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Expression of Tie-2 and angiopoietin-1 and -2 in early phase of ulcer healing

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Background. Angiogenesis is an important process in tissue development and wound healing. The Tie-2 receptor tyrosine kinases and ligands, angiopoietin (Ang)-1 and -2 have been postulated to play key roles in vascular development. The purpose of this study was to evaluate the expression of Tie-2 and Ang-1 and -2 in an acetic acid-induced gastric ulcer healing process in rats. ***Methods.*** Gastric specimens were obtained at 0 (control), 1, 3, 5, 7, and 14 days after ulcer induction for reverse transcription polymerase chain reaction (RT-PCR), Western blot analysis, and immunohistochemical analysis. ***Results.*** Expression of Tie-2 and Ang-1 and -2 mRNAs was detected in normal gastric tissue and ulcerative tissues by RT-PCR. Western blot analysis revealed that Tie-2 expression reached a maximum on the third to fifth days. Expression of Ang-1 and -2 peaked on the first day. Ang-1 expression gradually became weaker in 2 weeks, whereas Ang-2 expression returned to normal in a few days. Immunohistochemically, Tie-2 was expressed constitutively in the endothelial cells of pre-existing vessels of the gastric wall, and Tie-2 expression was increased in the new capillaries of the ulcer base. ***Conclusions.*** These findings suggest that Tie-2 and Ang-1 and -2 play an important role in angiogenesis in the early phase of ulcer healing.

Key words: angiogenesis, Tie-2, Ang-1, Ang-2, gastric ulcer, rat

Introduction

Angiogenesis, a complex multistep process of the formation of new blood vessels from pre-existing vessels,[1] is an important process in tissue development and wound healing. Angiogenesis is a key factor in ulcer healing, determining the quality of healing through nutrient and oxygen delivery to the healing site. Tie-2 (tek) is a member of the endothelial cell-specific receptor tyrosine kinase family,[2,3] and is essential for formation of the embryonic vasculature.[4] The ligands for Tie-2, angiopoietin (Ang)-1, and Ang-2 recently have been shown to be essential for normal vascular development in the mouse.[5,6] Several studies suggest that Ang-1 plays a role in pericyte recruitment by endothelial cells and in the maintenance of cell-cell and cell-matrix association in mature vessels.[7,8] Ang-2 also has been shown to function as a ligand for the Tie-2 receptor, but in contrast to Ang-1, Ang-2 does not phosphorylate the Tie-2 receptor and counteracts blood vessel maturation and stability, disrupting blood vessel structure.[6] In the present study, we examined the expression of Tie-2 and Ang-1 and -2 in gastric ulcer healing, as a model of angiogenesis in rats.

Materials and methods

This study was approved by the Animal Care Committee of Nagasaki University. Male Wistar rats were purchased from Charles River Japan (Atsugi, Japan) at 7 weeks of age ($n = 54$). The rats were housed in groups of 3 to 4 per cage in an air-conditioned room at 24°C (lights on from 7 am to 7 pm), and allowed free access to food (laboratory chow F2; Japan Clea, Tokyo, Japan) and tap water at the Laboratory Animal Center of Nagasaki University. Gastric ulcers were produced by luminal application of an acetic acid solution, as reported previously.[9] Briefly, with the rats under ether anesthesia, a midline incision was made and the stomach exposed. The anterior and posterior walls of the gastric fundus were clamped together with a pair of forceps with a round ring (interior diameter [ID], 6 mm). A 40% acetic

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acid solution was injected into the clamped portion through the forestomach, using a needle (21 gauge). Forty-five seconds later, the acid solution was removed and the abdomen was closed. Thereafter, the animals were fed normally. The rats were killed by deep anesthesia with ether. Gastric specimens were obtained at 0 (control), 1, 3, 5, 7, and 14 days after ulcer induction for immunohistochemical analysis, Western blot analysis, and reverse transcription polymerase chain reaction (RT-PCR) to evaluate the expression of Tie-2 and Ang-1 and -2 proteins and mRNAs. Ten to twelve rats were used for analysis on each experimental day. For Tie-2 immounohistochemistry, gastric tissues were fixed with 4% paraformaldehyde solution.

