Molecular mechanisms of bFGF effect on healing of ulcerative colitis in rats

Brankica Paunovic, Xiaoming Deng, Tetyana Khomenko, Amrita Ahluwalia, Ganna Tolstanova, Andrzej Tarnawski, Sandor Szabo, Zsuzsanna Sandor

Diagnostic & Molecular Medicine HCG (B.P., X.D., T.K., G.T., S.S.), and Medical HCG (A.A., A.T, Zs.S.), VA Long Beach Healthcare System

Departments of Pathology & Pharmacology (X.D., T.K., G.T., S.S.), and Medicine (A.T., Zs.S.), University of California-Irvine, Long Beach, CA, 90822, USA.
a) Running title: bFGF effect on healing of ulcerative colitis

b) Correspondence to:
   Sandor Szabo, MD, PhD
   VA Medical Center (05/113)
   5901 East 7th Street
   Long Beach, CA 90822, USA
   Tel.: 562-826-5403
   FAX: 562-826-5623
   e-mail: sandor.szabo@va.gov

c) Number of text pages: 23
   Number of figures: 8
   Number of references: 40
   Number of words in Abstract: 211
   Number of words in Introduction: 576
   Number of words in Discussion: 1338

d) Abbreviations: UC, ulcerative colitis; IA, iodoacetamide; bFGF, basic fibroblast growth factor; MPO, myeloperoxidase; TNF-α tumor necrosis factor-alpha.
Abstract
We previously demonstrated that bFGF accelerated healing of experimental duodenal ulcers and now hypothesized that bFGF might also accelerate 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 the colonic lesions were significantly reduced by bFGF treatment while neutralization of bFGF aggravated iodoacetamide (IA)-induced UC. Protein expression of bFGF was increased during the healing stage of UC. TNF-α levels and myeloperoxidase activity were significantly decreased in UC rats treated with bFGF, whereas increased in rats treated with anti-bFGF antibody. Real-time PCR and immunohistochemistry showed decreased levels of p27 in the UC compared to 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 were seen after bFGF treatment, indicating enhanced proliferation of fibroblasts, epithelial and endothelial cells, i.e., angiogenesis. Conclusions: bFGF plays a beneficial role in 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 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 U.S. is 205/100,000 persons that 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 pro-inflammatory cytokine tumor necrosis factor-alpha (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 degree of inflammatory cell infiltration in IBD (Ardizzzone et al., 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 due to 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.

Our and other publications suggest that angiogenesis may be an important component of UC pathogenesis (Sandor et al., 2006; Danese et al., 2006; Koutrobakis et al., 2006). Angiogenesis is critical to wound/ulcer healing and is closely controlled by growth factors such as VEGF, PDGF and bFGF (Carmeliet, 2003). Our and other studies 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 (Tolstanova et al., 2009; Scaldaferrri et al., 2009). As shown previously, these angiogenic growth factors accelerated healing of upper GI and skin ulcers by
promoting normal or physiologic angiogenesis (Szabo et al., 1994; Szabo and Sandor, 1996; Kusstatscher et al., 1995; Deng et al., 2004; Rico et al., 2009).

bFGF is an 18 kDa polypeptide which 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 lead to rapid healing of experimental UC (Szabo et al., 1993; Szabo and Sandor 1996; Satoh et al., 1997). However, its molecular mechanisms in promoting healing of UC have not been fully elucidated.

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

Methods

Animals. Female Sprague-Dawley rats (170-200 g) were obtained from Harlan Sprague-Dawley (San Diego, CA) and housed in the animal research facility at the VA Medical Center in Long Beach, CA. Animals had unlimited access to Purina chow and tap water throughout the study and were allowed to acclimatize for 3-5 days in cages (3 rats/cage) in a room with 12:12 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.

IA-induced colitis. Experimental UC was induced in rats by the SH alkylator IA (Sigma; St. Louis, MO, USA). 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 1-2 h after IA enema, leading to erosions and ulcers (6-12 h), followed by extensive acute and chronic inflammation (7-14 days). Briefly, 0.1 ml of 6% IA dissolved in 1% methylcellulose
(Sigma, St. Louis, MO, USA) was administrated once by enema (7 cm from anus) via rubber catheter Nelaton S-8 (Rüsch, Germany). Control rats were given 0.1 ml of 1% methylcellulose.

