

**APPROACHING THE MECHANISMS OF INDOMETHACIN
DELAYING GASTRIC RESTITUTION AND
GASTRIC CANCER GROWTH.**

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PH.D. THESIS

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PREFACE

In the scope of the present Thesis I would like to introduce two groups of experiments dealing with two major disorders of the stomach; gastric injury/ulceration and gastric cancer. It is known that nonsteroidal anti-inflammatory drugs interfere with gastric mucosal injury healing and with the proliferation of some GI cancers (e.g. colon). The first group of experiments (showed in Part I) searches after the molecular mechanisms involved in the delay of gastric restitution caused by a non-selective nonsteroidal anti-inflammatory drug, indomethacin. The role of actin cytoskeleton and focal adhesion kinase will be showed in the delay of gastric restitution. The second group of experiments (showed in Part II) was aimed to study the anti-proliferatory effect of nonsteroidal anti-inflammatory drugs (indomethacin and a cyclooxygenase-2 selective nonsteroidal anti-inflammatory drug, NS-398) and the proliferatory effect of a gastrointestinal hormone, gastrin on gastric cancer cells.

The molecular mechanisms involved in these two distinct areas of GI disorders will be discussed.

INDOMETHACIN-INDUCED DELAY IN GASTRIC RESTITUTION IS

ASSOCIATED WITH THE INHIBITION OF FOCAL ADHESION KINASE, TENSIN AND

ACTIN

OBJECTIVES AND AIMS

Gastric injury includes: a) *superficial injury* limited to exfoliation of the surface epithelium, b) *superficial mucosal injury* whereas in addition to superficial epithelium, microvessels are damaged and erosions are formed, and c) *deep injury* penetrating the mucosa and muscularis propria. Following superficial mucosal injury, the continuity of the surface epithelium is re-established by the process referred to as epithelial restitution. This process involves migration of the epithelial cells from the gastric pits and upper regions of the glands bordering the injury to cover the denuded mucosal surface. Restitution is independent of cell proliferation, but requires an intact basement membrane.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs with ~ 120 million prescriptions per year and over more than 200 over-the-counter products in the USA. NSAIDs such as aspirin, indomethacin, ibuprofen and others, control pain and inflammation by inhibiting cyclooxygenase (COX) or prostaglandin-endoperoxide synthase. NSAIDs, such as IND, delay gastric ulcer healing *in vivo*, in part by inhibiting re-epithelialization and also cause delayed

restitution of mucosal injury *in vivo* and *in vitro*. However, the subcellular targets and molecular mechanism(s) of IND-induced inhibition of restitution remain unknown.

In some cells, such as fibroblasts, characteristic changes occur at the wound edge during cell migration. These changes include the protrusions of the plasma membrane resulting in formation of lamellipodia and filopodia, and re-arrangements of focal adhesion complexes attaching cells to the extracellular matrix. At focal adhesions the polymerized actin filaments (stress fibers) interact with focal adhesion-associated proteins, such as FAK, tensin, paxillin, vinculin, Src and α -actinin. The focal adhesions serve as traction points over which the cell moves forward by the tension generated by contraction of the actin-myosin network. Cell migration is dependent on re-arrangement of cell cytoskeletal structures, predominantly actin filaments.

FAK - a cytoplasmic (non-receptor) tyrosine kinase, becomes activated upon autophosphorylation within focal adhesions and, in turn, phosphorylates several other substrate proteins, such as tensin and paxillin that are also localized to focal adhesions. In fibroblasts, FAK has been shown to play a central role in the regulation of cell proliferation, spreading and migration. Overexpression of FAK in Chinese hamster ovary (CHO) cells, for example, stimulates cell migration, while mutation of tyrosine phosphorylation site at tyrosine-397 of FAK inhibits cell migration.

Tensin is an actin capping protein, which anchors actin filaments to focal adhesion cellular structures. Tensin phosphorylation by FAK may affect its function. Tensin is a protein possessing both actin binding and phosphotyrosine kinase activities. It is considered to be a structural component of the cytoskeletal network, but also a coordinator of cytoskeletal signaling. The expression and the roles of FAK and tensin in gastric epithelial cells and in gastric wound restitution have not been studied. It is also unknown whether tensin phosphorylation is affected by EGF and/or IND. The aims of the present study were to determine whether EGF and/or IND affect restitution of wounded gastric epithelial monolayers and to examine the roles and temporal relationship of FAK and tensin phosphorylation and actin stress fiber formation during wound restitution.

MATERIALS AND METHODS

Cell Culture: Human gastric epithelial (MKN 28) cells derived from gastric adenocarcinoma were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator containing 5% CO₂. Subsequently, cells were starved and treated with either medium containing vehicle (controls) or 0.5 mM IND for 16 hrs. EGF (10 ng/ml) was

then added to the same medium and monolayers were incubated for further 24 hrs to assess wound restitution, actin, FAK localization, or for 5-60 min to assess phosphorylation levels of FAK, tensin. The optimal concentrations of IND and EGF used in the present study, were based on our previous dose-dependent studies and on dose-dependent studies.

Animals: Forty male Sprague-Dawley rats weighting 300-350 g were studied. Rats were fasted overnight and received, intragastrically, either vehicle or 5 mg/kg IND (1 ml) and 3 min later 2 ml 5% NaCl solution to produce exfoliation of gastric surface epithelium. At 1, 4 or 8 hrs after hypertonic saline administration, rats were anesthetized, their stomachs were excised, and the animals were euthanized. The stomachs were opened along the greater curvature, rinsed with 0.9% NaCl, and gastric tissues were excised and frozen immediately in liquid nitrogen and stored at -80°C for immunohistochemistry.

***In vitro* Restitution Assay:** Three 8 mm wide longitudinal wounds were made on confluent monolayers MKN 28 cells in 100-mm plastic dish using a razor blade similarly as described by Sato and Rifkin. The cells were washed and incubated with serum free media containing either vehicle or 0.5 mM IND for 16 hrs and then 10 ng/ml EGF or vehicle was added into the medium.

