Demonstration of Sucralfate-mediated Preservation of Growth Factor Bioactivity in the Presence of Low pH with a Human Gastric Epithelial Cell Line (AGS)

Huaibao Sheng,[1] Praful K. Shah,[2] and Kenneth L. Audus[1,3,4]

Received January 4, 1996; accepted March 30, 1996

KEY WORDS: gastric epithelium; AGS; sucralfate; epidermal growth factor; basic fibroblast growth factor.

INTRODUCTION

Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) have possible therapeutic use as wound healing agents (1). The potential of growth factors as therapeutic agents has prompted investigations into the development of suitable formulations for stabilization of the growth factors (2–4). These formulations are based on the observation that when bound to heparin-like molecules, bFGF and EGF are protected from thermal and low pH-induced degradation (3,5–9).

The structure of the sucrose octasulfate component of sucralfate is similar to the repeating disaccharide units of heparin and has a very high affinity for bFGF, EGF, and related growth factors (3,6,8). Both bFGF and EGF are effective in some degree in promoting *in vivo* gastric epithelial cell growth (9,10) and restitution in exised gastric mucosa (11). Sucralfate in *in vivo* studies has been shown to elevate bFGF levels in the gastric mucosa (6) and both bFGF and EGF factors are protected from thermal- and low pH-induced inactivation when co-administered with sucralfate *in vivo* (6,12). One of the proposed mechanisms of action for sucralfate as an antiulcer agent likely involves the preservation of growth factor bioactivity which in turn promotes the healing of gastroduodenal ulcers by increasing epithelial and endothelial cell proliferation (6).

The purpose of the current study was to develop a representative *in vitro* system that would allow evaluation of some of sucralfate's mechanisms of action as an antiulcer agent in the stomach. Specifically, an appropriate *in vitro* gastric epithelial cell system was desired that would be responsive to bFGF and EGF and that would be sensitive to changes in the bioactivity of peptides exposed to low pH conditions with or without different sucralfate formulations present. The availability of a convenient *in vitro* system would permit rapid evaluation of alternative drug formulations and chemically modified derivatives of sucralfate or related agents. A stable, mucus secreting human gastric epithelial cell line derived from a human adenocarcinoma, AGS (13), was chosen for evaluation in this study. We and others have shown (13,14) that this cell line retains some basic features of normal gastric epithelium.

[1] Department of Biochemistry, University of Kansas, Lawrence, Kansas 66045.

[2] Hoechst Marion Roussell, Kansas City, Missouri 64134.

[3] Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kansas 66045.

[4] To whom correspondence should be addressed.

ABBREVIATIONS: bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; MTT, 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide; KSOS, potassium sucrose octasulfate.

MATERIALS AND METHODS

Materials

Ham's F-12 was purchased from JRH Biosciences (Lenexa, KS). Fetal bovine serum was obtained from Hyclone Laboratories, Inc. (Logan, UT). Tris was supplied by Bio-Rad Laboratories (Richmond, CA.). 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was obtained from Eastman Kodak Company (Rochester, NY). The following reagents were purchased from Sigma company (St. Louis, MO): Dulbecco's Modified Eagle's medium, penicillin, streptomycin, HEPES buffer, and trypsin/EDTA. All other reagents were of the highest grade commercially available. All tissue culture plates and centrifuge tubes used in this study were purchased from Corning Costar (Cambridge, MA).

Drug Preparations

Sucralfate, sucralfate-xg (a xanthine gum-containing commercial formulation of sucralfate), potassium sucrose octasulfate (KSOS), and $Al(OH)_3$ were gifts from Marion Merrell Dow Laboratories (Kansas City, MO). These components were diluted in serum-free culture medium to desired concentrations before each experiment. Human recombinant basic fibroblast growth factor (bFGF; 17 kDa) and human recombinant epidermal growth factor (EGF; 6.2 kDa) were purchased from Promega company (Madison, WI), were prepared as a stock solutions in sterile PBS, and then were diluted in serum-free culture medium to desired concentrations before each experiment.

Cell Culture

The human adenocarcinoma cell line, AGS (14), was obtained from American Type Culture Collection (ATCC# CRL-1739). The cells (6×10^5 cells/plate) were grown in 100 mm Corning polystyrene tissue culture plates with 15 ml 1:1 (v:v) ratio of Ham's F12 and DME medium containing 100 μg/ml of penicillin, 100 μg/ml of streptomycin, 15 mM of HEPES, 1.2 g/L of sodium bicarbonate and 5% of fetal bovine serum, and were maintained in an incubator at 37°C, 95% humidity and 5% CO_2. The culture medium was changed two days after seeding and every other day, thereafter. The subcultured cells were harvested every five days with 0.25% trypsin plus 0.05% EDTA solution, and washed once with PBS before cells were seeded into other plates for experiments. A phase-contrast microscope (Nikon TMS) was used to observe the cell's growth and morphology throughout the culture period. Cells from passages 40–60 were used in these experiments.

