ROLE OF MACROPHAGE COLONY-STIMULATING FACTOR (M-CSF)-DEPENDENT MACROPHAGES IN GASTRIC ULCER HEALING IN MICE

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We examined the role of macrophage colony-stimulating factor (M-CSF)-dependent macrophages in the healing of gastric ulcers in mice. Male M-CSF-deficient (op/op) and M-CSF-expressing heterozygote (+/?) mice were used. Gastric ulcers were induced by thermal cauterization under ether anesthesia, and healing was observed for 14 days after ulceration. The numbers of macrophages and microvessels in the gastric mucosa were determined immunohistochemically with anti-CD68 and anti-CD31 antibodies, respectively. Expression of tumor necrosis factor (TNF)-α, cyclooxygenase (COX)-2, and vascular endothelial growth factor (VEGF) mRNA was determined via real-time reverse transcription-polymerase chain reaction (RT-PCR), and the mucosal content of prostaglandin (PG) E₂ was determined via enzyme immunoassay on day 10 after ulceration. The healing of gastric ulcers was significantly delayed in op/op mice compared with +/? mice. Further, significantly fewer macrophages were observed in the normal gastric mucosa of op/op mice than in +/? mice. Ulcer induction caused a marked accumulation of macrophages around the ulcer base in +/? mice, but this response was attenuated in op/op mice. The mucosal PGE₂ content as well as the expression of COX-2, VEGF, and TNF-α mRNA were all upregulated in the ulcerated area of +/? mice but significantly suppressed in op/op mice. The degree of vascularization in the ulcerated area was significantly lower in op/op mice than in +/? mice. Taken together, these results suggest that M-CSF-dependent macrophages play an important role in the healing of gastric ulcers, and that this action may be associated with angiogenesis promoted by upregulation of COX-2/PGE₂ production.

Key words: cyclooxygenase-2, gastric ulcer healing, macrophage-colony stimulating factor, op/op mice, prostaglandin E₂, vascular endothelial growth factor
In the present study, we examined the role of M-CSF-dependent macrophages in the healing of gastric ulcers by comparing the healing response between M-CSF-deficient (op/op) mice and M-CSF-expressing heterozygote (+/? ) mice, particularly with regard to angiogenic response and VEGF expression.

MATERIALS AND METHODS

Animals

M-CSF heterozygotes (+/?) breeding pairs were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and acclimated to standard laboratory conditions (12:12-h light-dark cycle, temperature 22 ±1°C). Homozygous op/op mice were produced by mating heterozygotes (+/? ) mice. Normal littermates (+/? ) were comprised of two-thirds +/op and one-third ++/ mice. Homozygous op/op mice lack incisors and develop a small body, domed skull, and a short tail at approximately 10 days after birth; in contrast, heterozygote +/op and wild-type (+/+ ) mice show no abnormalities and phenotypically indistinguishable (28). Op/op mice were fed powdered chow, whereas the ++/ mice received conventional chow. Both 8- to 10-week-old +/op and littermate ++/ mice were used in the present study. All experimental procedures were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

Induction of gastric ulcers

Chronic gastric ulcers were induced in mice by thermal cauterization, according to a method described previously (29). Briefly, under ether anesthesia, the stomach was exposed through a middle incision, and an electric probe (diameter: 6 mm, Biotex, Kyoto, Japan) was attached to the mid-corpus serosa. A gastric ulcer was induced by heating the probe tip at 70°C for 20 sec. Both ++/ and op/op mice were fed powder chow after induction of gastric ulcers. On various days (3, 7, 10, and 17 days) after ulceration, the animals were sacrificed under deep ether anesthesia. The stomach was removed, inflated by injection of 1 ml of 2% formalin for 10 min to fix the inner wall, and opened along the greater curvature. The ulcer area (mm²) of the stomach was measured under a dissecting microscope with a square grid (20X). The person measuring the ulcer area was blinded to animal grouping status.