**Experiments.** **Experiment 1:** Animals (n=5/group) were euthanized at 0, 1, 2, 6 h or 1, 3, 7, and 10 days after IA to examine protein expression of bFGF during development and healing of UC. **Experiment 2:** Rats were randomly divided into 4 groups (n=5/group) on the 2nd day after IA and were given intracolonically saline (0.1 ml/rat) or bFGF (2, 10, or 100 μg/rat, ProSpec Bio, Rehovot, Isreal), twice daily for consecutive 8 days. Rats were euthanized on the 10th day after IA. **Experiment 3:** Rats were randomly divided into 3 groups (n=5/group) on the 2nd day after IA and were given 50 μg/rat of neutralizing mouse monoclonal anti-bFGF antibody (Millipore's Corporate, Billerica, MA, USA), normal mouse IgG (Millipore) or 0.1 ml saline/rat, ×2, respectively, on the 3rd and 5th day after IA enema. Rats were euthanized on the 7th day after IA administration. During IA-induced colitis, well-formed necrotic lesions, extending into the submucosa and muscle layers, develop within 1-2 days. Therefore, we injected the first dose of anti-bFGF antibody on the 3rd day. All above 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 two largest diameters; subsequently quantified by computerized planimetry coupled with stereomicroscopy and expressed in mm². Colon wet weight (mg/100 g 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, USA) followed by purification with RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Concentrations of the total RNA were determined by a NanoDrop 2000c Spectrophotometer (Thermo Scientific, San Diego, CA, USA). For protein extraction, tissue specimens were homogenized in lysis buffer (2M NaCl, 10 mM Tris-HCl, pH 7.4) with protein inhibitors (1 mM phenylmethylsulfonyl fluoride - PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin; Sigma, St. Louis, MO, USA), sonicated and
centrifuged for 30 min at 16,000 G. Protein concentration was measured by the Bradford assay (VersaMax microplate reader, Molecular Devices, East Falmouth, MA, USA).

**Real-Time PCR.** For cDNA synthesis, 5 µg total RNA was used with the First-Strand cDNA Synthesis Super Mix (Invitrogen, Carlsbad, CA, USA). Expression of p27 mRNA was detected using TagMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and an iQ5 Real-Time PCR detection system (Bio-Rad iCycler Real-Time PCR instrument; Bio-Rad, Hercules, CA, USA). Each assay consists of two unlabeled PCR primers and a FAM™ dye labeled TaqMan® MGB (minor groove binder) probe. Specific primers were purchased from Applied Biosystems, (Foster City, CA, USA). 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^{-\Delta\Delta CP} method as described by Pfaffl (Pfaffl, et al., 2001). The fold changes of the treated over the control group were calculated.

**Western blotting.** Proteins (100 µg) which were extracted from colonic mucosal scrapings were subjected to sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) after dissociation by boiling (5 min) in 2x SDS-sample loading buffer (0.125 M Tris/HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.05% bromophenol blue). Discontinuous SDS-PAGE was used for assays of bFGF and TNF-α assay (15% gel), fibronectin (8% gel) and elastin (12% gel). After electrophoresis proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA). The membranes were incubated with 1:200 rabbit polyclonal bFGF antibody (Santa Cruz Scientifics, Santa Cruz, CA, USA), 1:500 mouse monoclonal TNF-α antibody (R&D Systems, Inc., Minneapolis, MN, USA), 1:200 mouse monoclonal fibronectin antibody (Santa Cruz scientifics) and 1:500 rabbit polyclonal elastin antibody (Abcam, San Francisco, CA). Loading controls were performed by using a mouse monoclonal antibody to GAPDH (1:2000; EnCor Biotech., Alachua, FL, USA). Every Western blot was repeated at least two times using protein from 2 rats/group. The density of Western blotting bands was measured by MetaMorph 7.5 Videoimage Analysis System (Molecular Devices, Downington, PA), 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 & eosin, and the histochemical trichrome techniques (to evaluate collagen deposition in the granulation tissues). 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, USA) at pH 10.00. An overnight incubation was performed using primary antibodies: human Ki67 (1:100; Chemicon Int., Temecula, CA, USA), human CD34 (1:100; Santa Cruz Biootech., Santa Cruz, CA, USA) and rabbit p27 (1:100; Santa Cruz Biootech., Santa Cruz, CA, USA), secondary antibodies and a peroxidase-labelled streptavidin-biotin and then developed using the DAB substrate detection method for examination under a Nikon microscope. To ensure specificity of the antibody, immunoabsorption of the antibodies was performed to provide controls.

