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

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PH.D. THESIS

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With this general view of the nature of Digestion before us, we can now examine more satisfactorily the structure and mode of action of the organs concerned in effecting it. Chymification being the first step in the complicated process, we shall begin with the organ by which it is performed, namely the STOMACH.

(The Physiology of Digestion by Andrew Combe, 1837)

Contents

1. List of Abbreviations

APC – adenomatous polyposis coli

ATCC – American Type of Culture Collection

COX – cyclooxygenase

DAG – diacylglycerol

DCC – deleted in colon cancer gene

DMEM – Dulbecco's modified Eagle's medium

EGF – epidermal growth factor

EGF-R – epidermal growth factor-receptor

ERK1/2 – extracellular sinal-regulated kinase-1/2

FAK – focal adhesion kinase

FCS – fetal calf serum

FGF-1/-2 – fibroblast growth factor-1/ -2

GDP / GTP- guanil diphosphate / triphosphate

GI – gastrointestinum / gastrointestinal

HGF – hepatocyte growth factor

IL-lα/β – interleukin-lalpha/beta

IND – indomethacin

IP_3 – inositol triphosphate

MCC – mutated in colorectal cancer gene

MAPK – mitogen activated protein kinase

MEK – MAP kinases kinase (or MAPKK)

MLCK – myosin light chain kinase

NSAID(s) – nonsteroidal anti-inflammatory drug(s)

PDGF – platelet-derived growth factor

PG – prostaglandin

PGE_2 – prostaglandin-E_2

PGI_2 – prostacyclin

PKC – protein kinase C

PLCγ – phospholipase C-gamma

PMN – polymorphonuclear

Raf – serine/theronine kinase

Ras – small G-protein, product of a proto-oncogene

Rb – retinoblastoma

SAPK – signal-activated protein kinase

SEM – standard error of mean

SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SH2 – Src homology-2

SOS – son of sevenless adapter protein

TGF-α/β – transforming growth factor-alpha/beta

TNFα – tumor necrosis factor-alpha

TP – trefoil peptid

TXA – tromboxane

VEGF – vascular endothelial growth factor

2. Preface

The gastrointestinal (GI) tract has an important function in the body. It stores ingested food and beverages for digestion, prepares the various components for absorption and transfers actively the physiologically necessary compounds into the blood stream and lymphatic system. On the other hand, the GI tract also performes excretory functions. Thus the GI tract contributes in many ways to homeostasis of the body. Stomach being an organ of GI tract possesses major roles. The primery function of the stomach is to serve as a storage reservoir. The hydrochloric acid in the stomach breaks food down into small particles and pepsin begins to process of breaking down protein molecules to amino acids. The stomach gradually empties its contents through the pyloric sphincter into the duodenum, the upper portion of the intestine, where most of the absorption takes place. Beside its food storage and digestive function, the stomach secretes bioactive peptides (intrinsic factor, gastrin).

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 the inhibition of 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 in gastric adenocarcinoma. The molecular

mechanisms involved in these two distinct areas of GI research seem to share quite a lot of common aspects, the basic mechanisms ivolved are common.

Part I.

Indomethacin-induced Delay in Gastric Restitution is Associated with the Inhibition of Focal Adhesion Kinase, Tensin and Actin

3. Backgound

3.1. Nonsteroidal anti-inflammatory drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are usually designated antipyretic or nonnarcotic analgestics to separate them from the more potent narcotic analgestics, but they are also highly effective anti-inflammatory agents. They relieve pain of diverse causes, including headache, joint and muscle pain. They lower the elevated body temperature and reduce inflammation, which is specially used in rheumatoid arthritis and rheumatic fever. 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 [Wolfe MM *et al*, 1999].

Aspirin is the far most important and most commonly used drug of the group. A German chemist, Kolbe, proposed in 1873 that salicylic acid be used to treat infections because it obviously would liberate phenol and therefore prove bactericidal. Then Swiss physician, Carl Buss, reported the antipyretic effect of willow bark and related it to its salicylate (salicin) content. The antirheumatic effect of pure salicin was described soon after. In 1883, Knorr, German chemist, discovered antipyrine, a second class of antipyretics. In 1886, in the school of the internist Kussmaul, two students gave acetanilide to a patient accidentally thinking that it was a parasiticide. Fortunately, the patient had fever and the beneficial effect was noted. This error

suggested to the Bayer organization that they might dispose of a surplus of para-aminophenol by converting it to some analog of acetanilide, and in this way ethoxyacetanilid (phenacetin) was prepared. The same laboratory, looking for an alternative to sodium salicylate, restudied acetylsalicylic acid and demonstrated the superiority of aspirin as an antipyretic in 1899.

NSAIDs such as aspirin, indomethacin, ibuprofen and others, control pain and inflammation by inhibiting cyclooxygenase (COX) or prostaglandin-endoperoxide synthase (Figure 1). COX first identified over 30 years ago, is the rate-limiting enzyme that catalyzes the formation of prostaglandin from arachidonic acid [Vane JR, 1971]. This enzyme exists as two isoforms, cyclooxygenase-1 and-2 (COX-1 and COX-2) [Yokoyama C *et al*, 1989; Hla T *et al*, 1992]. The constitutive isoform or COX-1 is expressed in most tissues [Vane J, 1994]. The inducible isoform, COX-2, is produced in response to proinflammatory cytokines and growth factors in a variety of cells and is constitutively expressed in brain and kidneys [Kubuju D *et al*, 1991; Jones DA *et al*, 1993; Dubois RN *et al,* 1994, 1998]. Both enzymes are inhibited by non-selective nonsteroidal anti-inflammatory drugs, while COX-1 and COX-2 are specifically inhibited by selective COX-1 and COX-2 inhibitors, respectively (Table 1).

Chronic use of NSAIDs causes gastric erosion in ~60 % of users, gastroduodenal ulcers in ~29 % of users, often with severe gastrointestinal (GI) bleeding and/or perforations and even leads to death. The costs of these side effects exceed $12 billion per year only in the US [Lee ER *et al*, 1975; Fries JF, 1991; Scheiman JM, 1996]. In Hungary ~1,5 % of the population regularly intakes NSAID, and ~30-37% of these users get GI complication [Nemesánszky E, 1995].

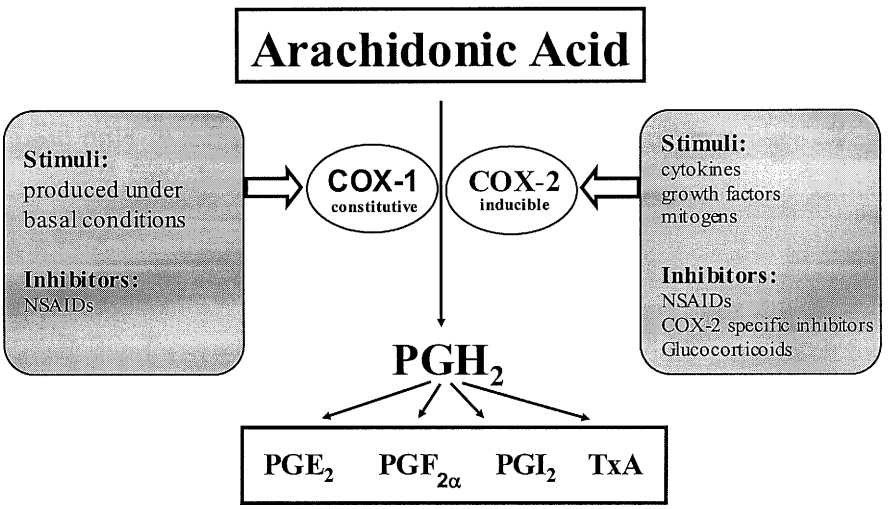

Figure 1. Role of cyclooxygenases. COX converts arachidonate to prostaglandin G_2 by inserting two oxygen molecules and then reducing this intermediate to prostaglandin H_2 (PGH_2) [Smith WL *et al*, 1991; Mernett L, 1992]. Prostaglandin H_2 is an unstable metabolite that is converted to an array of prostaglandins, including prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), and tromboxanes (TXA), which have both autocrine and paracrine functions. Citokines, growth factors and various mitogens stimulate inducible COX expression. PGE_2 is a potent vasodilative component resulting inflammatory oedema. Tromboxan (TXA) is a vasoconstrictor mainly produced in trombocytes, lymphocytes, monocytes and endothelial cells.

Table 1. Comparison of IC_{50} values of various NSAIDs on COX-1 and COX-2 inhibition [Jones RA, 1999]

NSAID	Ratio COX-1: COX-2
Aspirin	0.12
Diclophenac	38.00
Etodolac	179.00
Ibuprofen	0.86
Indomethacin	0.30
Loxoprofen-SRS	3.20
NS-398	1263.00
Oxaprozin	0.061
Zaltoprofen	3.80

3.2. Indomethacin

Indomethacin (IND), although it is certainly more toxic and not suggested as a simple analgestic, is used only in a few special situations and after safer drug have not given a desired effect. These special situations are acute gouty arthritis, rheumatoid spondylitis and osteoarthritis of the hip. In the laboratory, since IND seems to be more effective than aspirin in inhibition of prostaglandin synthesis, it is commonly used in a wide-range of studies representing the features of NSAIDs. IND was chosen for our experiment because of these above reasons.

3.3. Gastric Injury

Gastric mucosal injury by NSAIDs includes: *a)* ***superficial injury*** limited to exfoliation of the surface epithelium, *b)* deep mucosal injury whereas in addition to superficial epithelium, microvessels are also damaged and ***erosions*** are formed with the loss of its electrical and barrier function, and *c)* deep injury penetrating the mucosa and muscularis mucosa – an ***ulcer*** (Figure 2) [Cotran RS *et al*, 1994; Tarnawski AS *et al*, 1990, 1993].

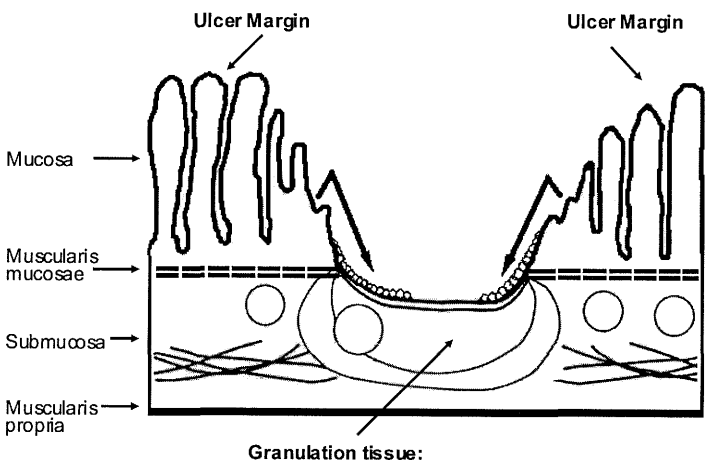

Figure 2. Diagram of a healing ulcer consisting of ulcer margin and granulation tissue

3.4. Superficial Injury Healing (Restitution)

Following superficial mucosal injury, the continuity of the surface epithelium is promptly reestablished within a few hours by the process referred to epithelial ***restitution*** [Lacy ER *et al*, 1984]. This process involves migration of the epithelial cells from the gastric pits and upper regions of the glands bordering injury to cover the denuded mucosal surface. Restitution is independent of cell proliferation since, but requires an intact basement membrane [Svanes K *et al*, 1982; Morris GP, 1986; Watanabe S *et al*, 1994].

3.5. Gastric Erosion Healing

Focal deep mucosal necroses are mucosal erosions, whereas in addition to superficial epithelium, microvessels are also damaged with the loss of its electrical and barrier function. The repair of such injury requires not only restoration of surface epithelium and glandular epithelial cells, but also the reestablishment of connective tissue and the microvascular network, crucial for delivery of oxygen and nutrients to the area. The molecular mechanisms involved in erosion healing are fairly identical with the mechanism of ulcer healing (described below).

3.6. Ulcer Healing

Gastric, duodenal or intestinal ulcers are deep necrotic lesions penetrating through the entire mucosal thickness and the muscularis mucosae (Figure 2). Healing of gastric ulcer is a kind of wound repair. Wound healing is consistant of three different elements. These are ***re-epithelialization***, ***angiogenesis***, and ***fibroplasia***. They are separate but interdependent processes that gradually restore tissue integrity to the wound.

Vascular and microvascular changes - endothelial injury, thrombi, formation and vascular constriction - are the earliest events occurring during formation of experimentally-induced

gastric ulcers [Tarnawski AS *et al*, 1990]. These vascular changes cause ischemic necrosis of the mucosa and muscularis mucosae. Polymorphonuclear (PMN) leukocytes and macrophages are attracted by a variety of signals, such as growth factors released by platelets and fibrin degradation products. PMN leukocytes phagocytize necrotic tissue and release pro-inflammatory cytokines [e.g. tumor necrosis factor alpha (TNFα), interleukin (IL) IL-lα and IL-lβ], which in turn activate local fibroblasts, endothelial and epithelial cells [Clark R, 1996; Martin P, 1997]. Initial infiltration of the ulcer with PMN leukocytes is reduced after several days and is followed by accumulation of macrophages via recruitment of blood monocytes and local tissue macrophages [Martin P, 1997]. These macrophages remove necrotic debris by phagocytosis, and upon activation, release a variety of growth factors and cytokines including transforming growth factors alpha (TGFα) and beta (TGFβ), fibroblast growth factors (FGFs) -1 and -2, platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) at the ulcer site [Bennett NT *et al*, 1993; Clark R, 1996; Martin P, 1997]. Macrophages are essential participants for ulcer healing as evidenced by the fact that inhibition of macrophage infiltration impairs and delays healing [Martin P, 1997].

Morphologically, an ulcer consists of a distinct (a) ***ulcer margin*** - the epithelial component - and of (b) ***granulation tissue*** at the ulcer base (Figure 2). The latter - the connective tissue component - is composed of fibroblasts, macrophages and proliferating endothelial cells which form microvessels [Cotran RS *et al*, 1994; Tarnawski AS *et al*, 1990, 1993].