0724-8741/96/0700-1122$09.50/0

MTT Colorimetric and Colony-forming Efficiency

Cells were seeded into 96-well Corning Costar polystyrene tissue culture plates (5,000 cells/well) with 100 μl culture medium. After two days, the cells were switched to serum-free culture medium with bFGF or EGF added. The cells were incubated for 44 hours and the MTT colorimetic assay was performed as previously detailed by Zheng et al. (15). Briefly, the medium with growth factors was changed to 100 μl fresh culture medium containing 10 μl of MTT solution (5 mg/ml in PBS) and returned to the incubator. After a 4 hour incubation, 100 μl acid isopropanol (0.04 N HCl in isopropanol) was added to all wells and the plate was incubated overnight at room temperature. The color changes were recorded on a microplate reader (Cambridge Technology, Inc. Watertown, MA) at 540 nm.

For colony forming efficiency, cells were seeded into 24-well Corning polystyrene plates. Each well received 100 cells with 1 ml culture medium containing 2.5% FBS. After two days in culture, the cells were exposed to either bFGF or EGF (0–10 ng/ml) for up to 8 days under standard culture conditions. In this 8-day period, the medium was not changed or supplemented. Resultant colonies that grew in each well were fixed by formalin-alcohol solution and dyed with 1% aqueous crystal violet. The number of colonies in each well was counted visually under a phase contrast microscope (14,15).

Low-pH Treatment of Growth Factors

A 100 μl aliquot of either bFGF or EGF (1 μg/ml) was mixed with 100 μl PBS or 100 μl sucralfate (10 μg/ml) (or, alternatively, 10 μg/ml of KSOS or sucralfate-xg, $Al(OH)_3$) for 10 minutes, then added to 100 μl of 1 M acetic acid, pH 2.2, and incubated 1 hour at 37°C. The pH was determined to be no higher than 3.0 for any of the mixtures except those with $Al(OH)_3$. Following the incubation, the mixture was neutralized by adding 5 ml culture medium, pH 7.4, and adjusting the pH to 7.4, as needed, with 5 N NaOH (about 10 μl). Finally, the solutions were diluted to desired concentrations and added to two-day cultures of AGS cells or two-day cultures of AGS colonies. Cell and colony numbers were determined by the MTT assay or colony-forming efficiency assay, respectively.

Data Analysis

Data were expressed as mean ± standard deviation for replicates of at least an n = 4. The differences between treatment and control groups were expressed as their units or as a percentage of corresponding control. A one-way ANOVA followed by the Scheff's multiple comparison test were applied to analyze the significance of differences between treatment and control groups of raw cell count data at the 0.05 level of significance.

RESULTS AND DISCUSSION

In this study, we were able to establish the sensitivity of AGS cell growth to two common gastric mucosal growth factors, EGF and bFGF. Both growth factors modestly stimulated AGS proliferation as measured by two independent tests (i.e., MTT, and colony forming efficiency) as shown in Figures 1A and 1B. Our results with EGF were consistent with Piontek et al. (16) who have demonstrated that AGS cells have EGF receptors and that the maximal stimulation of proliferation above controls, 140%, occurs in the presence of similar concentrations of this peptide. In a related study, Kuwayama et al. (10) also showed that 10 ng/ml EGF stimulates a maximum incorporation of thymidine of 170% of control in fetal rabbit gastric epithelial cell cultures. By contrast, neither receptor nor proliferation data for bFGF with AGS cells have apparently been reported. However, a recent study with two intestinal-derived epithelial cell types demonstrated a modest bFGF-

