Duodenal Ulcer

Discovery of a New Mechanism and Development of Angiogenic Therapy That Accelerates Healing

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The complete purification of the first angiogenic molecule, basic fibroblast growth factor (bFGF), was carried out in the authors' laboratory in 1983. Application of this peptide to chronic wounds enhances angiogenesis and accelerates wound healing. The authors showed that an acid-stable form of bFGF (*i.e.*, bFGF-CS23) could be administered orally to rats with duodenal ulcers. The peptide promoted a ninefold increase of angiogenesis in the ulcer bed and accelerated ulcer healing more potently than cimetidine. Basic fibroblast growth factor did not reduce gastric acid. The authors now show that bFGF exists as a naturally occurring peptide in rat and human gastric and duodenal mucosa. This endogenous bFGF is present also in the bed of chronic ulcers in rats. Sucralfate binds bFGF and protects it from acid degradation. The sucralfate is angiogenic, based on its affinity for bFGF. When sucralfate is administered orally to rats, it significantly elevates the level of bFGF in the ulcer bed. Cimetidine, by its capacity to reduce gastric acid, also elevates bFGF in the ulcer bed. A hypothetical model is proposed in which prevention of ulcer formation or accelerated healing of ulcers by conventional therapies may be FGF dependent. Acid-stable bFGF-CS23 may be considered as a form of replacement therapy in the treatment of duodenal ulcers.

THE FIELD OF angiogenesis research, which has been pursued in the laboratory during the past 20 years,[1-5] is beginning to yield clinical applications. In the area of cancer diagnosis, for example, the risk of distant metastasis in breast cancer now can be predicted by measurement of angiogenesis in biopsy specimens.[6] The first clinical use of an angiogenesis inhibitor has induced regression of life-threatening hemangiomas in children,[7,8] and a novel angiogenesis inhibitor is undergoing preclinical testing for use against solid tumors that have failed conventional therapy.[9] A clinical trial of "therapeutic angiogenesis" was recently reported in which healing was accelerated in chronic wounds by topical application of an angiogenic peptide.[10]

In this report we describe the development of another modality of therapeutic angiogenesis: the accelerated healing of experimental duodenal ulcers by an orally administered angiogenic growth factor.

Considerable evidence indicates that angiogenesis plays an important role in wound healing.[11-13] When neovascularization is suppressed or delayed, wound healing itself is retarded.[14,15] Recent experimental studies suggest that topical application of an angiogenic peptide such as basic fibroblast growth factor (bFGF) can promote the formation of vascularized granulation tissue.[16-19] Furthermore healing of experimental wounds can be accelerated by topical application of bFGF.[20-24]

Rationale for the Studies Reported Here

Chronic duodenal ulcers share many similarities with chronic wounds. Both lack an epithelial covering and contain inflammatory cells, exposed collagen, necrotic debris, and granulation tissue. Granulation tissue is composed mainly of capillary blood vessels, monocytes, and fibroblasts. It is essential for the healing of chronic ulcers. Therefore we asked, "Can the healing of chronic duodenal ulcers be accelerated by stimulating angiogenesis in the ulcer bed?"

To test this hypothesis we administered bFGF orally to rats with chronic duodenal ulcers to achieve topical application of the peptide to the ulcer bed. Basic fibroblast growth factor is one of the most potent of seven angiogenic

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FIG. 1. Degradation of bFGF by HCl at 2 hours. Recombinant human bFGF (= wild-type bFGF [bFGF-w]), was incubated at various pHs for 2 hours and its mitogenic activity on endothelial cells was measured. The bFGF CS23 mutein was incubated for the same time at similar pH levels, and its mitogenic activity was measured in the same manner on endothelial cells. The bFGF CS23 mutein is significantly more acid stable than the wild-type bFGF. Redrawn from Seno *et al.*[27]

molecules that have been completely sequenced and cloned.[3] It has a high affinity for heparin, and we originally purified it on the basis of its heparin-binding capacity.[25] Basic fibroblast growth factor is a direct mitogen for vascular endothelial cells as well as for fibroblasts, smooth muscle cells, and certain epithelial cells.[26] Because biologic activity of bFGF is rapidly degraded at pH 7.0 or below[27] (Fig. 1), we employed an acid-stable form of bFGF, produced by site-specific mutagenesis in which the second and third of the four cysteines within the peptide were substituted by serines (bFGF-CS23 mutein)[27] (Fig. 2).

Oral administration of bFGF-CS23 to rats with chronic duodenal ulcers over a period of 21 days stimulated a more than ninefold increase in angiogenesis in the ulcer bed and significantly accelerated ulcer healing compared with untreated animals or with animals treated with optimum doses of the H2-blocker cimetidine.[28,29] Gastric acid and pepsin levels were unaffected by bFGF-CS23 when the growth factor was administered as a single dose. When it was administered for 3 weeks, the volume of gastric juice and acid concentration were significantly increased. These results led to the question, "If the healing of duodenal ulcers can be accelerated without reduction

FIG. 2. Structure of bFGF-CS23. Recombinant human bFGF (*upper panel*) was made more acid stable by site-specific mutagenesis in which serine was substituted for the second and third cysteine (*lower panel*).

of gastric acid, what then is the role of gastric acid in duodenal ulceration?"

The widely used anti-ulcer drug Carafate® (sucralfate, *i.e.*, aluminum sucrose octasulfate), provided a clue to this puzzle, because it also accelerates ulcer healing but does not reduce gastric acid. Its mechanism of action is unclear, except that it is known to coat the ulcer bed. Because the chemical structure (Fig. 3) of sucralfate (*i.e.*, a disaccharide with 8 sulfate residues) resembled the repeating disaccharide units of heparin, we tested the affinity of sucralfate for bFGF. We found that sucralfate had a higher affinity to bFGF than heparin did and that it could protect bFGF from degradation by acid.[30]

This surprising result suggested that sucralfate could act by binding endogenous bFGF, protecting it from acid degradation, and delivering it to the ulcer bed. If supporting evidence could be found for this idea, then a new model of the mechanism of ulcer healing could be entertained in which endogenous bFGF, presumably existing in the ulcer bed and in the mucosa, might be critical for ulcer healing because of its angiogenic and other mitogenic properties. In this proposed model, gastric acid would contribute to ulcer formation by continually degrading and inactivating endogenous bFGF, but HCl may not be a primary cause of ulcer formation.

We show here, for the first time, that human and rat gastric and duodenal mucosa contain levels of biologically active bFGF. Basic fibroblast growth factor is present also in the duodenal ulcers of rats. We further demonstrate that oral administration of sucralfate elevates the level of available endogenous bFGF in the ulcer bed. To explain these findings, we propose a unifying hypothesis that suggests that antacids, H2-blockers, and sucralfate act through an FGF-dependent pathway to accelerate the healing of duodenal ulcers. Oral administration of bFGF thus may be considered as a natural form of replacement therapy to achieve accelerated healing of duodenal ulcers.

