barrier in both chemically induced and spontaneously developing models of UC [26]. These studies have generated important data and uncovered novel molecular mechanisms of VP regulation in early stages of UC. Increased VP is apparently initiated by early release of histamine from colonic mast cells and maintained/aggravated by VEGF-mediated VP pathways, leading to perivascular edema, vascular stasis, hypoxia, inflammatory cell infiltration, and colonic erosions/ulcers. Our experimental findings have a substantial clinical relevance. Extensive edema of the mucosal lamina propria and infiltration by inflammatory cells are constant features of human UC and recent studies using confocal endomicroscopy demonstrated the presence of significantly increased VP in patients with UC even among those in remission [27,28]. Increased VP is thus not only important for the initiation of inflammatory process in UC but also for maintaining its chronicity.

This review paper is focused on discussing new molecular and cellular targets such as anti-angiogenic therapies by inhibiting VEGF or PlGF, and angiogenic improvement by administrating bFGF, PDGF, and Rac-1 as well as options against increased VP by blockading mast cell degranulation, histamine- and/or VEGF-mediated VP pathways for an accelerated healing of UC. This would provide novel therapeutic approaches to prevent and treat UC.

ANGIOGENIC OR ANTI-ANGIOGENIC THERAPY: DIFFERENT ANGIOGENIC ACTIONS OF GROWTH FACTORS IN UC DEVELOPMENT AND HEALING

Angiogenesis in Wound/Ulcer Healing

Angiogenesis is the generation of new blood vessels from an existing vascular bed. The process involves extensive interaction between several cells and molecules. The switch to angiogenesis involves a change in the local equilibrium between positive and negative regulators of microvessels [29]. The proliferation of endothelial cells and tube formation are crucial elements in granulation tissue production [30]. Thus, vascular endothelial cells seem to represent a target for organoprotection against acute injury and for stimulating angiogenesis and granulation tissue production for wound/ulcer healing. Namely, prevention or reduction of endothelial damage results in maintenance of mucosal blood flow, which is essential for epithelial restitution to repair the superficial mucosal damage [31,32]. The formation of granulation tissue, i.e., angiogenesis followed by proliferation of fibroblasts and deposition of collagen, on the other hand, is a rate-limiting step in the repair of major damage to tissues that do not regenerate (e.g., after the loss of cardiac or gastric muscle). Only in certain organs such as liver, adrenal and renal cortex, the regeneration involves the proliferation of original parenchymal cells that rapidly replace the lost tissue. The healing process needs the granulation tissue, which forms the basis for proliferation and migration of epithelial cells in the GI tract. The migrating cells cannot grow over necrotic tissue, unless it is gradually replaced by angiogenesis-dependent granulation tissue. Thus, stimulation of only epithelial cell proliferation is counterproductive in the healing of internal and external wounds, unless it is accompanied by the expression of a loose or solid granulation tissue, which provides the basis and physical framework for the migration of epithelial cells to complete the healing process [32]. The healing of deep ulcers that reach or penetrate the muscularis propria almost always requires initial angiogenesis and proliferation of other elements of granulation tissue [33]. Endothelial cell migration, proliferation and microvascular structure formation are initiated and regulated by angiogenic growth factors, such as VEGF which is the most potent and specific regulator of angiogenesis [34].

Angiogenesis in UC

In normal wound/ulcer healing, angiogenesis, known as physiologic angiogenesis, is closely controlled by multiple growth and tissue factors resulting in minimal changes in microvascular permeability, proteolysis, and inflammation. However, development of new vasculature during chronic inflammation may play a negative "pathologic" role by contributing to increased inflammatory responses due to dysfunctional new vessel architecture and increase in the recruitment of inflammatory cell types. The abnormal or pathologic angiogenesis such as that observed in malignant tumors is characterized by its abnormal vasculature, which exhibits defective architecture, increased permeability, and increased inflammatory and thrombogenic potential [35]. Recent findings, both clinical and experimental, indicate that pathologic angiogenesis plays a crucial role in UC [12,13]. Examination of the relationship between angiogenesis and inflammation in experimental UC shows that initiating factors for these responses simultaneously increase as disease progresses. Recent data provide evidence that differential regulation of the angiogenic mediators involved in UC-associated chronic inflammation is the basis of this pathologic angiogenesis, and that angiogenic inhibition during chronic inflammatory diseases attenuates further inflammation and disease progression [36,37]. However, the microvascular changes that occur during UC have not been closely investigated, and we are only beginning to understand their involvement in disease processes. As such, expanding our knowledge of how angiogenesis occurs during UC, and understanding the specific molecular and pharmacologic roles of angiogenic growth factors in UC will provide new insights into the healing of UC.

Inhibiting Angiogenesis for UC Healing

Our recent studies demonstrated that differential expression of pro- vs. anti-angiogenic factors regulates pathologic angiogenesis by creating imbalances between these regulatory factors (i.e., up-regulation of pro- over anti-angiogenic factors or relative down-regulation of anti-angiogenic factors) [38,39]. Evidence indicates that some of the up-regulated angiogenic factors in UC may prevent the maturation of vessels, contributing to the pathologic nature of this angiogenesis. Recent studies have shown that VEGF links pathologic angiogenesis and refractory inflammation in UC, and that it is involved in prevention of vessel maturation and ulcer healing [13,40]. For example, HIF-1α-induced VEGF production has been shown to inhibit vessel maturation and pericyte stabilization, resulting in endothelial cell hyperplasia and abnormal angiogenesis [41,42]. These processes could contribute to a pathologic phenotype of neovascularization during UC.

Anti-VEGF Treatment in UC

VEGF is the most potent angiogenic factor in the growth factor family. After we demonstrated that therapy with peptide, especially, gene of VEGF produced fast healing of experimental DU [9,43], we predicted a similar beneficial effect of this angiogenic growth factor in experimental UC. However, we were not only surprised that we did not receive the predicted results, but gene therapy with VEGF even aggravated experimental UC. Thus, the next logical step was to determine the possible mechanistic role of VEGF in the patho-

genesis of UC, and in the first experiment we neutralized VEGF in rats with the specific anti-VEGF antibody. Treatment with anti-VEGF antibody had a beneficial effect on the course of iodoacetamide (IA)-induced UC, reducing diarrhea and lethargy scores and the macroscopic and microscopic lesions in the colon [10]. Neutralization of VEGF counteracted the increased VP in the early stages of experimental UC and decreased the number of infiltrated inflammatory cells in the pathogenesis of UC [10]. Moreover, we found significantly decreased number of leukocytes in the advanced stages of experimental UC after anti-VEGF therapy [10]. We also demonstrated a concurrently increased expression of angiogenic VEGF and anti-angiogenic endostatin and angiostatin in experimental UC, creating an "angiogenic imbalance" or inappropriate angiogenic response [38]. Furthermore, we realized that the initial name of this peptide was vascular permeability factor (VPF) because it induces increase in VP that represents the first step in inflammation [44].