Restitution of wounded monolayers was evaluated at 24 hrs after EGF treatment. After fixation in methanol and staining; the restitution rate was determined by measuring the area that the cells migrated into the wound using a video imaging system (Metamorph). The restitution was expressed as the mean \pm SD of area (mm²) of cells migrated into the wound in three separate experiments, each performed in triplicate. To determine specific contributions of cell migration and proliferation to restitution, some studies were also performed in the presence of mitomycin C (2 μ g/ml) added to the cultures 2 hrs before wounding to inhibit cell proliferation.

Cell Death Detection Assay: Cells were plated in six-well plates on glass coverslips. After reaching confluence, monolayers were wounded and cultured in serum-free medium containing either 0.5 mM IND or its vehicle for 16 and 40 hrs. Cell death was evaluated by terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) using a commercial kit (Roche Diag. Corp). Cell death was evaluated by randomly counting 1000 cells on coded coverslips. The results were expressed as apoptotic and necrotic indices, respectively, reflected as percentage of apoptotic or necrotic cells per total number of all counted cells.

Caspase-3 Activity Assay: MKN 28 cells were wounded, as in re-epithelialization assays, and incubated in serum free media containing either vehicle or IND (0.5 mM). After 16-hr or 40-hr incubation with IND or vehicle, caspase-3 activity was determined by colorimetric CaspACE Assay System (Promega). Caspase-3 specific activity was calculated by measuring the absorbance of

known amounts of p-nitroaniline (pNA) and determined protein concentration of each cell lysate sample.

Immunocytochemistry of Wounded MKN 28 Monolayers: Cells were plated in six-well plates on glass coverslips. After reaching confluence, monolayers were wounded and cultured in serum-free medium containing either vehicle or IND (0.5 mM) for 1, 4, 8 and 16 hrs. In some experiments EGF (10 ng/ml) or an equal volume of vehicle was added to 16-hr IND (0.1 mM, 0.25 mM or 0.5 mM) preincubated cells grown on coverslips, and further incubated for 24 hrs. After fixation cells were then incubated with either fluorescein isothiocyanate (FITC)-conjugated phalloidin (5 μ l/ml) which selectively binds to polymerized F-actin or with rabbit polyclonal anti-FAK antibody (2 μ g/ml). Coverslips for FAK staining were incubated with Alexa-conjugated anti-rabbit secondary antibody (1:200) for 30 min. High magnification images of random cells localized in the migrating front of wound edges were taken by a Nikon PCM confocal microscope. The evaluation of fluorescence labeling for F-actin was carried out by average intensity measurement of standard size cytoplasmic areas in individual cells on captured images. FAK localization to focal adhesion points was counted on coded slides as number of adhesion points stained for FAK on the perimeter of individual cells.

Immunocytochemistry of Rat Gastric Specimens: Frozen gastric specimens were cut with a cryostat (10 μ m thick), and permeabilized in acetone for 5 min at -20°C. They were then incubated with either fluorescein isothiocyanate (FITC)-conjugated phalloidin (5 μ l/ml) or with rabbit polyclonal anti-FAK antibody (2 μ g/ml). Slides were then incubated with FITC-conjugated anti-rabbit secondary antibody (1:100) for 30 min. Images of FAK staining and phalloidin labeling of rat gastric mucosa were viewed under a Nikon Optiphot microscope and captured using a Nikon DXM1200 digital camera.

Immunoprecipitation and Immunoblotting of FAK and Tensin: MKN 28 cells were wounded as described in restitution assays and cultured in serum free media containing either vehicle or IND (0.5 mM) for 1, 4, 8 and 16 hrs. EGF (10 ng/ml) or an equal volume of vehicle was then added to monolayers, and further incubated for 5-60 min. In some experiments, wounded monolayers were treated with vehicle or inhibitors of EGR-R kinase [PD 153035 (1 μ M) for 16 hrs or AG 1478 (250 nM) for 20 min] prior to EGF treatment. The cells were washed twice in ice-cold PBS and lysed in cold lysis buffer.

Frozen rat gastric mucosal tissue specimens were homogenized using a Polytron homogenizer in the same lysis buffer. One μ g anti-FAK polyclonal or 2 μ g anti-tensin monoclonal antibody was incubated with protein A-Sepharose beads (25 μ l and 50 μ l, respectively) for 2 hours. Aliquots of

clarified cell lysates containing equal amounts of protein (250 μ g for FAK and 1mg for tcnsin) were added to the protein A-Sepharose beads/antibody complex and incubated overnight. The beads were washed four times in lysis buffer, resuspended in SDS sample loading buffer and boiled for 5 min. Supernatants were subjected to 7.5% SDS-PAGE, and transferred to nitrocellulose membranes. The membranes were immunoblotted with anti-phosphotyrosine antibody. After washing, bound antibody was detected with a chemiluminescence (ECL) kit. Density of protein bands corresponding to 125 kDa (FAK) and 215 kDa (tensin) were analyzed using a video image system. The blots were stripped and re-probed with anti-FAK or anti-tensin antibodies to determine total protein levels. Phosphorylation levels were expressed as the density ratio of phosphorylated to total protein bands.

To determine whether IND affects cellular protein levels, aliquots of the same lysates (150 μ g protein) were subjected to SDS-PAGE and immunoblotted using anti-glucose-6-phosphate dehydrogenase (G6PDH) antibody following the same procedure described above. G6PDH is a constitutively expressed housekeeping protein and has been used as a marker of synthetic performance.

Statistical Analysis: Results were expressed as mean \pm standard deviation (SD). Statistical significance between differently treated groups was determined by analysis of variance followed by two-tailed Mann-Whitney *U*-test. A *p* value of <0.05 was considered statistically significant. Pearson's correlation coefficient between the inhibition of EGF-induced FAK or tensin phosphorylation and reduction of re-epithelialization or stress fibers were analyzed.

