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Evaluation of antiulcer agents with a human adenocarcinoma cell line (AGS)

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Abstract

A human gastric carcinoma epithelial cell line (AGS) was studied as an alternative in vitro model for evaluating the cytoprotective effects of antiulcer agents. Cytoprotection was measured by cleavage of 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) by surviving AGS cells following exposure to selected antiulcer agents and/or pH 3 medium, indomethacin, or ethanol. Sucralfate in three different preparations (including a sucralfate suspension, acid-solubilized sucralfate and sucralfate-xg, a sucralfate suspension formulated with xanthine gum) at concentrations of 2 and 5 mg/ml provided significant protection (40–99% of untreated control) against pH 3 medium-induced damage. Sucralfate-xg at 5 mg/ml almost completely blocked the toxic effect of 10 mM indomethacin. The survival of cells after indomethacin exposure was about 70 and 80% of untreated controls when the cells were pretreated with 5 mg/ml of either sucralfate or acid-solubilized sucralfate. Acid-solubilized sucralfate proved to be an effective protection against a 13% ethanol insult; about four times greater than the effect of a simple sucralfate suspension against the ethanol insult. Potassium sucrose octasulfate (KSOS) or aluminum hydroxide individually presented either partial or little protection against acid, indomethacin, or ethanol insults. However, a mixture of KSOS and aluminum hydroxide enhanced the AGS cells recovery from a pretreatment with acidified medium. Although not effective as a cytoprotective agent, 0.02–5 mg/ml KSOS stimulated AGS cell growth up to 40% over untreated controls. Overall results correlated well, qualitatively, with previous studies with primary cultures of rat gastric epithelial cells and rat and human studies, suggesting that the AGS cell line may also serve as a suitable model for preliminary evaluation of some antiulcer agents.

Keywords: Gastric epithelium; AGS cell line; Cell culture; Cytoprotection; Sucralfate

1. Introduction

Primary cultures of rat gastric cells have been successfully used to investigate the effects of antiulcer agents and cytotoxic agents on the gastric mucosa (Ota et al., 1988; Mutoh et al., 1990;

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Romano et al., 1990; Zheng et al., 1994), establishing that at least preliminary evaluation of antiulcer drugs with an in vitro model is reasonable. However, the primary cultures cannot be maintained for a long period or subcultured because of the potential for overgrowth of contaminating cells, e.g., fibroblasts. The presence of contaminating cells may also disturb the experiment which aims at observing biochemical changes specific to mucus-secreting epithelial cells (Romano et al., 1990).

The AGS cell line was derived from a specimen of human stomach adenocarcinoma consisting of mucus-secreting epithelial cells (Barranco et al., 1983). This cell line possesses some characteristics of normal gastric epithelial cells, such as epithelial-like morphology, microvilli, and the production of mucus (Sheng et al., 1996a). Unlike the primary cultures (Zheng et al., 1994), AGS can be conveniently and continuously maintained by subculturing. This cell line has been used to study the effect of calcium-modifying agents on cellular proliferation (Piontek and Hengels, 1992a), to examine the role of gastrin receptors in mediating growth stimulation (Piontek and Hengels, 1992b), and to study the in vitro gastric epithelial cell invasion by *Helicobacter pylori* (Bull-Henry et al., 1991; Nilius et al., 1994). Our aim in this study was to investigate the possibility that AGS cell line could serve as an in vitro model for evaluating drug-induced damage and cytoprotective effects of different sucralfate preparations or formulations.

