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The Effect of Nitric Oxide, Growth Factors, and Estrogen on Gastric Cell Migration

Outi Nylander-Koski, M.D., Tuula Kiviluoto, M.D., Ph.D., Pauli Puolakkainen, M.D., Ph.D., Eero Kivilaakso, M.D., Ph.D., and Harri Mustonen, D.Sc.[1]

Department of Surgery, Helsinki University Central Hospital, Helsinki, Finland

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Background. **To study gastric epithelial cell migration during nitric oxide (NO) and growth factor treatment, simulating inflammation and infection. Also, the effects of estrogen on migration of different malignant and nonmalignant gastric epithelial cell lines were explored.**

Material and methods. **Isolated primary cultured rabbit gastric epithelial cells, rat gastric mucosal cells, human gastric adenocarcinoma cells, and human colon adenocarcinoma cells (WiDr) were cultured to confluency in appropriate media (5% CO_2, 37°C). The cells were treated by hepatocyte growth factor (HGF), transforming growth factor-alpha (TGF-α) and keratinocyte growth factor (KGF), with and without sodium nitroprusside (SNP, NO donor) or 17β-estradiol. Caspase-3 activity and cell viability and migration speed after wounding were measured.**

Results. **HGF was the most potent growth factor to stimulate migration. SNP dose-dependently decreased the speed of migration. HGF and TGF-α were able to overcome the SNP-induced inhibition of migration, whereas KGF was not. SNP also induced caspase-3 activity, which was inhibited by HGF and TGF-α. 17β-estradiol decreased migration in all epithelial cells, but the decrease was more profound in malignant cell lines. HGF could overcome the estrogen retarded migration.**

Conclusions. **Growth factors can overcome NO-induced retardation of cell migration and inhibit NO-induced caspase-3 activity, which altogether might also have physiological significance in *in vivo* inflammation and in gastric cancer. The more profound decrease in migration speed of gastric adenocarcinoma cell line may suggest that estrogen might be one of the protective factor against female gastric adenocarcinoma before menopausal age.**

Key Words: **gastric mucosa; growth factors; estrogen; nitric oxide.**

INTRODUCTION

The gastric mucosa must have efficient mechanisms to defend itself against gastric acid, digestive enzymes, and endogenous and exogenous ulcerogenic agents. The gastric mucosa possesses an effective regeneration mechanism, and the surface epithelial cells are renewed every 3 to 5 days. The mitotic activity is located in the pits and the cells migrate along the wall of the pit to the luminal surface of the stomach [1]. All factors contributing to this rapid regeneration are not known, but growth factors are likely to have a crucial role. The effect of growth factors in wound healing and epithelial restitution is physiologically important in gastric mucosa, since growth factors are secreted into saliva and are also produced by the gastric mucosa. For example, transforming growth factor alpha (TGF-α) is produced by gastric mucosa and it binds to growth factor receptor of epithelial cells to stimulate cell proliferation, to maintain the gastric mucosal barrier, and to stimulate ulcer-healing [2]. Different growth factors (hepatocyte growth factor [3], epidermal growth factor [4], TGF-α [5], basic fibroblast growth factor [6], platelet-derived growth factor [7], and insulin-like growth factor [8]) facilitate wound healing in gastric epithelium [9]. The effects of different growth factors and hormones on cell migration are divergent, and these differences are poorly understood. We, therefore, have compared here the effect of different growth factors on gastric epithelial cell migration.

[1] To whom correspondence and reprint requests should be addressed at Helsinki University Central Hospital, Biomedicum Helsinki, Department of Surgery, Haartmaninkatu 8, 00290 Helsinki, Finland. E-mail: harri.mustonen@helsinki.fi.

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Nitric oxide (NO) is a short lived, readily diffusible messenger/signaling molecule regulating regional blood flow and increasing epithelial mucus production in the gastrointestinal tract [10–12]. These effects are caused, presumably, by calcium-dependent nitric oxide synthase, which is constitutively expressed in the stomach [13]. High levels of nitric oxide might be produced by inducible nitric oxide synthase during infection and inflammation. We have previously shown that nitric oxide inhibits migration and proliferation and induces apoptosis in healing gastric epithelium [14]. These effects seemed to be mediated by superoxide radicals or peroxynitrites. Nitric oxide also has protective influence on gastric mucosal defense, which is probably mediated by increased blood flow at the tissue level. Since nitric oxide has both beneficial and deleterious properties in gastric mucosa, we have here studied whether different growth factors, some of which are endogenously produced by gastric mucosa, might act as defensive agents against NO-induced damage at the gastric epithelium. This might enable NO to act as a protective and beneficial agent at the whole tissue level, since if the apoptotic effects of NO on gastric epithelium could be inhibited, the beneficial effects of increased blood flow induced by NO would strengthen even more the overall healing of damaged mucosa.