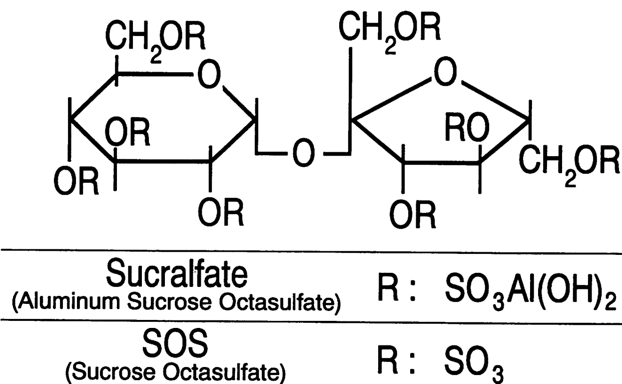


FIG. 3. Structure of sucralfate. Sucralfate (aluminum sucrose octasulfate) is poorly soluble compared with its soluble counterpart, potassium sucrose octasulfate (SOS).

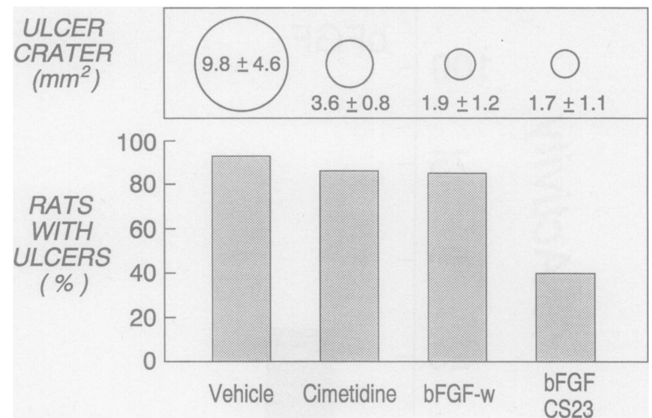


FIG. 4. Effect of orally administered bFGF on the healing of chronic duodenal ulcers. Duodenal ulcers were induced by cysteamine as reported by Szabo *et al.*[41] six rats per bar. Rats received bFGF-w or bFGF-CS23 by oral gavage, 100 ng/100 g twice daily, or vehicle alone, or cimetidine 10 mg/100 g twice daily. The rats were killed at 21 days, the ulcer crater was measured in two dimensions, and the area was calculated by the ellipse formula. The number of residual ulcers was also recorded. At the beginning of the experiment, all rats had penetrating or perforating ulcers deep into the muscularis propria. Redrawn from data in Szabo *et al.*[28]

Oral bFGF-CS23 Is Found to Accelerate Duodenal Ulcer Healing

Chronic duodenal ulcers were produced in rats by feeding cysteamine-HCl® (25 mg/100 g) by intragastric gavage for 3 doses on the first day, using a previously reported method.[31] On the third day, a laparotomy was carried out in all rats, and only those with ulcers deep into the muscularis propria or with penetrating or perforating ulcers were employed in the study.[28,29]

Rats received bFGF-CS23 100 ng/100 g by intragastric gavage twice daily for 21 days. A second group received wild-type bFGF (bFGF-w) that had not been mutagenized for acid stability. A third group received an optimum dose of cimetidine (10 mg/100 g), also by gavage twice daily. Control rats received vehicle alone (sodium citrate buffer 50 nmol/L, pH 7.0). There were six rats per group, and the experiment was repeated four times over a year.[28,29,32]

Treatment with bFGF-CS23 reduced the mean ulcer size (area in square millimeters) down to 17% of untreated controls at 3 weeks ($p = < 0.01$), whereas bFGF-w decreased ulcer size down to 23% of controls ($p = <0.05$) (Fig. 4). Cimetidine decreased ulcer size to 38% of untreated controls, an effect that was only at the margin of statistical significance. The number of rats with residual ulcers at 21 days was: vehicle, 93%; cimetidine, 86%; bFGF-w, 85%; and bFGF-CS23, 40%. Only in rats treated with bFGF-CS23 mutein was the incidence of residual ulcers significantly reduced. The potency of bFGF-CS23 was more than one million times greater than cimetidine on a weight-for-weight basis.

Histologic studies of duodenal ulcers in control rats disclosed a solitary ulcer crater with sparse granulation tissue, necrotic debris, and inflammatory cells. In contrast, animals treated with bFGF-CS23 had completely healed their ulcers or had a small residual ulcer crater with prominent neovascularization. Morphometric analysis of blood vessels stained with antibody to factor VIII-related antigen, a specific marker for vascular endothelial cells, demonstrated a greater than ninefold increase in the number of microvessels in rats treated with bFGF-CS23 compared with animals treated with vehicle alone (Table 1). Rats treated with bFGF-w showed approximately a twofold increase in the number of microvessels compared with rats receiving the vehicle alone. Thus the bFGF-CS23 mutein was superior to the natural form of bFGF in both stimulation of angiogenesis in the ulcer bed and acceleration of ulcer healing.

A single dose of bFGF-CS23 had no effect on gastric acid or pepsin levels. Chronic administration of bFGF-CS23 for 3 weeks increased the volume of gastric juice and acid concentration. Similarly pepsin outputs were increased after 3 weeks of treatment with bFGF-CS23. Interestingly bFGF-CS23 effectively accelerated healing of duodenal ulcers despite the high levels of luminal acid and pepsin. This ability of an angiogenic growth factor to accelerate healing of duodenal ulcers in the presence of high gastric acid and pepsin led us to rethink the role of acid in the pathogenesis of duodenal ulceration. Although most conventional anti-ulcer therapy depends on reduction of gastric acidity by drugs or surgical procedures, sucralfate does not reduce gastric acidity. We therefore directed our attention to elucidating the mechanism of action of sucralfate.

Sucralfate Binds to Fibroblast Growth Factor and Protects It from Acid Degradation

Sucralfate is a basic aluminum salt of sucrose octasulfate that is widely used for the treatment of gastric and duodenal ulcers.[33] This anti-ulcer action is achieved without decreasing gastric acidity.[33] The ability of sucralfate to coat an ulcer bed has been thought to be its principal mode of action. The active components of the molecule seem to be sulfate and sucrose octasulfate.[34] Based on our previous reports that other sulfated saccharides such as

TABLE 1. *Quantification of Vascularization in the Ulcer Bed Compared With Remote Submucosa*

	No. Microvessels/mm^2	
Treatment	Ulcer Bed	Submucosa Remote From Ulcer
Vehicle	9.9 ± 3.5	40.0 ± 3.0
bFGF-w	17.9 ± 4.3	43.5 ± 3.2
bFGF-CS23	92.0 ± 5.9	34.2 ± 3.6

