Selective Cyclooxygenase-2 Blocker Delays Healing of Esophageal Ulcers in Rats and Inhibits Ulceration-Triggered c-Met/Hepatocyte Growth Factor Receptor Induction and Extracellular Signal-Regulated Kinase 2 Activation

Dolgor Baatar,^{*†} Michael K. Jones,^{†‡} Rama Pai,^{†‡} Hirofumi Kawanaka,^{†‡} Imre L. Szabo,^{†‡} Woo S. Moon,^{†‡} Seigo Kitano,^{*} and Andrzej S. Tarnawski^{†‡}

From the Department of Veterans Affairs Medical Center,[†] Long Beach, California; the University of California,[‡] Irvine, California; and the Department of Surgery I,^{*} Oita Medical University, Oita, Japan

Nonsteroidal anti-inflammatory drugs, both nonselective and cyclooxygenase-2 (COX-2) selective, delay gastric ulcer healing. Whether they affect esophageal ulcer healing remains unexplored. We studied the effects of the COX-2 selective inhibitor, celecoxib, on esophageal ulcer healing as well as on the cellular and molecular events involved in the healing process. Esophageal ulcers were induced in rats by focal application of acetic acid. Rats with esophageal ulcers were treated intragastrically with either celecoxib (10 mg/kg, once daily) or vehicle for 2 or 4 days. Esophageal ulceration triggered increases in: esophageal epithelial cell proliferation; expression of COX-2 (but not COX-1); hepatocyte growth factor (HGF) and its receptor, c-Met; and activation of extracellular signal-regulated kinase 2 (ERK2). Treatment with celecoxib significantly delayed esophageal ulcer healing and suppressed ulceration-triggered increases in esophageal epithelial cell proliferation, c-Met mRNA and protein expression, and ERK2 activity. In an *ex vivo* organ-culture system, exogenous HGF significantly increased ERK2 phosphorylation levels in esophageal mucosa. A structural analog of celecoxib, SC-236, completely prevented this effect. These findings indicate that celecoxib delays esophageal ulcer healing by reducing ulceration-induced esophageal epithelial cell proliferation. These actions are associated with, and likely mediated by, down-regulation of the HGF/c-Met-ERK2 signaling pathway. (*Am J Pathol* 2002, 160:963–972)

Nonsteroidal anti-inflammatory drugs (NSAIDs) delay healing of experimental gastric ulcers by arresting epithelial proliferation in the ulcer margin, interfering with re-epithelialization, and inhibiting angiogenesis in the granulation tissue.^{1–4} Although cellular and molecular mechanisms of gastric ulcer healing have been extensively studied and well characterized,^{5–9} the mechanisms involved in the healing of esophageal ulcers remain virtually unexplored. Our preliminary study demonstrated that, similar to gastric ulcers, epithelial cell proliferation is increased at the esophageal ulcer margin.¹⁰ Whether NSAIDs affect esophageal ulcer healing, epithelial cell proliferation, and the molecular mechanisms involved in the healing process remain unknown.

NSAIDs inhibit cyclooxygenase (COX), a rate-limiting enzyme in prostaglandin synthesis.¹¹ Two isoforms of this enzyme are known: COX-1, constitutively expressed in most tissues, is responsible for the physiological production of prostaglandins; and COX-2, induced by cytokines, mitogens, and endotoxins in inflammatory cells, is responsible for the increased production of prostaglandins during inflammation.¹² Early studies examining the effect of NSAIDs on experimental gastric ulcer healing^{1–3} have been conducted with the use of nonselective NSAIDs (eg, indomethacin) that inhibit both COX-1 and COX-2 enzymes.¹³ However, more recent studies indicate that experimental gastric ulceration induces COX-2, but not COX-1, expression^{14,15} and that COX-2 selective NSAIDs delay gastric ulcer healing similarly to nonselective NSAIDs.¹⁶ The expression of COX in ulcerated esopha-

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Address reprint requests to Andrzej S. Tarnawski, M.D., D.Sc., Gastroenterology Section (111G) DVA Medical Center, 5901 E. Seventh St., Long Beach, CA 90822. E-mail: andrzej.tarnawski@med.va.gov.

geal mucosa has not been studied and it is not known whether the COX-2 selective inhibitors affect esophageal ulcer healing.

Various growth factors, including epidermal growth factor and hepatocyte growth factor (HGF), have been implicated in the stimulation of epithelial proliferation during gastric ulcer healing.^{9,17,18} Suppression of HGF production has been suggested as a key factor involved in the inhibitory action of NSAIDs on gastric ulcer healing.^{19,20} However, the role of endogenous HGF in the healing of esophageal ulcers remains unexplored. In regard to the esophagus, previous studies demonstrated that exogenous HGF is the most potent stimulator of proliferation and restitution of esophageal epithelial cells *in vitro*, suggesting that it might be involved in the repair process of esophageal mucosal damage.^{21,22}

In previous studies we have demonstrated that the extracellular signal-regulated kinases (ERKs) and their upstream kinase, Raf-1, which mediate the mitogenic effects of growth factors, are activated during gastric ulcer healing⁶⁻⁸ and that interruption of this signaling pathway dramatically delays the healing process.⁶ However, the signaling pathways involved in esophageal ulcer healing, and stimulation of esophageal epithelial cell proliferation essential for the healing, remain unknown.