RESULTS OF *IN VITRO* EXPERIMENTS

Restitution of Wounded MKN 28 Monolayers: EGF treatment stimulated re-epithelialization of wounded gastric epithelial monolayers by 59 \pm 8%, compared to vehicle treated monolayers. IND significantly inhibited wound re-epithelialization under the basal condition by 73 \pm 12% and inhibited EGF-stimulated re-epithelialization by 67 \pm 9% (both *p*<0.005).

To differentiate the effect of IND on cell migration and proliferation, we performed the restitution assay in the presence of mitomycin C (2 μ g/ml), which abolishes MKN 28 cell proliferation. In these experiments, EGF significantly stimulated the migration of MKN 28 cells by 53 \pm 7%, clearly indicating that cell migration is predominantly responsible for restitution, and that the possible induction of cell cycle arrest by IND does not play a major role in IND-induced inhibition of restitution of wounded monolayers. Treatment with IND in mitomycin C-treated monolayers significantly reduced both baseline and EGF-stimulated migration (72 \pm 11%, 65 \pm 9%,

respectively; both $p < 0.005$). Thus, cell proliferation had only a minor contribution to restitution measured at 24 hrs after EGF or its vehicle treatment.

Apoptotic/ Necrotic Indices and Caspase-3 Activity: Since IND can cause gastric cell apoptosis, we evaluated apoptosis in MKN 28 cells after IND treatment. We found a moderate but significant increase in apoptosis at the end of 16 and 40-hr time periods. The percentage of apoptotic cells was 4.37 ± 1.07 after 16-hr treatment with 0.5 mM IND, compared to $3.43 \pm 0.75\%$ in the vehicle treated groups ($p < 0.05$). After 40 hrs of IND treatment, we also found a moderate and significant increase in number of apoptotic cells (to $6.50 \pm 1.44\%$; $p < 0.01$), while monolayers incubated with vehicle had no further increase. The number of cells undergoing necrotic death was insignificant ($< 1\%$) in all experimental conditions.

IND did not significantly affect caspase-3 activity of MKN 28 monolayers after 16-hr incubation. Caspase-3 activity was significantly increased in experiments with 40-hr IND-treatment ($97 \pm 8\%$ increase, $p < 0.01$) vs. vehicle-treated controls).

Actin Stress Fiber Formation of MKN 28 Monolayers: Phalloidin labeling of actin filaments in cells lining at wounded margins demonstrated a significant enhancement of actin stress fiber formation after 24-hr EGF treatment ($43 \pm 11\%$ increase vs. baseline; $p < 0.05$). IND treatment caused a significant inhibition of actin stress fiber formation at baseline ($20 \pm 4\%$ inhibition; $p < 0.05$) as well as following 24-hr EGF stimulation ($24 \pm 7\%$ inhibition; $p < 0.05$).

Compared with controls, IND treatment did not affect stress fiber formation up to 16 hrs of IND treatment. The loss of actin stress fibers was apparent at 24 hrs and fully manifested at 40 hrs after IND incubation. The loss of stress fibers by IND was found dose dependent.

Cells localized inside the confluent monolayer showed qualitatively similar changes in stress fiber formation, but quantitatively they were significantly less expressed. At the baseline (non-stimulated condition), the number of actin filaments was less than in cells located at the wound edge. The effect of IND on baseline actin filaments in cells localized inside the confluent monolayer was not detectable by intensity measurement. However, these cells responded to EGF stimulation with increased stress fiber formation, but these changes were less prominent than in cells localized at the wound edge.

FAK Localization to Focal Adhesions: Immunostaining showed spotted localization of FAK in all groups. The major difference between vehicle-treated and E/EGF-treated monolayers was found at the periphery of cells as demonstrated by increased FAK localization to focal adhesion points. EGF treatment caused increased recruitment of FAK to focal adhesions in migrating cells at the wound edge - a $78 \pm 13\%$ increase compared to vehicle-treated monolayers ($p < 0.05$).

Monolayers treated with IND showed a significant loss of FAK recruitment to focal adhesion points in the periphery of cells at the wound edge – a $63 \pm 15\%$ reduction at baseline and a $62 \pm 14\%$ reduction (vs. EGF) in IND + EGF treated groups (both $p < 0.05$).

Tyrosine Phosphorylation of FAK and Tensin in MKN 28 Monolayers: EGF treatment of MKN monolayers increased tyrosine phosphorylation of FAK and tensin. The EGF-stimulated tyrosine phosphorylation of FAK peaked at 5 min ($45 \pm 7\%$; $p < 0.005$) and remained significantly elevated for 30 min. Pretreatment with EGF-R kinase inhibitors, PD 153035 and AG 1478, significantly prevented EGF-stimulated increase of FAK phosphorylation ($79 \pm 8\%$ and $92 \pm 9\%$ inhibition, respectively; both $p < 0.01$). The EGF treatment stimulated tensin phosphorylation within 5 min ($26 \pm 7\%$ increase; $p < 0.05$); this increase was sustained for 30 min.

Sixteen-hr IND pretreatment significantly inhibited the baseline and EGF-stimulated FAK phosphorylation ($19 \pm 7\%$ inhibition; $p < 0.05$; and $32.4 \pm 7\%$ inhibition; $p < 0.005$; respectively). IND did not affect the low baseline level of tensin phosphorylation, but significantly inhibited EGF-stimulated tensin phosphorylation by $18 \pm 5\%$ ($p < 0.05$). Inhibition of baseline FAK phosphorylation by IND was present after 4-hr incubation ($17 \pm 6\%$, $p < 0.05$) and 8-hr incubation ($19 \pm 7\%$; $p < 0.05$). The IND-induced inhibition of EGF-stimulated FAK and tensin phosphorylation strongly correlated with both the IND-induced inhibition of EGF-induced restitution ($r = 0.994$ and $r = 0.842$; respectively; $p < 0.05$) and the IND-induced inhibition of stress fiber formation ($r = 0.986$ and $r = 0.656$, respectively; $p < 0.05$).

