Indomethacin Delays Gastric Restitution: Association with the Inhibition of Focal Adhesion Kinase and Tensin Phosphorylation and Reduced Actin Stress Fibers

IMRE L. SZABÓ,† RAMA PAI,† MICHAEL K. JONES,*,† GEORGE R. EHRING,*
HIROFUMI KAWANAKA,† AND ANDRZEJ S. TARNAWSKI[1],*,†

*Medical Service, Department of Veterans Affairs Medical Center, Long Beach, California 90822; and †Department of Medicine, University of California, Irvine, California 92717

Repair of superficial gastric mucosal injury is accomplished by the process of restitution—migration of epithelial cells to restore continuity of the mucosal surface. Actin filaments, focal adhesions, and focal adhesion kinase (FAK) play crucial roles in cell motility essential for restitution. We studied whether epidermal growth factor (EGF) and/or indomethacin (IND) affect cell migration, actin stress fiber formation, and/or phosphorylation of FAK and tensin in wounded gastric monolayers. Human gastric epithelial monolayers (MKN 28 cells) were wounded and treated with either vehicle or 0.5 m*M* IND for 16 hr followed by EGF. EGF treatment significantly stimulated cell migration and actin stress fiber formation, and increased FAK localization to focal adhesions, and phosphorylation of FAK and tensin, whereas IND inhibited all these at the baseline and EGF-stimulated conditions. IND-induced inhibition of FAK phosphorylation preceded changes in actin polymerization, indicating that actin depolymerization might be the consequence of decreased FAK activity. In *in vivo* experiments, rats received either vehicle or IND (5 mg/kg i.g.), and 3 min later, they received water or 5% hypertonic NaCl; gastric mucosa was obtained at 1, 4, and 8 hr after injury. Four and 8 hr after hypertonic injury, FAK phosphorylation was induced in gastric mucosa compared with controls. IND pretreatment significantly delayed epithelial restitution *in vivo*, and reduced FAK phosphorylation and recruitment to adhesion points, as well as actin stress fiber formation in migrating surface epithelial cells. Our study indicates that FAK, tensin, and actin stress fibers are likely mediators of EGF-stimulated cell migration in wounded human gastric monolayers and potential targets for IND-induced inhibition of restitution.

Key words: indomethacin; FAK; tensin; actin; human gastric monolayers; restitution [Exp Biol Med Vol. 227(6):412–424, 2002]

This work was supported by a Veterans Affairs Merit Review Research Enhancement Award Program and by a Veterans Affairs Minority Initiative Awards (to A.S.T.).

[1] To whom requests for reprints should be addressed at Gastroenterology Section (111G), DVA Medical Center, 5901 East Seventh Street, Long Beach, CA 90822. E-mail: atarnawski@yahoo.com

Received January 26, 2002.
Accepted February 27, 2002.

1535-3702/02/2276-0412$15.00
Copyright © 2002 by the Society for Experimental Biology and Medicine

Gastric mucosal injury includes superficial injury limited to exfoliation of the surface epithelium; deep mucosal injury where, in addition to superficial epithelium, microvessels are also damaged and erosions are formed; and deep injury penetrating the mucosa and muscularis mucosa—an ulcer. Following superficial mucosal injury, the continuity of the surface epithelium is promptly reestablished by the process referred to as epithelial restitution (1). 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, but requires an intact basement membrane (2–4).

Non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin (IND) delay gastric ulcer healing *in vivo* (5–7), in part by inhibiting re-epithelialization, and they also cause delayed restitution of mucosal injury *in vivo* (8, 9) and *in vitro* (10). 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 rearrangements 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 focal adhesion kinase (FAK), tensin, paxillin, vinculin, Src, and α-actinin (11–13). The focal adhesions serve as traction points over which the cell moves forward by the tension generated by contraction of the actin-myosin network (14). Cell migration is dependent on rearrangement of cell cytoskeletal structures, predominantly actin filaments.

FAK—a cytoplasmic (non-receptor) tyrosine kinase, becomes activated upon autophosphorylation within focal adhesions (13) and, in turn, phosphorylates several other

substrate proteins such as tensin and paxillin, which are also localized to focal adhesions (15–18). In fibroblasts, FAK has been shown to play a central role in the regulation of cell proliferation, spreading, and migration (18–20). Overexpression of FAK in Chinese hamster ovary (CHO) cells, for example, stimulates cell migration, whereas mutation of tyrosine phosphorylation site at tyrosine-397 of FAK inhibits cell migration (20, 21).

Tensin is an actin-capping protein that anchors actin filaments to focal adhesions and other cellular structures (22). Tensin phosphorylation by FAK (23) may affect its actin-capping activity (24). 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 (25, 26). 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 epidermal growth factor (EGF) and/or IND. The aims of the present study were to determine whether EGF and/or IND affect migration of wounded gastric epithelial cell monolayers and to examine the roles and temporal relationship of FAK and tensin phosphorylation and actin stress fiber formation during wound restitution.

Materials and Methods

Cell Culture. Human gastric epithelial (MKN 28) cells derived from gastric tubular adenocarcinoma (27) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Atlanta Biological, Norcross, GA) at 37°C in a humidified incubator containing 5% CO_2.

Animals. This study was approved by the subcommittee for Animal Studies of the Long Beach Department of Veterans Affairs Medical Center. Forty male Sprague-Dawley rats weighing 300 to 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 of 5% NaCl solution to produce exfoliation of gastric surface epithelium. At 1, 4, or 8 hr after hypertonic saline administration, rats were anesthetized, their stomachs were excised, and the animals were euthanized. The stomachs were opened along the greater curvature, rinsed with 0.9% NaCl, and gastric tissues were excised and frozen immediately in liquid nitrogen and stored at −80°C for immunohistochemistry.

***In Vitro* Cell Migration Assay.** Three 8-mm-wide longitudinal wounds were made on confluent monolayers MKN 28 cells in a 100-mm plastic dish using a razor blade, as described previously (28). The cells were washed and incubated with serum-free media containing either vehicle or 0.5 m*M* IND (Sigma, St. Louis, MO) for 16 hr, and then 10 ng/ml EGF (R&D, La Jolla, CA) or vehicle was added into the medium. Optimal concentrations of EGF and IND used in the present study were based on our previous dose-dependent studies (29–31).

Re-epithelialization of wounded monolayers was evaluated at 24 hr after the addition of vehicle or EGF. Briefly, after fixation in methanol and staining with hematoxylin and eosine, the re-epithelialization 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). To determine specific contributions of cell migration and proliferation to re-epithelialization, some studies were also performed in the presence of mitomycin C (2 μg/ml; Calbiochem-Novabiochem, Minneapolis, MN) added to the cultures 2 hr before wounding in order to inhibit cell proliferation.

Cell Death Detection Assay. Cells were plated in six-well plates on glass coverslips. After reaching confluence, monolayers were wounded and cultured in serum-free medium containing either 0.5 m*M* IND or its vehicle for 16 and 40 hr. Cell death was evaluated by terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) using a commercial kit (Roche Diagnostic, Indianapolis, IN). 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 diamino benzidine substrate. Omission of the TdT enzyme in the TUNEL reaction served as a negative control and resulted in no staining. Because 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 cells 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 the percentage of apoptotic or necrotic cells per total number of all counted cells.

Caspase-3 Activity Assay. MKN 28 cells were wounded, as in cell migration assays, and incubated in serum-free media containing either vehicle or IND (0.5 m*M*). After a 16- or 40-hr incubation with IND or vehicle, caspase-3 activity was determined by colorimetric CaspACE Assay System (Promega, Madison, WI). Monolayers were washed twice in PBS and lysed in ice-cold caspase assay lysis buffer. The assays were performed in a total volume of 100 μl in 96-well plates for 4 hr, and absorbance was measured using a Vmax microplate reader (Molecular Devices, Sunnyvale, CA) at 405 nm. Relative absorbance was determined by using negative control (monolayers treated with 50 μ*M* Z-VAD-FMK caspase inhibitor for 16 hr) and positive controls (monolayers treated with 50 ng/ml anti-Fas antibody for 16 hr). Caspase-3-specific activity was calculated by measuring the absor-

bance of known amounts of p-nitroaniline (pNA) and protein concentration of each sample.

