MECHANISM BY WHICH INDOMETHACIN DELAYS THE HEALING OF ACETIC ACID-INDUCED ULCERS IN RATS. ROLE OF NEUTROPHIL ANTICHEMOTACTIC AND CHEMOTACTIC ACTIVITIES

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We have recently discovered a neutrophil antichemotactic factor in the normal gastric mucosa of rats. In this study, we examined whether this antichemotactic factor as well as chemotactic factors are involved in the indomethacin-induced delayed healing of experimental gastric ulcers. Ulcers were induced in male rats by a submucosal injection of 20% acetic acid into the gastric wall. Experimental rats received a subcutaneous injection of indomethacin at 2 mg/kg once daily for 28 days, starting at day 0 of ulceration (defined as 5 days after acetic acid injection). In the control group (without indomethacin) antichemotactic activity was not detected in the ulcerated tissues on day 0, but gradually increased for up to 28 days. This activity did not recover in the indomethacin-treated group by day 28. However, a high level of neutrophil chemotactic activity was observed in the extract of the ulcerated area in day 0. This activity gradually declined as the ulcers healed. In the control group, chemotactic activity was negligible after dialysis of the extract. In the indomethacin-treated group, however, chemotactic activity was maintained from the 10th day after treatment, even after the extract was dialyzed. Similar to chemotactic activity, myeloperoxidase activity was also augmented significantly in the indomethacin-treated group throughout the experiment. We conclude that the mechanism by which indomethacin delays ulcer healing is associated with reduced antichemotactic activity and increased chemotactic activity in the ulcerated tissues, resulting in the persistence of neutrophil infiltration.

Key words: acetic ulcer, indomethacin, antichemotactic activity, chemotactic activity, neutrophil infiltration

INTRODUCTION

Various nonsteroidal antiinflammatory drugs, are recognized as initiators of gastric ulcer formation and delayed ulcer healing (1, 2). Of these, indomethacin has also been shown to markedly delay the healing of experimental gastric ulcers (3, 4). The underlying mechanism seems to involve
reduced endogenous gastric mucosal prostaglandins (PG) (3), reduced mucosal blood flow at the ulcer margin (5), inhibited proliferation of gastric epithelial cells (6), and inhibited contraction of the ulcer base (7). To note, a marked infiltration of neutrophils has been observed in the rat gastric mucosa with acetic acid-induced ulcers (8). In addition, reduction of neutrophils in the ulcerated area by hydroxyurea (200 mg/kg/day, 2 wk) significantly facilitated ulcer healing. Thus, neutrophil activation is considered to play a crucial role in the aggravation of gastric ulcers.

To elucidate the role of neutrophil modulation we utilized a neutrophil antichemotactic factor (60 k-glycoprotein) which we have recently discovered in the gastric mucosa of normal rats (9). This neutrophil antichemotactic activity was measured throughout the course of indomethacin-delayed healing and spontaneous healing of gastric ulcers in rats. Chemotactic activity was also incorporated for comparison with antichemotactic activity, and we examined their responses as spontaneous and delayed healing progressed.

MATERIALS AND METHODS

Induction of gastric ulcers

Gastric ulcers were induced in male Donryu rats (Nihon SLC, Hamamatsu, Japan), weighing 260—280 g, by a previously reported method (3, 10). Under ether anesthesia, the abdomen was incised and the stomach exposed. Acetic acid (20%, 0.04 ml) was then injected into the submucosal layer between the antrum and corpus of the anterior gastric wall. After closure of the abdomen, the animals were maintained in a usual manner. Rats were killed at 7, 10, 14, and 28 days, and the stomachs were excised and opened along the greater curvature. After spread on a cork board, the ulcerated area (mm²) was then determined under a dissecting microscope (X10; Olympus, Tokyo, Japan). Since deep and well-defined ulcers were observed 5 days after the acid injection, we defined the 5th day as the day of ulceration (day 0). Indomethacin (Sigma, St Louis, MO), suspended in Tween 80/saline, was administered s.c. at 2 mg/kg once daily for 28 days from day 0.