RT-PCR

Total RNA was prepared from gastric tissue, using the acid guanidine phenol method. Total RNA (1 μg) was incubated at 37°C for 1 h in 50 μl of reverse transcriptase buffer containing 20 units of RNAsin (Promega, Madison, WI, USA), 100 pmol of random hexamer primers (Boehringer Mannheim, Mannheim, Germany), and 400 units of Moloney murine leukemic virus reverse transcriptase (GIBCO/BRL, Gaithersburg, MD, USA). Reverse transcription was terminated by heating to 95°C for 10 min, and 20% of the resulting cDNA was removed for PCR. PCR samples were incubated with 50 pmol of each primer and 2.5 units of Taq DNA polymerase. The rat Tie-2 and Ang-1 and -2 PCR primers were: 5′-TGTTCCTGTGCCACAGGCTG-3′ (sense) and 5′-CACTGTCCCATCCGGCTTCA-3′ (antisense); 5′-GCTGGCAGTACAATGACAGT-3′ (sense) and 5′-GTATCTGGGCCATCTCCGAC-3′ (antisense); and 5′-GAGATCAAGGCCTACAGT GA-3′ (sense) and 5′-AAGTTGGAAGGACCACA TGC-3′ (antisense), respectively. The human β-actin PCR primers were 5′-TCCTCCCTGGAGAAGA GCTA-3′ (sense) and 5′-AGTACT TGCGCTCAGGA GGA-3′ (antisense). The Tie-2 and Ang-1 and -2 and β-actin primers are predicted to amplify 317-, 339-, 443-, and 313-bp DNA products, respectively. Primer pairs were chosen to span introns of their respective rat genes. Samples were subjected to 40 cycles of PCR amplification, using a thermocycler. Each cycle included denaturation at 95°C for 3 min, annealing at 50°C for 1 min, and primer extension at 72°C for 1 min. An aliquot of each amplification mixture was subjected to electrophoresis on a 2% agarose gel, and DNA was visualized by ethidium bromide staining.

Western blot analysis of Tie-2 and Ang-1 and -2 expression

Western blot analyses for Tie-2 and Ang-1 and -2 expression were performed on ulcerated gastric tissues. Fresh gastric tissues were obtained from ulcerated areas and frozen immediately after tissue sampling. The tissues were then suspended in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40 [NP-40], 1% sodium deoxycholate, and 0.05% sodium dodecylsulfate [SDS]; pH 7.4), broken into pieces on ice, and subjected to three freeze-thaw cycles. The insoluble cell debris was removed by centrifugation at 14000 *g* at 0°C for 10 min. The supernatant was collected and the protein concentration was quantified using a protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). For each data point, two rats were used, and the assays were performed in duplicate. The proteins (30 μg) were separated by polyacrylamide gel electrophoresis (PAGE) under denaturing and reducing conditions, and transferred to a Hybond ECL Nitrocellulose Membrane (Amersham Life Science, Buckinghamshire, UK). The membranes were rinsed in Tris buffer saline (TBS), blocked with 5% low-fat dried milk in TBS containing 0.1% Tween 20 (TTBS), and then incubated for 2 h at room temperature with a 1:500 dilution of Tie-2 (as for immunohistochemistry) or Ang-1 (N-18, human origin; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or Ang-2 (F-18, mouse origin; Santa Cruz Biotechnology) polyclonal antibody. After extensive washing of the membranes with TTBS, they were incubated for 1 h with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Ig) G in TTBS containing 3% low-fat dried milk. The membranes were washed and developed with a horseradish peroxidase chemiluminescence detection reagent (ECL Plus System; Amersham Biosciences, Buckinghamshire, UK), and then exposed to Hyperfilm ECL (Amersham).

