Association of Heparin with Basic Fibroblast Growth Factor, Epidermal Growth Factor, and Constitutive Nitric Oxide Synthase on Healing of Gastric Ulcer in Rats

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ABSTRACT

The healing effect of heparin on gastric ulcer and its underlying mechanisms were studied. The influences of protamine on these effects were also investigated. Gastric ulcer was induced by acetic acid in rats. Heparin (100–1000 U/kg i.v.) was given once daily for 4 or 7 days. Ulcer area was measured; gastric mucosal regeneration, proliferation, and angiogenesis were determined by histological or immunohistochemical methods. Gastric mucosal basic fibroblast growth factor (bFGF) level was assessed by an enzyme-linked immunosorbent assay, and the mucosal epidermal growth factor (EGF) level and nitric oxide synthase (NOS) activity were measured by radioimmunoassay. The anticoagulant action of heparin was determined by the duration of bleeding time. The results showed that heparin given for 4 or 7 days significantly accelerated gastric ulcer healing in a dose-dependent manner. The three doses of heparin significantly stimulated mucosal regeneration and proliferation as well as angiogenesis but not the contraction of ulcer base. Similar effects were observed in gastric mucosal bFGF and EGF levels and constitutive NOS activity. Protamine not only abolished the anticoagulant action of heparin but also significantly potentiated its effects on ulcer healing, gastric mucosal proliferation, angiogenesis, and constitutive NOS activity. These findings indicate that heparin can accelerate gastric ulcer healing, which is associated with mucosal regeneration, proliferation, and angiogenesis. These actions are likely to be stimulated by bFGF, EGF, and constitutive NOS activity in the gastric mucosa. Protamine potentiates the ulcer-healing effect of heparin, which is probably acting through constitutive NOS activation.

Heparin, a known anticoagulant, has been recently studied for other actions and reported to have the ability to modulate cell proliferation, angiogenesis, wound healing, inflammation, and myocardial function (Folkman, 1985; D’Amore, 1990; Tirell et al., 1995; Galvan, 1996; Kouretas et al., 1998). An established effect of heparin, i.e., angiogenesis effect, is related to the action of basic fibroblast growth factor (bFGF) (Folkman, 1985; D’Amore, 1990; Folkman and Shing, 1992a), which belongs to the family of heparin-binding growth protein (D’Amore, 1980; Basilico and Moscatelli, 1992) and could modulate angiogenesis (Basilico and Moscatelli, 1992; Uchida et al., 1995), cell proliferation (Klagsbrun, 1989), neuronal regeneration (Anderson et al., 1988), and wound healing (Tsobo and Rifkin, 1990). A cardioprotective effect of heparin on myocardial ischemia-reperfusion injury has been reported (Black et al., 1995; Kouretas et al., 1998), which demonstrates further that this effect is acting through the stimulation of nitric oxide (NO) production.

Gastric ulcer is a common gastrointestinal disease. Its healing processes are associated with several factors, including luminal factors (H+ secretion, pepsin, mucus, and bicarbonate) and other factors promoting ulcer healing, such as different types of growth factors, angiogenesis, and oxygen and nutrient supply to the gastric mucosa (Tarnawski et al., 1991). Recently, studies have been focused on the role of growth factors such as epidermal growth factor (EGF) and bFGF in the process of ulcer healing. EGF acts as a stimulator of the restitution and proliferation of mucosal cells at the ulcer margin, which supplies cells for reepithelialization of the mucosal scar surface and reconstruction of glandular structure, and accelerates the healing of acute and chronic lesions (Tarnawski et al., 1991; Konturek et al., 1995). bFGF could accelerate the healing of gastric or duodenal ulcer in vitro and in vivo through the stimulation of gastric or duodenal epithelial cell migration and proliferation and regeneration of the microvascular system (angiogenesis) in the mucosal and submucosal layers (Folkman et al., 1991; Szabo et al., 1994, 1995; Schmassmann et al., 1995). Furthermore, the effect of endogenous NO on the healing process of gastric ulcer has been investigated (Konturek et al., 1993; Brzozo-
waki et al., 1997) by use of L-arginine, a substrate for NO synthase (NOS), and NG-monomethyl-L-arginine, an inhibitor of NOS. The results indicate that acceleration or delay of gastric ulcer healing could be affected by L-arginine or NG-
monomethyl-L-arginine, respectively, implicating that endog-
enous NO also plays an important role in the process of
gastric ulcer healing.

