Molecular Mechanisms of Basic Fibroblast Growth Factor Effect on Healing of Ulcerative Colitis in Rats

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ABSTRACT

We demonstrated previously that basic fibroblast growth factor (bFGF) accelerated the healing of experimental duodenal ulcers, and we now hypothesize that bFGF might also accelerate the healing of experimental ulcerative colitis (UC). We also explored the potential molecular mechanisms involved in the accelerated healing of UC in rats treated with bFGF. The results demonstrated that colonic lesions were significantly reduced by bFGF treatment, whereas neutralization of bFGF aggravated iodoacetamide-induced UC. Protein expression of bFGF was increased during the healing stage of UC. Tumor necrosis factor-α levels and myeloperoxidase activity were significantly decreased in UC rats treated with bFGF, whereas they increased in rats treated with anti-bFGF antibody. Real-time polymerase chain reaction and immunohistochemistry showed decreased levels of p27 in the UC rats compared with the healthy controls, which was reversed by bFGF treatment in a dose-dependent manner. By immunohistochemistry and double labeling of Ki-67 and CD34, prominent positive staining of Ki-67 and CD34 was seen after bFGF treatment, indicating the enhanced proliferation of fibroblasts and epithelial and endothelial cells, i.e., angiogenesis. We conclude that bFGF plays a beneficial role in the healing of UC in rats. The molecular mechanisms of bFGF in UC healing not only involve the expected increased cell proliferation, especially angiogenesis, but also encompass the reduction of inflammatory cytokines and infiltration of inflammatory cells. Thus, bFGF enema may be a new therapeutic option for UC.

Introduction

Ulcerative colitis (UC), a chronic inflammatory disorder, is the most prevalent form of inflammatory bowel disease (IBD) with a major clinical and economic impact. The incidence of UC in the United States is 205/100,000 people, which is the second highest in the world and steadily increasing (Herrinton et al., 2008). The pathogenesis and molecular mechanisms of UC are not fully understood. An enhanced expression of proinflammatory cytokine tumor necrosis factor-α (TNF-α) plays a crucial role in the pathogenesis of IBD, most likely because TNF-α disrupts the epithelial barrier, induces apoptosis of the villous epithelial cells, and stimulates the secretion of chemokines from the intestinal epithelial cells. TNF-α also activates neutrophils and macrophages to release acid hydrolase myeloperoxidase (MPO), the activity of which reflects the degree of inflammatory cell infiltration in IBD (Ardizzone and Bianchi Porro, 2005). However, the current anti-inflammatory therapy (e.g., treatment with anti-TNF-α antibody or glucocorticoids) does not cure the disease and results in long-term remission only in fewer than 34% of patients (Peyrin-Biroulet, 2010). This means that more than 60% of patients are always symptomatic or have frequent disease recurrences resulting in decreased quality of life and economic loss caused by the inability to work. Hence, anti-inflammatory therapy is not completely effective in eliminating the disease, indicating that other pathogenic factors may play important roles in UC (Stallmach et al., 2010). Also unknown are the molecular mechanisms that control and coordinate cell cycle and proliferation in the development and healing of UC. The main groups of molecules involved in the...
regulation of the cell cycle include the cyclin-dependent kinase inhibitors (e.g., p21 and p27) (Beauchamp et al., 1996). However, little is known about the mechanistic roles of p21 and p27 in UC healing.

We and others suggest that angiogenesis may be an important component of UC pathogenesis (Danese et al., 2006; Koutroubakis et al., 2006; Sandor et al., 2006). Angiogenesis is critical to wound/ulcer healing and is closely controlled by growth factors such as VEGF, platelet-derived growth factor, and bFGF (Carmeliet, 2003). We and others have demonstrated that VEGF plays a pathogenic role, e.g., in causing abnormal or pathologic angiogenesis and increasing vascular permeability that leads to enhanced release of inflammatory cytokines and infiltration of inflammatory cells in UC (Scaldaferri et al., 2009; Tolstanova et al., 2009). As shown previously, these angiogenic growth factors accelerated healing of upper gastrointestinal and skin ulcers by promoting normal or physiologic angiogenesis (Szabo et al., 1994; Kustatscher et al., 1995; Szabo and Sandor, 1996; Deng et al., 2004; Rico et al., 2009).