Detection of macrophages

Macrophages were examined immunohistochemically using anti-CD68 antibody in normal and ulcerated mucosa of +/? and op/op mice 10 days after ulceration. The animals were sacrificed under deep ether anesthesia and had their stomachs removed, washed in cold phosphate-buffered saline, and immersed in 4% paraformaldehyde for 48 h at 4°C. After treatment with 10% and 20% sucrose solutions, the tissue sample was embedded in O.C.T. compound (Miles, Elkhart, IN, USA) and frozen rapidly in liquid nitrogen. Cryostat sections (CM1510; Leica, Wetzlar, Germany) cut serially at a thickness of 10 µm were mounted on MAS-coated slides (Matsunami, Osaka, Japan) which were then incubated with rat monoclonal anti-mouse CD68 antibody (Serotec, Morpshos, UK) overnight at 4°C and then treated with Alexa Fluor 488-labeled donkey polyclonal anti-rat IgG antibody (Molecular Probes, Eugene, OR, USA) for 2 h at room temperature. Immunofluorescence was observed using a fluorescence microscope (200×, BX-51; Olympus, Tokyo, Japan). The number of CD68-positive macrophages in normal and ulcerated mucosa was measured across 3 randomly chosen 0.16-mm² (0.4×0.4 mm) fields, and the data were expressed as the number of macrophages per square millimeter of normal and ulcerated mucosa.

Determination of mucosal PGE₂ content

The content of PGE₂ in the normal and ulcerated gastric mucosa of +/? and op/op mice was determined 10 days after ulceration. Under deep ether anesthesia, the animals were sacrificed, the stomach was removed, and the whole layer of normal corpus mucosa or the ulcerated mucosa was isolated, weighed, and put in a tube containing 100% methanol plus 100 mM indomethacin (30). Tissues were then minced with scissors, homogenized with a polytron homogenizer (IKA, Tokyo, Japan), and centrifuged at 12,000 g for 10 min at 4°C. After the supernatant of each sample had been evaporated with N₂ gas, the residue was resolved in assay buffer and used for the determination of PGE₂. The concentration of PGE₂ was measured using a PGE₂ enzyme immunoassay kit (Cayman, Ann Arbor, MI, USA).

Determination of mucosal mRNA expression of TNF-α, COX-2 and VEGF

The mRNA expression of TNF-α, COX-2 and VEGF in the normal and ulcerated mucosa of +/? and op/op mice was examined 10 days after ulceration by real-time RT-PCR. The animals were sacrificed under deep ether anesthesia, the whole layer of stomach was removed, frozen in liquid nitrogen, and stored at -80°C until use. Total RNA was extracted using Separase RNA-1 (Nacalai Tesque) according to the manufacturer’s instructions. Reverse transcription was performed using ReverTra Ace-alpha with random hexamers (Toyobo, Osaka, Japan). Real-time polymerase chain reaction (PCR) amplification was performed using SYB Premix Ex Taq (Takara, Shiga, Japan) with specific primers sets, prepared using the Perfect real-time supporting system of Takara for TNF-α (Primer set ID: MA097070), COX-2 (Primer set ID: 029877), VEGF (Primer set ID: MA089111), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Primer set ID: MA050371) with an ABI PRISM 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Expression levels of TNF-α, COX-2, and VEGF mRNA were standardized to GAPDH mRNA and expressed as the ratio to the mean value for normal mucosa of +/? mice.

Evaluation of angiogenesis

Angiogenesis was examined immunohistochemically using anti-CD31 (platelet endothelial cell adhesion molecule-1, PECAM-1) antibody in the ulcerated mucosa of +/? and op/op mice on 10 days after ulceration. As described above, cryostat sections at a thickness of 10 µm were mounted on MAS-coated slides which were then incubated with rabbit polyclonal anti-human CD31 antibody (Abcam, Cambridge, UK) overnight at 4°C after deactivation of endogenous peroxidase with 0.3% H₂O₂. The immunocomplex was visualized via the avidin-biotin-peroxidase method using a Vectastain ABC-peroxidase kit (Vector, Burlingame, CA, USA). Sections were counter-stained with hematoxylin. Immunostaining was observed using a light microscope (200 ×, BX-50; Olympus). The degree of microvasculature in the ulcer base granulation tissues was determined across three randomly chosen 0.16-mm² (0.4×0.4 mm) fields and expressed as the number of macrophages per square millimeter of normal and ulcerated mucosa.
Analyses of data

Data are presented as the mean ±S.E.M. for 5-6 mice per group. Statistical analyses were performed using Student’s t-test and Dunnett’s multiple comparison test, with P values <0.05 regarded as significant.