Matsuura *et al.* [22] found that rectal administration of human recombinant bFGF to normal mice significantly increased expression of VEGF in colonic tissue, but expression of VEGF in mice with experimental UC after bFGF treatment was lower than in the untreated mice with colitis in a dose-dependent manner and directly correlated with a beneficial dose of bFGF. We proposed that the beneficial effect of anti-VEGF therapy in experimental UC is due to attenuation of VEGF-induced VP, resulting in reduced vascular leakage and inflammatory cell infiltration. Recently, it has been postulated that VEGF produces an abnormal "pathologic" angiogenesis in UC, which plays a critical role in the pathogenesis of UC [12]. More recently, Scaldaferri *et al.* [13] demonstrated that VEGF directly links angiogenesis and inflammation in the pathogenesis of both human and experimental UC. Increasing evidence suggests that VEGF is one of the major pro-angiogenic factors involved in pathologic angiogenesis. Blockade of VEGF, i.e., anti-VEGF-induced angiogenesis, may represent a new therapeutic option for UC.

However, recent studies have shown that the widely used anti-angiogenic agent bevacizumab (a clinically used anti-VEGF antibody) is associated with an increased risk of GI perforation and skin ulcers in patients [14,15]. Based on recent discovery of pathologic angiogenesis markers such as PlGF, we postulate that specific inhibition of pathologic angiogenesis by antagonizing the specific regulators of pathologic angiogenesis may not affect healthy blood vessel formation that is needed for tissue maintenance and repair.

Anti-PlGF Treatment in UC

PlGF is a pleiotropic cytokine that stimulates endothelial cell growth, migration, and survival; chemoattracts angiocompetent macrophages and bone marrow progenitors; and determines the metastatic niche. PlGF selectively binds to VEGFR-1. Besides indirect effects [17], PlGF signals directly via VEGFR-1, thus, acting independently of VEGF in endothelial cells, macrophages, bone marrow progenitors, and tumor cells, which primarily express VEGFR-1 [45]. Gene inactivation studies have revealed that PlGF deficient mice are viable and healthy, indicating that endogenous PlGF is redundant for vascular development. Genetic studies also show that PlGF participates in physiologic vessel maintenance in healthy adults and contributes to the angiogenic and inflammatory switch in various diseases, including tumor growth, ischemia, and chronic inflammation. Many cell types express PlGF in pathologic conditions, including endothelial cells, smooth muscle cells, fibroblasts, leukocytes, and bone-marrow progenitors, etc [17]. Furthermore, PlGF is readily upregulated in pathologic conditions by stimuli such as hypoxia, nitric oxide, inflammatory cytokines (e.g., IL-1 and TNFα), and growth factors (e.g., VEGF) [45]. Although initially controversial data have been reported on the pro-angiogenic role of PlGF, numerous studies of the last decade have clearly evidenced the crucial role of PlGF in modulating inflammation associated with pathologic angiogenesis [16].

To determine the role of PlGF in UC, we recently examined the expression of PlGF and the effect of neutralizing anti-PlGF antibody on IA-induced UC in rats. Western blotting showed that expression of PlGF was markedly increased on the 3rd and 7th days after IA in rats (Fig. **1A**). This is the first demonstration that PlGF is clearly expressed in rat intestines. The increased expression of PlGF in UC may indicate pathologic angiogenesis occurring during the development and healing of UC, respectively. The anti-PlGF treatment showed that inhibition of PlGF significantly reduced colonic lesions from 249.1±77.1 (control) to 110.1±73.7 mm^2 (Fig. **1B**). These data should stimulate the search for inhibitors of PlGF for therapeutic approaches. Overall, the expression profiling and genetic findings raise the question of whether PlGF is a specific pathologic angiogenic factor [16]. A neutralizing anti-PlGF antibody is now in Phase II of clinical trials [18]. If this is shown to be the case, PlGF could be an attractive drug target, as a PlGF inhibitor would be expected to selectively inhibit pathologic angiogenesis, without affecting the growth or maintenance of physiologic vessels, and, unlike VEGF/VEGFR inhibitors, would therefore be likely to cause fewer clinical side effects. This may lead to generate an attractive drug with a better safety profile [18,46].

Stimulating Angiogenesis for UC Healing

The assumption that wound healing-related angiogenesis can occur in the form of ulcer repair during remission of UC suggests a role for physiologic angiogenesis in UC and may also represent a return to normal regulation of angiogenic mediators during disease remission and may be the basis for ulcer recurrence. However, the majority of colonic tissue alterations that occur during UC are not fully normalized during remission. Thus, both angiogenic stimulation and inhibition may be conditionally needed in the process of UC healing.

bFGF Treatment in UC

bFGF is an 18-kDa polypeptide that was first isolated under this name from the brain as a fibroblast stimulator. It was later found to be identical to the most potent heparin-binding angiogenic stimulator. Indeed, bFGF is a direct mitogen for vascular endothelial cells, fibroblasts, smooth muscle cells, certain epithelial cells and neural cells. It has diverse roles in wound healing, tissue regeneration and embryonic development, and probably in carcinogenesis as well.

Our laboratory was the first to demonstrate that intracolonic treatment with bFGF peptide significantly accelerated the healing of experimental UC [6-8]. The effect of bFGF on UC healing was studied in a chemically induced animal model of UC, which was well established in our lab [47]. Both pharmacologic and histologic results demonstrated that bFGF treatment significantly improved UC healing which, on the other hand, was impaired by administration of anti-bFGF antibody [21]. The potential molecular mechanisms of the therapeutic actions of bFGF on healing of UC seem to involve increasing angiogenesis and mucosal regeneration, and reducing inflammatory response in colon. The finding of increased neovascularization indicated that bFGF facilitated healing via enhancing normal angiogenesis which is needed in UC healing. Surprisingly, bFGF markedly decreased TNF-α level and MPO activity in colonic tissues during healing of UC [21]. Matsuura *et al.* [22] also showed that gene expression of TNF-α was significantly reduced in dextran sulfate sodium (DSS)-induced UC after treatment with bFGF. The TNF-α levels and MPO activity in colonic tissues are strongly associated with the intensity of UC inflammation. Thus, reduced TNF-α levels and MPO activity after bFGF treatment indicate that bFGF plays anti-inflammatory role in UC. These findings suggest that bFGF, unlike VEGF, is a beneficial angiogenic growth factor that provides a promising option for the treatment of UC. bFGF enema may be a clinically safe and useful route that may provide as a new therapy way for UC. Moreover, our study demonstrated that the effective doses of bFGF we used were 4- to 10-folds