Quantitation of angiogenesis. For the evaluation of neovascularization, pictures were taken from CD34-stained IHC slides under light microscope at ×400 magnification by an imaging system (MetaMorph 7.5, BioImaging Solusions, San Diego, CA, USA). In each slide, 3 areas of maximal MVD, 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 3 areas was calculated according to Mazur (Mazur, et al., 2004).

Double labelling for CD34 and Ki-67. For double labelling, the same antibodies and concentrations were used as described above. Colonic tissue sections were de-paraffinized, hydrated, blocked for endogenous peroxidase using 3% H₂O₂/H₂O, and subsequently subjected to microwave antigen retrieval using a target retrieval solution (Dako, CA) at pH 10.00. After subsequent washing and blocking with protein block (Dako, CA), 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 DAB substrate detection method (brown staining). Subsequently, slides
were then washed and incubated with ant-CD34 antibody at 4°C overnight, washed and incubated with biotinylated secondary antibodies and a peroxidase-labeled streptavidin-biotin and then developed using the AEC substrate detection method (red staining). The tissue sections were counterstained with hematoxylin and examined under a Nikon microscope (Nikon, Tokyo, Japan). 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 0-3: 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 4-well chamber slides (Nunc) and treated with 50 ng/ml bFGF for 2 h. rMVECs were pulsed with 10mM BrdU (Upstate, Millipore, CA) for 60 minutes. The slides were then fixed in methanol for 10 minutes at 4°C and air dried. DNA was denatured with 2N HCl for 1 h followed by neutralization of acid with 0.1M borate buffer (pH8.5). Slides were washed with PBS and incubated with anti-BrdU antibody (MAB3424, Millipore, CA) for 1 h. After washing with PBS, slides were incubated with biotinylated anti-mouse IgG (1:500 dilution, DAKO) for 30 min. The slides were then washed with PBS, incubated with streptavidin/HRP conjugate (DAKO) for 30 minutes and developed with AEC chromogen. Counter staining 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 percent 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, St. Louis, MO, USA) 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 14000 g 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, St. Louis, Missouri, USA) and 0.005% hydrogen peroxide (Sigma, St. Louis, Missouri, USA). MPO activity was derived from an observed change in absorbance measured by spectrophotometry at 450 nm (VersaMax microplate reader, Molecular Devices, East Falmouth, MA, USA) and normalized to the total protein content of the supernatant.

Statistical analysis. Quantitative results are expressed as mean±SE. The statistical significance was determined by the non-parametric Mann-Whitney U-test or Student’s t test where appropriate and p values of <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 (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 bFGF compared to controls (Fig. 1B & C). Colon wet weight was also significantly reduced in group of animal treated with bFGF in dose of 10 µg/rat (Fig. 1D). Histologic examination showed that bFGF accelerated UC healing in a dose-dependent manner (Fig. 1F-H). Rats treated with 10 or 100 µg bFGF had much smaller ulcers and more regenerated mucosa covering the ulcer craters (Fig. 1G & 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 to both saline- (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. 2C, D & 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 to 6 h after IA) and increased during the healing stage of UC (7 and 10 days) (Fig. 3A). Since bFGF regulates matrix proteins such as collagen, 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 (blue color) in granulation tissues of UC treated with bFGF was markedly increased (arrows) at 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 0.01) in colonic granulation tissues of UC at 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 mostly located in the regenerated mucosa around the board of UC treated with 10 µg or 100 µg bFGF (Fig. 4A). Semi-quantitative evaluation showed that cell proliferation was significantly increased in colonic mucosa of UC rats treated with 10 or 100 µg bFGF compared to 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±1.8 vessels/area (control) to 5.7±3.1 (2 µg bFGF), 7.7±1.6 (10 µg, p< 0.05) and 13.3±3.5 (100 µg, 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 when compared to the control (Fig. 6A). The in vitro study further demonstrated that proliferation of rMVECs treated with bFGF was significantly increased (p < 0.05) compared to the control (Fig. 6B).