RESULTS

Gastric ulcer healing in +/? and op/op mice

On three days after the thermal cauterization, well-defined ulcers developed in the gastric mucosa of both M-CSF-expressing heterozygote +/? and M-CSF-deficient op/op mice, with ulcer scores of 15.3±0.8 and 13.7±1.0 mm², respectively. No significant differences were noted in the ulcer score between +/? and op/op mice. In +/? mice, gastric ulcers healed rapidly within 10 days and almost completely by 17 days after ulceration (Fig. 1). In contrast, healing was significantly impaired in op/op mice; the ulcer scores on day 10 and 17 following ulceration were approximately 3 and 10 times greater than those observed in +/? mice, respectively, at the same points.

Distribution of macrophages in the gastric mucosa of +/? and op/op mice

While CD68-positive macrophages were diffusely distributed in the stomach of both +/? and op/op mice, mostly in the lamina propria and submucosa, significantly fewer such cells were observed in op/op mice than in +/? mice (60.3±6.0 and 24.5±4.8 cells/mm², respectively; Fig. 2A, 2D and 3). On day 10 after ulceration, the number of macrophages was dramatically increased in both the ulcer base and margin of +/? mice, reaching a value of 355.5±59.9 and 813.3±128.0 cells/mm², respectively (Fig. 2B and 2C). While the increase in number of macrophages was observed in both ulcer base and margin of op/op mice, these increases were significantly less drastic (137.8±24.4 and 253.3±36.2 cells/mm², respectively) than those in +/? mice (Fig. 2E, 2F and 3). The number of macrophages in unaffected mucosa surrounding the ulcers (non-ulcerated) of +/? and op/op mice was almost similar with that in normal mucosa (Fig. 3).

PGE₂ content in the normal and ulcerated gastric mucosa of +/? and op/op mice

PGE₂ content in the normal gastric mucosa of +/? and op/op mice was 11.6±2.3 and 12.5±4.0 ng/tissue, respectively (Fig. 4). Values were markedly increased in the ulcerated mucosa of +/? mice on day 10 after ulceration, reaching a value of 44.7±5.2 ng/g tissue. Although the PGE₂ content in the ulcerated mucosa of op/op mice was slightly but significantly increased after ulceration (25.5±3.7 ng/g tissue), the value was still significantly less than that in +/? mice.

Expression of TNF-α, COX-2, and VEGF mRNA in the gastric mucosa of +/? and op/op mice

No differences were noted in the expression of TNF-α, COX-2, or VEGF mRNA in normal gastric mucosa between +/? and op/op mice (Fig. 5). On day 10 after ulceration, expression of TNF-α, COX-2, and VEGF mRNA was significantly enhanced around the ulcerated mucosa of +/? mice, with levels of 8.7±0.4, 3.2±0.5, and 4.3±0.9 times greater than those in the normal mucosa of +/? mice, respectively. In contrast, levels of these RNA around the ulcerated mucosa remained unchanged in op/op mice, and the degree of expression was almost equivalent to that in the normal gastric mucosa.

Angiogenesis in the ulcerated gastric mucosa of +/? and op/op mice

On Day 10 after ulceration, the ulcer base in +/? mice was spontaneously reconstructed by the growth of granulation tissue and newly formed microvasculature (angiogenesis); the number of microvessels was 69.3±2.9 per mm² when examined immunohistochemically using anti-CD31 (PECAM) antibody (Fig. 6A, 6C and 6E). In contrast, expression of CD31 in the ulcer base was reduced in op/op mice to 31.9±4.2/mm², a value significantly less than that in +/? mice (Fig. 6B, 6D and 6E).

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Fig. 1. Spontaneous healing of gastric ulcers in M-CSF-expressing heterozygote (+/? ) and M-CSF-deficient (op/op) mice. Gastric ulcers were produced by thermal cautery (70°C, 20 sec), and animals were sacrificed 3, 7, 10, and 17 days after ulceration. Data are presented as the mean ±S.E.M. from 5-7 mice. *Significant difference from control (+/? mice) at P<0.05.
DISCUSSION

In the present study, we investigated the role of M-CSF-dependent macrophages in the healing of gastric ulcers using M-CSF-deficient op/op mice. The healing of gastric ulcers was significantly impaired in op/op mice as compared to M-CSF-expressing heterozygote +/? mice. CD68-positive macrophages were markedly infiltrated and accumulated at the base and margin of gastric ulcers after ulceration in +/? mice but this response was dramatically suppressed in op/op mice. Furthermore, upregulation of COX-2 and VEGF expressions, and PGE2 production as well as angiogenesis in the ulcerated mucosa of +/? mice was also significantly attenuated in op/op mice. These findings suggest that M-CSF-dependent macrophages play an important role in the healing of gastric ulcers.