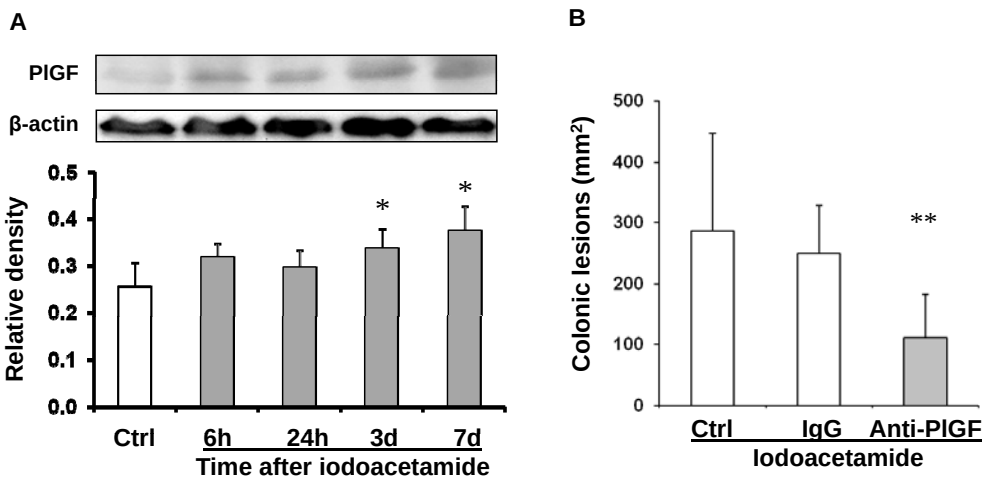

Fig. (1). Expression of PlGF and its pharmacologic role in development of UC induced by IA. **A:** Sustained expression of PlGF in colonic mucosa of rats with IA-induced UC. **B:** Neutralization of PlGF by a specific antibody significantly reduced the colonic lesions 7 days after IA enema in rats. Ctrl: control. Anti-PlGF: neutralizing anti-PlGF antibody. IA: iodoacetamide. * $p < 0.05$ and ** $p < 0.01$.

less than the doses others used to treat UC induced by DSS in rats and mice [22,23].

PDGF Treatment in UC

PDGF was originally described as a product of platelets, but it is also synthesized and secreted by activated macrophages. It consists of two disulphide-linked polypeptides: chain A (14 kDa) and chain B (17 kDa) sharing 60% similarity. Thus, the PDGF dimer has three isoforms: PDGF-AA, -AB and –BB [48]. PDGF plays a central role in tissue repair process, particularly by stimulating fibroblast and endothelial cell proliferation and angiogenesis following acute and chronic tissue injury. PDGF also signals and induces tissue remodeling, cellular differentiation and morphogenesis. In addition, PDGF directs the migration, differentiation and function of a variety of specialized mesenchymal and migratory cell types during wound healing. Clinical studies demonstrated that PDGF-BB (becaplermin) prevents formation of diabetic foot ulcers and accelerates healing of chronic lower extremity diabetic ulcers [49].

We also tested the effect of PDGF-BB on healing of UC induced by IA in rats. PDGF-BB treatment significantly decreased the severity of colitis and extent of adhesions after 5 days of treatment. The colonic lesions were significantly improved 10 days after treatment (Fig. **2A**). Histologically, the ulcer size was smaller, the signs of inflammation were reduced, and in the majority of cases extensive re-epithelization was seen. Furthermore, we tested the effect of gene therapy with adenoviral vector (AV) encoding PDGF-BB on healing of UC. The results showed that treatment with a single dose of AV-PDGF-BB significantly reduced colonic lesions (Fig. **2B**). The colonic dilation, colonic thickness, colon wet weight, pericolonic adhesions and body weight loss were significantly diminished in the group treated with double doses of AV-PDGF-BB. Histology showed extensive re-epithelialization in ulcer areas after AV-PDGF-BB treatment (Fig. **2C**). This is the first demonstration that intracolonic PDGF-BB significantly accelerates healing of UC in rats.

Angiogenic Effect of Gastric Pentadecapeptide BPC 157 on UC Healing

Pentadecapeptide BPC157 (PL-10, PLD-116, PL 14736) is a partial (15 amino acid fragment) of sequence of human gastric juice protein BPC, thought to be essential for its activity. The pentadecapeptide BPC 157 is stable in human gastric juice. Although the mechanisms of action is not fully understood, it has exhibited a particular wound healing effect [50] and interaction with the NO-system [51] providing endothelial protection and an angiogenic effect, even in severely impaired conditions such as advanced and poorly controlled IBD including failure of intestinal anastomosis healing [54], short bowel syndrome and fistulas [50-52]. Veljaca *et al.* demonstrated that BPC 157 significantly reduced the extent of TNBS-induced UC, which was associated with a dose-dependent reduction of MPO activity in colonic tissue [53]. Our laboratory was the first to show that the healing effect of BPC 157 in experimental UC was associated with increased angiogenesis in colonic mucosa [54,55]. Our studies demonstrated that BPC 157 increased expression of early growth response 1 (egr-1) gene responsible for generation of cytokine and angiogenic growth factors. Recently, this peptide has been successfully studied in clinical trials for IBD patients [56,57], showing that BPC 157 has a safe profile [58,59]. In agreements with our previous results, recently, Brcic *et al.* [60] revealed that pentadecapeptide BPC 157 promotes angiogenesis in various tissues, resulting in adequate healing. Therefore the therapeutic potential of BPC 157 seems to be closely related to the healing stimulated by angiogenesis *in vivo*. However, unlike VEGF, BPC 157 has no effect on angiogenesis *in vitro*.

Pharmacologic Role of Rac-1 in UC

Rac1 is a key member of the Rho GTPase family involved in the regulation of cell migration, proliferation, survival, and cell-cell junctions by virtue of its ability to regulate the actin-based cytoskeleton and nuclear gene expression. Activation of Rac1 is mediated by guanine nucleotide exchange factors in response to cytokine stimulation. Rac1 is essential for embryonic angiogenesis and selective deletion of the Rac1 gene in endothelial cells caused defective neovascularization which was incompatible with life. Recent endothelial cell culture studies demonstrated essential roles for Rac1 in stretch-provoked proliferation, directed migration toward the gradient of thrombin, sphingosine 1-phosphate, VEGF, and Ephrin-A1 and tube formation in three-dimensional gel environment [24]. The genetic deletion of Rac1 caused reduced endothelial cell migration, tube formation, adhesion, and increased vascular permeability. Rac1 has been also suggested to regulate the functions of endothelial progenitor cells, which contribute to neovascularization [61]. Two recent studies showed that Rac1 was required for epithelial stem cell function during dermal and oral mucosal wound healing in mice [62], and active Rac1 improves pathologic VEGF neovessel architecture and reduces vascular leak [25]. It was shown that the expression of a dominant inhibitory mutant of Rac1 delays epidermal wound-healing [62]. In these studies, excisional