Protein level of glucose-6-phosphate-dehydrogenase (house-keeping) protein: Western blot analysis using anti-glucose-6-phosphate dehydrogenase antibody (housekeeping protein) demonstrated that IND did not inhibit protein synthesis indicating that IND-induced inhibition of phosphorylation is not due to altered protein synthesis.

RESULTS OF *IN VIVO* EXPERIMENTS

F-actin Labeling in Rat Gastric Mucosa: In rat gastric mucosa injured by hypertonic saline, surface epithelial continuity was almost completely restored at 8 hrs after injury. In the gastric mucosa of rats pretreated with IND, epithelial restitution was delayed. The number of epithelial cells migrating from the glandular pits to the surface was significantly reduced in the gastric mucosa of rats pretreated with IND vs. vehicle-pretreated controls at 4 hrs after injury. In the gastric mucosa of IND-pretreated rats, the migrating epithelial cells showed less intracellular actin filaments than epithelial cells of vehicle-treated rats.

FAK Localization to Adhesion Points in Rat Gastric Mucosa: Immunostaining for FAK showed spotted localization of intracellular FAK in superficial migrating epithelial cells. The number FAK recruited to the periphery (adhesion points) of the migrating cells was significantly reduced in gastric mucosa of rats pretreated with IND than in vehicle-treated at 8 hrs after injury.

Tyrosine Phosphorylation of FAK in Rat Gastric Mucosa: Tyrosine phosphorylation of FAK was significantly increased in rat gastric mucosa at 4 and 8 hrs after injury ($74 \pm 10\%$ increase at 4 hrs, $p < 0.001$; $42 \pm 11\%$ increase at 8 hrs, $p < 0.05$; respectively). IND pre-treatment significantly inhibited the injury-induced FAK phosphorylation at 4 hrs ($53 \pm 8\%$; $p < 0.001$) and 8 hrs ($55 \pm 8\%$; $p < 0.001$) compared to vehicle.

DISCUSSION

The present study shows that IND, a non-selective cyclooxygenase inhibitor, significantly impairs both basal and EGF-stimulated restitution of human gastric monolayers. Furthermore, IND-induced inhibition of *in vitro* restitution is associated with the early inhibition of FAK phosphorylation and late disruption of actin stress fiber formation, as well as inhibition of FAK recruitment of to focal adhesions. The inhibition of actin stress fiber formation showed a strong correlation with reduced phosphorylation of FAK and tensin. IND pretreatment also delayed *in vivo* restitution of rat gastric mucosa injured by hypertonic saline inhibited injury-induced FAK phosphorylation and recruitment of FAK to focal adhesions.

Our study shows that IND inhibits epithelial wound restitution *in vitro*, both under basal and EGF-stimulated conditions. Furthermore, it showed that this action is accomplished predominantly by inhibition of cell migration rather than cell proliferation or enhancement of apoptosis or cell cycle arrest. In this regard, our studies are in agreement with previous studies demonstrating that migration is the major component of the early phase of restitution (up to 24 hrs) and that cell proliferation becomes more important only during later 24-48 hr period.

EGF-induced actin polymerization and stress fiber formation depend on EGF-R tyrosine kinase activity, since the selective tyrosine kinase inhibitors abolish EGF-induced actin polymerization. Moreover, EGF-R has been shown to bind directly to actin and induce serine phosphorylation. In this context, our findings suggest that the action of EGF on FAK phosphorylation requires EGF-R activation, and that EGF-R can mediate signals downstream via activating FAK.

Cell migration requires a proper reorganization and re-assembly of actin filaments. Studies *in vitro* in several cell lines have shown that EGF-induced activation of EGF-R leads to reorganization

of the actin cytoskeleton. McCormack and colleagues demonstrated *in vitro* that migration of IEC-6 cells depends on actin polymerization and other studies have demonstrated that EGF induces rapid reorganization of actin in human A431 cells. Our present study demonstrated in human gastric-derived MKN 28 cells that EGF significantly increases actin stress fiber formation and that IND reduces both basal and EGF-stimulated actin stress fiber formation, which closely correlates with and likely results in reduced wound re-epithelialization.

Increased expression and/or activation of FAK have been shown to increase cell motility. Withers and colleagues have shown that there is a direct correlation between FAK activity and phosphotyrosine content. In migrating cells, phosphorylated FAK localizes to focal adhesions. In contrast, inhibition of FAK phosphorylation has been shown to decrease both cell proliferation and motility. Tyrosine de-phosphorylation of focal adhesion proteins is correlated with their reduced recruitment to focal adhesions and decrease in the length and number of actin stress fibers in keratinocytes and Swiss 3T3 cells.

Other investigators found that the elevation of FAK's phosphotyrosine content following cell adhesion to the extracellular matrix, directly correlates with increased FAK activity. Our study demonstrated that IND-induced inhibition of restitution involves reduced FAK phosphorylation and reduced FAK recruitment to focal adhesions in wounded gastric monolayers under both basal and EGF-stimulated conditions and *in vivo* in migrating epithelial cells to reconstitute injured rat gastric mucosa.