The present study was aimed to: 1) explore molecular events associated with esophageal ulcer healing: expression of COX-1, COX-2, HGF and its receptor c-Met, and ERK2 phosphorylation levels and activity; and, 2) determine whether the selective COX-2 inhibitor, celecoxib, affects esophageal ulcer healing, ulceration-triggered cell proliferation and the above stated molecular events.

Materials and Methods

The RNeasy Mini Kit was purchased from Qiagen (Valencia, CA), the bicinchoninic acid protein assay kit was purchased from Pierce Chemical (Rockford, IL), and the enhanced chemiluminescence Western blotting detection reagents were purchased from Amersham Life Science (Arlington Heights, IL). Monoclonal mouse anti-COX-1 and polyclonal rabbit anti-COX-2 antibodies were purchased from Cayman Chemical Co. (Ann Arbor, MI), polyclonal rabbit anti-HGF- α , anti-c-Met p140, anti-ERK2 antibodies and monoclonal mouse anti-pERK antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) antibody and 3,3'-diaminobenzidine tetrahydrochloride were purchased from DAKO (Carpinteria, CA). [γ -³²P]ATP was purchased from Dupont NEN Research Products (Boston, MA). Keratinocyte basal media was purchased from Clonetics (San Diego, CA). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Induction of Esophageal Ulcers

This study was approved by the Subcommittee for Animal Studies of the Long Beach Department of Veterans Af-

fairs Medical Center. Male Sprague-Dawley rats (weighing 225 to 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.8504; Harlan Teklad, Madison, WI) and water. The animal room was illuminated on 12-hour light-dark cycles. Room temperature was kept at 18 to 22°C and humidity at 60 to 70%. Rats fasted for 12 hours underwent laparotomy under ketamine-xylazine anesthesia (40 mg/kg body wt of ketamine and 5 mg/kg body wt of xylazine, intraperitoneally). Esophageal ulcers were induced by modification of the method described by Tsuji and colleagues.²³ In brief, the stomach was pulled down and the intra-abdominal esophagus was exposed. The length of esophagus, brought below the diaphragm by this procedure, was ~1 cm providing sufficient space for application of acetic acid. After careful separation of vessels and nerves, 100% acetic acid (30 μ l) was applied through a polyethylene tube (3-mm inner diameter) to the anterior wall of intra-abdominal esophagus for 3 minutes. The area was then washed with isotonic saline and the abdomen was closed. The rats undergoing the operation as described above but without application of acetic acid (sham operation) served as controls. All rats survived the scheduled study design.

Study Design

To evaluate the effects of ulceration on the studied parameters, rats with esophageal ulcers and sham-operated (SO) rats were euthanized 3 or 7 days after ulcer induction. To evaluate the effects of celecoxib on ulcer healing, rats with esophageal ulcers were treated once daily with either 10 mg/kg of celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide; Pfizer Inc., New York, NY) or its vehicle (1 ml of 1% carboxymethyl cellulose sodium salt) starting 3 days after ulcer induction. Celecoxib was given at 10 mg/kg because this dose has been shown to significantly inhibit the COX-2-induced generation of prostaglandin E₂ in the rat air pouch without affecting whole-blood thromboxane synthesis, an index of COX-1 activity.²⁴ Treatments were given intragastrically for 2 or 4 days, and rats were euthanized 5 and 7 days after ulcer induction. The starting of treatment (day 3) and ending (day 7) time points were chosen because our previous preliminary studies demonstrated that esophageal ulcers are fully developed by day 3 after application of acetic acid and that ~60% of ulcers are completely re-epithelialized by day 9.¹⁰ A one-cm long segment of the lower esophagus (including ulcer if present) was excised, opened longitudinally, and photographed. The ulcer area was measured using a computerized video-image analysis system (Image 1/FL; Universal Imaging Corp., Westchester, PA). Esophageal tissue samples including ulcer and immediately adjacent tissue (ulcerated tissue) or esophageal tissue from SO rats (normal tissue) were snap-frozen in liquid nitrogen, and stored at -80°C for RNA isolation and protein extraction or fixed in 10% formalin. In addition, a 1-cm long segment of the esophagus distal to the resected segment was

excised and fixed in formalin for immunohistochemical staining.