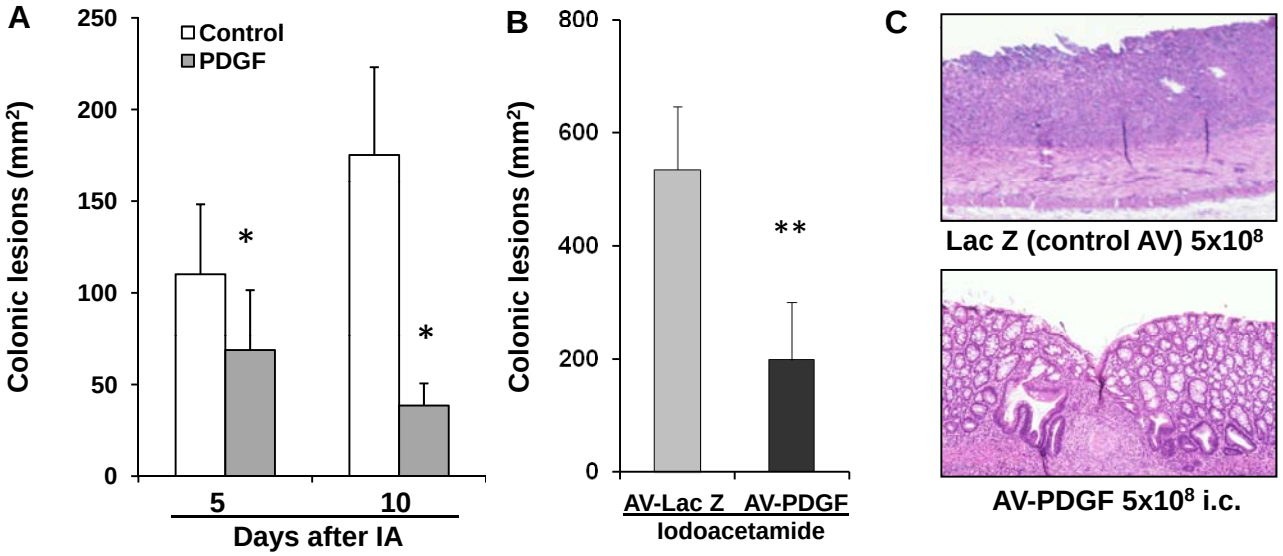

Fig. (2). Peptide or gene therapy with PDGF in IA-induced UC in rats. **A:** Daily PDGF peptide therapy for 5 or 10 days after IA enema in rats. **B:** A single dose gene therapy with adenoviral vector encoding PDGF in IA-induced UC in rats. **C:** Light microscopy of colonic mucosal healing after treatment with control Lac Z gene or PDGF gene in IA-induced UC in rats. IA: iodoacetamide. AV: adenoviral vector. * $p < 0.05$; ** $p < 0.01$.

wound healing of the skin was also delayed by conditional deletion of the Rac1 gene. More recently, the development of the Rac1 specific inhibitor (NSC23766) and agonist (SEW2871) supports the investigation of the roles of Rac1 [63].

The technical advance made with these new tools to specifically stimulate or inhibit Rac1 function *in vivo* provides opportunity for us to reveal the roles of Rac1 in UC. We therefore tested a hypothesis whether inhibition of rac1 would affect healing of experimental UC induced by IA. The results showed that treatment with a selective inhibitor of Rac1 (NSC23766) delayed UC healing. Quantitative data showed that UC lesions were increased from 68.3±9.4 (controls) to 102.6±11.7mm^2. We thus concluded that, in contrast to VEGF, Rac-1 seems to play a beneficial role in UC healing, indicating that Rac-1 may reverse VEGF actions in this disease (Fig. **3**).

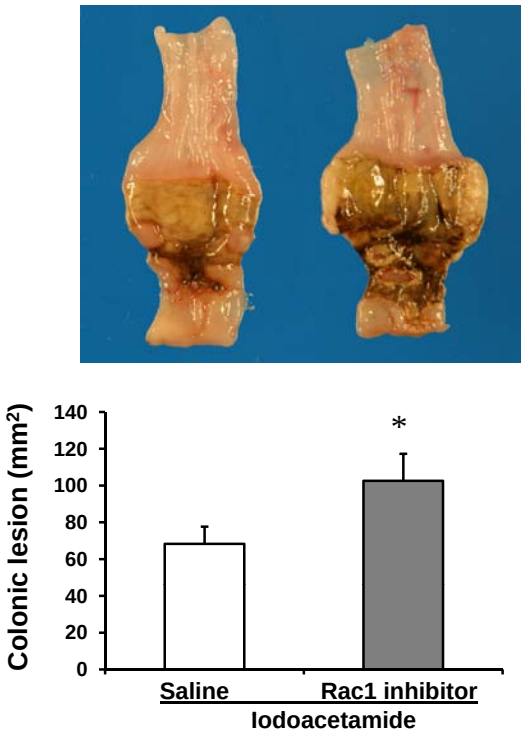

Fig. (3). Effects of blocking Rac1 by a specific inhibitor (NSC23766) on healing of experimental UC induced by IA in rats. IA: iodoacetamide. * $p < 0.05$.

BLOCKADE OF EARLY INCREASED VP IN COLONIC MUCOSA: TARGETING VP MEDIATORS TO PREVENT UC

Early Increased VP is a Key Event in Pathogenesis of UC

Endothelial Microvascular Barrier and VP

The mucosal microvascular network plays a critical role for all mucosal constituents by supplying oxygen and nutrients and by eliminating metabolic products. To accomplish these functions, the mucosal blood microvessels, composed of a continuous layer of endothelial cells and a basement membrane, must be sufficiently permeable to allow free, bidirectional transport of small molecules, plasma proteins and even inflammatory cells [64]. Microvascular endothelial barrier dysfunction and increased VP represent critical events in the development of a variety of pathologic processes. In general, VP is significantly increased in acute and chronic inflammation, in malignant tumors, as well as during wound healing. Three different types of permeability can be distinguished based on the specific mechanisms: 1) basic VP of normal tissues, 2) acutely increased VP which occurs in response to a single, brief exposure to VEGF/VPF or other factors increasing VP, and 3) chronically increased VP which characterizes pathologic angiogenesis found in tumors and chronic inflammatory diseases such as UC [54]. In contrast to basic VP, acute and chronic, sustained increase in VP occurs only in pathologically altered vessels [65].

Early Injury in Endothelial Barrier and Increased VP in UC

Recent studies of human and experimental colitis have shown increased microvascular density in human UC and experimental colitis [66-68]. Although these data provide evidence that vascular changes are important components of UC, the specific role and molecular mechanism of endothelial barrier changes and VP have not been explored in depth. Endothelial cells in intestinal mucosa play important roles via: 1) forming a mechanical barrier serving as

an component of intestinal mucosal defense, 2) maintaining capillary blood flow, 3) functioning as a "gatekeeper" preventing the extravasation of circulating white and red blood cells, and 4) rapidly mounting an innate immune response, synthesis and release of pro-inflammatory cytokines and expression of adhesion molecules [69]. Our recent studies demonstrated for the first time that increased colonic VP precedes the elevation of colonic epithelial permeability and development of colonic lesions in experimental UC induced by IA in rats (Fig. **4A**). We also found edema of the lamina propria - indicating increased VP - even in the mucosa covered with uninterrupted normal layer of surface epithelial cells in both chemically IA-induced UC in rats and spontaneously developing UC in Gα-i2$^{-/-}$ mice (Fig. **4B**). Our transmission electron microscopy studies confirmed that before EP was increased, epithelial TJs stayed intact, despite an underlying lamina propria edema which is most likely of vascular origin (Fig. **4C**). These observations serve as the basis of our conclusion that early increased VP and vascular injury may be an essential element in UC pathogenesis. More recently, we demonstrated that early increased VP in experimental UC is initiated by histamine and exaggerated/sustained by upregulated VEGF.