Immunohistochemistry

Paraformaldehyde-fixed and paraffin-embedded tissues were cut into 4-μm sections, deparaffinized in xylene, and rehydrated in phosphate-buffered saline. The deparaffinized sections were preincubated with normal bovine serum to prevent nonspecific binding after 3% H_2O_2 treatment for 30 min, and then incubated overnight at 4°C with an optimal dilution (2 μg/ml) of a primary polyclonal antibody against Tie-2 (C-20, mouse origin; Santa Cruz Biotechnology). The slides were incubated with an anti-rabbit immunoglobulin antibody (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) and the reaction products were resolved using diaminobenzidine (DAB) colorization.

Results

Pathological changes in the ulcer

One day after ulcer induction, the gastric mucosa was necrotic macroscopically, and bleeding, edema, and inflammatory cell infiltration were observed in the submucosa microscopically. From the second day, fresh granulation was formed and new capillaries appeared in the ulcer margins. On the third and the fifth days, there was an increase in the amount of granulation tissue in the ulcer bed. On the seventh day, absorption of granulation and stromal fibrosis occurred and the regenerative epithelium expended from the margin. Mild inflammatory cell infiltration was observed. On the fourteenth day, the ulceration appeared to be healed macroscopically. The submucosal granulation was organized, with fibrosis, and regenerative epithelium covered the area of ulceration completely.[10]

RT-PCR

Expression of Tie-2 and Ang-1 and -2 mRNAs was detected in normal gastric tissue and ulcerative tissues, irrespective of the time point (Figs. 1, 2). β-Actin mRNA, a control to demonstrate the equivalent amounts of tissue RNA, was detected in all the samples.

Western blotting

Tie-2 expression was detected weakly in the normal gastric tissue, but increased on the first day. Tie-2 was expressed at high levels on the third to fifth days, while its expression became weak again on the seventh day (Fig. 3). Weak expression of Ang-1 and Ang-2 was detected in normal gastric tissue and the peak expression of both factors was observed on the first day. Ang-1 expression gradually became weaker up to the fourteenth day, whereas Ang-2 expression became weak on the third day (Fig. 4).

Immunohistochemistry

Immunohistochemical staining of Tie-2 was weakly positive in the endothelial cells of pre-existing vessels of the gastric wall (Fig. 5A). In the basic and marginal granulation tissues, Tie-2 expression in the endothelial cells of new capillaries increased on the third to the fifth days after ulcer induction (Fig. 5B) and returned to normal levels on the seventh day.

Discussion

Angiogenesis occurs in many physiological and pathological processes, including embryonic development, wound healing, and tumor growth.[11,12] Ulcer healing consists of two processes, epithelial regeneration and mesenchymal reconstruction. Mesenchymal reconstruction comprises angiogenesis, fibrosis, and smooth muscle regeneration. Angiogenesis is central to granulation tissue formation, because the ingrowth of newly formed vessels is needed to ensure the supply of oxygen

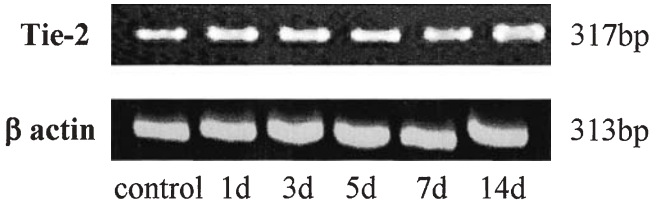

Fig. 1. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of Tie-2 mRNA expression in normal and ulcerative gastric tissues, using the specific primer pairs predicted to amplify the fragment sizes shown *on the right* (Tie-2, 317 bp; β-actin as internal control, 313 bp). *Lane 1*, Normal gastric tissue. *Lanes 2–6*, basic and marginal tissues of the gastric ulcer on the first (*lane 2*), third (*lane 3*), fifth (*lane 4*), seventh (*lane 5*), and fourteenth days (*lane 6*) of ulceration. *d*, Day