Determination of COX-1, COX-2, and c-Met mRNAs by RT-PCR

RNA was isolated using the RNeasy Mini Kit according to the manufacturer's instructions. Reverse transcription (RT) and polymerase chain reaction (PCR) were performed to determine mRNA levels. RT was performed using a GeneAmp RNA PCR kit and a DNA thermal cycler (Perkin Elmer, Norwalk, CT), which were also used for PCR. Total RNA (0.3 μ g) was used as the template to synthesize complementary DNA (cDNA) with 2.5 U of Moloney murine leukemia virus reverse transcriptase in 10 μ l of buffer containing 10 mmol of Tris-HCl, pH 8.3, 50 mmol KCl, 5 mmol random hexamer, 1.4 U of ribonuclease inhibitor. RT was performed at 42°C for 15 minutes. The resulting cDNA was used as a template for subsequent PCR. The specific primer set for rat COX-1 was 5'-ACGCCCTCATTACCCATTT-3' (sense) and 5'-CACGGACGCCTGTTCTACGG-3' (anti-sense) and for COX-2 was 5'-TGGTGCCGGGTCTGATGATG-3' (sense) and 5'-GCAATGCGGTTCTGATACTG-3' (anti-sense), and the sizes of amplified fragments were 561 bp for COX-1 and 253 bp for COX-2, respectively.²⁵ The primers for rat c-Met were 5'-GCGAACTAATTCAGTCCCA-3' (sense) and 5'-GCATCTGTGTTGTGTACGGT-3' (antisense), and the size of the amplified fragment was 242 bp.²⁶ The PCR for β -actin was used as a positive control and an internal standard. The specific primer set for rat β -actin was purchased from Clontech Laboratories, Inc., Palo Alto, CA. The PCR was performed in 50 μ l of buffer containing 10 mmol Tris-HCl, pH 8.3, 2 mmol $MgCl_2$, 50 mmol KCl, 0.2 mmol each of deoxyribonucleoside triphosphates, 0.4 μ mol of each primer, 2 U of *Taq* DNA polymerase. For the amplification of COX-1, COX-2, and rat c-Met cDNAs, 35 cycles of 1 minute at 94°C for denaturing, 1 minute at 55°C for annealing, and 2 minutes at 72°C for extension were performed. Nine- μ l aliquots of the PCR products were subjected to electrophoresis on a 1.25% agarose gel, and the DNA was visualized by ethidium bromide staining. Location of the products and their sizes were determined using a 100-bp ladder (Life Technologies, Inc., Gaithersburg, MD). The gel was then photographed under UV transillumination. For the quantitative assessment of the PCR products, a computerized video analysis system (Image-1/FL, Universal Imaging Corp.) was used. The results are expressed as target cDNA/ β -actin ratio.

Protein Extraction

Esophageal tissues were homogenized with a Polytron homogenizer (Kinematica, Littau, Switzerland) in a lysis buffer containing 62.5 mmol ethylenediaminetetraacetic acid, 50 mmol Tris, pH 8.0, 0.4% deoxycholic acid, 1% Nonidet P-40, 0.5 mg/ml leupeptin, 0.5 mg/ml pepstatin, 0.5 mg/ml aprotinin, 0.2 mmol phenylmethylsulfonyl fluoride, and 0.05 mmol aminoethyl benzene sulfonyl fluo-

ride. The homogenates were then centrifuged at 14,000 rpm for 10 minutes at 4°C. The protein concentration of the supernatant was determined by the bicinchoninic acid protein assay kit.

Determination of COX-1, COX-2, HGF, and c-Met Protein Levels by Western Blotting

Equal amounts of protein (0.15 mg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with specific antibodies at room temperature for 1 hour. The membranes were washed and incubated with corresponding IgG peroxidase conjugates at room temperature for 1 hour. The signal of the bound antibody was visualized using enhanced chemiluminescence Western blotting detection reagents. Protein expression was measured using a computerized video analysis system (Image-1/FL, Universal Imaging Corp.). COX-1 and COX-2 protein levels were determined using monoclonal mouse anti-COX-1 antibody and polyclonal rabbit anti-COX-2 antibody (Cayman Chemical Co.) diluted 1:1000. For the determination of HGF and c-Met protein expression, polyclonal rabbit anti-HGF- α antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and polyclonal rabbit anti-c-Met p140 antibody (Santa Cruz Biotechnology) were used at 1:250 dilution.

Determination of ERK2 Activity and Phosphorylation Levels

ERK2 activity was determined as described previously.⁶ Briefly, 50 μ g of protein from tissue lysates was added to a conjugate of protein A Sepharose and 1 μ g of polyclonal rabbit anti-ERK2 antibody and mixed at 4°C for 2 hours. The conjugates were then pelleted by centrifugation and washed four times. After the final wash, buffer was removed completely and 40 μ l of protein kinase assay mixture (10 mmol/L HEPES, pH 7.5, 10 mmol/L $MgCl_2$, 50 μ mol/L ATP, 30 μ g myelin basic protein, and 4 μ Ci [γ -³²P]ATP) were added to each sample. The samples were incubated at 30°C for 20 minutes and the reaction was terminated by the addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. The samples were then electrophoresed on 15% acrylamide gels. After electrophoresis, the gels were stained with Coomassie brilliant blue and dried. The gels were autoradiographed; the myelin basic protein bands were cut out and the radioactivity was counted in a scintillation counter. The activity was expressed as pmol of [γ -³²P]ATP incorporated into 1 mg of MBP protein (pmol/mg). ERK2 and phosphorylated ERK (pERK) protein levels were determined by Western blot analysis using polyclonal rabbit anti-ERK2 antibody and monoclonal mouse anti-pERK antibody. The ERK2 phosphorylation levels were expressed as a percentage of total ERK2 protein levels.