Estrogen, the female steroid hormone, has been proposed to afford protection against gastric adenocarcinoma in women before menopause. The incidence of gastric cancer is higher in males than in females before menopausal age, and this difference has been shown also in experimental rat gastric cancer models [15]. Menopause increases the incidence of gastric cancer in women to the same level as in men [16]. Therefore, we have measured here the effects of estrogen on migration speed in primary cultured rabbit gastric surface epithelial cells and in different immortalized or carcinoma-derived cell lines. Our hypothesis is that estrogen would possibly decrease cellular migration more in gastric cancer cell lines than in other cell lines and thereby reduce the invasiveness of cancer cells.

MATERIALS AND METHODS

Rabbit gastric epithelial cells were isolated and cultured as described by Watanabe *et al*. [14]. Fasting male white rabbits, weighting 2.0 to 3.0 kg, were anesthetized and sacrificed with intraperitoneal administration of Nembutal (pentobarbital sodium, 50 mg/kg). The stomach was removed and opened along the greater curvature and rinsed carefully with ice-cold isotonic saline. The corpus part of the mucosa was quickly separated with a razor blade and minced into small pieces (2 to 3 mm^2). The pieces were incubated in a medium containing 0.07% collagenase (Type 1; WAKO Chemical Inc, Japan), 130 mM NaCl, 12 mM $NaHCO_3$, 3 mM NaH_2PO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1% bovine serum albumin, and 0.2% glucose for 15 min in a shaker bath at 37°C. After incubation, the minced tissue was washed with Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution with 1 mM ethylenediamine tetraacetic acid. These procedures were repeated twice before the tissue was filtered through a metal mesh (pore size 300 μm). The cells were then washed in a Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution containing 1 mM ethylenediamine tetraacetic acid and 0.1% bovine serum albumin. As shown before, 90% of the cells were gastric surface epithelial cells [17]. All of the animals received good care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals formulated and prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication no. 86-23, revised 1985). The authorization to perform this study was given by the Provincial Government of Uusimaa in accordance to Finnish legislation.

Cell Culture

The isolated gastric epithelial cells were inoculated on collagen Type 1 coated culture dishes (Biocoat Collagen I Cellware; Falcon, Becton Dickinson Labware, Bedford, MA) and cultured in Coon's modified Ham's F-12 medium supplemented with inactivated 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin. Rat gastric mucosal (RGM) cells [18] were obtained from Riken BioResource Center (RCB0876; RIKEN BRC, Ibaraki, Japan) and grown in F12:DMEM 1:1. Human gastric adenocarcinoma cells (AGS, CRL-1739) and human colon adenocarcinoma cells (WiDr, CCL-218) were obtained from ATCC (Manassas, VA) and grown in F12K medium. RGM, AGS, and WiDr media were supplemented with inactivated 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin. All cells were incubated at 37°C in humidified atmosphere containing 5% CO_2–95% O_2 and grown to confluency.

Wounding and Measurement of Migration Speed

A round artificial wound with a cell-free area of constant size (3.0 mm^2) was made in the center of the mucosal cell sheet using a modified pencil type mixer with a rotating silicon tip, without damaging the coated dish surface. The test agents, as described under "Chemicals", were added immediately after the artificial wounding of the monolayer cell culture and their effects were monitored by taking phase-contrast micrographs at 12 to 24 h intervals for 48 h, but migration speeds only up to 24 h were taken into account in the results. We have previously shown with primary cultured rabbit gastric epithelial cells by BrdU staining that the migration speed measured during the first 24 h is not affected significantly by proliferation [14]. The cell-free area on the epithelial cell sheet was measured and the change in cell-free area (round-shaped) was converted to average migration speed (v) with the following formula: $v = (\sqrt{A_1/\pi} - \sqrt{A_2/\pi})/t$, where A1 and A2 are the cell-free areas separated by time interval t.

Cell Viability Assay

The tetrazolium salt, sodium 3′-[1-(phenylaminocarbonyl)-3,2. tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) is cleaved by mitochondrial dehydrogenases to formazan salts. Formazan salts can be detected with spectrophotometrical absorbance measurements; thus the mitochondrial activity can be measured with this technique and has been used as a cell proliferation and cytotoxicity assay. The cells were grown on 96 well plates to confluent monolayers at 37°C in humidified atmosphere containing 5% CO_2 in air. The total volume in each well was 150 μL including 50 μL XTT labeling mixture from the Cell Proliferation Kit II (Roche, Mannheim, Germany) giving final XTT concentration of 0.3 mg/mL. The plate was incubated for 4 h in the incubator (37°C, 5% CO_2) and the absorbance was measured with a Victor^2 plate reader (Wallac, Turku, Finland) at 490 nm. The reference absorbance at 650 nm was subtracted from the results. Results from blank samples were subtracted from the measurements. The percentage mitochondrial activity was intercepted as cell viability and calculated from the ab-