3.7. Ulcer margin

Ulcer margin is a zone of normal mucosa adjacent to the ulcer. In this region, the epithelial cells bordering the ulcer de-differentiate, express EGF-receptor (EGF-R), and then

actively proliferate [Tarnawski AS *et al*, 1992, 1993]. Simultaneously, epithelial cells start to migrate toward the ulcerated area. This process is called ***re-epithelialization***, which is endoscopically the disappearance of whitish exudates, and has been suggested to indicate the healing of gastric ulcer [Basson MB *et al*, 1992; Martin P, 1997; Tarnawski AS *et al*, 1993, 1997]. Cell proliferation supplies the epithelial cells necessary for reconstruction of the gastric glands, re-epithelialization is dependent on cell proliferation contradictory to restitution. Without restoration of a continuous epithelial "barrier", granulation tissue (the other essential component) would be vulnerable to mechanical or chemical injury and infections thereby preventing ulcer healing [Bennett NT *et al*, 1994; Tarnawski AS *et al*, 1993, 1997].

3.8. Granulation Tissue

Granulation tissue develops at the ulcer base within 48-72 hrs after ulceration [Cotran RS *et al*, 1994; Tarnawski AS *et al*, 1990, 1991, 1993, 1997], consisting of proliferating connective tissue cells, including macrophages, fibroblasts, and proliferating endothelial cells. Proliferating connective tissue cells restore the lamina propria, endothelial cells form microvessels through the process of ***angiogenesis*** [Bennett NT *et al*, 1993; Cook GA *et al*, 1997; Martin P, 1997; Tarnawski AS *et al*, 1990, 1991, 1993, 1997] , supplying oxygen and nutrients for proliferating tissue components (Figure 3, Table 2).

3.9. Regulation of Re-epithelialization

Cell proliferation, division, migration and re-epithelialization are activated by growth factors (Figure 3.) [Bennett NT *et al*, 1993; Cotran RS *et al,* 1994; Tarnawski AS *et al*, 1993]. In addition to initial pool of growth factors derived from the platelets, macrophages and injured tissue activates genes encoding for the growth factors such as, EGF, bFGF, PDGF, Trefoil

peptids (TP), and hepatocyte growth factor (HGF) in a well coordinated, sequential manner in the mucosa of the ulcer margin [Cook GA *et al*, 1997; Taupin D *et al*, 1999]. These growth factors produced locally, activate migration and proliferation and accelerate gastrointestinal mucosal healing (Table 2).

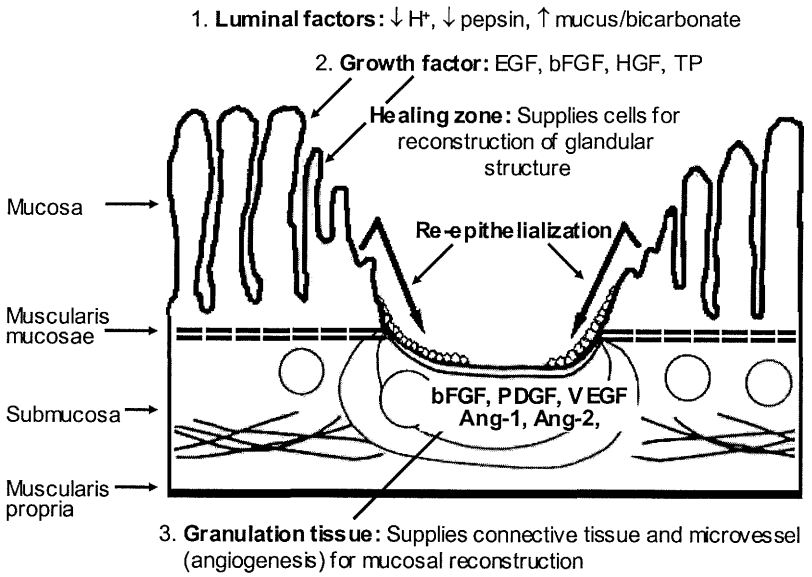

Figure 3. Diagram of components participating in the healing of ulcer. Healing of the ulcer is accomplished by filling the mucosal defect with: 1) cell migrating from the healing zone and replicating under influence EGF, trefoil peptides, HGF, bFGF and other growth factors and cytokines as well as 2) connective tissue cells including microvessels originating from the granulation tissue and growing under influence of bFGF, VEGF, PDGF and angiopoietins (Ang1, Ang2). (Modified from Tarnawski AS *et al*, 1991).

Table 2. Structural, cellular and molecular changes in the epithelial component of the ulcer (ulcer margin)

Cellular changes:	1) Cell de-differentiation 2) Cell proliferation 3) Re-epithelialization (migration onto granulation tissue) 4) Tube formation and gland regeneration & gland reconstruction in the ulcer scar
Molecular changes:	Activation of genes encoding for: • EGF and EGF-R • Trefoil peptides • PDGF • Hepatocyte growth factor (HGF) and c-met/HGF-R • Early response genes: c-fos, c-jun, egr-1, SP-1 • Other growth factors and cytokines

Activation of epithelial cells is accomplished by binding growth factors to their specific receptors (e.g. EGF to EGF-R) on the epithelial cell membrane, this process triggers a number of intracellular signaling events that culminate in cell migration and proliferation [Basson *et al*, 1992; Bennett NT, 1993; Ciacci C *et al*, 1993; Dignass AU *et al*, 1994, Tarnawski AS, 1993; Majumdar APN *et al*, 1996; Martin P, 1997; Podolsky DK, 1994, 1997; Takahashi M *et al*, 1997; Watanabe S *et al*, 1995, 1996]. Growth factors stimulate cell migration by inducing actin cytoskeleton re-arrangements: actin stress fiber assembly, lamelipodia and focal adhesion formation, which are all crucial for cell motility [Santos MF *et al*, 1997]. Phospholipase C-γ (PLCγ) activation is required for EGF- and PDGF-induced cell motility [Polk DB, 1998; Bornfeldt KE *et al*, 1995]. The molecular basis of how PLCγ modulates actin filament rearrangement allowing cell migration is not well understood. One possible mechanism of migration may involve the direct hydrolysis of PIP_2 by PCLγ and subsequent mobilization of membrane-associated actin modifying proteins, such as tensin, paxillin, focal adhesion kinase (FAK) and/or myosin light chain kinase (MLCK) [Chen HC *et al*, 1994]. PLCγ activity may

also promote cell motility secondary to the generation of second messengers or by altering the membrane-cytoskeleton associations in focal adhesions.

An attractive hypothesis proposes that cytoskeleton, especially the actin microfilament system, mediates a signal transduction cascade inside the cell [Boonstra J *et al*, 1995]. The actin binding proteins appear to interact with metabolites of the phosphoinositide pathway [van Delft S *et al*, 1995], which results in actin polymerization. It has also been reported that formation of membrane ruffles as a result of actin polymerization is associated with activation of the Ras pathway and also involves phosphatidyl inositol 3-kinase (PI-3K) [Auger KR *et al*, 1996; Chen HC *et al*, 1994]. Moreover, studies in A431 cells indicate that EGF-R is associated with the actin microfilament system [den Harting JC *et al*, 1992; van Bergen en Henegouwen PM *et al*, 1992; van Delft S *et al*, 1995] and, more importantly, biochemical analysis demonstrated that EGF-R itself is an actin binding protein [den Harting JC *et al*, 1992]. The relationship and interactions between the cytoskeleton and the EGF-triggered signaling cascade may explain the mechanisms of EGF-induced cell migration, which involves cytoskeletal rearrangements as the major moving force.

The mechanisms involved in regulation of restitution and re-epithelialization diverse on the scope of growth factors present for stimulation of migration. Restitution can be triggered by only those growth factors, which are present in time of or shortly following the injury (do not have to be synthesized). The saliva and other GI juices (gastric, intestinal) play a major role in continuous supply of growth factors for the stomach and other part of the GI tract (e.g. EGF, trefoil peptides) [Podolsky DK *et al*, 1994, 1997], which can easily stimulate epithelial restitution upon acute injury within minutes.

3.10. Signaling Pathways in the Mucosa of the Ulcer Margin

Several studies demonstrated that gastric ulceration triggers activation of genes encoding for EGF, its receptor - EGF-R, trefoil peptides and other regulatory peptides in epithelial cells of the ulcer margin [Bennett NT *et al*, 1993; Ferrara N, 1999; Ciacci C *et al*, 1993; Clark R, 1996; Cook GA *et al*, 1997; Dignas AU *et al*, 1997; Martin P, 1997; Podolsky KD, 1994, 1997; Tarnawski AS *et al*, 1990, 1993]. Tarnawski and colleagues [1993], utilizing acetic acid-induced gastric ulcers in rats, demonstrated *in vivo* that gastric ulceration triggers overexpression of EGF and its receptor, EGF-R, in epithelial cells of the ulcer margin and that healing of the epithelial component of ulcers involves activation of the EGF-R – MAPK (Erk) signal transduction pathway (Figure 4) [Pai R *et al*, 1998]. In the mucosa of the ulcer margin there is a marked (440 - 900 %) increase in Erk1 and Erk2 activities and phosphorylation [Pai R *et al*, 1998]; phosphorylated Erk1 and -2 translocate to the nuclei of epithelial cells and activate *c-fos* gene [Tarnawski AS *et al*, 1998b]. Blockage of this pathway with a specific inhibitor of EGF-R kinase, tyrphostin A46, dramatically delayed ulcer healing [Pai R *et al*, 1998]. Subsequent observations demonstrated that during experimental gastric ulcer healing, EGF-R associates with the adapter proteins, Grb2, Shc, and the guanidine nucleotide exchange factor SOS, leading to activation of Raf-1 kinase [Pai R *et al*, 1997]. These studies have delineated the importance of signaling pathway: EGF-R, adapter proteins (Grb2, Shc, SOS), Ras, Raf-1 kinase and MAP (Erk1/2) kinase for the restoration of the epithelial component during gastric ulcer healing [Pai R *et al*, 1997, 1999; Tarnawski AS *et al*, 1998, 1998b]. Other regulatory peptides (e.g. trefoil peptides and endothelin) may utilize EGF-R signaling pathway by transactivating the EGF receptor [Prenzel N *et al*, 1999].

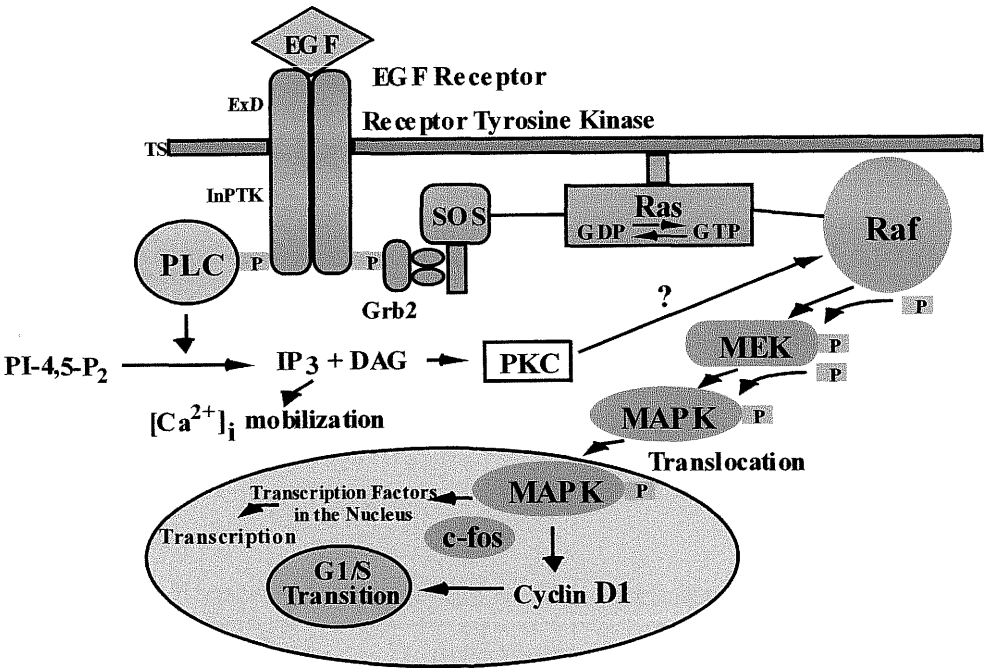

Figure 4. Diagrammatic representation of EGF-R-mediated activation of Ras and PLC signaling pathways. EGF-R consists of three major domains: the extracellular domain (ExD), the transmembrane segment (TS), and the intracellular protein tyrosine kinase domain (InPTK). Binding of EGF to EGF-R leads to receptor dimerization followed by autophosphorylation [P] on specific tyrosine residues of the intrinsic protein tyrosine kinase (InPTK). The Growth factor receptor binding protein 2 (Grb2) is bound to the nucleotide exchange factor, Son of Sevenless (SOS), in the cytoplasm through the interaction of two SH3 domains on Grb2 with a proline-rich region in the C-terminus of SOS. This Grb2-SOS complex is recruited to the plasma membrane by binding of an SH2 domain on Grb2 to one of the autophosphorylated tyrosine residues on the activated EGF-R. Recruitment of the Grb2-SOS complex to the plasma membrane brings it into the proximity of GDP-bound Ras (inactive), which is associated with the plasma membrane by a lipid modification. SOS is then able to mediate the exchange of GTP for the GDP bound to Ras, thereby leading to Ras activation. Activated Ras participates in the activation of serine/theronine kinase, Raf, which in turn activates, through phosphorylation, the MAP kinases kinase (MEK, MAPK/ERK kinase). Activated MEK then phosphorylates MAPK, which is translocated into the nucleus where it activates transcriptional factors. Another pathway of EGF-R signaling is through PLCγ. The products of PLCγ are two second messengers: inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is release into the cytoplasm, where it increases cytosolic free calcium. An increase in free cytosolic calcium activates Ca^{2+} - and calmodulin-dependent CAM kinases and triggers cytoskeletal changes (e.g. polymerization of F-actin). DAG acts on the cell membrane, where it activates protein kinase C (PKC). Activation of the latter by EGF can cause, at least in some cells, elevation of intracellular pH.

3.11. Regulation of Angiogenesis

Angiogenesis - formation of new microvessels - is essential for delivery of oxygen and nutrients to the healing site and thus for ulcer healing and tissue regeneration. The growth of granulation tissue and formation of new microvessels through angiogenesis is stimulated by bFGF, VEGF, PDGF, angiopoietins (Ang-1 and Ang-2) (Table 3) [Folkman J *et al*, 1992, 1996; Santos MF *et al*, 1997] and possibly by other growth factors and cytokines, including IL-1 and TNFα.

