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Increased expression of epidermal growth factor receptor and betacellulin during the early stage of gastric ulcer healing

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Abstract. Epidermal growth factor receptor (EGFR) is important for the proliferation and differentiation of gastric mucosal cells. Betacellulin (BTC) is a novel ligand for EGFR Since their role is unclear in the ulcer healing process, we investigated their expression. Gastric ulcers in 30 Sprague-Dawley rats were induced by acetic acid. RT-PCR and Western blotting were performed to detect EGFR and BTC. Immunohistochemical studies were performed to detect EGFR, BTC and proliferating cell nuclear antigen (PCNA). The expression of EGFR and the BTC gene was significantly increased at 12 h, 24 h and 3 days after ulcer induction ($P<0.05$). The expression of EGFR and BTC protein was significantly increased at 24 h and 3 days and at 12 h, 24 h and 3 days after ulcer induction, respectively ($P<0.05$). The phosphorylation of EGFR also increased significantly and reached a maximum 24 h after ulcer induction ($P<0.05$). Immunostaining for EGFR and BTC was observed in numerous epithelial cells from the ulcer margin and ulcer bed, and corresponded to the localization of PCNA. To conclude, there was an increase in EGFR and BTC expression in the early stages of ulcer healing, localized in the epithelial cells of the ulcer margins and regenerating glands with proliferating activity. BTC/EGFR may play an important role in the early stages of ulcer healing.

Introduction

Gastric ulcers are defined as a breach in the mucosa that extends through the muscularis mucosae into the submucosa or even deeper (1). Ulcers can be distinguished from erosions, in which there is epithelial disruption within the mucosa but no breach of the muscularis mucosae (1). The ulcer results from tissue necrosis triggered by mucosal ischemia, free radical formation and the cessation of nutrient delivery, which are caused by vascular and microvascular injury such as thrombi, constriction or other occlusions (2). Tissue necrosis and the release of leukotriene B attract leukocytes and macrophages, which release pro-inflammatory cytokines (e.g. TNFα, IL-1α, and IL-1ß). These in turn activate local fibroblasts, endothelial and epithelial cells. Histologically, an ulcer has two characteristic structures: a distinct ulcer margin formed by the adjacent non-necrotic mucosa, and granulation tissue composed of fibroblasts, macrophages and proliferating endothelial cells forming microvessels at the ulcer base (1,3). Ulcer healing is a complicated process that involves cell migration, proliferation, re-epithelialization, angiogenesis and matrix deposition, all ultimately leading to scar formation (1-4). The regulatory mechanism of ulcer healing is modulated by growth factors, transcription factors and cytokines (2-5).

The epidermal growth factor (EGF) family and growth factors with structural similarity to EGF have been detected in the normal human gastric mucosa (6-9). They bind to their specific cell surface receptor, EGF receptor (EGFR), which has intrinsic tyrosine kinase activity. EGRF can be activated by the binding of its ligands, including EGF (10), transforming growth factor-α (TGF-α) (11), heparin-binding EGF-like growth factor (HB-EGF) (12), amphiregulin (AR) (8), betacellulin (BTC) (13) and epiregulin (14). The effects of these growth factors are pleiotropic, ranging from the induction of DNA synthesis and changes in cell adhesion and motility to the stimulation of the differentiated cell function (15). In particular, EGF (10,16) as well as TGF-α (17) play important roles in the proliferation and differentiation of mucosal cells in the gastrointestinal tract, including the stomach. In addition, several studies have demonstrated that EGF and EGFR are overexpressed in the epithelial cells in the ulcer margin during ulcer healing, which requires epithelial migration and proliferation (18-20).