Molecular and Pharmacologic Roles of VP Mediators in Experimental UC

Histamine: An Initiating Factor for Increased VP in UC

The elevated mucosal histamine levels are present in patients with UC [70] and increased level of N-methylhistamine, a stable metabolite of the mast cell mediator, is detected in the urine of patients with active UC [71]. Since increased level of N-methylhistamine is significantly correlated with clinical disease activity, this finding further suggests the active role of histamine in the pathogenesis of UC. Interestingly, mast cells originating from the resected colon of patients with active CD or UC release more histamine than those from normal colon when stimulated with an antigen [71]. Also, cultured colorectal endoscopic biopsy samples from patients with inflammatory bowel disease secreted more histamine in response to substance P alone or substance P plus anti-IgE than the samples from normal control subjects under the same stimulation [72]. As a pro-inflammatory mediator, histamine is selectively located within the granules of mast cells and basophils and released from these cells upon degranulation. Histamine causes dissociation of interendothelial junctions as well as cytoskeleton contraction, resulting in a widening of intercellular spaces that fa-

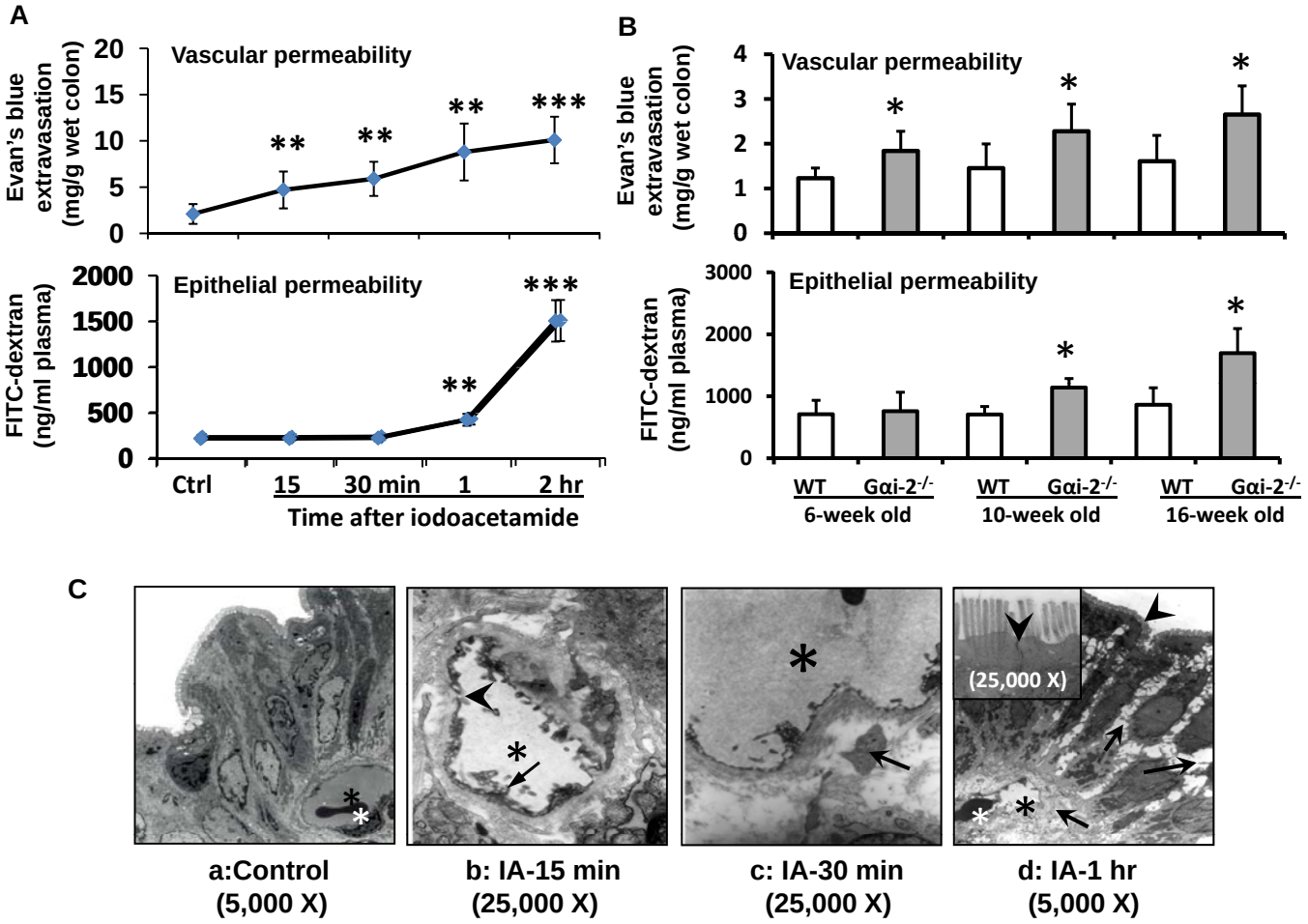

Fig. (4). Changes of vascular and epithelial permeability in early stages of experimental UC. **A:** IA-induced UC in rats and **B**: Gα-i2$^{-/-}$ spontaneously developing UC in mice. **C**: Ultrastructural changes in colonic mucosa at the early stages of IA-induced UC in rats. The transmission electron microscopy reveals at 15 minutes following IA-enema small breaks in endothelial lining (arrowheads) and platelets adhering to endothelial cells (arrow), indicating a very early endothelial injury (**b**). At 30 min following IA, subepithelial capillaries show platelets attached to endothelial cells, and platelets also are present in the perivascular space (marked by arrow) which is enlarged and filled with fibrillar structures (mostly likely fibrin). Intravascular congestion also is readily evident [*] (**c**). At 1 hr after IA enema, (at which time increased EP was detected) a huge perivascular edema containing fibrin deposits and intravascular platelet aggregation (arrows) is present. Most of the epithelial cells are separated by edema (arrows) with apparently intact tight junctions (arrowheads) and intact microvilli (**d**). IA: iodoacetamide.

cilitate trans-endothelial flux. These structural changes initiated by agonist receptor binding are followed by activation of intracellular signaling molecules such as tyrosine kinases and myosin light chain kinase. These kinases then phosphorylate or alter the conformation of different cytoskeletal elements, e.g., VE-cadherin, that control endothelial cell–cell adhesion, resulting in increased paracellular permeability.