Although heparin was reported to increase ulcer healing (Li et al., 1998), its relationship with cell proliferation and angiogenesis in the gastric mucosa is still undefined. We hypothesized that heparin could have a beneficial effect on gastric ulcer healing through its stimulatory action on bFGF levels and NO production. We investigated the healing effect of heparin on the acetic acid-induced gastric ulcer and its underlying mechanisms related to bFGF and constitutive
NOS (cNOS), the enzyme to stimulate NO production. Be-
cause it has been demonstrated that EGF is a crucial factor for gastric ulcer healing, the relationship between heparin and EGF on gastric ulcer healing was also studied. However, the anticoagulation effect of heparin could be detrimental to gastric ulcer healing; we therefore examined whether block-
ing of such an effect by a heparin antagonist, protamine sulfate, could promote ulcer healing.

**Materials and Methods**

**Animals.** The use of animals in this study was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong. Male Sprague-Dawley rats (180–200 g) were reared on a standard laboratory diet (Ralston Purina Co., St. Louis, MO) and given tap water. They were kept in a room where temperature (22 ± 1°C), humidity (65–70%), and day/night cycle were controlled. Rats were fasted for 24 h but had free access to water before being subjected to acetic acid to produce gastric ulcer. These rats were then given heparin 1 day after ulcer induction.

**Drugs.** Heparin (sodium salt, produced from porcine intestinal mucosa, 174 USP U/mg; Sigma, St. Louis, MO) or protamine sulfate (Sigma) was prepared in 0.9% w/v NaCl (British Drug House, Poole, Dorset UK) solution (normal saline) for i.v. or s.c. injection.

**Induction and Measurement of Acetic Acid-Induced Gas-
tric Ulcer and Heparin Treatment.** Twenty-four hours after star-
vation, gastric ulcers were produced by luminal application of an acetic acid (E. Merek, Darmstadt, Germany) solution to rats as previously described (Tsukimi and Okabe, 1994a), with modifica-
tions. Briefly, the abdomen was opened under ether anesthesia and the stomach exposed. The anterior and posterior walls of the stomach were clamped together with a pair of forceps with a round ring (i.d. 11 mm) situated between the two arms of the forceps. A 60% acetic acid solution of 0.12 ml was injected into the clamped portion through the forestomach via a 21-gauge needle. Forty-five seconds later, the acid solution was removed and the abdomen closed. There-
after, rats were fed normally. Heparin in doses of 100, 500, and 1000 U/kg i.v. or its vehicle (normal saline) was administered through the tail vein once daily starting 1 day after ulceration for 4 or 7 days to observe the ulcer-healing effect. After the drug treatment, rats were sacrificed and stomachs were removed, opened along the greater curvature, and spread on a glass board. The ulcers in the anterior and posterior walls were determined and summed blinded in each stomach. The ulcer area 1 day after ulcer induction was served as a starting point for subsequent ulcer-healing assessment.

After measuring the ulcers, gastric tissues were excised for histo-
logical and immunohistochemical analysis. Gastric glandular mu-
cosa was then removed by scraping with a glass slide and immedi-
ately frozen in liquid nitrogen and stored at −70°C until determinations for different parameters.

**Histological Studies.** Histological sections (5 mm thick) were stained with H&E for measurement of the length of regenerated gastric mucosa at the ulcer edge, the length of the ruptured musculo-
laris mucosae, and the thickness of the ulcer base under a light
microscope (Nikon, 40×) according to the method described by Ogi-
haru and Okabe (1993). Simultaneously, the thickness of the regen-
erated mucosa was measured in the area 500 mm distant from the ulcer margin.

