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Keratinocyte Growth Factor Is Highly Overexpressed in Inflammatory Bowel Disease

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Recently we demonstrated an important function of keratinocyte growth factor (KGF) in wound re-epithelialization. As KGF is mitogenic for various epithelial cells, we speculated about a role of KGF in epithelial repair processes of other organs as seen in a variety of inflammatory diseases. Here we demonstrate a strikingly increased expression of KGF in surgical specimens from patients suffering from Crohn's disease and ulcerative colitis. The levels of KGF expression strongly correlated with the degree of inflammation as assessed by histological analysis of adjacent tissue and expression analysis of the pro-inflammatory cytokine interleukin-1β. The highest levels of KGF mRNA and protein were found in mesenchymal cells of the lamina propria, particularly in highly inflamed areas. As the KGF receptor is expressed in intestinal epithelial cells, KGF seems to act in a paracrine manner to stimulate proliferation of these cells. These data suggest a crucial role of KGF in epithelial repair after injury caused by inflammatory processes. (Am J Pathol 1996, 149:521–529)

Keratinocyte growth factor (KGF), a member of the fibroblast growth factor (FGF) family, is a potent and highly specific mitogen for epithelial cells.[1,2] Recently we demonstrated an important role of this growth factor in cutaneous wound repair. KGF is highly overexpressed in the mesenchyme below the wound and at the wound edge during normal wound healing in mice and humans.[3,4] The highest levels of KGF mRNA were found in dermal fibroblasts below the wound and at the wound edge, whereas the receptor for KGF, a splice variant of FGF receptor 2 (FGFR2)[5,6] was expressed only in keratinocytes of the epidermis and the hair follicles. The distribution of KGF and its receptor in the wound suggested a paracrine stimulation of epithelial cell proliferation by KGF. This hypothesis was supported by our recent *in vivo* studies, in which inhibition of KGF receptor signaling in the epidermis of transgenic mice disturbed epithelial morphogenesis and inhibited wound re-epithelialization.[7] Furthermore, reduced expression of KGF after injury is associated with wound healing defects as seen in genetically diabetic or glucocorticoid-treated mice.[8,9]

The factors responsible for KGF induction *in vivo* have not been identified. However, *in vitro* studies have demonstrated a positive regulation of KGF expression by serum growth factors and pro-inflammatory cytokines.[10,11] As the temporal and spatial expression patterns of these factors correlate with the pattern of KGF expression in the wound,[12] they might likely be responsible for KGF induction *in vivo*.

Besides in the skin, KGF expression has also been demonstrated in the mesenchymal compartments of other tissues, including the gastrointestinal tract.[13] Therefore, we speculated about a possible role of KGF in repair processes of the gut epithelium as seen, for example, in inflammatory bowel disease. The latter encompasses at least two forms of severe intestinal inflammation: Crohn's disease (CD) and ulcerative colitis (UC). Both disorders are characterized by their chronic and unpredictable course and the fact that acute inflammatory episodes are followed by remissions. Patients suffering from these diseases display local and systemic symptoms including abdominal pain, diarrhea, weight loss, and fever. Long-term complications such as fistulae formation and obstruction are frequently observed in

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CD patients, and UC patients have an increased risk of developing colon cancer. Histologically, inflammatory bowel disease is characterized by infiltration with activated neutrophils, macrophages, and lymphocytes. In addition, granulomas, fibrosis, and smooth muscle hyperplasia are present in CD. Epithelial damage is frequently seen in inflammatory bowel disease and is particularly severe in UC.[14,15]

Despite many clinical and experimental studies, the etiology of these diseases is still unknown, and the factors involved in the pathogenesis of these disorders are poorly characterized.[15,16] Most importantly, the mitogens responsible for the extensive epithelial repair caused by acute inflammatory episodes have not been identified.

Because of the potent mitogenic effect of KGF on intestinal epithelial cells[13] and to its inducibility by pro-inflammatory cytokines,[10,11] we speculated about a possible role of this growth factor in inflammatory bowel disease. Here we demonstrate a strong overexpression of KGF in the intestine of patients suffering from UC and CD, particularly in highly inflamed areas. This finding suggests that KGF is not only involved in cutaneous wound healing but also in repair processes of the intestinal epithelium as seen in inflammatory bowel disease.

