Bacterial colonization and healing of gastric ulcers: the effects of epidermal growth factor

SUSAN N. ELLIOTT,1 J. L. WALLACE,1 W. MCKNIGHT,1
D. G. GALL,2 J. A. HARDIN,2 M. OLSON,2 AND A. BURET3
Department of 1Pharmacology and Therapeutics, 2Gastrointestinal Research Group, and
3Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4

EGF receptor has been found throughout the fetal and adult gastrointestinal tract, liver, and pancreas (10). Binding of the EGF receptor activates the intrinsic tyrosine kinase, which then leads to a complex cascade of cellular events that ultimately result in DNA synthesis and cellular growth (2). Chronic administration of EGF produces a significant increase in mucosal DNA, RNA, and protein content (19). This proliferative action of EGF is believed to contribute to the normal maintenance of mucosal integrity within the gastrointestinal tract (35). EGF has also been shown to be beneficial in pathophysiological processes in the gastrointestinal tract by either reducing injury (22, 27, 36) or accelerating repair (37, 41, 51). EGF administration is capable of providing protection against a variety of gastrointestinal insults, both acid dependent (25, 27) and acid independent (22, 25). The importance of EGF in ulcer healing is demonstrated by the marked increase of EGF receptors and EGF-producing cells around experimental gastric ulcers in rats induced by acetic acid (41, 43) or cryoprobe (1). Furthermore, the removal of submandibular glands in rodents significantly reduces the rate of healing of experimentally induced gastric ulcers, and exogenous administration of EGF to these animals greatly enhances healing (41, 42). In addition, skin wounds in mice heal more slowly when the animals are caged individually than when the mice are caged in groups in which communal licking occurs. Salivary gland extirpation in these animals retarded wound closure, and the topical application of EGF to these wounds accelerated healing (17, 31). EGF also increases epithelial brush-border surface area (15), electrolyte and nutrient uptake (34), phospholipid synthesis (6), and glucose (16), galactose, (39) and glycine (39) uptake.

It is well recognized that EGF is critical to mucosal protection and repair by decreasing acid secretion (5), increasing gastric blood flow (44), improving restitution (37), increasing both the synthesis and secretion of mucus (36, 46), and stabilizing the actin cytoskeleton (15, 37). However, recent work has demonstrated that EGF may play a protective role in the gastrointestinal tract by preventing bacterial colonization of the normal intestinal mucosa (4). EGF has been shown to decrease the incidence of burn-induced bacterial translocation in mice (52). Liu et al. (29), using a model of acute pancreatitis in rats, demonstrated that EGF treatment could also significantly decrease bacterial translocation while restoring and/or maintaining intestinal mucosal struc-
Gastric ulcer induction results in markedly elevated levels of bacterial colonization at the ulcer site, which in turn delays ulcer healing (7). It having been previously demonstrated that EGF administration could significantly prevent bacterial colonization of healthy intestinal mucosa (4), experiments were needed to determine if accelerated gastric ulcer healing observed with EGF administration may be due to decreases in bacterial colonization. Therefore, using experimental gastric ulcers in rats, we aimed in this study to examine the effects of oral EGF administration in a model of mucosal injury with preexisting bacterial colonization.

**MATERIALS AND METHODS**

Animals. Male Wistar rats weighing 175–200 g were obtained from Charles River Laboratories (St. Constant, PQ, Canada). The animals were housed within the animal care facility of the University of Calgary in polycarbonate cages. The rats had free access to standard pellet chow and tap water throughout the experiment, with the exception of the overnight fast in which the animals were deprived of food, but not water, for 18–24 h before the experiment was performed. The experimental protocol was approved by the Animal Care Committee at the University of Calgary, and the experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

Induction of ulcers. Rats were fasted overnight with free access to water. Ulcers were induced using a model (8) modified from that originally described by Okabe and Pfeiffer (32). Briefly, under halothane anesthesia, a midline laparotomy was performed and the stomach was gently exteriorized. The barrel of a 3-ml syringe, which had been cut and filed smooth, was placed on the serosal surface of the stomach in the corpus region. Half a milliliter of 80% acetic acid (vol/vol) was instilled into the barrel of the syringe and allowed to remain in contact with the stomach for 1 min, after which time it was aspirated off and the area was gently rinsed with sterile saline. The area exposed to acetic acid was 59.7 mm². Gastric ulcer area was determined as follows. The rats were killed by cervical dislocation, and the stomach was removed for ulcer area determination, and tissue samples were taken for bacterial culturing. The difference between bacterial levels recovered from vehicle and EGF or antibiotic-treated ulcer beds was calculated and expressed as a percentage of the average number of bacteria recovered from the vehicle-treated group. This value was referred to as percent clearance. Body weight was measured daily throughout the study.