**Assessment of Epithelial Proliferation at Ulcer Margin.** A single dose of 100 mg/kg i.p. 5-bromo-2-deoxyuridine (BrdU) (Sigma) was injected 1 h before the animals were sacrificed. The cell prolif-
eration was assessed by immunohistochemical staining with anti-
BrdU antibody as described previously (Lacy et al., 1991). Briefly, after being incubated with 0.5% H2O2 in methanol solution, tissue
sections were denatured for DNA in 2 N HCl, followed by a rinse in
0.1 M sodium borate, pH 8.6. Sections were subsequently digested in 0.1% trypsin (Sigma) solution. After washing in Tris/HCl-buffered saline and being incubated in 1.5% normal horse serum, anti-BrdU monochonal antibody (mouse IgG; Sigma) was applied overnight at 4°C. The sections were then incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA), followed by an applic-
ation of streptavidin-biotinylated horseradish peroxidase complex (Dako, A/S, Glostrup, Denmark). Diaminobenzidine tetrahydro-
chloride (Sigma) was used for color development. The sections were counterstained with Mayer’s hematoxylin.

The percentage of cells labeled with BrdU relative to the total number of mucosal cells at a field of vision of 0.142 mm2 (100× magnification) was counted in both sides of ulcer margin of the ulcer pit, via a Leica image processing and analysis system (Leica, Cam-
bridge, UK) for each rat and finally expressed as the labeling index
by the average of both margins.

**Determination of Angiogenesis at Ulcer Margin and Base.** Immunohistochemical staining of microvessels at the ulcer margin and base in the granulation tissue of submucosa was performed with von Willebrand factor antibody (Augustin et al., 1995). In brief, the tissue sections were incubated with 0.3% H2O2 in methanol and then trypsinized in 0.1% trypsin. After incubation in 1.5% normal goat serum, polyclonal rabbit anti-human von Willebrand factor antibody (Dako) was applied to the sections overnight at 4°C. The sections were then incubated with biotinylated goat anti-rabbit immunoglo-in antibody (Vector Laboratories). After rinsing in PBS, avidin-biotinylated peroxidase complex (ABC Elite; Vector Laboratories) was applied, and the antibody location was determined with a per-
oxidase reaction with diaminobenzidine tetrahydrochloride solution as chromogen.

The microvessels stained with the antibody were quantitated at the two sides of the ulcer margin and at the base of the ulcer pit in a microscopic field (0.8956 mm2) with the Leica image processing and analysis system. The number of blood vessels at the ulcer margin was expressed by taking the average of both sides of ulcer margin.

**Measurement of bFGF Level in Gastric Mucosa.** The gastric mucosal bFGF levels were quantitated by an enzyme-linked immu-
nosorbent assay (ELISA) measurement (Kaye et al., 1996) with a bFGF ELISA kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to the manufacturer’s instructions. The mucosal samples (100 mg) were homogenized (Ultra-Turrax, Staufen, Ger-
many) for 30 s under ice-cold conditions, followed by centrifugation (Beckman J2–21 centrifuge; Beckman Instruments, Berkeley, CA) at 20,000g for 20 min at 4°C. The supernatant was assayed for bFGF with the bFGF ELISA kit and then counted with an automatic microplate reader (Bio-Rad model 3500; Bio-Rad Laboratories, Rich-
mond, CA). The amount of bFGF was determined by comparison with a standard curve constructed on the same 96-well plate by use of a purified recombinant human bFGF and expressed as picograms per milligram of protein. Protein assay was performed with the method developed by Lowry et al. (1951).