BTC is a 32-kDa glycoprotein, synthesized as a large transmembrane precursor molecule, which can be cleaved proteolytically to the soluble form of BTC. It functions as a membrane-anchored growth factor in paracrine signaling, and was originally identified as a growth-promoting factor in the conditioned medium of a mouse and human pancreatic ß-cell carcinoma (insulinoma) cell line (13,21). To date, BTC has been shown to be a potent mitogen for fibroblasts, vascular

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smooth muscle cells and retinal pigment epithelial cells. It is synthesized in a wide variety of adult tissues, including the liver, kidney, lung, small intestine and colon and in many cultured cells, including smooth muscle cells and epithelial cells (13,21). We hypothesized that BTC may also play an important role in the gastric ulcer healing process because EGFR and its ligands, EGF and TGF-α, are involved in gastric ulcer healing and BTC is one of the EGFR ligands. BTC has a proliferative effect on fibroblasts and vascular smooth muscle cells, which are characteristic features of ulcer healing.

Therefore, this study examined i) the level of EGFR and BTC expression in the gastric ulcer and ii) activation of EGFR during gastric ulcer healing. In addition, an immunohistochemical study was performed to determine the distribution of EGFR and BTC in rat gastric ulcer tissue as well as to evaluate the relationship between cell proliferation and expression of EGFR and BTC in rat gastric mucosa.

Materials and methods

Induction of chronic gastric ulcers by acetic acid. The Subcommittee for Animal Studies of the Chonbuk National University Medical School (Jeonju, Korea) approved this study. Thirty male Sprague-Dawley rats (Samtako, Osan, Korea) weighing 220-250 g were used in the experiments. The rats were fasted for 16 h before undergoing a laparotomy under pentobarbital anesthesia (50 mg/kg body weight, administered intraperitoneally). The gastric ulcers were induced by the topical application of 100% acetic acid (50 μl) through a polyethylene tube (4-mm inner diameter) to the anterior wall of the stomach for 60 sec. The area was then washed with phosphate-buffered saline (PBS), and the abdomen was closed. The control rats (n=4) underwent the operation as described above without the application of acetic acid (sham operation). The rats were sacrificed at 12 h, 24 h, 3 days, 7 days or 14 days after ulcer induction. The anterior wall of the stomach including the ulcer was excised, rinsed in PBS, snap-frozen in liquid nitrogen for the extraction of RNA and protein, or fixed in 10% formalin for immunohistochemical staining.

Determination of EGFR and BTC mRNA by RT-PCR. Reverse transcription polymerase chain reaction (RT-PCR) was carried out to determine the levels of EGFR and BTC mRNA in the rat gastric ulcer tissues using a GeneAmp RNA-PCR Kit and a DNA thermal cycler (Perkin-Elmer, Foster, CA). The RNA was isolated using an Ultra-Turrax homogenizer (Ika, Staufen, Germany) and TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quality of the isolated RNA was verified by electrophoresis on 1% agarose-formaldehyde gels, and its quantity was determined by measuring its absorbance at 260 and 280 nm. Total-RNA (5 μg) was used as the template to synthesize the complementary DNA (cDNA) with the Moloney murine leukemia virus (M-MLV, USB, Cleveland, OH) in 10 μl of buffer containing 10 mM Tris-HCl, 50 mM KCl, 1 mM dNTP and 5 mM random hexamers. RT was performed for 1 h at 42˚C, followed by 10 min at 95˚C to denature the enzyme. The resulting cDNA (2 μg) was used as a template for the subsequent PCR. The specific primer set for EGFR was 5'-CAACCCTGAGTATCTCAACA-3' (sense) and 5'-CTG GAAAGTCCGGTTTGTAA-3' (antisense). The specific primer set for BTC was 5'-CTTCGTGGTGGACGAGCAA-3' (sense) and 5'-AGCAGACCACCAGGATCTGC-3' (antisense). The sizes of the amplified fragments were 260 bp for EGFR and 122 bp for BTC, respectively. ß-actin was used as the loading control for PCR. Thirty cycles of the following were carried out for the amplification of the cDNAs of EGFR and BTC: 5 min at 95˚C for initial denaturing, 30 sec at 55˚C for annealing, 60 sec at 72˚C for extension, 30 sec at 95˚C for denaturing, and 10 min at 72˚C for a final extension. All experiments were carried out using the conditions optimized for linear amplification. The PCR products were then subjected to electrophoresis on a 1% agarose gel with ethidium bromide. A luminescent image analyzer (LAS-3000, Fuji, Tokyo, Japan) and Multigauge software V3.0 (Fuji, Tokyo, Japan) were used to make a quantitative assessment of the PCR products. The results were expressed as the target cDNA/ß-actin ratio.