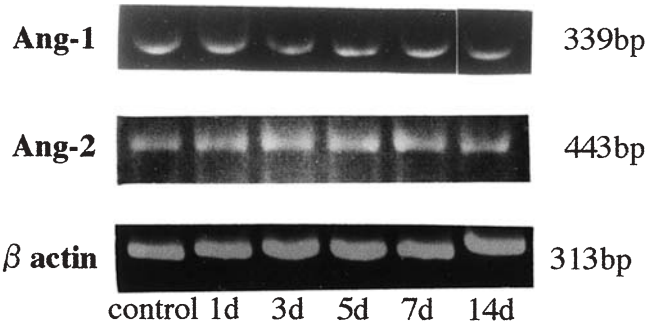

Fig. 2. RT-PCR analysis of angiopoietin-1 (Ang-1) and -2 mRNA expression in normal and ulcerative gastric tissues, using the specific primer pairs predicted to amplify the fragment sizes shown *on the right* (Ang-1, 339 bp; Ang-2, 443 bp; β-actin as internal control, 313 bp). *Lane 1*, Normal gastric tissue. *Lanes 2–6*, Basic and marginal tissue of the gastric ulcer on the first (*lane 2*), third (*lane 3*), fifth (*lane 4*), seventh (*lane 5*), and fourteenth days (*lane 6*) of ulceration

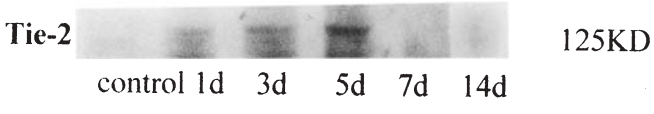

Fig. 3. Western blotting of Tie-2 proteins. Tie-2 was detected (*lane 1*) in normal gastric tissue. On the first day (*lane 2*) the expression levels increased, and Tie-2 was expressed at high levels on the third to the fifth days (*lanes 3, 4*), while its expression became weak on the seventh day (*lane 5*)

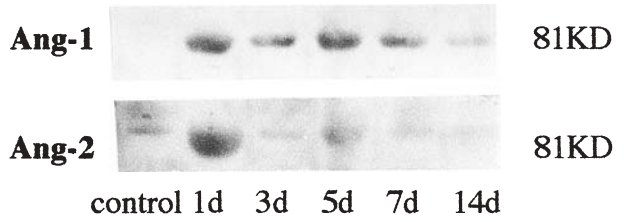

Fig. 4. Western blotting of Ang-1 and -2 proteins. Ang-1 and -2 were detected weakly (*lane 1*) in normal gastric tissue. Peak expression was observed on the first day (*lane 2*). Ang-1 expression gradually became weaker until the fourteenth day (*lane 6*), whereas Ang-2 expression became weaker on the third day (*lane 3*)