Materials and Methods

RNA Isolation and RNAse Protection Assays

Fresh surgical specimens were frozen in liquid nitrogen and used for RNA isolation.[17] RNAse protection mapping of KGF and KGF receptor transcripts were performed as recently described.[3] Briefly, antisense transcripts were synthesized *in vitro* by using T3 RNA polymerase and [^{32}P]UTP (800 Ci/mmol; Amersham, Arlington Heights, IL). Samples of 30 μg of total RNA were hybridized at 42°C overnight with 100,000 cpm of the labeled antisense transcript. Hybrids were digested for 60 minutes at 30°C with RNAses A and T1. Protected fragments were separated on 5% acrylamide/8 mol/L urea gels and analyzed by autoradiography. The same RNA preparations were used for the KGF, KGF receptor, and IL-1β RNAse protection assays. The following templates were used: a 240-nucleotide fragment encoding the carboxy terminus of human KGF[2] and a 291-bp fragment corresponding to nucleotides 1629 to 1920 of the human KGF receptor cDNA.[18] The latter includes 53 nucleotides that are specific for the KGF receptor variant of FGFR2 (FGFR2-IIIb). The other nucleotides are also part of other FGFR2 splice variants. Therefore, the transcripts that encode the KGF receptor give rise to a 291-bp protected fragment whereas other FGFR2 transcripts generate a shorter 238-bp protected fragment. A 149-bp fragment corresponding to nucleotides 389 to 565 of the human IL-1β cDNA[19] was also used.

In Situ *Hybridization*

Antisense riboprobes were made by *in vitro* transcription with T3 RNA polymerase and ^{35}S-labeled UTP (1000 Ci/mmol; Amersham) using the templates described above. *In situ* hybridization was performed on 6-μm frozen sections as described.[20] After hybridization, sections were coated with NTB2 nuclear emulsion (Kodak, Rochester, NY) and exposed in the dark at 4°C for 4 weeks. After development, the sections were counterstained with hematoxylin and eosin (H&E).

Western Blot Analysis of KGF Protein

For the detection of KGF protein, intestinal specimens were homogenized in 4 ml of 2× lysis buffer (1× lysis buffer contained 1% Triton X-100, 20 mmol/L Tris-HCl, pH 8.0, 137 mmol/L NaCl, 10% glycerol, 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1% aprotinin (0.15 U/ml), 15 μg/ml leupeptin, 10 μg/ml pepstatin). The tissue extract was cleared by centrifugation, and the supernatant was diluted 1:1 with water. The amount of protein in the lysate was determined using the Bio-Rad protein assay (Bradford method). Lysates containing 2 mg of total protein were incubated overnight at 4°C with 100 μl of heparin-Sepharose beads and subsequently washed three times with 1 ml of 20 mmol/L Tris-HCl, pH 7.4 and 0.3 mol/L NaCl. Heparin-Sepharose-bound proteins were eluted from the beads with 40 μl of 2× Laemmli sample buffer and analyzed by Western blotting for the presence of KGF proteins, using a KGF-specific polyclonal rabbit antiserum[10] (1:1000 diluted) and an alkaline phosphatase detection system (Promega, Madison, WI).

Histological Analysis

Fresh surgical specimens were fixed in 4% paraformaldehyde, paraffin embedded or frozen in OCT, and sectioned. The 6-μm sections were stained with H&E.

Immunohistochemistry

Fresh surgical specimens were frozen in OCT and sectioned. The 6-μm frozen sections were fixed with

acetone and treated for 10 minutes at room temperature with 1% H_2O_2 in phosphate-buffered saline to block endogenous peroxidase activity. They were subsequently incubated for 60 minutes at room temperature with a 1:40 dilution of a KGF-specific rabbit polyclonal antiserum that had been affinity purified on a KGF column or with a 1:250 dilution of a polyclonal antiserum directed against the kinase insert region of FGFR2. Antibody-binding cells were stained with the avidin-biotin-peroxidase complex system (Vector Laboratories, Burlingame, CA) using 3-amino-9-ethylcarbazole (AEC) as a chromogenic substrate. After development, slides were rinsed with water, counterstained with hematoxylin, and mounted.

Results

To determine a potential role of KGF in inflammatory bowel disease, surgical specimens from patients suffering from CD (15 patients) or UC (7 patients) were analyzed for expression of KGF and its receptor by RNAse protection analysis. Tissue specimens taken from the vicinity of these biopsies were analyzed histologically to examine the degree of inflammation. In most cases, specimens from differently affected areas of the same patient were taken to correlate the levels of KGF expression with the degree of inflammation. Tissue specimens from 11 patients without intestinal inflammation were used as controls.

A basal level of KGF expression was found in the bowel of all control patients. However, a strikingly increased expression of this growth factor was seen in specimens obtained from highly inflamed areas of patients with inflammatory bowel disease. In 10 of 15 CD patients, KGF mRNA levels were 50 to 150-fold higher compared with control patients (Figure 1, upper panel) and a 5 to 50-fold induction was seen in another 4 CD patients (Figure 2A). Only 1 patient had normal KGF mRNA levels. Most importantly, expression levels of KGF mRNA strongly correlated with the degree of inflammation as assessed by histological analysis of adjacent tissue (Figure 2C). Thus, expression of KGF mRNA was highest in highly inflamed areas that are characterized by epithelial destruction, smooth muscle hyperplasia, and the presence of multiple inflammatory cells (Figure 2, A and C, sample 4). To further compare the level of KGF expression to the degree of inflammation, we used RNAs from the same tissue specimens to determine the expression of the pro-inflammatory cytokine interleukin (IL)-1β (Figure 2B). The latter has