Sucralfate, an aluminum hydroxide complex of sucrose octasulfate, has been shown in clinical studies to be effective in the treatment of duodenal and gastric ulcers (Martin et al., 1982; Lam et al., 1985; Folkman et al., 1991) and in the prevention of acute gastric mucosal lesions induced by alcohol (Hollander et al., 1985; Tarnawski et al., 1985; Cohen et al., 1989; Konturek et al., 1989; Morris et al., 1989) and aspirin or bile salts (Konturek et al., 1986; Danesh et al., 1988; Morris et al., 1989). The in vitro protective effect of sucralfate has been observed in similar studies with primary and passaged cultures of rat gastric cells (Romano et al., 1988; Romano et al., 1989; Romano et al., 1990; Zheng et al., 1994). The capacity of the AGS cell model to distinguish between the efficacy of different formulations of sucralfate, including sucralfate-xg, a sucralfate suspension formulated with xanthine gum, and acid-solubilized sucralfate was examined in this study. The possible efficacy of the major components of sucralfate, potassium sucrose octasulfate (KSOS) and aluminum hydroxide, as cytoprotectants, was also tested at the same time. Finally, the stimulatory effect of KSOS on the growth and proliferation of rat gastric cells observed in our previous work (Zheng et al., 1994) was further investigated with the AGS cell line.

2. Materials and methods

2.1. Chemicals

Sucralfate (aluminum sucrose octasulfate), sucralfate-xg, KSOS (potassium sucrose octasulfate heptahydrate), and aluminum hydroxide ($Al(OH)_3$) were supplied by Marion Merrell Dow Laboratories (Kansas City, MO). 3-(4,5-Dimethyl-2-thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) was purchased from Eastman Kodak Company (Rochester, NY). Hydrochloric acid (HCl) and isopropanol were obtained from Fisher company (St. Louis, MO). Fetal bovine serum was purchased from Hyclone Laboratories, Inc. (Logan, UT). F-12 medium and all other reagents were supplied by Sigma Company (St. Louis, MO).

2.2. AGS cell culture

The AGS cell line was derived from a well-differentiated human gastric adenocarcinoma consisting of mucus-secreting epithelial cells (Barranco et al., 1983) and was obtained from American Type Culture Collection (CRL # 1739). The cells were incubated in Ham's F-12 culture medium containing 100 μg/ml of penicillin, 100 μg/ml of streptomycin, 15 mM Hepes, 1.2 g/l sodium bicarbonate, and 10% fetal bovine serum. The cell monolayer in a 25-cm^2 flask was subcultured at a 1:5 ratio every 3 days by treatment with 0.1% trypsin and 0.03% EDTA. The flask was maintained in a

Steri-Cult incubator at 37°C in a humidified atmosphere with 5% CO_2. For most experiments, 5×10^3 cells/well were seeded into 96-well culture plates and grown under the conditions above. Cells were from passages 40–60. Culture medium was changed every other day.

2.3. Phase contrast microscopy

The AGS cells were observed under the phase-contrast microscope to confirm general epithelial cell morphology as described by Terano et al. (1982) and Sheng et al. (1996a). Histochemical identification of the gastric cells was performed with the periodic acid-Schiff (PAS) reaction which produces granules in the mucus-producing cells (Sigma Kit # 395-B; Sigma Chemical Co.) (Terano et al., 1982; Sheng et al., 1996a).

2.4. Drug preparation and experimental protocols

To determine the effect of acid, indomethacin and ethanol insults on AGS cells, the cells were exposed to pH 3 medium for 3–30 min, or incubated in serum-free medium containing either 0–12 mM indomethacin for one hour, or 0–15% ethanol for 30 min. Indomethacin was prepared as 50 mM solution in 0.2 M Na_2CO_3, then diluted in serum-free culture medium to desired concentrations; pH was adjusted to 7.4 with 1 N HCl before each experiment. Acid treatment conditions were based on preliminary studies which showed that pH 3 medium produced about a 50–60% reduction in AGS cell viability after a 3 min exposure. Cells surviving under those conditions continued to be viable when returned to normal culture medium and assayed at later times. On the other hand, those AGS cells surviving exposure to a lower pH or pH 3.0 for longer treatement periods were not viable for an extended period after return to normal culture medium (data not shown). Incubation times and concentrations used in experiments with indomethacin and ethanol were selected on the basis of precedent literature (Romano et al., 1988; Romano et al., 1989).