Histamine is a main factor contributing to increase in VP mainly from venules in acute inflammatory response associated with trauma, burns, allergy and acute inflammation. Four subtypes of G-protein-coupled histamine receptors (H1, H2, H3 and H4) are responsive to histamine action [73-75], among which H1 is considered the most important with respect to VP [75]. Increased endothelial paracellular permeability resulting from intercellular gaps has been recognized as the major cellular mechanism underlying histamine-induced endothelial barrier dysfunction and tissue edema.

Since we found that increased VP and hypoxia in colonic mucosa in experimental UC occurred earlier than elevation of colonic VEGF [26], we tested a hypothesis that histamine plays an initiating role in the early increased VP in a rat model of UC induced by IA. The results showed that histamine in plasma was increased as early as 15 min and had a peak value at 30 min after IA (Fig. **5**). To determine whether colonic mast cells and increased release of histamine mediate the increased VP in the early stage of UC, we tested effects of a mast cell stabilizer doxantrazole and H1 receptor antagonists mepyramine and diphenhydramine in IA-induced UC in rats. We demonstrated that pretreatment with the mast cell stabilizer or H1 antagonists 30 min before IA enema significantly reduced colonic VP (Table **1**). Since the above studies showed that histamine release occurred very early, and inhibition of mast cell secretion and H1 receptor significantly reduced the early increase of VP in IA-induced UC, we tested another hypothesis whether stabilizing mast cells and antagonizing H1 receptor prevent/attenuate development of UC. Rats were pretreated with a mast cell stabilizer doxantrazole and two H1 antagonists mepyramine and diphenhydramine 30 min before IA enema. The results showed that colonic lesions and colonic dilation were significantly reduced in the rats pretreated with mepyramine, diphenhydramine or dozantrazole (Table **1**). We thus conclude for the first time that histamine plays an initiating role in the early increase in colonic mucosal VP leading to colonic mucosal edema and hypoxia, which in turn activates VEGF gene expression. Blockade of mast cell secretion and H1 receptor reduces increased VP and attenuates development of key features of IA-induced UC in rats.

VEGF/VPF-mediated VP

VEGF is key regulator of VP under both physiologic and pathologic conditions. Typically, VEGF mediates VP via activating downstream signaling factors such as tyrosine phosphorylation of Src kinase, leading to changes in VP (Fig. **6**). Mechanistically, knockout and pharmacologic inhibitor studies point to the importance of Src family protein tyrosine kinase activation by VEGF in promoting phosphorylation of endothelial adherens junction (AJ) molecule VE-cadherin and endothelial transport protein caveolin-1 [76]. Very recently, two new downstream pathways are demonstrated showing that VEGF-induced VP is also regulated by focal adhesion tyrosine kinase (FAK) and an orphan nuclear transcription factor TR3/Nur77 (Fig. **6**). VEGF promotes tension-independent FAK activation, rapid FAK localization to cell-cell junctions, binding of the FAK FERM domain to the vascular endothelial cadherin (VE-cadherin) cytoplasmic tail, and direct FAK phosphorylation of β-catenin at tyrosine-142 facilitating VE-cadherin-β-catenin dissociation and AJ breakdown [77]. It was also recently reported that VEGF activates expression of TR3/Nur77 which has a more general role in regulating VP. TR3/Nur77 modulated VP increasing endo-

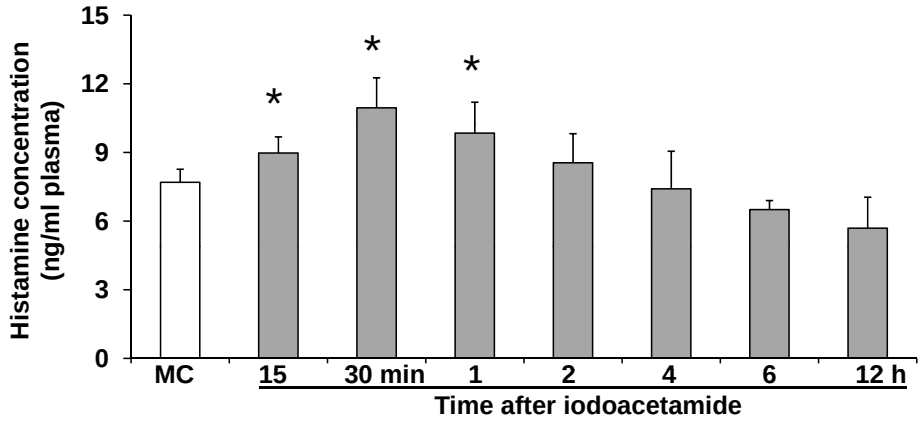

Fig. (5). Plasma levels of histamine during development of IA-induced UC in rats. MC: methylcellulose. IA: iodoacetamide. * $p < 0.05$.

Table 1. Effects of H1 Antagonists Mepyramine and Diphenhydramine, and Mast Cell Stabilizer Doxantrazole on Colonic Vascular Permeability and Lesions in Iodoacetamide (IA)-induced UC in Rats

Treatment	Vascular Permeability (E-blue: mg/g wet colon)	Colonic Lesions (mm^2)	Colonic Dilation (mm)
Saline + IA (controls)	16.7±4.7	285.5±61.1	19.0±2.2
Mepyramine 10 mg + IA	6.5±1.3**	133.0±57.7*	14.2±2.7*
Diphenhydramine 10 mg + IA	8.7±3.5*	121.3±59.6*	12.5±1.0*
Doxantrazole 10mg + IA	8.3±2.5*	117.4±39.8*	12.5±0.6*

* $p < 0.05$; ** $p < 0.01$ compared to controls.

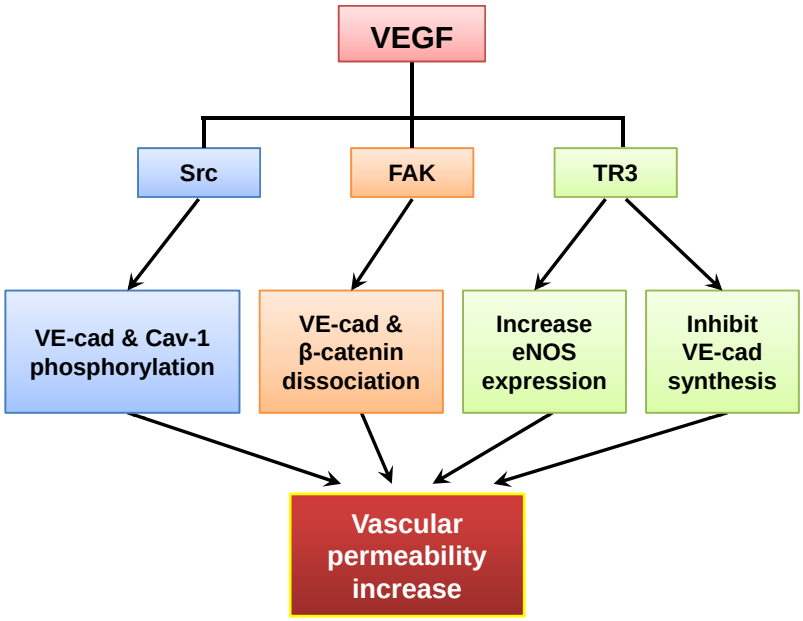

Fig. (6). A diagram of VEGF-mediated pathways for vascular permeability. Src: Src kinase. FAK: focal adhesion tyrosine kinase. TR3: an orphan nuclear transcription factor. eNOS: endothelial nitric oxide synthase. VE-cad: VE-cadherin.

thelial nitric-oxide synthase expression and by down-regulating AJ proteins that maintain vascular homeostasis [78]. However, none of these VEGF-related pathways of increased VP has been investigated in the pathogenesis of UC.