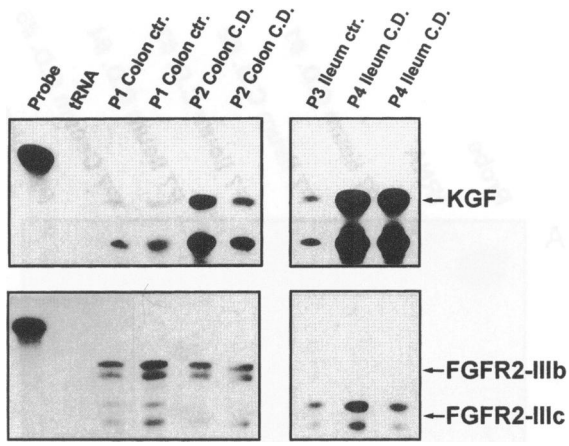

Figure 1. *Expression of KGF and FGFR2 mRNA in the ileum and the colon of CD patients and control patients. Surgical specimens were obtained from different areas of the ileum and colon of control patients (P1 and P3) and CD patients (P2 and P4). Tissue specimens were frozen in liquid nitrogen after surgery and used for RNA isolation. Total cellular RNA (30 μg) from these specimens was analyzed by RNAse protection assay for expression of KGF* (**upper panel**) *and FGFR2* (**lower panel**). *The upper bands in the FGFR2 protection assay were generated by transcripts encoding the KGF receptor splice variant of FGFR2 (FGFR2-IIIb). The lower bands were protected by transcripts encoding the IIIc splice variant of FGFR2, which does not bind KGF. Hybridization probes (1000 cpm) were used as size markers, and 50 μg of tRNA was used as a control.*

been shown to be strongly overexpressed in UC and CD, whereby the degree of overexpression correlated to the activity index of the disease.[21–24] We found a strong correlation between KGF and IL-1β expression (Figure 2, A and B, Figure 3, A and D). As this cytokine is a potent inducer of KGF expression *in vitro*,[10,11] it might also induce expression of KGF in the gut of CD and UC patients.

In contrast to KGF, KGF receptor expression levels in CD patients were similar or even lower compared with control patients (Figure 1, lower panel). The KGF receptor is a splice variant of FGFR2 (FGFR2-IIIb), which is specifically expressed in epithelial cells.[6,25,26] In addition, FGFR2-IIIc, an alternative splice variant of this gene, was expressed at similar levels in biopsies from CD patients and control patients (Figure 1B). This form of receptor binds other members of the FGF family but not KGF.[6] In contrast to the KGF receptor, FGFR2-IIIc is expressed in mesenchymal cells but not in epithelial cells.[6,25,26]

In addition to CD, a dramatic overexpression of KGF mRNA was also seen in all tested UC patients. In five of these patients, KGF mRNA levels were 50 to 150-fold higher compared with the control (Figure 3A and B) and a 5- to 50-fold induction was seen in the other two patients. Similar to CD, KGF mRNA levels strongly correlated with the degree of inflammation