bFGF is an 18-kDa polypeptide that was first isolated from brain as a fibroblast stimulator (Gospodarowicz et al., 1978) and was later found to be identical to the most potent heparin-binding angiogenic stimulator (Shing et al., 1984). Indeed, bFGF is a direct mitogen for vascular endothelial cells, fibroblasts, smooth muscle cells, epithelial cells, and neural cells. It plays a major role in wound healing, tissue regeneration, and embryonic development, and probably in cancerogenesis as well (Barrientos et al., 2008). Pharmacologic studies also demonstrated that bFGF treatment led to rapid healing of experimental UC (Szabo et al., 1993; Szabo and Sandor 1996; Satoh et al., 1997). However, its molecular mechanisms in promoting the healing of UC have not been fully elucidated.

In this study we tested the hypothesis that bFGF may accelerate the healing of chemically induced UC, whereas inhibition of bFGF may aggravate UC in rats. We also explored the molecular mechanisms of bFGF effect on the healing of experimental UC.

**Materials and Methods**

**Animals.** Female Sprague-Dawley rats (170–200 g) were obtained from Harlan (Indianapolis, IN) and housed in the animal research facility at the VA Medical Center in Long Beach, CA. Animals had unlimited access to Purina (St. Louis, MO) chow and tap water throughout the study and were allowed to acclimatize for 3 to 5 days in cages (three rats/cage) in a room with a 12-h light/dark cycle at a constant temperature of 22°C. They were randomly divided into groups. These studies were approved by the Subcommittee for Animal Studies of the R&D Committee of the VA Medical Center in Long Beach, CA.

**Iodoacetamide-Induced Colitis.** Experimental UC was induced in rats by the SH alkylator iodoacetamide (IA) (Sigma, St. Louis, MO). A single dose of 6% IA induces well reproducible colonic lesions, with the initial manifestations (e.g., increased vascular permeability, massive mucosal edema) seen in 1 to 2 h after IA enema, leading to erosions and ulcers (6–12 h), followed by extensive acute and chronic inflammation (7–14 days). In brief, 0.1 ml of 6% IA dissolved in 1% methylcellulose (Sigma) was administered once by enema (7 cm from anus) via a rubber catheter Nelaton S-S (Rüsch, Kernen, Germany). Control rats were given 0.1 ml of 1% methylcellulose.

**Experiments.** In experiment 1 animals (n = 5/group) were euthanized at 0, 1, 2, and 6 h or 1, 3, 7, and 10 days after IA to examine the protein expression of bFGF during development and healing of UC. In experiment 2 rats were randomly divided into four groups (n = 5/group) on the second day after IA and given intracolonically saline (0.1 ml/rat) or bFGF (2, 10, or 100 μg/rat; ProSpec Bio, Rehovot, Israel) twice daily for 8 consecutive days. Rats were euthanized on the 10th day after IA. In experiment 3 rats were randomly divided into three groups (n = 5/group) on the second day after IA and given 50 μg/rat of neutralizing mouse monoclonal anti-bFGF antibody (Millipore Corporation, Billerica, MA), normal mouse IgG (Millipore Corporation), or 0.1 ml of saline/rat, ×2, respectively, on the third and fifth days after IA enema. Rats were euthanized on the seventh day after IA administration. During IA-induced colitis, well formed necrotic lesions, extending into the submucosa and muscle layers, developed within 1 to 2 days. Therefore, we injected the first dose of anti-bFGF antibody on the third day. All three experiments were repeated two times, and data were pooled.

**Evaluation of UC Healing and Sample Preparation.** At autopsy, the area of colonic lesions (e.g., extent of tissue damage) and loss of rugae (as an indicator of recently healed, regenerated mucosa) were measured in millimeters in the two largest diameters, subsequently quantified by computerized planimetry coupled with stereomicroscopy, and expressed in square millimeters. Colon wet weight (milligrams per 100 g of body weight) and severity of colitis (scale: 0, none; 1, mild; 2, moderate; 3, severe) were also measured. The distal colon (7 cm from anus) was harvested and either frozen in –80°C for biochemical and molecular analysis or fixed in 10% buffered formalin for histology. Total RNA were isolated by TRIzol reagent (Invitrogen, Carlsbad, CA) followed by purification with a RNaseasy Mini Kit (QIAGEN, Valencia, CA). Concentrations of the total RNA were determined by using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). For protein extraction, tissue specimens were homogenized in lysis buffer (2 M NaCl and 10 mM Tris-HCl, pH 7.4) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin; Sigma), sonicated, and centrifuged for 30 min at 16,000g. Protein concentration was measured by the Bradford assay (VersaMax microplate reader; Molecular Devices, Sunnyvale, CA).