Localization of COX-2 and c-Met Protein Expression by Immunohistochemical Staining

Immunohistochemical staining with specific antibodies was performed to determine localization of COX-2 and

c-Met expression in normal and ulcerated esophageal sections. Deparaffinized sections were incubated overnight at 4°C with polyclonal rabbit anti-COX-2 antibody diluted 1:300 in phosphate-buffered saline (PBS) or polyclonal rabbit anti-c-Met antibody diluted 1:50 in PBS. After washing, sections were then incubated for 30 minutes with rabbit fluorescein-conjugated IgG diluted 1:100 in PBS. Immunofluorescence signal was evaluated under a Nikon Optiphot epifluorescence microscope with B-filter composition (Nikon, Garden City, NY).

Evaluation of Esophageal Epithelial Cell Proliferation

Expression of PCNA in formalin-fixed paraffin-embedded esophageal tissue sections was determined by the enhanced polymer one-step staining method.²⁷ Deparaffinized sections were incubated with monoclonal mouse anti-PCNA antibody for 1 hour at room temperature. The color was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAKO) and the sections were counterstained with Mayer's hematoxylin. Coded specimens were evaluated quantitatively under $\times 400$ microscopic magnification by two investigators unaware of the code. Cell nuclei that stained dark brown were considered as labeled. Labeled cells were counted in the epithelium above the edge of interrupted muscularis mucosa at each ulcer margin corresponding to 700 μm of mucosal section length, in the epithelium distant from the ulcer, and in normal esophageal epithelium of SO rats. The length of the basement membrane was measured on the photographed images of corresponding hematoxylin and eosin (H&E) sections using a computerized video analysis system (Image-1/FL, Universal Imaging Corp.) and the number of PCNA-labeled cells per 100- μm length of the basement membrane was calculated. The results are expressed as percentage of increase in the number of labeled cells in the epithelium of the ulcer margin over the number of labeled cells in the epithelium distant from the ulcer. Sections from six rats per group were evaluated and the mean $\pm$ SD was calculated.

Assessment of Angiogenesis in Granulation Tissue at the Ulcer Bed

To assess angiogenesis, enhanced polymer one-step immunostaining with monoclonal mouse anti-factor VIII-related antigen antibody (DAKO) that visualizes endothelial cells of vessels was used. Five days after ulcer induction, only a few endothelial cells forming microvessels were present in granulation tissue at the ulcer bed. Therefore, microvessels were counted only in sections obtained 7 days after ulcer induction. Coded specimens were evaluated quantitatively under $\times 200$ microscopic magnification by two investigators unaware of the code. Microvessels with distinct lumen were counted in granulation tissue below the regenerating epithelium of the ulcer margin at each side. The results are expressed as a number of microvessels (mean $\pm$ SD) per $\times 200$ micro-

scopic field. Sections from six rats per group were evaluated.

Effects of Exogenous HGF on Esophageal Mucosal ERK2 Phosphorylation Levels

Rats ($n = 18$) were euthanized and a 1-cm long segment of the lower esophagus was excised and opened longitudinally. The esophageal mucosa was stripped from its muscle layers by sharp dissection as described previously.²⁸ Explants consisted of squamous epithelium, lamina propria, and muscularis mucosa as determined by H&E staining. Esophageal mucosal explants were incubated in serum-free keratinocyte basal medium at 37°C with 5% CO₂ and 95% air in a humidified incubator in the presence of 10 $\mu\text{mol/L}$ or 100 $\mu\text{mol/L}$ of SC-236 (Searle, Skokie, IL), a structural analog of celecoxib,²⁹ or its vehicle (dimethyl sulfoxide) for 6 hours. SC-236 was used because of its better solubility compared to celecoxib. Then explants were treated with either human recombinant HGF (100 ng/ml) or its vehicle (PBS) and were incubated for an additional 30 minutes. Protein extraction and Western blotting for pERK2 and ERK2 were performed as described above.

Statistical Analysis

Values are expressed as the mean $\pm$ SD. Student's *t*-test was used to determine the statistical significance of the differences. One-way analysis of variance followed by Bonferroni correction was used for multiple comparisons. A *P* value of <0.05 was considered statistically significant.

Results

Esophageal Ulceration Triggers Increases in COX-2 (but not COX-1) mRNA and Protein Expression

Three days after ulcer induction, COX-2 mRNA and protein levels in ulcerated esophageal tissue were increased ≈ 2.5 -fold and $\approx$ threefold, respectively, versus normal esophageal tissue (Figures 1 and 2). COX-2 protein levels in ulcerated esophageal tissue were decreased at 7 days versus 3 days after ulcer induction, but remained elevated versus normal tissue. COX-1 mRNA and protein levels were not significantly affected by esophageal ulceration (Figures 1 and 2).