Fig. 2. Fluorescence immunohistochemical study for macrophages in the gastric mucosa of +/? and op/op mice with or without ulceration. Gastric ulcers were produced by thermal cautery (70°C, 20 sec), and animals were sacrificed 10 days after ulceration. Frozen sections were prepared, and fluorescence immunostaining with anti-CD68 antibody was performed for detection of macrophages. A-E: +/? mice; D-E: op/op mice; A and D: normal mucosa; B and E: ulcer base; C and D: ulcer margin.

Fig. 3. Numbers of macrophages in the gastric mucosa of +/? and op/op mice with or without ulceration. Gastric ulcers were produced by thermal cautery (70°C, 20 sec), and animals were sacrificed 10 days after ulceration. Macrophages were detected immunohistochemically using anti-CD68 antibody. The number of macrophages was counted in normal mucosa (without ulceration), ulcer base, ulcer margin, and unaffected mucosa surrounding the ulcer (non-ulcerated), respectively. Data are presented as the mean ±S.E.M. from 6 mice. Significant difference at P<0.05, * from corresponding values in control (+/? mice); # from corresponding values in normal mucosa.
Fig. 4. PGE₂ content in the gastric mucosa of +/- and op/op mice with or without ulceration. Gastric ulcers were produced by thermal cauterization (70°C, 20 sec), and animals were sacrificed 10 days after ulceration. The PGE₂ content was determined by enzyme immunoassay. Data are presented as the mean ±S.E.M. from 6 mice. Significant difference at P<0.05, * from corresponding values in control (+/? mice); # from corresponding values in normal mucosa.

Fig. 5. Expression of COX-2, TNF-α, and VEGF mRNA in the gastric mucosa of +/- and op/op mice with or without ulceration. Gastric ulcers were produced by thermal cauterization (70°C, 20 sec), and animals were sacrificed 10 days after ulceration. mRNA expression was determined using real-time RT-PCR. Data are presented as the mean ±S.E.M. from 6 mice. Significant difference at P<0.05, * from corresponding values in control (+/? mice); # from corresponding values in normal mucosa.

healing of gastric ulcers by promoting angiogenesis via upregulation of COX-2/PGE₂ production and VEGF expression. M-CSF is a critical trophic and differentiation factor for monocyte lineage cells (20-22), and M-CSF-deficient op/op mice have been reported to lack not only monocytes and tissue macrophages but also osteoclasts (23). Cecchini et al. (21) demonstrated an almost complete absence of macrophages in the stomach and small intestine of op/op mice, in stark contrast to
findings in M-CSF-expressing +/? mice. Here, we used anti-CD68 antibody to immunohistochemically examine macrophages in the stomachs of op/op and +/? mice and confirmed that op/op mice had significantly fewer such cells than +/? mice.

Macrophages can be divided into several subsets based on exposure to panels of cytokines and growth factors such as M-CSF and granulocyte macrophage colony-stimulating factor (GM-CSF) (31, 32). As such, while macrophages located in the gastric mucosa are likely primarily dependent on M-CSF, some of these cells may be differentiated under the influences of other growth factors and cytokines, including GM-CSF.

The most important finding in the present study was that the healing of gastric ulcers was significantly impaired in op/op mice. We further showed marked infiltration and accumulation of macrophages at the ulcerated mucosa is dependent on M-CSF and that these M-CSF-dependent macrophages play a critical role in the healing process of gastric ulcers.

Many studies have demonstrated the upregulation of COX-2 expression in the ulcerated mucosa, concomitant with an increase in endogenous PG production, and suggested that COX-2/PGs play an important role in promoting the healing of gastric ulcers (33-37). Indeed, healing was significantly impaired when COX-2-derived PG production was inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors (34-37). Schmassmann et al. (38) reported that while COX-1 plays no significant role in gastric ulcer healing when COX-2 is unimpaired, it becomes important when COX-2 is impaired.