**Real-Time PCR.** For cDNA synthesis, 5 μg of total RNA was used with First-Strand cDNA Synthesis Super Mix (Invitrogen). Expression of p27 mRNA was detected using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and an iQ5 Real-Time PCR detection system (Bio-Rad iCycler Real-Time PCR instrument; Bio-Rad Laboratories, Hercules, CA). Each assay consisted of two unlabeled PCR primers and a 5-carboxyfluorescein dye-labeled TaqMan minor groove binder probe. Specific primers were purchased from Applied Biosystems. The thermal profile was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, finally holding at 4°C. The relative quantification of expression of the gene was normalized to the internal control gene GAPDH and determined using the ratio = 2−ΔΔCT method as described by Pfaffl (2001). The fold changes of the treated over the control group were calculated.

**Western Blotting.** Proteins (100 μg) that were extracted from colonic mucosal scrapings were subjected to SDS-polyacrylamide gel electrophoresis after dissociation by boiling (5 min) in 2× SDS-sample loading buffer (0.125 M Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.05% bromphenol blue). Continuous SDS-polyacrylamide gel electrophoresis was used for assays of bFGF and TNF-α (15% gel), fibronectin (8% gel), and elastin (12% gel). After electrophoresis proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories). The membranes were incubated with 1:200 rabbit polyclonal bFGF antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:500 mouse monoclonal TNF-α antibody (R&D Systems, Minneapolis, MN), 1:200 mouse monoclonal fibronectin antibody (Santa Cruz Biotechnology, Inc.), and 1:500 rabbit polyclonal elastin antibody (Abcam Inc., Cambridge, MA). Loading controls were performed by using a mouse anti-β-actin antibody.
monoclonal antibody to GAPDH (1:2000; EnCor Biotechnology, Alachua, FL). Every Western blot was repeated at least two times using protein from two rats/group. The density of Western blots bands was measured by using the MetaMorph 7.5 Videoimage Analysis System (Molecular Devices) and presented as relative density against density of GAPDH bands.

**Histology, Trichrome Technique, and Immunohistochemistry.** To assess histologic damage, full-thickness colonic tissue samples fixed in 10% buffered formalin were cut, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, and the histochmical trichrome techniques (to evaluate collagen deposition in the granulation tissue) were used. Immunostaining was performed using paraffin-embedded, 5-μm-thick intestinal sections. Sections were deparaffinized, hydrated, blocked for endogenous peroxidase using 3% H2O2/H2O, and subsequently subjected to microwave antigen retrieval using a Dako target retrieval solution (BD Pharmingen, San Diego, CA) at pH 10.00. An overnight incubation was performed using the primary antibodies human Ki-67 (1:100; Millipore Bioscience Research Reagents, Temecula, CA), human CD34 (1:100; Santa Cruz Biotechnology, Inc.), and rabbit p27 (1:100; Santa Cruz Biotechnology, Inc.), secondary antibodies, and a peroxidase-labeled streptavidin-biotin and then developed using the 3,3′-diaminobenzidine substrate detection method for examination under a Nikon (Tokyo, Japan) microscope. To ensure specificity of the antibody, immunoabsorption of the antibodies was performed to provide controls.

**Quantification of Angiogenesis.** For the evaluation of neovascularization, pictures were taken from CD34-stained immunohistochemistry slides under a light microscope at 400× magnification by an imaging system (MetaMorph 7.5; BioImaging Solutions, San Diego, CA). In each slide, three areas of maximal microvessel density, so-called hot spots, were identified. Microvessels (capillaries and small venules) were counted in each area. For each slide, the mean number of microvessels from these three areas was calculated according to Mazur et al. (2004).