Immunocytochemistry of Wounded MKN 28 Monolayers. Cells were plated in six-well plates on glass coverslips. After reaching confluence, monolayers were wounded and cultured in serum-free medium containing either vehicle or IND (0.5 m*M*) for 1, 4, 8, and 16 hr. In some experiments, EGF (10 ng/ml) or an equal volume of vehicle was added to 16-hr IND (0.1, 0.25, or 0.5 m*M*) preincubated cells grown on coverslips, and they were incubated for an additional 24 hr. 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), which selectively binds to polymerized F-actin or with rabbit polyclonal anti-FAK antibody (2 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA). Cells for FAK staining were grown on coverslips and were incubated with Alexa-conjugated anti-rabbit secondary antibody (Molecular Probes; 1:200) for 30 min. After washing with PBS, 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 PCM confocal microscope (Nikon, Tokyo, 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 following the method described previously (32). FAK localization to focal adhesion points was counted on coded slides as the number of adhesion points stained for FAK on the perimeter of individual cells.

Immunocytochemistry of Rat Gastric Specimens. Frozen gastric specimens were cut with a cryostat (10 μm thick; Jung Cryostat; Leica, Deerfield, IL), and were permeabilized in acetone for 5 min at –20°C. Slides were then incubated with either FITC-conjugated phalloidin (5 μl/ml in PBS; Molecular Probes) or with rabbit polyclonal anti-FAK antibody (2 μg/ml; Santa Cruz Biotechnolgy). After washing with PBS, coverslips 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 were captured using a Nikon DXM1200 digital camera.

Immunoprecipitation and Immunoblotting of FAK and Tensin. MKN 28 cells were wounded as described earlier and were cultured in serum-free media containing either vehicle or IND (0.5 m*M*) for 1, 4, 8, and 16 hr. EGF (10 ng/ml) or an equal volume of vehicle was then added to monolayers and was further incubated for 5 to 60 min. In some experiments, wounded monolayers were treated with vehicle or inhibitors of EGR-R kinase PD 153035 (1 μ*M* for 16 hr) or AG 1478 (250 n*M* for 20 min; both Calbiochem-Novabiochem, La Jolla, CA) prior to EGF treatment. The cells were washed twice in ice-cold PBS and lysed in cold lysis buffer containing 50 m*M* Tris-HCl (pH 7.5), 150 m*M* NaCl, 0.5% Nonidet P-40, 5 m*M* NaF, 1 m*M* Na_3VO_4, 1 m*M* 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 Biotechnolgy) and anti-tensin (Transduction Laboratories, Lexington, KY) antibodies, respectively, followed by immunoblotting using anti-phosphotyrosine monoclonal antibody (Santa Cruz Biotechnolgy). In brief, 1 μg of anti-FAK polyclonal or 2 μg of anti-tensin monoclonal antibody was incubated with protein A-Sepharose beads (25 and 50 μl, respectively) for 2 hr at 4°C under constant stirring. Aliquots of clarified cell lysates containing equal amounts of protein (250 μg for FAK and 1 mg for tensin) were added to the protein A-Sepharose beads/antibody complex and were 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 were transferred to nitrocellulose membranes. The membranes were immunoblotted with anti-phosphotyrosine antibody. After washing, bound antibody was detected with an enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL). 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 of protein) were subjected to SDS-PAGE and were 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 (33, 34).

Statistical Analysis. Results were expressed as mean ± SD. Statistical significance between differently treated groups was determined by analysis of variance (ANOVA) 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 fiber formation were analyzed.

Results

***In Vitro* Experiments.** *IND inhibits EGF-stimulated gastric epithelial cell migration.* In wounded gastric epithelial cell monolayers, EGF treatment stimulated cell migration by 59% ± 8% compared with vehicle-treated monolayers. IND significantly inhibited gastric epithelial cell migration under the basal condition by 73% ± 12%, and significantly inhibited EGF-stimulated cell migration by 67% ± 9% (both $P < 0.005$; Table I).

To differentiate the effect of IND on cell migration and proliferation, we performed the cell migration assay in the presence of mitomycin C (2 μg/ml for 2 hr), which abolishes MKN 28 cell proliferation (35). In these experiments, EGF significantly stimulated the migration of MKN 28 cells by 53% ± 7%, clearly indicating that cell migration is predominantly responsible for re-epithelialization, and that the possible induction of cell cycle arrest by IND does not play a major role in IND-induced inhibition of re-epithelialization of wounded monolayers. Treatment with IND of mitomycin C-treated monolayers significantly reduced both baseline and EGF-stimulated migration (72% ± 11% and 65% ± 9%, respectively; both $P < 0.005$). Thus, cell proliferation had only a minor contribution to re-epithelialization at 24 hr after EGF or its vehicle treatment.

Effect of IND on apoptotic/necrotic indices and caspase-3 activity. Because IND can induce gastric cell apoptosis, we determined apoptosis in MKN 28 cells after IND treatment. We found a moderate but significant increase in apoptosis at the end of 16- and 40-hr time periods. The percentage of apoptotic cells was 4.37 ± 1.07 after a 16-hr treatment with 0.5 m*M* IND compared with 3.43% ± 0.75% in the vehicle-treated groups ($P < 0.05$). After 40 hr of IND treatment, we also found a moderate and significant increase in number of apoptotic cells (to 6.50% ± 1.44%; $P < 0.01$), whereas monolayers incubated with vehicle had no further increase (Table II). 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 of incubation. Caspase-3 activity was significantly increased in experiments with 40 hr of IND-treatment (97% ± 8% increase, $P < 0.01$) versus vehicle-treated controls (Table II).

IND reduces actin stress fiber formation in gastric epithelial cell monolayers. Phalloidin labeling of actin filaments in cell lining at wounded margins demonstrated a significant enhancement of actin stress fiber formation after 24 hr of EGF treatment (43% ± 11% increase vs. baseline; $P < 0.05$; Fig. 1). 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 of EGF stimulation (24% ± 7% inhibition; $P < 0.05$; Fig. 1).

Compared with controls (Fig. 2A), IND treatment did not affect stress fiber formation up to 16 hr of IND treatment (Fig. 2B). After 24 hr of IND treatment, alterations in actin immunoreactivity within the cells occurred. The immunoreactivity became more diffuse and less intense (Fig. 2, C and F). This reduction in actin immunoreactivity in response to IND was dose dependent (Fig. 2, D–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 intensity measurement. However, these cells responded to EGF stimulation with increased stress fiber formation, but these changes were less prominent than in cells localized at the wound edge.

Effect of IND on FAK immunoreactivity in gastric epithelial cell monolayers. Immunostaining for FAK showed coarse punctate immunoreactivity at the lamellipodial edge of cells in all groups. The major difference between vehicle-treated and EGF-treated monolayers was found at the periphery of cells, as demonstrated by increased FAK immunoreactivity. EGF treatment caused increased immunoreactivity to FAK in migrating cells at the wound edge—a 78% ± 13% increase compared with vehicle-treated monolayers ($P < 0.05$; Fig. 3). Monolayers treated with IND showed a significant loss of immunoreactivity to

Table I. Re-Epithelialization of Wounded Human Gastric Epithelial Cell Monolayers Cultured without and with Mitomycin C and Effects of EGF and Indomethacin

Treatment	Re-epithelialization		Re-epithelialization in presence of mitomycin C (2 μg/ml)	
	Area[a]	Percentage of control	Area[a]	Percentage of control[b]
Control (vehicle)	11.5 ± 2.1	100	10.7 ± 2.0	93.4
EGF (10 ng/ml)	18.4 ± 2.7[c]	159.0	16.5 ± 2.5[c]	143.0
Indomethacin (0.5 m*M*)	3.1 ± 0.5[c]	27.0	3.0 ± 0.5[c]	26.0
Indomethacin + EGF	6.1 ± 1.0[c,d]	52.5	5.8 ± 0.9[c,d]	50.5

Note. Data are expressed as means ± SD.
[a] Area of cells is expressed in mm^2 per 2 cm wounding edge.
[b] Compared with control (vehicle-treated) monolayers without mitomycin C cotreatment.
[c] $P < 0.005$ vs. control (vehicle-treated) monolayers.
[d] $P < 0.005$ vs. EGF-treated monolayers.

Table II. Induction of Apoptosis by Indomethacin in Human Gastric Monolayers

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

Note. Data are expressed as means ± SD.
[a] Apoptotic index is calculated as percentage of apoptotic cells per total number of cells.
[b] Caspase-3 activity is expressed in picomoles per hour per milligram of protein. Calculated as percentage of control (40-hr vehicle-treated) monolayers.
[c] $P < 0.05$ vs. control (16-hr vehicle-treated) monolayers.
[d] $P < 0.01$ vs. control (40-hr vehicle-treated) monolayers.