Preparation of gastric extract from the ulcerated tissues

The animals were anesthetized with ether and perfused systemically via the heart with saline (200 ml). Their stomachs were excised, opened along the greater curvature, and washed with saline. The ulcerated portion was punched out with a cork borer (10 mm, ID), and then homogenized with 10 mM sodium phosphate buffer (PB; pH 7.2) containing 1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 20,000 g for 60 min and the supernatant was recovered as gastric extract. In order to detect high molecular weight (M.W.) chemoattractants, the extract was dialyzed against 10 mM PB through a cut off membrane (M.W. < 10,000). The extracts before and after dialysis were used for chemotaxis assay.
To determine the antichemotactic activity, the extract was applied to a Wheat Germ Lectin (WGL)-Sepharose 6MB column (Pharmacia Biotec, Uppsala, Sweden) preequilibrated with 10 mM PB containing 0.5 M NaCl. The column was washed with the equilibration buffer; then the absorbed materials were eluted with 0.1 M N-acetyl glucosamine (Glc NAc) at a flow rate of 0.2 ml/min. The active fraction from WGL-Sepharose was used for determination of antichemotaxis.

Isolation of neutrophil

Rat peritoneal neutrophils were purified according to the methods of Watt et al. (11) and Kudo et al. (12). In brief, 15 ml of 3% casein (Nacalai Tesque, Kyoto, Japan) was injected into the peritoneal cavity of a rat. Fifteen hours later, peritoneal exudated cells were collected and suspended in a Krebs-Ringer solution containing 1% glucose. The cells were treated with hypotonic buffer to lyse erythrocytes and washed twice with Krebs-Ringer solution. The cells were then placed on a Percoll density gradient and centrifuged at 1,500 x g for 15 min. Cells at the boundary between the specific gravities of 1.04 and 1.08 were collected and washed with Dulbecco modified eagle's medium (MEM) containing 1% bovine serum albumin. Both the purity and viability of the neutrophils obtained were over 95%, as estimated by Diff Quick staining (International Reagents, Kobe, Japan) and the trypan blue dye exclusion test (13) respectively.

Determination of chemotaxis

The chemotaxis assay was performed following the modified Boyden’s method (14), using a membrane filter of 5 mm pore size (Neuro Probe Inc., Cabin John, MD). The gastric extract or vehicle (10 mM PB) was added to the lower compartment of the chemotaxis chamber. Neutrophils were placed in the upper compartment at 1 x 10^6 cells/well in MEM. To determine the antichemotactic activity, neutrophils were mixed with the extract for 5 min before assaying the formyl-methionyl-leucyl-phenylalanine (Sigma) (FMLP) induced chemotaxis. The chamber was incubated for 60 min at 37°C to allow neutrophil migration. The filter was then removed and the cells were stained with Diff Quick. Neutrophils, which migrated to the lower face of the filter, were counted under a light microscope (x 400; Olympus, Tokyo, Japan) in five randomly chosen fields. In preliminary experiments, we examined the maximal effect of FMLP on neutrophil migration. FMLP caused neutrophil chemotaxis in the ranges of 5 nM to 100 nM; the maximal response was observed at 50 nM FMLP. Therefore, we used FMLP at 50 nM in the present study. Data is expressed as an average number for each of the five fields.

Determination of myeloperoxidase activity

Myeloperoxidase (MPO) activity was determined by the method of Krawisz et al. (15). In brief, a portion of the ulcerated tissue was punched out with a cork borer (10 mm, I.D.), homogenized in 50 mM PB (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma), and freeze-thawed three times. The homogenate was then centrifuged at 1,600 x g for 10 min. MPO activity in the supernatant was assayed spectrophotometrically at 450 nm using a microplate reader (THERMO Max; Molecular Devices, Sunnyvale, CA) after the addition of 0.167 mg/ml o-dianisidine dihydrochloride (Sigma) and 0.0005% H_2O_2. The activity was expressed as μ mol H_2O_2 per min per g tissue. Horseradish peroxide (Sigma) was used as a standard.
Histological studies

Gastric tissues were fixed in 5% paraformaldehyde and 10 μm-frozen sections were mounted on zelatin-coated glass slides. Sections were washed in phosphate-buffered saline containing 3% Triton-X100 and then stained with 3,3'-diaminobenzidin (DAB; Dojindo Laboratories, Kumamoto, Japan) in the presence of 0.005% H₂O₂.

Neutrophil counting

To assess numbers of neutrophil in the blood of normal rats and ulcerated rats with or without indomethacin treatment, 50 μl of blood from each animal was mixed with 1% crystal violet (Merck, Darmstadt, Germany) dissolved in 3% acetic acid. Cells were then counted using a hemocytometer (Erma, Tokyo, Japan) and expressed as cells per ml of blood.