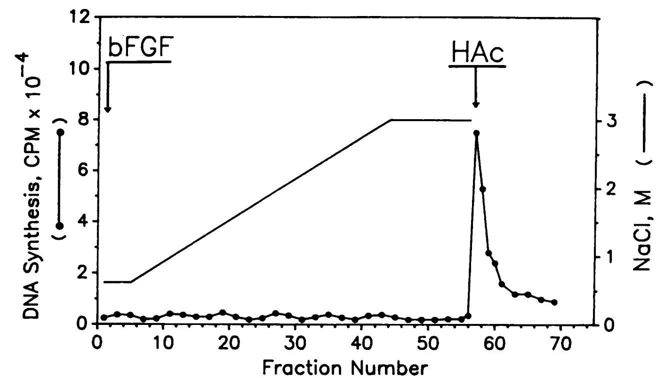


FIG. 5. Elution of bFGF from a sucralfate-Sepharose column by acetic acid. A sucralfate-Sepharose column was prepared by mixing 0.1 g of sucralfate and 0.8 ml (bed volume) of Sepharose CL-6B (Pharmacia) in Tris buffer with 0.6 M NaCl; 2 μg of bFGF in 0.2 ml of saline containing 0.1 mg of BSA was loaded onto the sucralfate column. The column was rinsed with 10 ml Tris buffer and 0.6 M NaCl and eluted consecutively at a flow rate of 15 ml/hr: (1) A gradient of NaCl (80 ml) from 0.6 M to 3.0 M, (2) 20 ml of 3 NaCl, and (3) 30 ml of 1.5 M acetic acid (pH 2.2). Fractions of 2 ml were collected. Aliquots from each fraction were diluted, neutralized with Tris buffer, and analyzed for growth factor activity. The bFGF could not be eluted from a sucralfate-Sepharose column even with 3.0 M NaCl. However, biologically active bFGF was eluted by 1.5 M acetic acid.

heparin[25,35] and beta-cyclodextrin tetradecasulfate[36] have high affinity for fibroblast growth factors, and because the structure of sucrose octasulfate (Fig. 3) resembles the repeating disaccharide structure of heparin, we tested the affinity of sucralfate and sucrose octasulfate for bFGF.

Our previous studies showed that bFGF adhered tightly to heparin immobilized on Sepharose beads and could be eluted with a high concentration of NaCl of approximately 1.5 M.[25] In the present experiment, however, when insoluble granules of sucralfate (0.1 g), were mixed with 0.8 ml Sepharose beads to form a chromatographic column and bFGF was loaded onto the column, the peptide bound so avidly to sucralfate that it could not be removed by elution with up to 3 mol/L NaCl (Fig. 5). Thus sucralfate has a higher affinity for bFGF than heparin. Nevertheless bFGF was recovered from this column by elution with acetic acid 1.5 mol/L (pH 2.2). Acetic acid dissolved the sucralfate and bFGF came out in the acidic solution. When this eluate was neutralized with TRIS buffer, diluted 20-fold, and added directly to cultures of 3T3 fibroblasts, the eluted bFGF was highly mitogenic for these cells and retained its full biologic activity. Thus although bFGF is normally degraded and inactivated by acid conditions (see Fig. 1), sucralfate protects bFGF from stronger acid conditions than would normally be found in the stomach (pH 2.2). Water-soluble sucrose octasulfate also protected the biologic activity of bFGF against acid degradation (Fig. 6). If bFGF was denatured by acid conditions before incubation with potassium sucrose octasulfate, however, bFGF biologic activity could not be recovered by addition

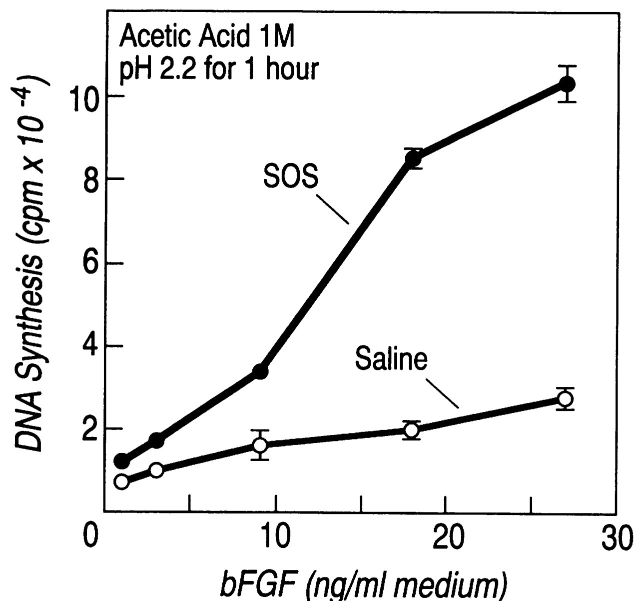


FIG. 6. Stabilization of bFGF by potassium sucrose octasulfate under acidic conditions. The bFGF (0.25 μg/60 μL) was incubated at pH 2.2 with 1 M acetic acid in the presence of (●) and absence of (O) 2 mg potassium sucrose octasulfate at 37 C for 1 hour. At the end of incubation, bFGF samples were diluted and analyzed for their ability to stimulate DNA synthesis on 3T3 fibroblasts.

of the sucrose octasulfate to the treated samples. This indicates that the mechanism of bFGF protection by sucrose octasulfate probably does not involve a renaturation process. Sucrose octasulfate (up to 200 μg/mL) neither increased nor decreased the mitogenic effect of bFGF under neutral pH conditions.

Although these results do not indicate whether the sucrose octasulfate is dissociated from bFGF, such dissociation may not be necessary for the maintenance of biologic activity of bFGF. It is probable that the small sulfated disaccharides attach themselves to specific domains along the full length of the bFGF peptide and protect it from degradation by some as yet unknown mechanism. Nevertheless this protective property of sucralfate for bFGF strongly suggested that if bFGF was naturally present in the stomach, a novel mechanism of action could be assigned to sucralfate.

Evidence that Endogenous bFGF Is Present in the Rat and Human Stomach and Duodenum

Growth Factor Activity in Gastric Mucosa

To determine if any growth factor activity could be extracted from the rat gastric mucosa, sucrose octasulfate was used as the extraction solution. The glandular mucosa of rat stomachs was excised, weighed, and incubated in either saline or sucrose octasulfate (Fig. 7). After centrifugation, an aliquot of the supernatant was assayed for mitogenic activity on 3T3 fibroblasts. Sucrose octasulfate efficiently extracted potent mitogenic activity from the rat gastric mucosa.

The Growth Factor Extracted from Gastric Mucosa Contains bFGF

To demonstrate that this extractable growth factor activity contained biologically active basic fibroblast growth factor (bFGF), glandular mucosa from the rat stomach was extracted in 2 mol/L NaCl and purified by heparin-affinity chromatography. An eluted fraction that coincided with bFGF then was confirmed to contain bFGF by Western blot analysis (see Fig. 9 legend for method). A similar extraction procedure and Western blot analysis was carried out on human gastric mucosa obtained from