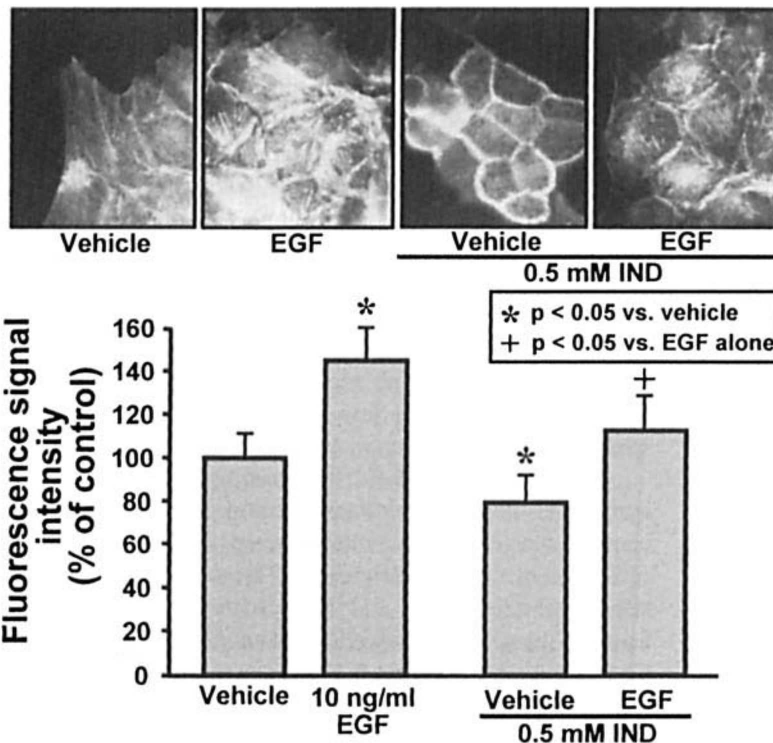

Figure 1. Phalloidin labeling of F-actin in wounded MKN 28 monolayers. Wounded cell monolayers were pretreated with either vehicle or 0.5 m*M* IND for 16 hr and were incubated with EGF (10 ng/ml) or vehicle for a further 24 hr. Intensity of fluorescence signal was measured by confocal microscopy. Values are expressed as the mean ± SD (percentage of control, $n = 12$) of three independent experiments.

FAK 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 $P < 0.05$; Fig. 3).

IND inhibits tyrosine phosphorylation of FAK and tensin in gastric epithelial cell 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% increase; $P < 0.005$) and remained significantly elevated for 30 min (Fig. 4A) vs. control. 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 $P < 0.01$; Fig. 5). The EGF treatment stimulated tensin phosphorylation within 5 min (26% ± 7% increase; $P < 0.05$); this increase was sustained for 30 min (Fig. 4B).

Sixteen-hour 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; Fig. 6A). IND did not affect the low baseline level of tensin phosphorylation, but significantly inhibited EGF-stimulated tensin phosphorylation by 18% ± 5% ($P < 0.05$; Fig. 6B). Inhibition of baseline FAK phosphorylation by IND was present after 8 hr of incubation (17% ± 6%, $P < 0.05$) and 16 hr of incubation (19% ± 7%; $P < 0.05$; Fig. 7).

The IND-induced inhibition of EGF-stimulated FAK and tensin phosphorylation strongly correlated with both the IND-induced inhibition of EGF-induced cell migration (r = 0.994 and 0.842; respectively; $P < 0.05$) and the IND-induced inhibition of stress fiber formation (r = 0.986 and 0.656, respectively; $P < 0.05$).

Western blot analysis using anti-G6PDH antibody (housekeeping protein) demonstrated that IND did not in-

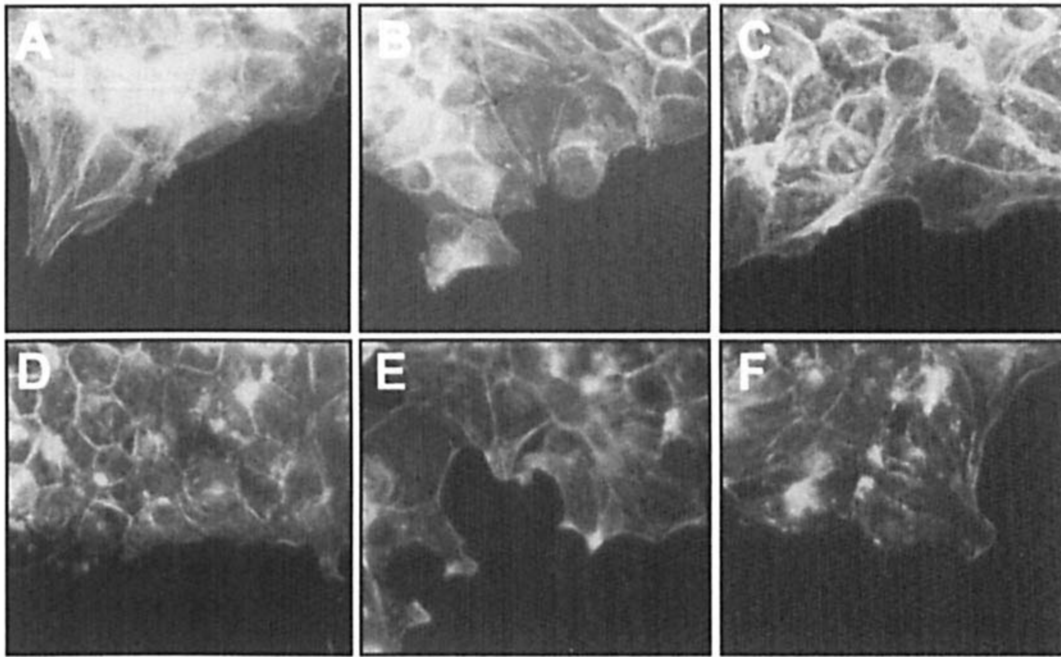

Figure 2. Phalloidin labeling of F-actin in wounded MKN 28 monolayers. Wounded cell monolayers were treated with vehicle (A) or 0.5 m*M* IND (B) for 16 hr or 0.5 m*M* IND (C) for 24 hr. To determine the dose-relation of IND treatment to the loss of stress fiber formation, monolayers were treated with 0.1 (D), 0.25 (E), or 0.5 m*M* (F) IND for 40 hr. Sixteen hours after IND treatment (B), the F-actin filaments were not changed compared with vehicle-treated monolayers (A), and the loss of stress fiber formation was apparent after 24 hr (C). The various concentrations of IND (0.1, 0.25, and 0.5 m*M*) caused a dose-dependent loss of stress fibers in human gastric monolayers (D–F).

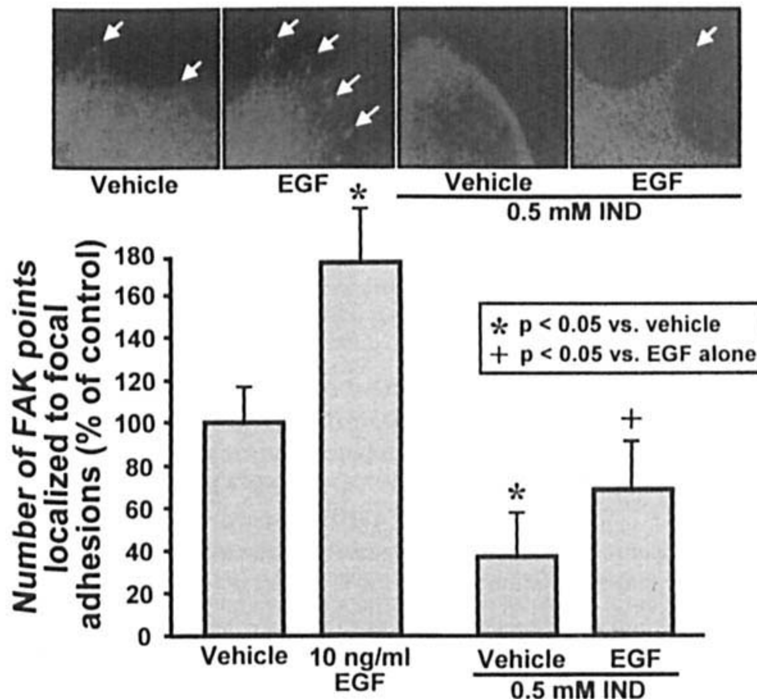

Figure 3. Immunoreactivity of FAK in wounded MKN 28 monolayers. Wounded cell monolayers were pretreated with either vehicle or 0.5 m*M* IND for 16 hr and were incubated with EGF (10 ng/ml) or vehicle for a subsequent 24 hr. FAK was visualized by immunostaining with specific antibody as described in "Materials and Methods." Arrows indicate FAK immunoreactivity clustered at the cell periphery. The number of FAK fluorescence clusters was evaluated by randomly counting 50 cells at the wound edge on each coded coverslip. Values are expressed as the mean ± SD (percentage of control, n = 12) of three independent experiments.

hibit protein synthesis, indicating that IND-induced inhibition of phosphorylation is not due to altered protein synthesis (data not shown).