**Determination of NOS Activity in Gastric Mucosa.** NOS activity in gastric mucosa was determined as described by Tepper-
man et al. (1993) from the conversion of [3H]-arginine to the NO coproduct [3H]citrulline (Knowles et al., 1990). Briefly, the mucosal samples, 100 to 150 mg, were homogenized for 20 s at 0°C in homogenizing buffer (pH 7.2) followed by centrifugation at 20,000g for 30 min at 4°C. The reaction mixture, comprised of 100 µl of supernatant and 150 µl of buffered solution (pH 7.2) containing 10 mM HEPES, 0.7 mM NADPH, 150 µM CaCl2, 7 mM L-valine to inhibit any arginase, and 1 µCi [3H]-arginine (1 mCi/ml; specific activity 36.1 Ci/mmol; New England Nuclear, Boston, MA) was incubated at 37°C for 30 min. For determining the activity of inducible NOS (iNOS), EGTA (1 mM) was used to inhibit the activity of calcium-dependent eNOS. After the reaction was stopped, the resulting mixture was applied to a column containing Dowex AG50WX-8 (Na form, Bio-Rad, Hercules, CA) resins. Scintillation fluid (Biodegradable Counting Scintillant; Amersham, Buckinghamshire, UK) was mixed with the eluent at a ratio of 9:1, and the mixture was counted with a scintillation counter (Beckman). The final result was expressed as picomoles of [3H]citrulline formed per minute per gram of protein.

Determination of EGF Concentration in Gastric Mucosa. Concentrations of EGF in the gastric mucosa were measured by radioimmunoassay according to the methods of Okita et al. (1991) and Zandomeneghi et al. (1992) with modifications. Mucosal samples of 100 mg were homogenized in 0.5 ml of 0.01 M PBS (pH 7.0) for 20 s at 0°C, followed by centrifugation at 20,000g for 20 min at 4°C. The assay was conducted in a mixture containing 100 µl of supernatant, 50 µl of 125I-labeled EGF (mouse, final dilution 1:2,000, -10,000 cpm; Amersham), and 50 µl of EGF antiserum (Amersham) for overnight incubation at room temperature. Afterward, the mixture was reacted with 0.1 M EDTA and 300 µl of anti-mouse EGF antiserum (Amersham) for 15 min at room temperature. After centrifugation at 10,000g for 10 min at 4°C, the pellets were monitored with an LKB 1442 gamma counter (Beckman) equipped with a radioimmunoassay calculating program. The final result was expressed as nanograms EGF per milligram of protein.

Assessment of Coagulation Function. The bleeding time was used as a determinant of coagulation function (Ogle et al., 1977). Rats were anesthetized under pentobarbitone sodium (Abbott Laboratories, Abbott Park, IL), and the abdomen was opened to expose the liver. A piece of liver was excised from the edge of a lobe, and then pieces of filter paper were dipped at 5-s intervals into the blood oozing from the cut surface until the endpoint was reached, indicated by a piece of blood clot clinging to the filter paper. The bleeding time was taken as the time elapsing between cutting the liver edge and the endpoint. Three separate consecutive readings were taken from three lobes in each rat, and the values were averaged.

Effects of Protamine Sulfate on Heparin. In a separate experiment, 40 mg/kg s.c. of protamine sulfate was used to neutralize 100 U/kg of heparin given once daily. Protamine sulfate was given immediately after heparin injection and 12 h after heparin for 4 days. The same parameters were measured as for heparin treatment alone.

Statistical Analysis. All data are presented as means ± S.E. Statistical analysis was performed with an ANOVA followed by Dunnett’s t test or Student’s two-tailed unpaired t test. p < .05 was considered statistically significant.

Results

Effect of Heparin on Gastric Ulcer Healing. The gastric ulcer areas in the vehicle control were spontaneously reduced with time after ulcer induction. Four days after heparin administration, the ulcer areas were smaller in all the drug-treated groups than in the control group. A significant difference, compared with the control, was found at the two higher doses. A similar effect was observed in rats after 7 days of heparin treatment. All three doses of heparin produced a significant decrease of ulcer area (Fig. 1).