The cytoprotective effects of different sucralfate formulations (sucralfate-xg, sucralfate suspension, acid-solubilized sucralfate), and aluminum hydroxide ($Al(OH)_3$) on acid-induced damage were investigated by incubating the cells with the agents for one hour and then exposing to pH 3 medium for 25 min. The treatment effects of sucralfate and the related agents were tested by treating the cells with pH 3 medium for 3 min and followed by the agents for 24 h. These conditions were based on earlier work with the primary cultures (Zheng et al., 1994).

For measuring the cytoprotective effects of sucralfate-xg, sucralfate suspension, acid solubilized sucralfate and KSOS on indomethacin-induced damage, cells were incubated in culture medium containing these drugs for one hour and then followed by a second hour of exposure to 10 mM indomethacin (Zheng et al., 1994).

The cytoprotective role of different sucralfate preparations and aluminum hydroxide against an ethanol insult was assayed by exposing AGS cells to 13% ethanol for 30 min following a one-hour treatment with these agents (Zheng et al., 1994).

To simulate the acid conditions, the drug normally encountered in the stomach, acid-solubilized sucralfate was prepared immediately prior to each experiment by dissolving the drug in 1 N HCl, and then diluting in culture medium before being adjusted back to pH 7.4.

2.5. Cell survival

Cells grown in 96-well plates were treated as described in the protocols above and then bathed in 100 μl of culture medium containing 10 μl of an MTT solution (5 mg/ml in PBS) for 4 h at 37°C according to the method of Mosmann (1983) and as previously described in our laboratory (Zheng et al., 1994). This assay measures the activity of the remaining dehydrogenase enzymes in the active mitochondria present only in living cells (Mosmann, 1983). Following the incubation, 100 μl acid-isopropanol (0.04 N HCl in isopropanol) was added to wells and the plate was incubated overnight at room temperature. The color changes were recorded at 540 nm on a microplate reader (Cambridge Series 700, Cambridge Technology, Inc., Watertown, MA). In order to exclude the disturbance of precipitates in

some samples, samples were centrifuged and only the supernatants were read. A standard curve was generated by measuring the absorbance of varying numbers of stained cells counted with a hemacytometer (r^2 = 0.99–1.00).

2.6. Statistical analysis

Data were expressed as the mean ± standard error (SE) of mean from eight replicates. A one-way analysis of variance (ANOVA) followed by Scheffe's posthoc test (ABSTAT, Anderson Bell software) was used to test the significance between control and drug-treated samples. Differences were considered significant if $p < 0.05$.

3. Results

AGS cells grow rapidly in F-12 medium; a morphologically epithelial-like confluent monolayer of polygonal-shaped cells was obtained in 3 days following a 1:5 subculture split. The presence of histochemically identifiable PAS staining for cytoplasmic mucin was observed in these cell cultures as previously reported (Barranco et al., 1983). Qualitative and quantitative descriptions of AGS morphology and mucin secretions are presented in a related report (Sheng et al., 1996a).

Treatment with any of three insults, acidified medium, indomethacin, or ethanol, produced a statistically significant reduction of cell viability when measured with the MTT assay. Incubation of AGS cells in acidified medium (pH 3.0) for 3–30 min reduced viability to less than 10% as seen in Fig. 1. Under the phase-contrast light microscope, the AGS cell monolayers have an altered morphology and appear grainy following the acid medium treatment (results not shown). Although still attached to the cell surface, the MTT assay confirmed that the AGS cells have limited viability following the treatment with acidified medium. Indomethacin produced a dose-dependent cytotoxicity over the concentration range of 9–12 mM as shown in Fig. 2. After treatment with 10 mM indomethacin for about 60 min, most AGS cells detach from the cell surface (results not shown). Ethanol treatment resulted in a significant linear loss of the living cells over the concentration range of 10–14% as illustrated in Fig. 3. After treatment with 13% ethanol for 25 min, the AGS cell monolayer appeared disorganized under the phase-contrast microscope and significant numbers of cells were detached from the growth surface (results not shown).