***In Vivo* Experiments.** *IND affects actin stress fibers in rat gastric mucosa.* In rat gastric mucosa injured by hypertonic saline, surface epithelial continuity was almost completely restored at 8 hr 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 (Fig. 8B) versus vehicle-pretreated controls at 4 hr after injury (Fig.

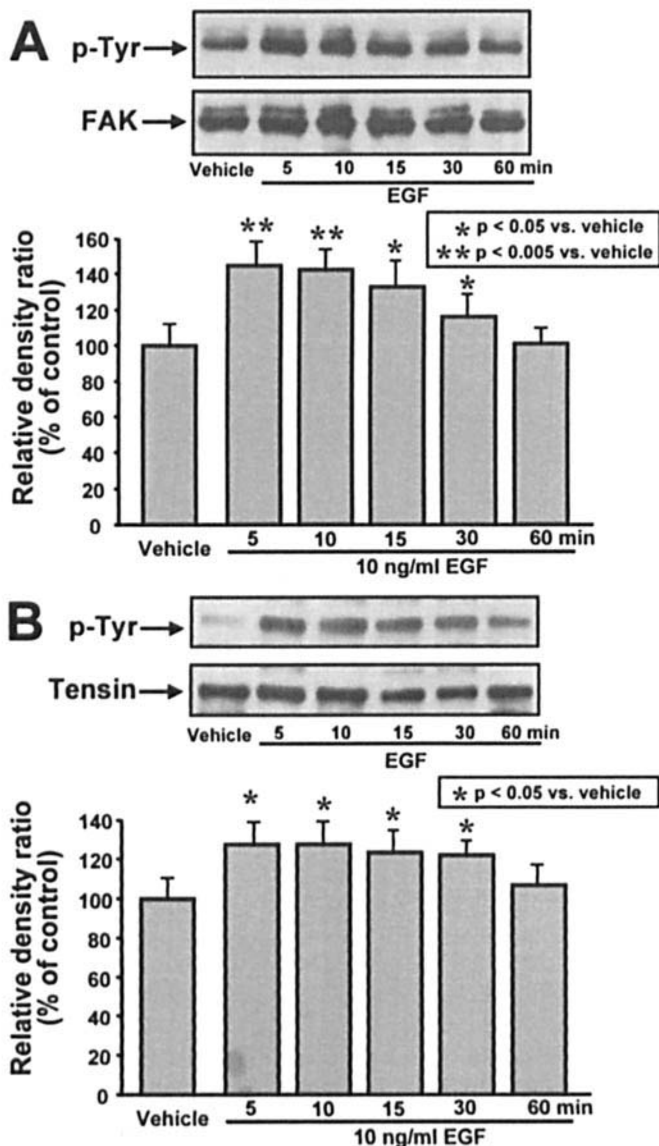

Figure 4. EGF stimulation of tyrosine phosphorylation of FAK and tensin in wounded MKN 28 monolayers. Wounded cell monolayers were cultured in serum-free medium for 16 hr and were incubated with EGF (10 ng/ml) or vehicle for 5 to 60 min. (A) Tyrosine phosphorylation of FAK was determined by immunoprecipitation of FAK using specific antibody followed by Western blot analysis using phosphotyrosine antibody (p-Tyr). The same membrane was stripped and reprobed 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 (percentage 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 p-Tyr. The same membrane was stripped and reprobed 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 (percentage of control, $n = 9$) of phosphorylated to total tensin protein levels.

8A). In the gastric mucosa of IND-pretreated rats, the migrating epithelial cells showed less intracellular actin filaments (Fig. 8C) than epithelial cells of vehicle-treated rats (Fig. 8D).

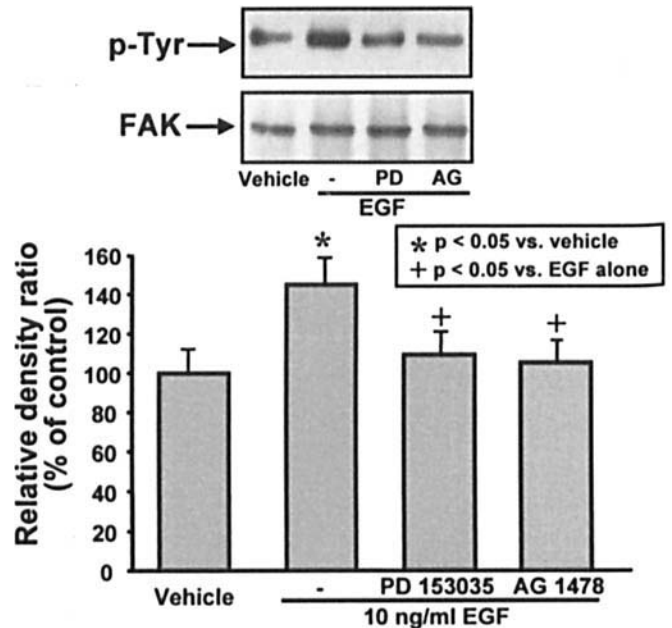

Figure 5. Inhibition of EGF receptor kinase inhibits FAK phosphorylation in wounded MKN 28 monolayers. Wounded cell monolayers were pretreated with vehicle or PD 153035 (1 μ*M*) for 16 hr or AG 1478 (250 n*M*) for 30 min, and then EGF (10 ng/ml) or vehicle was then added to the media for 5 min. Tyrosine phosphorylation of FAK was determined by immunoprecipitation as described in "Materials and Methods." (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 (percentage of control, $n = 9$) of phosphorylated to total FAK protein levels.

IND reduces FAK immunoreactivity in rat gastric mucosa. Immunostaining of injured gastric mucosa showed coarse punctate FAK immunoreactivity in the cell edge (Fig. 9A). The number of FAK (adhesion points) in migrating cells was significantly reduced in gastric mucosa of rats pretreated with IND than in vehicle-treated at 8 hr after injury (Fig. 9B).

Effect of IND on tyrosine phosphorylation of FAK in rat gastric mucosa. Tyrosine phosphorylation of FAK was significantly increased in rat gastric mucosa at 4 and 8 hr after injury (74% ± 10% increase at 4 hr, $P < 0.001$; 42% ± 11% increase at 8 hr, $P < 0.05$; respectively; Fig. 10). IND pretreatment significantly inhibited the injury-induced FAK phosphorylation at 4 hr (53% ± 8%; $P < 0.001$) and 8 hr (55% ± 8%; $P < 0.001$) compared with vehicle (Fig. 10).