Table 3. Structural, cellular and molecular changes in granulation tissue

Structural and cellular changes	1) Angiogenesis endothelial cell proliferation, migration and formation new capillary vessels. 2) Fibroblast proliferation and matrix formation.
Molecular changes:	Activation of genes encoding for: • bFGF & FGF-R 1, 2, 4 • VEGF and flk-1/KDR • Angiopoietin 1 (Ang-1) • Angiopoietin 2 (Ang-2) • Tie 2 receptor (common receptor of angiopoietins) • Tie 1 receptor (ligand not identified yet)

4. Introduction and Aims

NSAIDs, such as IND, delay gastric ulcer healing *in vivo* [Wang JY *et al*, 1989; Inauen W *et al,* 1988; Tarnawski AS *et al*, 1991], in part by inhibiting re-epithelialization and also cause delayed restitution of mucosal injury *in vivo* [Erikson RA, 1988; Takeuchi K *et al*, 1989] and *in vitro* [Giap AO *et al*, 1999]. 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 [Smilenov LB *et al*, 1999; Bockholt SM *et al*, 1992; Ilic D *et al*, 1997]. The focal adhesions serve as traction points over which the cell moves forward by the tension generated by contraction of the actin-myosin network [Lauffenburger DA *et al*, 1996]. 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 [Ilic D *et al*, 1997] and, in turn, phosphorylates several other substrate proteins, such as tensin and paxillin that are also localized to focal adhesions [Bockholt SM *et al*, 1993; Schaller MD *et al*, 1995; Hildebrand JD *et al*, 1995; Gilmore AP *et al*, 1996]. In fibroblasts, FAK has been shown to play a central role in the regulation of cell proliferation, spreading and migration [Gilmore AP *et al*, 1996; Ilic D *et al*, 1995; Cary LA *et al*, 1996]. 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 [Cary LA *et al*, 1996; Tamura M *et al*, 1997].

Tensin is an actin capping protein, which anchors actin filaments to focal adhesions and other cellular structures [Lin S *et al*, 1989]. Tensin phosphorylation by FAK [Davis S *et al*, 1991] may affect its actin capping activity [Auger KR *et al*, 1996]. Tensin by possessing both actin binding and phosphotyrosine binding properties, is considered to be not only a structural component of the cytoskeletal network, but also a coordinator of cytoskeletal signaling [Chuang JZ *et al*, 1995; Zhu T *et al*, 1998]. 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.

5. Materials and Methods

5.1. Cell Culture

Human gastric epithelial (MKN 28) cells derived from gastric tubular adenocarcinoma [Hoyo H *et al*, 1977] were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum (Atlanta Biol., Norcross, GA) at 37°C in a humidified incubator containing 5 % CO_2. 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. After incubation cells were washed in ice-cold PBS, lysed and analyzed as described below. The optimal concentrations of IND and EGF used in the present study, were based on our previous dose-dependent studies [Szabó I *et al*, 1996, 1997; Sasaki E *et al*, 1998; Pai R *et al,* 1999; Jones MK *et al*, 1999a] and on dose-dependent studies (0.1 - 0.5 mM for IND).

5.2. Animals

This study was approved by the subcommittee for Animal Studies of the Long Beach (CA, USA) Department of Veterans Affairs Medical Center. Fourty male Sprague-Dawly 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.

5.3. *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 [Sato Y *et al*, 1989]. The cells were washed and incubated with serum free media containing either vehicle or 0.5 mM IND (Sigma, St. Louis, MO, USA) for 16 hrs and then 10 ng/ml EGF (R&D, La Jolla, CA, USA) or vehicle was added into the medium.

Restitution of wounded monolayers was evaluated at 24 hrs after EGF treatment. Briefly, after fixation in methanol and staining with hematoxylin and eosin; the restitution rate was determined by measuring the area that the cells migrated into the wound using a video imaging system (Metamorph; Universal Imaging Corp., West Chester, PA, USA). To eliminate potential observer's bias, the plates were coded and evaluated by an observer unaware of the code. The restitution was expressed as the mean ±SD of area (mm^2) 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 μg/ml) (Calbiochem-Novabiochem, Minneapolis, MN, USA) added to the cultures 2 hrs before wounding to inhibit cell proliferation.

5.4. 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 Diagnostic

Corp., Indianapolis, IN, USA). Briefly, monolayers were air-dried and fixed with 4 % paraformaldehyde for 1 hr. After blocking with 3 % H_2O_2, coverslips were incubated with the TUNEL reaction mixture. Following incubation with anti-fluorescein antibody conjugated with horse-radish peroxidase (POD), monolayers were developed with DAB substrate. Omission of the TdT enzyme in the TUNEL reaction served as a negative control and resulted in no staining. Since this staining visualizes both apoptotic and necrotic cells, discrimination of apoptosis from necrotic cell death was assessed based on cellular morphology. Morphological features of apoptotic cell death were: cell shrinkage, condensation of chromatin, cytoplasmic budding and formation of apoptotic bodies. Cells displaying cytoplasmic and nuclear swelling or membrane rupture were classified as cell undergoing necrotic cell death. 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.

5.5. 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 Corp., Madison, WI, USA). Monolayers were washed twice in ice-cold PBS and lysed in cold caspase assay lysis buffer. The assays was perfomed in a total volume of 100 μl (cell lysayes, caspase assay buffer, DMSO, dithioethritol, destilled water) in 96-well plates for 4 hrs, absorbance was measured using a Vmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm. Relative absorbance was determined by using negative control (monolayers treated with 50 μM Z-VAD-FMK caspase inhibitor for 16 hrs) and positive controls (monolayers treated with 50 ng/ml anti-Fas antibody for 16 hrs). 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.

5.6. 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. Cells were washed twice in phosphate-buffered saline (PBS), fixed in 4 % paraformaldehyde solution for 20 min at 4°C, and permeabilized with acetone for 5 min at –20°C. The cells were then incubated with either fluorescein isothiocyanate (FITC)-conjugated phalloidin (5 μl/ml in PBS, Molecular Probes, Eugene, OR, USA) which selectively binds to polymerized F-actin or with rabbit polyclonal anti-FAK antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) (2 μg/ml). Coverslips for FAK staining were incubated with Alexa-conjugated anti-rabbit secondary antibody (Molecular Probes) (1:200) for 30 min. After washing with PBS, the all coverslips were mounted onto glass slides using ProLong Antifade Kit (Molecular Probes). Omission of the primary FAK antibody in immunocytochemistry served as a negative control and resulted in no staining. High magnification images of random cells localized in the migrating front of wound edges were taken by a Nikon PCM confocal microscope (Nikon Corp., Japan). All images were captured under the same parameters (magnification, brightness and frame average). 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.

5.7. Immunocytochemistry of Rat Gastric Specimens

Frozen gastric specimens were cut with a cryostat (10 μm thick; Jung Cryostat, Leica, Deerfield, IL, USA), and permeabilized in aceton for 5 min at –20°C. Slides were then incubated with either fluorescein isothiocyanate (FITC)-conjugated phalloidin (5 μl/ml in PBS, Molecular Probes) or with rabbit polyclonal anti-FAK antibody (Santa Cruz Biotech.) (2 μg/ml). Coverslips for FAK staining were incubated with FITC-conjugated anti-rabbit secondary antibody (Sigma) (1:100) for 30 min. After washing with PBS, all slides were mounted using a ProLong Antifade Kit. 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.

5.8. 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 μM) for 16 hrs or AG 1478 (250 nM) for 20 min (both Calbiochem-Novabiochem, La Jolla, CA, USA)] prior to EGF treatment. The cells were washed twice in ice-cold PBS and lysed in cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 % Nonidet P-40, 5 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin.

Frozen rat gastric mucosal tissue specimens were homogenized using a Polytron homogenizer (Kinematica AG, Litau, Switzerland) in the same lysis buffer. FAK and tensin phosphorylation levels were determined by immunoprecipitation with anti-FAK (Santa Cruz

Biotech.) and anti-tensin (Transduction Laboratories, Lexington, KY, USA) antibodies, respectively, followed by immunoblotting using antiphosphotyrosine monoclonal antibody (Santa Cruz Biotech.). In brief, 1 μg anti-FAK polyclonal or 2 μg anti-tensin monoclonal antibody was incubated with protein A-Sepharose beads (25 μl and 50 μl, respectively) for 2 hours at 4°C under constant stirring. Aliquots of clarified cell lysates containing equal amounts of protein (250 μg for FAK and 1mg for tensin) were added to the protein A-Sepharose beads/antibody complex and incubated overnight at 4°C under constant stirring. The beads were washed four times in lysis buffer, resuspended in SDS sample loading buffer and boiled for 5 min. After centrifugation for 5 min, the 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 (Amersham Pharmacia Biotech, Buckinghamshire, UK). Density of protein bands corresponding to 125 kDa (FAK) and 215 kDa (tensin) were analyzed using a video image system (Metamorph, Universal Imaging). 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 μg 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 [Kletzien RF *et al*, 1994; De Hause RB *et al*, 1996].

5.9. Statistical Analysis

Results were expressed as mean ± 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.

6. Results

6.1. Results of *In vitro* Experiments

6.1.1. Restitution of Wounded MKN 28 Monolayers

EGF treatment stimulated re-epithelialization of wounded gastric epithelial monolayers by 59 ± 8 %, compared to vehicle treated monolayers. IND significantly inhibited wound re-epithelialization under the basal condition by 73 ± 12 % and inhibited EGF-stimulated re-epithelialization by 67 ± 9 % (both $p<0.005$) (Figure 5) (Table 4).

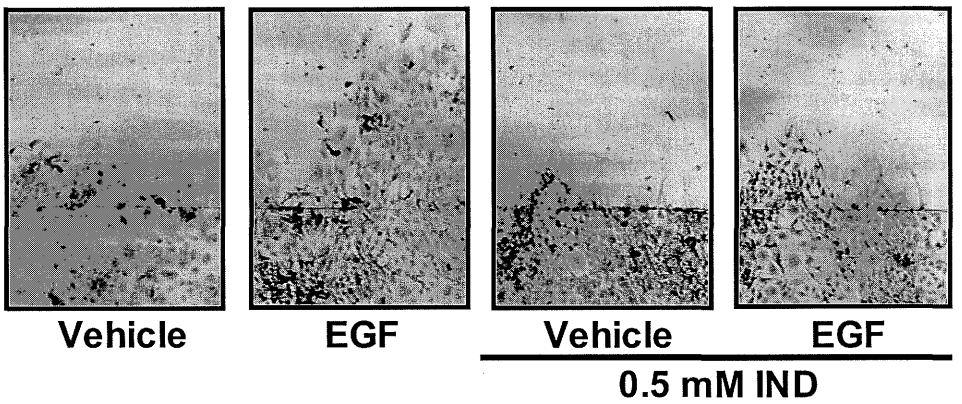

Figure 5. Restitution of wounded MKN 28 monolayers. Wounded cell monolayers were pretreated with either vehicle or 0.5 mM indomethacin (IND) for 16 hrs and incubated with EGF (10 ng/ml) or vehicle for a further 24 hrs. After fixation and staining, the restitution rate was determined by measuring the area that the cells migrated into the wound using a video imaging system

To differentiate the effect of IND on cell migration and proliferation, we performed the restitution assay in the presence of mitomycin C (2 μg/ml), which abolishes MKN 28 cell proliferation [Ricci V *et al*, 1996]. In these experiments, EGF significantly stimulated the

migration of MKN 28 cells by 53 ± 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 ± 11 %, 65 ± 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.

Table 4. Restitution of wounded human gastric monolayers cultured without and with mitomycin C and effects of EGF and indomethacin

Treatment	*Restitution*		*Restitution in present of mitomycin C (2 μg/ml)*	
	*Area**	*% of control*	*Area**	*% of control***
Control (vehicle)	11.5 ± 2.1	100	10.7 ± 2.0	93.4
EGF (10 ng/ml)	18.4 ± 2.7[a]	159.0	16.5 ± 2.5[a]	143.0
Indomethacin (0.5 mM)	3.1 ± 0.5[a]	27.0	3.0 ± 0.5[a]	26.0
Indomethacin + EGF	6.1 ± 1.0[a,b]	52.5	5.8 ± 0.9[a,b]	50.5

NOTE. Data are expressed as means ± SD, n=12.
* Area of cells is expressed in mm^2 per 2 cm wounding edge.
** Compared to control monolayers without mitomycin C co-treatment.
[a] $p<0.005$ vs. vehicle treated monolayers.
[b] $p<0.005$ vs. EGF treated monolayers.

6.1.2. Apoptotic/ Necrotic Indices and Caspace-3 Activity

Since IND can cause gastric cell apopotosis, we evaluated apotosis 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 ± 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 ± 1.44 %; $p<0.01$), while monolayers incubated with vehicle had no further increase

(Table 2). 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 ± 8 % increase, $p<0.01$) vs. vehicle-treated controls (Table 5).

Table 5. Induction of apoptosis by indomethacin in human gastric monolayers

Treatment	*TUNEL assay*		*Caspase-3 activity assay*	
	*Apoptotic index**	*% of control*	*Activity***	*% of control*
Control (vehicle) 16 hrs	3.43 ± 0.75	100	0.072 ± 0.01	100
Indomethacin (0.5 mM) 16 hrs	4.37 ± 1.07 [a]	127.4	0.079 ± 0.02	109.6
Control (vehicle) 40 hrs	3.45 ± 0.75	100.6	0.076 ± 0.01	105.6
Indomethacin (0.5 mM) 40 hrs	6.50 ± 1.44 [b]	188.4¤	0.149 ± 0.03[b]	196.9¤

NOTE. Data are expressed as means ± SD, n=12
* Apoptotic index is calculated as percentage of apoptotic cells per total number of cells.
** Caspase-3 activity is expressed in pmol/hr/mg protein.
¤ Calculated as percentage of control (40 hr vehicle-treated) monolayers.
[a] $p<0.05$ vs. control (16 hr vehicle-treated) monolayers.
[b] $p<0.01$ vs. control (40 hr vehicle-treated) monolayers.

6.1.3. 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 ± 11 % increase vs. baseline; $p<0.05$) (Figure 6). IND treatment caused a significant inhibition of actin stress fiber formation at baseline (20 ± 4 % inhibition; $p<0.05$) as well as following 24-hr EGF stimulation (24 ± 7 % inhibition; $p<0.05$) (Figure 6).