We thus investigated the role of VEGF and Src-dependent downstream signaling pathway in increased VP in experimental UC based on our previous and recent studies.

Increased Levels of VEGF in UC

Both serum and tissue levels of VEGF were significantly higher in patients with active UC than in the controls [79-81]. Griga *et al*. reported increased serum VEGF levels in patients with active UC but not in patients with inactive disease, and identified the colonic mucosa as the source of the increased serum level of VEGF [81]. We and others also found that VEGF levels were significantly elevated in chemically induced (IA or TNBS) and also in genetic (IL-$10^{-/-}$ or Gα-$i2^{-/-}$) animal models of UC [82-84]. Although these reports indicate increased VEGF levels in both animals and patients with UC, the precise role of VEGF in UC is not well understood. Our recent study showed that neutralization of VEGF with specific antibody reduces increased VP and attenuates chemically induced experimental UC [10].

Src Kinase-dependent Increase in VP in UC

Recent *in vitro* studies demonstrated that VEGF-induced increased VP requires a Src family protein, tyrosine kinase [85]. There is no increase in VP in response to an enhanced level of VEGF in Src-deficient mice [86]. The mechanism by which VEGF increases VP through Src *in vivo* has not been studied. Some, exclusively *in vitro* studies in aortic cell lines showed that unstimulated endothelial junction contains a protein complex composed of VEGFR2, VE-cadherin, and β-catenin, all involved in maintenance of endothelial barrier integrity. This molecular complex immediately dissociates following VEGF stimulation, an event that depends on Src kinase activity [86]. Whether any of these events take place in microvessels of colonic mucosa during VEGF stimulation and/or in mucosa during development and progression of UC is not known. In our recent publication we showed that increased VEGF levels were associated with increased phosphorylation of Src in IA-induced UC in rats [10]. Based on a literature search, the mechanistic role of Src in UC has not been investigated, with the exception of our recent report demonstrating that Src inhibitor reduces increased VP in IA-induced UC in rats [10]. Since Src plays a key role in mediation of VEGF-induced colonic VP, we further tested whether inhibition of Src attenuates UC development and accelerates UC healing. We examined the effect of Src inhibition using a specific Src inhibitor PP1 in IA-induced UC in rats. The results showed that inhibition of Src significantly reduced colonic lesions and accelerated healing (Fig. **7**) of UC compared to controls 7 days after IA. These results indicate that Src kinase plays a key role in VEGF-mediated VP signaling pathways and inhibition of Src improves healing of experimental UC.

AJs Molecule VE-cadherin (Para-endothelial Permeability)

VP and endothelial barrier function are modulated mainly by the AJs proteins, e.g., VE-cadherin. Dynamic interactions between the AJs proteins and the actin cytoskeleton are crucial for the regulation of junction permeability. Namely, the cortical actin band stabilizes AJs and re-organization of actin into contractile stress fibers disrupts them. Actin-mediated endothelial cell contraction is the result of myosin light chain phosphorylation which drives myosin-actin cross-bridge cycling [87,88].

VE-cadherin is localized in interendothelial AJs where they are linked in the cytoplasm to β-, γ-, and p120-catenins, and in turn to α-catenin and the actin cytoskeleton. Once phosphorylated, VE-cadherin is dissociated from catenins, which can cause intercellular gap formation, leading to an increase in VP [13]. In some endothelial cells, e.g., cardiovascular and renal endothelial cells, VEGF causes up-regulation of junctional adhesion molecule-C expression at cell junctions and stimulates VE-cadherin phosphorylation via Src, leading to decreased occludin and VE-cadherin. Gavard and Gutkind [89] recently demonstrated that the endpoint of this signaling pathway is the β-arrestin2-dependent endocytosis of VE-cadherin, thereby disrupting the endothelial barrier function.

We investigated whether Src-dependent downstream mechanism mediates VEGF-induced colonic VP during experimental UC. In our study, we demonstrated increased interactions of β-arrestin2 and VE-cadherin following its phosphorylation activated by Src in colonic mucosa, and inhibition of Src markedly recduced phosphrylation of VE-cadherin in colonic mucosa after IA enema, which were detected by Western blot with co-immunoprecipitation (Fig.

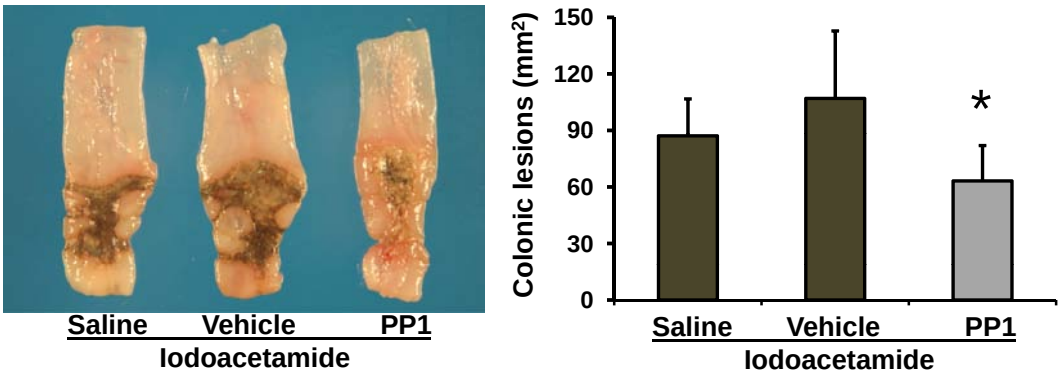

Fig. (7). Effect of blocking Src kinase by a specific inhibitor PP1 on healing of UC induced by IA in rats. IA: iodoacetamide. * $p < 0.05$.