**Double Labeling for CD34 and Ki-67.** For double labeling, the same antibodies and concentrations were used as described above. Colonic tissue sections were deparaffinized, hydrated, blocked for endogenous peroxidase using 3% H2O2/H2O, and subsequently subjected to microwave antigen retrieval using a target retrieval solution (Dako North America, Inc., Carpinteria, CA) at pH 10.00. After subsequent washing and blocking with protein block (Dako North America, Inc.), tissue sections were incubated with anti-Ki-67 antibody at 4°C overnight. The sections were then washed, incubated with biotinylated secondary antibodies and a peroxidase-labeled streptavidin-biotin, and then developed using the 3,3′-diaminobenzidine substrate detection method for examination under a Nikon microscope. To ensure specificity of the antibody, immunoabsorption of the antibodies was performed to provide controls.

**Evaluation of In Vivo Cell Proliferation and Cell Cycle Inhibition.** Cell proliferation and cell cycle inhibition were evaluated in paraffin-fixed tissue slides by immunostaining with human Ki-67 and rabbit p27 antibodies, respectively. Slides were reviewed blindly by two pathologists, and the degree of immunopositivity was determined as scales 0 to 3: 0, negative staining; 1, weakly positive staining; 2, moderately positive staining; and 3, strongly positive staining.

**Measurement of In Vitro Endothelial Cell Proliferation.** In vitro cell proliferation was determined using bromodeoxyuridine (BrdU) assay. Rat microvascular endothelial cells (rMVECs) were cultured on four-well chamber slides (Nalge Nunc International, Rochester, NY) and treated with 50 ng/ml bFGF for 2 h. rMVECs were pulsed with 10 mM BrdU (Millipore Corporation) for 60 min. The slides were then fixed in methanol for 10 min at 4°C and air-dried. DNA was denatured with 2 N HCl for 1 h followed by neutralization of acid with 0.1 M borate buffer, pH 8.5. Slides were washed with PBS and incubated with anti-BrdU antibody (MAB3424; Millipore Corporation) for 1 h. After washing with PBS, slides were incubated with biotinylated anti-mouse IgG (1:500 dilution; Dako North America, Inc.) for 30 min. The slides were then washed with PBS, incubated with streptavidin/horseradish peroxidase-durate conjugate (Dako North America, Inc.) for 30 min, and developed with 3-amo-9-ethylcarbazole chromogen. Counterstaining of nuclei was achieved using Mayer’s hematoxylin. Images were captured using a Nikon digital camera DXM1200 attached to a Nikon Optiphot. Six areas were randomly selected on each slide, and cells were counted. Cell proliferation (BrdU-positive cells) was determined as the percentage of total cells in each area.

**MPO Assay.** Distal colon samples were suspended in 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide (Sigma) followed by sonication on ice for 10 s. Suspensions were then freeze-thawed three times, and the supernatant was separated from the solid phase by centrifugation at 14,000g for 15 min. A total of 14 μl of the supernatant was mixed with 200 μl of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml o-dianisidine dihydrochloride (Sigma) and 0.005% hydrogen peroxide (Sigma). MPO activity was derived from an observed change in absorbance measured by spectrophotometry at 450 nm (VersaMax microplate reader; Molecular Devices) and normalized to the wet colon weight.

**Statistical Analysis.** Quantitative results are expressed as mean ± S.E. The statistical significance was determined by the nonparametric Mann-Whitney U test or Student’s t test where appropriate, and p values <0.05 were considered statistically significant.

**Results**

**Accelerated Healing of Experimental UC by Intracolonic Administration of bFGF.** bFGF treatment significantly accelerated healing of IA-induced UC in a dose-dependent manner (Fig. 1). Colonic lesions were decreased from 114 ± 30 mm2 (controls) to 94 ± 36 mm2 (2 μg/rat of bFGF), 41 ± 12 mm2 (10 μg/rat, p < 0.05), and 22 ± 9 mm2 (100 μg/rat, p < 0.02) (Fig. 1A). Severity of colitis and loss of rugae were significantly decreased in the rats treated with 10 and 100 μg of bFGF compared with controls (Fig. 1, B and C). Colon wet weight was also significantly reduced in groups of animal treated with bFGF in doses of 10 μg/rat (Fig. 1D). Histologic examination showed that bFGF accelerated UC healing in a dose-dependent manner (Fig. 1, F–H). Rats treated with 10 or 100 μg of bFGF had much smaller ulcers and more regenerated mucosa covering the ulcer craters (Fig. 1, G and H) than controls (Fig. 1E).

