Keratinocyte growth factor-2 (FGF-10) promotes healing of experimental small intestinal ulceration in rats

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Han, Dong Soo, Fengling Li, Lisa Holt, Kevin Connolly, Melissa Hubert, Renée Miceli, Zebedee Okoye, Gemma Santiago, Kathleen Windle, Eling Wong, and R. Balfour Sartor. Keratinocyte growth factor-2 (FGF-10) promotes healing of experimental small intestinal ulceration in rats. Am J Physiol Gastrointest Liver Physiol 279: G1011–G1022, 2000.—Keratinocyte growth factor-2 (KGF-2, repifermin) is a homolog of KGF-1 with epithelial mitogenic activities. We investigated the therapeutic role of KGF-2 in intestinal ulceration and its mechanisms of protection. KGF-2 (0.3–5 mg/kg) was administered before or after induction of small intestinal ulceration by indomethacin (Indo) in prevention and treatment protocols. In acute studies, KGF-2 was injected for up to 7 days before or daily for 5 days after Indo. In a 15-day chronic study, KGF-2 was injected intravenously daily beginning before or 7 days after Indo. Injury was evaluated by blinded microscopic and macroscopic inflammatory scores, epithelial BrdU staining, tissue IL-1β, PGE2, and hydroxyproline concentrations, and collagen type I RNA expression. In vitro effects of KGF-2 were evaluated by epithelial cellular proliferation, restitution of wounded monolayers, PGE2 secretion, and expression of COX-2 and collagen mRNA. Intravenous KGF-2 significantly decreased acute intestinal injury by all parameters and significantly decreased chronic ulceration. Pretreatment, daily infusion, and delayed treatment were effective. KGF-2 promoted in vitro epithelial restitution with only modest effects on epithelial cell proliferation, stimulated COX-2 expression in cultured epithelial cells, and upregulated in vitro and in vivo PGE2 production. KGF-2 did not affect in vivo fibrosis, although it induced collagen expression in cultured intestinal myofibroblasts. These results suggest that KGF-2 inhibits intestinal inflammation by stimulating epithelial restitution and protective PGs.

immunoregulation; inflammatory bowel disease; cytokines; prostaglandins

THE INTESTINAL EPITHELIUM is composed of a dynamic single layer of epithelial cells that separates the highly concentrated bacterial antigens and toxins in the lumen of the distal intestinal tract from the gut-associated lymphoid tissue (21). Defective mucosal barrier function either caused by an intrinsic defect or the result of a secondary consequence of inflammation can lead to unrestrained luminal antigen uptake, which overwhelms the innate host defense mechanisms of the mucosal immune response (12, 14, 31). Therefore, efficient epithelial repair is an essential component in the resolution of intestinal inflammation.

Growth factors, which are a class of soluble mediators that promote mitogenicity (17), chemotaxis (42), cytokine release (28), and fibrogenesis (4) in a number of cells, are important contributors to tissue repair, remodeling, and fibrosis (4). Keratinocyte growth factor-1 (KGF-1) is the seventh member of the fibroblast growth factor family and is a polypeptide mitogen secreted by fibroblasts and endothelial cells that acts in a paracrine fashion primarily on epithelial cells, which do not express this molecule but bear its receptor (11). KGF-1 stimulates mitogenic and motogenic activity in epithelial cells in vitro. In vivo administration of recombinant KGF-1, which was first isolated from human lung fibroblasts, was shown to induce proliferation of type II pneumocytes (38) and to stimulate proliferation of epithelial cells throughout the gastrointestinal tract and liver (15). The role of KGF-1 in intestinal inflammation is unclear, but increased expression of KGF-1 mRNA has been reported in the mucosa of patients with inflammatory bowel diseases (IBD) (2, 10) and in a T lymphocyte-mediated model of in vitro crypt hyperplasia (3). In this in vitro model, KGF-1 expression by mesenchymal cells was upregulated by tumor necrosis factor-α (TNF-α), and neutralization of endogenous KGF-1 partially inhibited T cell-mediated crypt hyperplasia (3). Exogenous injection of recombinant KGF-1 into rats significantly reduced the severity and extent of mucosal injury induced by trinitrobenzene sulfonic acid (TNBS) (45). Although intra-peritoneal injection of KGF-1 promoted epithelial proliferation in normal rats and potentiated healing of TNBS-induced colitis, it is uncertain whether the protective effect of KGF-1 is a result of its mitogenic properties or is due to an anti-inflammatory effect.
The recently cloned KGF-2 (fibroblast growth factor-10, repifermin) (5) was found to have homology with KGF-1 and to bind to the same fibroblast growth factor receptor IIb on epithelial cells (16). However, KGF-2 also binds to an additional receptor, fibroblast growth factor receptor IIIb (23). Fibroblast growth factor-10 knockout mice demonstrate that this molecule functions as a regulator of embryonic brain, lung, and limb development (34). The role of KGF-2 in the gastrointestinal tract is not well studied, although it attenuated colitis induced by dextran sulfate sodium in mice in a recent study (25). In the present study, we investigated the protective and therapeutic effects of exogenous KGF-2 on indomethacin-induced small intestinal ulceration in rats. Furthermore, we explored the effects of KGF-2 on in vivo and in vitro wound healing, epithelial proliferation, and prostaglandin production to determine its mechanisms of protection and quantified fibrosis to explore potential detrimental effects of this molecule.