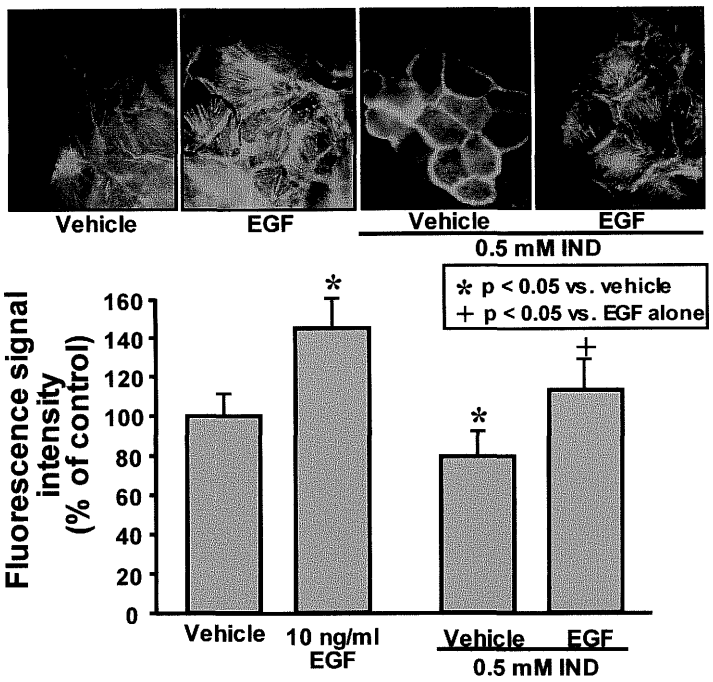

Figure 6. Phalloidin labeling of F-actin in wounded MKN 28 monolayers. Wounded cell monolayers were pretreated with either vehicle or 0.5 mM indomethacin (IND) for 16 hrs and incubated with EGF (10 ng/ml) or vehicle for a further 24 hrs. Intensity of fluorescence signal was measured by confocal microscopy. Values are expressed as the mean ± SD (% of control, n=12) of three independent experiments.

Compared with controls (Figure 7A), IND treatment did not affect stress fiber formation up to 16 hrs of IND treatment (Figure 7B). The loss of actin stress fibers was apparent at 24 hrs and fully manifested at 40 hrs after IND incubation (Figure 7C and F). The loss of stress fibers by IND was found dose dependent (Figure 7D – F).

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 intesity 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.

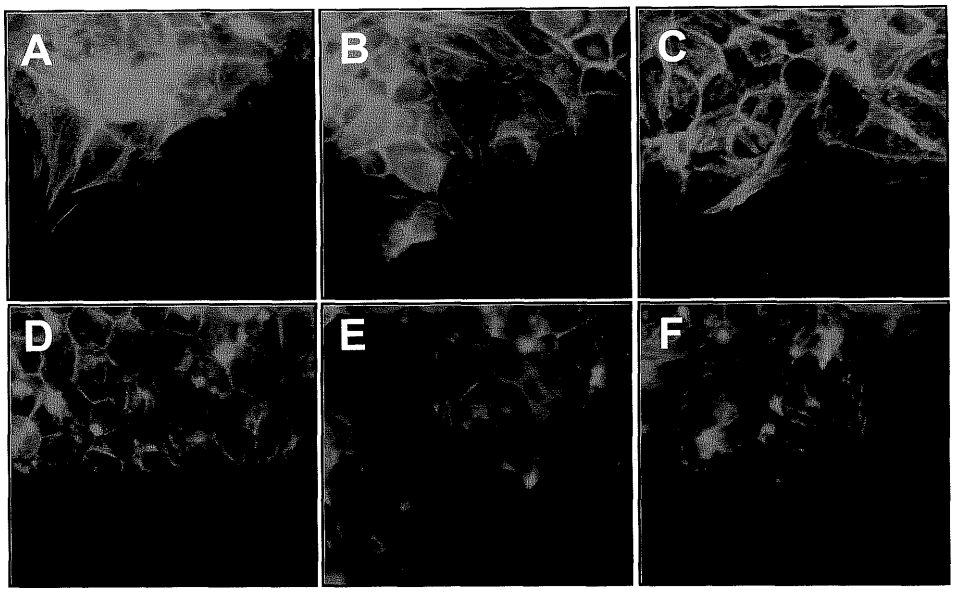

Figure 7. Phalloidin labeling of F-actin in wounded MKN 28 monolayers. Wounded cell monolayers were treated with **(A)** vehicle or **(B)** 0.5 mM indomethacin (IND) for 16 hrs or **(C)** 0.5 mM IND for 24 hrs. To determine the dose-relation of IND treatment to the loss of stress fiber formation, monolayers were treated with **(D)** 0.1mM or **(E)** 0.25mM or **(F)** 0.5 mM IND for 40 hrs. Sixteen hrs after IND treatment (B), the F-actin filaments were not changed compared to vehicle-treated monolayers (A), the loss of stress fiber formation was apparent after 24 hrs (C). The various concentrations of IND (0.1 mM, 0.25 mM and 0.5 mM) caused a dose-dependent loss of stress fibers in human gastric monolayers (D-F).

6.1.4. FAK Localization to Focal Adhesions

Immunostaining showed spotted localization of FAK in all groups. The major difference between vehicle-treated and E/GF-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 ± 13 % increase compared to vehicle-treated monolayers ($p<0.05$) (Figure 8). 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 ± 15 % reduction at baseline and a 62 ± 14 % reduction (vs. EGF) in IND + EGF treated groups (both $p<0.05$) (Figure 8).

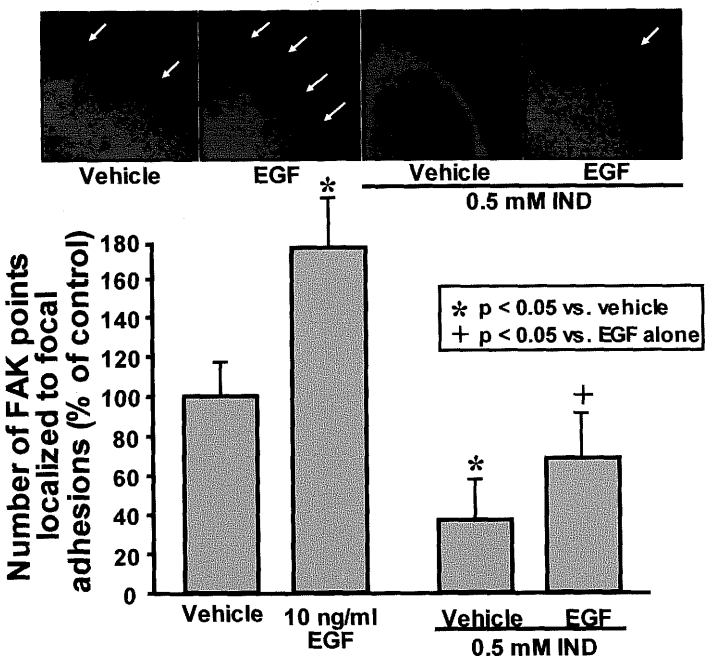

Figure 8. FAK localization to focal adhesions in wounded MKN 28 monolayers. Wounded cell monolayers were pretreated with either vehicle or 0.5 mM indomethacin (IND) for 16 hrs and incubated with EGF (10 ng/ml) or vehicle for a subsequent 24 hrs. FAK was visualized by immunostaining with specific antibody as described under the Methods section. Arrows indicate FAK signal localized to focal adhesion points at the cell periphery. The number of FAK fluorescence clusters was evaluated by randomly counting 50 cells at wound edge on each coded coverslip. Values are expressed as the mean ± SD (% of control, n=12) of three independent experiments.

6.1.5. 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 ± 7 %; $p<0.005$) and remained significantly elevated for 30 min (Figure 9A). Pretreatment with EGF-R kinase inhibitors, PD 153035 and AG 1478, significantly prevented EGF-stimulated increase of FAK phosphorylation (79 ± 8 % and 92 ± 9 % inhibition, respectively; both $p<0.01$) (Figure 10.). The EGF treatment stimulated tensin phosphorylation within 5 min (26 ± 7 % increase; $p<0.05$); this increase was sustained for 30 min (Figure 9B).

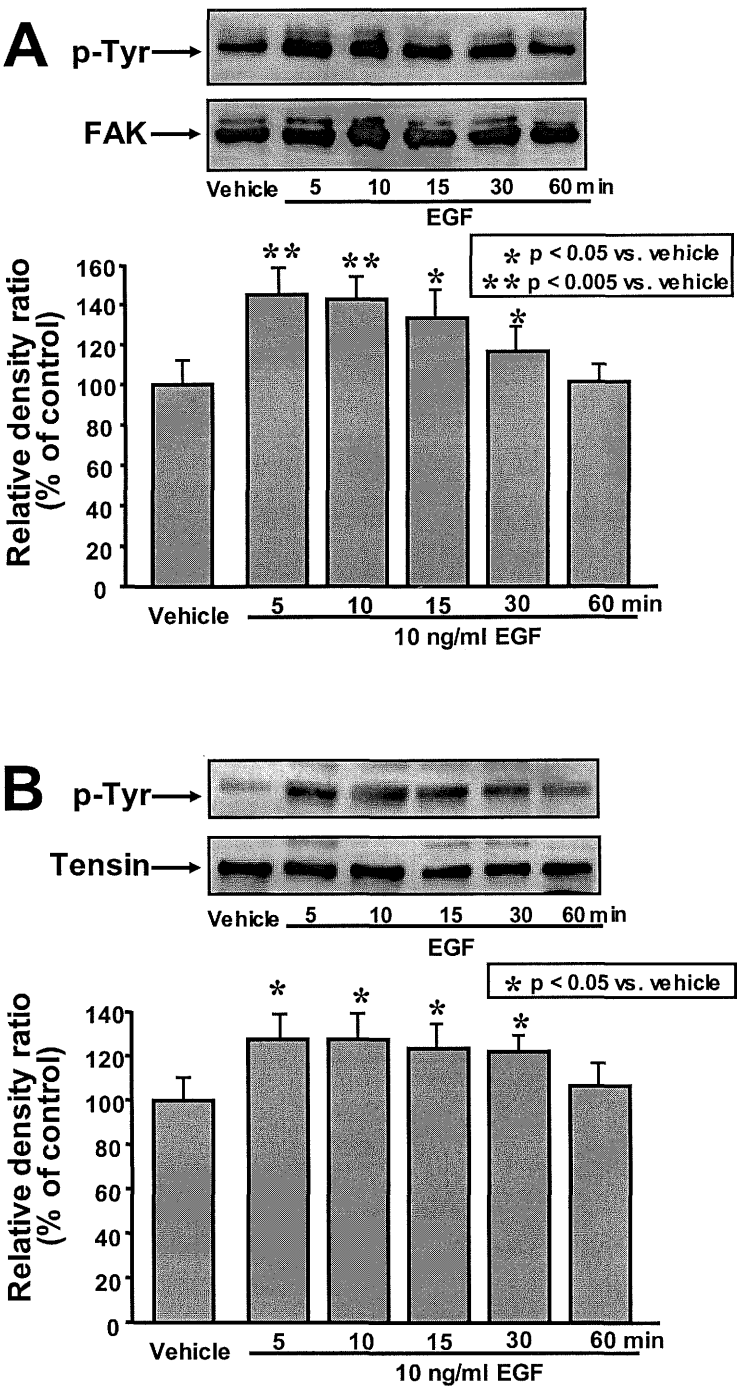

Figure 9. EGF stimulation of tyrosine phosphorylation of focal adhesion kinase (FAK) and tensin in wounded MKN 28 monolayers. Wounded cell monolayers were cultured in serum-free medium for 16 hrs and incubated with EGF (10 ng/ml) or vehicle for 5-60 min.

(A) Tyrosine phosphorylation of FAK was determined by immunoprecipitation of FAK using specific antibody followed by Western blot analysis using phosphotyrosine antibody (p-Tyr). The same membrane was stripped and re-probed for total FAK protein using anti-FAK antibody. *Upper panel:* Representative Western blots showing phosphorylated and total FAK protein

levels. *Lower panel:* Quantification of FAK phosphorylation levels in MKN 28 cells expressed as relative density ratios ± SD (% of control, n=9) of phosphorylated and total FAK protein levels.

(B) Tyrosine phosphorylation of tensin was determined by immunoprecipitation of tensin using specific antibody followed by Western blot analysis using phosphotyrosine antibody (p-Tyr). The same membrane was stripped and re-probed for total tensin protein using anti-tensin antibody. *Upper panel:* Representative Western blots showing phosphorylated and total tensin protein levels. *Lower panel:* Quantification of tensin phosphorylation levels in MKN 28 cells expressed as relative density ratios ± SD (% of control, n=9) of phosphorylated to total tensin protein levels.

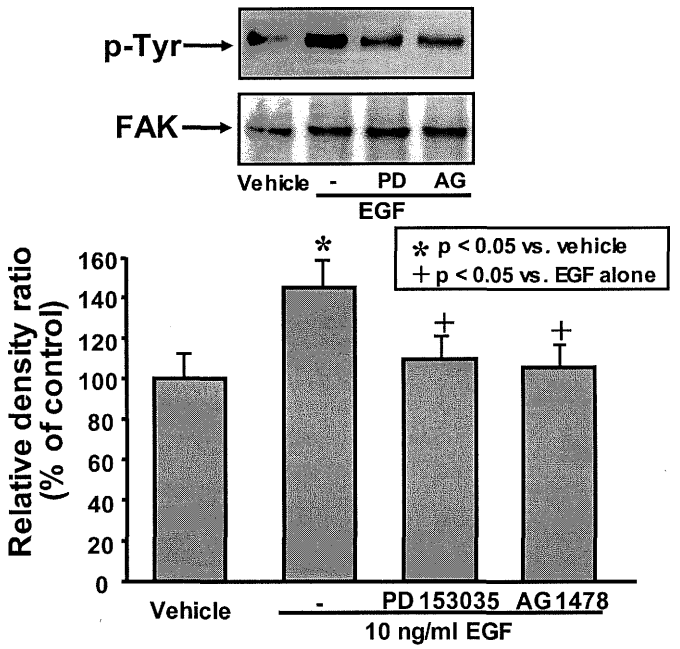

Figure 10. Inhibition of EGF receptor kinase inhibits focal adhesion kinase (FAK) phosphorylation in wounded MKN 28 monolayers. Wounded cell monolayers were pretreated with vehicle or PD 153035 (1 μM) for 16 hrs or AG 1478 (250 nM) for 30 min, and then EGF (10 ng/ml) or vehicle was then added to the media for 5 min. Tyrosine phosphorylation of FAK was determined by immunoprecipitation as described in Methods section. *Upper panels:* Representative Western blots showing phosphorylated and total protein levels. *Lower panels:* Quantification of phosphorylation levels in MKN 28 cells expressed as relative density ratios ± SD (% of control, n=9) of phosphorylated to total FAK protein levels.