Determination of PGE₂ production by the ulcerated tissues

PGE₂ production assay was performed according to the method of Lee and Feldman (16). Three hours after the administration of indomethacin, the animals were killed and their stomachs were excised. The ulcerated portion was punched out with a cork borer (10 mm, ID), placed in ice-cold 50 mM Tris-HCl (pH 8.4) buffer, and then finely minced with scissors. The minced tissues were transferred into microtubes and centrifuged at 10,000 x g for 15 seconds; the supernatants were discarded. After 1 ml of the buffer was added to the tubes, each tube was subjected to vortex mixing at room temperature for 1 min to stimulate PGE₂ production, followed by centrifugation at 10,000 x g for 15 seconds. The amounts of PGE₂ in the resulting supernatants were determined by enzyme immunoassaying (PGE₂ EIA kit; Cayman Chemicals, Ann Arbor, MI). PGE₂ production was expressed as pg PGE₂/mg tissue/min.

Statistical analysis

Statistical differences were evaluated using the Student's t-test, with a P value of < 0.05 regarded as significant.

RESULTS

Healing of gastric ulcers in rats

Round and deep ulcers were observed in all rats, with a mean ulcerated area of 40.7 ± 2.9 mm² (n = 8) on the day of ulceration (day 0). Ulcers spontaneously healed with time, the ulcerated areas on days 7, 10, 14, and 28 averaging 19.9 ± 2.2 mm², 20.0 ± 3.1 mm², 5.7 ± 1.2 mm², and 2.1 ± 0.5 mm² (n = 6—8) respectively (Fig. 1). Daily administration of indomethacin for 7 days had no effect on ulcer healing; but, treatment for 14 and 28 days significantly delayed the ulcer healing, the ulcerated areas decreasing to 18.1 ± 1.8 mm² and 17.2 ± 3.1² (n = 8) respectively.
Fig. 1. Time course changes in the healing of acetic acid-induced gastric ulcers in rats. Indomethacin (2 mg/kg) was s.c. administered once daily for 28 days starting from day 0 after ulceration. Data are means ± 1 S.E.M. for 6—8 rats. * Significantly different from the control group at P < 0.05.

**Neutrophil antichemotactic activity in the ulcerated tissues**

Neutrophil antichemotactic activity was observed in the mucosa of normal rats at a value of 71.0 ± 5.5% (% inhibition of FMLP-stimulated migration). Antichemotactic activity was markedly reduced by ulceration, the value at day 0 being −3.5 ± 6.6%. In the control group, the reduction of antichemotactic activity returned to the normal level as the gastric ulcers healed; its activity on day 14 was almost the same as the normal value (Fig. 2). In contrast, a low level...
of antichemotactic activity persisted in the indomethacin-treated group. From day 7, the antichemotactic activity in the indomethacin-treated group was significantly lower than that of the control group, at values below 30%. Indomethacin administered to normal rats for 14 days at 2 mg/kg did not affect antichemotactic activity in the normal mucosa (data not shown).

**Neutrophil chemotactic activity in the ulcerated tissues**

The extract from the gastric mucosa of normal rats did not promote neutrophil chemotaxis (data not shown). The extract from the ulcerated tissues on day 0 significantly promoted neutrophil chemotaxis by nearly 5-fold, compared with the vehicle alone (Fig. 3A). In the control group, neutrophil migration was significantly stimulated by treatment with the extract on day 7, with a percent activity of 247.2 ± 19.0%. Thereafter, the extract did not significantly induce neutrophil chemotaxis. In contrast, in the indomethacin-treated group, chemotactic activity in the extract was clearly observable until day 28, the activity on days 10, 14, and 28 averaging 285.4 ± 17.8%, 244.1 ± 15.4%, and 280.2 ± 15.2%, respectively. When indomethacin was administered at 2 mg/kg once daily for 14 days to normal rats (without ulcers), the gastric extract did not exhibit chemotactic activity (data not shown).

![Fig. 3. Time course changes in neutrophil chemotactic activity during spontaneous and indomethacin-delayed ulcer healing in rats. Neutrophil chemotactic activity in the extract of ulcerated tissues (A) and after dialysis of the extract (B). Data are expressed as percentages of the vehicle and are means ± 1 S.E.M. for 6—8 rats. * or #: Significantly different from the vehicle (extraction buffer) or control group, respectively at P < 0.05.](image)

After dialysis of the extract, chemotactic activity was remained negligible throughout the 28 days in the control group (Fig. 3B). In the indomethacin-treated group, chemotactic activity was negligible through day 7.
However, chemotactic activity was observed on days 10, 14, and 28; with values of $168.6 \pm 13.5\%$, $310.5 \pm 43.2\%$, and $301.6 \pm 71.0\%$ respectively. There were significant differences between the control and indomethacin-treated groups on these days.