Tensin, actin capping protein, links actin filaments to focal adhesions and is phosphorylated by FAK. Lo and colleagues have shown that tensin plays a crucial role in the actin-dependent maintenance of cell structural integrity in fibroblasts and is involved in the transmission of signals regulating fibroblast spreading and growth. Therefore, our present finding that IND inhibits EGF-stimulated tyrosine phosphorylation of FAK and tensin, implicates the reduced phosphorylation of these proteins in the mechanism of NSAID-induced disruption of actin stress fibers and thereby inhibition of restitution. Our present study demonstrated the presence of tensin in human gastric epithelial cells, its increased phosphorylation by EGF and reduction of phosphorylation by IND. The level of tensin phosphorylation at baseline was low and therefore the effect of IND on basal levels was undetectable by our methodology. Our observation is supported by a study in rat embryonic fibroblasts showing that phosphorylation of tensin cannot be detected under an unstimulated (baseline) condition. In our present study we found strong correlation between EGF-stimulated wound restitution, increased actin stress fiber formation and enhanced FAK and tensin phosphorylation. While these strong correlations indicate a linear relationship, they do not prove a

causal relationship. However, these results should be considered in the context of the known (described above) crucial roles of actin, FAK, and tensin in cell motility and thus re-epithelialization. Furthermore, IND-induced inhibition of FAK phosphorylation precedes its inhibitory effect on actin polymerization. Therefore, actin depolymerization cannot be the cause of FAK dephosphorylation, but rather is the consequence of decreased FAK activity. Ridyard and colleagues showed that treatment of primary cultures of chick embryo cells with antisense oligonucleotides to FAK reduces the level of FAK protein expression, which causes the loss of stress fibers.

In conclusion, our data show for the first time that FAK and tensin are expressed in human gastric epithelial cells and are phosphorylated in response to EGF. IND treatment inhibits stress fiber formation and phosphorylation of FAK and tensin, suggesting a possible mechanism for interference with mucosal restitution. Since actin polymerization (stress fiber formation) and focal adhesion function are crucial for cell migration, these findings provide a new insight into the mechanism for NSAIDs' interference with restitution following acute gastric mucosal injury.

PART II. EFFECT OF GASTRIN AND A SELECTIVE AND NONSELECTIVE COX-2 INHIBITOR ON THE GROWTH OF GASTRIC ADENOCARCINOMA

BACKGROUND AND INTRODUCTION

Gastric cancer is the seventh most frequent cause of cancer mortality in the US. It has projected for 2002, approximately 21,600 Americans will be diagnosed with gastric cancer and 12,400 will die of it. There are about 700,000 new cases diagnosed each year worldwide. It is still the second most common cancer and the second most common cause of cancer death in the world, surpassed only by the rapid increase in lung cancer since the 1970s. Although a large number of risk factors have been associated with gastric adenocarcinoma, a definite etiology for the majority of gastric adenocarcinomas is still unknown. The pathogenesis is most likely multifactorial. Most epidemiologic studies suggest that gastric adenocarcinoma is increased in areas of lower socioeconomic status, higher use of tobacco and alcohol, limited access to refrigeration and proper food storage, and limited access to fresh fruits and vegetables (vitamin C, vitamin B₆, folate, β -carotene). Patients with *H. pylori* infectio, chronic atrophic gastritis, pernicious anemia, Ménétrier's

disease or patients who have undergone gastrectomy for benign disease are also at an increased risk of developing gastric adenocarcinoma. Although many investigators have postulated a sequence of histologic events in the progression to gastric adenocarcinoma, definitive experimental or clinical evidence confirming this progression does not exist.

Gastrin: The gastrointestinal peptid gastrin has been implicated in a wide variety of functions, including secretion of gastric and pancreatic juices, satiety, and the growth of the gastrointestinal tract. Gastrin is an important growth factor for gastrointestinal tract (GI) and has been reported to be trophic to different GI tissues including parietal cells, enterochromaffin cells, colonic and pancreatic epithelial cells. The receptors for gastrin have been identified in most of these tissues. Besides of these main functions of gastrin, it has been shown to have function on the stimulating of growth colonic, pancreatic cancers. Gastrin administration is able to enhance the growth of carcinogen-induced colonic cancer in rats, human colon cancer cells in culture and tumor xenografts to nude mice, therefore it would be interesting to see whether gastrin enhances the growth of gastric cancer.

NSAIDs and Gastric Cancer: Epidemiological studies have reported a decrease in the incidence of gastric cancer in chronic users of NSAIDs. Many tumors, including gastric carcinoma, contain high concentrations of PGs which promote cellular proliferation, tumor growth and angiogenesis. Using APC knockout mice, Oshima and colleagues provided genetic evidence linking COX-2 expression to colonic tumor promotion. Upregulation of COX-2 has also been demonstrated in human gastric adenocarcinomas as well as in pre-cancerous lesions of the stomach such as metaplasia and gastric adenoma cells vs. normal gastric. It has therefore been postulated that NSAIDs exert their cancer chemopreventive properties through inhibition of COX-2 and thus inhibition of prostaglandins. The possible anti-neoplastic actions of NSAIDs include the induction of apoptosis and/or inhibition of cellular proliferation. IND, a non-selective NSAID, induces apoptosis in human gastric cancer cells (AGS).

EGF and other growth factors upon binding to their receptors activate receptor protein tyrosine kinases, which trigger signaling cascades transmitting mitogenic and proliferative signals to the nucleus. This is accomplished by sequential activation of various cytoplasmic protein kinases including mitogen activated protein kinase (MAPK). There are several isoforms of MAPK (ERK1, ERK2, SAPK, p38, HOG1 kinase and ERK5). The ERK2 (extra-cellular regulated kinase) controls cellular growth and differentiation. Constitutive over-expression of MAPK promotes cellular transformation. Pharmacological inhibition or mutational inactivation of MAPK blocks neuronal differentiation, and results in cell cycle arrest in fibroblasts. Sebolt-Leopold and colleagues

demonstrated an 80% inhibition of colon carcinoma growth in rodents treated with an oral MAPK inhibitor (PD 184352). The involvement of MAPK/ERK2 in the progression of gastric cancer however remains unknown.

AIMS

Since the gastrin administration seems to be able to enhance the growth of carcinogen-induced colonic cancer, we examined *in vitro* (I) the stimulating effect of gastrin on tumor proliferation of human gastric adenocarcinoma and compared that effect of pentagastrin, which is usually used *in vivo* animal experiments, with the stimulating effect of human gastrin-17, that is more often used *in vitro* circumstances.