COX-2 and c-Met Protein Localization by Immunohistochemical Staining

In normal esophageal epithelium, faint COX-2 signal was detected predominantly in the cells of the basal zone (Figure 3A). Three days after ulcer induction, a strong COX-2 fluorescence signal was present in all epithelial cells constituting the ulcer margin (Figure 3B). In the

Figure 1. COX-1 and COX-2 mRNA expression in normal esophageal tissue from sham-operated (SO) rats and ulcerated esophageal tissue (UL) 3 days after sham operation or ulcer induction. **A:** RT-PCR products obtained with use of specific primers for COX-1 (561 bp) and COX-2 (253 bp) and β -actin. **B:** Quantitative data for COX-1 and COX-2 mRNA expression. Data were obtained by computerized analysis of the optical intensity of amplified PCR products; each signal was normalized against corresponding β -actin signal and the results are expressed as COX-1/ β -actin and COX-2/ β -actin ratio. NS, not significant. Values are means $\pm$ SD. For each column, $n = 6$.

normal esophageal epithelium of sham-operated rats, c-Met signal was predominantly localized to the membranes of cells in basal and stratified zones (Figure 4A). Three days after ulcer induction, c-Met signal was

Figure 2. COX-1 and COX-2 protein expression in normal esophageal tissue from sham-operated (SO) rats and ulcerated esophageal tissue (UL) 3 and 7 days after sham operation or ulcer induction. **A:** Immunoblotting with specific antibodies detected the specific 70-kd band for COX-1 and the specific 72-kd band for COX-2. **B:** Quantitative data for COX-1 and COX-2 protein expression. Data were obtained by a computerized video analysis of the Western blots. NS, not significant; *, $P < 0.05$ versus UL at 3 days. Values are expressed as optical intensity units and represent means $\pm$ SD. For each column, $n = 6$.

present in all epithelial cells constituting ulcer margin; cells in the stratified zone displayed strong membrane and cytoplasmic staining, whereas, basal cells showed mainly diffuse cytoplasmic staining (Figure 4B).

Esophageal Ulcer Healing Dynamics: Effects of Celecoxib

The mean $\pm$ SD area of the ulcers 3 days after ulcer induction was 7.1 ± 1.5 mm². Five days after ulcer induction, the mean ulcer area was slightly but significantly decreased (*versus* 3 days) in vehicle-treated but not in celecoxib-treated rats (Figure 5). Seven days after ulcer induction, the mean ulcer area was significantly decreased (*versus* 3 days) in both vehicle-treated and celecoxib-treated rats. However, the mean ulcer area in celecoxib-treated rats was nearly twofold larger than in vehicle-treated rats, demonstrating that celecoxib treatment significantly delays esophageal ulcer healing.

Esophageal Epithelial Cell Proliferation: Effects of Celecoxib

Seven days after ulcer induction, in the esophageal epithelium distant from the ulcer, PCNA staining was present only in cells of the basal zone (Figure 6, A and B). Epithelium of the ulcer margin in rats treated with vehicle showed a dramatic increase in the number of cells expressing PCNA (Figure 6C). This increase was significantly diminished in rats treated with celecoxib (Figure 6D). The quantitative data are shown in Figure 7. The number of PCNA-labeled epithelial cells in normal esophageal mucosa (mucosa of SO rats) was not significantly different between the lower (corresponding to the ulcer site in rats with ulcers) and upper (corresponding to the area distant from the ulcer) esophageal segments (18.5 ± 1.1 versus 17.9 ± 2.2 , respectively; $P = 0.501$). Three days after ulcer induction, the number of PCNA-labeled epithelial cells in the mucosa distant from the ulcer (17.2 ± 2.1) was not significantly ($P = 0.55$) different from that in the mucosa of the upper esophageal segment of SO rats. Three days after ulcer induction, the number of PCNA-labeled epithelial cells at the ulcer margin was increased to 180% over that in the epithelium distant from the ulcer. In vehicle-treated rats, this increase reached 390% at 7 days after ulcer induction (Figure 7). In rats treated with celecoxib, the number of PCNA-labeled epithelial cells in the epithelium of the ulcer margin was also increased (*versus* epithelium distant from the ulcer), however, this increase was significantly reduced (*versus* vehicle-treated rats) both 5 and 7 days after ulcer induction (Figure 7).

Angiogenesis in Granulation Tissue at the Ulcer Bed: Effects of Celecoxib

Seven days after ulcer induction, the number of microvessels in granulation tissue at the ulcer bed was slightly, but not significantly, lower in celecoxib-treated

Figure 3. Photomicrographs of the esophageal epithelium showing immunofluorescence staining for COX-2 (3 days after sham operation or ulcer induction). **A:** Epithelium of sham-operated rats, a faint COX-2 signal is detected in the cells of the basal zone. **B:** Epithelium of the ulcer margin, a strong COX-2 signal is present in the cytoplasm of almost all cells constituting ulcer margin. BZ, basal zone; SZ, stratified zone. Scale bars, 50 μ m.

rats *versus* vehicle-treated rats (21.7 ± 1.7 *versus* 23.7 ± 3.0 , respectively; $P = 0.18$).