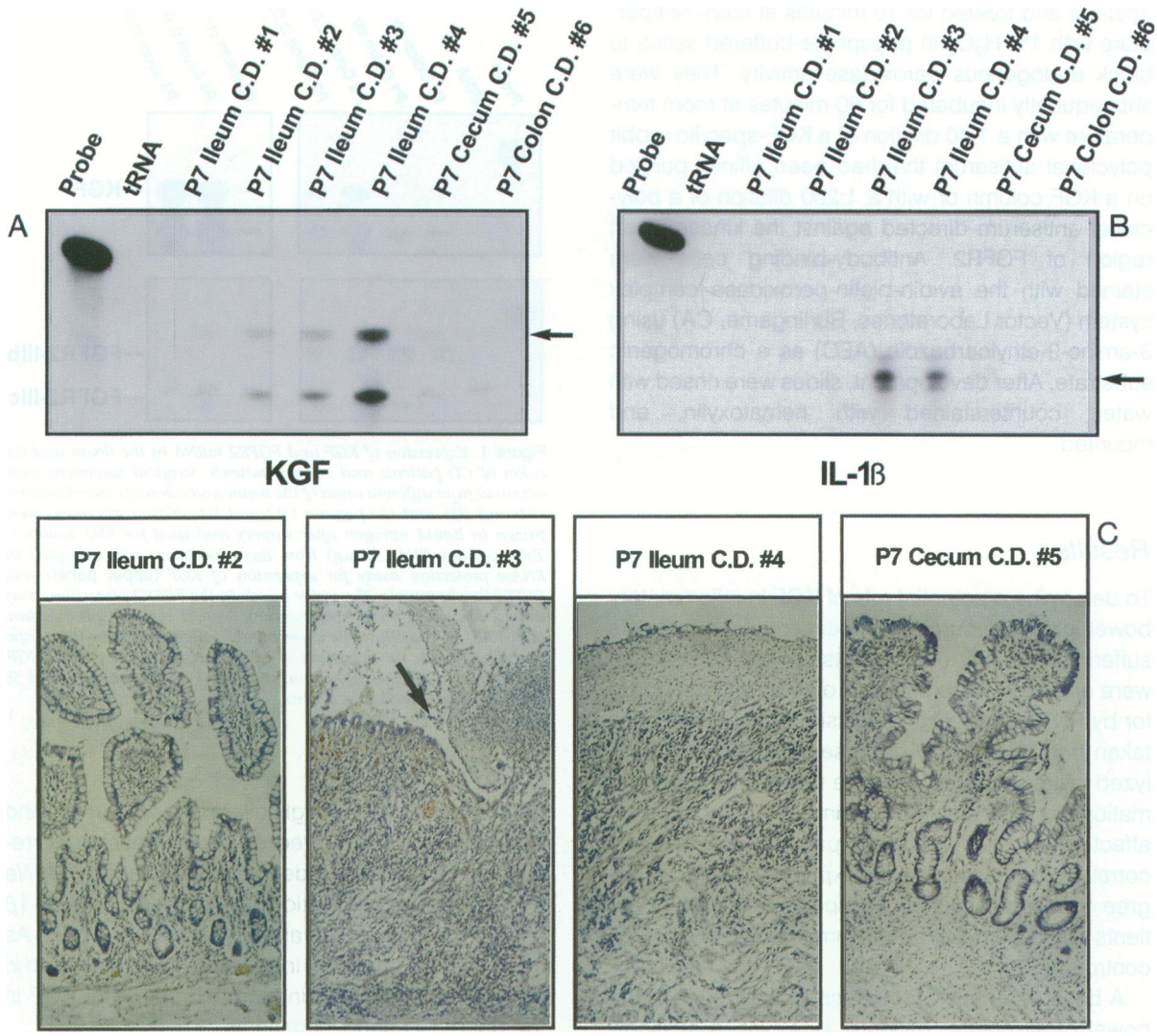

Figure 2. *Expression of KGF mRNA in the ileum of a patient with CD (P7) correlates with the degree of inflammation. Surgical specimens from different areas of the ileum and cecum were obtained from a CD patient. Total cellular RNA (30 μg) was analyzed by RNAse protection assay for expression of KGF mRNA* (A) *and IL-1β mRNA* (B). *To determine a possible correlation between KGF/IL-1β expression with pathological features, adjacent tissue specimens were fixed in 4% paraformaldehyde, frozen in OCT, and analyzed by H&E staining* (C). *Samples 2 and 5 were taken from non-inflamed tissue of the cecum or ileum, respectively. Sample 3 was taken from a region of the ileum where highly inflamed tissue* (right side) *can be clearly distinguished from relatively normal tissue* (left side). *The* arrow *indicates the transition site from normal to affected tissue. Sample 4 was taken from a highly inflamed area of the ileum. Magnification, ×100.*

as assessed by analysis of IL-1β expression (Figure 3D) and by histological analysis of adjacent tissue (data not shown). Expression of the KGF receptor was significantly reduced in highly affected areas of all UC patients (Figure 3C). As the KGF receptor is expressed only in epithelial cells,[6,25,26] this finding most likely reflects the gradual destruction of the intestinal epithelium seen in these regions. A comparison of pathological data with the levels of KGF receptor expression suggests a correlation between KGF receptor expression and the degree of epithelial cell damage.

To determine whether increased expression of KGF mRNA correlates with elevated levels of KGF protein, tissue lysates from the ileum of a patient with CD and a control patient as well as lysates from differently affected areas of the colon of an UC patient were prepared. KGF protein was enriched by its capacity to bind to heparin-Sepharose beads and subsequently detected by Western blot analysis. We found a 24-kd KGF-specific protein in tissue specimens from UC and CD patients, particularly in highly inflamed areas (Figure 4). By contrast, it was not detected in the bowel of control patients (Figure 4). The size of this protein corresponds to the expected size of glycosylated KGF.[10] This finding demonstrates a strong correlation between KGF mRNA and protein expression.