Protein extraction and determination of EGFR, BTC and phospho-EGFR protein levels by Western blotting. The tissues were homogenized using an Ultra-Turrax homogenizer in an ice-cold lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Nonidet p-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mM sodium fluoride and 1 mM sodium orthovanadate. The homogenates were then centrifuged at 14,000 rpm for 10 min at 4˚C. The protein concentration in the supernatant was determined using a protein assay reagent kit (Pierce, Rockford, IL). Equal amounts of protein (50 μg) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were incubated at 4˚C overnight with EGFR (Sigma, St. Louis, MO), phospho-EGFR (Cell Signaling, Danvers, MA) and BTC (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies, then washed and incubated with the corresponding anti-IgG peroxidase conjugates at room temperature for 1 h. The signal of the bound antibodies was visualized by chemi-luminescence (Amersham Bioscience, Buckinghamshire, UK). The membranes were stripped and reprobed with the monoclonal anti-ß-actin antibody (Sigma) as a control for the protein loading and transfer. The data was quantified using a luminescent image analyzer.

Determination of EGFR, BTC and proliferating cell nuclear antigen expression by immunohistochemical staining. Immunohistochemical staining with anti-EGFR, anti-BTC and anti-proliferating cell nuclear antigen (PCNA) antibodies was carried out to determine their expression in the gastric ulcers. Briefly, after deparaffinization, the tissue sections underwent a microwave antigen retrieval procedure in 0.01 M sodium citrate buffer (pH 6.0) for 10 min. After blocking the endogenous peroxidase, non-specific staining was blocked by incubating the sections with Protein Block Serum-Free (Dako, Carpinteria, CA) at room temperature for 10 min. The sections were then incubated with anti-BTC (1:100, Santa Cruz Biotechnology), anti-EGFR (1:1000, Sigma) and anti-PCNA (1:50, Dako) antibodies for 2 h at room temperature. After washing, the sections were incubated for 30 min with biotin-