Sixteen-hr IND pretreatment significantly inhibited the baseline and EGF-stimulated FAK phosphorylation (19 ± 7 % inhibition; $p<0.05$; and 32.4 ± 7 % inhibition; $p<0.005$;

respectively) (Figure 11A). IND did not affect the low baseline level of tensin phosphorylation, but significantly inhibited EGF-stimulated tensin phosphorylation by 18±5 % ($p<0.05$) (Figure 11B). Inhibition of baseline FAK phosphorylation by IND was present after 4-hr incubation (17±6 %, $p<0.05$) and 8-hr incubation (19±7 %; $p<0.05$) (Figure 12).

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; $p<0.05$).

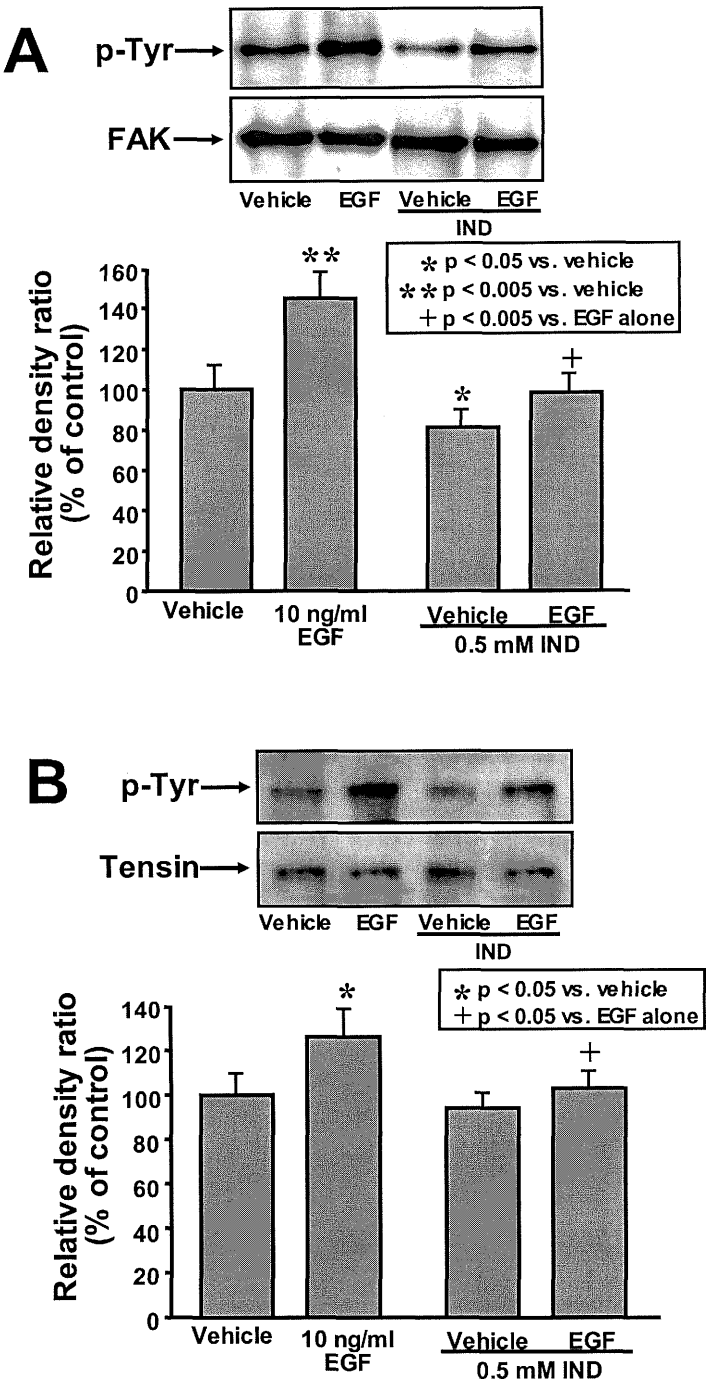

Figure 11. Inhibition of tyrosine phosphorylation of focal adhesion kinase (FAK) and tensin by indomethacin (IND) in wounded MKN 28 monolayers. Wounded cell monolayers were pretreated with either vehicle or IND (0.5 mM) for 16 hrs and incubated with EGF (10 ng/ml) or vehicle for 5 min. **(A)** Tyrosine phosphorylation of FAK. **(B)** Tyrosine phosphorylation of tensin. Phosphorylation levels was determined by immunoprecipitation as described in the Methods section. *Upper panels:* Representative Western blots showing

phosphorylated and total protein levels. *Lower panels:* Quantification of phosphorylation levels in MKN 28 cells expressed as relative density ratios ± SD (% of control, n=9) of phosphorylated to total FAK or tensin protein levels.

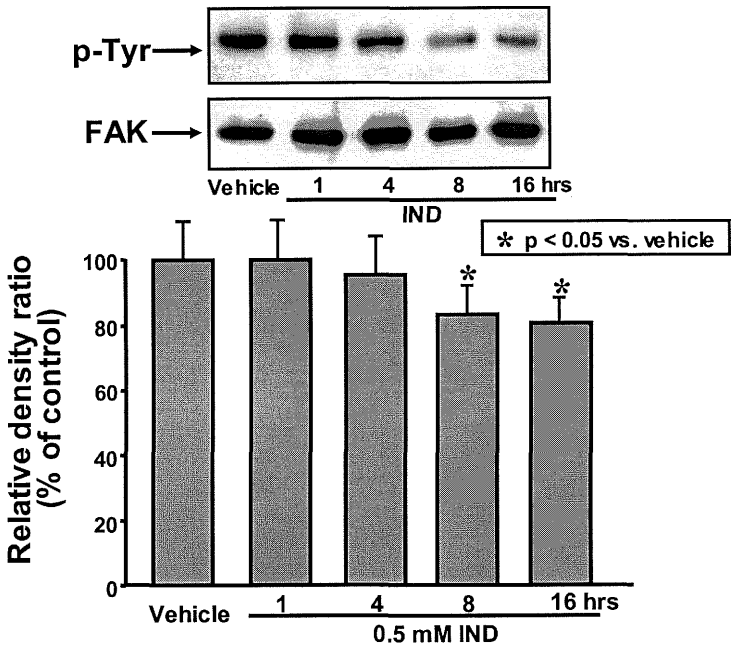

Figure 12. Time line of indomethacin-induced inhibition in tyrosine phosphorylation of FAK in wounded MKN 28 monolayers. Wounded cell monolayers were treated with either vehicle or indomethacin (0.5 mM) for 1, 4, 8 and 16 hrs. Tyrosine phosphorylation of FAK was determined by immunoprecipitation as described in the Methods section. *Upper panels:* Representative Western blots showing phosphorylated and total protein levels. *Lower panels:* Quantification of phosphorylation levels in MKN 28 cells expressed as relative density ratios ± SD (% of control, n=9) of phosphorylated to total FAK protein levels.

6.1.6. 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 (Figure 13).

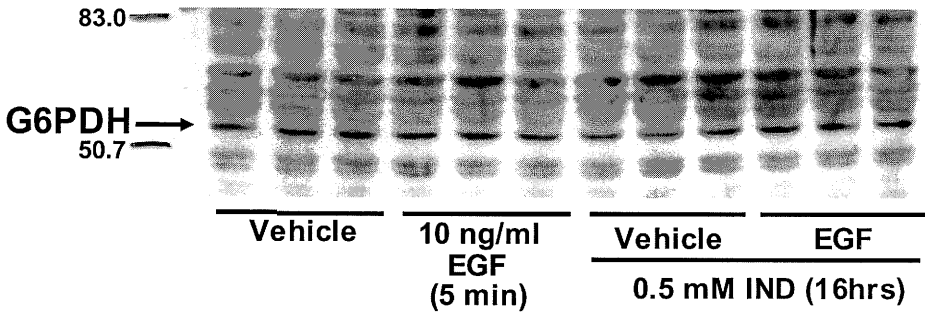

Figure 13. IND does not effect the protein level of glucose-6-phosphate-dehydrogenase house-keeping protein in wounded MKN 28 monolayers. Wounded cell monolayers were cultured in serum-free medium.

6.2. *In vivo* Experiments

6.2.1. 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 (Figure 14). In the gastric mucosa of IND-pretreated rats, the migrating epithelial cells showed less intracellular actin filaments than epithelial cells of vehicle-treated rats (Figure 14).

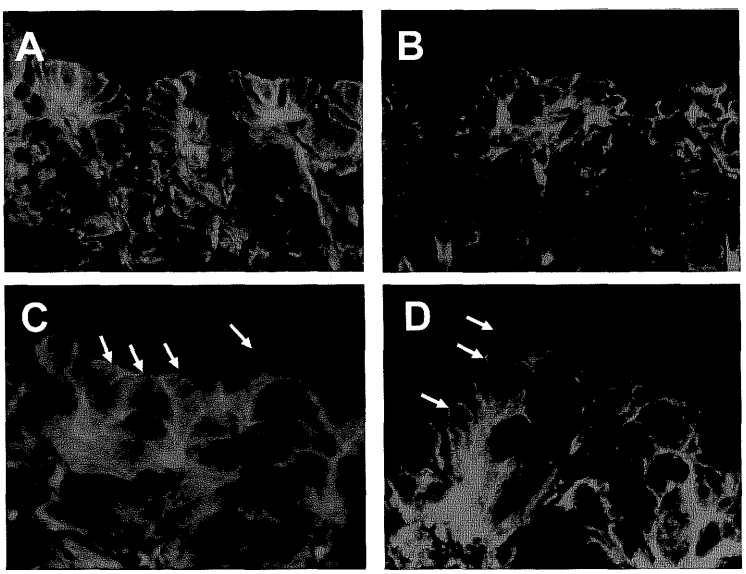

Figure 14. Phalloidin labeling of F-actin in migrating surface epithelial cells of rat gastric mucosa at 4 hrs after injury. Rats received either **(A and C)** vehicle or **(B and D)** indomethacin (5 mg/kg, 1ml) intragastrically (i.g.), and 3 min later rats were given 2 ml 5 % NaCl i.g. Arrows indicate the migrating surface epithelial cells. Original magnification: A and B X200; C and D X400.

6.2.2. FAK Localization to Adhesion Points in Rat Gastric Mucosa

Immunostaining for FAK showed spotted localization of intracellular FAK in superficial migrating epithelial cells (Figure 15). 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 (Figure 15).

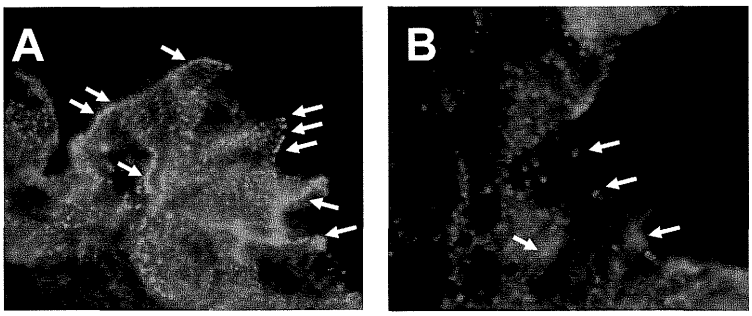

Figure 15. Focal adhesion kinase (FAK) distribution in migrating surface epithelial cells of rat gastric mucosa at 8 hrs after injury. Rats received either **(A)** vehicle or **(B)** indomethacin (5 mg/kg, 1 ml) intragastrically (i.g.), and 3 min later rats were given 2 ml 5 % NaCl i.g. FAK was visualized by immunostaining with specific antibody as described in the Methods section. Arrows indicate FAK signal localized to the cell periphery. Original magnification: X1000.

6.2.3. 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 ± 10 % increase at 4 hrs, $p<0.001$; 42 ± 11 % increase at 8 hrs, $p<0.05$; respectively) (Figure16). IND pre-treatment significantly inhibited the injury-induced FAK phosphorylation at 4 hrs (53 ± 8 %; $p<0.001$) and 8 hrs (55 ± 8 %; $p<0.001$) compared to vehicle (Figure 16).

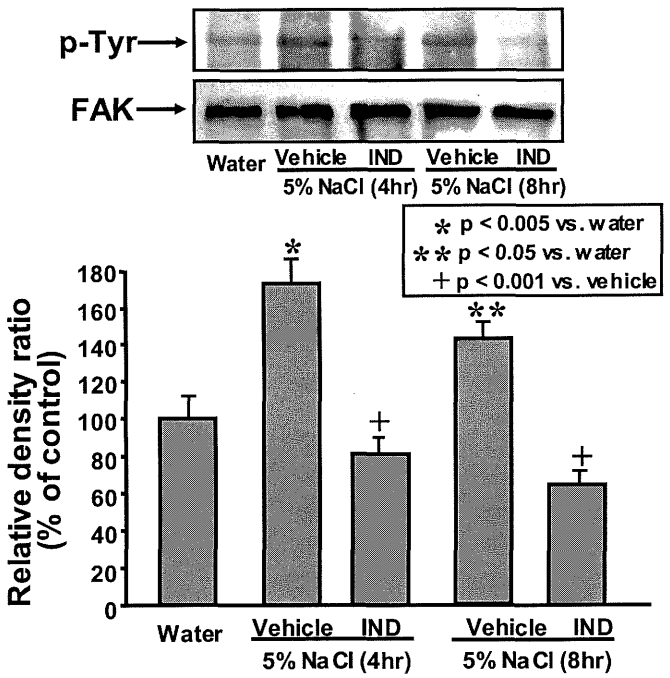

Figure 16. Focal adhesion kinase (FAK) phosphorylation in injured and non-injured rat gastric mucosa. Rats received either **(A and C)** vehicle or **(B and D)** indomethacin (IND) (5 mg/kg, 1ml) intragastrically (i.g.), and 3 min later rats were given either 2 ml 5 % NaCl or water i.g. Tyrosine phosphorylation of focal adhesion kinase (FAK) was determined by immunoprecipitation as described in the Methods section. *Upper panels:* Representative Western blots showing phosphorylated and total FAK protein levels. *Lower panels:* Quantification of phosphorylation levels in gastric mucosa expressed as relative density ratios ± SD (% of control, n=9) of phosphorylated to total FAK protein levels.

7. 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 phoshorylation 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.

Mucosal restitution is an early phase of superficial injury repair. It involves migration of viable cells from the area bordering necrosis into the damaged surface to cover the denuded basement membrane and to re-establish epithelial continuity [Jones MK *et al*, 1999b]. This process is stimulated by a number of growth factors, including EGF, TGF-α, PDGF and trefoil peptides [Jones MK *et al*, 1999b; Kato K *et al*, 1999].