**Aggravation of UC by Neutralizing Anti-bFGF Antibody.** To determine the effect of bFGF neutralization on experimental UC, rats were treated with either neutralizing anti-bFGF antibody, mouse IgG, or saline. The lesion areas of UC in animals treated with neutralizing anti-bFGF antibody were markedly larger than those treated with saline or IgG (Fig. 2A). Quantitative analysis of colonic lesions were significantly increased at 392 ± 74 mm2 in the group treated with neutralizing anti-bFGF antibody compared with both saline-treated (147 ± 32 mm2) and IgG-treated (210 ± 49 mm2) groups (p < 0.05) (Fig. 2B). bFGF neutralization also significantly increased severity of colitis, colon wet weight, and loss of rugae in UC (Fig. 2, C–E).
Increased Levels of bFGF and Matrix Proteins in Experimental UC. Western blot demonstrated that levels of bFGF protein were significantly decreased early in UC development (1–6 h after IA) and increased during the healing stage of UC (7 and 10 days) (Fig. 3A). Because bFGF regulates matrix proteins such as collage, fibronectin, and elastin, we also assessed the levels of these matrix proteins in the colonic tissues during the healing stage (7 days after IA). Trichrome staining showed that collagen (Fig. 3B, blue) in granulation tissues of UC treated with bFGF was markedly increased (Fig. 3B, arrows) in a dose-dependent manner (Fig. 3B). Western blotting indicated that other two matrix proteins, e.g., fibronectin and elastin, were also significantly increased \( p < 0.05 \) or \( p < 0.01 \) in colonic granulation tissues of UC in a dose-dependent manner (Fig. 3C).

Increased Cell Proliferation after bFGF Treatment in UC. Immunohistochemistry showed increased positive staining of cells for Ki-67 that reflected increased cell proliferation in UC treated with bFGF in a dose-dependent manner (Fig. 4A). The increased positive staining of Ki-67 was located primarily in the regenerated mucosa around the board of UC treated with 10 or 100 \( \mu \)g of bFGF (Fig. 4A). Semiquantitative evaluation showed that cell proliferation was significantly increased in colonic mucosa of UC rats treated with 10 or 100 \( \mu \)g of bFGF compared with control \( p < 0.05 \) (Fig. 4B).

Increased Angiogenesis after bFGF Treatment in UC. Immunostaining demonstrated a dose-dependent increase of CD34 (a specific marker of vascular endothelial cells)-positive staining in UC after bFGF treatment, indicating increased angiogenesis (Fig. 5A). Blood vessel counts based on CD34-positive staining indicated that microvascular density increased from \( 2.0 \pm 1.8 \) vessels/area (control) to \( 5.7 \pm 3.1 \) (2 \( \mu \)g of bFGF), \( 7.7 \pm 1.6 \) (10 \( \mu \)g, \( p < 0.05 \)), and \( 13.3 \pm 3.5 \) (100 \( \mu \)g, \( p < 0.05 \)) in colonic tissues with IA-induced UC (Fig. 5B).

Increased Endothelial Cell Proliferation In Vivo and In Vitro after bFGF Treatment. Double labeling of Ki-67 and CD34 showed that bFGF treatment markedly increased proliferation of colonic microvascular endothelial cells in the newly formed microvessels in the granulation tissues compared with the control (Fig. 6A). The in vitro study further demonstrated that proliferation of rMVECs treated with bFGF was significantly increased \( p < 0.05 \) compared with the control (Fig. 6B).

Effect of bFGF and Anti-bFGF Treatment on TNF-\( \alpha \) Levels and MPO Activity in Colonic Mucosa during Experimental UC. Both TNF-\( \alpha \) expression and MPO activity were significantly increased in UC induced by IA (Fig. 7). bFGF treatment significantly decreased the levels of TNF-\( \alpha \) in colonic mucosa at a dose-dependent manners (Fig. 7B), whereas treatment with neutralizing antibody significantly increased TNF-\( \alpha \) expression compared with both control.
groups treated with saline or IgG antibody (Fig. 7B). MPO assay showed significantly decreased MPO activity in the group treated with 100 μg/rat of bFGF (p < 0.04) (Fig. 7C) and increased MPO activity in the group treated with anti-bFGF antibody compared with saline and IgG treatment (p < 0.04) (Fig. 7D).