Since increased and unregulated cell proliferation and reduced cell apoptosis are important features of cancer growth, we also aimed determine whether (II) NS-398, a selective COX-2 inhibitor and/or IND, a non-selective COX inhibitor: 1) inhibit gastric cancer cell proliferation, 2) if this inhibition is mediated via MAPK(ERK2), and 3) whether NSAIDs trigger apoptosis in gastric cancer cells.

OBSERVATIONS

I. EFFECT OF GASTRIN OF GASTRIC CANCER GROWTH

Gastrin possesses the same five-amino acid carboxy terminal end as cholecystokinin (CCK). Originally two types of CCK receptors have been described (CCK-A and CCK-B). Recently the CCK-B receptor and gastrin receptor have been cloned, and they appeared to be identical. This CCK-B/gastric receptor has been reported to be present on the surface of cancer cells isolated from humans surgical specimens and on rat and human colonic (WiDr, HT-29, CoLo 205, T84, YAMC), pancreatic (PANC-1, BON) cell lines.

Nowadays, a new era has started when an incomplete growth inhibition by highly selective CCK-A and CCK-B receptor antagonists was found on pancreatic tumor cell line, AR4-2J and on fibroblast cell line, Swiss-3T3 cells after the incubation with glycine-extended gastrin. The mouse colon cell line, YAMC, was also found to express surface receptor selective for glycine-extended gastrin-17.

Materials and Methods

Cell Culture: The human gastric adenocarcinoma cell line (AGS, CRL-1739) was obtained from American Type Culture Collection. The cells were cultured in DMEM containing 10% FCS at

37 °C in a humidified (95%) incubator containing the atmosphere of 95% air and 5% CO₂. The cells were seeded on incubating plates in 10⁴ initial numbers. After the 24 hrs, the growth media of the cells were changed to serum-free medium containing vehicle or pentagastrin (10⁻⁹ – 10⁻⁵ M) or gastrin-17 (10⁻¹⁰ – 10⁻⁷ M).

Detection of Proliferation: For the measurement of cell line proliferation we preceed several methods following a 5-day incubation in serum-free medium containing vehicle or pentagastrin or gastrin-17. For the detection of the metabolically active cells we used Cell Titer 96Aqueous Assay purchased from Promega. The absorbency of the produced formazan was measured at 490 nm with Dynatech 6000 Elisa Reader directly from 96 well assay plates without additional processing.

The changes of total protein content of the wells were measured by the method of Bradford using Coomassie Brilliant G-250 for another method for the determination of cell proliferation. Firstly, the cells were seeded onto 96 well assay plates, and incubated with gastrin/pentagastrin concentrations. After 5-day growth, the cells were incubated with 0.1% Triton X-100. Fifteen µL of sample were put onto 96 well assay plates together with 200 µL Bradford reagent. Then, the measurement was carried out using Dynatech 6000 Elisa Reader at 595 nm within 5 min.

Cell growth measurements were also evaluated by performing cell counting in hemocytometer.

Statistical analysis: Student's *t* test was used to compare data between two groups. Values are expressed as mean ± standard deviation (SD). *P* values less than 0.05 were considered significant.

Results

Results of Proliferation Assay: 10⁻⁵ M pentagastrin incubation caused the lowest extinction detected. Actually, it slightly inhibited the growth of the cell line (88% of control). Under smaller concentrations of pentagastrin (10⁻⁹ – 10⁻⁶ M), the propagation of the cell line increased parallel with the decrease of pentagastrin concentration. The maximal effect (120.5% of control on the 5th day) could be reached with 10⁻⁸ M pentagastrin concentration.

In case of human gastrin-17, the cell proliferation changed similarly with the decrease of concentration. The 10⁻⁹ M dose caused the maximal absorbency (123.7% of control) at the end of the 5th day. Otherwise, similar and almost equal changes could be detected.

Changes of Protein Content: The method of Bradford led us to similar results, although, the standard errors were the highest in these measurements. The total protein contents in wells of assay plates containing gastrin/pentagastrin were compared to that value measured in wells of controls with no gastrin/pentagastrin in the medium. The maximal effects were reached with the same concentrations of gastrin-17 and pentagastrin as in case of the proliferation assay (gastrin: 10^{-9} M; pentagastrin: 10^{-8} M) ($p < 0.001$). However, it may be predicted that the gastrin-17 was found to be more capable to increase the protein content of the AGS cells (27.5mg/ml vs. 25 mg/ml, detected on the 5th day).

Changes of Cell Number: Similar tendencies were found in the results of cell counting. The extents of stimulation were similar to the results of proliferation assays (pentagastrin: 122%; gastrin-17: 120% on the 5th day).

Discussion

The aim of our study was to compare the tumor growth-promoting effect of pentagastrin and gastrin-17 *in vitro* on human cultured gastric adenocarcinoma cells. Pentagastrin is typically used in experiments involving animals because it is more available and less costly than gastrin-17. Human gastrin-17 is mostly used *in vitro* experiments.

We chose gastric cancer, AGS cell line. AGS cells contain Alcian blue and PAS positive granules in their cytoplasm, which are consistent with cells that secrete mucopolysaccharides. Results of electron microscopic analysis revealing junctional complexes and rudimentary apical microvillae, also suggest that AGS cells possess the ability to differentiate morphologically and have characteristic traits consistent with mucus secreting cells. Sharma et al. demonstrated that this cell line to be an excellent model for studying *H. pylori* infection under *in vitro* conditions. These results and our personal experience with this cell line led us to believe that this cell line is an excellent model for the study of the propagation of gastric cancer.