MATERIALS AND METHODS

Animals

Specific pathogen-free, inbred female Lewis rats (180–200 g) were obtained from Charles River Laboratories (Raleigh, NC). Rats were fed standard rat chow ad libitum and were weighed daily. All rat experiments were conducted according to the highest standards of humane animal care as outlined in the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85–23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20892] and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Experimental Design

Prevention of acute indomethacin-induced small intestinal ulceration by KGF-2. Indomethacin (Sigma Chemical, St. Louis, MO) was dissolved in absolute ethanol (50 mg/ml) by intermittent vortexing for 1 h at room temperature, then diluted 1:4 (vol/vol) in 5% sodium bicarbonate to produce a stock solution of 10 mg/ml. A final dilution of 7.5 mg indomethacin/kg body wt in 0.3 ml of sodium bicarbonate was administered subcutaneously once daily for 2 days. Negative control rats received 0.3 ml of vehicle containing a mixture of 80% sodium bicarbonate and 20% ethanol. Recombinant KGF-2 was supplied by Human Genome Sciences (Rockville, MD). KGF-2 (1 or 5 mg/kg) was administered intravenously or subcutaneously for 6 days beginning 1 day before indomethacin injection. Animals were divided into five groups: 1) 1 mg/kg iv KGF-2 (n = 7); 2) 1 mg/kg sc KGF-2 (n = 7); 3) 5 mg/kg sc KGF-2 (n = 7); 4) 5 mg/kg iv human serum albumin (HSA) (n = 7) as a positive control (all rats receiving indomethacin subcutaneously); and a negative control group receiving vehicle subcutaneously and HSA (5 mg/kg) intravenously (n = 5).

In a separate dose-ranging study, rats received intravenous KGF-2 (0.3, 1.0, or 3.0 mg/kg) for 6 days beginning 1 day before subcutaneous indomethacin (n = 12/group). Positive control rats received indomethacin subcutaneously and HSA (1 mg/kg) intravenously, and negative controls received vehicle subcutaneously and HSA intravenously.

To explore the ability of KGF-2 to prevent subsequent intestinal injury, KGF-2 (0.3, 1.0, or 3.0 mg/kg) was administered intravenously on days −3, −2, and −1 before subcutaneous indomethacin (n = 12/group). In a separate study, KGF-2 (1.0 mg/kg) was injected intravenously 1–7 days before subcutaneous indomethacin (12 rats/group). In both protocols, rats were killed 4 days after indomethacin challenge.

Prevention and treatment of chronic indomethacin-induced intestinal ulceration by KGF-2. Indomethacin (7.5 mg/kg) was delivered by the same schedule as described in Prevention of acute indomethacin-induced small intestinal ulceration by KGF-2, and KGF-2 (0.3 or 1 mg/kg) was delivered once daily by intravenous injection in the tail vein beginning 1 day before indomethacin for 14 days after the first indomethacin injection (prevention protocol). In a treatment protocol, KGF-2 (1 mg/kg) was delivered intravenously daily beginning 7 days (7 rats) after initiation of indomethacin treatment and continued for 14 days. Positive control rats received HSA (1 mg/kg iv) for 15 days, beginning 1 day before indomethacin (n = 7); negative controls received HSA (1 mg/kg) beginning 1 day before buffer injection (n = 5).

Clinical Assessment of Inflammation

Clinical manifestations of inflammation were assessed by monitoring the body weight of animals daily. Rats were euthanized by inhalation of 100% CO₂. Cardiac blood was obtained for cell counts, which were processed using an automated cell counter (CDC Technology, Oxford, CT). Liver, spleen, and intestine weights were recorded and normalized for the body weight of each rat. Gross intestinal inflammatory scores were quantified by blinded observers at the time of necropsy using a previously validated scale (32). Values of 0–4 (4 as the most severe) were assigned based on the severity of mesenteric contraction, the severity of adhesions, and the extent of intestinal wall thickening. The resulting gross gut score is the sum of these values, the maximum possible being 12. Also, the percentage of the surface area covered by ulceration and the number of ulcers in the most involved 10-cm length of small intestine were determined; this segment of involved intestine was weighed after gently removing the luminal contents. Samples of intestine, liver, and spleen were fixed in 10% formalin, embedded in paraffin, and sectioned for histochemical staining with hematoxylin and eosin, Masson trichrome, and Alcian blue stains. A histological inflammatory score was evaluated for each animal by a blinded observer as previously described and validated (24). In brief, values from 0 to 4 (4 as the most severe) were assigned for both acute and chronic inflammation in coded multiple cross sections of the mid small intestine. The acute and chronic scores of at least three histological segments were averaged, a score of 8 (4 acute, 4 chronic) representing the maximum possible combined total histological inflammatory score. The percentage of surface area covered by microscopic ulceration was also measured in at least three sections of small intestine from each rat. Immunologic assessment of inflammation was performed by determining the intestinal concentration of immunoreactive rat interleukin-1β (IL-1β) using an ELISA as previously described (29). The supernatant was frozen at −70°C until being assayed.

5-Bromo-2′-Deoxyuridine Staining for Assessment of Proliferating Cells

Rats in the chronic indomethacin-induced colitis experiment were injected intraperitoneally with 5-bromo-2′-deoxyuridine (BrdU; 50 mg/kg; Sigma Chemical) 1 h before necropsy. Paraffin-embedded sections of 8-μm thickness
were deparaffinized and pretreated with 2 N hydroxychloride and pepsin (Sigma Chemical). Endogenous peroxidase was quenched with 1% H2O2. Slides were blocked with 1% normal horse serum in PBS with 0.1% BSA and then incubated with anti-BrdU (DAKO, Carpinteria, CA) in a 1:100 dilution. After being washed with PBS, sections were incubated with biotinylated antimouse IgG (Vector Laboratories, Burlingame, CA) and then with a peroxidase-linked avidin-biotin complex (Vector Laboratories) diluted 1:100 in PBS with 0.1% BSA. Slides were exposed to 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical) substrate for 3 min. Sections were counterstained with hematoxylin. The total number of proliferating cells per crypt was counted at a magnification of ×100 in 10 crypts by two independent investigators blinded to treatment.