Esophageal Ulceration Induces c-Met mRNA and Protein Expression: Effects of Celecoxib

Three days after ulcer induction, c-Met mRNA levels in ulcerated tissue were increased twofold *versus* normal esophageal tissue (Figure 8). In vehicle-treated rats, c-Met mRNA levels in ulcerated tissue remained high at 5 days and were decreased at 7 days *versus* 3 days after ulcer induction (Figure 8). Celecoxib significantly reduced (*versus* vehicle) c-Met mRNA expression in ulcerated tissue both 5 and 7 days after ulcer induction (Figure 8). Western blot analysis demonstrated that HGF protein levels in ulcerated tissue were significantly increased *versus* normal esophageal tissue 3 days after ulcer induction (Figure 9). In vehicle-treated rats, HGF protein levels remained high 5 and 7 days after ulcer induction (Figure 9). Celecoxib (*versus* vehicle) did not significantly affect the ulceration-induced increase in HGF protein levels at either time point. In vehicle-treated rats, c-Met protein

levels in ulcerated tissue were significantly increased at both 5 and 7 days *versus* 3 days. Celecoxib treatment (*versus* vehicle treatment) significantly reduced c-Met protein levels in ulcerated tissue both 5 and 7 days after ulcer induction.

Esophageal Ulceration Induces ERK2 Phosphorylation and Activity: Effects of Celecoxib

Three days after ulcer induction, ERK2 phosphorylation levels and ERK2 activity were increased more than fourfold and fivefold, respectively, in ulcerated tissue *versus* normal esophageal tissue (Figure 10). In vehicle-treated rats, ERK2 phosphorylation levels and ERK2 activity remained high at both 5 and 7 days after ulcer induction. In celecoxib-treated rats, ERK2 phosphorylation levels and ERK2 activity in ulcerated tissue were significantly reduced *versus* vehicle-treated rats at both 5 and 7 days. ERK2 total protein levels were not significantly different between any of the groups studied, demonstrating that

Figure 4. Photomicrographs of esophageal epithelium showing immunofluorescence staining for c-Met (3 days after sham operation or ulcer induction). **A:** In the normal esophageal epithelium of sham-operated rats, c-Met staining is present mainly in membranes of cells in basal and stratified zones (green fluorescence signal). **B:** In the epithelium of the ulcer margin, cells in the stratified zone display strong membrane and cytoplasmic staining, whereas, basal cells show mainly diffuse cytoplasmic staining. BZ, basal zone; SZ, stratified zone. Scale bars, 50 μ m.

Figure 5. Effects of celecoxib on esophageal ulcer healing. Ulcer area was measured 3, 5, and 7 days after ulcer induction. Starting from 3 days after ulcer induction, rats were treated with either celecoxib (CEL) (10 mg/kg, once a day) or its vehicle (VHC) for 2 or 4 days. Ulcer area was measured by a computerized video analysis of the ulcers. NS, not significant; *, $P < 0.05$ versus 3 days. Values are means $\pm$ SD. For each column, $n = 6$.

celecoxib does not reduce ERK2 protein expression but inhibits its activation directly.

Exogenous HGF Increases ERK2 Phosphorylation Levels in Esophageal Mucosa Ex Vivo: Effects of SC-236

Dose response studies using 1 to 100 ng/ml of HGF revealed that the 100-ng/ml dose significantly increased ERK2 phosphorylation levels in esophageal mucosal explants. A thirty-minute incubation with 100 ng/ml of HGF significantly increased ERK2 phosphorylation levels in esophageal mucosal explants (Figure 11). For *ex vivo* studies we used a structural analog of celecoxib, SC-236, because of its better solubility compared to celecoxib. Pretreatment with both 10 μ mol/L and 100 μ mol/L SC-236

Figure 7. Quantitative data for PCNA expression in normal esophageal epithelium of sham-operated (SO) rats and rats with esophageal ulcers (UL). Starting from 3 days after ulcer induction, rats with esophageal ulcers were treated intragastrically with either celecoxib (CEL) (10 mg/kg, once a day) or its vehicle (VHC) for 2 or 4 days. The results are expressed as the percentage of increase in the number of PCNA-labeled cells in the epithelium of the ulcer margin (epithelium of the lower esophageal segment in SO rats) over that in the epithelium distant from the ulcer (epithelium of the upper esophageal segment in SO rats). *, $P < 0.01$ versus UL at 3 days. Values are means $\pm$ SD. For each column, $n = 6$.

completely blocked the HGF-induced increase in ERK2 phosphorylation levels. Basal ERK2 phosphorylation levels were not significantly altered by 10 μ mol/L of SC-236, however, they were slightly, but significantly reduced by 100 μ mol/L of SC-236. ERK2 total protein levels were not significantly different between the studied groups.

Discussion

This study demonstrated for the first time that esophageal ulceration triggers overexpression of COX-2, HGF and its receptor c-Met, and induces ERK2 activation. Moreover, it demonstrated that the selective COX-2 inhibitor, celecoxib, significantly delays esophageal ulcer healing and reduces ulceration-triggered increases in esophageal epithelial proliferation, c-Met mRNA and protein expression, and ERK2 phosphorylation and activity.

