Effects of H₂-Receptor Antagonists on Matrix Metalloproteinases in Rat Gastric Tissues with Acetic Acid-Induced Ulcer

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To investigate the destructive process of connective tissues in gastric ulcer, both collagenolytic and gelatinolytic activities were examined in the homogenates of rat acetic acid-induced gastric ulcer, a typical model of chronic ulcer. Gelatinolytic activity in the ulcerous lesion was significantly higher than that in the normal tissue. However, collagenase was not detected in both normal and ulcerated tissues either by the enzyme assay or by the immunoblotting. By gelatin-gel-zymographic analyses, the gelatinolytic activity was found to be composed of a number of species, mainly 60-, 72- and 92-kDa, all of which were inhibited by ethylendiaminetetraacetic acid. Among the induced matrix metalloproteinases, one crossreacted with a sheep anti-(rabbit prostromelysin)antibody. Thus, in chronic gastric ulcer, it is likely that several metalloproteinases participate in degradation of connective tissue matrices including components of basement membranes. The elevated levels of gelatinolytic activities in the ulcerous tissues and ulcer index were significantly suppressed by treating the animals with famotidine or a new H₂-receptor antagonist, 3-amino-4-[4-(1-piperidinomethyl)-2-pyridyloxoy]-cis-2-butenylamino]-3-cyclobutene-1,2-dione hydrochloride (IT-066).

Keywords — H₂-receptor antagonist; matrix metalloproteinase; gelatinase; stromelysin; gastric ulcer; acetic acid-induced ulcer

Introduction

Neutral matrix metalloproteinases (MMPs) are generally known to participate in physiological turnover and pathological destruction of various connective tissue matrix macromolecules. Mammalian tissue collagenases (MMP-1, EC 3.4.24.7) participate in the breakdown of interstitial collagens. During the progression of gastric ulcer, it is proposed that collagenase plays an important role in the destruction of extracellular matrix in ulcerous lesions. Collagenase activity has been found in the media of the cultured human gastric mucosa and in the homogenates of biopsy specimens of gastric tissues associated with ulcer or chronic gastritis.1-3 In addition to the destruction of interstitial collagens, degradation of basement membrane collagen, type IV collagen, in gastric tissue is considered to be important in the development of gastric ulcer.

Among MMPs characterized, 72-kDa gelatinase/type IV collagenase (MMP-2), stromelysin (MMP-3, EC 3.4.24.17) and 92-kDa gelatinase/type IV collagenase (MMP-9) are shown to degrade type IV collagen.4-9

In this report, we have examined the MMP activities in the gastric ulcerous tissue induced by acetic acid, a typical experimental model for chronic ulcer. In addition, we have tested the effect of new H₂-receptor antagonist, 3-amino-4-[4-(1-piperidinomethyl)-2-pyridyloxoy]-cis-2-butenylamino]-3-cyclobutene-1,2-dione hydrochloride (IT-066) on the ulcer-induced animals and examined then MMP activities characteristically increased in the gastric ulcer lesion of rats. IT-066 effectively suppressed the activity of MMPs along with ulcer index. The possible relationship of gelatinolytic enzymes elevated in gastric ulcerous tissue with well-characterized MMPs is discussed.

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Materials and Methods

Materials — Bacto gelatin for the gelatin-gel-zymography was purchased from Difco Lab., Detroit, MI., U.S.A. Alkaline phosphatase-conjugated donkey anti-(sheep immunoglobulin G (IgG))IgG, 5-bromo-4-chloro-3-indoyl phosphate and Nitro Blue Tetrazolium were from Sigma Chemical Co., St Louis, MO., U.S.A. Monospecificities of sheep anti-(rabbit synovial procollagenase)antibody and sheep anti-(rabbit synovial prostromelysin)antibody were demonstrated previously. Prior to the immunoblotting for procollagenase and prostromelysin, each antibody was confirmed for their crossreactivity with the corresponding antigen in culture media of rat skin fibroblasts stimulated with interleukin-1. Famotidine is a product of Yamanouchi Pharmaceutical Co., Chuo, Tokyo, Japan. IT-066, a new H2-receptor antagonist is synthesized by Taisho Pharmaceutical Co., Ltd., Ohmiya, Saitama, Japan. Other reagents used were the same as described previously.