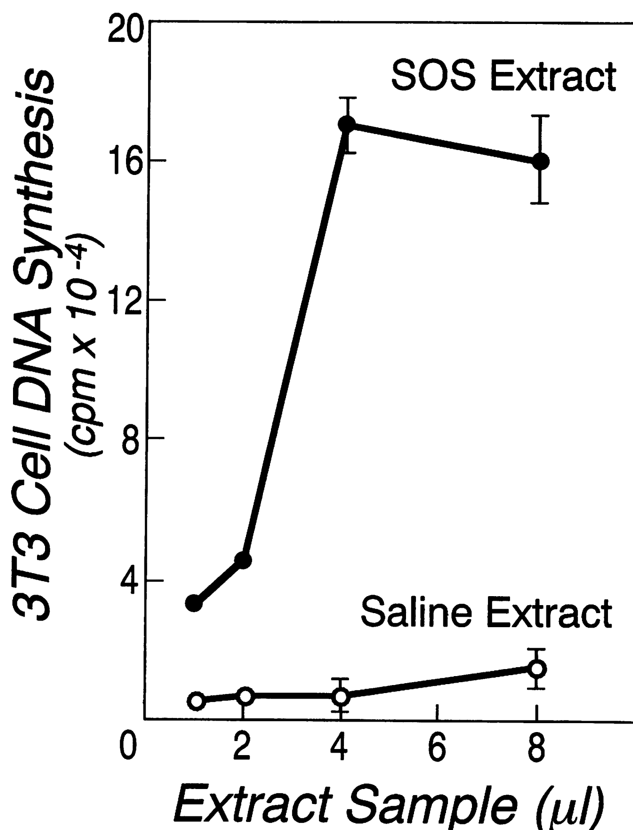


FIG. 7. Elution of growth factor activity from gastric mucosa by sucrose octasulfate (SOS). Sixteen normal rats were killed, and the glandular mucosa of the stomach was excised. The stomachs were randomly divided into two groups. Eight stomachs were individually placed in a volume of either saline or sucrose octasulfate (SOS, 10 mg/ml) to make a weight/volume ratio of 1 g stomach/5 ml solution. Each stomach was then incubated for 12 hours at 4 C with gentle mixing, the extractant centrifuged at 3000× *g* for 30 minutes, and the supernatant assayed on 3T3 fibroblasts for DNA synthesis in a dose-dependent manner. The open circles represent stomachs extracted only in saline. The closed circles show stomachs extracted by SOS. Each point represents eight individual stomachs. Each sample was assayed on 3T3 fibroblasts in triplicate ($p = <0.001$). Sucrose octasulfate efficiently extracts mitogenic activity from the rat gastric mucosa.

the pyloric antrum. This also confirmed the presence of bFGF.

Evidence that Endogenous bFGF Is Present in the Ulcer Bed and Is Increased by Orally Administered Sucralfate

To determine if the ulcer bed *per se* contained bFGF, rats with penetrating duodenal ulcers were treated for 7 days with either oral sucralfate by gavage or with vehicle alone (Fig. 8). The rats were killed and sucralfate was gently removed from the ulcer bed. The ulcer bed was excised, weighed, and extracted in 2 mol/L NaCl for 12 hours. After centrifugation, the supernatant was diluted and applied to a heparin-affinity column. The eluted peak of bFGF was assayed for mitogenic activity on 3T3 fibroblasts. Basic fibroblast growth factor also was extracted from proximal stomach and duodenum. Figure 8 demonstrates a significant increase in extractable bFGF in the ulcer bed of sucralfate-treated animals compared with the untreated rats ($p = <0.001$). Moreover untreated ulcers contained significantly more bFGF than normal mucosa from stomach or duodenum. In Figure 9A, Western blot analysis of bFGF extracted from the ulcer beds and purified by heparin-affinity chromatography confirms its identity to pure recombinant bFGF having a molecular weight of 18,000. Lane 1 shows human recombinant bFGF. Lane 2 shows bFGF extracted from untreated ulcer beds. Lane 3 shows bFGF extracted from ulcer beds after treatment with sucralfate. The band in lane 3 is significantly stronger than the band in lane 2. This is consistent with the much higher level of growth factor activity observed in the sucralfate-treated ulcer bed in Figure 8.

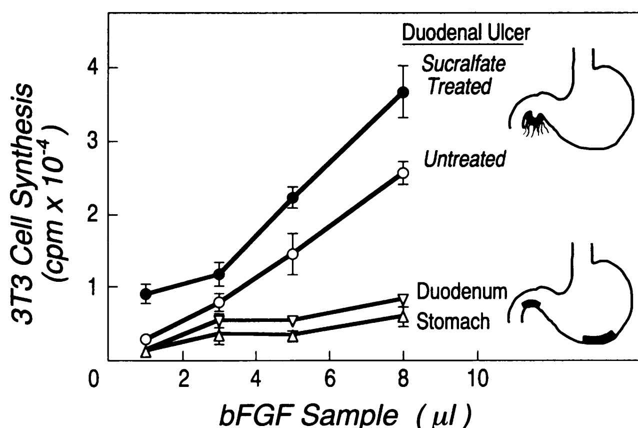


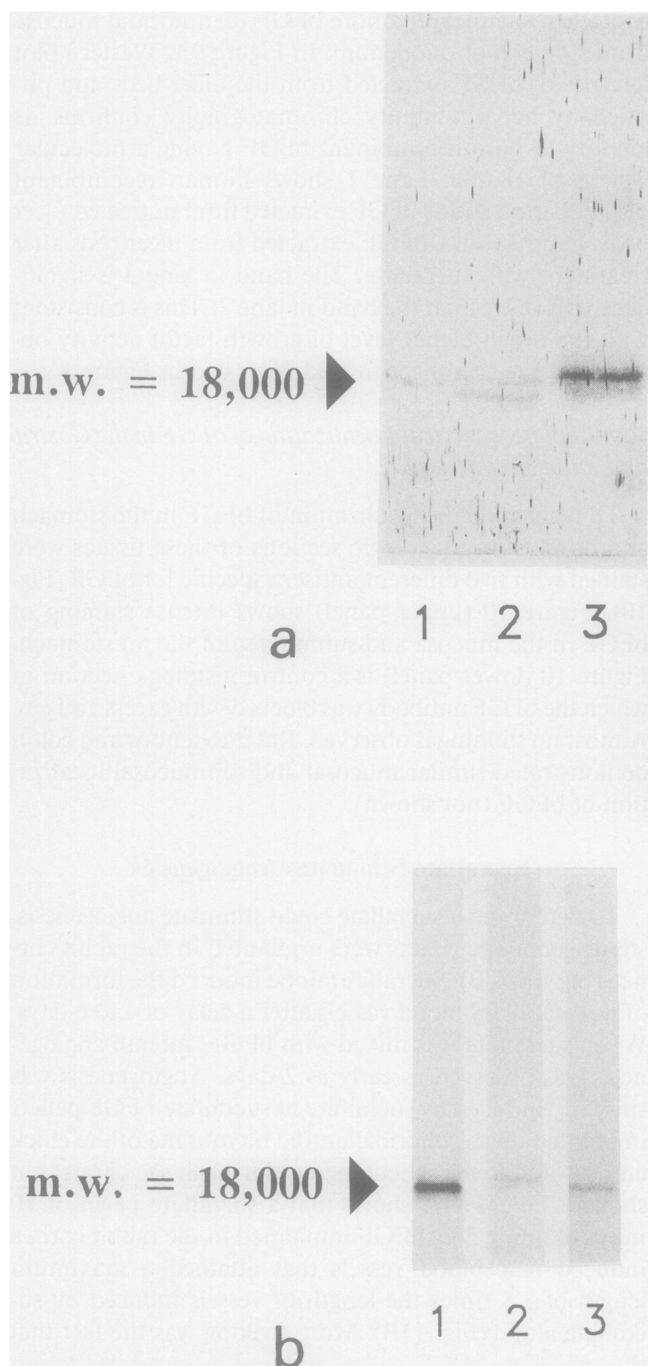
FIG. 8. Effect of sucralfate on endogenous bFGF in duodenal activity from the rat gastric mucosa. Duodenal ulcers were induced in 20 rats by cysteamine. Ten rats received oral sucralfate by gavage, 20 mg/100 g, twice daily for 7 days. Ten rats received the vehicle solution (water) by gavage. Both groups of rats were killed on day 8. The sucralfate was gently washed away from where it had coated the ulcer bed, one gram of stomach was then incubated in 5 ml NaCl 2.0 M (*e.g.*, 500 mg stomach in 2.5 ml NaCl), plus 10 mM Tris at pH 7.0 to extract FGF from the tissues at 4 C for 12 hours. The extractant was centrifuged at 3000× *g* for 30 minutes, and the supernatant was diluted fourfold with 10 mM Tris buffer to lower the salt concentration. The diluted supernatant was then applied to a heparin-affinity column. The column was rinsed with 0.6 M NaCl and eluted with NaCl 2.0 M, and the fractions were assayed for mitogenic activity on 3T3 fibroblasts. All of the mitogenic activity was in the 2.0 M NaCl fraction. For control subjects, proximal portions of stomach and duodenum were also sampled and weighed in the same way and analyzed by heparin-affinity chromatography. There is a significant increase of bFGF in the ulcer bed of sucralfate-treated animals compared with the untreated animals ($p = <0.001$). Furthermore, the ulcer beds of untreated animals had significantly more bFGF than normal duodenal or stomach mucosa ($p = <0.005$). Each point represents the mean triplicate 3T3 assay from an aggregate of 10 animals. Error bars SEM. The identity of the bFGF in the ulcer bed and in the mucosa was confirmed by Western blot analysis (Fig. 9).