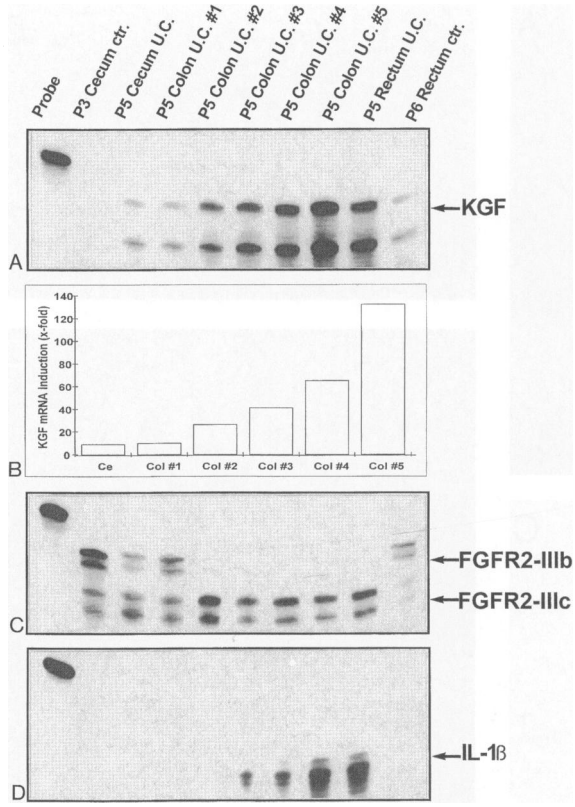

Figure 3. *Expression of KGF and FGFR2 mRNA in the colon of a patient with UC. Surgical specimens from different areas of the cecum, colon, and rectum were obtained from a patient suffering from UC (P5). Tissue specimens from the cecum and rectum of control patients (P3 and P6) were used as controls. Total cellular RNA (30 μg) was analyzed by RNAse protection assay for expression of KGF* (**A**), *FGFR2* (**C**), *and IL-1β* (**D**). *The degree of KGF overexpression in the cecum and in different areas of the colon of patient P5 (colon specimens 1 to 5) in comparison with the cecum of control patient P3 was assessed by laser scanning densitometry of the autoradiograms and is shown schematically in* **B**. *In* **C**, *transcripts encoding FGFR2-IIIb (KGF receptor) and FGFR2-IIIc give rise to different protected fragments as indicated on the* **right side** *of the figure. Hybridization probes (1000 cpm) were used as size markers.*

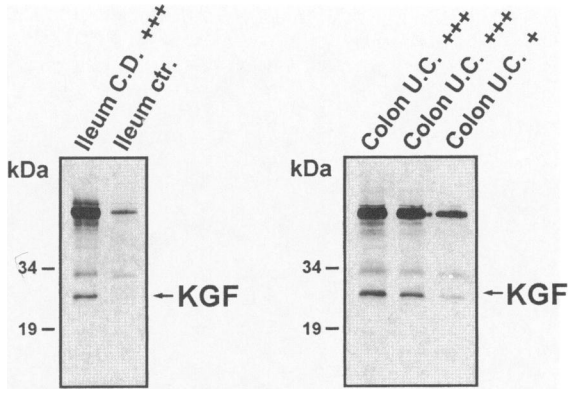

Figure 4. *Expression of KGF protein in the intestine of UC and CD patients. Surgical specimens from the ileum of a CD patient and a control patient* (**left side**) *and from different areas of the colon of a UC patient* (**right side**) *were frozen in liquid nitrogen and homogenized in lysis buffer. KGF proteins were enriched by their capacity to bind to heparin-Sepharose and analyzed by Western blotting using a KGF-specific polyclonal antiserum. The number of asterisks indicates the degree of inflammation: ***specimens from highly inflamed areas; *specimens from areas with a low degree of inflammation.*

To further define the sites of KGF action, we analyzed KGF mRNA and protein expression in the colon of a patient with UC using *in situ* hybridization (Figure 5, A and B) and immunohistochemical staining with a KGF-specific antiserum (Figure 5C). The highest levels of KGF mRNA and protein were found in a certain population of mesenchymal cells in the intestinal mucosa, immediately below the intestinal epithelium (indicated with a arrow in Figure 5A). Interestingly, expression of this gene was particularly high in areas that were characterized by epithelial damage. This expression pattern of KGF is comparable to the localization of KGF mRNA in wounded skin.[3] In addition, high levels of KGF mRNA were also detected in mesenchymal cells of the submucosa (Figure 5B).

To determine the localization of the KGF receptor, we stained the tissue with a polyclonal antibody directed against the intracellular domain of FGFR2. As this part of the receptor is identical in the KGF receptor (FGFR2-IIIb) and in the IIIc splice variants of FGFR2, this antibody recognizes both forms of FGFR2. However, use of the IIIb exon has been shown to be specific for epithelial cells, whereas the IIIc variant has never been detected in these cells. The opposite is true for mesenchymal cells.[6,25,26] Therefore, FGFR2 proteins in epithelial cells are likely to be KGF receptors, whereas FGFR2-IIIc proteins are expressed in mesenchymal cells. As shown in Figure 5D, we detected FGFR2 protein in gastrointestinal epithelial cells. Given the cell type specificity of the IIIb and IIIc splice variants, these receptors are likely to represent KGF receptors. In addition to the epithelium, FGFR2 was also found in the intestinal mesenchyme, particularly in the muscularis mucosae. In contrast to epithelial cells, these proteins are likely to be encoded by the IIIc variant of FGFR2, which does not bind KGF.