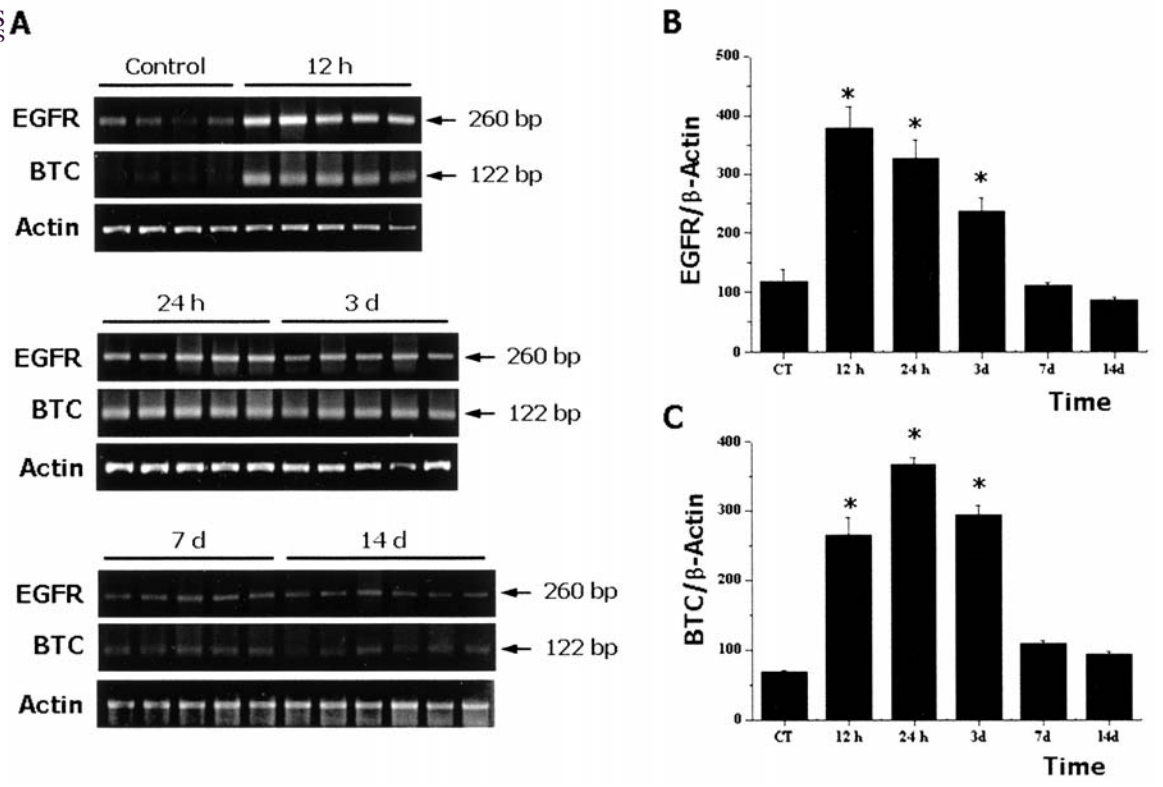

Figure 1. Time course changes of EGFR and BTC mRNA expression in experimental gastric ulcer. (A) RT-PCR products were obtained using the specific primers for EGFR (260 bp), BTC (122 bp) and ß-actin. Quantitative data for the mRNA expression of EGFR (B) and BTC (C). Data were obtained by computerized analysis of the optical intensity of the amplified PCR products. Each signal was normalized against the corresponding ß-actin signal. Results are expressed as the EGFR/ß-actin and BTC/ß-actin ratios, and are represented as the mean ± SE. *P<0.05 versus the control.

conjugated IgG and for 30 min with peroxidase-conjugated streptavidin at room temperature. The peroxidase activity was detected using the enzyme substrate 3-amino-9-ethyl carbazole.

Statistical analysis. Values were expressed as the mean ± SE. The one-way ANOVA test was used to determine the statistical significance of the differences between the control and ulcer tissues with time. A P-value <0.05 was considered significant.

Results

Time course changes of EGFR and BTC mRNA expression in gastric ulcers. EGFR is required for gastric ulcer healing and BTC is a novel ligand for EGFR. The time course of the changes in mRNA expression of EGFR and BTC was examined by RT-PCR. The EGFR mRNA level of the ulcer group 12 h, 24 h and 3 days after ulcer induction was significantly increased compared with that of the control group (P<0.05) (Fig. 1). In the ulcer group, EGFR expression reached a maximum at 12 h and declined thereafter in a time-dependent manner, but remained higher than that of the control until day 3. The level of BTC gene expression was also significantly higher in the ulcer group at 12 h, 24 h and 3 days than in the control (P<0.05). BTC expression reached a maximum at 24 h and declined thereafter in a time-dependent manner, but also remained higher than that of the control until day 3. These results suggest that EGFR and BTC may be involved in the early stages of ulcer healing.

