
**TRANSLOCATION OF MAP (ERK-1 and -2) KINASES TO CELL NUCLEI AND ACTIVATION OF c-FOS GENE DURING HEALING OF EXPERIMENTAL GASTRIC ULCERS**

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We examined localization of extracellular signal regulated kinases (Erk) 1 and 2, and c-fos mRNA expression in normal and ulcerated gastric mucosa in rats at 1, 3 and 7 days after gastric ulcer induction. In normal gastric mucosa immunofluorescence signal for Erk-1 and Erk-2 was detectable in surface epithelial, neck and some glandular cells. In gastric mucosa of the ulcer margin, almost all epithelial cells displayed strong Erk-1 and Erk-2 immunoreactivity in the basolateral membranes and the cytoplasm. In addition 19 ± 3% of cells showed nuclear localization of the Erk-1 and -2 signal. The c-fos mRNA expression was increased by 790 ± 14% and 220 ± 10%, respectively in gastric ulcer at 3 and 7 days after ulcer induction. Since in in vitro models nuclear translocation of Erk-1 and -2 triggers cell proliferation, our finding indicates relevance of this mechanism to gastric ulcer healing.

**Key words:** ulcer healing, signal transduction, protooncogenes, mitogen activated protein (MAP) kinase, Erk-1, Erk-2

**INTRODUCTION**

Chronic gastric ulcer is a deep necrotic lesion involving the entire mucosal depth and penetrating through the muscularis mucosae (1, 2). Ulcer healing is a dynamic process of filling mucosal defect with proliferating and migrating epithelial and connective tissue cells resulting in reconstruction of the mucosal architecture (3, 4). Growth factors and their receptors are known to play important roles in cell proliferation, tissue repair and ulcer healing (5-8). However, the molecular mechanisms of growth factor-triggered cell proli-
feration during ulcer healing *in vivo* and the biochemical and signaling events that regulate the ulcer healing processes have not been fully elucidated.

Exogenous epidermal growth factor (EGF) accelerates healing of gastric and duodenal ulcers in experimental models and in humans (5-8). Several studies have demonstrated that gastric ulceration triggers a dramatic overexpression of EGF receptor (EGF-R) in epithelial cells of the ulcer margin (i.e. "healing zone") and the numerous cells (ulcer-associated cell lineage) initiate synthesis of EGF, which most likely stimulates cell proliferation and migration (9-11). The cellular events and the signal transduction pathways triggered by EGF binding to its receptor, have been studied *in vitro* in various cell systems, predominantly in neoplastic or transformed cells (8, 12-14). Therefore, the relevance of these findings to *in vivo* condition is uncertain. Also, involvement of the signal transduction pathways in ulcer healing has not been extensively studied. Our recent study has shown increased expression of EGF-R in the epithelial cells of gastric ulcer margins (9), increased EGF-R levels and its phosphorylation leading to a dramatic increase in MAP (Erk-1 and Erk-2) kinase activities (by more than 440% and 880%, respectively) and their phosphorylation levels during early stages of experimental gastric ulcer healing (15). Moreover, tyrphostin A46, an inhibitor of EGF-R kinase and EGF-R kinase - dependent cell proliferation, significantly inhibited the above signaling pathways and ulcer healing (15).

Mitogen-activated protein (MAP) kinases are important mediators involved in transducing extracellular cues, such as evoked by growth factors, to the intracellular signal - regulated kinase cascade (16-19). So far, several MAP kinase isoforms (Erk1, Erk2, SAPK, p38 HOG1 kinase and Erk5) have been described in various mammalian cell types (16-19). These cells include Swiss 3T3 cells, A431 epidermal carcinoma cells, PC12 cells, 3Y1 fibroblasts and vascular smooth muscle cells (20-26). These MAP kinases respond to distinct extracellular stimuli and have different intracellular substrates. A diverse array of extracellular signals utilizes MAP kinase signaling cascades to initiate a variety of cells signaling outcomes. The targets for MAP kinases include other kinases, nuclear transcription factors and cell cycle activator proteins including the protooncogenes *fos*, *myc* and *jun* (27-32), which ultimately relay the information to the nucleus. The localization of MAP kinases in normal and ulcerated gastric mucosa remains unexplored.

Cellular proto-oncogenes such as *c-fos* and *c-myc* may play important roles in controlling gastric mucosal regeneration following injury. In situ hybridization and immunohistochemical studies have shown the sequential increase in expression of the *c-myc* and the *c-Ha-ras* genes during gastric mucosal regeneration following indomethacin injury in rats (33). Wang et al has shown increased expression of *c-fos* and *c-myc* in polyamine stimulated healing of stress ulcers in rats (34). However, the expression of *c-fos* during healing of
chronic gastric ulcer remains unknown. This study was aimed to determine whether healing of experimental gastric ulcer involves translocation of Erk-1 and -2 MAP kinases to the nuclei and activation of c-fos gene.