**MPO activity in the ulcerated tissues**

MPO activity was negligible in the gastric mucosa of normal rats (6.8 $\pm$ 1.2 $\mu$mol H$_2$O$_2$/mg tissue per min). Its activity was markedly elevated by gastric ulceration, the value in the ulcerated tissue in day 0 being 485.2 $\pm$ 45.4 $\mu$mol H$_2$O$_2$/mg tissue per min ($n = 6$). This activity spontaneously declined to 71.9 $\pm$ 18.6, 49.5 $\pm$ 14.6, and 33.2 $\pm$ 7.4 $\mu$mol H$_2$O$_2$/mg tissue per min ($n = 6$) on days 7, 10, and 14, respectively (*Fig. 4*). However the reduction in MPO activity in the indomethacin treatment group was significantly lesser than the control group at all points (124.7 $\pm$ 27.2, 121.2 $\pm$ 23.5, and 124.1 $\pm$ 20.5 $\mu$mol H$_2$O$_2$/mg tissue per min, $n = 6$).

Fig. 4. Effect of repeated administrations of indomethacin (2 mg/kg, s.c.) on myeloperoxidase (MPO) activity in the ulcerated tissues of rats over a 28 day period. Data are means $\pm$ 1 S.E.M. for 6 rats. Note that indomethacin significantly increased MPO activity. * or #: Significantly different from vehicle or control group, respectively at $P < 0.05$.

**Histological analysis**

Although DAB-positive cells were not found in the gastric mucosa of normal rats, they appeared in the ulcerated gastric tissue (*Fig. 5A*). In the control group, the number of DAB-positive cells decreased as the ulcers healed. In contrast, treatment with indomethacin inhibited the reduction of DAB-positive cells from day 10. Numerous DAB-positive cells were observed in the ulcer base and margin of the indomethacin-treated group in comparison with the control (*Fig. 5B*).
Fig. 5. Microscopic observation of the ulcerated area in rats on day 10 after ulceration. Note that while there were a few DAB-positive cells in the ulcerated area of a control rat (A), there was an abundance of cells in the submucosa and ulcer base in an indomethacin-treated rat (B).

Number of neutrophil in blood

The numbers of circulating neutrophil were $4.3 \times 10^6$ cells/ml in normal rats and $4.4 \times 10^6$ cells/ml in rats with ulcers on day 14. Treatment with indomethacin for 14 days did not affect the numbers of neutrophil in blood.
either in normal rats ($4.5 \times 10^6$ cells/ml) or in rats with ulcers ($4.6 \times 10^6$ cells/ml).

**Production of PGE$_2$ by ulcerated tissues**

Production of PGE$_2$ was $69.1 \pm 6.6$ pg/min/mg in normal rats. In control rats, production of PGE$_2$ in ulcerated tissues was elevated compared with that in normal tissues, the values at day 7 and 14 with values of $275.8 \pm 36.5$ pg/min/mg and $119.9 \pm 18.4$ pg/min/mg respectively. Treatment with indomethacin significantly reduced mucosal PGE$_2$ production, the values at day 7 and 14 being $38.8 \pm 11.4$ pg/min/mg and $13.4 \pm 3.3$ pg/min/mg, respectively.

**DISCUSSION**

This data clearly shows that although antichemotactic activity was reduced in the ulcerated tissues on day 0, it gradually increased as the ulcers healed in the control group. Yet significantly, the activity remained low for up to 28 days in the indomethacin-treated rats with delayed ulcer healing. It is hard to determine whether this decreased antichemotactic activity is related to the unhealed gastric mucosa or the potential suppressive response to indomethacin. As expected, a high level of chemotactic activity was observed in the ulcerated tissue on day 0. The activity gradually declined as the ulcers healed spontaneously. In the indomethacin-treated group, however, this reduction in chemotactic activity was significantly prevented. These findings suggest that the persistence in activated neutrophil chemotaxis around ulcers is partly involved in the causal mechanism of indomethacin-delayed ulcer healing.