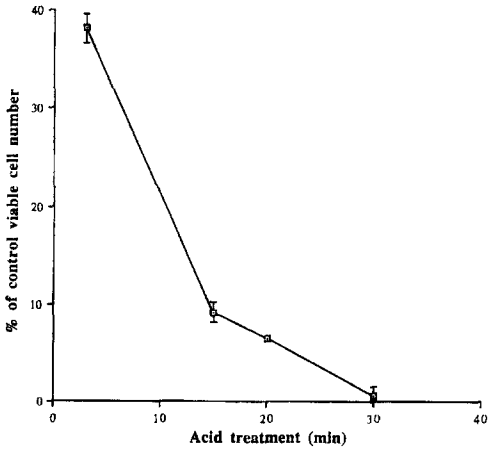

Fig. 1. Effects of acidified medium on the viability of AGS cell monolayers. AGS monolayers were incubated in pH 3.0 medium for 3–30 min before determining cell survival with the MTT assay. Data represent means ± SE for n = 8.

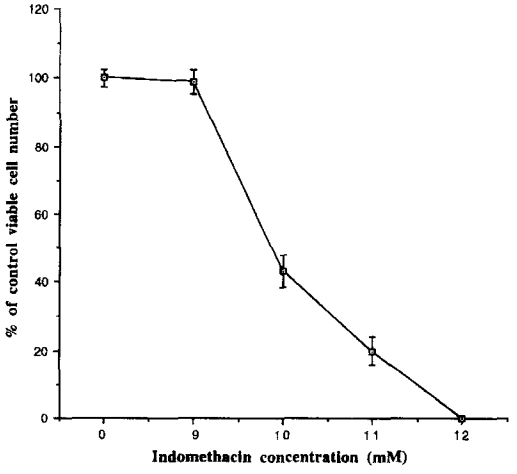

Fig. 2. Effects of indomethacin on the viability of AGS cell monolayers. AGS monolayers were treated with 0–12 mM indomethacin in culture medium for one hour before determining cell survival with the MTT assay. Data represent means ± SE for n = 8.

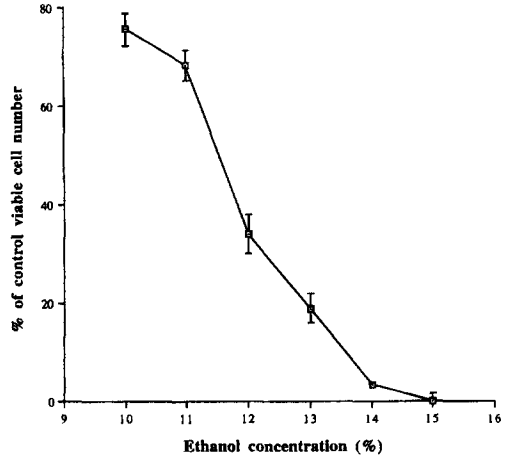

Fig. 3. Effects of ethanol on the viability of AGS cell monolayers. AGS monolayers were treated with 10–15% of ethanol in culture medium for 30 min before determining cell survival with the MTT assay. Data represent means ± SE for $n = 8$.

As shown in Fig. 4, sucralfate in three different preparations reduced the loss of cell viability induced by pH 3 medium in a dose-dependent manner. Relative to untreated control cells, 84 and 68% of cells survived pH 3.0 medium-induced damage with the preincubation of 5 and 2 mg/ml sucralfate-xg (while only 4% of the cells survived in placebo wells which contained only supplemental reagents in sucralfate-xg but not sucralfate; data not shown). The sucralfate suspension and acid-solubilized sucralfate similarly provided for 79 and 99% cell survival at 5 mg/ml. Aluminum hydroxide exerted a lower but still significant protective effect with 52% cell survival (relative to untreated control) at 5 mg/ml. KSOS failed to prevent acidified medium-induced injury (data not shown).