MATERIAL AND METHODS

This study was approved by the Institutional Subcommittee for Animal Studies. Forty eight male Sprague-Dawley rats (weighing 225-250 g) were used in the experiments. Rats were kept individually in wire bottom cages with free access to a standard rat chow (Rodent Diet No, 8594; Harlan Teklad, Madison, WI) and water. The animal room was illuminated on 12 hours light-dark cycle. Room temperature was kept at 18-22°C and humidity at 60-70%.

Rats fasted for 12 hrs underwent laparotomy under Nembutal anesthesia (60 mg/kg body weight). One hundred percent acetic acid (50 μl) was applied to the serosa of lower corpus at the posterior wall through a polyethylene tube (4.0 mm i.d.) for 90 seconds. The serosal area was then washed with isotonic saline and the abdomen closed. Sham operated rats underwent similar procedures without acetic acid administration. Rats with gastric ulcers and sham operated rats were killed 1, 3 and 7 days after operation.

Immunostaining for ERK1 and ERK2:

Coded cryostat sections (10 μm thick; Jung CRYOCUT 1800, Leica Inc., IL, USA) were fixed in cold acetone and preincubated with superblock (Sigma Chemicals Co; St. Louis, Missouri, USA). Then, they were incubated in a humidified chamber for 1 hr with either polyclonal ERK1 or monoclonal ERK2 antibody diluted 1:100 (Santa Cruz Biotech; Santa Cruz, CA). The sections were washed and then incubated in a humidified chamber with fluorescein-conjugated secondary antibody diluted 1:200 for 30 min. Immunofluorescence was evaluated qualitatively and quantitatively using a Nikon Optiphot epifluorescence microscope with B filter composition and an Image 1, videoimage analysis system similarly as previously described (9).

Northern blot analysis for c-fos mRNA:

Frozen tissues were homogenized with a Polytron homogenizer in 4 mol/L guanidium isothiocyanate and total RNA was prepared with the phenol-chloroform procedure (35). The amount of RNA was quantified by measuring the absorbance at 260 nm using a spectrophotometer. Twenty-five micrograms of total RNA were loaded into individual wells of a 1.2% agarose gel containing formaldehyde and electrophoresis was performed. The RNA from the gel was transferred onto MSI nylon membrane using a capillary trans-blotting technique. The nylon membrane was UV-cross linked using a UV cross linker (Fisher Scientific, Pittsburgh, PA). The cDNA probes for c-fos and GAPDH (ATCC, Rockville, MD) were used for hybridization after [α-32P]dCTP-labeling by random oligonucleotide priming. The membranes were washed three times for 30 min: first 2X SSC with 0.1% SDS at room temperature, second in 0.1 X SSC with 0.1% SDS at room temperature and third with 0.1X SSC with 0.1% SDS at 58°C.

Autoradiography was performed by exposing the blots to Kodak X-ray film with intensifying screens at -70°C. The blots were then re-hybridized with 32P-labeled GAPDH cDNA probe as an internal control to assess RNA quantity and integrity. Quantification of mRNA signals was performed by densitometric scanning of autoradiographs and normalization with GAPDH mRNA signals.
RESULTS

Immunostaining for ERK1 and ERK2

In normal gastric oxyntic mucosa, immunoreactivity for Erk1 and Erk2 was detectable in the surface epithelial, neck and in some glandular cells (Fig. 1A). Immunofluorescence signal was localized predominantly to the

![Photomicrographs illustrating immunofluorescence staining of ERK1. A) In normal gastric mucosa, green fluorescence signal is localized to the surface epithelial and neck cells, to their basolateral membranes and cytoplasm and to some parietal and chief cells (200X). B and C) Mucosa of the ulcer margin 7 days after ulcer induction. Almost all the epithelial cells lining mucosa of ulcer margin and regenerating glands show strong fluorescence signal localized to the cytoplasm, basolateral and luminal membranes and to some nuclei (B-200X; c-400X). C) Higher magnification of the regenerating gland in the mucosa of the ulcer margin. Strong fluorescence signal of ERK1 is localized to basolateral and luminal membranes, cytoplasm and nuclei of some cells (arrows) 400X. D) Control staining of the gastric mucosa of the ulcer margin performed identically as A-C, except that instead of primary ERK1 antibody, a nonimmune serum was used. Immunofluorescence signal for ERK1 antigen is absent (200X). Immunofluorescence staining for ERK2 showed a very similar pattern of distribution to that of ERK1.](image-url)
Table 1. The percent of mucosal section (area) expressing Erk-1 immunoreactivity and the percentage of cells demonstrating nuclear translocation of Erk-1. The data are expressed as mean ±SE% of total mucosal section area.