Induction of Gastric Ulcer — Male Sprague-Dawley rats (SPF grade and approx. 250 g body weight) were used. According to the method of Takagi et al., the acetic acid-induced gastric ulcer was produced by the injection of 50 μl of 20% acetic acid into the subserosal layer at the junction of glandular stomach and antrum of the anterior wall. After the operation for the ulcer induction, animals were maintained in a temperature- and humidity-controlled room (22—24 °C and 50—60%), and allowed free access to food and water.

Morphologic Examination of the Acetic Acid-Induced Ulcer — The ulcerous tissues were fixed in 10% buffered-formalin, and paraffin sections were stained with hematoxylin and eosin.

Extraction of Metalloproteinases in the Gastric Tissues and Assay for Metalloproteinases — The tissues of the acetic acid-induced ulcerous regions were punched out in circular form with 10 mm diameter to make them similar in size. Extraction of metalloproteinases from ulcer tissues was carried out as follows: the tissue was homogenized in 10 volumes of 50 mM Tris-HCl/0.15 M NaCl/100 mM CaCl2 (pH 7.5) and then centrifuged at 10000 × g for 10 min at 4 °C. The supernatant was dialyzed against 9 volumes of 50 mM Tris–HCl/0.15 M NaCl (pH 7.5) and used as the enzyme source. Collagenolytic activity was measured by the reconstituted fibril assay using 14C-acetylated guinea pig skin type I collagen and gelatinolytic activity was also determined using heat-denatured 14C-acetylated collagen (gelatin) as described previously. One unit of enzyme is defined as the amount that hydrolyzes 1 μg of respective substrate/min at 37 °C. The total metalloproteinase activity was assayed after the activation of proenzymes with 1 mM 4-aminophenylmercuric acetate (APMA).

Protein Determination — This was performed by the method of Lowry et al. using bovine serum albumin as a standard.

Western Blotting for Collagenase and Stromelysin — Western blotting for collagenase and stromelysin in the ulcerated tissue homogenates was carried out as described by Ito and Nagase. Briefly, the samples concentrated with trichloroacetic acid were first subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% (w/v) acrylamide slab gel under reducing conditions, and then electrotransferred to a nitrocellulose filter. The filter paper was reacted with sheep anti-(rabbit synovial procollagenase)antibody or anti-(rabbit synovial prostromelysin)antibody which was then complexed with alkaline phosphatase-conjugated donkey anti-(sheep IgG)IgG. Immunoreactive enzymes were visualized indirectly using 5-bromo-4-chloro-3-indoyl phosphate and Nitro Blue Tetrazolium.

Gelatin Gel-Zymography — This zymography was performed using a 0.03% (w/v) gelatin–12.5% (w/v) acrylamide slab gel as described by Hibbs et al. After electrophoresis, gels were first washed to remove SDS with 50 mM Tris–HCl/5 mM CaCl2/1 μM ZnCl2/2.5% (v/v) Triton X-100/0.02% (w/v) NaN3 (pH 7.5) for 1 h and then incubated in the same buffer without Triton X-100 at 37 °C for 18 h. Proteins were stained with Coomassie Brilliant Blue R250.

Effect, in Vivo, of H2-Receptor Antagonists
Metalloproteinases in Rat Gastric Ulcer

Fig. 1. Microphotographs of Rat Gastric Ulcers Observed on the 5th Day after the Injection of 20% Acetic Acid
A, intact gastric tissue of Sprague-Dawley rat (original magnification, ×90), and B, ulcerous tissue at 5th day after the acetic acid-injection (×90) and C, mucosal layer of ulcerous tissue in panel B (×360). Tissues were stained with hematoxylin-eosin.

on the Gelatinolytic Activity in the Acetic Acid-Induced Ulcer — After 24 h operation for the ulcer induction, oral administration of IT-066 or famotidine (5 mg/kg body weight) was started and continued once a day for 14 d. Control animals were administered the vehicle similarly.

The animals were killed 24 h after the final administration. After the estimation of ulcer index, tissues of the ulcerated portion were punched out for the assay of gelatinolytic activity as described above.