Discussion

The present study shows that IND, a non-selective cyclooxygenase inhibitor, significantly impairs both basal and EGF-stimulated cell migration in wounded human gastric epithelial monolayers. Furthermore, IND-induced inhibition of *in vitro* cell migration is associated with the early inhibition of FAK phosphorylation and late disruption of actin stress fiber formation, as well as inhibition of FAK immunoreactivity. The inhibition of actin stress fiber formation showed a strong correlation with reduced phosphorylation

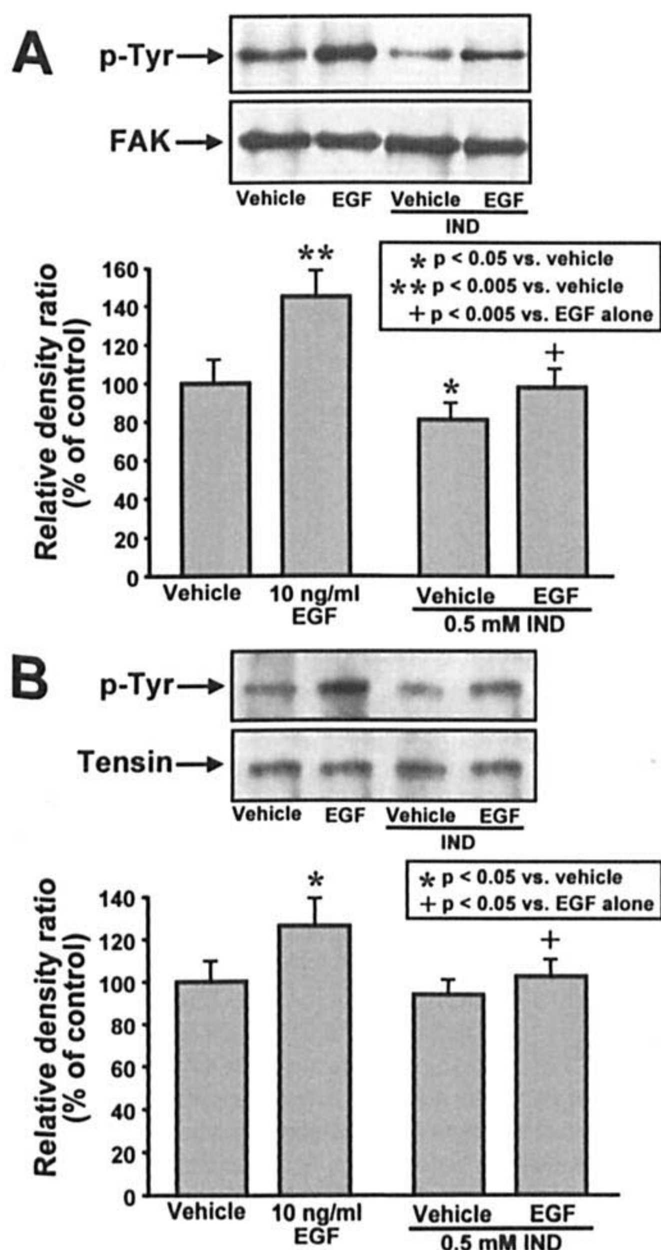

Figure 6. Inhibition of tyrosine phosphorylation of FAK and tensin by IND in wounded MKN 28 monolayers. Wounded cell monolayers were pretreated with either vehicle or IND (0.5 m*M*) for 16 hr and were incubated with EGF (10 ng/ml) or vehicle for 5 min. (A) Tyrosine phosphorylation of FAK. (B) Tyrosine phosphorylation of tensin. Phosphorylation levels was determined by immunoprecipitation as described in "Materials and Methods." (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 (percentage of control, $n = 9$) of phosphorylated to total FAK or tensin protein levels.

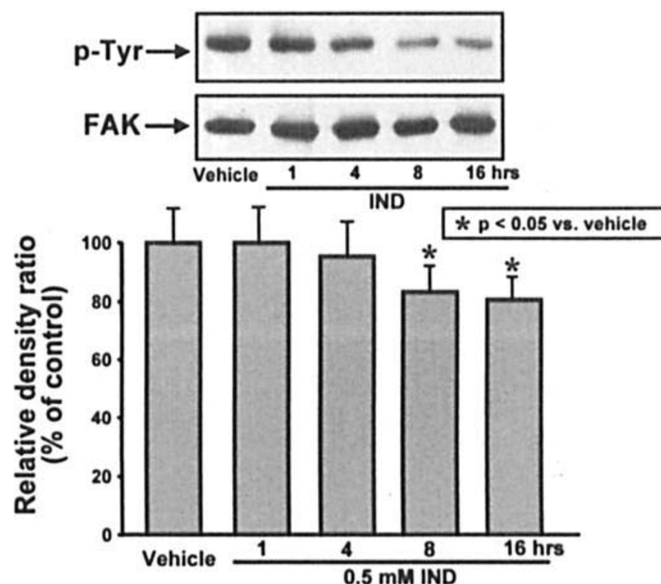

Figure 7. Time line of IND-induced inhibition of tyrosine phosphorylation of FAK in wounded MKN 28 monolayers. Wounded cell monolayers were treated with either vehicle or IND (0.5 m*M*) for 1, 4, 8, and 16 hr. Tyrosine phosphorylation of FAK was determined by immunoprecipitation as described in "Materials and Methods." (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 (percentage of control, $n = 9$) of phosphorylated to total FAK protein levels.

of FAK and tensin. IND pretreatment also delayed *in vivo* restitution of rat gastric mucosa injured by hypertonic saline, and inhibited injury-induced FAK phosphorylation and immunoreactivity to FAK.

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 reestablish epithelial continuity (36). *In vivo*, epithelial restitution can be accomplished rapidly within 15 to 60 min (37), and is independent of cell proliferation or differentiation, but dependent on uninterrupted blood flow (38, 39). A previous study has demonstrated that cell motility without proliferation can substantially expand an intestinal epithelial monolayer in culture even over a 6-day period if proliferation is pharmacologically ablated (40). Cell migration involves specific matrix interactions that generate physical forces to move the mucosal cells across the defect (40). Several growth factors, including EGF, TGF-α, PDGF, and trefoil peptides have been shown to play important roles in mucosal restitution (41, 42).

The *in vitro* model of gastrointestinal cell migration used in the present study has been shown to be compatible with *in vivo* conditions (28). Some important aspects of this model that resemble the early phase of epithelial cell restitution in the gastrointestinal tract *in vivo* include independence from DNA synthesis, complete dependence on actin polymerization, and accelerated cell migration in the presence of extracellular matrix (28).

Our study shows that IND inhibits gastric epithelial cell migration *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 by 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

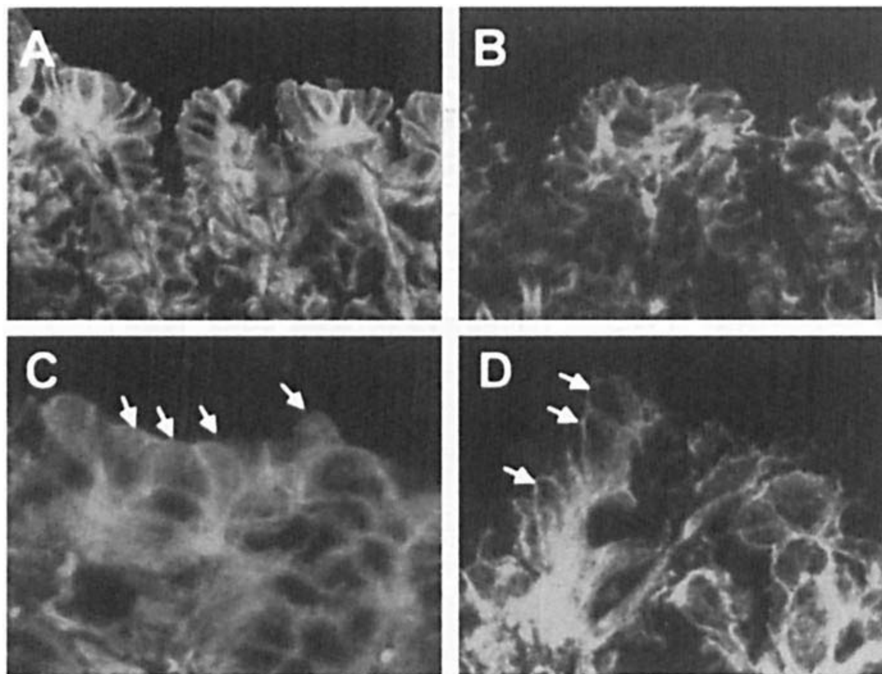

Figure 8. Phalloidin labeling of F-actin in migrating surface epithelial cells of rat gastric mucosa at 4 hr after injury. Rats received either vehicle (A and C) or IND (B and D; 5 mg/kg, 1 ml) i.g., and 3 min later, rats were given 2 ml of 5% NaCl (i.g). Arrows indicate the migrating surface epithelial cells. Original magnification: A and B, ×200; C and D, ×400.