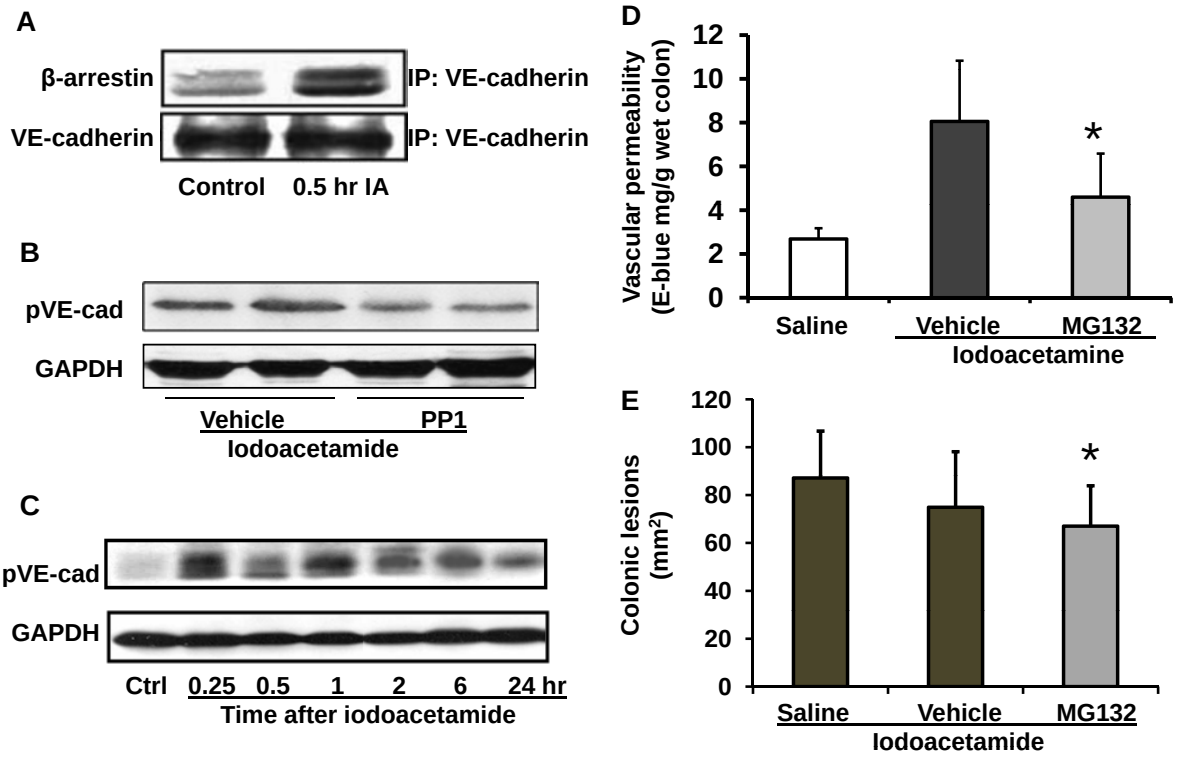

Fig. (8). Phosphorylation of VE-cadherin and its roles in regulation of vascular permeability and healing of IA-induced UC. **A**: Interactions of β-arrestin2 and VE-cadherin following its phosphorylation activated by Src in colonic mucosa. **B**: Reduced phosphorylation by inhibition of Src. **C:** Increased levels of VE-cadherin phosphorylation during development of IA-induced UC. **D:** Decreased vascular permeability after inhibition of VE-cadherin phosphorylation. **E:** Accelerated healing by blocking phosphorylation of VE-cadherin. IA: iodoacetamide. Ctrl: control. PP1: Src inhibitor. MG132: a proteasome inhibitor for inhibition of VE-caherin phosphorylation. * $p < 0.05$.

8A, **B**). Reduction of VE-cadherin in endothelial junctions may lead to increase in VP. We therefore performed a further study to examine whether phosphorylation of VE-cadherin occurs and whether it is involved in mediating increased VP in colonic mucosa during development of IA-induced UC. Phosphorylation of VE-cadherin was examined by Western blot in colonic mucosa of rats with IA-induced UC. The role of VE-cad in increased VP was determined by inhibition of its phosphorylation using a proteasome inhibitor MG132 which has been reported to effectively inhibit VE-cadherin phosphorylation [90]. The results showed that phosphorylation of VE-cad occurred as early as 15 min and lasted through the entire study course after IA enema (Fig. **8C**). The early phosphorylation of VE-cadherin (e.g., 15 and 30 min after IA) may be mediated by histamine, and likely followed by VEGF-Src-induced VE-cadherin phosphorylation. Inhibition of VE-cadherin phosphorylation showed a significant reduction of VP in IA-induced UC (Fig. **8D**). We also tested a hypothesis that inhibition of VE-cadherin prevents or reduces UC development. UC was induced by IA enema in rats. The rats were given MG132 subcutaneously 1 hr before and 2 days after IA administration. Rats were euthanized 7 days after IA. The results showed that colonic lesions were improved by treatment with MG132 in IA-induced UC in rats (Fig. **8E**). These studies demonstrated that VE-cadherin is involved in mediating the increased VP likely initiated by histamine and exaggerated by VEGF. Inhibition of VE-cad phosphorylation reduced the increase in VP in colonic mucosa and attenuated development of IA-induced UC.

SUMMARY

Increasing evidence indicates that angiogenesis is a novel and crucial element in the pathogenesis of UC. In this review, we first

summarized mostly our previous data on healing of experimental UC with angiogenic therapies using bFGF, PDGF or Rac1, and anti-angiogenic therapies by neutralizing pathologic angiogenesis inducers VEGF or PlGF. These studies indicate that the above angiogenic growth factors have different biologic features and actions regulating different types of angiogenesis in healing of UC. Namely, rectal enemas containing bFGF, PDGF or Rac1 stimulate angiogenesis which indeed accelerates healing of UC, while VEGF or PlGF induce pathologic angiogenesis that impairs UC healing. Thus, it seems that either anti-pathologic angiogenic or angiogenic therapies are needed for healing of UC. PlGF has been suggested to be a marker of pathologic angiogenesis and may play critical roles in abnormal angiogenesis only, e.g., inhibition of PlGF did not affect quiescent vessels in healthy organs. In addition, recent publications showed that activation of Rac1 markedly improved VEGF-induced neovessel architecture defect and reduced vascular leakage. Since anti-VEGF therapy often causes gastric ulcer perforation and development of skin ulcers in patients, and pathologic angiogenesis is associated with architecturally defective and leaky blood vessels, PlGF and Rac1 may be selected as attractive therapeutic targets for the development of safe anti-angiogenic drug in UC. Secondly, we reviewed our most recent demonstrations for the first time that increased VP in colonic mucosa is an early event initiated by histamine and exaggerated/sustained by VEGF, preceding the increase in epithelial lining permeability, and play an important pathogenic role in UC initiation and progression. The impairment of microvascular endothelial barrier facilitates initiation and maintenance of inflammatory cell infiltration, resulting in colonic mucosal erosions/ulcers and UC. This VP inducing action of VEGF is entirely different from its stimulatory effect on pathologic angiogenesis in UC. Moreover, we also demonstrated that anti-histamine and anti-VEGF treatment markedly reduced increase in VP as well as the extent and severity of ulceration in experimental UC. These findings provide the potential to identify novel cellular and molecular targets (e.g., histamine/mast cells, VEGF, Src, and VE-cadherin) for more effective preventive and therapeutic interventions in UC.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENT

These studies were supported by VA Merit Review grants to Dr. Zs. Sandor.

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Received: May 14, 2012 **Accepted: July 30, 2012**