Cell Culture

Nontransformed rat intestinal epithelial cells IEC-6 (ATCC CRL 1592) used between passages 8 and 18 were cultured with DMEM (GIBCO BRL, Long Island, NY) with 5% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine, antibiotics, and insulin (4 U/ml). Caco-2 epithelial cells (ATCC HBT-37) were also used. Caco-2 cells were cultured in MEM (GIBCO BRL) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1% nonessential amino acids, and antibiotics. HT-29 epithelial cells (ATCC HTB-38) were cultured in DMEM (GIBCO BRL) with 10% FBS and antibiotics. Rat subepithelial intestinal myofibroblasts, passages 3–10, isolated from the colon of neonatal Lewis rats (19,41) were cultured in Dulbecco's MEM/F-12 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics.

RNA Preparation and RT-PCR

Intestinal tissues were snap-frozen in isopentane and kept at −80°C for later mRNA and protein analysis. Total tissue RNA was prepared using a standard method with TRIzol (GIBCO BRL). Total RNA was quantified by a spectrophotometer (A260/A280). The integrity and quality of each RNA sample was checked by electrophoresis on a 1% agarose gel containing ethidium bromide. RNA was reverse transcribed using 1 μg of total RNA, 15 U of RNA inhibitors, 1× first-strand buffer (Life Technologies, Long Island, NY), 5 mM dNTP (Pharmacia, Uppsala, Sweden), 125 pmoL random hexamer primers (Pharmacia), and 125 U of Moloney murine leukemia virus RT (Life Technologies) in a final volume of 25 μl. The reaction was carried out for 1 h at 39°C followed by 7 min at 93°C and 1 min at 24°C and then slowly cooled to 4°C for 20 min. PCR was carried out in a volume of 50 μl containing 5 μl of RT mixture, 1× Thermos aquaticus (Taq) buffer, 5 pmoL of each primer, 2.5 mM dNTP, and 1 U of Taq polymerase. The primers used for human cyclooxygenase-2 (COX-2) and actin and rat COX-2 and IL-1β were previously described (18,19,29). PCR was carried out in a Perkin-Elmer 9600 cycler set for various cycles to assess linearity of the amplification.

Northern Blot Analysis

Total RNA (5 μg/ lane) was electrophoresed through 1% agarose/formaldehyde gels. The size-fractioned RNA was transferred to a nylon membrane (Hybond-N, Amersham) for 2–3 h at 65°C followed by washing under high-stringency conditions to reduce background using variable (0.1–2×) concentrations of saline-sodium phosphate-EDTA buffer with 0.1% SDS. The membranes were exposed to Kodak-X-OMAT film (Rochester, NY) at −80°C using an intensifier screen.

Hydroxyproline Content of Rat Small Intestine

To determine whether administration of KGF-2 (1 mg/kg iv, days 0–7) altered the collagen content of intestines from normal or indomethacin-treated rats, tissue hydroxyproline content was measured using modifications of the procedure of Deterting et al. (8). Briefly, 10 cm of normal or ulcerated rat mid small intestine were lyophilized, and the dry weight was recorded. The dry samples were minced and hydrolyzed with 6 N HCl for 18 h at 120°C. Oxidation of the samples at room temperature was made with chloramine T solution (44). Ehrlich's reagent was used to develop the color reaction of 30 min at 65°C (44). The reaction was read at 560 nm, and the amount of hydroxyproline was determined from the hydroxyproline standards (Sigma Chemical).

Western Blot Analysis for COX-2 Concentrations

For analysis of COX-2 expression, Caco-2 cells (5 x 105/well) were stimulated with various concentrations of KGF-2 (1.0–100 ng/ml), medium alone, TNF-α (2 ng/ml), or IL-1β (2 ng/ml) for 24 h. After removal of media, cells were lysed in 1× Laemmli buffer. The protein concentration was determined using a protein assay kit (Bio-Rad, Richmond, CA). Samples (20 μg of protein) were electrophoresed on 10% SDS-polyacrylamide gels overlayed with 4% of acrylamide stacking gel. The proteins were transferred to Hybond-C nitrocellulose paper (Amersham) using a mini-Tran-Blot (Bio-Rad) according to the manufacturer's directions. Transfers were carried out for 1 h at 4°C with 0.200 mA of current. Nitrocellulose blots were blocked with a solution of 10% (wt/vol) nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 30 min at room temperature. Anti-COX-2 antibody (N20; Santa Cruz Biotechnology, Santa Cruz, CA) was added at a dilution of 1:1,000, and blots were incubated for 1 h as previously described (18). Blots were then rapidly washed three times in TBS-T followed by two 15-min washes in TBS-T. Blots were incubated for 45 min in 5% milk containing anti-rabbit IgG conjugated with horseradish peroxidase at a dilution of 1:1,000. Immunoreactive bands were visualized using chemiluminescence agents (Renaissance kit; NEN Research Products, Boston, MA) and recorded on film (Kodak scientific imaging film).

Cell Proliferation Assay

Cells (IEC-6 or Caco-2) were seeded into 24-well plates (5 x 104 cells/well) in the presence of DMEM containing 5% FBS. When ~50% confluent, cells were washed three times and then cultured for an additional 24 h in DMEM containing 0.1% FBS. Cultures were then supplemented with KGF-2 in concentrations ranging from 0.1 to 100 ng/ml. After 20 h at 37°C, 1 μCi/well of [3H]thymidine was added; after 4 h, cells were washed with cold PBS three times and precipitated with 10% TCA; acid-insoluble materials were lysed with 0.1 N NaOH. Incorporation of radio labeled thymidine was determined by a liquid scintillation counter. [3H]thymidine incorporation in KGF-2-related cultures was expressed as a percentage of [3H]thymidine incorporation in control cultures.
Continuous KGF-2 Treatment Reduces Acute Indomethacin-Induced Injury