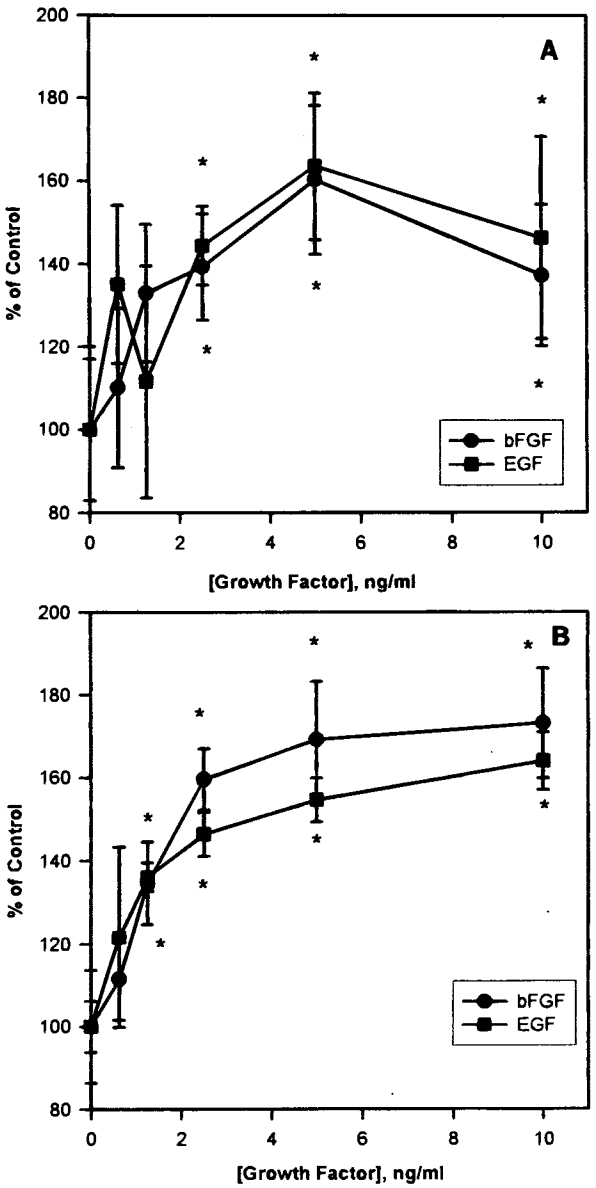

Fig. 1. Effects of bFGF and EGF on the A) Proliferation and B) Colony-forming efficiency of AGS Cells. For the proliferation measures, AGS cells were incubated with bFGF and EGF for 44 hours and viable cell numbers were quantitated with the MTT assay (n = 8). For colony forming efficiency, the AGS cells were incubated either bFGF or EGF for 6 days in 24-well plates and the colonies formed in each well counted (n = 4). Data points are means ± SD. *P < 0.05 versus the control cells which were not treated with a growth factor.

induced increase in cell proliferation above controls of 180% (17), which again, was consistent with our findings with bFGF.

The growth stimulating activity of bFGF and EGF is pH-dependent and abolished by low pH conditions in a time-dependent manner (6,12). We similarly confirmed that the proliferation of AGS cells was reduced in the presence of growth factors pretreated with solutions with pHs lower than pH 7.4 for one hour (data not shown). The critical point for loss of all growth stimulating activity of EGF appeared to be at about pH 4, and about pH 5 for bFGF, suggesting that bFGF was more sensitive to low pH conditions (data not shown). These findings were also in agreement with other studies (6,12). In experiments where 10–1000 μg/ml sucralfate was mixed with the growth factors prior to exposure to low pH conditions, the bioactivity of both EGF and bFGF was retained and is illustrated in Figure 2. In the other experiments, the ratio of sucralfate to EGF (or bFGF) was kept constant (100 μg sucralfate/5 ng growth factor) and the stimulatory activity of the growth factor was determined at selected concentrations. As shown in Figures 3A and 3B, EGF or bFGF alone and not exposed to low pH treatment produced typical concentration-dependent stimulatory effects on AGS growth as described earlier. Similarly, EGF or bFGF mixed with sucralfate had stimulatory effects after exposure to low pH conditions. Finally, neither EGF nor bFGF, after exposure to low pH conditions, had significant growth enhancing properties at any of the concentrations tested in the assays. Sucralfate alone had no effect on AGS cell growth (14).