<table>
<thead>
<tr>
<th>Ulcer</th>
<th>Expression of Erk-1 signal (%)</th>
<th>Nuclear translocation (%)</th>
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<tbody>
<tr>
<td>1 day</td>
<td>56 ± 4%*</td>
<td>8 ± 1*</td>
</tr>
<tr>
<td>3 days</td>
<td>91 ± 8*</td>
<td>22 ± 2*</td>
</tr>
<tr>
<td>7 days</td>
<td>96 ± 6*</td>
<td>19 ± 3*</td>
</tr>
<tr>
<td>Normal mucosa</td>
<td>31 ± 2</td>
<td>1 ± 0.2</td>
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* p < 0.001 vs. normal mucosa

basolateral membranes and the cytoplasm. In gastric mucosa of the ulcer margin, almost all epithelial cells lining ulcer margin displayed Erk1 and Erk2 immunoreactivity (Fig. 1B) at both 3 and 7 days after ulcer induction. Immunoreactivity was localized to basolateral membranes and the cytoplasm, but in addition, numerous cells 19 ± 3 showed nuclear localization (Fig. 1C). Mucosal sections incubated with PBS or normal serum (instead of Erk1/2 antibody) did not give any staining reaction (Fig. 1D). Also, at 14 days, cells lining dilated gland of the ulcer scar demonstrated strong signal for Erk1 and Erk2 immunoreactivity (Fig. 2).

Fig. 2. Photomicrograph illustrating immunofluorescence staining Erk-1 in the mucosal scar 14 days after ulcer induction. Strong signal is still present in epithelial cells lining dilated glands.
Northern blot analysis for c-fos mRNA

We studied steady-state mRNA expression levels of c-fos in gastric specimens during the healing of chronic gastric ulcers (Fig. 3). Quantitative analysis by densitometric scanning of Northern blot autoradiographs and normalization with GAPDH signals showed significant increase in the c-fos mRNA transcripts of the predicted size 2.2 kb, after 3 and 7 days, but not at 1 day following ulcer induction. The c-fos mRNA expression increased by $790\% \pm 14\%$ ($p<0.03$) at 3 days and $200\% \pm 10\%$ at 7 days ($p<0.0003$), when compared with their corresponding controls.

![Diagram](image)

**Fig. 3.** Time course changes in c-fos mRNA expression in normal mucosa of sham operated rats (controls) and in gastric ulcers. A) Autoradiograph of the representative Northern blot showing c-fos mRNA expression in the sham operated controls (lane 1) and in the ulcerated gastric mucosa 1 day (lane 2), 3 days (lane 3) and 7 days (lane 4) after ulcer induction. B) Relative density of c-fos/GAPDH mRNA ratio demonstrate significant overexpression of c-fos mRNA at 3 and 7 days ulcer vs. control.

DISCUSSION

The present study demonstrated that gastric ulceration triggers overexpression of Erk-1 and -2, their nuclear translocation and enhancement of c-fos mRNA expression in the mucosa of the ulcer margin. Immunoreac-
tivity of Erk1 and -2 was expressed predominantly in epithelial cells of the ulcer margin, where virtually all cells demonstrated a strong fluorescence signal and numerous cells displayed translocation of the signal to the nucleus. Several studies have shown that during ulcer healing, a healing zone is formed in the mucosa at the ulcer margin (4, 36). Gastric glands in this area become dilated and are lined with poorly differentiated cells that vigorously proliferate (37). Increased cell proliferation results in an increased supply of cells utilized for re-epithelialization and reconstruction of mucosal structures. The regulatory mechanisms of the ulceration — triggered mucosal proliferation and the biochemical events that regulate this process remain unexplored. A number of growth factors including EGF, TGF-α and basic fibroblast growth factor have been shown to participate in ulcer healing. Exogenous EGF has been shown to stimulate DNA synthesis in the gastroduodenal mucosa of rats and to accelerate ulcer healing (37). Immuno-histochemical studies have shown that epithelial cells in the healing zone have increased EGF and EGF-R expression (9-11), indicating a major target for the proliferation-stimulating action of EGF.

In response to extracellular stimuli, such as growth factors, MAP kinase (including Erk1 and Erk2) signaling pathway is activated and it regulates the transcriptional activity of several transcription factors via phosphorylation of either stimulatory or inhibitory regulatory sites, thereby initiating the expression of a variety of immediate early and delayed response genes (21-23, 27). This regulation of gene expression and the phosphorylation and regulation of cytosolic, as well as, nuclear targets by MAP kinase are critical for cell signaling outcome (27). Several studies have shown that expression of proto-oncogenes is enhanced during regeneration of damaged tissues (38, 39) or after wounding of cultured cell monolayers (40). The c-fos is implicated early in the process of cell proliferation and differentiation (41, 42). The oncogene products fos has a well defined function as a transcription factor in a variety of cell types (43). Yoshiura and co-workers have shown that EGF triggers increase in c-fos expression in rabbit gastric epithelial cells (44). In the present study we demonstrated a significant increase in c-fos mRNA expression in ulcerated gastric mucosa that peaked at 3 days and sustained high levels after 7 days, in parallel to Erk1 and Erk2 activity increase found in our previous study (15). This clearly indicates that during ulcer healing, c-fos expression is likely stimulated by translocation of Erk-1 and Erk-2 to cell nuclei.

In summary, this study demonstrates that gastric ulceration triggers translocation of Erk1 and Erk2 to the nuclei of epithelial cells of the ulcer margin and increased c-fos mRNA expression.
REFERENCES


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