In an experiment investigating activities of KGF-2 administered intravenously or subcutaneously, indomethacin-injected rats developed progressive weight loss after 2 days that was almost completely prevented by daily intravenous injection of KGF-2 beginning 1 day before indomethacin administration and continuing for 5 days (Fig. 1). There were significant differences in weight loss between the indomethacin plus HSA group and the groups treated with intravenous KGF-2 or high-dose subcutaneous KGF-2 (5 mg/kg) from day 3 to day 5. Low-dose subcutaneous KGF-2 (1 mg/kg) did not prevent weight loss. The extent of mucosal ulceration in the most affected 10-cm segment of mid small bowel, as determined by the percentage of surface area ulcerated, was markedly reduced in rats treated with intravenous KGF-2, compared with indomethacin-injected rats treated with HSA or subcutaneous KGF-2 (Table 1). The number of ulcers was also decreased in rats receiving intravenous KGF-2 compared with those receiving indomethacin plus HSA (3.7 ± 1.1 vs. 8.7 ± 1.7, respectively; P < 0.01). There were highly significant differences in intestinal adhesions (P = 0.019), mesenteric contractions (P = 0.0003), intestinal thickness (P = 0.00002), and the total gross gut score (P = 0.0003) between the indomethacin plus HSA-treated group and rats treated with 1 mg/kg iv KGF-2. Subcutaneous injection of KGF-2 was less effective in preventing grossly evident intestinal injury, although the average wet weights and percent ulceration of surface area of the most affected 10-cm length of small intestine in all treated groups were significantly decreased compared with positive controls. IL-1β concentration in tissue homogenates was significantly reduced in the 1 mg/kg iv KGF group (Table 1). In addition, hemoglobin levels were decreased in the indomethacin plus HSA-treated control group, but anemia was attenuated with intravenous KGF-2 administration. Subcutaneous injection of KGF-2 or high-dose subcutaneous KGF-2 (5 mg/kg) did not prevent weight loss. The extent of mucosal ulceration in the most affected 10-cm segment of mid small bowel, as determined by the percentage of surface area ulcerated, was markedly reduced in rats treated with intravenous KGF-2, compared with indomethacin-injected rats treated with HSA or subcutaneous KGF-2 (Table 1). The number of ulcers was also decreased in rats receiving intravenous KGF-2 compared with those receiving indomethacin plus HSA (3.7 ± 1.1 vs. 8.7 ± 1.7, respectively; P < 0.01). There were highly significant differences in intestinal adhesions (P = 0.019), mesenteric contractions (P = 0.0003), intestinal thickness (P = 0.00002), and the total gross gut score (P = 0.0003) between the indomethacin plus HSA-treated group and rats treated with 1 mg/kg iv KGF-2. Subcutaneous injection of KGF-2 was less effective in preventing grossly evident intestinal injury, although the average wet weights and percent ulceration of surface area of the most affected 10-cm length of small intestine in all treated groups were significantly decreased compared with positive controls. IL-1β concentration in tissue homogenates was significantly reduced in the 1 mg/kg iv KGF group (Table 1). In addition, hemoglobin levels were decreased in the indomethacin plus HSA-treated control group, but anemia was attenuated with intravenous KGF-2 administration. Subcutaneous injection of

Table 1. Reduction of acute Indo-induced small intestinal injury and systemic manifestations by continuously administered KGF-2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gross Gut Score</th>
<th>% Ulceration</th>
<th>Intestinal Weight, g</th>
<th>IL-1β/Tissue Weight, ng/mg</th>
<th>Liver/Body Weight, mg/g</th>
<th>Hemoglobin, g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle + HSA</td>
<td>0±</td>
<td>0±</td>
<td>0.6 ± 0.03*</td>
<td>2.2 ± 0.7*</td>
<td>39 ± 2*</td>
<td>13.5 ± 0.39*</td>
</tr>
<tr>
<td>Indo + HSA</td>
<td>7.9±</td>
<td>24.4±</td>
<td>21.0±</td>
<td>29.1±</td>
<td>49±</td>
<td>10.2±</td>
</tr>
<tr>
<td>Indo + KGF-2 (1 mg/kg iv)</td>
<td>1.5±</td>
<td>2.9±</td>
<td>0.9±</td>
<td>18.0±</td>
<td>51±</td>
<td>11.9±</td>
</tr>
<tr>
<td>Indo + KGF-2 (1 mg/kg sc)</td>
<td>4.2±</td>
<td>8.8±</td>
<td>1.3±</td>
<td>25.8±</td>
<td>47±</td>
<td>10.3±</td>
</tr>
<tr>
<td>Indo + KGF-2 (5 mg/kg sc)</td>
<td>5.4±</td>
<td>9.9±</td>
<td>1.5±</td>
<td>24.4±</td>
<td>48±</td>
<td>9.9±</td>
</tr>
</tbody>
</table>

Values are means ± SE. Female Lewis rats (n = 7/group) were treated daily with varying doses of keratinocyte growth factor-2 (KGF-2) or human serum albumin (HSA) administered subcutaneously or intravenously beginning 1 day before subcutaneous indomethacin (Indo; 7.5 mg/kg) injected on 2 sequential days. Rats were killed 5 days after Indo injection (6 days after 1st KGF-2 injection) and blinded assessment of grossly evident mid small intestinal inflammation, liver weights, and cardiac blood hemoglobin concentrations. The maximally involved 10-cm segment of mid small intestine was weighed and blindly assessed for the % surface area ulcerated. IL-1β, interleukin-1β. *P < 0.001, †P < 0.01, §P < 0.05 vs. Indo + HSA.
KGF-2 did not significantly improve hemoglobin levels or reduce tissue IL-1β concentrations.