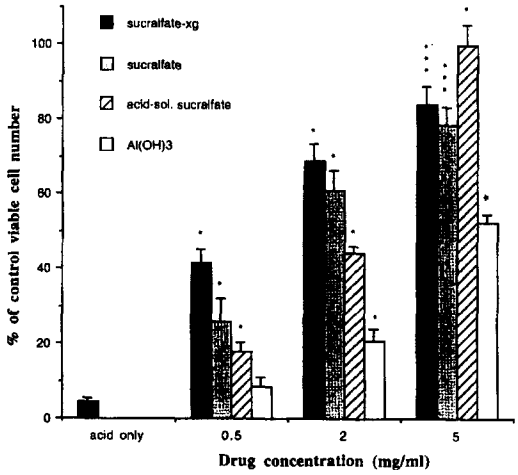

Fig. 4. Survival of AGS cell monolayers in the presence of acidified medium following a pretreatment with sucralfate-xg, sucralfate, acid-solubilized sucralfate, or aluminum hydroxide. The cells were incubated with the indicated agents for one hour and then exposed to pH 3.0 medium for 25 min before determining survival with the MTT assay. Data represent means ± SE for $n = 8$. Significant difference from treatment with acidified medium in the absence of any pretreatment agent: $^*p < 0.05$.

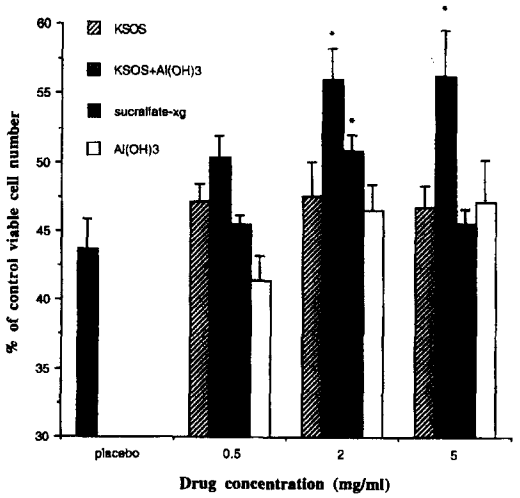

Fig. 5. Survival of AGS cell monolayers exposed to selected agents following pretreatment with acidified medium. AGS monolayers were incubated in pH 3.0 medium for 3 min and then exposed to indicated concentrations of sucralfate-xg, KSOS, aluminum hydroxide, or a mixture of KSOS plus aluminum hydroxide for one hour before determining cell survival with the MTT assay. The Placebo contains only the excipients of sucralfate-xg. Data represent means ± SE for $n = 8$. Significant difference from pretreatment with acidified medium in the absence of any subsequent treatment with the indicated agents: $^*p < 0.05$.

When the cells were damaged by pH 3 medium for 3 min prior to the treatment with these drugs, acid-solubilized sucralfate and sucralfate-xg facilitated an apparent cell recovery as shown in Fig. 5. At 2 mg/ml sucralfate-xg, cell recovery was increased. Neither KSOS alone nor $Al(OH)_3$ alone showed any effect on the cell recovery. However,

as illustrated in Fig. 5, a combination of KSOS and $Al(OH)_3$ exerted significant promotion of cell recovery and survival.

Results summarized in Fig. 6, showed that sucralfate-xg provided the best protection for AGS cells against an indomethacin-induced damage. Sucralfate-xg almost completely blocked the effect of indomethacin, while only 25% of cells survived indomethacin exposure in drug-free controls. Survival in the presence of the sucralfate suspension was about 70% (relative to untreated controls) at 5 mg/ml and was slightly less than for the acid-solubilized sucralfate. The latter agent provided the cell protection in a dose-dependent manner with 80% of the cells surviving. KSOS protected about 50% of the cells at 5 mg/ml, but also had small but significant protective effects at lower concentrations.