**Increased Cell Cycle Inhibitor p27 in UC Treated with bFGF.** To examine whether bFGF influences the regulation of cell cycle inhibitors in UC healing, we detected p21 and p27 mRNA expression and their potential correlation with cell proliferation. p27 mRNA was decreased approximately 9-fold in UC rats treated with saline compared with the healthy control, whereas bFGF treatment increased p27 mRNA to 2- to 4-fold in a dose-dependent manner compared with the saline-treated control (Fig. 8A). Immunohistochemistry of p27 showed increased staining of p27-positive cells in the colonic mucosa of UC rats treated with bFGF in a dose-dependent manner (Fig. 8B). Semiquantitative evaluation of the immunohistochemistry demonstrated that p27 positivity was significantly increased in colonic mucosa of UC rats treated with 10 or 100 μg of bFGF compared with controls (p < 0.05) (Fig. 8C). Neither p21 mRNA nor protein showed any changes during development and healing of UC induced by IA (data not shown).

**Discussion**

In the present study we demonstrated that intracolonic administration of bFGF improved healing of UC induced by IA, extending our previous demonstration that intragastric administration of bFGF accelerated healing of chronic duodenal ulcers induced by cysteamine in rats (Szabo et al., 1994; Kusstatscher et al., 1995). Moreover, our study demonstrated that the effective doses of bFGF we used were 4- to 10-fold less than the doses others used to treat UC induced by dextran sulfate sodium (DSS) in rats and mice (Matsuura et al., 2005; Kojima et al., 2007). This difference is especially important in light of the fact that the DSS induced UC-like superficial lesions are much less severe than the deep ulcers and severe inflammation caused by IA (Sandor et al., 2006; Tolstanova et al., 2009). To gain further insights into the beneficial role of bFGF in the healing of UC we inhibited bFGF by using a specific neutralizing anti-bFGF antibody in the same model of UC. The results for the first time showed that neutralization of bFGF significantly impaired healing of experimental UC. This finding clearly confirmed that endogenous bFGF played a beneficial role in UC healing and bFGF may have both physiologic and pharmacologic roles in the pathogenesis of UC. Previously, we demonstrated that neutralization of bFGF or VEGF worsened experimental duodenal ulcers (Kusstatscher et al., 1994; Szabo et al., 1997). However, our study demonstrated that neutralizing VEGF.
improved healing of UC induced by IA in rats (Carmeliet, 2003), indicating that, unlike VEGF, bFGF plays protective roles in the pathogenesis of both upper and lower gastrointestinal ulcers.

Our results also demonstrated that the levels of bFGF protein were significantly decreased during UC development and progression (1–6 h after IA) and increased during the healing stage of UC (7–10 days). After the increased levels of bFGF protein during the healing stage of UC, we also found that the matrix proteins, e.g., collagen, fibronectin, and elastin, were increased in the colonic granulation tissues of UC rats treated with bFGF. These results indicated that bFGF may play an important role in the healing of UC at least partially via increasing the levels of matrix components such as collagen, fibronectin, and elastin. The late increased bFGF levels are opposite to the changes of VEGF in the same model of UC, which indicated increased levels of VEGF protein as early as 0.5 h after IA enema, with a continued rise in disease progression (Tolstanova et al., 2009). We and others have demonstrated that VEGF plays a pathogenic role in development of UC apparently without much effect on the healing of these colonic lesions (Scaldalferri et al., 2009; Tolstanova et al., 2009). Although VEGF and bFGF are both angiogenic factors, they seemed to play different roles in UC.

Because bFGF acts as a direct mitogen for vascular endothelial cells, fibroblasts, smooth muscle cells, certain epithelial cells, and neural cells, we further tested the hypothesis whether bFGF accelerates healing of UC via increasing angiogenesis and cell proliferation in colonic mucosa. Our results showed that neovascularization determined by CD34-positive staining and proliferating cells determined by Ki-67-
positive staining were significantly increased around the ulcer craters in colonic mucosa treated with bFGF. The endothelial cell cultures and double labeling of Ki-67 and CD34 further identified that bFGF treatment significantly stimulated the proliferation of endothelial cells both in vitro and in vivo. The finding of increased neovascularization indicated that bFGF facilitated healing via enhancing “normal” angiogenesis, which is still needed in UC healing, although some investigations have demonstrated that increased pathologic “abnormal” angiogenesis, especially VEGF-induced, may be a critical element in UC pathogenesis (Danese et al., 2006; Scaldaferri et al., 2009). The increased Ki-67-positive cells in bFGF-treated rats with UC in this study indicates that bFGF stimulates cell proliferation in colonic mucosa. Previous studies have shown that the increase of Ki-67-positive cells directly correlates with crypt cell proliferation in the intestinal mucosa (Gerdes et al., 1984; Johnston et al., 1989). It is noteworthy that it was reported that exogenous bFGF markedly enhanced crypt stem cell survival in the mouse after radiation injury (Houchen et al., 1999) and fibroblast growth factor receptor-3, whose ligand includes bFGF, was expressed prominently in the epithelial stem cell compartment (Vidrich, 2003). Taken together, bFGF might enhance epithelial cell proliferation primarily through its direct effect on intestinal epithelial cells, including stem cells.