Each method for detection of proliferation stimulated by gastrins resulted analogue trends to the results of both other methods. We found that pentagastrin is 10 times less potent stimulator of tumor growth of the AGS cells than gastrin-17. It suggests that smaller gastrin forms are more readily metabolized or degraded than the larger forms. Because of these pharmacological differences, dosages of pentagastrin are needed to be higher than gastrin-17. The AGS cells might be CCK-B/gastrin like receptor positive. Our results are in correlate with results of other studies. Majumdar et al. found similar differences in the effective dosages of pentagastrin and gastrin on rat

pancreatic tissue. Smith and colleagues proceeded observations suggesting similar results on human pancreatic adenocarcinoma cell line (PANC-1).

II. EFFECT OF NSAIDS ON GASTRIC CANCER GROWTH

Materials and Methods

Cell lines: Human gastric epithelial (MKN 28) cells derived from gastric tubular adenocarcinoma were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator containing 5% CO₂. Cells were incubated for 6, 16, 24 and 48 hr in media containing either vehicle, IND at concentrations of 0.25-0.5 mM, or NS-398 at concentrations of 50-100 µM.

Cell Proliferation Assay: The effect of IND and NS-398 on MKN 28 cell proliferation was studied by determining the incorporation of ³H-thymidine into cellular DNA. Cells (4 x 10⁴ cells/well) were growing for 24 hr in 24-well culture plates until 70 - 75 % confluence. Cells were serum-starved for 24h and treated for 16 hr with either vehicle, IND or NS-398. Three hours prior to termination of the experiment, 0.5 µCi of [methyl-³H]thymidine was added to each well. After incubation, the cells were washed three times with PBS, lysed with 0.5 N NaOH, neutralized with 0.5 N HCl, and the radioactivity was measured. The optimal concentrations of IND and NS-398 were determined by our previous dose-dependent studies. These studies also revealed that these doses of IND do not reduce cell viability below 90%.

Apoptosis/Cell Death Detection Assay: MKN 28 cells were plated in 6-well plates on glass coverslips. After reaching confluence, monolayers were wounded and cultured in serum-free medium containing either 0.5 or 0.25 mM IND or 50 or 100 µM NS-398 or their vehicles for 24 hrs. Cell death was evaluated by terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) described under the Methods Section of Part I.

Determination of Mitogen-activated Protein Kinase Activity: ERK2 activity was determined as described in our previous study. Briefly, cells were incubated in medium containing either vehicle or the indicated concentration of IND or NS-398. The cells were then lysed on ice; 30 µg of total protein from each experimental sample was added to a conjugate of protein A Sepharose and 1 µg anti-ERK2 antibody and mixed at 4°C for 2 hrs. The conjugates were then pelleted by centrifugation and washed four times. After the final wash, buffer was removed completely and 40 µl of MAPK assay mixture (10 mM HEPES, pH 7.5; 10 mM MgCl₂; 50 µM ATP; 30 µg myelin basic protein; and 4 µCi [³²P] ATP) was added to each sample. The samples were incubated at 30°C

for 20 min and the reaction was terminated by the addition of SDS-polyacrylamide gel electrophoresis sample buffer. The samples were then electrophoresed on 15% acrylamide gels. After electrophoresis, the gels were stained with Coomassie brilliant blue and dried. The gels were autoradiographed; the myelin basic protein bands were cut out and radioactivity was counted in a scintillation counter.

Ras Activation Assay: MKN 28 cells were plated in 100-mm tissue culture dishes and grown until ~80% confluent in RPMI 1640 supplemented with 10 % fetal bovine serum. The cells were serum-starved for 16 hrs and metabolically labeled for an additional 8h in serum-free, phosphate-free RPMI containing 200 $\mu\text{Ci/ml}$ $^{32}\text{PO}_4$. During the serum starvation and metabolic labeling, cells were incubated with either vehicle, 0.5mM IND, or 100 μM NS-398. Ras activation was determined as described. Briefly, cells were washed with ice-cold PBS and lysed on ice in 1 ml of lysis buffer. Ras proteins contained in the cell lysates were immunoprecipitated with rat monoclonal anti-Ras antibody. The guanine nucleotides bound to the Ras proteins were eluted in 16 μl of 2 mM EDTA; 5 mM dithiothreitol, 1 mM GTP, 1 mM GDP, 0.2% SDS at 68°C for 20 min and fractionated by thin-layer chromatography. Quantification was performed using a phosphorimager. The percent of GTP bound to Ras (as an indicator of Ras activation) was calculated as $\text{cpm in GTP}/(\text{cpm in GTP} + \text{cpm in GDP})$ normalized for moles phosphate in each nucleotide.

Statistical analysis: Student's *t* test was used to compare data between two groups (e.g., control and growth factor-treated group). One-way ANOVA and Bonferroni correction were used to compare data between three or more groups. Values are expressed as mean \pm standard deviation (SD). *P* values less than 0.05 were considered significant.

Results

Effects of IND and NS-398 on proliferation of MKN 28 cell lines: To evaluate the effects of IND and NS-398 on proliferation of gastric cancer cells, various concentrations of IND (0.25 mM - 0.5 mM) or NS-398 (50 μM - 100 μM) were added to the medium for 24 and 48 hrs. Compared to control, both the higher and lower concentrations of IND and NS-398 significantly inhibited cell proliferation at 24 hrs.

Effect of IND and NS-398 on MAP (ERK2) kinase activity: Various concentrations of IND (0.25 mM-0.5 mM) and NS-398 (50 μM -100 μM) were added to MKN 28 cell lines for 24 hrs.

Cells treated with vehicle served as controls. Compared to control, both NSAIDs significantly inhibited ERK2 activity.

Effects of IND and NS-398 on Ras activation in MKN28 cells: IND (0.5 mM) and NS-398 (100 μ M) were added to the MKN 28 cell media, and Ras activation was evaluated. Compared to control treatment with vehicle, neither IND nor NS-398 (at their higher concentration) significantly affected Ras activation in MKN 28 cells.