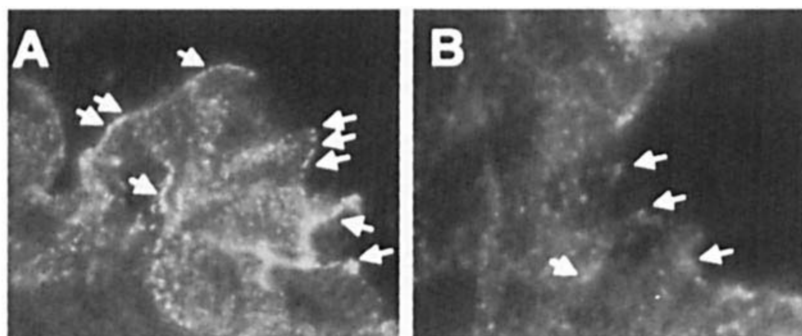

Figure 9. FAK distribution in migrating surface epithelial cells of rat gastric mucosa 8 hr after injury. Rats received either vehicle (A) or IND (B; 5 mg/kg, 1 ml) i.g., and 3 min later, rats were given 2 ml of 5% NaCl i.g. FAK was visualized by immunostaining with specific antibody as described in "Materials and Methods." Arrows indicate FAK signal localized to the cell periphery. Original magnification: ×1000.

24 hr) and that cell proliferation becomes more important only during the later 24- to 48-hr period (43, 44). EGF-induced actin polymerization and stress fiber formation depends on EGF-R tyrosine kinase activity because the selective tyrosine kinase inhibitors abolish EGF-induced actin polymerization (45). Moreover, EGF-R has been shown to bind directly to actin (46) and to induce serine phosphorylation (47). In this context, our findings suggest that the action of EGF on FAK phosphorylation requires EGF-R activation, and that EGF-R can mediate downstream signals via activating FAK.

It is known that in polarized epithelia, EGF acts predominantly on the basolateral EGF receptors. In our experiments, basolateral EGF-Rs 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 (48). Although Kuwada *et al.* (49) 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 (48).

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 (50) 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. 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 (21, 51). 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, expressions of FAK and tensin have not been demonstrated in gastric cells.

Although we recognize the important roles of the above mechanisms, in this paper we focused our attention on F-actin, FAK and tensin for the following reasons. Cell migration requires a proper reorganization and re-assembly of

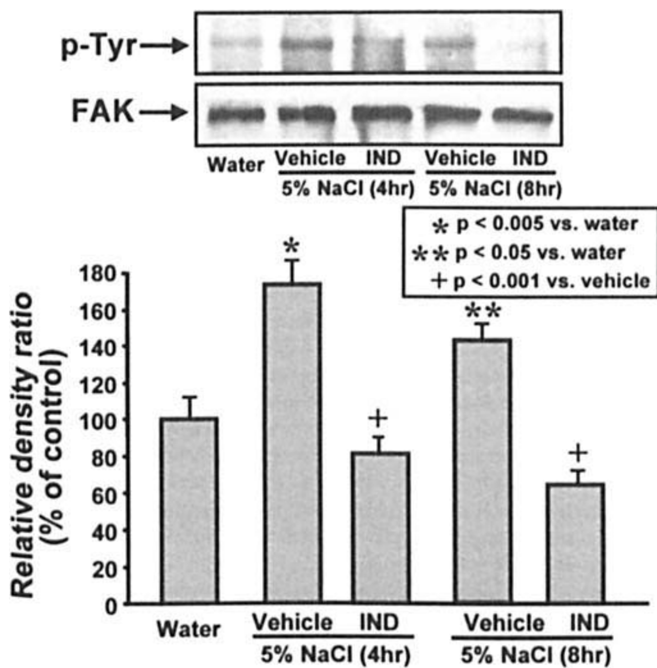

Figure 10. FAK phosphorylation in injured and noninjured rat gastric mucosa. Rats received either vehicle (A and C) or IND (B and D; 5 mg/kg, 1 ml) i.g., and 3 min later, rats were given either 2 ml of 5% NaCl or water i.g. Tyrosine phosphorylation of FAK was determined by immunoprecipitation as described in "Materials and Methods." (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 (percentage of control, $n = 9$) of phosphorylated to total FAK protein levels.

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 (52, 53). McCormack *et al.* (28, 54) demonstrated *in vitro* that migration of IEC-6 cells depends on actin polymerization, and other studies have demonstrated that EGF induces rapid reorganization of actin in human A431 cells (55). 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 (19, 56). Withers *et al.* (57) have shown that there is a direct correlation between FAK activity and phosphotyrosine content. In migrating cells, phosphorylated FAK localizes to focal adhesions (58, 59). In contrast, inhibition of FAK phosphorylation has been shown to decrease both cell proliferation and motility (60, 61). 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 (58, 59, 62, 63). Other investigators found that the elevation of FAK's phosphotyrosine content following cell adhesion to the extracellular matrix directly correlates with increased FAK activity (64). Yu *et al.* (65) demonstrated in migrating colon cancer cells that although membrane/cytoskeletal FAK, paxillin, and cytosolic FAK proteins are reduced in a matrix-dependent manner (65), their phosphorylation is increased in proportion to their proteins. This suggests that matrix may regulate intestinal epithelial cell motility by modulating amounts and distribution of focal adhesion plaque proteins available for phosphorylation (65). EGF has been shown to exert a matrix-specific effect on cell migration by modulating integrin expression and organization (40, 66). FAK (expression and activity) is suggested to function as an important signaling point to coordinate EGF-stimulated migration (67). Our study demonstrated that IND-induced inhibition of cell migration involves reduced FAK phosphorylation and reduced FAK immunoreactivity in the lamellipodial edge under both basal and EGF-stimulated conditions *in vitro* and *in vivo* in migrating epithelial cells; however, we did not find any significant changes in FAK protein levels. It is likely that the coarse punctate immunoreactivity we observed at the lamellipodial edges of FAK-stained cells represents FAK clustering at focal adhesion complexes. Because our study did not characterize other focal adhesion complex proteins such as paxillin and vinculin, it is also possible that this staining may represent only clusters of FAK without actual focal adhesion complex formation. The method used to quantify fluorescence intensity on histologic slides used in the present study has been previously published (32, 68, 69). This technique gives reproducible results with almost linear correlations in the range of antigen concentration of 1 to 10, but tends to loose its sensitivity over the 15- to 20-fold concentration.

Although our study did not establish a direct EGF-R-FAK interaction, FAK as an important contiguous link between EGF-R and integrin signaling pathways is implicated by the following observations: our finding that inactivation of EGF-R kinase with specific inhibitors inhibit FAK activation, that loss of FAK from the focal adhesions inhibits EGF-R signaling at the cell membrane and attenuates EGF-dependent migration (70), and that cells lacking FAK have reduced motility signals from EGF-R (71).

Our present findings that IND causes loss of actin stress fibers, reduces FAK phosphorylation and immunofluorescence signal, and inhibits gastric epithelial cell migration is in agreement with an earlier study demonstrating that treatment of colon cancer cells (DLD-1 and HT-29) with sub-apoptotic doses of sulindac sulfide (a non-selective NSAID) causes rearrangement of actin cytoskeleton and subsequent loss of focal adhesion plaques (72). The latter study also showed that changes in the actin cytoskeleton and focal adhesion plaques were associated with reduced tyrosine phosphorylation of FAK and reduced cell invasion (72).

Tensin, an actin-capping protein, links actin filaments to focal adhesions (73) and is phosphorylated by FAK. Lo

et al. (73) have shown that tensin plays a crucial role in the actin-dependent maintenance of cell structural integrity in fibroblasts and is involved in the transmission of signals regulating fibroblast spreading and growth. Therefore, our present finding that IND inhibits EGF-stimulated tyrosine phosphorylation of FAK and tensin implicates the reduced phosphorylation of these proteins in the mechanism of NSAID-induced disruption of actin stress fibers and thereby inhibition of cell migration. Our present study demonstrates 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 (15). In our present study, we found strong correlations between EGF-stimulated wound restitution, increased actin stress fiber formation, and enhanced FAK and tensin phosphorylation. Although 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 *et al.* (72) showed that treatment of primary cultures of chick embryo cells with antisense oligonucleotides to FAK reduces the level of FAK protein expression, which causes the loss of stress fibers.

In conclusion, our data show for the first time that FAK and tensin are expressed in human gastric epithelial cells and are phosphorylated in response to EGF. IND treatment inhibits stress fiber formation and phosphorylation of FAK and tensin, suggesting a possible mechanism for interference with mucosal restitution. Because 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.

Drs. Imre Szabó and Hirofumi Kawanaka were visiting scientists from the First Department of Medicine (University of Pécs, Hungary) and from the Second Department of Surgery (Kyushu University, Japan), respectively.