**Effect of bFGF and anti-bFGF treatment on TNF-α levels and MPO activity in colonic mucosa during experimental UC.** Both TNF-α expression and MPO activity were significantly increased in UC induced by IA (Fig. 7). bFGF treatment significantly decreased the levels of TNF-α in colonic mucosa at a dose-dependent manners (Fig. 7A), whereas treatment with neutralizing antibody significantly increased TNF-α 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 to saline and IgG treatment (p<0.04) (Fig.7D).

**Increased cell cycle inhibitor p27 in UC treated with bFGF.** To examine whether bFGF influences 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 about 9-folds in UC treated with saline compared to the healthy control, whereas bFGF treatment increased p27 mRNA to 2-4-folds in a dose-dependent manner compared to the saline-treated control (Fig. 8A). Immunohistochemistry of p27 showed increased staining of p27 positive cells in colonic mucosa of UC rats treated with bFGF in a dose-dependent manner (Fig. 8B). Semi-quantitative evaluation on the immunohistochemistry demonstrated that p27 positivity was significantly increased in colonic mucosa of UC rats treated with 10 or 100 µg bFGF compared to 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-folds 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). Thus difference is especially important in the light of 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). In order to gain further insights into the beneficial role of bFGF in 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 that bFGF may have both physiologic and pharmacologic role 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 recent 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 levels of bFGF protein were significantly decreased during UC development and progression (1 to 6 h after IA) and increased during the healing stage of UC (7-10 days). Following 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 also increased in the colonic granulation tissues of UC 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). Our and other studies have demonstrated that VEGF plays a pathogenic role in development of UC apparently without much effect on the healing of these colonic lesions (Tolstanova et al., 2009; Scaldaferrri et al. 2009). Although VEGF and bFGF are both angiogenic factors, they seemed to play different roles in UC.
Since 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 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 (Johnston et al., 1989; Gerdes et al., 1984). Interestingly, it was recently reported that exogenous bFGF markedly enhanced crypt stem cell survival in the mouse after radiation injury (Houchen et al., 1999) and that FGF 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 cell cycle inhibitor p27 was significantly decreased in UC compared to the healthy control, while bFGF treatment reversed the decreased levels of p27 by about 50% in the present study. Ioachim (Ioachim et al. 2004) demonstrated that p27 expression was also decreased in both UC and Crohn disease patients compared with healthy controls. Furthermore, a significantly lower p27 expression was found in colorectal carcinomas compared with UC and Crohn 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 non-dysplastic 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, upregulated 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 also found that bFGF markedly decreased TNF-α level and MPO activity in colonic tissues during healing of UC. Matsuura (Matsuura et al., 2005) also showed that gene expression of TNF-α was significantly reduced in DSS-induced UC after treatment with bFGF. TNF-α is one of the most important pro-inflammatory cytokines, and directly influences intestinal epithelial cells. TNF-α disrupts the epithelial barrier, and 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 (Peterson et al., 2007; Naito 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 healing of UC indicate that bFGF plays 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), while TGF-β may inhibit the production of pro-inflammatory cytokines, such as TNF-α and interferon gamma 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 on 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 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 as a new therapy way for UC.

Authorship Contributions

Participated in research design: Szabo, Sandor.

Conducted experiments: Paunovic, Deng, Khomenko, Tolstanova, and Ahluwalia

Contributed new reagents or analytic tools: None.

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

Wrote or contribute to the writing of the manuscript: Paunovic, Deng, Khomenko, Ahluwalia, Szabo and Sandor
References


Ioachim EE, Katsanos KH, Michael MC, Tsianos EV, and Agnantis NJ (2004) Immunohistochemical expression of cyclin D1, cyclin E, p21/waf1 and p27/kip1 in
inflammatory bowel disease: correlation with other cell-cycle-related proteins (Rb, p53, ki-67 and PCNA) and clinicopathological features. Int J Colorectal Dis 19: 325-333.


Kusstatscher S, Sandor Zs, Satoh H, and Szabo S (1994) Inhibition of endogenous basic fibroblast growth factor (bFGF) delays duodenal ulcer healing in rats: implications for a physiologic role of bFGF. Gastroenterology 106: A113.


*Gastroenterology* **106**: 1106-1111.


Footnotes
The present study was supported by a Department of Veterans Affairs, Veterans Health Administration Merit Review grants to Drs. Zsuzsanna Sandor & Sandor Szabo.