Fig. 1. Effects of different doses of heparin given for 4 or 7 days on the healing of acetic acid-induced gastric ulcer in rats. Open columns represent groups without heparin treatment. Hatched, cross-hatched, and solid columns represent groups with heparin at doses of 100, 500, and 1000 U/kg, respectively. Each column represents the mean ± S.E. of eight animals. *p < .05, **p < .01, ***p < .001, versus corresponding group without heparin treatment.

Fig. 2. Effects of different doses of heparin given for 4 or 7 days on the regeneration (A) and thickness (B) of the gastric mucosa at ulcer margin. Open columns represent groups without heparin treatment. Hatched, cross-hatched, and solid columns represent groups treated with heparin at doses of 100, 500, and 1000 U/kg, respectively. Each column represents the mean ± S.E. of eight animals. *p < .05, **p < .01, ***p < .001, versus corresponding group without heparin treatment.

Effects of Heparin on Histological Changes. The gastric mucosa at ulcer margin regenerated with time in the control group after ulcer induction. Heparin dose-dependently accelerated this process, as reflected by an increase in


the length of regenerated gastric mucosa, and the significant effects were found in the two higher doses after 4 days and in the three doses after 7 days of treatment (Figs. 2a and 3, a and b). A similar effect of heparin was also observed on the thickness of gastric mucosa at the ulcer edge (Fig. 2b). However, the length of ruptured muscularis mucosae was not significantly affected by heparin regardless of duration of treatment (Fig. 4a). Heparin treatment increased the thickness of the ulcer base, but only the highest dose of heparin in the 7-day group had a significant effect compared with the corresponding control (Fig. 4b).

**Effect of Heparin on Gastric Mucosal Proliferation.** In the control group, the BrdU-labeling index was the highest on the first day of heparin therapy (0 day). It was then gradually decreased and maintained with the healing of ulcers. All three doses of heparin produced significantly higher BrdU-labeling indices after 4 or 7 days of treatment in a dose-dependent manner (Figs. 3, c and d, and 5).

**Fig. 3.** Photomicrographs of gastric mucosa. Mucosal regeneration at ulcer margin in control (a) and heparin (b) groups (40×, bars indicate length of the regenerated mucosa); immunohistochemical staining of BrdU at the ulcer margin in control (c) and heparin (d) groups; immunohistochemical staining of microvessels at the ulcer margin in control (e) and heparin (f) groups; microvessels at the ulcer base in control (g) and heparin (h) groups (c–h, 100×).
Effect of Heparin on Gastric Angiogenesis. The number of microvessels in the granulation tissues both at the ulcer margin and base was increased with time after ulceration. Heparin stimulated this response. At the ulcer margin, the microvessels were not only increased in number but also dilated after heparin treatment (Fig. 3, e and f). The degree of angiogenesis was increased after 4 or 7 days of treatment (Fig. 6a). At the ulcer base, the microvessel number after the three doses of heparin, regardless of the treatment duration, was also significantly higher than that in the control group (Figs. 3, g and h, and 6b).

Effect of Heparin on Gastric Mucosal bFGF Level. In the controls, ulcer induction increased the mucosal bFGF level. The three doses of heparin dose-dependently increased the gastric mucosal bFGF levels. These increases were more marked after 4 days of treatment, with a significant difference at the two higher doses, whereas significance was only reported at the highest dose after 7 days of treatment (Fig. 7).

Effect of Heparin on Gastric Mucosal EGF Level. The three doses of heparin treated for 4 or 7 days enhanced the gastric mucosal EGF levels in a dose-dependent manner. All three doses of the drug after 4 days or the two higher doses after 7 days of treatment significantly elevated the mucosal EGF (Fig. 8).

Effect of Heparin on Gastric Mucosal cNOS Activity. Gastric mucosal cNOS activity was activated with time after ulcer induction in the control group. Heparin significantly augmented this change after 4 or 7 days of treatment in a dose-dependent manner (Fig. 9). The gastric mucosal inducible NOS activity was also activated after ulcer induction, but it was not affected by heparin (data not shown).