Direct effects of EGF on bacteria in vitro. The effects of EGF on bacterial growth were determined in vitro. Three bacterial isolates were used for these studies: 1) gram-positive Enterococcus faecalis isolated from fresh rat feces as a single colony grown on a TSB agar plate for 18 h at 37°C, 2) gram-negative Escherichia coli isolated from fresh rat feces as a single colony grown on a TSB agar plate for 18 h at 37°C, and 3) a streptomycin-resistant strain of E. coli (C-25) that has previously been shown to delay healing of gastric ulcers in rats (7). The E. faecalis and E. coli isolated from fresh feces were identified as such by the Veterinary Pathology Laboratory (Edmonton, AB, Canada) using standard bacterial identification sensitivity assays. All bacterial stock cultures were stored at −70°C in TSB (Difco Laboratories, Detroit, MI) coated onto Microbank porous beads (Pro-Labs Diagnostics, Richmond Hill, ON, Canada). In a series of three experiments, log phase bacteria (10⁵ CFU/ml) were added in duplicate to wells on a 96-well plate containing TSB with either no EGF (control) or 10 µM EGF, in a total volume of 100 µl/well. This concentration was chosen to reveal the higher end of EGF levels that may be encountered by gastrointestinal bacteria in vivo (11) and is consistent with previous studies using similar experimental protocols of oral EGF administration in infected animals (4). At 1-h intervals (0–5 h postinoculation), viable bacterial cells in each well were counted by serial dilution and culture on TSB agar plates (for E. faecalis) or MacConkey agar plates (for E. coli) for 18 h at 37°C. Bacterial numbers are expressed as log₁₀ CFU per milliliter.

Myeloperoxidase assay. Myeloperoxidase (MPO) is an enzyme that is found predominantly in the azurophilic granules of neutrophils and may be used as a quantitative index of inflammation (28). An additional group of rats (n = 5–10/group) that had ulcers induced and underwent treatment with either vehicle, EGF (100 µg/kg), or the streptomycin/penicillin combination had tissue samples from the ulcer site significantly prevent bacterial colonization of healthy intestinal mucosa. Enterobacteriaceae and gram-positive Staphylococci were isolated from the normal stomach and from the ulcer bed. The bacterial load was determined as log₁₀ CFU per gram of tissue.

Effect of EGF treatment. The effects of daily treatment with EGF were assessed. Ulcers were induced in the rats as described above. On the seventh day after ulcer induction, a 7-day treatment period was initiated, during which EGF (1 or 100 µg/kg) was orally administered once daily. The vehicle for EGF was sterile water; control rats received the same volume of vehicle. For comparison, the effect of twice-daily oral treatment with the combination of streptomycin (336 mg/ml; 0.25 ml) and penicillin (168 mg/ml; 0.25 ml) was also determined. This treatment regimen has previously been shown to be capable of significantly reducing total aerobic bacterial levels and significantly accelerating healing using this ulcer model (7). Fourteen days after ulcer induction, the rats were killed by cervical dislocation, the stomach was removed for ulcer area determination, and tissue samples were taken for bacterial culturing. The difference between bacterial levels recovered from vehicle and EGF or antibiotic-treated ulcer beds was calculated and expressed as a percentage of the average number of bacteria recovered from the vehicle-treated group. This value was referred to as percent clearance. Body weight was measured daily throughout the study.

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taken and processed for the MPO assay as previously described (30). The results were compared with MPO activity levels measured in control animals or animals without ulcers. Results are expressed as units of MPO activity per milligram of tissue.

Transmission electron microscopy. Tissue samples taken from the ulcer bed were fixed in 5% gluteraldehyde in 5% PBS, pH 7.4, at 20°C. Specimens were washed in buffer, postfixed in 1% OsO4, and dehydrated in increasing concentrations of distilled ethanol. Samples were cleared with propylene oxide, infiltrated, and embedded in Spurr’s low-viscosity medium. Thin sections (90 nm) were double stained with saturated uranyl acetate in 50% ethanol, followed by 0.4% lead citrate (49). Specimens were examined on a Hitachi 7000 transmission electron microscope.