Immunohistochemical Localization of bFGF in the Gastric and Duodenal Mucosa

To determine the localization of bFGF in the stomach and duodenum, histologic sections of these tissues were stained with two different antisera specific for bFGF (Fig. 10). Figure 10 (upper panel) shows intense staining of bFGF in the mucosa and submucosa of the rat stomach. Figure 10 (lower panel) is a control histologic section in which the bFGF antibody was blocked with excess antigen. Almost no staining is observed. Rat duodenum and colon demonstrated similar mucosal and submucosal localization of bFGF (not shown).

Sucralfate Stimulates Angiogenesis

To determine if sucralfate could stimulate angiogenesis, sterile sucralfate pellets were implanted in the rabbit cornea (Fig. 11A, B). Sucralfate alone induced the formation of new capillary blood vessels after a delay of 4 to 6 days. When sucralfate was mixed with bFGF, intense angiogenesis was observed as early as 2 days. Angiogenesis was similarly induced by sucralfate or sucralfate-bFGF pellets implanted on the chorioallantoic membrane of the chick embryo and in the subcutaneous dorsal air sac of rats (not shown). Figure 11A shows that a sucralfate pellet of 10 mg containing 5 μg bFGF implanted in the rabbit cornea induced new blood vessels that attained a maximum length of 2.8 times the length of vessels induced by sucralfate alone (Fig. 11B). More striking was the fact that the sucralfate-bFGF pellets induced neovascularization over the full circumference of the cornea. In contrast sucralfate alone induced only a narrow sector of neovascularization. Even in the presence of neovascularization, the corneas remained clear and transparent. Histologic sections after 12 days showed virtually no inflammatory cells in the corneas. Sucralfate-bFGF mixtures induced the most intense and prolonged angiogenesis of any noninflammatory angiogenic compound that has been tested in the cornea. By approximately 25 to 30 days, the sucralfate pellets had begun to disappear. We speculate that implanted sucralfate pellets extract and bind available



FIGS. 9A and B. Western blot analysis of bFGF in rat and human stomach. (A) The extractions from the sucralfate-treated ulcers and the untreated rat ulcers described in Fig. 8, were then purified on heparin-affinity chromatography as previously described by Shing *et al.*[25] Fractions eluted from the heparin-affinity column at approximately 1.5 M NaCl, which coincides with elution for pure bFGF, were then analyzed by Western blot analysis using a polyclonal antibody produced against the internal synthetic peptide of bFGF.

Briefly, the active fraction from heparin-affinity chromatography was dialyzed against water (changing twice overnight) in 4 L of water and then lyophilized. The lyophilizate was applied to a 15% SDS polyacrylamide electrophoresis gel, as described by Lemmeli. The SDS gel was then transferred to a nitrocellulose paper that was then incubated with a polyclonal antibody produced in rabbits against the synthetic internal sequence of human bFGF. This blot was then incubated with a secondary antibody (goat anti-rabbit IgG) conjugated to alkaline phosphatase. The blot was then stained for alkaline phosphatase.

In (A), Lane 1 shows bFGF (18,000 molecular weight). Lane 2 shows bFGF extracted from the duodenal ulcer of an untreated rat. Lane 3 shows bFGF isolated from the ulcer bed of a sucralfate-treated rat.

In (B), Lane 1 shows bFGF. Lane 2 shows bFGF isolated from rat stomach mucosa. Lane 3 shows bFGF isolated from human stomach mucosa (discarded at the time of gastrectomy).

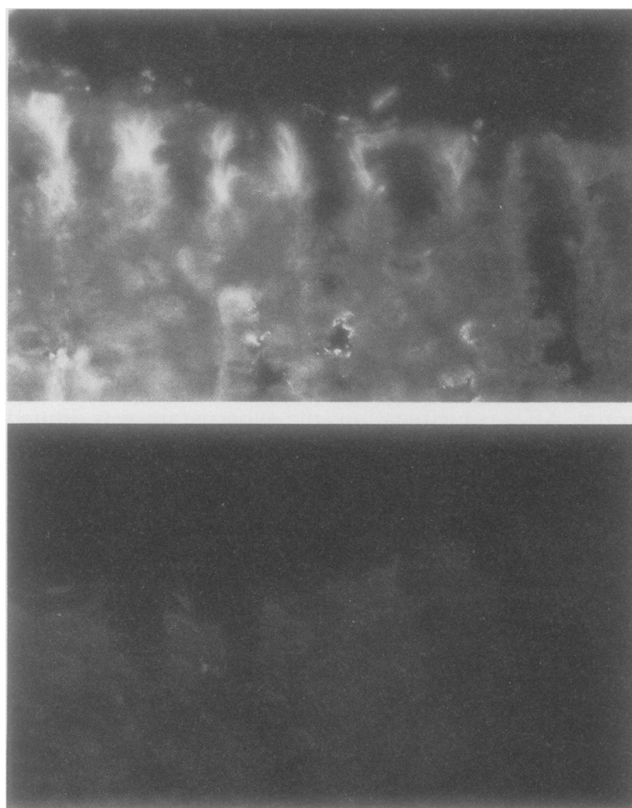
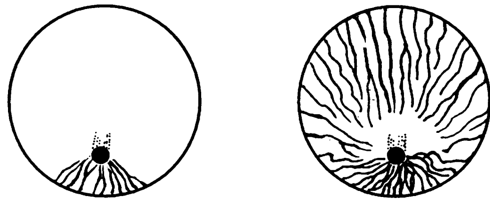
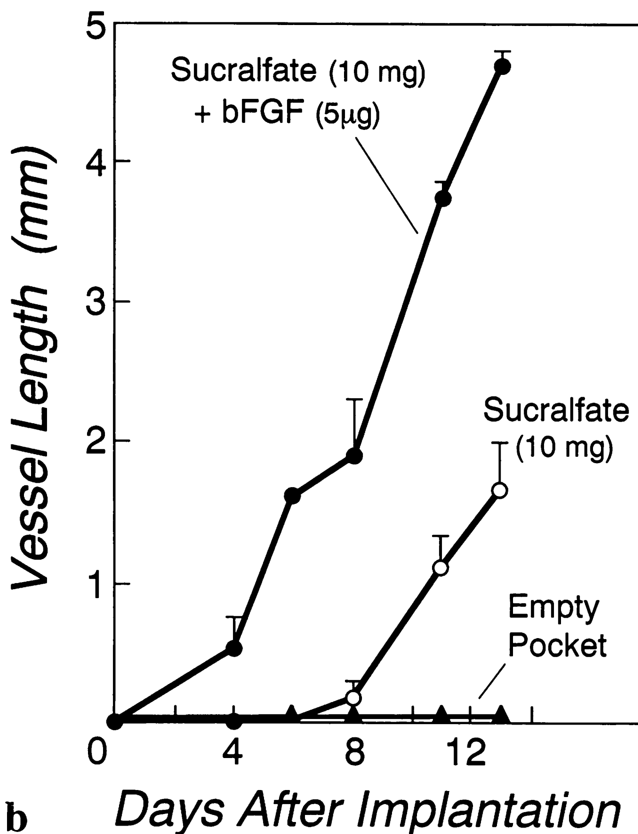