A similar expression pattern of KGF and its receptor was seen in CD patients. As shown in Figure 6, A–C, KGF transcripts were abundant in inflamed areas, particularly in mesenchymal cells that are surrounded by many inflammatory cells (Figure 6C). The receptor for KGF was expressed in the intestinal epithelium (data not shown), suggesting that mesenchyme-derived KGF stimulates proliferation of intestinal epithelial cells in a paracrine manner.

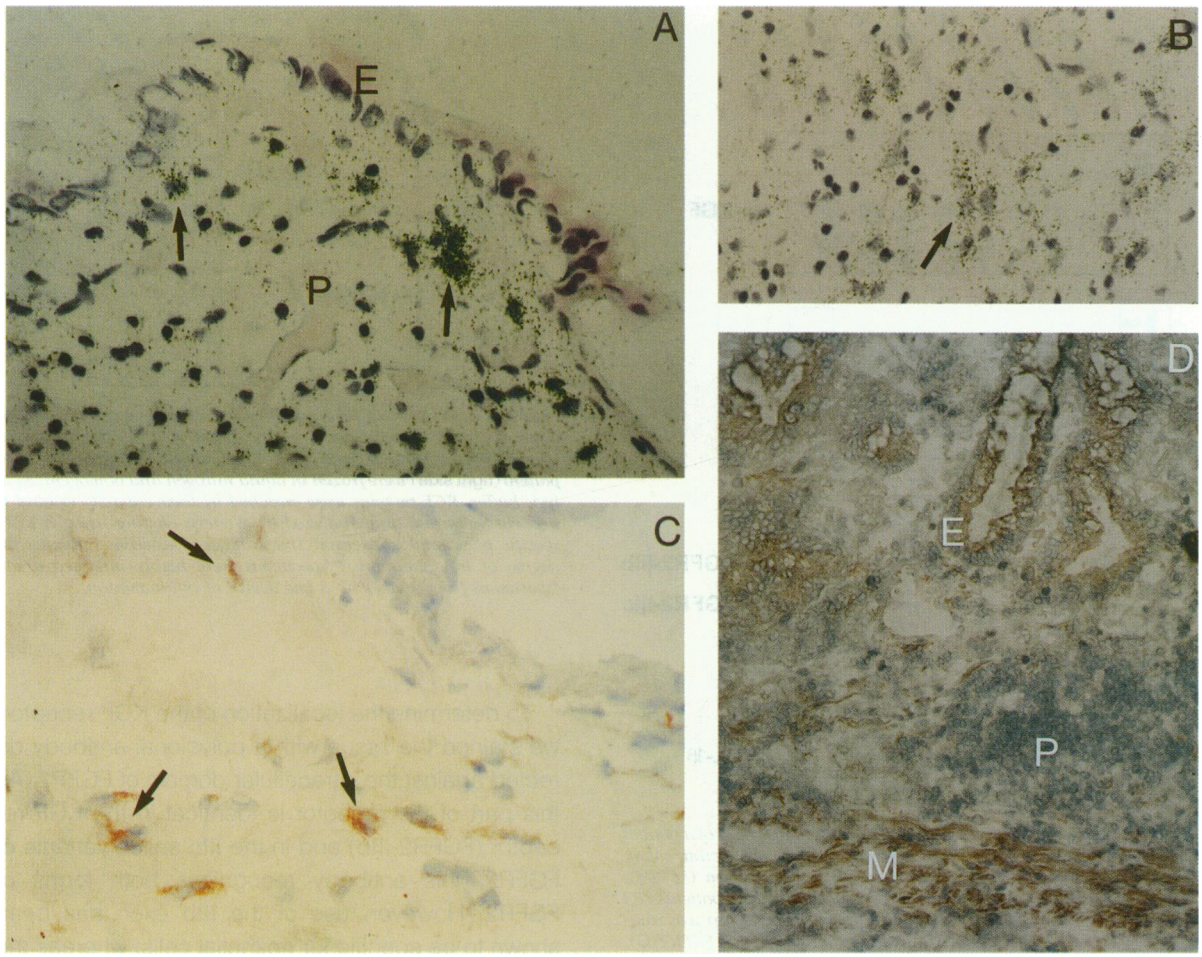

Figure 5. In situ *detection of KGF and FGFR2 in the colon of a patient with UC.* **A** *and* **B:** In situ *hybridization demonstrating KGF mRNA in the colon of a patient with UC. Surgical specimens were fixed in 4% paraformaldehyde and frozen in OCT. Frozen sections (6 μm) were hybridized with a* ^{35}*S-labeled human KGF riboprobe and counterstained with H&E.* **A:** *KGF expression in mesenchymal cells of the lamina propria.* **B:** *KGF expression in mesenchymal cells of the submucosa. E, intestinal epithelium; P, lamina propria. KGF-expressing fibroblasts are indicated with an* arrow. **C** *and* **D:** *Immunohistochemical staining of the colon of a patient with UC. Surgical specimens were frozen in OCT. Sections (6 μm) were fixed with acetone and incubated with an affinity-purified KGF-specific antiserum* (**C**) *or with a polyclonal antiserum directed against the kinase insert region of FGFR2* (**D**). *This antibody is specific for FGFR2 and does not recognize other FGF receptors (data not shown). Antibody-binding cells were identified using a peroxidase detection system and AEC as chromogen. The latter causes a red stain in KGF-expressing cells. Slides were counterstained with hematoxylin. KGF-expressing cells in the intestinal mesenchyme are indicated with an* arrow *in* **C**. *E, intestinal epithelium; M, muscularis mucosae; P, lamina propria. Magnification, ×200* (**A–C**) *and ×100* (**D**).

Discussion

Inflammatory bowel disease is characterized by severe intestinal inflammation that results in destruction of the intestinal epithelium. However, this damage is at least partially reversible, suggesting the existence of soluble factors that mediate epithelial repair. The study described in this manuscript suggests an important role of KGF in this process.

We found a basal expression of KGF in the small and large bowel of all control patients, suggesting a role of this growth factor in the continuous renewal of the intestinal epithelium. However, a strong overexpression of KGF mRNA and protein was detected in the intestine of UC and CD patients as assessed by the highly sensitive RNAse protection assay and also by Western blot analysis. The latter revealed the presence of a 24-kd KGF-specific protein in inflamed areas of the bowel but not in normal intestine. A protein of similar size had also been found in the conditioned medium of murine and human fibroblasts *in vitro*[10] and most likely corresponds to a glycosylated form of KGF. *In situ* hybridization studies demonstrated highest levels of KGF mRNA and protein in a certain population of cells in the intestinal mesenchyme that probably represent fibroblasts. By contrast, expression of KGF was not seen in inflammatory cells. KGF-producing mesenchymal cells were particularly abundant in highly inflamed areas. They were found in close proximity to inflammatory cells, including polymorphonuclear leukocytes and