Effect of Heparin on Blood Coagulation. The three doses of heparin dose-dependently and significantly prolonged the bleeding time 1 h after injection. The bleeding times of the control and the three doses of heparin were $48.3 \pm 3.9$, $82.5 \pm 6.7$, $127.5 \pm 5.1$, and $185.0 \pm 20.1$, respectively.

Effects of Protamine Sulfate on Pharmacological Actions of Heparin. In this experiment, 40 mg/kg of protamine sulfate was used together with the lowest dose of heparin (100 U/kg) for 4 days. This dose of heparin produced marginal effect on ulcer healing (Fig. 1). Protamine sulfate not only completely neutralized the anticoagulation action of heparin but also enhanced the ulcer-healing effect of heparin. The thickness of regeneration, the length of the ruptured muscularis mucosae, and the thickness of the ulcer base were not affected further by the drug. Protamine sulfate significantly potentiated the proliferative effect of heparin and increased the angiogenic effect of heparin on the gastric mucosa at both the ulcer margin and base. The mucosal cNOS activity was also significantly increased. Protamine sulfate, however, did not significantly enhance the stimulatory action of heparin on gastric mucosal EGF and bFGF levels (Table 1).

Discussion

The results of our study indicate that heparin accelerated the healing of gastric ulcer after treatment for 4 or 7 days. The histological study showed a dose-related and significant
increase in length of regeneration and thickness of gastric mucosa at the ulcer margin after heparin treatment (Fig. 2), which reflected the stimulatory action of heparin on mucosal regeneration and proliferation. However, the length of the ruptured muscularis mucosae, an indicator of contraction of ulcer base, was not significantly affected by heparin regardless of the dose and time of drug treatment (Fig. 4A). Because regeneration of gastric mucosa at ulcer margin and contraction of ulcer base played a pivotal role during the ulcer-healing process (Tarnawski et al., 1990; Ogihara and Okabe, 1993; Tsukimi and Okabe, 1994b), our results demonstrated that the healing effect of heparin on acetic acid-induced gastric ulcer was related to the increase in gastric mucosal regeneration but not to the contraction of ulcer base.

Tarnawski et al. (1990) indicated that the healing process of acetic acid-induced gastric ulcer involved migration of new cells from the ulcer margin and from the ulcer base in which the granulation tissue was transitionally turned into a scar and covered the ulcer with regenerated mucosa. In our experiment, heparin increased gastric mucosal regeneration and proliferation not only at the ulcer margin (Fig. 5) but also at the ulcer base (Fig. 4b). There was relatively less action on ulcer base, suggesting that heparin acted preferably on the ulcer margin to promote ulcer healing in the stomach.