Effects of EGF on acid secretion. EGF is able to regulate gastric acid secretion, which may in turn affect both the levels of bacteria colonizing the ulcer site and the rate of ulcer healing. To determine the effect of EGF on acid secretion, a continuous gastric perfusion system was used to measure gastric output (50). Rats were anesthetized with urethan (250 mg/kg) and placed on homeothermic blankets to maintain their body temperature at 37°C. A jugular vein was cannulated for pentagastrin administration, and a tracheostomy was performed. An orogastric catheter was inserted and secured with two ligatures around the esophagus. A midline laparotomy was performed, and duodenogastric cannalua were inserted and secured with ligatures. The stomach was gently flushed with ~20 ml of 37°C saline to rinse it clean of any residual matter.

Ulcers were induced in rats by serosal application of acetic acid. On the seventh day after ulcer induction, daily oral administration of EGF (100 µg/kg) or vehicle began. Basal and pentagastrin-stimulated acid secretion were measured in rats that had received EGF for either 3 or 7 days. Acid output was also measured in rats that had received an equal volume of vehicle (sterile water) for the same amount of time. Each test group consisted of 4 or 5 rats. Rats were fasted for 24 h, with free access to drinking water before the experiment. During the experiment, the stomach was perfused with isotonic saline (37°C, 3.0 ml/h). After a 15-min stabilization period, perfusate was collected for three 30-min periods. After the first period (basal secretion), a bolus dose of 20 µg/kg pentagastrin was administered intravenously, followed by continuous infusion of 20 µg·kg⁻¹·h⁻¹ for 60 min. Perfusates were collected, and the concentration of acid in each sample, expressed as microequivalents per 30 min, was determined by titration to pH 7.0 using a Metrohm automated titrator (Brinkmann Instruments).

Statistical analysis. All data are expressed as means ± SE. Comparisons among groups of data were made using a one-way ANOVA and a Dunnet’s multiple-comparisons test. Values of P < 0.05 were considered significant.

Materials. Human recombinant EGF was obtained from Austral Biologicals (San Ramon, CA). E. coli C-25 was generously provided by Dr. E. Deitch (New Jersey Medical School). The bacterial culturing materials were from Becton-Dickinson (Cockeysville, MD). All transmission electron microscopy reagents and the Spurr’s low-viscosity medium were obtained from J. B. EM Services (Dorval, PQ, Canada). Streptomycin sulfate, penicillin G, urethan, pentagastrin, and all reagents utilized in the MPO assay were obtained from Sigma Chemical (St. Louis, MO).

RESULTS

Induction of ulcers and gastric ulcer healing. Serosal application of acetic acid results in the reproducible formation of spherical ulcers that persist for several weeks. Previous histological assessment of the ulcers 7–14 days after acetic acid application revealed a thick layer of granulation tissue with glandular disorganization at the ulcer margins. Furthermore, these lesions involved the full thickness of the mucosa and penetrated into the muscularis mucosae (8). Perforation was not observed with this model.

Rats treated with vehicle from day 7 to day 14 after ulcer induction had a mean ulcer area of 38.1 ± 6.4 mm² (Fig. 1). Daily administration of EGF significantly reduced ulcer area. Treatment with EGF at a dose of 1 µg/kg resulted in a mean ulcer area, on day 14, of 16.1 ± 5.3 mm² (P < 0.05 compared with vehicle), whereas EGF at 100 µg/kg resulted in a mean ulcer area of 12.1 ± 3.1 mm² (P < 0.01 compared with vehicle). Consistent with previous findings, daily treatment with the streptomycin/penicillin combination significantly reduced ulcer area to a mean of 12.6 ± 2.6 mm² (P < 0.01 compared with vehicle-treated animals). The reduction in gastric ulcer area observed with EGF...
administration at both doses of EGF was not significantly different from that observed with antibiotic administration.

Bacterial colonization of gastric ulcers. Bacterial numbers in gastric ulcer samples after 7-day treatment with either vehicle, EGF, or streptomycin/penicillin are illustrated in Fig. 1. Rats receiving vehicle over the 7-day treatment period had a mean aerobic bacterial level of $3.2 \times 10^6$ CFU/g tissue at the ulcer site, a level significantly ($P < 0.01$) elevated over those seen in tissue cultures taken from the stomach of rats without ulcers ($-3 - 4 \log_{10}$ CFU/g tissue; Ref. 7). Administration of EGF at both 1 µg/kg and 100 µg/kg significantly ($P < 0.01$) reduced aerobic bacterial levels (5.0 ± 0.4 and 5.3 ± 0.3 log$_{10}$ CFU/g tissue, respectively) relative to the rats receiving vehicle. Treatment with the streptomycin/penicillin combination also resulted in a marked reduction in aerobic bacterial colonization at the ulcer site (4.9 ± 0.3 log$_{10}$ CFU/g tissue; $P < 0.01$ vs. vehicle-treated animals). Weight gain over the seven-day treatment period did not significantly differ among any of the groups (data not shown).