Sucralfate has a high affinity for growth factors and the binding of growth factors is pH-dependent. At pHs around 7.0, about 10% of either EGF (12) or bFGF (8) will bind to sucralfate. At around pH 2.0, about 90% of either EGF (12) or bFGF (8) will bind to sucralfate. The results obtained with the AGS cells would suggest that growth factor association with sucralfate occurred at low pHs and preserved peptide bioactivity. From the perspective of the *in vivo* situation, the pH in the lumen of stomach approaches 2, at this pH sucralfate becomes a highly viscous gel-like substance which adheres to the ulcer crater and likely binds secreted growth factors, protecting them from the low pH environment. On the interface with the gastric epithelial cells, where the pH is near 7, sucralfate becomes insoluble and releases bound growth factors which are then free to diffuse to and interact with appropriate receptors on target endothelial and epithelial cells. Sucralfate apparently

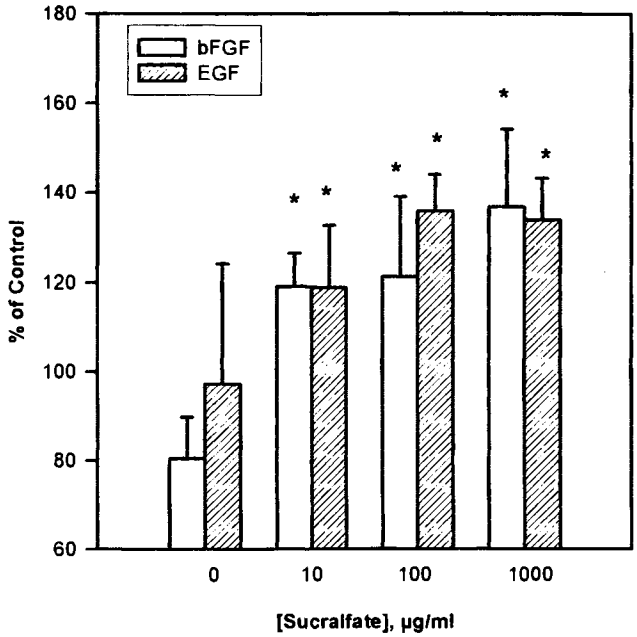

Fig. 2. Concentration-dependent effect of sucralfate for preserving bFGF and EGF bioactivity against low pH treatment. Either bFGF or EGF at 10 ng/ml were premixed with sucralfate before exposing to acetic acid (pH = 2.2). Cell numbers were measured by the MTT assay. Data points are means ± SD for n = 8. *P < 0.05 versus data from cells which were not treated with either growth factor or sucralfate.

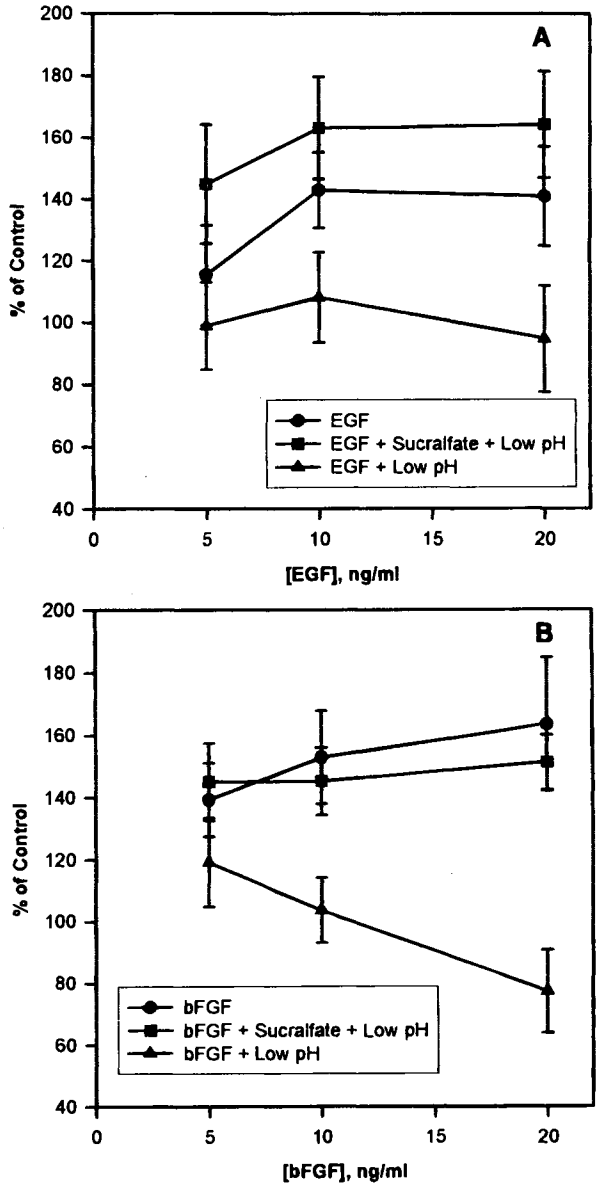

Fig. 3. Protective effect of sucralfate for either A) EGF or B) bFGF against low pH exposure. The growth factors were premixed with buffer or sucralfate (100 μg sucralfate/5 ng growth factor) before low pH (pH = 2.2) exposure. Viable cell numbers were measured by the MTT assay. Data points are means ± SD for n = 8.