1. Lacy ER, Ito S. Rapid epithelial restitution of the rat gastric mucosa after ethanol injury. Lab Invest **51**:573–583, 1984.
2. Svanes K, Ito S, Takeuchi K, Silen W. Restitution of the surface epithelium of the in vitro frog gastric mucosa after damage with hyperosmolar sodium chloride: morphologic and physiologic characteristics. Gastroenterology **82**:1409–1426, 1982.
3. Morris GP. Prostaglandins and cellular restitution in the gastric mucosa. Am J Med **81**:23–29, 1986.
4. Watanabe S, Hirose M, Yasuda T, Miyazaki A, Sato N. Role of actin and calmodulin in migration and proliferation of rabbit gastric mucosal cells in culture. J Gastroenterol Hepatol **9**:325–333, 1994.
5. Wang JY, Yamasaki S, Takeuchi K, Okabe S. Delayed healing of acetic acid-induced gastric ulcers in rats by indomethacin. Gastroenterology **96**:393–402, 1989.
6. Inauen W, Wyss PA, Kayser S, Baumgartner A, Schurer-Maly CC, Koelz HR, Halter F. Influence of prostaglandins, omeprazole, and indomethacin on healing of experimental gastric ulcers in the rat. Gastroenterology **95**:636–641, 1988.
7. Tarnawski A, Stachura J, Douglass TG, Krause WJ, Gergely H, Sarfeh IJ. Indomethacin impairs quality of experimental gastric ulcer healing: a quantitative histological and ultrastructural analysis. In: Garner A, O'Brien PE, Eds. Mechanisms of Injury, Protection and Repair of the Upper Gastrointestinal Tract. Chichester, UK: Wiley & Sons, pp521–531, 1991.
8. Erickson RA. Effect of 16,16-dimethyl PGE2 and indomethacin on bile acid-induced intestinal injury and restitution in rats. J Lab Clin Med **112**:735–744, 1988.
9. Takeuchi K, Ishihara Y, Okada M, Niida H, Okabe S. A continuous monitoring of mucosal integrity and secretory activity in rat stomach: a preparation using a Lucite chamber. Jpn J Pharmacol **49**:235–244, 1989.
10. Giap AO, Ma TY, Hoa NT, Tarnawski AS. Does specific COX-2 inhibitor interfere with re-epithelialization of wounded gastric epithelial monolayers? Gastroenterology **116**:A576, 1999.
11. Smilenov LB, Mikhailov A, Pelham RJ, Marcantonio EE, Gundersen GG. Focal adhesion motility revealed in stationary fibroblasts. Science **286**:1172–1174, 1999.
12. Bockholt SM, Otey CA, Glenney JR Jr, Burridge K. Localization of a 215-kDa tyrosine-phosphorylated protein that cross-reacts with tensin antibodies. Exp Cell Res **203**:39–46, 1992.
13. Ilic D, Damsky CH, Yamamoto T. Focal adhesion kinase: at the crossroads of signal transduction. J Cell Sci **110**(Pt 4):401–407, 1997.
14. Lauffenburger DA, Horwitz AF. Cell migration: a physically integrated molecular process. Cell **84**:359–369, 1996.
15. Bockholt SM, Burridge K. Cell spreading on extracellular matrix proteins induces tyrosine phosphorylation of tensin. J Biol Chem **268**:14565–14567, 1993.
16. Schaller MD, Otey CA, Hildebrand JD, Parsons JT. Focal adhesion kinase and paxillin bind to peptides mimicking β-integrin cytoplasmic domains. J Cell Biol **130**:1181–1187, 1995.
17. Hildebrand JD, Schaller MD, Parsons JT. Paxillin, a tyrosine phosphorylated focal adhesion-associated protein binds to the carboxyl terminal domain of focal adhesion kinase. Mol Biol Cell **6**:637–647, 1995.
18. Gilmore AP, Romer LH. Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. Mol Biol Cell **7**:1209–1224, 1996.
19. Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature **377**:539–544, 1995.
20. Cary LA, Chang JF, Guan JL. Stimulation of cell migration by overexpression of focal adhesion kinase and its association with Src and Fyn. J Cell Sci **109**:1787–1794, 1996.
21. Tamura M, Gu J, Danen EH, Takino T, Miyamoto S, Yamada KM. PTEN interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway. J Biol Chem **274**:20693–20703, 1999.
22. Lin S, Risinger MA, Butler JA. A model for protein-protein interactions involved in the linking of the actin cytoskeleton to transmembrane receptors for extracellular matrix. In: Aebi U, Engel J, Eds. Cytoskeletal and Extracellular Matrix Protein: Springer Series in Biophysics. Berlin: Springer-Verlag, Vol **3**:pp341–344, 1989.