Direct effects of EGF on bacteria in vitro. To assess whether the EGF-induced reduction in bacterial colonization observed in vivo was due to a direct antibacterial effect of EGF, growth of three bacterial isolates was assessed in vitro in the presence or absence of EGF. Endogenous E. faecalis and E. coli, as well as the laboratory strain E. coli C-25, have been shown to delay healing of experimental gastric ulcers in rats (7). In medium without EGF, mean bacterial growth between 0 and 5 h for fecal gram-negative E. coli, gram-positive E. faecalis, and E. coli (C-25) was 2.13, 1.31, and 1.84 log$_{10}$ CFU/ml, respectively. No difference in bacterial proliferation was observed when 10 µM EGF was added to the medium for either isolate (Figs. 2 and 3).

Transmission electron microscopy and MPO assay. Significant inflammatory cell infiltrate was observed in ulcers of each group. Cells involved were neutrophils, eosinophils, and mast cells. A representative micrograph is shown in Fig. 4. Neutrophil infiltration was confirmed using the MPO assay. Control gastric tissue taken from rats without ulcers had a very low MPO level (13.1 ± 2.4 U/mg tissue) (Fig. 5). Ulcer induction resulted in a significant elevation of MPO levels. Vehicle-treated animals had an MPO level of 113.1 ± 15.4 U/mg tissue ($P < 0.05$ vs. control animals), and treatment with either the streptomycin/penicillin combination (129.6 ± 28.2 U/mg tissue) or 100 µg/kg EGF (112.4 ± 20.0 U/mg tissue) did not affect MPO concentrations compared with vehicle-treated animals.

Effects on acid secretion. Figure 6 summarizes the results of the in vivo perfused stomach preparations in which basal and pentagastrin-stimulated acid secretion was measured. Pentagastrin infusion significantly increased acid output during the 30- to 60-min period in both the 3- and 7-day vehicle and EGF treatment groups compared with the corresponding levels of basal secretion. No difference in acid secretion was observed between the vehicle- and EGF-treated animals during any of the collection periods.

DISCUSSION

The epithelium of the gastrointestinal tract is one of the most rapidly proliferating tissues in the body. EGF is intimately involved in the maintenance of mucosal integrity because of its potent ability to stimulate DNA synthesis and subsequently regulate the proliferation and differentiation of a wide variety of cell types in the gastrointestinal tract. Extensive work has detailed the
effects of EGF and its ability to prevent and accelerate the healing of gastric ulcers (14, 18, 43).

Findings from this study demonstrate that, together with its healing properties, EGF inhibits bacterial colonization in gastric ulcers. Daily oral administration of recombinant human EGF, at 1 and 100 µg/kg for 1 wk, resulted in a reduction in gastric ulcer area (Fig. 1). Consistent with previous findings, daily treatment with the streptomycin/penicillin antibiotic combination also significantly reduced gastric ulcer area after a 1-wk dosing regimen (7), and the degree of healing was similar to that seen in EGF-treated rats. Together, these results suggest that the anti-infective effects of EGF, at least in part, contribute to gastric ulcer healing.

Endogenous EGF is critical in the healing of chronic ulceration because the extirpation of the salivary glands in several different models results in a significant delay in wound healing (1, 17, 31, 41, 43). Furthermore, Wright et al. (51) found that ulceration of the human epithelium in the gastrointestinal tract induces the development of a novel cell lineage from stem cells that contains and secretes abundant amounts of immunoreactive EGF. It has been suggested (47) that EGF responds to the presence of an ulcer because there is an observable increase in the EGF concentration in the gastric juice of patients with duodenal ulcers. Working in concert with the increased secretion of EGF when ulceration is present, there is a notable increase in EGF receptor numbers adjacent to the ulceration (14, 21, 43). The enhanced gastric ulcer healing observed with exogenous administration of EGF is consistent with previous observations that luminal EGF is capable of stimulating growth and repair when given to the damaged bowel of rats and humans (18, 22).