Microscopic interpretation by a blinded observer showed marked reduction of acute and chronic histological inflammatory scores in the intravenous KGF-2-treated group compared with the indomethacin plus HSA group (Fig. 2). Subcutaneous KGF-2 (1 mg/kg) reduced acute and total inflammatory scores but did not significantly inhibit chronic inflammation, and the 5 mg/kg sc dose had no beneficial effect. Similar to the macroscopic observations, KGF-2 at the 1 mg/kg iv (1.0% of surface area) and 1 mg/kg sc doses (1.6% of surface area) decreased the extent of microscopic mucosal ulceration compared with the indomethacin plus HSA positive control group (8.9%) (P = 0.001 and 0.002, respectively). Mucosal ulcers in indomethacin plus HSA-treated rats (Fig. 3A) were active with exudate and necrosis. In some KGF-2-treated rats, ulcers were beginning to heal by the time of necropsy (5 days after beginning indomethacin) as shown by reepithelialization of the ulcer base and margins (Fig. 3, B and C).

**Dose-Dependent Effects of Continuous KGF-2 in Reducing Acute Intestinal Ulceration**

In a separate experiment to determine the optimal dose of intravenous KGF-2, rats were pretreated intravenously with KGF-2 at 0.3, 1, or 3 mg·kg⁻¹·day⁻¹ beginning 1 day before indomethacin injection and continuing for 6 days. The most effective dose for reducing intestinal ulceration and inflammation was 1 mg·kg⁻¹·day⁻¹ KGF-2 (Table 2). This intermediate dose significantly and consistently attenuated percent mid small bowel ulceration, gross gut score, intestinal weight, body weight loss, and anemia. Interestingly, the high dose (3 mg·kg⁻¹·day⁻¹) was the least effective, whereas the low dose of KGF-2 (0.3 mg·kg⁻¹·day⁻¹) exhibited an intermediate effect (Table 2). These results established an optimal dose of KGF-2 in this model and demonstrated consistent reduction of acute inflammation in the small intestine with 1 mg·kg⁻¹·day⁻¹ iv KGF-2 in two separate studies.

**Pretreatment with KGF-2 Prevents Acute Intestinal Injury**

Because daily administration of KGF-2 in the protocol discussed in *Dose-Dependent Effects of Continuous KGF-2 in Reducing Acute Intestinal Ulceration* could manifest its protective effects by treating the early phase of indomethacin-induced intestinal injury as well as preventing the onset of intestinal ulceration, we next determined whether pretreatment alone with KGF-2 could prevent acute intestinal ulceration and weight loss. In a purely prophylactic study, we demonstrated that KGF-2 injected intravenously daily for 3 days before indomethacin decreased all parameters of intestinal injury and systemic weight loss in a dose-dependent fashion (Table 3). Of interest, 3 mg/kg KGF-2 was more effective than 1 mg/kg in this prevention protocol, in contrast to the continuous treatment study depicted in Table 2.

In a second purely prophylactic experiment, we sought to determine whether a single injection of KGF-2 could prevent indomethacin-induced injury and, if so, to identify the duration of this protective effect. A single injection of KGF-2 (1 mg/kg iv) significantly attenuated acute mid small bowel ulceration and inflammation 1, 3, or 5 days, but not 7 days, before indomethacin administration (Table 4). No significant improvement in weight loss was noted after pretreatment for longer than 3 days. Together, these studies demonstrate that short-term prophylactic administra-
Table 2. Dose-dependent reduction of acute Indo-induced small intestinal injury and systemic manifestations by daily continuous KGF-2 administration

<table>
<thead>
<tr>
<th>Group</th>
<th>% Ulceration</th>
<th>Gross Gut Score</th>
<th>Intestinal Weight, g</th>
<th>% Body Weight Change</th>
<th>% Hematocrit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle + HSA</td>
<td>0</td>
<td>0</td>
<td>0.6 ± 0.1</td>
<td>7.4 ± 1.3</td>
<td>44.5 ± 0.3</td>
</tr>
<tr>
<td>Indo + HSA</td>
<td>9.7 ± 2.5</td>
<td>7.6 ± 1</td>
<td>1.2 ± 0.2</td>
<td>-21.3 ± 2.3</td>
<td>27.7 ± 16</td>
</tr>
<tr>
<td>KGF-2 (0.3 mg/kg iv)</td>
<td>3.7 ± 1.0*</td>
<td>3.6 ± 0.6†</td>
<td>0.9 ± 0.1*</td>
<td>-5.7 ± 1.1‡</td>
<td>34.5 ± 12.2†</td>
</tr>
<tr>
<td>KGF-2 (1 mg/kg iv)</td>
<td>2.5 ± 0.6*</td>
<td>2.9 ± 0.5†</td>
<td>0.9 ± 0.1*</td>
<td>-8.9 ± 2.1*</td>
<td>36.3 ± 16*</td>
</tr>
<tr>
<td>KGF-2 (3 mg/kg iv)</td>
<td>6.3 ± 2.3</td>
<td>5.0 ± 0.7*</td>
<td>1.1 ± 0.1</td>
<td>-10.6 ± 2.1*</td>
<td>31.3 ± 12</td>
</tr>
</tbody>
</table>

Values are means ± SE. Female Lewis rats (n = 12/group) were treated daily with KGF-2 (0.3–3 mg/kg iv) beginning 1 day before subcutaneous Indo injection on 2 sequential days. Rats were killed 5 days after the first Indo injection (6 days after initial KGF-2 injection) and blindly assessed for mid small intestinal and systemic inflammation as outlined in Table 1 legend. % Body weight change was measured at day 5. *P < 0.05, †P < 0.01, ‡P < 0.001 vs. Indo + HSA.