FIG. 10. Demonstration of bFGF in rat gastric mucosa by immunohistochemical staining. Female Sprague-Dawley rats 200–250 g were killed with sodium pentobarbital, and sections of the forestomach, glandular stomach, duodenum, and colon were obtained. The tissues were fixed with 4% paraformaldehyde cryoprotected in 30% sucrose. Immunohistochemical staining was performed with a murine monoclonal antibody directed against the N-terminal sequence of human bFGF (gift from Takeda Chemical Industries, Ltd., Osaka, Japan). This antibody reacts with bFGF but not aFGF on Western blots and not with other proteins. The cryostat sections were fixed for 10 minutes with 10 mM NaCl in PBS. Tissue sections were washed three times with PBS, permeabilized in 0.2% Triton, and then incubated with normal goat serum for 60 minutes. The blocking buffer was then drained off and the sections were incubated for 12 hours with the primary antibody of 1:400 dilution.

Control sections were stained with antisera preincubated in the presence of an excess (50×) of the immunizing peptide for 3 hours 37 C or with nonimmune serum alone. The sections were washed with PBS and incubated for 3 hours with secondary antisera (a fluorescein-labeled goat anti-murine IgG). The tissue sections were washed, mounted in an antibleaching agent, and examined by fluorescence microscopy.

Intense staining (*upper panel*) for bFGF in the gastric mucosa taken from a region in the rat antrum. Absence of staining *lower panel* when anti-bFGF antibody was blocked by excess antigen. Duodenal and colon mucosa also demonstrated significant staining for the presence of bFGF. A similar staining pattern was obtained using a polyclonal antibody directed against human bFGF (gift from Dr. Patricia D'Amore, Harvard Medical School).





FIGS. 11A and B. Angiogenic activity of sucralfate and sucralfate complexed with bFGF. (A) Drawing from Ektachrome photographs. Pure sucralfate was sterilized by boiling for 1 minute. A portion of the sucralfate was complexed with bFGF (10 mg sucralfate plus 5 μg of bFGF by gentle mixing for 1 hr at room temperature). A 10-mg pellet of sucralfate was implanted into a rabbit corneal pocket 2 mm from the limbal edge by a technique previously reported.[1]

Sucralfate complexed to bFGF was implanted in the opposite cornea or in the cornea of a different rabbit. Five eyes were used from each group. (B) Mean maximum vessel length and SEM measured at intervals from the time of implantation.

Sucralfate plus bFGF induced intense angiogenesis around the entire cornea without inflammation or edema. Sucralfate alone had no effect until about day 6, after which neovascularization appeared in a narrow sector closest to the implanted pellet.

bFGF that is produced in the local tissue. The bound bFGF then is protected from degradation and slowly released as the sucralfate gradually dissolves. In contrast sucralfate that is precomplexed with bFGF acts as a slow release depot for the biologically active peptide, leading to the induction of more intense angiogenesis. Preliminary data from the chick embryo support this notion.

A Unifying Hypothesis for the Mechanism of Accelerated Ulcer Healing

These experiments suggest a model in which conventional anti-ulcer therapies may operate through a common mechanism. In this model naturally occurring endogenous bFGF is produced by gastric and duodenal mucosa as well as within the ulcer bed (possibly by macrophages and mast cells, etc.) (Fig. 12A, B). This peptide may be necessary for ulcer healing by virtue of its angiogenic activity as well as its mitogenic activity for fibroblasts, smooth muscle, and epithelium. This endogenous bFGF, however, is highly susceptible to continuous degradation and inactvation by gastric acid.

According to this hypothetical model, anti-ulcer therapies that reduce acid (*e.g.*, antacids, H2-antagonists, surgical procedures) would retard degradation of endogenous bFGF (Fig. 12C, D). Sucralfate therapy would protect bFGF from acid degradation, without affecting gastric acidity *per se* (Fig. 12E). Oral administration of acid-stable bFGF (*e.g.*, bFGF-CS23) could be considered as a form of replacement therapy (Fig. 12F). Such therapy would overcome the problem of steady degradative losses of endogenous bFGF.

The role of hydrochloric acid in the pathogenesis of peptic ulcer would, in the context of this model, depend on the extent to which HCl degraded endogenous bFGF.

The Effect of Cimetidine on bFGF Levels in the Ulcer Bed

One important prediction that arises from this hypothetical model is that cimetidine therapy should elevate available levels of bFGF in the ulcer bed as a result of a significant reduction in gastric acidity. To test this prediction, rats with penetrating duodenal ulcers induced by Cysteamine were treated with high doses of cimetidine by intragastric gavage (20 mg/100 g, twice daily) to maximally raise gastric pH. Control rats received vehicle alone. The animals were killed after 5 days, and the gastric pH was measured. The ulcer beds were excised and the bFGF in the ulcer bed was extracted, purified, and quantitated (as described in the legend of Fig. 8). The cimetidine-treated animals had a pH range of 6.4 to 7.2, and their ulcer beds contained significantly higher levels of biologically active bFGF than did those of untreated animals (pH 3.4–4.3)