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 [Watanabe S *et al*, 1995b; Murai T *et al*, 1996].

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 [Rijken PJ *et al*, 1995]. Moreover, EGF-R has been shown to bind directly to actin (van Bergen en Henegouwen PM *et al*, 1992) and induce serine phosphorylation [van Delft S *et al*, 1995]. 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.

It is known that in polarized epithelia EGF acts predominantly on the basolateral EGF receptors. In our experiments, basolateral EGF-R are exposed in the cells localized at the wound edge. Moreover, apical EGF receptors have been described on the cell surfaces in several cell lines, and in primary gastric mucosal cells [Chen MC *et al*, 2001]. While Kuwada and colleagues [Kuwada SK *et al*, 1998] demonstrated in EGF receptor-transfected polarized kidney epithelial cells that FAK is tyrosine phosphorylated more by basolateral than apical EGF exposure, other studies describe that both apical and basolateral EGF-R mediate ligand-induced tyrosine phosphorylation of junction proteins [Chen MC *et al*, 2001].

Several groups of investigators have made major contributions to the understanding of some of the mechanisms of EGF-induced intestinal epithelial cell migration and wound healing. Polk demonstrated that intestinal epithelial cell migration requires intact EGF-R tyrosine kinase, phospholipase C, and protein kinase C activities, and that phospholipase C may play a key regulatory role in this process [Polk DB, 1998]. Other groups demonstrated the interactions of FAK with the extracellular matrix proteins and the involvement of phosphatidylinositol 3-kinase in this process in fibroblasts and pancreatic cells [Tamura M *et al*, 1999; Tapia JA *et al*, 1999]. However, those studies were performed in non-gastric cells and it is not certain whether the same mechanisms will apply to gastric mucosal epithelial cells. Furthermore, expression of FAK and tensin have never been demonstrated in gastric cells.

While we recognize the important roles of the above mechanisms, in our experiments we focused our attention on F-actin, FAK and tensin for the following reasons.

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 [Matrisian LM *et al*, 1990; Yoshida KT *et al*, 1990]. McCormack and colleagues demonstrated *in vitro* that migration of IEC-6 cells depends on actin polymerization [McCormick SA *et al*, 1992, 1998] and other studies have demonstrated that EGF induces rapid reorganization of actin in human A431 cells [Rijken PJ *et al*, 1991]. 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 [Ilic D *et al*, 1995; Romer LH *et al*, 1994]. Withers and colleagues have shown that there is a direct correlation between FAK activity and phosphotyrosine content [Withers BE *et al*, 1996]. In migrating cells, phosphorylated FAK localizes to focal adhesions [Weiner TM *et al*, 1993; Gates RE *et al*, 1994]. In contrast, inhibition of FAK phosphorylation has been shown to decrease both cell proliferation and motility [Guan JL *et al*, 1997; Burridge K *et al*, 1992]. 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 [Weiner TM *et al*, 1993; Gates RE *et al*, 1994; Casamassima A *et al*, 1997; Rakin S *et al*, 1994].

Other investigators found that the elevation of FAK's phosphotyrosine content following cell adhesion to the extracellular matrix, directly correlates with increased FAK activity [Calalb MB *et al*, 1995]. 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 restitute injured rat gastric mucosa.

Tensin, actin capping protein, links actin filaments to focal adhesions [Lo SH *et al*, 1994] 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 [Lo SH *et al*, 1994]. 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 [Bockholt SM *et al*, 1993]. 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 [Ridyard MS *et al*, 2001].

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

8. Background and Introduction

8.1. Gastric Cancer

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 [American Cancer Society, 2002]. The disease is much more common in other countries, principally Japan, Central Europe, Scandinavia, Hong Kong, South and Central America, China, Korea and on the territory of the former Soviet Union [American Cancer Society, 2002]. 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 [Parkin DM *et al*, 1999]. All over the world, wherever data are reliably available, there has been a marked divergence in the prevalence of and death from stomach and lung cancer, with a marked continued decline in stomach cancer and a marked and persistent increase in lung cancer, which is almost universally associated with the increasing use of tobacco. The striking association of gastric adenocarcinoma with lower socioeconomic status, the evidence linking *Helicobacter pylori* with gastric atrophy, intestinal metaplasia, and gastric adenocarcinoma, and the declining incidence of gastric adenocarcinoma worldwide suggest that gastric adenocarcinoma is a worthwhile model with which to explore the role of environmental factors in human cancer and

the potential of preventing and decreasing the prevalence of such environmentally linked cancers [Craanen ME *et al*, 1992; Mayne ST *et al*, 2001].

Although a large number of risk factors have been associated with gastric adenocarcinoma (Table 6), a definite etiology for the majority of gastric adenocarcinomas is still unknown [Rustgi AK *et al*, 1995; Ramon JM *et al*, 1993; Correa P *et al*, 1994; Hanson LE *et al*, 1994; You WC *et al*, 2000; Mayne ST *et al*, 2001]. The pathogenesis is most likely multifactorial. Although it has long been postulated that there is a sequence of histologic premalignant changes, progressing from atrophic gastritis to intestinal metaplasia and ultimately to gastric adenocarcinoma, these premalignant histologic changes may be necessary but clearly not sufficient for the development of adenocarcinoma. [Wee A *et al*, 1992; Correa P, 1992; Rokkas T *et al*, 1991]. Although genetic abnormalities occur in gastric adenocarcinoma (Table 7), current knowledge of them does not yet permit the formulation of a sequence of progression or accumulation of genetic abnormalities analogous to that described for the progression of colorectal adenocarcinoma [Correa P *et al,* 1994; Uchino S *et al*, 1992; Joypaul BU *et al*, 1994; Tamura G *et al*, 1994, 1996; Mironov NM *et al*, 1994; Rhyu MG *et al*, 1994; Chong JM *et al*, 1994; Tahara E, 1995, Chae KS *et al*, 2002; Testino G *et al*, 2002; Testino G *et al*, 2002]. Nevertheless, gastric adenocarcinoma forms a component of Lynch syndrome II, one of the hereditary nonpolyposis colorectal cancer syndromes [La Vecchia C *et al*, 1992; Lynch HT *et al*, 1993]. Some patients with familial adenomatous polyposis (FAP) develop gastric adenomas and gastric adenocarcinomas [Luk GD, 1995]. Extensive epidemiologic data strongly suggest the importance of environmental factors, especially dietary factors [Ramon JM *et al*, 1993]. Evidence suggests that infection with *H. pylori*, particularly *cag*A+ strains infection may be an important etiologic agent [The Eurogast Study Group, 1993; Hanson LE *et al*, 1993]. The World Health Organization declared, in 1994, *H. pylori* infection a Group 1 carcinogen, definitive cause of human neoplassias, similar to tobacco. Helicobacter and Gastric Collaborative Group [2001]

found that 5.9 is the best estimate of relative risk of non-cardiac carcinoma associated with *H. pylori* infection. In gastric carcinogenesis H. pylori might cause the severe imbalance of proliferation and apoptosis resulting to precancerous lesions (intestinal metaplasia, severe dysplasia), leading to p53-Rb tumor suppressor system mutation and telomerase reactivation, finally causing cancer [Lan J *et al*, 2003].

Table 6. Risk factors of gastric adenocarcinoma

Low socioeconomic status	Postgastrectomy (>20 years)
Tobacco smoking	Pernicious anemia
High intake of salted, pickled, smoked foods, cholesterol, animal protein	Peutz-Jeghers syndrome
	Ménétrier's disease
	Familial adenomatous polyposis
High intake of poorly preserved foods	Adenomas
	Intestinal metaplasia
Low intake of fruits and vegetables, folate, vitamin C, B_6	Chronic atrophic gastritis
	High-grade dysplasia
High intake of alcohol, vitamin B_{12}	Hamartomas
Helicobacter pylori infection	Hyperplastic polyps
Hereditary nonpolyposis colorectal cancer (Lynch II)	Fundic gland polyps
	Benign gastric ulcers
Barrett's esophagus (cardia and gastroesophageal junction)	

Table 7. Genetic abnormalities in gastric adenocarcinoma
[modified from Feldman M *et al*, 1996]

Abnormalities	Gene	Approximate frequency %)
DNA aneuploidy		70
Tumor suppressor gene (allelic loss)	*p53*	65 [Testino G *et al*, 2002]
	APC	35 [Jin Z *et al*, 2002]
	MCC	30
	DCC	27 [Cho JH *et al*, 1996]
Microsatellite instability		25 [Fang DC *et al*, 2001]
Oncogene amplification	*k-sam*	15
	c-met	7
	c-erb B-2	7
Oncogene mutations	*ras*	8 [Testino G *et al*, 2002]

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 [Genta RM *et al*, 2001]. Dysplasia is not found in the normal stomach, but it can be a precursor lesion for gastric adenocarcinoma. Dysplasia in about 10 % of patients may progress in severity over the course of 5 to 15 years, but in the majority of people, dysplasia either regresses or remains stable [Clouston AD, 2001]. High-grade dysplasia may be only a transient phase in the progression to gastric adenocarcinoma, or it may relentlessly progress to adenocarcinoma in virtually all cases [Rugge M *et al*, 1994; You WC *et al*, 1994]. Gastric epithelial dysplasia usually occurs in the setting of atrophic gastritis or intestinal metaplasia and is frequently found coincidentally and concurrently with gastric adenocarcinoma. Thus there may not be a true dysplasia-carcinoma sequence in the stomach, but the occurrence of dysplasia and adenocarcinoma may be merely the end result of common etiologic factors [Rugge M *et al*, 1994].

Most epidemiologic studies suggest a contributory role for dietary factors. Although studies are seldom conclusive or consistent, the available data suggest that the risk of 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 B_6, folate, β-carotene) [Kabat G *et al*, 1993; Hanson LE *et al*, 1994; Mayne ST *et al*, 2001].

Although there is a strong association among chronic atrophic gastritis, intestinal metaplasia, and adenocarcinoma, and although populations with high prevalence of chronic atrophic gastritis and intestinal metaplasia have up to a 25-fold increase in gastric adenocarcinoma, chronic atrophic gastritis and intestinal metaplasia may be so common so as to render this finding of little predictive importance [Rokkas T *et al*, 1991; Kato I *et al*, 1992]. Even in areas of high prevalence, probably less than 1 % of patients with chronic atrophic

gastritis or intestinal metaplasia develop adenocarcinoma each year (lifetime risk of gastric adenocarcinoma is about 1 in 15 in the general worldwide population).

Patients with pernicious anemia are also at an increased risk of developing gastric adenocarcinoma [Hsing AW *et al*, 1993; Affronti J *et al*, 1994]. However, only about 5 - 10 % of patients eventually develop gastric adenocarcinoma; these proportions are not substantially different from the lifetime risk of gastric adenocarcinoma in high-risk areas worldwide.

Patients who have undergone gastrectomy for benign disease are at increased risk of developing adenocarcinoma 15 or more years after gastrectomy. Studies with long follow-up show 50 - 70 % increased risk of gastric adenocarcinoma after 15 to 25 years. In patients undergone subtotal or near total gastrectomies, dysplasia occur mostly near the anastomotic site [Dubrow R, 1993; Fisher SG *et al*, 1993].

Patients with Ménétrier's disease also appear to have an increased risk of gastric adenocarcinoma, as high as 10 - 15 % in some reports. But the condition is extremely rare, and the reported studies may reflect referral bias or other confounding factors [Hsu CT *et al*, 1991].

8.2. Gastrin

The gastrointestinal peptid gastrin has been implicated in a wide variety of functions, including secretion of gastric [Forte JG *et al*, 1987] and pancreatic [Solomon TE, 1987] juices, satiety [Moran TH *et al*, 1992], and the growth of the gastrointestinal tract [Dembinski AB *et al*, 1980]. Gastrin is an important growth factor for gastrointestinal tract (GI) and has been reported to be trophic to different GI tissues [Majundar A *et al*, 1982; Dembinski AB *et al*, 1980] including parietal cells [Majumdar A *et al*, 1982], enterochromaffin cells [Hakanson R *et al*, 1991], colonic [Johnson LR, 1987] and pancreatic [Dembinski AB, 1980] 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 in several neoplasms including colonic [McGregor DB *et al*, 1982; Sirinek KR *et al*, 1985; Winsett OE *et al*, 1986], pancreatic [Smith JP *et al*, 1995] and gastric cancers [Watson SL *et al*, 1989]. Gastrin administration is able to enchance the growth of carcinogen-induced colonic cancer in rats [Winsett OE *et al*, 1986], human colon cancer cells in culture and tumor xenografts to nude mice [Smith JP *et al*, 1988], therefore it would be interesting to see whether gastrin enhances the growth of gastric cancer.

8.3. NSAIDs and Gastric Cancer

Epidemiological studies have reported a decrease in the incidence of gastric cancer in chronic users of NSAIDs [Gridley G *et al*, 1993; Isomaki H *et al*, 1978; Laakso M *et al*, 1986; Coogan PF *et al*, 2000; Farrow DC *et al*, 1998; Langham MJS *et al*, 2000; Thun MJ *et al*, 1991]. Many tumors, including gastric carcinoma, contain high concentrations of PGs which promote cellular proliferation, tumor growth and angiogenesis [Rigas B *et al*, 1993; Bennett A *et al*, 1987; Shiff SJ *et al*, 1996; Lupulescu A, 1996; Form DM *et al*, 1983]. Using APC knockout mice, Oshima and colleagues provided genetic evidence linking COX-2 expression to colonic tumor promotion [Oshima M *et al*, 1996]. 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 tissue [Lim HY *et al*, 2000; Ristimaki A *et al*, 1997; Uefuji K *et al*, 1998]. 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 [Schiff SJ *et al*, 1995]. IND, a non-selective NSAID, induces apoptosis in human gastric cancer cells (AGS) [Zhu GH *et al*, 1999a, 1999b].

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) [Davis RJ, 1993]. There are several isoforms of MAPK (ERK1, ERK2, SAPK, p38, HOG1 kinase and ERK5) [Davis RJ, 1993]. 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 [Cowley S *et al*, 1994; Pang L *et al*, 1995; Pages G *et al*, 1993]. Sebolt-Leopold and colleagues demonstrated an 80 % inhibition of colon carcinoma growth in rodents treated with an oral MAPK inhibitor (PD 184352) [Sebolt-Leopold JS *et al*, 1999]. The involvement of MAPK/ERK2 in the progression of gastric cancer however remains unknown.