Reprints request to: Zsuzsanna Sandor, MD, PhD. VA Medical Center, 5901 East 7th Street, Long Beach, CA 90822. Email: zsuzsanna.sandor@va.gov
Legends of figures

Figure 1. The effect of treatment with bFGF on UC induced by IA in rats. Healing of UC was evaluated by measurements of colonic lesions (A), severity of colitis (B), loss of rugae (C), and colon wet weight (D), and histology (H&E staining) of the rat colonic tissues in IA-induced UC treated with saline (control) (E), bFGF 2 µg/rat (F), bFGF 10 µg/rat (G), bFGF 100 µg/rat (H). IA: iodoacetamide; * p <0.05.

Figure 2. The effect of treatment with neutralizing anti-bFGF antibody on UC induced by IA in rats. Gross appearances of representative colonic lesions in rats after saline, IgG or anti-bFGF antibody (A). Quantitative evaluation of UC healing by measurements of colonic lesions (B). Severity of colitis (C). Loss of rugae (D). Colon wet weight (E). Anti-bFGF: Neutralizing anti-bFGF antibody; * p <0.05. IA: iodoacetamide; UC: ulcerative colitis.

Figure 3. Measurement of bFGF protein and matrix components. Protein expression of bFGF measured by Western blotting in a time-course study of IA-induced UC in rats (A). Levels of collagen measured by trichrome staining in colonic tissue with IA-induced UC 10 days after IA (B). Expression of fibronectin and elastin measured by Western blotting in colonic tissue with IA-induced UC 10 days after IA (C). Ctrl: control; * p < 0.05 and ** p < 0.01. IA: iodoacetamide; UC: ulcerative colitis.

Figure 4. Immunohistochemistry of Ki-67. Levels of positive staining of Ki67 representing cell proliferation in rat colon with IA-induced UC treated with saline or bFGF (A). Semi-quantitative evaluation of Ki-67 immunohistochemistry (B). IA: iodoacetamide. UC: ulcerative colitis.

Figure 5. Immunohistochemistry of vascular marker CD34. Microscopical appearance of CD34-positive staining blood vessels (arrow heads) in granulation tissues of UC (A). Microvascular density determined by counts of CD34-positive staining blood vessels in rat colon with IA-induced UC treated with saline or bFGF (B). * p <0.05; IA: iodoacetamide. UC: ulcerative colitis.
**Figure 6.** Measurement of in vivo and in vitro endothelial cell proliferation after bFGF treatment. Immunohistochemical double labeling of Ki-67 and CD34 on microvascular endothelial cells in colonic mucosa of rat with IA-induced UC treated with saline (control) and bFGF (A). *In vitro* evaluation of bFGF effect on proliferation of microvascular endothelial cells (B). * p < 0.05.

**Figure 7.** Measurement of TNF-α and MPO. Levels of TNF-α (23 kDa) measured by Western blotting (A,B) and MPO activity measured by a specific MPO assay (C,D) in colonic tissues of healthy rats or rats with IA-induced UC treated with saline, bFGF, IgG or anti-bFGF antibody Ab: anti-bFGF antibody; * p <0.05; IA: iodoacetamide; UC: ulcerative colitis.

**Figure 8.** Measurement of cell cycle inhibitor p27. Gene expression of p27 (A). Immunohistochemistry of p27 in colonic tissues of healthy rats or rats treated with saline or bFGF in IA-induced UC (B). Semi-quantitative evaluation of p27 immunohistochemistry (C). **p<0.01, * p <0.05; IA: iodoacetamide. UC: ulcerative colitis.
Figure 1
Figure 3
Figure 4

A  

Ki-67 immunohistochemistry

IA + saline (40X)  
IA + bFGF 2 µg (40X)  
IA + bFGF 10 µg (40X)  
IA + bFGF 100 µg (40X)

B

Ki-67 immunopositivity (scales: 0-3)

Saline  
2  
10  
100 µg

bFGF  
Iodoacetamide

*
Figure 5
A Double labeling of Ki-67 and CD34 on microvascular endothelial cells in colonic mucosa

Control (200x)  
bFGF 10 μg (200x)

B In vitro rat microvascular endothelial cell proliferation

Brd U positive cells (% of total cells in the areas)

0 20 40 60 80 100

Control  
bFGF 50 ng

Figure 6
Figure 7
Figure 8