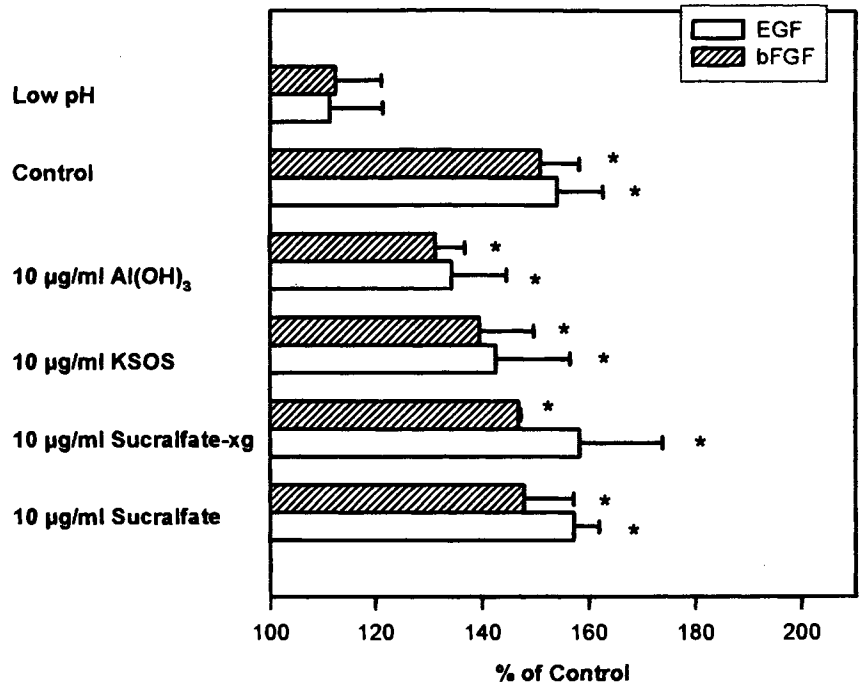

Fig. 4. Protective effects of sucralfate, sucralfate-xg (a commercial sucralfate formulation containing xanthine gum), potassium sucrose octasulfate (KSOS), or aluminum hydroxide ($Al(OH)_3$) for 10 ng/ml of either EGF or bFGF against a low pH (pH = 2.2) treatment. The control received only normal growth factor and the low pH data was for growth factor exposed to pH 2.2 only. Viable cell numbers were quantitated by the colony-forming efficiency assay. Data points are means ± SD for n = 6. *$P < 0.05$ indicates a significant difference from cell growth in the absence of any growth factor treatments.

facilitates an increase in the level of growth factors in the ulcer bed and cell proliferation, mucosal repair and gastroprotection are promoted (6,12). Our *in vitro* system effectively mimics these conditions and our results collectively support a protective role for sucralfate towards the bioactivities of EGF and bFGF under low pH conditions.

Volkin et al. (3) have shown that both sucralfate (i.e., aluminum sucrose octasulfate), and the soluble potassium salt of sucrose octasulfate, KSOS, bind and stabilize acidic fibroblast growth factor against thermal-, urea-, and low pH-induced unfolding. In results from our studies summarized in Figure 4, we observed that sucralfate, KSOS and sucralfate-xg (a xanthine gum-containing commerical sucralfate formulation) comparably preserved the biological functions of bFGF and EGF during exposure to low pH conditions. Although a component of sucralfate is aluminum, an acid neutralizer, aluminum is not believed to contribute significantly to the drug's overall activity as an antiulcer agent (18). Incubating the growth factors with an aluminum salt prior to exposure of the peptides to low pH preserved some bioactivity. However, at the concentration used, aluminum hydroxide alone neutralized the low pH solution to which the growth factors were exposed. By contrast, concentrations of sucralfate that preserved growth factor bioactivity did not alter the pH of the test solutions. Thus, the mechanism of growth factor protection in the presence of sucralfate was different from an aluminum salt alone. These findings were consistent with the observation that a variety of polyanionic heparin-like agents can signficantly protect growth factors from thermal- and low-pH conditions (2). Since there were differences in effects of a given agent, it appeared that the AGS cell line may be useful in distinguishing among formulations for optimal preservation of peptide bioactivity.