Table 3. Prophylactic effect of KGF-2 pretreatment on Indo-induced small intestinal inflammation

<table>
<thead>
<tr>
<th>Group</th>
<th>% Ulceration</th>
<th>Gross Gut Score</th>
<th>Intestinal Weight, g</th>
<th>% Body Weight Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0*</td>
<td>0</td>
<td>0.8 ± 0.1*</td>
<td>+5.6 ± 1.0*</td>
</tr>
<tr>
<td>Indo + KGF-2</td>
<td>16.9 ± 4.2</td>
<td>5.5 ± 1.2</td>
<td>1.4 ± 0.1</td>
<td>-7.4 ± 1.5</td>
</tr>
<tr>
<td>Indo + KGF-2 (0.3 mg/kg)</td>
<td>5.5 ± 1.9†</td>
<td>2.2 ± 0.8‡</td>
<td>1.0 ± 0.1‡</td>
<td>-0.1 ± 3.1†</td>
</tr>
<tr>
<td>Indo + KGF-2 (1 mg/kg)</td>
<td>3.1 ± 2.1‡</td>
<td>0.5 ± 0.3*</td>
<td>0.9 ± 0.1*</td>
<td>+1.6 ± 1.5‡</td>
</tr>
<tr>
<td>Indo + KGF-2 (3 mg/kg)</td>
<td>0.6 ± 0.6ª</td>
<td>0.4 ± 0.4</td>
<td>0.9 ± 0.1‡</td>
<td>+1.8 ± 1.0ª</td>
</tr>
</tbody>
</table>

Values are means ± SE. Indo-treated female Lewis rats (n = 12/group) were dosed with KGF-2 (0.3, 1, and 3 mg/kg iv) on days −3, −2, and −1. Indo was injected at a dose of 8 mg/kg sc on days 0 and 1. Animals were weighed on days 0 and 4, and the change in body weight was calculated. On day 4 of the experiment, animals were euthanized, gross intestinal pathology was blindly evaluated, and a 10-cm section of the intestine was taken for ulcer assessment and weight. *P < 0.001, †P < 0.05, ‡P < 0.01 treated groups vs. Indo control.
Moreover, the single layer of cells reepithelializing ulcers (Fig. 3C) did not exhibit BrdU staining. We demonstrated little or no evidence of KGF-2-induced gastrointestinal mucosal proliferation in normal (noninflamed) rats. A single injection of KGF-2 (1 mg/kg iv) followed by BrdU injection 24 h later failed to show enhanced proliferation of enterocytes. However, periodic acid-Schiff staining did show an increase in goblet cell number. In a second, long-term study KGF-2 (0.01, 0.1, and 1 mg/kg) was injected intravenously daily into normal rats for 28 days. Histopathological analysis demonstrated no microscopic changes in the intestinal mucosa with no demonstrable epithelial hyperplasia. To quantitate the ability of KGF-2 to induce goblet cell hyperplasia, six normal rats were injected with KGF-2 (100 ng/ml) for 6 days. Image analysis demonstrated a significant (P < 0.05) increase in the percentage of the epithelial surface area occupied by goblet cells in the ileum (17.7 ± 2.0 vs. 7.0 ± 0.5 for control) and colon (27.4 ± 2.7 vs. 18.9 ± 0.5 for control).

In vitro studies provided evidence of a modest proliferative effect of KGF-2 in some, but not all, colonic cell lines. Addition of KGF-2 (1–100 ng/ml) to serum-deprived medium slightly enhanced growth of Caco-2 cells in a dose-dependent manner as determined by [3H]thymidine incorporation (Fig. 5). KGF-2 (100 ng/ml) increased cellular proliferation in Caco-2 cells by twofold. A slight proliferation was noted in human HT-29 epithelial cells in response to KGF-2, with no effect on rat IEC-6 cells (Fig. 5).

KGF-2 Promotes Caco-2 Monolayer Restitution

To investigate the effect of KGF-2 on migration of cells after in vitro “wounding,” Caco-2 cells were grown to confluence and then changed to serum-deprived conditions. The width of the acellular region was measured at various time intervals after creation of linear wounds, and data were calculated as the percent change over baseline values. As demonstrated in Fig. 6, KGF-2 significantly enhanced wound healing by cellular migration in a dose- and time-dependent fashion. The effect of KGF-2 (10 ng/ml) was equivalent to that of TGF-β1 (2 ng/ml) and 10% FBS in this assay (Fig. 6).

**Table 5. Prevention and treatment of chronic Indo-induced small intestinal injury by continuously administered KGF-2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Gross Gut Score</th>
<th>% Ulceration</th>
<th>Intestinal Weight, g</th>
<th>Liver Weight, mg/g body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle + HSA</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0.6 ± 0.1</td>
<td>38.0 ± 0.8</td>
</tr>
<tr>
<td>Indo + HSA</td>
<td>7</td>
<td>6.2 ± 1.5</td>
<td>8.3 ± 2.4</td>
<td>1.4 ± 0.2</td>
<td>39.7 ± 0.8</td>
</tr>
<tr>
<td>Indo + KGF-2 (0.3 mg iv)</td>
<td>7</td>
<td>4.2 ± 1.9</td>
<td>5.0 ± 2.1</td>
<td>1.4 ± 0.3</td>
<td>49.4 ± 1.5*</td>
</tr>
<tr>
<td>Indo + KGF-2 (1 mg iv)</td>
<td>8</td>
<td>3.9 ± 1.4</td>
<td>2.5 ± 1.0*</td>
<td>1.5 ± 0.3</td>
<td>52.9 ± 1.5*</td>
</tr>
<tr>
<td>Indo + delayed KGF-2 (1 mg iv)</td>
<td>7</td>
<td>3.4 ± 0.8*</td>
<td>1.6 ± 0.3*</td>
<td>1.4 ± 0.1</td>
<td>47.0 ± 0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Female Lewis rats were treated daily with intravenous KGF-2 beginning 1 day before or 7 days after (delayed) subcutaneous 7.5 mg/kg Indo for 2 days. Rats were killed 14 days after the first Indo injection and blindly assessed for mid small bowel inflammation. *P < 0.05 vs. Indo + HSA.
KGF-2 increases expression and activity of COX

KGF-2 increased expression of COX-2 mRNA in Caco-2 cells in a dose-dependent fashion (Fig. 7A). Also, Western blotting showed a dose-dependent increase of COX-2 protein after incubation with KGF-2 (Fig. 7B). PGE₂ levels in supernatants of cultured epithelial cells were also increased by KGF-2 (100 ng/ml) to levels comparable with TNF-α stimulation (Fig. 7C).