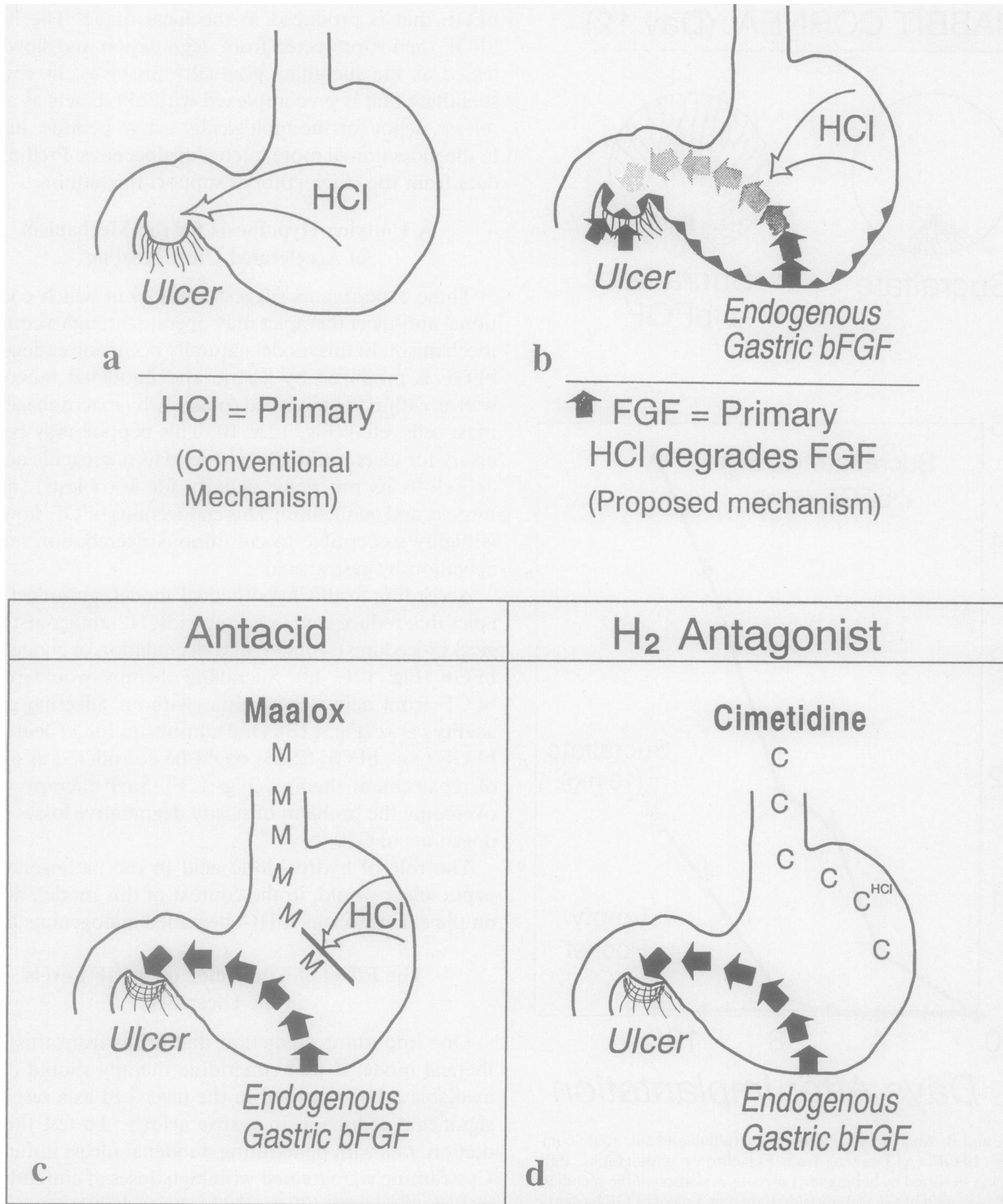


FIG. 12. Models of ulcer formation and anti-ulcer therapy. (a) A conventional model in which HCl is believed to directly cause duodenal ulcers. (b) A proposed mechanism in which endogenous bFGF in the stomach and duodenum may act to prevent ulceration unless this peptide is excessively degraded by HCl. (c) A hypothetical model of anti-ulcer therapy in which neutralization of HCl permits increased availability of endogenous bFGF in the ulcer bed. (d) A hypothetical model of anti-ulcer therapy in which an H2 antagonist decreases secretion of HCl, thus interfering with degradation of bFGF in the gastric mucosa in the ulcer bed. (e) A hypothetical model of anti-ulcer therapy in which sucralfate binds and protects available endogenous bFGF without affecting levels of HCl. (f) A hypothetical model of anti-ulcer therapy by oral delivery of an angiogenic peptide that has been made acid resistant (bFGF-CS23). In this model, exogenous bFGF can be considered as replacement therapy for endogenous bFGF undergoing degradation by HCl.

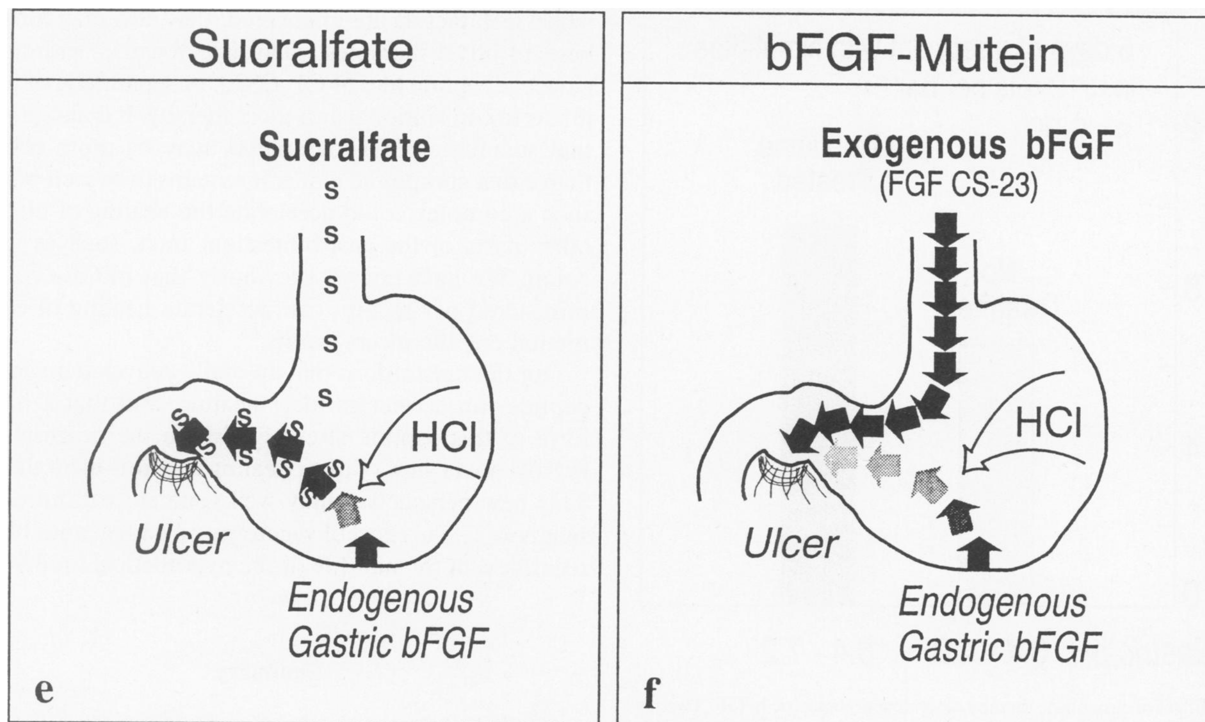


FIG. 12. (*Continued*)

($p < 0.001$), (Fig. 13). These results suggest that cimetidine anti-ulcer therapy may be FGF dependent.

Discussion

These studies show that oral administration of an acid-stable form of bFGF (bFGF-CS23) stimulates angiogenesis in the ulcer bed and significantly accelerates the healing of chronic duodenal ulcers in rats. Although our studies do not prove a causal relationship between enhanced angiogenesis and accelerated ulcer healing, neovascularization is a critical event in wound healing, and peptic ulcers are not dissimilar from chronic wounds. Furthermore bFGF is a growth factor for fibroblasts, smooth muscle cells, and epithelium.[26] Its ability to stimulate proliferation of these cells also could contribute to accelerated ulcer healing.