Time course changes of EGFR and phospho-EGFR protein expression in gastric ulcers. The time course changes of the protein expression of EGFR and phospho-EGFR in the gastric ulcer were examined using Western blotting. As shown in Fig. 2, EGFR protein expression began to increase at 12 h after ulcer induction and reached significant levels at 24 h and 3 days (P<0.05). The level of EGFR protein expression reached a maximum at 24 h and declined thereafter. The phosphorylation of EGFR began to increase at 12 h after ulcer induction and reached significant levels at 24 h (P<0.05) and declined thereafter. These results show that the expression of the EGFR protein and its phosphorylation reached a maximum within 24 h, which is regarded as the early stages of ulcer healing.

Time course changes of BTC protein expression in gastric ulcers. Since increased expression of the BTC gene was observed after ulcer induction, the time course changes in the expression of BTC protein during ulcer healing were examined using Western blotting. As shown in Fig. 2, the level of BTC protein was significantly higher in the ulcer group than in the control at 12 h, 24 h and 3 days after ulcer induction (P<0.05), and reached a maximum level at 24 h. From day 7 to 14, the expression of the BTC protein decreased markedly.

EGFR, BTC and PCNA immunohistochemistry in rat gastric ulcers. To examine EGFR and BTC localization during ulcer healing, the level of EGFR and BTC expression in formalin-fixed paraffin-embedded gastric tissue sections was determined