Consistent with the ability of KGF-2 to upregulate prostaglandins in vitro, tissue PGE₂ levels in the small intestine of rats treated with KGF-2 (1 mg/kg iv) were twofold higher compared with the indomethacin plus HSA injection group (Fig. 8A, 1,338 ± 281 vs. 696 ± 145 pg/mg tissue wt; P = 0.04) in the acute continuous treatment study, despite significantly lower inflammatory scores (Fig. 2) and IL-1β tissue concentrations (Fig. 8B) in the KGF-2-treated rats. The ratio of tissue PGE₂ to IL-1β was >10-fold higher in the KGF-2 (1 mg/kg iv)-treated group compared with the indomethacin plus HSA control group (0.27 ± 0.17 vs. 0.02 ± 0.003, P = 0.08) (Fig. 8C). The PGE₂-to-IL-1β ratio in the vehicle plus HSA control group was quite high (2.76 ± 1.27) because of the very low tissue IL-1β concentrations. This ratio of protective to proinflammatory molecules correlated well with tissue injury as manifested by the gross gut score (r = 0.94, P < 0.03). In keeping with these results, the ratio of COX-2 to IL-1β mRNA expression in mid small intestinal tissues was slightly higher in KGF-2-treated rats (3.00 ± 0.95 in indomethacin plus KGF-2-injected rats vs. 2.39 ± 0.47 in the indomethacin plus HSA controls).

KGF-2 Promotes Collagen Type I Synthesis in Cultured Intestinal Myofibroblasts Without Stimulating In Vivo Fibrosis

To investigate whether KGF-2 promotes collagen matrix synthesis, a rat intestinal myofibroblast cell line (41) was stimulated with various concentrations of KGF-2 for 24 h. There was a dose-dependent increase in collagen type I mRNA expression in KGF-2-treated cells compared with control cells and TGF-β-stimulated cells (Fig. 9A). However, when collagen type I mRNA expression was compared in the intestines of
rats treated in vivo with KGF-2, there was no evidence of stimulation of collagen synthesis in areas of mucosal inflammation. Intravenous injection of KGF-2 (1 mg/kg) for 14 days did not increase mid small intestinal collagen type I mRNA synthesis compared with the indomethacin plus HSA group (ratio of collagen type I to actin: 0.61 HSA vs. 0.47 KGF-2) (Fig. 9B). The depth of submucosal collagen deposits measured in Masson trichrome-stained tissues was not significantly different in the KGF-2-injected group compared with the indomethacin plus HSA group (data not shown), fur-

**DISCUSSION**

This study shows that exogenous KGF-2 administration improved wound healing in acute and chronic small intestinal ulceration in rats and suggests that these effects are mediated by enhanced epithelial migration as well as upregulation of protective prostaglandins. KGF-2 had beneficial effects on restoring weight loss, improving macroscopic and microscopic small intestinal inflammation and ulceration, decreasing anemia, and lowering tissue IL-1β levels in an acute, continuous administration protocol. Continuous treatment with KGF-2 (1 mg/kg) was also effective in attenuating chronic intestinal ulceration and, in contrast to KGF-1 in the TNBS model (45), KGF-2 exhibited therapeutic activity by reversing established small intestinal ulceration in a 7-day delayed-treatment protocol. Of interest, prophylactic administration of KGF-2 for up to 5 days before indomethacin prevented
intestinal injury. Optimal doses of KGF-2 were 1 mg·kg\(^{-1}\)·day\(^{-1}\), with higher doses being less effective, consistent with the bell-shaped dose-response curve of several biological molecules, including recombinant IL-10 (33) and anti-TNF-\(\alpha\) monoclonal antibody (36). Intravenous administration was more effective than subcutaneous delivery, possibly because of differences in pharmacokinetics between the two routes of administration.

The role of endogenous KGF-2 in intestinal inflammation is still unknown, but our results suggest a potential therapeutic activity of this growth factor with several observed mechanisms of action that appear to be largely independent of cellular proliferation. In our experiments, high concentrations of KGF-2 (100 ng/ml) had only a modest effect on in vitro epithelial proliferation. Chronic in vivo administration of KGF-2 normalized epithelial proliferation in the antimesenteric region of the small intestine but had almost no proliferative effect in areas of active ulceration, as measured by BrdU incorporation. Furthermore, the regions of reepithelialization of ulcer bases showed no cell division. Previous studies (39) have shown that indomethacin induces transmural ulceration on the mesenteric border of the mid small intestine and decreases epithelial cell proliferation with certain dose ranges in rats. These findings suggest that KGF-2 stimulates epithelial healing by a mechanism independent of cellular proliferation.