ACKNOWLEDGMENTS

This research was supported by a grant from Marion Merrell Dow, Inc. (presently known as Hoechst Marion Roussel), Kansas City, Missouri.

REFERENCES

1. P. ten Dijke and K. K. Iwata. Growth factors for wound-healing. *Biotechnology* 7:793–798 (1989).
2. P. K. Tsai, D. B. Volkin, J. M. Dabora, K. C. Thompson, M. W. Bruner, J. O. Gress, B. Matuszewska, M. Keogan, J. V. Bondi, and C. R. Middaugh. Formulation design of acidic fibroblast growth factor. *Pharm. Res.* **10**:649–659 (1993).
3. D. B. Volkin, A. M. Verticelli, K. E. Marfia, C. J. Burke, H. Mach, and C. R. Middaugh. Sucralfate and soluble sucrose octasulfate bind and stabilize acidic fibroblast growth factor. *Biochim. Biophys. Acta* **1203**:18–26 (1993).
4. K. Fukunaga, S. Hijikata, K. Ishimura, R. Sonoda, T. Irie, and K. Uekama. Aluminum β-cyclodextrin sulphate as a stabilizer and sustained-release carrier for basic fibroblast growth factor. *J. Pharm. Pharmacol.* **46**:168–171 (1994).
5. D. Gospodarowicz and J. Cheng. Heparin protects basic and acidic FGF from inactivation. *J. Cell. Physiol.* **128**:475–484 (1986).
6. J. Folkman, S. Szabo, M. Stovroff, P. Mcneil, W. Li, and Y. Shing. Duodenal ulcer: discovery of a new mechanism and development of angiogenic therapy that accelerates healing. *Ann. Surg.* **214**:414–427 (1991).

7. S. J. Prestrelski, G. M. Fox, and T. Arakawa. Binding of heparin to basic fibroblast growth factor induces a conformational change. *Arch. Biochem. Biophys. Acta* **293**:314–319 (1992).
8. S. Faham, R. E. Hileman, J. R. Fromm, R. J. Linhardt, and D. C. Rees. Heparin structure and interactions with basic fibroblast growth factor. *Science* **271**:1116–1120 (1996).
9. S. J. Konturek, T. Brzozowski, J. Majka, A. Szlachcic, W. Bielanski, J. Stachura, and W. Otto. Fibroblast growth factor in gastroprotection and ulcer healing:interaction with sucralfate. *Gut* **34**:881–887 (1993).
10. H. Kuwayama, T. Naito, and Y. Kojima. Proliferative response of rabbit and rat gastric epithelial cells to human epidermal growth factor. *Digestion* **55**:108–114 (1994).
11. H. Paimela, P. J. Goddard, K. Carter, R. Khakee, P. L. McNeil, S. Ito, and W. Silen. Restitution of frog gastric mucosa *in vitro:* Effect of basic fibroblast growth factor. *Gastroenterology* **104**:1337–1345 (1993).
12. E. Nexo and S. S. Poulsen. Does epidermal growth factor play a role in the action of sucralfate? *Scand. J. Gastroent.* **22** (**Suppl 127**):45–49 (1987).
13. S. C. Barranco, C. M. Townsend, C. Casartelli, Jr., B. G. Macik, N. L. Burger, W. R. Boerwinkle, and W. K. Gourley. Establishment and characterization of an *in vitro* model system for human adenocarcinoma of the stomach. *Cancer Res.* **43**:1703–1709 (1983).
14. H. Zheng, P. K. Shah, and K. L. Audus. Evaluation of antiulcer agents with a human adenocarcinoma cell line (AGS). *Int. J. Pharm.,* **129**:103–112 (1996).
15. H. Zheng, P. K. Shah, and K. L. Audus. Primary culture of rat gastric epithelial cells as an *in vitro* model to evaluate antiulcer agents. *Pharm. Res.* **11**:77–82 (1994).
16. M. Piontek, K. J. Hengels, R. Porschen, and G. Strohmeyer. Antiproliferative effect of tyrosine kinase inhibitors in epidermal growth factor-stimulated growth of gastric cancer cells. *Anticancer Res.* **13**:2119–2123 (1994).
17. A. U. Dignass, S. Tsunekawa, and D. K. Podolsky. Fibroblast growth factors modulate intestinal epithelial cell growth and migration. *Gastroenterology* **106**:1254–1262 (1994).
18. R. Nagashima. Development and characteristics of sucralfate. *J. Clin. Gastroenterol.* **3**(**suppl. 2**):103–110 (1981).