Fig. 2. Gelatinolytic Activities in Stomach Tissues after Gelatin-SDS-PAGE
Enzyme preparations were first incubated with 1 mM APMA in 50 mM Tris–HCl/0.15 mM NaCl/10 mM CaCl₂/0.02% (w/v) NaN₃ (pH 7.5) or the same buffer for 30 min at 37°C, and then mixed with the same volume of non-reducing sample buffer. Each 10 μl of the sample was subjected to SDS-PAGE. After electrophoresis, gels were washed with 50 mM Tris–HCl/5 mM CaCl₂/1 μM ZnCl₂/2.5% Triton X-100/0.02% (w/v) NaN₃ (pH 7.5) to remove SDS from gels. Then the gels were incubated in the same buffer without Triton X-100 at 37°C for 18 h. Lanes 1, 3 and 5 are 5th, 10th and 15th day’s samples after the acetic acid injection, respectively, and lanes 2, 4 and 6 are those samples with the APMA-treatment.
Results

Morphologic Observation for the Acetic Acid-Induced Ulcer

Figure 1 shows the histological observations of rat gastric tissues after the 5 d-acetic acid injection. Destruction and partial lack of mucus layer were observed (Fig. 1B) and exudation of numerous polymorphonuclear leukocytes into the basal layer was the other characteristic change observed in ulcerous sites (Fig. 1C). These changes, but with less extent are still observed at day 10 after the acetic acid injection. These morphologic observations are very similar to those reported by Takagi et al. 13

Characterization of Gelatinolytic Activities in Rat Acetic Acid-Induced Ulcer

To characterize extracellular matrix-degrading metalloproteinases in the acetic acid-induced ulcerous regions in the gastric tissue, both gelatinolytic and collagenolytic activities were measured in the homogenates of normal and ulcerous tissues. Figure 2 shows gelatinolytic activities detected by zymography. The normal tissues showed low levels of gelatinolytic activity around 60- and 72-kDa. On the 5th, 10th and 15th day after the acetic acid injection, gelatinolytic activity in the ulcerous lesion was obviously higher than that in normal tissues, and the considerably high level of activity was observed with ulcerous regions at day 5 and 10 after induction of ulcer. Further studies are needed to clarify the precise changes in gelatinolytic activity in the progressing and repairing phases of gastric ulcers. Figure 2 also indicates that the gelatinolytic activity in the lesion was composed of several major species of enzymes with molecular mass of 60-, 72- and 92-kDa. Some of them were found to be proenzymes since their molecular masses decreased upon APMA treatment (Fig. 2). All the gelatinolytic activities were completely inhibited by ethylenediaminetetraacetic acid (EDTA). Data not shown, indicating that the enzymatic activities detected by zymography in both normal and ulcerated tissues are due to metalloproteinases.

These gelatinolytic enzymes in the ulcerous tis-

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**Table I. Effects of IT-066 and Famotidine on the Acetic Acid-Induced Gastric Ulcers**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Ulcer index (mm²)</th>
<th>Gelatinolytic activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Active form (munits/100 mg wet wt)</td>
</tr>
<tr>
<td>Control</td>
<td>9</td>
<td>19.6±9.5</td>
<td>278.4±130.4 1430.9±307.8</td>
</tr>
<tr>
<td>IT-066</td>
<td>11</td>
<td>7.6±7.5b</td>
<td>143.8±49.0 1000.5±187.6b</td>
</tr>
<tr>
<td>Famotidine</td>
<td>13</td>
<td>6.3±4.1b</td>
<td>184.1±53.3 1075.8±411.3</td>
</tr>
</tbody>
</table>

After 24 h laparotomy for the ulcer induction, oral administration of IT-066 or famotidine (5 mg/kg body weight) was started and continued once a day for 14 d. The animals were killed 24 h after the final administration. After estimation of the ulcer index, tissues of the ulcerated portion were punched out. Preparation of enzyme extracts and assay for gelatinolytic activity were done as described in the text. Values are shown as the mean ± S.D. a, b Significantly different from untreated control (p<0.05 and 0.01).
sues were further characterized by Western blotting. As shown in Fig. 3, an antigen that crossreacted with anti-(rabbit prostromelysin)antibody was detected only in the ulcerous tissues at day 5 and 10 after the induction of ulcer. Takagi et al. have reported the progression and healing process of acetic acid-induced ulcer, in detail, and shown by means of histological observation and ulcer index that the most severe tissue destruction in the ulcerous regions is observed on the 5th day after the operation, and then the ulcer recovered gradually.\(^{13}\) However, we confirmed the high levels of gelatinolytic activities and stromelysin(-like enzyme) in ulcerous tissues even on the 10th day, repairing phase, after the operation. These results indicate that stromelysin or a stromelysin-like enzyme and gelatinolytic enzymes mainly participate in the destruction of the tissues in chronic gastric ulcer since they can hydrolyze type IV collagen, a main component of basement membranes, and that they also accelerate the wound healing to remove tissue debris during the repairing process of the ulcer. On the other hand, collagenase was not detected in both normal and ulcerous regions either by the collagen fibril assay or by the Western blotting. Nevertheless the enzyme was identified in human gastric tissues.\(^{1-3}\) This difference might have been due to a low amount of detectable collagenase presented in the homogenates of rat ulcerous tissue, or to a coexistence of excess amount of metalloproteinase inhibitor(s) presented in the homogenates, and/or a low sensitivity of anti-(rabbit collagenase)antibody used. More sensitive methods may be needed to clarify the above discrepancy in the future.