Ethanol-induced cell loss was reduced by the acid-solubilized sucralfate, sucralfate-xg and, to a lesser degree, KSOS and sucralfate suspension, as illustrated in Fig. 7. Maximum survival of cells was 76% of untreated controls at best, under 5 mg/ml acid-solubilized sucralfate. The sucralfate

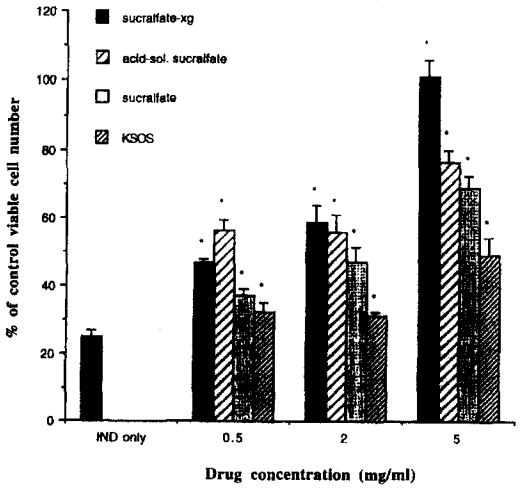

Fig. 6. Survival of AGS cell monolayers treated with indomethacin (IND) following a pretreatment with sucralfate-xg, acid-solubilized sucralfate, sucralfate, or KSOS. The cells were incubated with these drugs for one hour and then with 10 mM indomethacin for additional hour before determining cell survival with the MTT assay. Data represent means ± SE for $n = 8$. Significant difference from indomethacin treatment in absence of pretreatment with the indicated agent: $^*p < 0.05$.

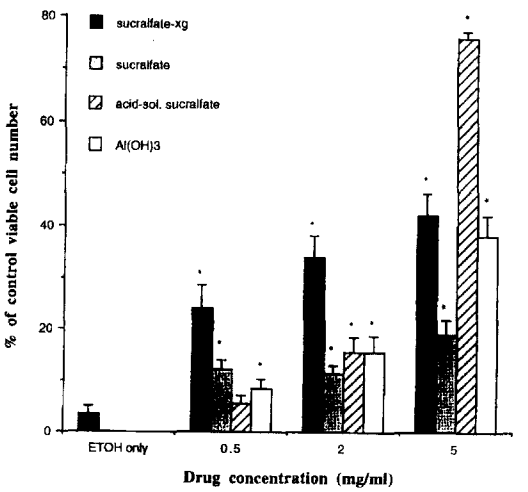

Fig. 7. Survival of AGS cell monolayers treated with ethanol following a pretreatment with sucralfate-xg, sucralfate, acid-solubilized sucralfate, or aluminum hydroxide. The cells were incubated in drugs for one hour and then with 13% ethanol for 30 min before determining cell survival with the MTT assay. Data represent means ± SE for $n = 8$. Significant difference from ethanol treatment in absence of pretreatment with the indicated agent: $^*p < 0.05$.

suspension did not reduce the insult as dramatically as acid-solubilized sucralfate (less than 20 versus 3% drug-free control). Sucralfate-xg and aluminum hydroxide partially prevented the damage with 42 and 38% of cells surviving in the presence of these agents.

KSOS concentrations of 0.02–5 mg/ml were applied to a nonconfluent monolayer of AGS cells for 24 h prior to cell number counts with the MTT assay. A dose-related stimulation of cell growth was observed in the presence of KSOS. A maximum increase of around 40% was observed at 5 mg/ml KSOS as presented in Fig. 8. None of the other agents tested, sucralfate-xg, acid-solubilized sucralfate, sucralfate suspension, or aluminum hydroxide, promoted cell growth under similar conditions (not shown).