Effects of IND and NS-398 on apoptosis of MKN28 cells: IND (0.25 mM - 0.5 mM) and NS-398 (50 μ M - 100 μ M) were added to the MKN 28 cell media, and apoptosis was evaluated using the TUNEL assay. Compared to control treatment with vehicle, both concentrations of IND and NS-398 significantly induced apoptosis in MKN 28 cells. Necrotic index was <1% in all experiments.

Discussion

This study demonstrated that both the non-selective NSAID, IND, and the selective COX-2 inhibitor NS-398 significantly inhibit proliferation of the human gastric cancer cell line MKN 28, reduce ERK2 activity and enhance apoptosis. Our results differ from those found in the limited literature on the role of NSAIDs in gastric cancer. Sawaoka H *et al* and Tsuji S *et al* demonstrated inhibition of cell proliferation by both selective and non-selective NSAIDs in gastric cancer cell lines that over-expressed COX-2. However, cell lines such as MKN 28 and KATO III which express significantly more COX-1 than COX-2 were less suppressed by these same agents. These results would indicate that the anti-neoplastic properties of NSAIDs are dependent on COX-2 rather than COX-1 inhibition. The discrepancy between their findings and ours may be due to the concentrations of IND and NS-398 utilized for the respective experiments. We used significantly higher concentrations of both agents compared to previous studies, though it should be noted that even at these higher concentrations, cell viability was not reduced below 90%. It is very likely that the anti-proliferative properties of both NSAIDs are dose dependent. It has been previously demonstrated that IND inhibits KATO III cell growth. This gastric cancer cell line expresses much less COX-2 than COX-1 and does not produce prostaglandins. The anti-proliferative action of IND on these cells was not reversed by the administration of exogenous prostaglandins. Therefore, the anti-neoplastic properties of NSAIDs on gastric cancer can not be explained by COX inhibition alone.

MAP (ERK2) kinase signaling pathway is essential for cell proliferation and may be one possible COX- independent target for NSAIDs. We have shown significant inhibition of MAP (ERK2) kinase activity and phosphorylation in gastric cancer cells treated with 0.5 mM IND, and 50 μ M and 100 μ M NS-398 without a significant change in the protein levels of this enzyme. The activity of other upstream proteins in this pathway such as Ras is not markedly affected by NSAIDs treatment. These data suggest that the NSAID-induced inhibition of gastric cancer proliferation and growth is likely mediated by the inhibition of MAP (ERK2) kinase signaling pathway. Therefore, this study demonstrated for the first time that in addition to inhibition of cyclooxygenase, NSAIDs also inhibit phosphorylating enzymes-kinases such as ERK2 essential for transduction of mitogenic signals in gastric cancer cells.

Our results also demonstrated the induction of apoptosis by both IND and NS-398 in the MKN 28 cell line. Treatment with both NSAIDs significantly increased the apoptotic index by up to three fold. These results are consistent with those reported in other gastric cancer cell lines. However, this increase in cell apoptotic death was from ~3% at baseline to 4.5-8.5% and the majority of the cells (>90%) remained viable. Therefore, such modest reduction of viable cells cannot account for very significant reductions of cell proliferation and MAPK activity. The signaling pathways regulating NSAID-induced apoptosis in gastric cancer are poorly understood. Zhu GH *et al* has suggested that IND-induced apoptosis of gastric cancer cell lines is associated with either constitutive expression of wild-type p53 and/or increased expression of the proto-oncogene *c-myc*. Protein kinase C has also been shown to modulate NSAID-induced apoptosis by changing the expression of pro-apoptotic genes.

We conclude from our experiments that both non-selective and selective-COX-2 inhibitors significantly suppress cellular proliferation and growth of the MKN 28 gastric cancer cell line. This inhibitory effect is likely mediated by inhibition of MAP (ERK2) kinase signaling pathway, which is the key pathway responsible for cell proliferation. In addition we have also shown that NSAIDs increase apoptosis in gastric cancer cells.

SUMMARY OF NEW RESULTS

The results of the present Thesis can provide a new insight in the molecular mechanism of gastric injury healing and cancer research. Summarizing these new results:

1. In wounded gastric epithelial monolayers EGF significantly stimulated wound restitution, actin stress fiber formation, increased FAK localization to focal adhesions, and phosphorylation of FAK and tensin,
2. IND inhibited wound restitution at the baseline and EGF-stimulated conditions.
3. IND-induced inhibition of FAK phosphorylation preceded changes in actin polymerization, indicating that actin depolymerization might be the consequence of decreased FAK activity.
4. In surface epithelial cells of rat gastric mucosa, FAK phosphorylation is induced following injury.
5. IND significantly delays epithelial restitution *in vivo*, reduces FAK phosphorylation and recruitment to adhesion points as well as actin stress fiber formation in migrating surface epithelial cells.
6. Our study indicates that FAK, tensin and actin stress fibers are likely mediators of EGF-stimulated restitution in wounded human gastric monolayers and potential targets for IND-induced inhibition of restitution.
7. Human gastrin-17 and pentagastrin stimulate the proliferation of human gastric cancer cells in correlation with their concentration, indicating a possible disadvantage for long-term proton pump inhibitor (PPI) treatment.
8. Pentagastrin is 10 times less effective stimulator of proliferation of the gastric cancer than gastrin-17, and human adenocarcinoma cell line might be CCK receptor positive.
9. Nonsteroidal anti-inflammatory drugs, IND and NS-398 significantly inhibit proliferation and growth of human gastric cancer cell line MKN 28. This effect is mediated by NSAID-induced inhibition of MAPK (ERK2) kinase signaling pathway, essential for cell proliferation. NSAIDs also increase apoptosis in MKN 28 cells.
10. In addition to inhibiting cyclooxygenase, NSAIDs inhibit a phosphorylating enzymes – kinases essential for migration and for signaling cell proliferation.

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