Epithelial cell proliferation is a crucial component of gastric ulcer healing because it provides the cells necessary to fill the mucosal defect and to restore the mucosa within the scar.⁵ It has been suggested that inhibition of COX activity and the resulting inhibition of prostaglandin synthesis may play a role in NSAID-induced inhibition of epithelial cell proliferation during gastric ulcer healing.⁴ Prostaglandins have been demonstrated to induce HGF expression in gastric fibroblasts *in vitro*¹⁹ and, conversely, inhibition of prostaglandin synthesis by indomethacin significantly reduced HGF production in these cells.²⁰ Therefore, it has been suggested that effects of NSAIDs on gastric ulcer healing may be mediated by suppression of prostaglandin-dependent HGF expression.²⁰ However, whether NSAIDs affect HGF protein levels *in vivo* in the stomach or in the esophagus remains unknown. In the present study, HGF protein levels were increased during esophageal ulcer healing but

Figure 6. Photomicrographs of esophageal epithelium showing immunostaining for PCNA (7 days after ulcer induction). Starting from 3 days after ulcer induction, rats were treated intragastrically with either celecoxib (10 mg/kg, once a day) or its vehicle for 4 days. **A:** Epithelium distant from the ulcer in rats treated with vehicle. **B:** Epithelium distant from the ulcer in rats treated with celecoxib. **C:** Epithelium of the ulcer margin in rats treated with vehicle. **D:** Epithelium of the ulcer margin in rats treated with celecoxib. PCNA staining is present in the cell nuclei (dark color). E, epithelium; MM, muscularis mucosae; SM, submucosa. Scale bars, 100 μ m.

Figure 8. c-MET mRNA expression in normal esophageal tissue from sham-operated (SO) rats and ulcerated tissue (UL) 3, 5, and 7 days after sham operation or ulcer induction. Starting from 3 days after ulcer induction, rats were treated with either celecoxib (CEL) (10 mg/kg, once a day) or its vehicle (VHC) for 2 or 4 days. **A:** RT-PCR products obtained with specific primers for rat c-MET (320 bp) and rat β-actin. **B:** Quantitative data for c-MET mRNA expression. Data were obtained by computerized analysis of the optical intensity of amplified PCR products; each signal was normalized against corresponding β-actin signal and the results are expressed as c-MET/β-actin ratio. *, $P < 0.05$ versus UL at 3 days. Values are means ± SD. For each column, $n = 6$.

were not affected by treatment with celecoxib, suggesting that effects of celecoxib on esophageal epithelial proliferation are not mediated via inhibition of HGF expression. On the other hand, there is evidence indicating an interaction between HGF and COX-2. Previously, we have demonstrated that HGF induces COX-2 expression in gastric epithelial cells *in vitro*.²⁵ A recent *in vivo* study has also demonstrated that exogenous HGF up-regulates COX-2 expression in ulcerated gastric mucosa in rats.³⁰ Moreover, the acceleration of gastric ulcer healing by HGF was significantly attenuated by selective COX-2 inhibitors, suggesting that COX-2 may mediate effects of HGF on gastric ulcer healing.³⁰ However, the molecular mechanisms involved in this phenomenon remain unclear.

HGF elicits its biological effects by binding to and activating its receptor, c-Met,³¹ which is present on the majority of epithelial cells, including esophageal epithelial cells.²² The present study demonstrated that c-Met is expressed in esophageal epithelial basal and squamous cells and that its expression is induced by esophageal ulceration. Furthermore, we show that celecoxib significantly suppresses c-Met mRNA and protein expression

Figure 9. HGF and c-Met protein expression in normal esophageal tissue from sham-operated (SO) rats and ulcerated tissue (UL) 3, 5, and 7 days after sham operation or ulcer induction. Starting from 3 days after ulcer induction, rats were treated with either celecoxib (CEL) (10 mg/kg, once a day) or its vehicle (VHC) for 2 or 4 days. **A:** Immunoblotting with anti-HGF-α antibody detected the specific 69-kd band for α chain of HGF protein and immunoblotting with anti-c-MET antibody detected a 140-kd band for β-subunit of c-MET protein. **B:** Quantitative data for HGF protein expression. **C:** Quantitative data for c-MET protein expression. Data were obtained by a computerized video analysis of the Western blots. NS, not significant; *, $P < 0.05$ versus UL at 3 days. Values are expressed as optical intensity units and represent means ± SD. For each column, $n = 6$.

during esophageal ulcer healing. Previous *in vitro* studies demonstrated that c-Met mRNA is inducible and that its expression is increased in response to HGF in cell lines derived from human lung adenocarcinoma and glioblastoma.^{32,33} Because inhibition of ERK activity completely prevented HGF-mediated c-Met induction in human glioblastoma cells,³³ it is possible that celecoxib-induced inhibition of ERK2 activity is involved in the down-regula-