Mucosal restitution occurs by rapid migration of viable epithelial cells from adjacent areas to cover the damaged area without proliferation (4, 6, 43). Subsequent restoration of the normal epithelial architecture is mediated by stem cell proliferation, forming regenerative crypts, with crypt-to-surface cell migration and differentiation (4, 6, 43). Our in vitro results demonstrate that KGF-2 promotes cellular migration in a dose-dependent fashion. Many growth factors, including TGF-\(\alpha\), TGF-\(\beta\), acidic or basic fibroblast growth factor, hepatocyte growth factor, and intestinal trefoil peptides, promote epithelial restitution (9), with TGF-\(\beta\) playing a central role in all but trefoil peptide-stimulated repair (4). A dose of 1 ng/ml of KGF-2 was equivalent to 2 ng/ml of TGF-\(\beta\) in the epithelial cell migration in vitro assay. The lack of BrdU staining in the single layer of cells reepithelializing ulcer bases further supports epithelial migration as the major mechanism of initial ulcer healing and provides in vivo confirmation of the observation that growth factor-induced cell migration is independent of cell proliferation (1). Promotion of epithelial restitution by KGF-2 with subsequent ulcer healing would prevent further influx of bacteria, toxins, and antigens from the gut lumen. A number of rodent models demonstrate that normal luminal bacteria and bacterial products are essential for chronic intestinal inflammation (31). Decreased stimulation of lamina propria macrophages by luminal bacterial cell wall polymers due to ulcer healing may account for the observed decrease in IL-1\(\beta\) tissue concentrations, despite the lack of a direct effect of KGF-2 on cytokine-stimulated epithelial cell nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) activation (D. S. Han and R. B. Sartor, unpublished results).

In addition to promotion of cellular migration, KGF-2 upregulated COX-2 expression, thereby stimulating the production of protective prostaglandins that could inhibit the onset of intestinal injury and enhance mucosal healing. Prostaglandin synthesis is an important mediator of mucosal integrity and homeostasis in the gastrointestinal tract (37). Mucosal prostaglandin production is regulated by constitutive COX-1 and inducible COX-2, which is upregulated in intestinal epithelial cells in active IBD (35) through the activation of NF-\(\kappa\)B by proinflammatory cytokines (18). We demonstrated that KGF-2 induces the expression of COX-2 in cultured intestinal epithelial cells and stimulates PGE\(_2\) production in vitro and in vivo, consistent with upregulation of COX-2 expression in endothelial cells by basic fibroblast growth factor (20). Stenson et al. (7, 27, 37) showed the key role of epithelial PGE\(_2\) in mucosal protection and healing in radiation-induced enteritis and dextran sulfate sodium-induced colitis and the importance of this molecule in oral tolerance. In the dextran sulfate sodium model, another model of experimental intestinal inflammation attenuated by KGF-2 (25), PGE\(_2\) reversed injury-induced defective epithelial cell proliferation (37), which is a feature of indomethacin-induced mucosal injury (39). The importance of prostaglandins, especially those induced by upregulation of COX-2, in mucosal protection is further illustrated by more aggressive dextran sulfate sodium-induced colitis in COX-2\(^{-/-}\) mice (26). In addition to effects on mucosal cytoprotection and epithelial proliferation, PGE\(_2\) inhibits macrophage activation and proinflammatory cytokine production (22), consistent with our observation of decreased tissue IL-1\(\beta\) concentrations and enhanced tissue PGE\(_2\)-to-IL-1\(\beta\) ratios after KGF-2 administration. Furthermore, the correlation of tissue PGE\(_2\)-to-IL-1\(\beta\) ratios with intestinal injury supports the key role of balanced inflammatory mediators and cytokines in mucosal protection. Although these results concentrate on COX-2 and PGE\(_2\), it is quite probable that KGF-2 induces expression of additional protective molecules that diminish tissue injury by additive or synergistic interactions with PGE\(_2\). For example, in preliminary studies, we have documented upregulation of the intracellular isotype form of IL-1 receptor antagonist in cultured colonic epithelial cells with KGF-2 (Han and Sartor, unpublished data). In addition, long-term KGF-2 administration stimulated goblet cell hyperplasia, similar to the effects of its homologue KGF-1 (15), presumably resulting in enhanced mucin secretion, which could provide additional mucosal protection.

Growth factors are integrally involved in mucosal homeostasis and tissue repair. For example, TGF-\(\beta\) regulates epithelial proliferation, differentiation, and restitution; inhibits T lymphocyte proliferation while promoting oral tolerance; and stimulates extracellular matrix deposition (4, 42). In contrast, KGF-2 has no known activities on T cell regulation. However, one potential detrimental consequence of treatment with
growth factors is stimulation of fibrogenesis, because TGF-β, insulin-like growth factor, and KGF-1 have been implicated in collagen deposition in the intestine through direct effects on intestinal mesenchymal cells (40, 41, 45, 46). Excessive collagen deposition can lead to clinically significant fibrosis, as demonstrated by colonic obstruction after mucosal administration of a TGF-β1 plasmid expressed in an adenoviral vector (40). Our in vitro results using cultured rat intestinal myofibroblasts show increased expression of collagen type I RNA by KGF-2. However, extracts from the small intestines of rats with indomethacin-induced ulceration exhibited no significant upregulation of collagen type I RNA or hydroxyproline concentrations by a therapeutically active dose of KGF-2, and serum hydroxyproline measurements were not increased after KGF-2 administration. The absence of detectable in vivo fibrogenesis offers a conceptual advantage of KGF-2 over other growth factors as a therapeutic candidate for IBD.

In summary, in vivo beneficial effects of KGF-2 in preventive, continuous treatment and therapeutic protocols with no apparent toxicity or induction of fibrosis suggest potential therapeutic application of this molecule to human IBD. Possible mechanisms of protection include accelerated wound healing by increased migration of epithelial cells, with or without enhanced proliferation, and increased mucosal PGE2 production. The latter mechanism is more likely to be involved in the ability of KGF-2 pretreatment to attenuate intestinal injury up to 5 days before indomethacin injection. It is likely that optimal therapeutic effects of protective growth factors may be achieved in conjunction with simultaneous use of immunosuppressive agents and broad-spectrum antibiotics to synergistically restore mucosal barrier function while decreasing antigenic stimulation and blocking activation of regulatory macrophages and TH1 lymphocytes.

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