These experiments demonstrate for the first time that biologically active bFGF is present in human and rat gastric and duodenal mucosa, and also in the bed of duodenal ulcers in rats. Our studies do not define which cells are the source of bFGF in the stomach or duodenum. A recent report suggests that bFGF may be present also in normal human colon, but the method of detection was by immunostaining alone; biologic activity was not measured.[37]

Because sucralfate is now shown to be capable of binding and protecting bFGF from acid degradation, this finding may explain its mechanism of action. When this result is taken together with the finding that both sucralfate and cimetidine elevate levels of endogenous bFGF in the stomach and in the ulcer bed, it appears that the anti-ulcer properties of these drugs may be FGF dependent. We have extended this concept to construct a hypothetical model of ulcer formation and ulcer therapy in which endogenous bFGF is continually produced by gastric and duodenal mucosa, and may play a protective or preventive role in ulcer formation. Production of endogenous bFGF is balanced, however, by continuous inactivation of the peptide because of its acid sensitivity. Once an ulcer has formed, bFGF also appears to be produced in the ulcer bed, and may be a possible contributing factor in cases of spontaneous healing.

The preventive role of bFGF in ulcer formation is speculative, and we currently have no direct evidence for it. It remains to be seen whether endogenous bFGF eventually will be considered as a component of the cytoprotective mechanism in gastric mucosa.[38–40]

We wish to emphasize that the hypothesis proposed here attempts to provide a unifying mechanism for the anti-ulcer effect of H2-antagonists, antacids and sucralfate, based on our data. This hypothesis does not take into account other anti-ulcer therapies, such as prostaglandins,[41] and it may not apply to stress ulcers.[42]

Furthermore bFGF is not the only endogenous growth factor likely to be important in healing of duodenal ulcers or in their prevention. Epidermal growth factor (EGF),

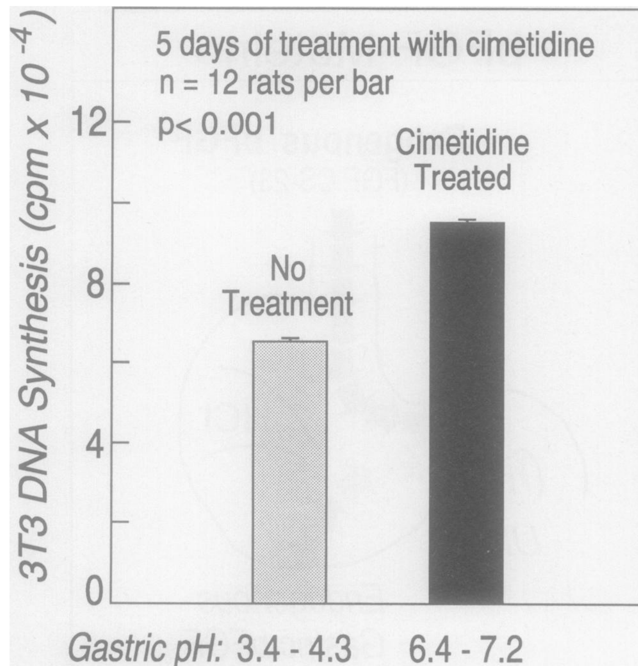


FIG. 13. Effect of cimetidine therapy on endogenous gastric bFGF. Twenty four rats with chronic duodenal ulcers induced by cysteamine were divided randomly into two groups of 12 rats each. The first group received no treatment except for gavage with vehicle (water) twice daily. The second group received cimetidine, 20 mg/100 g by intragastric gavage twice daily for 5 days. All animals were killed on day 6, and the pH of the gastric juice was measured. The untreated animals had a pH range of 3.4 to 4.3 compared with the cimetidine-treated animals, which had a pH of 6.4 to 7.2. The ulcer beds were excised, and the bFGF in the ulcer bed was analyzed as described in the legend of Figs. 8 and 9. The extracted bFGF was analyzed for DNA synthesis activity on the 3T3 fibroblasts. Cimetidine-treated animals had significantly higher levels of biologically active bFGF in the ulcer bed than untreated animals ($p = <0.001$).

found in saliva, can accelerate healing of experimental ulcers and is about as effective as cimetidine.[43-45] It also reduces gastric acid.[46] Transforming growth factor-alpha (TGF-α), shares about 35% amino acid sequence homology with EGF and binds to the same cell surface receptor as EGF. Transforming growth factor-alpha also inhibits gastric acid secretion.[47] It is angiogenic,[48] although it may be less potent than bFGF. Expression of mRNA for TGF-α has been found in the gastric mucosa of guinea pig, rat, and dog, and also in the gastric mucosa of patients undergoing gastric resection for peptic ulcer or for cancer.[49]

Studies of healing wounds show a cascade of growth factors released at first by platelets (*i.e.*, platelet-derived growth factor and TGF-β), and later by macrophages and other cells that enter the wound. If the principles of peptic ulcer healing are similar to the general rules of chronic wound healing, then one might predict that multiple endogenous growth factors may be cooperating to bring about spontaneous healing of a duodenal ulcer. If and when such factors are uncovered, they also may form the basis of novel therapies when used together with an angiogenic peptide like bFGF-CS23, or separately, or as adjuncts to conventional anti-ulcer therapy. It is also possible that sucralfate-bFGF complexes may be more effective than either compound alone. It remains to be seen whether such a complex could accelerate the healing of ulcers in other parts of the gastrointestinal tract, such as in the colon. We have shown previously that bFGF-CS23 administered per rectum can accelerate healing of experimental colonic ulcers in rats.[50]

Our demonstrations that an orally delivered angiogenic peptide can accelerate ulcer healing, and that a natural form of this peptide already exists in the mucosa, have led to a novel view of ulcer healing and anti-ulcer therapy. This new perspective may well generate fruitful experiments, (*e.g.*, the effect of vagotomy on endogenous bFGF), regardless of the half-life of the hypothetical model itself.

Summary

Oral administration of an acid-stable form of the angiogenic peptide, basic fibroblast growth factor (bFGF-CS23) stimulates angiogenesis in experimental duodenal ulcers and accelerates their healing more potently than cimetidine. Naturally occurring endogenous bFGF is present in the rat and human gastric and duodenal mucosa, but is very sensitive to acid degradation.

Sucralfate and sucrose octasulfate have a high affinity for bFGF and protect bFGF from acid degradation and inactivation. Oral administration of sucralfate elevates local levels of bFGF in the ulcer bed. Sucralfate is also a potent angiogenesis stimulator, apparently on the basis of its ability to stabilize and slowly release locally available bFGF. This may be the mechanism of its anti-ulcer efficacy, because it does not reduce gastric acid.

Cimetidine therapy, which does reduce gastric acid, also significantly increases the level of biologically active bFGF in the ulcer bed. The mechanism here may be related to the longer survival of endogenous bFGF at slightly higher pH.

A unifying hypothesis is proposed to provide a common mechanism for conventional anti-ulcer therapy. In this model, endogenous bFGF plays a central role, and oral administration of exogenous bFGF-CS23 provides a form of replacement therapy as a new approach to the problem of duodenal ulcer.

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