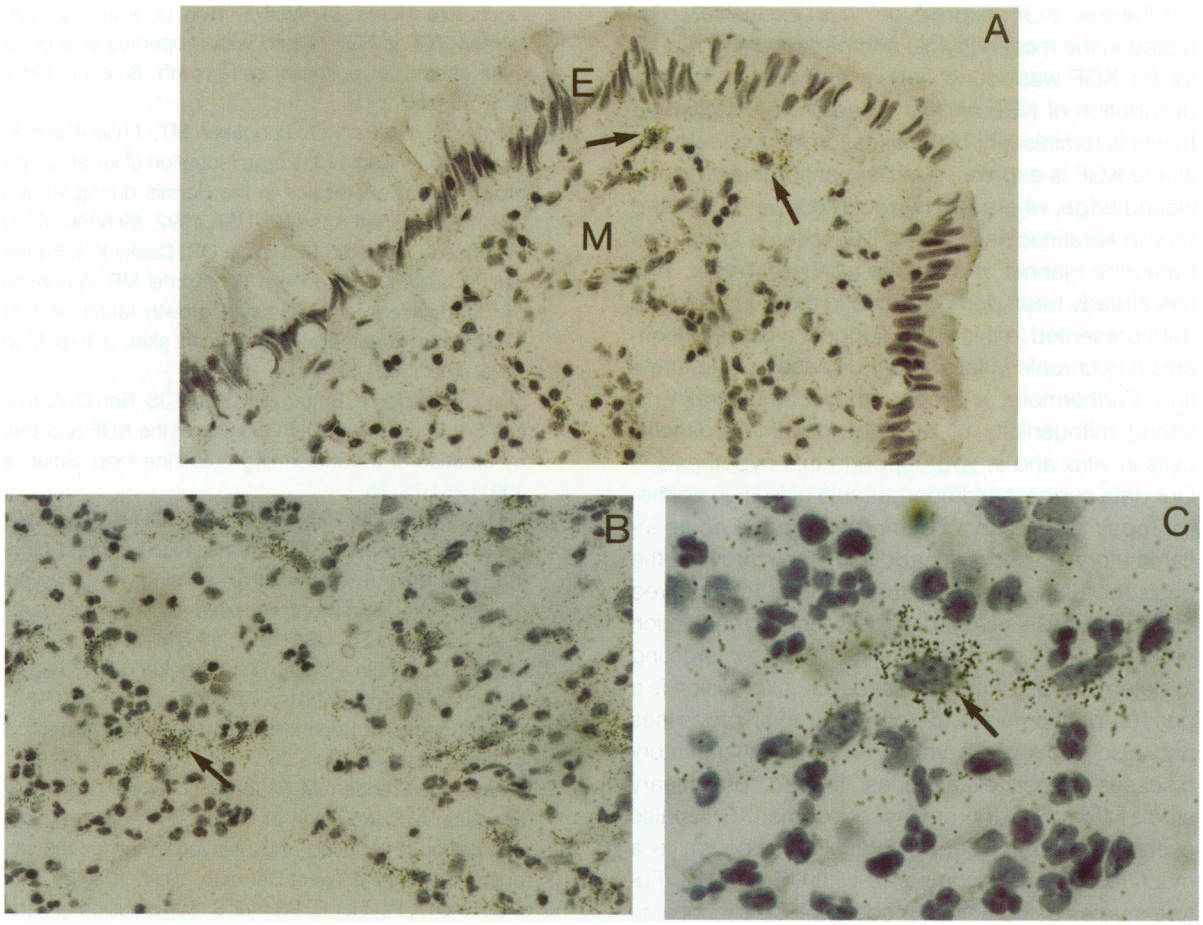

Figure 6. In situ *detection of KGF mRNA in the ileum of a CD patient. Surgical specimens were fixed in 4% paraformaldehyde and frozen in OCT. Frozen sections (6 μm) were hybridized with a ^{35}S-labeled human KGF riboprobe and counterstained with H&E. KGF-expressing cells are indicated with an* arrow. **A:** *KGF expression in a moderately inflamed area of the ileum. Note the presence of KGF-expressing cells below the epithelium. Magnification, ×200. A highly inflamed area of the same patient is shown at a ×200 and ×400 magnification in* **B** *and* **C**. *Note the presence of KGF-expressing cells in the vicinity in inflammatory cells. M, mesenchyme; E, intestinal epithelium.*

macrophages, suggesting that these inflammatory cells produce and secrete factors that stimulate KGF expression. This hypothesis is supported by recent *in vitro* studies that demonstrated a strong induction of KGF expression by pro-inflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor-α. These cytokines are produced at high levels by polymorphonuclear leukocytes and activated macrophages, which are abundant in the intestine of UC and CD patients.[15,16,27,28] Furthermore, overexpression of these cytokines has been demonstrated in inflammatory bowel disease,[21–24,28–31] and the expression level of IL-1β correlates with the level of KGF expression (this study). Therefore, these factors might likely be responsible for the increased expression of KGF in patients suffering from CD and UC. The strong inducibility of KGF expression by pro-inflammatory cytokines provides the most likely explanation for the strong correlation between the degree of inflammation and the levels of KGF expression. Besides pro-inflammatory cytokines, up-regulation of KGF expression can also occur in response to serum growth factors such as platelet-derived growth factor and epidermal growth factor.[10] These mitogens could be released in the inflamed bowel upon local hemorrhage, a process that is frequently seen in UC patients[15] and could therefore enhance the induction of KGF expression.