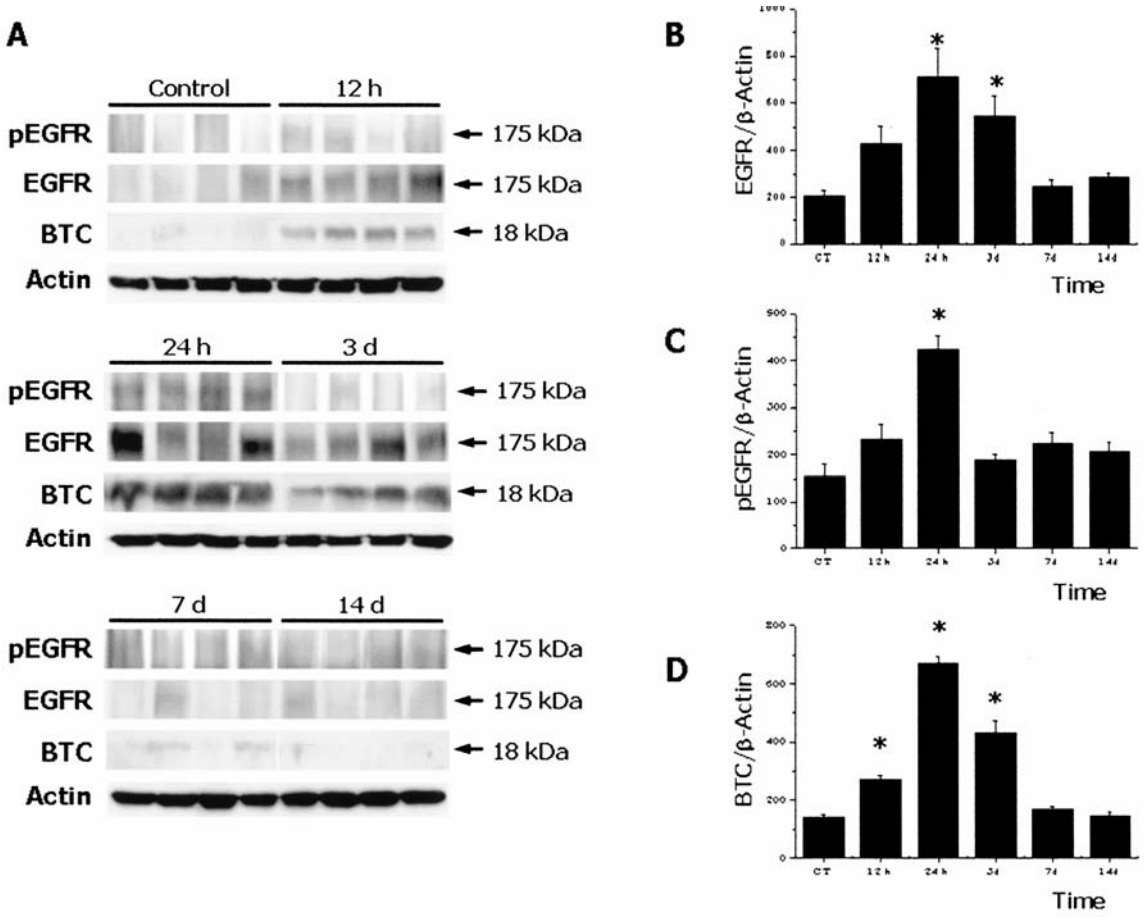

Figure 2. Time course changes in the protein expression of EGFR, phospho-EGFR and BTC in an experimental gastric ulcer. (A) Immunoblotting with the specific antibodies detected the specific 175-kDa band for EGFR and the 18-kDa band for BTC. The phosphorylation of EGFR was detected by the phospho-EGFR antibody. Quantitative data of the protein expression for EGFR (B), phospho-EGFR (C) and BTC (D). The data was obtained by computerized analysis of the Western blots. Each signal was normalized to the corresponding ß-actin signal. Results are expressed as the EGFR/ß-actin, phospho-EGFR/ß-actin and BTC/ß-actin ratios, and are represented as the mean ± SE. $^{*}P<0.05$ versus the control.