Recent work using the acetic acid ulcer model revealed elevated levels of bacterial colonization within the gastric ulcer bed as early as 12 h after ulcer induction (7). The presence of bacteria in the ulcer bed prolongs the chronicity of the ulcer (7). Previous work, in which EGF was administered to rabbits before oral inoculation with enteropathogenic E. coli (4), demon-
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It was demonstrated that EGF inhibits bacterial colonization, which in turn reduces the extent of microvillus injury and disaccharidase deficiencies caused by the infection. The present study sought to determine whether the EGF-induced gastric ulcer healing was due to a reduction of bacterial colonization in the ulcer bed. Consistent with the previous study (4), we observed a significant reduction in bacterial colonization of ulcers after the 7-day dosing with EGF at both 1 and 100 μg/kg (Figs. 2 and 3). Furthermore, previous studies (4) have shown that EGF at lower (0.1 or 1 μM) and higher (100 μM) concentrations does not affect bacterial growth in vitro. Thus EGF did not reduce bacterial colonization and accelerate gastric ulcer healing via a bactericidal action. This is consistent with results reported by Okuyama et al. (33), who examined the effect of EGF on bacterial translocation in newborn rabbits. These authors reported that EGF was capable of significantly reducing spontaneous bacterial translocation without significantly affecting small bowel bacteria.

Neutrophil infiltration is the hallmark of inflammatory disorders of the gastrointestinal tract. With the use of transmission electron microscopy, we observed a pronounced neutrophil-predominant inflammatory response in tissues taken from the gastric ulcer bed of vehicle-treated animals, an observation confirmed by high levels of MPO activity in ulcer tissue samples. Because neutrophils are capable of releasing a wide array of substances that can destroy cells and dissolve connective tissue, and thereby retard wound healing, the effect of EGF treatment on neutrophil infiltration needed to be determined. Results from the MPO assay clearly demonstrated that neutrophil infiltration in ulcer tissues was not different among the EGF-, antibiotic-, or vehicle-treated animals (Fig. 5). These results are consistent with the inability of EGF administration, after the induction of experimental colitis in rats, to reduce the amount of intestinal inflammation as assessed by the MPO assay (36). This suggests that the effects of EGF were not attributable to altered neutrophil infiltration into the inflamed and damaged tissue.

In addition to its anti-infective properties and proliferative effects on tissues, EGF is capable of regulating gastric acid secretion. After short-term administration of EGF, inhibition of agonist-stimulated acid secretion has been observed in vivo in humans (24), dogs (26), and rats (12) and in vitro in parietal cells or gastric glands from guinea pigs (9), rats (40), and rabbits (5). In vitro findings suggest that EGF exerts a direct effect on the parietal cell to reduce acid secretion (48). Our results failed to demonstrate an inhibition of acid secretion. In contrast, Chew et al. (5) demonstrated, using rabbit gastric parietal cells, that long-term administration of EGF can stimulate acid secretion. Multiple studies have demonstrated an increase in EGF levels when ulceration is present (14, 21, 43, 51). Together with the daily oral administration of EGF during the study, the concentrations of EGF in gastric tissue may have reached levels sufficient to stimulate, rather than inhibit, acid secretion. The primary role of acid in the stomach is to kill bacteria. Indeed, a prolonged reduction in acid secretion predisposes to infection with a variety of bacteria (20, 45). In this context, an increase in acid secretion could theoretically aid in ulcer healing. Of course, there is substantial evidence from both animal models and human studies to demonstrate that suppression of acid secretion results in accelerated ulcer healing (3, 13, 38).

In the present study, we found that EGF administration did not influence gastric acid secretion. Treatment with EGF for 3 or 7 days, starting on the seventh day after ulcer induction, did not significantly alter acid secretion compared with animals that had been administered vehicle over the same time period. Thus the accelerated healing observed with EGF administration in this study was independent of altered acid secretion.

Findings from this study indicate that the EGF-induced reduction in ulcer size was associated with a significant decrease in bacterial colonization of the ulcer bed. We have previously demonstrated that the presence of bacteria within the ulcer inhibits healing of gastric ulcers (7). From these results, we speculate that the parallel decrease in ulcer area and bacterial levels observed after EGF treatment suggests that EGF may promote healing via an anti-infective mechanism. Although we are unable to conclusively delineate the mechanism of the EGF-induced reduction in bacterial colonization, the present study provides evidence to rule out some alternative potential mechanisms, i.e., a direct antibacterial effect, altered acid secretion, and modulation of neutrophil infiltration.

In summary, the present study demonstrated that oral administration of recombinant human EGF significantly reduced bacterial colonization of gastric ulcers. This effect is not due to a direct antibacterial effect of EGF. EGF treatment did not affect neutrophil infiltration or acid secretion. Together, these findings show that EGF enhances ulcer healing, at least in part, by inhibiting bacterial colonization in the damaged mucosa via mechanisms that are independent of a bactericidal or anti-inflammatory effect. Together with our previous findings (4), we speculate that EGF may have potential as an oral therapeutic agent to control infection-induced gastrointestinal pathophysiology.

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