9. Aims

Since the gastrin administration seems to be able to enhance the growth of carcinogen-induced colonic cancer, we examined

- *in vitro* 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

- 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.

10. Observations

10.1. Effect of Gastrin of Gastric Cancer Growth

Gastrin possesses the same five-amino acid carboxy terminal end as cholecyctokinin (CCK). Originally two types of CCK receptors have been described (CCK-A and CCK-B) [Wank SA *et al*, 1992]. 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 [Frucht H *et al*, 1992; Singh P *et al*, 1985; Upp JR *et al*, 1989] and on rat and human colonic (WiDr, HT-29, CoLo 205, T84, YAMC) [Hoosen NM *et al*, 1990; Smith JP *et al*, 1996], pancreatic (PANC-1, BON) [Smith JP *et al*, 1994], and gastric (MKN 95) [Weinstock J *et al*, 1988; Watson SL *et al*,1989] 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 [Seva C *et al*, 1994] and on fibroblast cell line, Swiss-3T3 [Singh P *et al*, 1995] cells after the incubation with glycine-extented gastrin. The mouse colon cell line, YAMC, was also found to express surface receptor selective for glycine-extended gastrin-17 [Seva C *et al*, 1994].

10.1.1. Materials and Methods

10.1.1.1. Cell Culture

The human gastric adenocarcinoma cell line (AGS, CRL-1739) was obtained from American Type Culture Collection (ATCC, Rockville, MA, USA), originally isolated in1979 by Barranco SC and Townsend CM jr, from an adenocarcinoma of the stomach resected from a 54-

year-old Causasian female person. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FCS, L-glutamine, penicillin and streptomycin in usual doses at 37 °C in a humidified (95 %) incubator containing the atmosphere of 95 % air and 5 % CO_2. The DMEM was purchased from Sigma-Aldrich (Budapest, Hungary), the FCS was from Gibco BRL (Life Technologies Ltd., Gaithersburg, MA, USA). Pentagastrin and human gastrin-17 were from Zeneca Limited (Macclasfield, UK) and Sigma-Aldrich (Budapest, Hungary). The cells were seeded on incubating plates in 10^4 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).

10.1.1.2. 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, which is composed of solutions of a novel tetrazolium compound (MTS) and an electron coupling reagent (PMS). 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 [Bradford M, 1976] using Coomassie Brillant 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 [Bradford M, 1976]. 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 counts of treated cells. Cells were harvested after 5-day incubation with various concentrations of gastrin or pentagastrin or their vehicle on 6 well assay plates, and counted in hemocytometer.

10.1.1.3. 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.

10.1.2. Results

10.1.2.1. Results of Proliferation Assay

10^{-5} M pentagastrin incubation caused the lowest extinction detected (Figure 17). 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 5^{th} 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 5^{th} day. Otherwise, similar and almost equal changes could be detected (Figure 18).

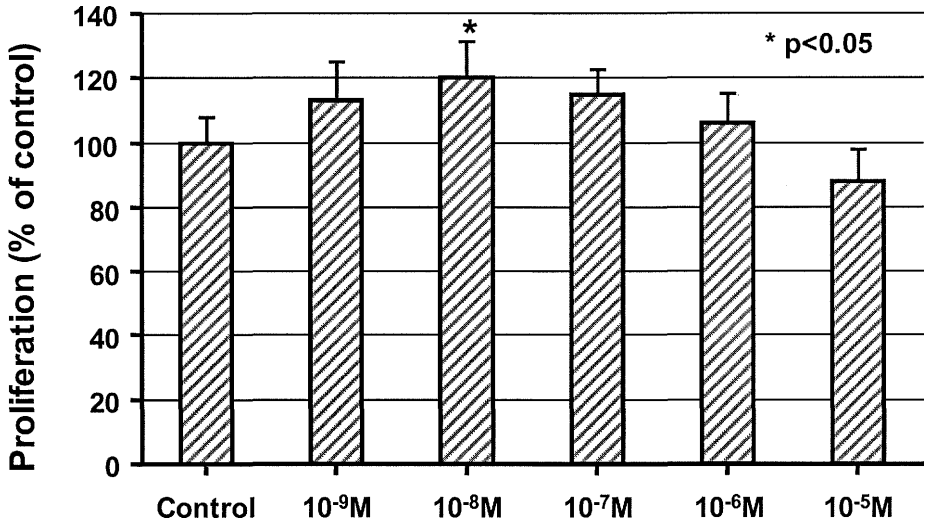

Figure 17. Proliferation of AGS cells incubated with either vehicle or pentagastrin (10^{-9} – 10^{-5} M) for 5 days detected by proliferation assay. Significant difference (*) in cell proliferation vs. control ($p<0.05$).

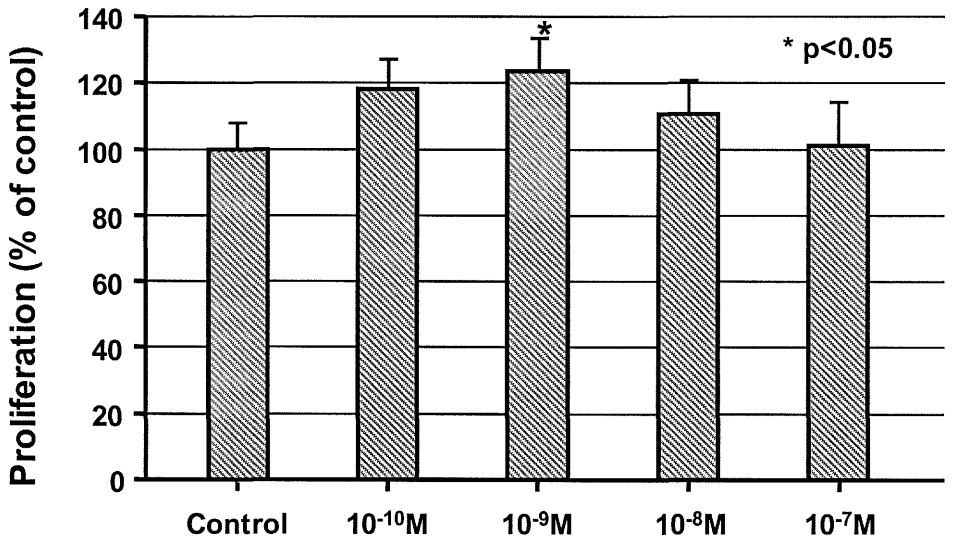

Figure 18. Proliferation of AGS cells incubated with either vehicle or gastrin-17 (10^{-10} – 10^{-7} M) for 5 days detected by proliferation assay. Significant difference (*) in cell proliferation vs. control ($p<0.05$).

10.1.2.2. 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 (27.5 mg/ml vs. 25 mg/ml, detected on the 5th day).

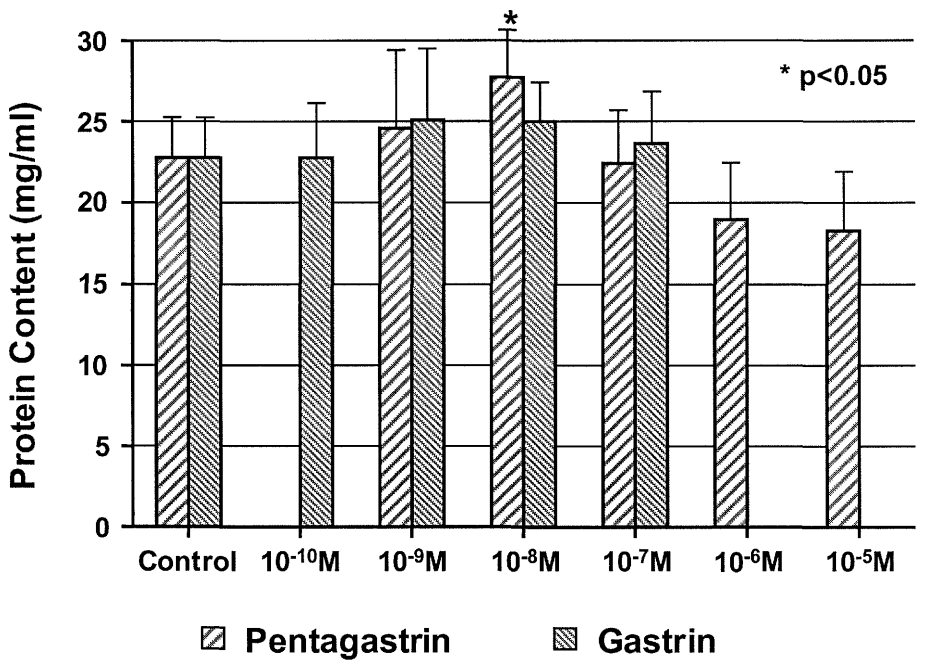

Figure 19. Changes of total protein content pro well containing AGS cells incubated with vehicle or pentagastrin (10^{-9} – 10^{-5} M) or gastrin-17 (10^{-10} – 10^{-7} M) for 5 days. The protein content was determined by the method of Bradford described under Materials and Methods. Significant difference (*) in cell proliferation vs. control ($p < 0.05$).

10.1.2.3. 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).

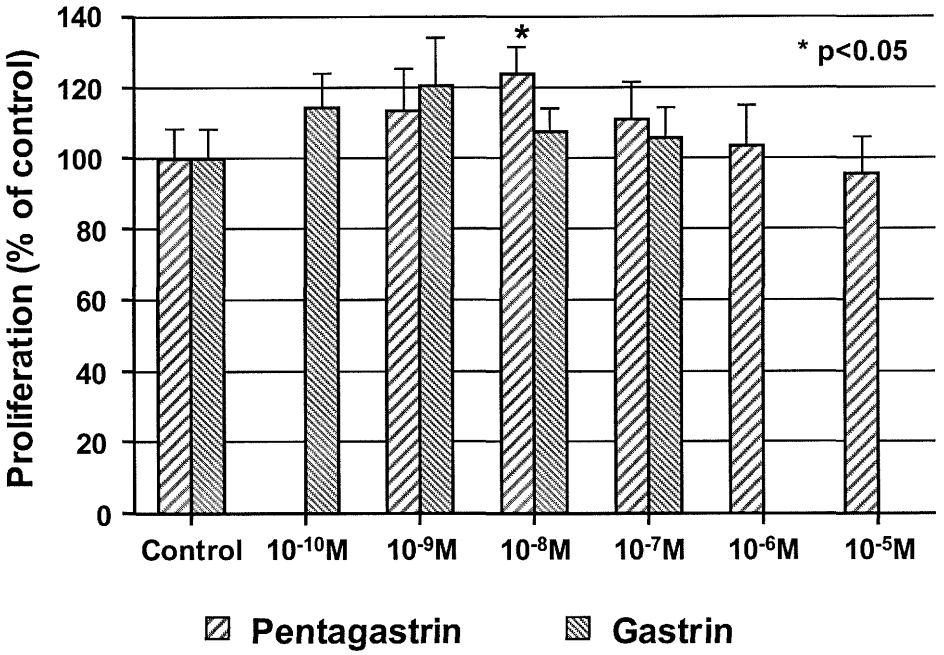

Figure 20. Proliferation of AGS cells incubated with vechicle or pentagastrin (10^{-9} – 10^{-5} M) or gastrin-17 (10^{-10} – 10^{-7} M) for 5 days determined by cell counting in haemocytometer. Significant difference (*) in cell number vs. control ($p<0.05$).

10.1.3. 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 [Kokoska EK *et al*, 1998] in their cytoplasm, which are consistent with cells that secrete mucopolysaccharides. Results of electron microscopic analysis revealing junctional complexes and rudimentary apical microvillae [Kokoska EK *et al*, 1998], also suggest that AGS cells possess the ability to differentiate morphologically and have characteristic traits consistent with mucus secreting cells. Sharma and colleagues demonstrated that this cell line to be an excellent model for studying *H. pylori* infection under *in vitro* conditions [Sharma SA *et al*, 1995]. 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 and colleagues found similar differences in the effective dosages of pentagastrin and gastrin on rat pancreatic tissue [Majumdar A *et al*, 1979]. Smith and colleagues proceeded observations suggesting similar results on human pancreatic adenocarcinoma cell line (PANC-1) [Smith JP *et al*, 1995].

10.2. Effect of NSAIDs on Gastric Cancer Growth

10.2.1. Materials and Methods

10.2.1.1. Cell lines

Human gastric epithelial (MKN 28) cells derived from gastric tubular adenocarcinoma [Hoyo H, 1977] were grown in RPMI 1640 medium supplemented with 10 % fetal bovine serum (Atlanta Biol., Norcross, GA) at 37°C in a humidified incubator containing 5 % CO_2. 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.

10.2.1.2. Cell Proliferation Assay

The effect of IND and NS-398 on MKN 28 cell proliferation was studied by determining the incorporation of ^{3}H-thymidine into cellular DNA. Cells (4×10^{-4} 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 for 24 or 48 hr. Three hours prior to termination of the experiment, 0.5 μCi of [methyl-^{3}H]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 [Fujiwara Y *et al*, 1995]. These studies also assured that these doses of IND do not reduce cell viability below 90 %.

10.2.1.3. 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) using a commercial kit (Roche Diagnostic Corp.), and following manufacturer's instructions. Briefly, monolayers were air-dried and fixed with 4 % paraformaldehyde for 1 hr. After blocking with 3 % H_2O_2, coverslips were incubated with the TUNEL reaction mixture. Following incubation with anti-fluorescein antibody conjugated with horseradish peroxidase (POD), monolayers were developed with DAB substrate. Omission of the TdT enzyme in the TUNEL reaction served as a negative control and resulted in no staining. Since this staining visualizes both apoptotic and necrotic cells, discrimination of apoptosis from necrotic cell death was assessed based on cellular morphology. Morphological features of apoptotic cell death were: cell shrinkage, condensation of chromatin, cytoplasmic budding and formation of apoptotic bodies. Cells displaying cytoplasmic and nuclear swelling or membrane rupture were classified as cell undergoing necrotic cell death. Cell death was evaluated by randomly counting 1000 cells on each coverslip. 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.

10.2.1.4. Determination of Mitogen-activated Protein Kinase Activity

ERK2 activity was determined as described in our previous study [Jones MK *et al*, 1999c]. Briefly, cells were incubated in medium containing either vehicle (controls) 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 (Santa Cruz Biotech.) 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 $MgCl_2$; 50 µM ATP; 30 µg myelin basic protein; and 4 µCi [^{32}P] 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 sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer [Jones MK *et al*, 1999c]. 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.