4. Discussion

The morphology and histochemical characteristics of AGS culture were consistent with a homogenous mucus producing gastric epithelial cell

monolayer as detailed by other investigators (Barranco et al., 1983; Bull-Henry et al., 1991; Piontek and Hengels, 1992a; Piontek and Hengels, 1992b; Nilius et al., 1994) and as we have presented in a related report (Sheng et al., 1996a). Our results also indicated that the viability of the AGS cell line was sensitive to acidified medium, ethanol and indomethacin, agents used to experimentally induce gastric lesions in vitro and in vivo. Qualitatively and quantitatively, the survival of the AGS cell line on exposure to acidic medium, ethanol and indomethacin was comparable to other primary and passaged cell cultures described by Romano et al. (1988); Romano et al. (1989) and primary cultures developed in our own laboratory (Zheng et al., 1994).

Sucralfate has been shown in many in vivo studies to be an effective treatment for ulcers (Martin et al., 1982; Okabe et al., 1983; Lam et al., 1985; Folkman et al., 1991) and to prevent the formation of acute gastric mucosal injury induced by alcohol (Hollander et al., 1985; Tarnawski et al., 1985; Cohen et al., 1989; Konturek et al., 1989; Morris et al., 1989) and aspirin or bile salts (Konturek et al., 1986; Danesh et al., 1988; Morris et al., 1989). On a qualitative basis, our observations of sucralfate's efficacy in cytoprotection with the AGS cells were complimentary and consistent with the drug's activity in prevention and treatment of acid-, alcohol- or indomethacin-induced lesions in rats and humans.

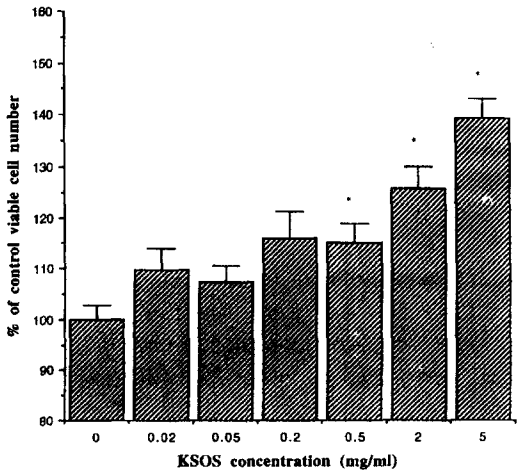

Fig. 8. The stimulatory effect of potassium sucrose octasulfate (KSOS) on the growth of AGS cells. Cell numbers were determined after a 24-h incubation of AGS cells with KSOS in culture medium before determining cell survival with the MTT assay. Data represent means ± SE for $n = 8$. Significant difference from AGS cells receiving no KSOS treatment: $*p < 0.05$.

Multiple mechanisms are involved in the development of ulcers (Szabo and Nagy, 1992). Perhaps not coincidentally, the mechanism(s) of the gastroprotective and anti-ulcer effects of sucralfate has not been fully explained. An acid environment in the stomach was considered to be the important factor in facilitating the protective effect of sucralfate (Nagashima, 1981a; Nagashima, 1981b). In some of our investigations, sucralfate was dissolved in acid first and then adjusted to neutral pH before applying to AGS cell monolayers. In the same neutral environment, the acid-solubilized sucralfate seemed to produce a different effect than when first dissolved in neutral medium, also suggesting an acid-enhanced protective effect. In addition, the results here indicated that under most conditions, acid-solubilized sucralfate generally exerted a greater protective effect than the sucralfate suspension, especially against the ethanol insult. The number of surviving cells after a 30-min incubation in 13% ethanol, for example, was four times higher in the presence of acid-solubilized sucralfate versus the suspension. This observation complements the in vivo results from Konturek et al. (1989), and supports the conclusion that an acid milieu may be important in sucralfate-induced gastric protection against various insults.