The angiogenesis at the ulcer margin after heparin treatment (Fig. 6) showed a similar time course to that of gastric mucosal proliferation. This observation reflected that the gastric mucosal proliferation and angiogenesis stimulated by heparin at the ulcer margin took place in a synchronized manner. Increased microvessel formation could supply more oxygen and nutrients to support the tissue proliferation and maintain the migration of new cells from the ulcer margin into the ulcer crater, to fully cover or reepithelialize the ulcer base.
Combined effects of protamine sulfate (s.c. twice daily) and heparin (i.v. once daily) on the stomach during ulcer-healing process for 4 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (Vehicle)</th>
<th>Heparin (100 U/kg)</th>
<th>Heparin (100 U/kg) + Protamine (40 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bleeding time, s</strong></td>
<td>40.5 ± 3.7</td>
<td>81.7 ± 8.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.7 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td><strong>Uler area, mm&lt;sup&gt;2&lt;/sup&gt;</strong></td>
<td>77.8 ± 3.9</td>
<td>69.6 ± 3.9</td>
<td>59.2 ± 2.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>LRGM, mm</strong></td>
<td>0.51 ± 0.04</td>
<td>0.67 ± 0.02&lt;sup*a&lt;/sup&gt;</td>
<td>0.71 ± 0.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>TGM, mm</strong></td>
<td>0.67 ± 0.02</td>
<td>0.76 ± 0.02&lt;sup*e&lt;/sup&gt;</td>
<td>0.82 ± 0.03&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>LRMM, mm</strong></td>
<td>7.7 ± 0.4</td>
<td>7.9 ± 0.2</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td><strong>TUB, mm</strong></td>
<td>1.67 ± 0.09</td>
<td>1.69 ± 0.06</td>
<td>1.74 ± 0.04</td>
</tr>
<tr>
<td><strong>BrdU LI, %</strong></td>
<td>6.8 ± 0.8</td>
<td>10.2 ± 0.7&lt;sup&gt;e&lt;/sup&gt;</td>
<td>14.7 ± 0.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>MVN/mm&lt;sup&gt;2&lt;/sup&gt; (margin)</strong></td>
<td>76.8 ± 3.3</td>
<td>124.5 ± 4.9&lt;sup&gt;e&lt;/sup&gt;</td>
<td>149.6 ± 9.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>MVN/mm&lt;sup&gt;2&lt;/sup&gt; (base)</strong></td>
<td>77.1 ± 5.1</td>
<td>126.1 ± 10.2</td>
<td>151.8 ± 6.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>bFGF, pg/mg protein</strong></td>
<td>150.2 ± 12.1</td>
<td>168.1 ± 32.3</td>
<td>173.2 ± 28.6</td>
</tr>
<tr>
<td><strong>EGF, ng/mg protein</strong></td>
<td>0.35 ± 0.02</td>
<td>0.50 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.78 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>cNOS, pmol · min&lt;sup&gt;-1&lt;/sup&gt; · g&lt;sup&gt;-1&lt;/sup&gt; protein</strong></td>
<td>2.17 ± 0.37</td>
<td>3.54 ± 0.40&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.91 ± 0.28&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
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</table>

**LRGM**, length of regenerated gastric mucosa; **TGM**, thickness of gastric mucosa; **LRMM**, length of ruptured muscularis mucosae; **TUB**, thickness of ulcer base; **MVN**, microvessel number; **BrdU LI**, BrdU-labeling index. Results are presented as means ± S.E. of eight animals per group.

<sup>a</sup>p < .01, <sup>b</sup>p < .001, <sup>c</sup>p < .05, versus control.

<sup>e</sup>p < .01, <sup>f</sup>p < .001, <sup>c</sup>p < .05, versus heparin.

In contrast to the ulcer margin, the angiogenesis at the ulcer base continued even though the ulcer crater became smaller by epithelialization from the ulcer margin. This effect could be explained by the fact that granulation tissue continuously needed a favorable environment to grow until the ulcer crater was completely replaced by newly formed granulation tissue. Indeed, granulation tissue formation is an important component in the healing process because it supplies connective tissue cells for the restoration of lamina propria and endothelial cells for restoration of the microvasculature within the mucosal scar (Tarnawski et al., 1991). Completeness in granulation tissue formation at the ulcer base represents a good quality of ulcer healing.

Based on the above-mentioned findings, we studied the underlying mechanisms for these phenotypes. The results indicate that heparin increased the gastric mucosal bFGF level in a dose-dependent manner. It has been established (Folkman et al., 1991; Szabo et al., 1994; Schmassmann et al., 1995) that bFGF is a proliferating and angiogenic factor that has also been reported to accelerate healing of gastric and duodenal ulcers. Our findings suggest that the ulcer-healing effect of heparin was probably caused by an increase of bFGF level in the gastric mucosa. Although the exact mechanism of how heparin increased bFGF was not defined, it is likely that heparin could stabilize bFGF and act as a natural chaperone to shuttle bFGF from its stored site in the extracellular matrix to cellular compartments (Folkman and Shing, 1992b). Moreover, heparin could stimulate the proliferation of bovine aortic endothelial cells through activation of endogenous bFGF (Tazawa et al., 1993). Therefore, the stimulatory action of heparin on bFGF might induce cell proliferation and angiogenesis and promote granulation tissue formation as well as accelerate re-epithelialization at the ulcer site.