**Suppression of the Gelatinolytic Activities in Acetic Acid-Induced Ulcer in the H\(_2\)-Receptor Antagonist-Treated Animals**

Effects of H\(_2\)-receptor antagonists, IT-066 and famotidine, on animals with the acetic acid-induced gastric ulcer were evaluated in terms of ulcer index and gelatinolytic activities in the lesion. As shown in Table I, repeated oral administration of both IT-066 and famotidine significantly decreased the ulcer index, and these drugs effectively reduced both active and total gelatinolytic activity in the ulcerous lesion as compared with the untreated control. As demonstrated by gelatin zymography, the suppressive effect of H\(_2\)-receptor antagonists was primarily on 72- and 92-kDa gelatinolytic enzymes (Fig. 4).

**Discussion**

In this communication, we have demonstrated that MMP activities were characteristically increased in the acetic acid-induced gastric ulcers, and one of them crossreacted with anti-(rabbit prostromelysin)antibody. The latter enzyme is likely to be similar, if not identical, to MMP-3. These observations are important to understand the destructive nature of the tissue in chronic gastric ulcers, since stromelysin is known to degrade basement membrane type IV collagen, more effectively than 72-kDa gelatinase.\(^{8,18}\) The destruction of basement membranes is recognized to be one of the major features during the development of ulcer in gastric tissue.

To date, the presence of two kinds of gelatinases with molecular masses of about 72- and 92-kDa has been reported. Both enzymes have the ability to degrade type IV collagen. The former is produced mainly by connective tissue cells such as fibroblasts and epithelial cells,\(^{19,20}\) and the latter is found in polymorphonuclear leukocytes,\(^{17,21-23}\) macrophages\(^{24}\) and metastatic tumor cells,\(^{25-28}\) although some connective tis-
Tissue cells produce 92-kDa gelatinase as well as 72-kDa species. In the acetic acid-induced gastric ulcers in rats the levels of both 72- and 92-kDa species were significantly elevated as compared with those in the normal tissues. We have not clarified the origins of the 92-kDa gelatinase, but it may be derived primarily from inflammatory cells since our morphologic observation demonstrated the infiltration of a large number of polymorphonuclear leukocytes into acetic acid-induced ulcer tissues (Fig. 1). However, it is also possible that the connective tissue cells under these pathological conditions may produce both 92- and 72-kDa gelatinases.

Repeated oral administration of IT-066 and famotidine depressed the apparent activity of metalloproteinase in the ulcerated tissues as well as ulcer index. The mechanism of action, however, of these H2-receptor antagonists in the present study is obscure. It may be that the lowered gelatinolytic activities by these antagonists accelerate the healing process of gastric ulcer and/or associate with the acceleration of repairing ulcerous tissue. In this point of view, Takagi et al. suggest that the inhibition of gastric acid or pepsin secretion may not accelerate the healing of the acetic acid-induced ulcer, and the agents stimulating the epithelization or granulation of the gastric tissue exert an obvious acceleration of the repair in the early phase of ulceration. Further studies, however, are needed to explain the action mechanism of H2-receptor antagonists on the suppression of metalloproteinases in the ulcerous tissues.

In conclusion, we have provided the first evidence that gelatinolytic activities and stromelysin are characteristically identified and increased in acetic acid-induced gastric ulcers, and that oral administration of H2-receptor antagonists significantly depressed their activities as well as the ulcer index. These observations suggest that quantification of gelatinolytic activity in ulcerous lesions may be an additional useful parameter for the evaluation of the curative effects of drugs.

References

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