10.2.1.5. 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 µCi/ml $^{32}PO_4$. During the serum starvation and metabolic labeling, cells were incubated with either vehicle (control), 0.5mM IND, or 100 µM NS-398. Ras activation was determined as described [Downward J *et al*, 1990]. Briefly, cells were washed with ice-cold PBS and lysed on ice in 1 ml of lysis buffer (50 mM Hepes, pH 7.5; 500 mM NaCl; 5 mM $MgCl_2$; 1 % Triton X-100; 0.5 % deoxycholate; 0.05 % SDS; 1 mM EGTA; 10 mM benzamidine; and 10 µg/ml each of aprotinin, leupeptin and soybean trypsin inhibitor). Ras proteins contained in the cell lysates were immunoprecipitated with rat monoclonal anti-Ras antibody (Y13-259; Santa Cruz Biotech.). 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 (Molecular Dynamics, Sunnyvale, CA, USA). The percent

of GTP bound to Ras (as an indicator of Ras activation) was calculated as cpm in GTP/(cpm in GTP + cpm in GDP) normalized for moles phosphate in each nucleotide.

10.2.1.6. 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 ± standard deviation (SD). *P* values less than 0.05 were considered significant.

10.2.2. Results

10.2.2.1. 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 culture medium for 24 and 48 hrs. Control group consisted of cells treated with vehicle. Cell proliferation was determined using ^{3}H-thymidine uptake. Compared to control, both the higher and lower concentrations of IND and NS-398 significantly inhibited cell proliferation at 24 and 48 hrs (Figure 21).

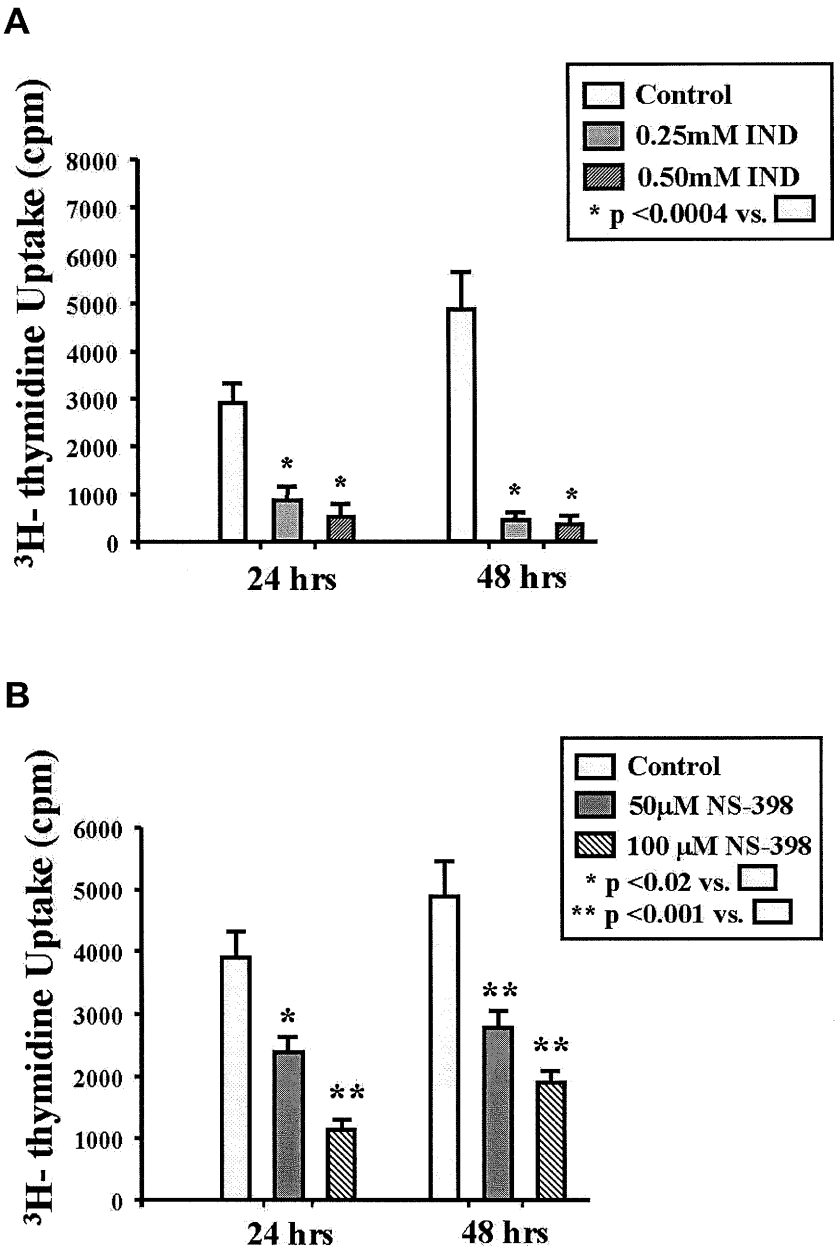

Figure 21. Effect of indomethacin (A) and NS-398 (B) on DNA synthesis in MKN28 cells reflecting cell proliferation. MKN 28 cells ($2.5x10^{-4}$ cells/well) were grown for 24 hrs in 24-well culture plates to attain 70-75 % confluence and subjected to reduced serum (5 % FBS) condition for 24 hrs. Subsequently, cells were treated with indomethacin (0.25 mM or 0.5 mM) or NS-398 (50 μM or 100 μM) for 24 and 48 hrs. Three hours prior to termination of the experiment, 0.5 μCi of [methyl-^{3}H] thymidine was added to each well. After incubation, the cells were washed with phosphate buffered saline (PBS), lysed with 0.5 N NaOH, neutralized with 0.5 N HCl, and the radioactivity was measured. Data are expressed as cpm (mean ± SD) from three separate experiments performed in triplicate.

10.2.2.2. 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. MAP (ERK2) kinase activity was measured by incorporation of radiolabeled phosphate into myelin basic protein. Compared to control, both NSAIDs significantly inhibited ERK2 activity (Figure 22).

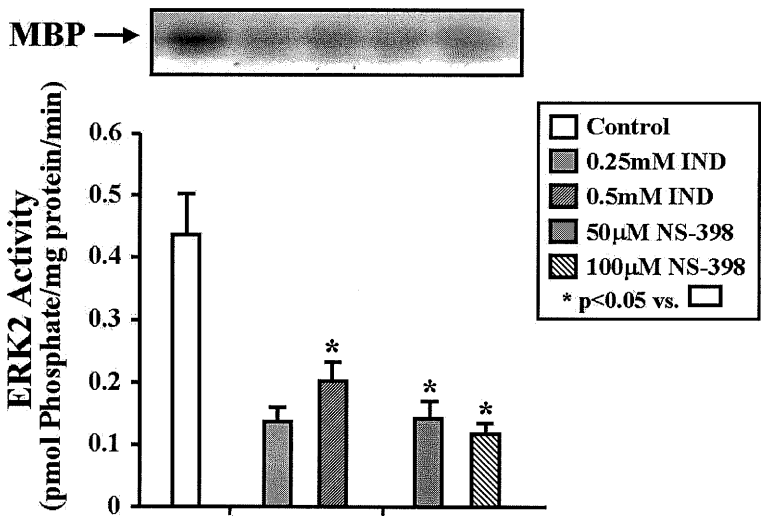

Figure 22. Effect of indomethacin and NS-398 on ERK2 activation in MKN28 cells. Top panel: autoradiography from representative experiment showing the *in vitro* phosphorylation of the substrate, myelin basic protein (MBP), by ERK2 immunoprecipitated from cytosolic lysates. Lanes represent ERK2 activity in cells incubated with medium containing vehicle (*lane 1, left*); 0.25 mM indomethacin (IND) (*lane 2*); 0.5 mM IND (*lane 3*); 50 μM NS-398 (*lane 4*) and 100 μM NS-398 (*lane 5, right*). B) The ERK2 activity was determined by measuring the levels of radiolabeled [γ-^{32}P] ATP incorporated into MBP (pmole/mg protein) by ERK2 immunoprecipitated from cell lysates. Values represent mean ± SD of 3 experiments performed in triplicates.

10.2.2.3. Effects of IND and NS-398 on Ras activation in MKN28 cells

IND (0.5 mM) and NS-398 (100 μM) 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 (Figure 23).

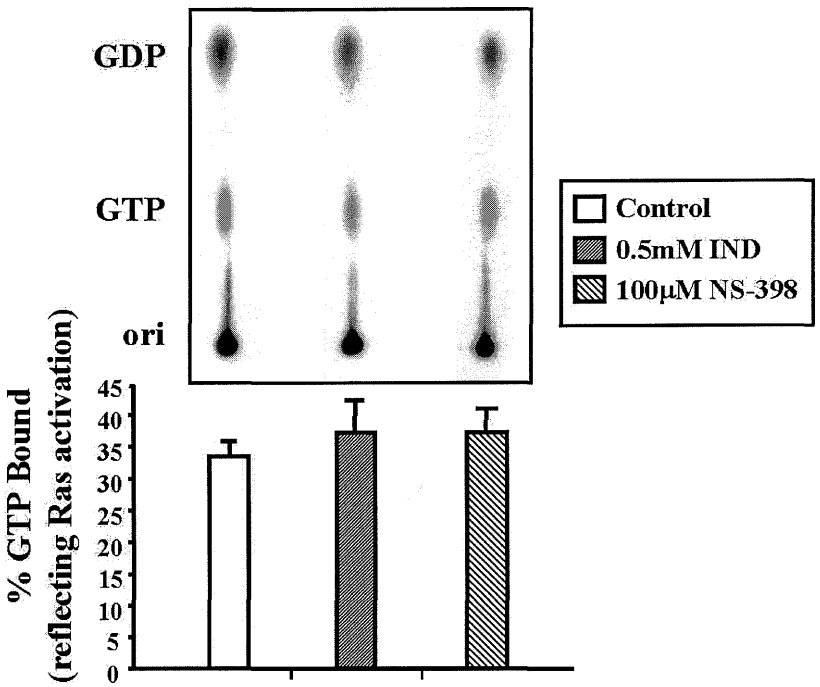

Figure 23. Effect of indomethacin and NS-398 on Ras activation in MKN 28 cells. Ras activation was determined as described in Materials and Methods. MKN 28 cells were serum-starved for 24 hrs and treated with either vehicle (control), 0.5 mM indomethacin, or 100 μM NS-398 for the final 16 hrs of the serum starvation. *Upper panel:* representative autoradiograph of the eluted GTP and GDP from Ras immunoprecipitated from MKN 28 cells cultured under the above conditions. *Lower panel:* quantitative data of the percent of GTP binding as a measure of the percent of Ras activation. Results are the mean ± SD of three independent determinations. % GTP bound is calculated as [cpm in GTP/(cpm in GTP + cpm in GDP)] x 100 normalized for moles phosphate in each nucleotide.

10.2.2.4. Effects of IND and NS-398 on apoptosis of MKN28 cells

IND (0.25 mM - 0.5 mM) and NS-398 (50 μM - 100 μM) 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 (Figure 24). Necrotic index was < 1 % in all experiments.

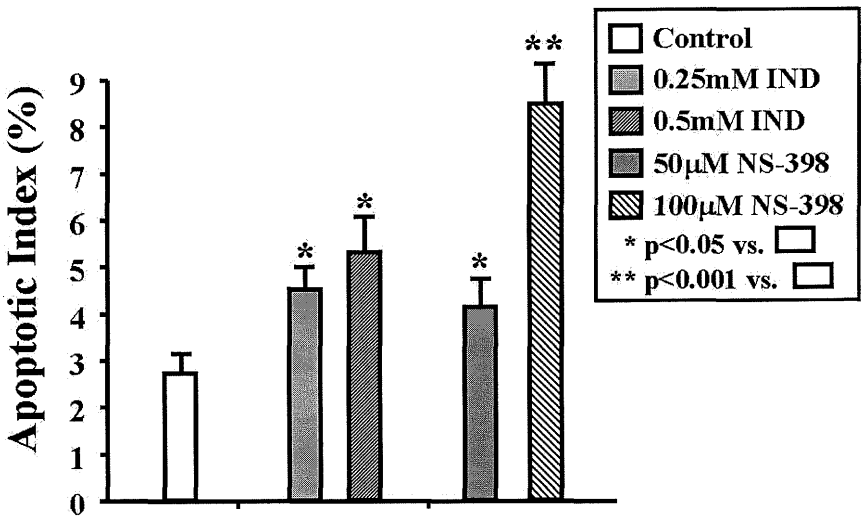

Figure 24. Effect of indomethacin and NS-398 on apoptosis in MKN 28 cells. MKN 28 cells were grown on collagen coated glass coverslips to attain confluence. Cell monolayers were incubated with either vehicle, indomethacin (0.25 mM and 0.5 mM) or NS-398 (50 μM and 100 μM) for 24 hrs. Cell apoptosis was evaluated by the TUNEL assay as detailed in the Methods Section. The data are expressed as apoptotic index reflecting percentage of apoptotic cells per total number of all cells counted (1000 cells/condition).

10.2.3. 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* [1998] and Tsuji S *et al* [1996] 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 [Sawaoka H *et al*, 1998; Tsuji S *et al*, 1996]. 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 [Fujiwara Y *et al*, 1993]. This gastric cancer cell line expresses much less COX-2 than COX-1 and does not produce prostaglandins [Sawaoka H *et al*, 19998; Tsuji S *et al*, 1996]. 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 μM and 100 μM 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 [Zhu GH *et al*, 1999a, 1999b]. 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* [1999a] 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 [Zhu GH *et al*, 1999b].

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.

11. 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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List of Author's Peer-reviewed Papers Published Related to Thesis

1. Szabó I, Bódis B, Nagy L, Mózsik Gy. Evidence for the direct protective and toxic effect of ethanol, prostacyclin and indomethacin in isolated gastric mucosal mixed cells. Hungarian Weekly Medical Journal 137: 2687-2689, 1996.

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5. Tarnawski AS, Arakawa T, Sekhon S, Ichikawa Y, Szabó IL, Szabo S, Sarfeh IJ. Isolation of morphologically and functionally intact gastric mucosal microvessels. J Physiol Pharmacol 51: 167-175, 2000.

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14. Szabó IL, Kawanaka H, Jones MK, Pai R, Soreghan B, Baatar D, Tarnawski AS. Activation of hypoxia-inducible factor-1α in gastric mucosa in response to ethanol injury. A trigger for angiogenesis. Life Sci 69: 3035-44, 2001.

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