However, up-regulation of KGF expression in CD and UC is unlike to be a simple consequence of the increased levels of cytokines and serum growth factors in the affected tissue, as expression of other mitogens, eg, vascular endothelial growth factor, which is also induced by pro-inflammatory cytokines and serum growth factors *in vitro*,[32] is not increased in inflammatory bowel disease (M. Brauchle, unpublished data). Thus, additional, more specific stimuli might also be responsible for the strikingly increased KGF levels in the intestine of CD and UC patients.

Whereas KGF expression was exclusively detected in the mesenchymal compartment, the receptor for KGF was found only in epithelial cells. This distribution of KGF and its receptor in the inflamed bowel is reminiscent of the situation in wounded skin where KGF is expressed in dermal fibroblasts at the wound edge, whereas KGF receptors were detected only in keratinocytes.[3] Thus, KGF seems to act in a paracrine manner to stimulate epithelial repair. This has already been demonstrated in the skin,[7] and the data presented in this study suggest a similar mechanism in chronic inflammatory diseases of the intestine. Furthermore, a recent study demonstrating a strong mitogenicity of KGF for intestinal epithelial cells *in vitro* and *in vivo* supports this hypothesis.[13] Our data suggest an important role of KGF in epithelial repair after injury occurring during the acute inflammatory episode. In addition, they provide the basis for future studies that might lead to improved therapy of these severe disorders, eg, by modulation of KGF expression or availability. It will be interesting to determine whether increased KGF expression is a general phenomenon of epithelial repair processes and also occurs in a variety of other inflammatory diseases of epithelial tissues. Indeed, preliminary studies from our laboratory suggest that KGF is also overexpressed in acute diverticulitis, although to a much lesser extent compared with UC and CD. Future studies using animal models will be essential to further clarify the role of KGF in various inflammatory diseases.

Note Added in Proof

During the review process of this manuscript, Finch et al also reported on increased expression of KGF in inflammatory bowel disease (Gastroenterology 1996, 110:441–451).

Acknowledgments

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