Considering the marked actions of heparin on cell proliferation and angiogenesis, which could not be fully explained by an increase in bFGF level in the gastric mucosa, we therefore investigated the effect of heparin on mucosal EGF, a known gastroprotective factor, to stimulate restitution and proliferation in gastric mucosal cells (Konturek et al., 1988, 1995; Tarnawski et al., 1991). Heparin treatment enhanced gastric mucosal EGF levels and promoted mucosal proliferation and angiogenesis, which could be correlated to the ulcer-healing effect of heparin. The underlying mechanisms of how heparin increases the gastric mucosal EGF level remain to be investigated.

We also studied the involvement of gastric mucosal NOS activities in ulcer healing. It was reported (Konturek et al., 1993; Brzozowski et al., 1997) that L-arginine, a substrate for NOS, accelerated ulcer healing and increased gastric blood flow and angiogenesis at the ulcer margin, whereas treatment with NG-nitro-L-arginine or NG-monomethyl-L-arginine, the inhibitors of NOS, resulted in a delay in ulcer healing and a reduction in blood flow at the ulcer margin and the number of capillaries in the granulation tissue at the ulcer bed. These results indicate that NO plays a key role in ulcer healing. Furthermore, NO produced by cNOS was considered to be beneficial in maintaining the mucosal integrity of the stomach (Lopez-Belmonte et al., 1993). In this study,
heparin significantly increased the gastric mucosal cNOS activity. The actions of NO derived from this enzyme not only contribute to ulcer healing but also work with EGF and bFGF to facilitate regeneration and proliferation as well as angiogenesis in the gastric mucosa. Although heparin could increase gastric mucosal cNOS activity, the exact isoform of cNOS, i.e., whether neuronal cNOS or endothelial cNOS is involved in these actions, remains to be further investigated.

Ma et al. (1999) demonstrated that endothelial cNOS could be detected in blood vessels and parietal cells in the gastric mucosa and submucosa by using anti-endothelial cNOS antibody. Furthermore, heparin markedly increased the number of microvessels in the same tissue, suggesting that this type of cNOS was, at least in part, related to the effect of heparin. However, the iNOS activity that was not affected by heparin (data not shown) might not participate in the action of heparin to promote ulcer healing.

Although the anticoagulation action of heparin is detrimental to gastric ulcer formation and healing, our data showed that a prolonged bleeding time after heparin treatment did not seem to impede its ulcer-healing property. Nevertheless, blockage of such anticoagulation action should be beneficial to ulcer healing. We used protamine sulfate, the antagonist of heparin, to study whether it could promote the ulcer-healing effect of heparin while the anticoagulant effect of heparin was neutralized. It was found that protamine sulfate not only completely neutralized the anticoagulation effect of heparin but also potentiated its ulcer-healing activity. Moreover, the drug also enhanced significant gastric mucosal proliferation, angiogenesis, and cNOS activity. These findings are interesting and suggest that protamine sulfate could potentiate the ulcer-healing effect of heparin through cNOS activation. We hypothesize that the action of heparin and protamine on NO synthesis is an independent process, because protamine itself was able to stimulate NO production (Li et al., 1996). The combination of both drugs would therefore produce more NO and further enhance ulcer healing.

In summary, we conclude that heparin could accelerate gastric ulcer healing, and this effect is mediated through the stimulation of mucosal bFGF, EGF, and cNOS followed by increases in regeneration and proliferation and by angiogenesis. Protamine sulfate neutralizes the anticoagulant action of heparin and potentiates its ulcer-healing effect through the enhancement of gastric mucosal cNOS activity.

References


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