Figure 10. Phosphorylated ERK (pERK), total ERK2 protein expression, and ERK2 activity in normal esophageal tissue from sham-operated (SO) rats and ulcerated tissue (UL) 3, 5, and 7 days after sham operation or ulcer induction. Starting from 3 days after ulcer induction, rats were treated with either celecoxib (CEL) (10 mg/kg, once a day) or its vehicle (VHC) for 2 or 4 days. **A:** Immunoblotting with anti-phosphorylated Tyr 204 ERK antibody detected the specific 44-kd band for pERK1 protein and the specific 42-kd band for pERK2 protein. Stripping and reprobing with anti-ERK2 antibody showed total ERK2 protein levels. **Bottom:** Myelin basic protein (MBP) phosphorylated by ERK2 immunoprecipitated from esophageal tissue samples. **B:** Quantitative data for pERK2 levels. Data were obtained by a computerized video analysis of the Western blots. The pERK2 protein levels are expressed as a percentage of total ERK2 protein levels. **C:** ERK2 activity determined by measuring levels of radiolabeled [γ -³²P]ATP incorporated to MBP by ERK2 (pmol/mg). Values represent means ± SD. For each column, $n = 6$.

Figure 11. Phosphorylated ERK (pERK) and ERK2 protein expression in esophageal mucosal explants pretreated with either 10 $\mu\text{mol/L}$ of SC-236 (SC 10), 100 $\mu\text{mol/L}$ of SC-236 (SC 100), or dimethyl sulfoxide (DMSO) for 6 hours and treated with either HGF (100 ng/ml) or its vehicle (PBS) for 30 minutes. **A:** Immunoblotting with anti-phosphorylated Tyr 204 ERK antibody detected the specific 44-kd band for pERK1 protein and the specific 42-kd band for pERK2 protein. Stripping and reprobing with anti-ERK2 antibody showed total ERK2 protein levels. **B:** Quantitative data for pERK2 levels. Data were obtained by a computerized video analysis of the Western blots. The pERK2 protein levels are expressed as a percentage of total ERK2 protein levels. NS, not significant; *, $P < 0.05$ versus DMSO + PBS group. Values represent means $\pm$ SD. For each column, $n = 6$.

tion of c-Met mRNA expression during esophageal ulcer healing. Because c-Met mediates the mitogenic effects of HGF, suppression of its expression may be one of the mechanisms by which celecoxib inhibits epithelial proliferation during esophageal ulcer healing.

HGF binding and activation of its receptor, c-Met, triggers activation of ERKs and cell proliferation in many cell types.^{34–36} Our previous studies demonstrated that HGF activates ERK2 in gastric epithelial cells.²⁵ Inhibition of enhanced epithelial proliferation during gastric ulcer healing by NSAIDs has been well documented in experimental models and in humans.^{3,4,16} Previous studies demonstrated that gastric ulceration activates epidermal growth factor/mitogen-activated protein kinase (ERK2) mitogenic signaling pathway,⁶ but no studies have evaluated the effects of NSAIDs on this pathway during gastric or esophageal ulcer healing. Our present study showed that ERK2 is also activated during esophageal ulcer healing. Moreover, it demonstrated for the first time that celecoxib suppresses ERK2 activity up-regulated by esophageal ulceration and that a structural analog of

celecoxib, SC-236, inhibits basal and HGF-induced ERK2 phosphorylation in esophageal mucosal explants. Taken together, these results indicate that COX-2 inhibitors may directly or indirectly (via interference with upstream events of ERK signaling pathway) inhibit ERK2 activation in esophageal mucosa. Because activation of the ERK2 pathway is essential for cell proliferation, the inhibitory action of celecoxib on esophageal epithelial proliferation is likely mediated by suppression of the ulceration-triggered ERK2 activation.

Studies of experimental gastric ulcers demonstrated that NSAIDs, including COX-2-selective inhibitors, in addition to inhibition of epithelial cell proliferation at the ulcer margin, also interfere with angiogenesis (formation of new capillary blood vessels) in granulation tissue at the ulcer bed.^{3,16} In the present study, celecoxib, slightly ($\approx 8\%$) but not significantly, inhibited angiogenesis in granulation tissue during esophageal ulcer healing. This finding suggests that inhibition of epithelial proliferation may be more important for celecoxib-induced delay in esophageal ulcer healing than inhibition of angiogenesis. This finding is in contrast to NSAID-induced inhibition of gastric ulcer healing, whereas NSAIDs markedly inhibit angiogenesis and suggests that angiogenesis may be more important for healing of gastric ulcers than for healing of esophageal ulcers. However, more detailed sequential studies specifically assessing angiogenesis, as well as using angiogenic growth factors are necessary to determine the precise role of angiogenesis in esophageal ulcer healing.

In conclusion, esophageal ulceration triggers increases in esophageal epithelial proliferation; COX-2, HGF, and c-Met expressions; and ERK2 activity. A selective COX-2 inhibitor, celecoxib, delays healing of experimental esophageal ulcers and suppresses ulceration-induced increase in epithelial cell proliferation, c-Met induction, and ERK2 activation.

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