We also found that expression of the cell cycle inhibitor p27 was significantly decreased in UC rats compared with the healthy control, whereas bFGF treatment reversed the decreased levels of p27 by approximately 50% in the present study. Ioachim et al. (2004) demonstrated that p27 expression was also decreased in patients with both UC and Crohn’s disease compared with healthy controls. Furthermore, significantly lower p27 expression was found in colorectal carcinomas compared with UC and Crohn’s disease (Vidrich, 2003). p27 is a negative regulator of cell cycle and a potential tumor suppressor gene, and decreased levels of p27 expression are associated with colonic dysplasia in UC and IBD-related carcinoma (Mikami et al., 2006). Down-regulated p27 expression in inflamed and IBD-associated nondysplastic mucosa is indistinguishable from that in transitional mucosa adjacent to sporadic colorectal carcinomas. In contrast, p27 is overexpressed in dysplastic lesions, perhaps as an attempt to counterbalance proliferative stimuli (Wong and Harrison, 2001). Thus, up-regulated p27 expression by bFGF treatment seems to recover a balance between cell cycle inhibition and cell proliferation, preventing loss of control on the cell cycle progression during UC healing.

In addition, we found that bFGF markedly decreased the TNF-α level and MPO activity in colonic tissues during healing of UC. Matsuura et al. (2005) also showed that the gene expression of TNF-α was significantly reduced in DSS-induced UC after treatment with bFGF. TNF-α is one of the most important proinflammatory cytokines and directly influences intestinal epithelial cells. TNF-α disrupts the epithelial barrier, induces apoptosis of epithelial cells, and stimulates the secretions of chemokines from intestinal epithelial cells. It also activates the adaptive immune system of the bowel by recruiting and activating neutrophils and macrophages (Papadakis and Targan, 2000; Shih and Targan, 2009). MPO is an enzyme found in neutrophils and to a lesser extent in monocytes and certain types of macrophages (Hoy et al., 2002). MPO activity reflects degree of neutrophil infiltration, and therefore, is a specific biomarker of inflammation (Naito et al., 2007; Peterson et al., 2007). The concentrations of both TNF-α and MPO in colonic tissues are strongly associated with the intensity of UC inflammation. Thus, reduced TNF-α and MPO levels by bFGF in the healing of UC indicate that bFGF plays an anti-inflammatory role in UC. Previous studies demonstrated that bFGF up-regulated TGF-β production in both epithelial cells and fibroblasts in colonic tissues of UC (Szabo and Sandor, 1996), whereas TGF-β may inhibit the production of proinflammatory cytokines, such as TNF-α and interferon-γ in activated immune cells (Letterio and Roberts, 1998). TGF-β may also have a role in down-regulating inflammatory responses in DSS-induced colitis (Letterio and Roberts, 1998).

Although increased levels of bFGF have been reported to be a potential marker for variant tumor development and prognosis, including colorectal cancer, it still remains unclear whether bFGF is involved in the pathogenesis of UC-associated colorectal cancer. Future studies are needed to figure out its role in the relationship between UC and colorectal cancer.

In summary, our study for the first time demonstrated that bFGF protein was involved in the pathogenesis of UC and its synthesis markedly increased in the healing stages of experimental UC. 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. The potential molecular
mechanisms of the therapeutic actions of bFGF on the healing of UC seem to involve increasing angiogenesis and mucosal regeneration and reducing inflammatory response in colon. 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 a new therapy for UC.

Authorship Contributions

Performed data analysis: Paunovic, Deng, Khenomenko, Ahluwalia, Tostanova, Tarnawski, Szabo, and Sandor.

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