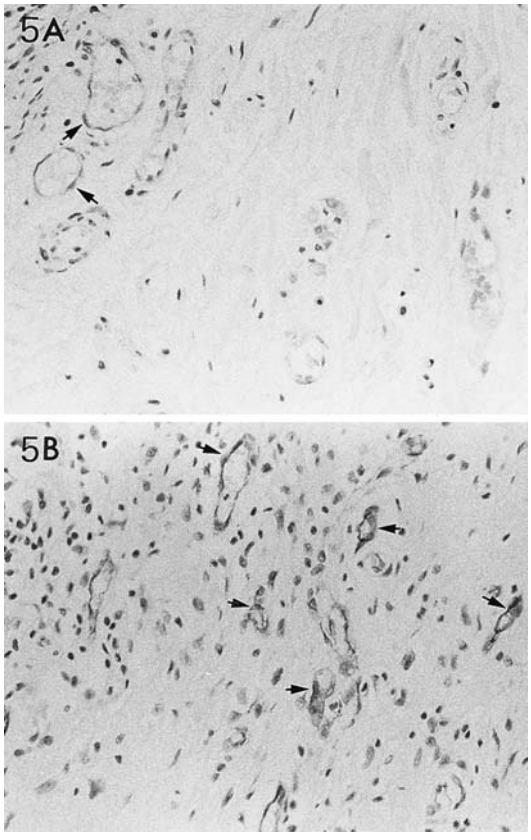

Fig. 5. **A** Tie-2 was weakly positive in the endothelial cells of pre-existing vessels of the gastric wall (*arrowheads*) **B** Tie-2 expression in ulcerative lesions. Tie-2 expression in the endothelial cells of new capillaries was increased on the third day after ulcer induction (*arrowheads*). **A,B** DAB staining, ×200

and nutrients to the regenerating tissue. Tie-2 is a one of the receptor tyrosine kinases that are expressed by endothelial cells,[2,13,14] and these kinases have been reported to play important roles in embryonic blood vessel development.[4,7,15] Ang-1 and -2, the ligands for Tie-2, have also been reported to be important in the development of the embryonic vasculature and in mouse kidney maturation.[7,16] Expression of the Tie-2 receptor has been reported to be upregulated in endothelial cells in angiogenesis associated with wound healing and breast cencer.[17–19] The expression of Ang-2 has also been reported to be upregulated in human hepatocellular carcinoma and astrocytomas.[20,21] These studies all point to an important role for the Tie-2 receptor and angiopoietins in vascular biology and pathology. Although the Tie-2 receptor and angiopoietins have been studied in angiogenesis, their roles in the process of gastric ulcer healing remain largely unknown. The present study is the first to demonstrate the expression of the Tie-2 receptor and angiopoietins by gastric tissues in an ulcer model. Tie-2 receptor expression was detected in the endothelial cells of the ulcer bases and margins. Its expression reached a maximum on the third to fifth days after ulcer induction. Upregulation of Tie-2 expression has also been reported to be involved in angiogenesis in cutaneous wound healing.[22] The intensity of expression of the Tie-2 receptor suggests that it could play an important role in the early phase of ulcer healing.

Ang-1 induces sprouting and branching in vitro[23] and is hypothesized to be required for the stabilization of peri-endothelial contacts with surrounding smooth muscle cells in mature vessels.[24] A recent report suggested that Ang-1 and -2 were colocalized in the microvessels of granulation tissues and macrophages.[25,26] In our study, a marked upregulation of Ang-1 was observed in the ulcer bases and margins. Furthermore, its expression reached a maximum on the first day and remained at relatively high levels until the fourteenth day. These observations suggest that it plays a protracted role in angiogenesis (branching and stabilizing) in ulcer healing.

Ang-2 loosens capillary structure and sensitizes endothelial cells to angiogenic stimuli by blocking the stabilizing effect of Ang-1 and, consequently, disrupting the contacts between endothelial cells, pericytes, and extracellular matrix.[5–7] We found that its expression reached a maximum on the first day and was stronger than Ang-1 expression. Our observations support the hypothesis that Ang-2 induction leads to vessel proliferation in the early phase of angiogenesis.

We have reported here the localization of the Tie-2 receptor. We observed a time-dependent change in Tie-2 receptor and angiopoietins in ulcer lesions. The present results are not consistent with findings in the wound healing of skin,[22] because the surroundings are quite different between stomach and skin. In the stomach, the mucosa is exposed to acid, and is controlled by the autonomic nervous system. Gastric ulcer and skin ulcer are different events. Our results demonstrated that Tie-2 and Ang-1 and -2 increased rapidly during the early phase of ulcer healing. These results suggest that

Tie-2 receptor and angiopoietins play an important role in the early phase of ulcer healing.

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