In their studies, Konturek et al. (1989) demonstrated in humans and in rats that sucralfate was relatively more effective in mucosal protection against ethanol when administered in formulations of lower pH (1 or 2) than at neutral pH. When encountering the acid environment in the stomach, sucralfate becomes a highly condensed, viscous adhesive substance which readily adheres tightly to the ulcer bed, and does not lose this property in the duodenum (about pH 6.5). This 'coating' is believed to, in part, exert a 'barrier' function for the ulcer bed. However, on solubilization at low pHs, sucralfate also dissociates, releasing aluminum and sucrose octasulfate ions

(Nagashima, 1981a). By analogy, on solubilization under acidic conditions, aluminum-containing antacids have substantially enhanced gastroprotective activity, due to an increased activated aluminum cation concentration (DiJoseph et al., 1989). In the case of sucralfate, then, low pH exposure could possibly increase the availability of sucrose octasulfate and aluminum ions in the preparation. Potassium sucrose octasulfate (KSOS) and aluminum hydroxide are the two major components of sucralfate. KSOS or aluminum hydroxide alone presented either partial or little protection against acid, indomethacin or ethanol insults when compared to sucralfate preparations. On the other hand, a mixture of these two agents enhanced the AGS cell recovery from the acid-pretreatment. We also observed in this study and in a rat gastric cell model that KSOS alone enhanced AGS cell growth. This interesting phenomenon may suggest that there are some mutual supplementary effects between sucrose octasulfate and aluminum ions which could contribute to the special role of sucralfate in ulcer therapy or cytoprotection. Therefore, both acid neutralization and cell growth-promoting properties of the agents, combined, may contribute to the rescue and recovery of gastric cells from an acid insult. Collectively, then, our in vitro results provide a further indication that the potential of an acid-enhanced effect on sucralfate observed in vivo, may result from changes in the solubility characteristics of sucralfate, i.e., an increased concentration of sucrose octasulfate and aluminum ions, and could form a basis for further examination of the role of pH in the efficacy of the drug.

Aside from the 'barrier' effect and the neutralization potential, sucralfate inhibits the action of pepsin, and adsorbs bile salts (Nagashima, 1981b) and growth factors (Folkman et al., 1991) in the ulcer bed. Folkman et al. (1991) reported that basic fibroblast growth factor (bFGF) promoted a significant increase in angiogenesis in the ulcer bed. Paimela et al. (1993) have also shown that endogenous bFGF promotes gastric mucosal restitution. Naturally occurring endogenous bFGF is present in the rat and human gastric and duodenal mucosa, but is subject to acid degradation. Folkman et al. (1991) found that sucralfate has a relatively high affinity for bFGF and protected bFGF from acid degradation and inactivation. Subsequently, Volkin et al. (1993) confirmed these observations. A possibility exists that sucralfate could promote, bind, and protect growth factors and other secretions in the enhancement of cell survival. In our laboratory, the AGS cell line has also been used to demonstrate the ability of sucralfate and related formulations to preserve the bioactivity of bFGF and epidermal growth factor under acidic conditions in vitro (Sheng et al., 1996b), also consistent with Folkman's hypothesis (Folkman et al., 1991).

Our results demonstrated the capacity of the AGS cell line in distinguishing, to some degree, between the efficacies of different preparations of sucralfate. The potential commercial formulation, sucralfate-xg, provided significant protection for AGS cells against all of the insults. When compared with a simple sucralfate suspension, the surviving cell numbers were almost doubled under the protection of sucralfate-xg against indomethacin- and ethanol-induced damage. The AGS cell line possesses the histochemical and biochemical characteristics of a mucus-producing gastric epithelial cell, is sensitive to relevant growth factors, appears homogenous, and can be conveniently subcultured, all advantages over primary culture systems (Sheng et al., 1996a; Sheng et al., 1996b). Moreover, the cytoprotective activity of sucralfate and related formulations against acid-, ethanol-, and indomethacin-induced injury demonstrated with the AGS cell line were qualitatively in good agreement with and complement rat and human studies performed in other laboratories (Danesh et al., 1988; Cohen et al., 1989; Morris et al., 1989; Konturek et al., 1989; Folkman et al., 1991). Therefore, the results of the present study suggests the AGS cell line, a human-derived gastric epithelial cell line, may be a suitable in vitro model for preliminary evaluation of certain antiulcer agents. The use of this in vitro system, though, does not preclude or eliminate the eventual need for in vivo studies.

Acknowledgements

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