23. Davis S, Lu ML, Lo SH, Lin S, Butler JA, Druker BJ, Roberts TM, An Q, Chen LB. Presence of an SH2 domain in the actin-binding protein tensin. Science **252**:712–715, 1991.
24. Auger KR, Songyang Z, Lo SH, Roberts TM, Chen LB. Platelet-derived growth factor-induced formation of tensin and phosphoinositide 3-kinase complexes. J Biol Chem **271**:23452–23457, 1996.
25. Chuang JZ, Lin DC, Lin S. Molecular cloning, expression, and mapping of the high affinity actin-capping domain of chicken cardiac tensin. J Cell Biol **128**:1095–1109, 1995.
26. Zhu T, Goh EL, Lobie PE. Growth hormone stimulates the tyrosine phosphorylation and association of p125 focal adhesion kinase (FAK) with JAK2: FAK is not required for STAT-mediated transcription. J Biol Chem **273**:10682–10689, 1998.
27. Hoyo H. Establishment of cultured lines of human stomach cancer: origin and their morphological characteristics. Niigata Igakuki Zasshi **91**:737–752, 1977.
28. McCormack SA, Viar MJ, Johnson LR. Migration of IEC-6 cells: a model for mucosal healing. Am J Physiol **26**:G426–G435, 1992.
29. Sasaki E, Pai R, Halter F, Komurasaki T, Arakawa T, Kobayashi K, Kuroki T, Tarnawski AS. Induction of cyclooxygenase-2 in a rat gastric epithelial cell line by epiregulin and basic fibroblast growth factor. J Clin Gastroenterol **27**(Suppl 1):S21–S27, 1998.
30. Pai R, Jones MK, Tomikawa M, Tarnawski AS. Activation of Raf-1 during experimental gastric ulcer healing is Ras-mediated and protein kinase C-independent. Am J Pathol **155**:1759–1766, 1999.
31. Jones MK, Wang H, Peskar BM, Levin E, Itani RM, Sarfeh IJ, Tarnawski AS. Inhibition of angiogenesis by nonsteroidal anti-inflammatory drugs: insight into mechanisms and implications for cancer growth and ulcer healing. Nat Med **5**:1418–423, 1999.
32. Irwin FL, Sarfeh IJ, Tanoue K, Chaurasia OP, Tarnawski AS. The role of extracellular matrix in injury to gastric mucosa by indomethacin. J Clin Gastroenterol **21**:S18–S23, 1995.
33. Kletzien RF, Harris PK, Foellmi LA. Glucose-6-phosphate dehydrogenase: a "housekeeping" enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. FASEB J **8**:174–181, 1994.
34. De Heus RB, Diegenbach PC, Van Raamsdonk W, Roberts BL. Changes in enzyme histochemical profiles of identified spinal motoneurons of the European eel, *Anguilla anguilla*, following cordotomy. Histochem J **28**:335–340, 1996.
35. Ricci V, Ciacci C, Zarrilli R, Sommi P, Tummuru MK, Del Vecchio Blanco C, Bruni CB, Cover TL, Blaser MJ, Romano M. Effect of *Helicobacter pylori* on gastric epithelial cell migration and proliferation in vitro: role of VacA and CagA. Infect Immun **64**:2829–2833, 1996.
36. Lacy ER, Ito S. Microscopic analysis of ethanol damage to rat gastric mucosa after treatment with a prostaglandin. Gastroenterology **83**:619–625, 1982.
37. Wallace JL, McKnight GW. The mucoid cap over superficial gastric damage in the rat: a high-pH microenvironment dissipated by nonsteroidal anti-inflammatory drugs and endothelin. Gastroenterology **99**:295–304, 1990.
38. Konturek SJ. Role of growth factors in gastroduodenal protection and healing of peptic ulcers. Gastroenterol Clin North Am **19**:41–65, 1990.
39. Wallace JL, Granger DN. The cellular and molecular basis of gastric mucosal defense. FASEB J **10**:731–740, 1996.
40. Basson MD, Modlin IM, Madri JA. Human enterocyte (Caco-2) migration is modulated in vitro by extracellular matrix composition and epidermal growth factor. J Clin Invest **90**:15–23, 1992.
41. Jones MK, Tomikawa M, Mohajer B, Tarnawski AS. Gastrointestinal mucosal regeneration: role of growth factors. Front Biosci **4**:D303–D309, 1999.
42. Kato K, Chen MC, Nguyen M, Lehmann FS, Podolsky DK, Soll AH. Effects of growth factors and trefoil peptides on migration and replication in primary oxyntic cultures. Am J Physiol **276**:G1105–G1116, 1999.
43. Watanabe S, Hirose M, Wang XE, Maehiro K, Murai T, Kobayashi O, Mikami H, Otaka K, Miyazaki A, Sato N. A new model to study repair of gastric mucosa using primary cultured rabbit gastric epithelial cells. J Clin Gastroenterol **21**(Suppl 1):S40–S44, 1995.
44. Murai T, Watanabe S, Hirose M, Kobayashi O, Maehiro K, Ohkura R, Miwa H, Kitamura T, Ogihara T, Oide H, Miyazaki A, Sato N. Evaluation of ethanol on gastric epithelial restoration in vitro. Alcohol Clin Exp Res **20**:45A–46A, 1996.
45. Rijken PJ, Post SM, Hage WJ, van Bergen en Henegouwen PM, Verkleij AJ, Boonstra J. Actin polymerization localizes to the activated epidermal growth factor receptor in the plasma membrane, independent of the cytosolic free calcium transient. Exp Cell Res **218**:223–232, 1995.
46. van Bergen en Henegouwen PM, den Hartigh JC, Romeyn P, Verkleij AJ, Boonstra J. The epidermal growth factor receptor is associated with actin filaments. Exp Cell Res **199**:90–97, 1992.
47. van Delft S, Verkleij AJ, Boonstra J, van Bergen en Henegouwen PM. Epidermal growth factor induces serine phosphorylation of actin. FEBS Lett **357**:251–254, 1995.
48. Chen MC, Goliger J, Bunnett N, Soll AH. Apical and basolateral EGF receptors regulate gastric mucosal paracellular permeability. Am J Physiol **280**:G264–G272, 2001.
49. Kuwada SK, Lund KA, Li XF, Cliften P, Amsler K, Opresko LK, Wiley HS. Differential signaling and regulation of apical vs. basolateral EGFR in polarized epithelial cells. Am J Physiol **275**: C1419–C1428, 1998.
50. Polk DB. Epidermal growth factor receptor-stimulated intestinal epithelial cell migration requires phospholipase C activity. Gastroenterology **114**:493–502, 1998.
51. Tapia JA, Camello C, Jensen RT, Garcia LJ. EGF stimulates tyrosine phosphorylation of focal adhesion kinase (p125FAK) and paxillin in rat pancreatic acini by a phospholipase C-independent process that depends on phosphatidylinositol 3-kinase, the small GTP-binding protein, p21rho, and the integrity of the actin cytoskeleton. Biochim Biophys Acta **1448**:486–499, 1999.
52. Matrisian LM, Hogan BL. Growth factor-regulated proteases and extracellular matrix remodeling during mammalian development. Curr Top Dev Biol **24**:219–259, 1990.
53. Yoshida KT, Tsujino T, Yasui W, Kameda T, Sano T, Nakayama H, Toge T, Tahara E. Induction of growth receptor and metalloproteinases genes by epidermal growth factor and/or transforming growth factor-α in human gastric carcinoma cell line MKN 28. Jpn J Cancer Res **81**:793–798, 1990.
54. McCormack SA, Blanner PM, Zimmerman BJ, Ray R, Poppleton HM, Patel TB, Johnson LR. Polyamine deficiency alters EGF receptor distribution and signaling effectiveness in IEC-6 cells. Am J Physiol **274**:C192–C205, 1998.
55. Rijken PJ, Hage WJ, van Bergen en Henegouwen PM, Verkleij AJ, Boonstra J. Epidermal growth factor induces rapid reorganization of the actin microfilament system in human A431 cells. J Cell Sci **100**:491–499, 1991.
56. Romer LH, McLean N, Turner CE, Burridge K. Tyrosine kinase activity, cytoskeletal organization, and motility in human vascular endothelial cells. Mol Biol Cell **5**:349–361, 1994.
57. Withers BE, Hanks SK, Fry DW. Correlations between the expression, phosphotyrosine content and enzymatic activity of focal adhesion kinase, pp125FAK, in tumor and nontransformed cells. Cancer Biochem Biophys **15**:127–139, 1996.
58. Weiner TM, Liu ET, Craven RJ, Cance WG. Expression of focal adhesion kinase gene and invasive cancer. Lancet **342**:1024–1025, 1993.
59. Gates RE, King LE Jr, Hanks SK, Nanney LB. Potential role for focal adhesion kinase in migrating and proliferating keratinocytes near epidermal wounds and in culture. Cell Growth Differ **5**:891–899, 1994.
60. Guan JL. Role of focal adhesion kinase in integrin signaling. Int J Biochem Cell Biol **29**:1085–1096, 1997.
61. Burridge K, Petch L, Romer LH. Signals from focal adhesions. Curr Biol **2**:537–539, 1992.
62. Casamassima A, Rozengurt E. Tyrosine phosphorylation of p130(cas)

by bombesin, lysophosphatidic acid, phorbol esters, and platelet-derived growth factor: signaling pathways and formation of a p130(cas)-Crk complex. J Biol Chem **272**:9363–9370, 1997.
63. Rankin S, Rozengurt E. Platelet-derived growth factor modulation of focal adhesion kinase (p125FAK) and paxillin tyrosine phosphorylation in Swiss 3T3 cells: bell-shaped dose response and cross-talk with bombesin. J Biol Chem **269**:704–710, 1994.
64. Calalb MB, Polte TR, Hanks SK. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. Mol Cell Biol **15**:954–963, 1995.
65. Yu CF, Sanders MA, Basson MD. Human Caco-2 motility redistributes FAK and paxillin and activates p38 MAPK in a matrix-dependent manner. Am J Physiol **279**:G952–G966, 2000.
66. Basson MD. Role of integrins in enterocyte migration. Clin Exp Pharmacol Physiol **25**:280–285, 1998.
67. Hauck CR, Sieg DJ, Hsia DA, Loftus JC, Gaarde WA, Monia BP, Schlaepfer DD. Inhibition of focal adhesion kinase expression or activity disrupts epidermal growth factor-stimulated signaling promoting the migration of invasive human carcinoma cells. Cancer Res **61**:7079–7090, 2001.
68. Tanoue K, Ohta M, Tarnawski AS, Wahlstrom KJ, Sugimachi K, Sarfeh IJ. Portal hypertension activates the nitric oxide synthase genes in the esophageal mucosa of rats. Gastroenterology **110**:549–557, 1996.
69. Ohta M, Tanoue K, Tarnawski AS, Pai R, Itanin RM, Sander FC, Sugimachi K, Sarfeh IJ. Overexpressed nitric oxide synthase in portal-hypertensive stomach of rat: a key to increased susceptibility to damage? Gastroenterology **112**:1920–1930, 1997.
70. Jones G, Machado J Jr, Merlo A. Loss of focal adhesion kinase (FAK) inhibits epidermal growth factor receptor-dependent migration and induces aggregation of nh(2)-terminal FAK in the nuclei of apoptotic glioblastoma cells. Cancer Res **61**:4978–4981, 2001.
71. SeigDJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH, Schlaepfer DD. FAK integrates growth factor and integrin signals to promote cell migration. Nat Cell Biol **2**:249–256, 2000.
72. Weyant MJ, Carothers AM, Bertagnolli ME, Bertagnolli MM. Colon cancer chemopreventive drugs modulate integrin-mediated signaling pathways. Clin Cancer Res **6**:949–956, 2000.
73. Lo SH, Weisberg E, Chen LB. Tensin: a potential link between the cytoskeleton and signal transduction. Bioessays **16**:817–823, 1994.
74. Ridyard MS, Sanders EJ. Inhibition of focal adhesion kinase expression correlates with changes in the cytoskeleton but not apoptosis in primary cultures of chick embryo cells. Cell Biol Int **25**:215–226, 2001.