Some groups (17, 18) reported that lipid mediators such as leukotriens (LTs) and platelet activating factor (PAF) function as chemotactic factors, recruiting phagocytes to the site of damage, resulting in an increased susceptibility of the mucosa to injury. Cytokine-induced neutrophil chemoattractant (CINC), a chemotactic molecule of the interleukin 8 (IL-8) family, reportedly causes dermal and pleural inflammation in rats (19, 20). Although CINC has not been reported to cause gastric mucosal inflammation, it is possible that CINC levels are elevated in the ulcerated tissues. The molecular weights of LTs, PAF, and CINC are all < 10,000. In the present study, neutrophil chemotactic activity during spontaneous ulcer healing completely disappeared after dialysis, suggesting that its activity is mainly due to low molecular substances such as these. In contrast, activity in the indomethacin-treated group on days 14 and 28 was not affected by dialysis,
suggesting that the persistent increase in chemotactic activity in response to indomethacin is caused by substances with molecular weights > 10,000.

Appleyard et. al (21) reported that tumor necrosis factor (TNF)-α (> 10,000 in molecular weight), having neutrophil chemotactic activity, was significantly increased in the indomethacin-damaged gastric mucosa in rats. In addition, Kozol et al. (22) discovered a neutrophil chemotactic factor whose molecular weight was > 100,000 in rabbit gastric tissue. Our group recently found that the mRNAs of extracellular matrix proteins, such as collagen and fibronectin, were highly expressed in the ulcerated tissues of rats treated with indomethacin for 10 days (unpublished data). Fibronectin (> 10,000 in M.W.), a component of proteoglycan, reportedly stimulates neutrophil chemotaxis in a similar manner as IL-8 (23). Therefore, it is probable that the above factors are involved in the persistence of neutrophil activation in the ulcerated tissues of indomethacin-treated animals. At present, the mechanism by which high molecular chemotactic substances respond to indomethacin in ulcerated tissues remains unknown.

Neutrophil activation results in the production of reactive oxygen species, MPO, and proteases which exert a noxious effect on the gastric mucosa (24). Increased MPO activity is considered to reflect an increase in granulocyte numbers (15). Therefore, we investigated the relation of MPO activity to the delayed healing of gastric ulcers caused by indomethacin. As expected, MPO activity on day 0 was significantly greater in ulcerated tissues than in normal tissues. Increases in MPO activity gradually declined as the ulcers healed. On the other hand, repeated treatment with indomethacin significantly sustained an increased level of MPO activity for 28 days. At day 10, a histological study showed that DAB positive cells, most likely neutrophils, increased in the ulcerated areas of indomethacin-treated rats. These findings suggest that sustained inflammation at the ulcerated areas may worsen the healing of ulcers in indomethacin-treated animals.

It is reported that the numbers of neutrophil in gastric microcirculation in normal rats increases with > 10 mg/kg of indomethacin treatment (25). Our present data shows that the numbers of circulating neutrophil does not change with the induction of the gastric ulcers. Furthermore, we demonstrate that treatment with 2 mg/kg of indomethacin for 14 days does not affect the numbers of neutrophil in blood either in normal rats or ulcerated rats. We attribute this disparity in data to the difference in the dosage of indomethacin applied. In fact, our results suggest that indomethacin at the dose of 2 mg/kg does not stimulate the growth or differentiation of precursor cells of neutrophil. Taken together, promoted infiltration of neutrophil into the ulcerated tissues in the indomethacin-treated rats is not due to an increase in blood neutrophil.

We have previously reported that indomethacin causes delayed ulcer healing through inhibition of mucosal PGE₂ production and that
exogeneously administered PGE₂ inhibits indomethacin-delayed ulcer healing (3). These results indicate that the deficiency of mucosal PGs might result in the delay of ulcer healing. In this study we reconfirmed our previous finding that indomethacin significantly reduced the production of PGE₂ in ulcerated tissues. With the persistent inhibition of PGE₂ production in the ulcerated tissues by indomethacin, the chemotactic activity, derived from a high molecular weight substance, increased, while antichemotactic activity did not recover. We have proposed that the delay in ulcer healing by indomethacin is due primarily to inhibition of PG production in the ulcerated tissues. In other words, inhibition of PG production is regarded as an initiator of the indomethacin-induced delay of ulcer healing. In addition of PGs deficiency, the chemotactic factor, having a high molecular weight, and the antichemotactic factor is strongly involved in the delayed ulcer healing caused by indomethacin. Additionally, the extract, prepared from normal rats treated with 2 mg/kg of indomethacin for 14 days did not exhibit the increase in chemotactic activity or the decrease in antichemotactic activity. Therefore, it is unlikely that the increase in the chemotactic activity and the decrease in the antichemotactic activity is related to the depletion of PG production.

We conclude that, in addition to endogeneous deficiency of PGs, decreased antichemotactic activity as well as increased chemotactic activity in the ulcerated tissues greatly contribute to delayed ulcer healing in indomethacin-treated rats.

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