We also previously showed that healing of gastric ulcers was significantly impaired by administration of indomethacin (a non-selective COX inhibitor) and rofecoxib (a selective COX-2 inhibitor) but not SC-560 (a selective COX-1 inhibitor) (14). In addition, we obtained similar findings using COX-1 and COX-2 inhibitors.
knockout mice. These findings suggest that PGs derived from COX-2, but not COX-1, likely play a pivotal role in the mechanism of gastric ulcer healing. Here, we confirmed a marked increase in PGE₂ production with enhanced COX-2 mRNA expression in the ulcerated mucosa of +/? mice. Interestingly, this upregulation of COX-2/PGE₂ expression in the ulcerated mucosa was significantly suppressed in op/op mice, suggesting that the impaired ulcer healing in op/op mice may be a result of suppression of PGE₂ production due to downregulation of COX-2. M-CSF-dependent macrophages may therefore be responsible for upregulation of COX-2/PGE₂ in the mechanism of gastric ulcer healing.

COX-2 is induced in many types of cells, including fibroblasts, monocytes/macrophages, neutrophils, and granulocytes at inflammatory sites (39, 40), its downregulation in ulcerated areas of op/op mice may be in turn due to depletion of macrophages responsible for COX-2 expression. On the other hand, Takahashi et al. (41) reported that COX-2 was locally induced in the ulcer base and upregulated by inflammatory cytokines such as TNF-α. In the present study as well, we confirmed increased expression of TNF-α at the ulcerated mucosa of +/? mice, but this response was significantly suppressed in op/op mice. Taken together, these results suggest that the lack of COX-2 expression in op/op mice may be due not only to suppression of macrophage accumulation but also to expression of cytokines, such as TNF-α, which are responsible for COX-2 induction at the ulcerated mucosa. However, Shimizu et al. (42) demonstrated that early-phase inhibition of the inflammatory response, including TNF-α production, actually accelerated healing of gastric ulcers. This seemingly conflicting finding suggests that TNF-α likely has a dual influence, deleterious and beneficial, depending on the phase of ulcer healing. Particularly in the later phase, TNF-α may play an important role in the healing mechanism of gastric ulcers through upregulation of COX-2PG production.

The healing mechanism in wounded tissues is multiple steps, involving formation of granulation tissue, contraction of the ulcerated tissue, and reepithelialization (1), and these processes are regulated by growth factors, including VEGF, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and other cytokines produced locally regenerating cells (2-4). Angiogenesis, the essential component of wound healing (1), is induced by VEGF, which is known to be a fundamental regulator of angiogenesis (5, 6). Indeed, Szabo et al. (5) reported that exogenous VEGF enhanced the healing of duodenal ulcers, and Johnes et al. (6) demonstrated that gene therapy with VEGF significantly promoted the healing of gastric ulcers, a response which was inhibited by neutralization of endogenous VEGF with anti-VEGF antibody. These findings clearly indicate an essential role for VEGF and angiogenic response in ulcer healing. As expected, we found in the present study that the angiogenic response in the ulcerated mucosa was significantly impaired in op/op mice compared with +/? mice, as evidenced by both results of immunohistochemical staining with anti-CD31 antibody and determination of the number of microvessels. Further, we showed that the increase in VEGF mRNA expression in the gastric mucosa after ulceration was potently suppressed in op/op mice. Given the above, depletion of M-CSF-dependent macrophages in op/op mice is therefore likely to impair the angiogenic response in the process of gastric ulcer healing via downregulation of VEGF through suppression of COX-2/PGE₂ production.

However, despite the above-mentioned findings, we found in the present study that healing of gastric ulcers definitely occurred, albeit significantly delayed, even in op/op mice with downregulated COX-2/PGE₂ production and VEGF expression. A similar phenomenon was observed in several previous studies in COX-2 inhibitor-treated and COX-2-deficient mice (33-37). While the precise mechanism of gastric ulcer healing under such adverse conditions is unknown, COX-1-dependent mechanisms may be involved, at least in part. Further studies are needed to clarify this point.

In summary, we found that healing of gastric ulcers induced by thermal cauterization was significantly impaired in M-CSF-deficient op/op mice as compared to M-CSF-expressing heterozygote +/? mice. While marked infiltration and accumulation of macrophages were observed at the ulcerated mucosa after ulceration in +/? mice, this response was dramatically suppressed in op/op mice. Upregulation of COX-2, TNF-α, and VEGF expression in the ulcerated mucosa of +/? mice was also significantly attenuated in op/op mice, as was the angiogenic response at the ulcer base. We therefore conclude that M-CSF-dependent macrophages play an important role in the healing of gastric ulcers and that the role macrophages play may be associated with angiogenesis promoted by upregulation of COX-2/PGE₂ production.

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