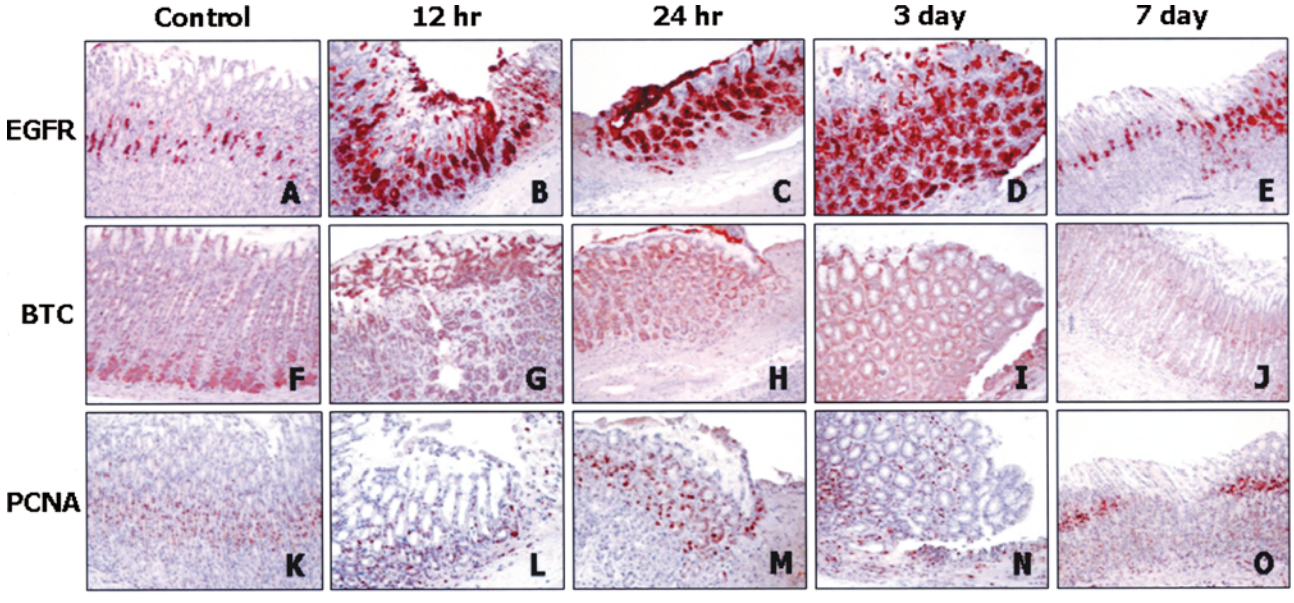

Figure 3. The localization of EGFR (A-E), BTC (F-J) and PCNA (K-O) in the experimental gastric ulcer determined by immunohistochemistry. In the normal gastric tissue, EGFR (A) was mainly localized in mucous neck cells of the proliferation zone, whereas BTC (F) was mainly localized in the base of the gastric mucosa. From 12 to 24 h after ulcer induction, EGFR (B and C) was present in the epithelial cells of the ulcer margin and the ulcer bed, whereas BTC (G and H) was diffusely localized in epithelial cells and the ulcer margin. At day 3, EGFR (D) and BTC (I) were observed in the epithelial cells lining the regenerating gastric glands, but the intensity was weaker than that observed at 12 and 24 h. At day 7, EGFR (E) and BTC (J) were mainly localized in the mucous neck cells of the proliferation zone. The areas expressing PCNA (K-O) also expressed EGFR and BTC at the same time points. (Magnification of the control up to day 3, x100; at day 7, x40).

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nohistochemistry. As shown in Fig. 3, EGFR was calized in some mucous neck cells of the proliferation zone in normal gastric mucosa. EGFR expression was also observed in some parietal cells, but was weaker than in the neck cells. On the other hand, BTC was mainly localized in the base of the gastric mucosa in normal gastric tissue (Fig. 3). From 12 to 24 h after ulcer induction, EGFR immunoreactivity was diffuse and strong in the mucosa of the ulcer margin and ulcer bed. At this time, BTC was diffusely localized in the epithelial cells of the ulcer margin. Three days after ulcer induction, EGFR immunoreactivity was observed in the epithelial cells lining the regenerating gastric glands, but the staining intensity was weaker than that observed at 12 and 24 h. At this time, the immunoreactivity of BTC was also localized in the epithelial cells lining the regenerating gastric glands with weaker staining intensity. Seven days after ulcer induction, the immunoreactivity of EGFR was mainly localized in the mucous neck cells of the proliferation zone, as well as in the regenerating gastric glands in the ulcer scars. At this time, BTC was localized mainly in the mucous neck cells of the proliferation zone and at the base of the gastric mucosa.

Localization of the proliferating activity was determined by immunohistochemistry for PCNA and was compared with the areas expressing EGFR and BTC in the same specimens. As shown in Fig. 3, the areas expressing PCNA were similar to those expressing EGFR and BTC. These findings suggest a positive relationship between levels of EGFR and BTC expression and proliferating activity.

Discussion

An ulcer is a deep defect in the esophageal, gastric, duodenal or intestinal wall that involves the entire mucosal thickness and penetrates the muscularis mucosae. Ulcer healing is an active and complex process that requires the coordinated interaction of various cellular and connective tissue components. In addition, ulcer healing involves the reconstruction of the glandular structures, the re-epithelialization of the mucosal surface, angiogenesis, and the estoration of connective tissue components (3). During ulcer healing, processes including mucosal cell migration, proliferation and biochemical events are modulated by various growth factors, transcription factors and cytokines (2-5).

Previous studies have shown that a number of growth factors, including EGF, TGF-α, trefoil growth peptides and bFGF, participate in the repair of tissue injury by stimulating cell proliferation and migration, an essential step for re-epithelialization and ulcer healing (18,20,22-25). Gastric ulceration also induces the overexpression of EGF and its receptor EGFR (18,22).

EGFR is a 170-kDa receptor tyrosine kinase that has been detected in a variety of tissues and cells (6-9). The EGFR family includes four homologous transmembrane receptor protein kinases, EGFR, c-erbB-2, c-erbB-3 and c-erbB-4. This receptor family plays an important role in regulating cell proliferation, differentiation and transformation (24,25). EGFR binds several ligands, including EGF, TGF-α, HB-EGF, AR, BTC and epiregulin (10-14). Binding affinities vary among these ligands. Ligand-induced activation of these receptors results in the formation of homodimers and heterodimers, which undergo transphosphorylation and transactivation (26,27). It has been reported that EGFR is involved in organ morphogenesis, the maintenance and repair of tissues, and epithelial migration and proliferation (18-20). This study examined the time course change of EGFR mRNA and protein expression in gastric tissue after ulcer induction. The results show that expression of EGFR mRNA and protein reached a maximum level at 12 and 24 h, respectively. In addition, the level of EGFR phosphorylation also began to increase at 12 h and reached a maximum at 24 h after ulcer induction. Overall, EGFR may play an important role in the early stages of ulcer healing due to its maximal expression and phosphorylation occurring within 24 h after ulcer induction. These results are consistent with previous studies showing the increase in EGFR expression in the ulcer healing process (18-20).

BTC is a member of the EGF family that binds to EGFR with a similar affinity to EGF. Its soluble active forms are produced by proteolytic cleavage, and it functions as a potent mitogen factor for fibroblasts, vascular smooth muscle cells and retinal pigment epithelial cells (13). It was reported that BTC induces proliferation and migration of vascular smooth muscle cell through various signal transduction pathways, including ERK 1/2, Akt and p38 MAPK (28), and that it is synthesized in a wide range of adult tissues, including the liver, kidney, lung, small intestine and colon (13,21). Because EGFR and its ligands, EGF and TGF-α, play an important role in gastric ulcer healing through the migration and proliferation of epithelial cells, it could be assumed that BTC is also involved in the ulcer healing process. However, there have been no reports on this hypothesis. Therefore, this study undertook to examine whether or not the expression of BTC in gastric tissue is altered after ulcer induction. The results show that the expression of both the mRNA and protein of BTC reached a maximum level at 24 h. Thus, due to their similar mRNA and protein expression patterns, both BTC and EGFR appear to play important roles in the early stages of gastric ulcer healing.

The time course changes in the *in vivo* localization of EGFR and BTC expression in gastric ulcer tissue using immunohistochemistry were significant. After the stomach was treated with acetic acid, the gastric walls showed characteristic sequential morphological changes as reported previously (29). Ulcer craters without necrotic tissue developed within 2 days after ulcer induction, and the gastric mucosa of the ulcer margin formed a transitional (or healing) zone by day 3 (20). Seven days after ulcer induction, the size of the ulcer had decreased and its margin was more clearly delineated. In normal gastric tissues, EGFR and BTC expression was mainly localized in the mucous neck cells and the base of the gastric mucosa, respectively. At 12 and 24 h after ulcer induction, immunoreactivities for EGFR and BTC were diffusely and strongly localized in the numerous epithelial cells lining the gastric glands of the ulcer margin and in the base. The results also show that the localization of EGFR and BTC at day 3 after ulcer induction was similar to that observed at 12 and 24 h, but with weak staining intensity. These results are consistent with those of RT-PCR and Western blotting. In addition, the question of whether increased expression of EGFR and BTC in ulcer healing is involved in mucosa cell proliferation was examined using immunohistochemistry for PCNA. The results

indicate a positive correlation between increased EGFR and BTC expression in ulcer healing and the proliferation of gastric epithelial cells.

In summary, this study demonstrated for the first time that gastric ulceration triggers an increase in the mRNA and protein expression of EGFR and BTC in the early stages of ulcer healing. EGFR and BTC mRNAs were overexpressed as early as 12 h after ulcer induction, which lasted for 3 days. Moreover, the cells overexpressing EGFR and BTC had high proliferating activity. Immunohistochemical staining revealed that the epithelial cells in the ulcer margin overexpressed BTC and its receptor EGFR. These cells are major targets for the proliferation-stimulating action of BTC. Therefore BTC, a member of the EGFR ligands, may play an important role in the early stages of ulcer healing. However, further studies are needed to clarify its precise role as a mitogenic factor in ulcer healing.

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