Monocyte chemotactic protein-1 regulates leukocyte recruitment during gastric ulcer recurrence induced by tumor necrosis factor-α

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Watanabe, Toshibio, Kazuhide Higuchi, Masaki Hamaguchi, Masatsugu Shiba, Kazunari Tominaga, Yasuhiro Fujiwara, Takayuki Matsumoto, and Tetsuo Arakawa. Monocyte chemotactic protein-1 regulates leukocyte recruitment during gastric ulcer recurrence induced by tumor necrosis factor-α. Am J Physiol Gastrointest Liver Physiol 287: G919–G928, 2004. First published June 17, 2004; 10.1152/ajpgi.00372.2003.—TNF-α has numerous biological activities, including the induction of chemokine expression, and is involved in many gastric injuries. C-C chemokines [monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α] and C-X-C chemokines [MIP-2 and cytokine-induced neutrophil chemoattractant (CINC)-2α] mediate chemotaxis of monocytes and neutrophils, respectively. We examined the roles of TNF-α and dynamics of chemokine expression in gastric ulceration including ulcer recurrence and indomethacin-induced injury. Rats with healed chronic gastric ulcers received intraperitoneal TNF-α to induce ulcer recurrence. Some rats were given neutralizing antibodies against neutrophils or MCP-1 together with TNF-α. In a separate experiment, rats were orally administered 20 mg/kg indomethacin with or without pretreatment with pentoxifylline (an inhibitor of TNF-α synthesis) or anti-MCP-1 antibody. TNF-α (1 μg/kg) induced gastric ulcer recurrence after 48 h, which was completely prevented by anti-neutrophil antibody. TNF-α increased the number of macrophages and MCP-1 mRNA expression in scarred mucosa from 4 h, whereas it increased MPO activities (marker of neutrophil infiltration) and mRNA expression of MIP-2 and CINC-2α from 24 h. Anti-MCP-1 antibody inhibited leukocyte infiltration with reduction of the levels of C-X-C chemokines and prevented ulcer recurrence. Indomethacin treatment increased TNF-α/chemokine mRNA expression from 30 min and induced macroscopic erosions after 4 h. Pentoxifylline inhibited the indomethacin-induced gastric injury with reduction of neutrophil infiltration and expression of chemokine (MCP-1, MIP-2, and CINC-2α). Anti-MCP-1 antibody also inhibited the injury and these inflammatory responses but did not affect TNF-α mRNA expression. In conclusion, increased MCP-1 triggered by TNF-α may play a key role in gastric ulceration by regulating leukocyte recruitment and chemokine expression.

ulceration; cytokine; macrophage; real-time reverse transcriptase-polymerase chain reaction

RECENT CLINICAL AND EXPERIMENTAL studies (7, 12, 29) have demonstrated that the accumulation and activation of leukocytes such as neutrophils and monocytes/macrophages is closely associated with a variety of gastric injuries. The chemokine family plays a crucial role in the regulation of leukocyte recruitment at sites of inflammation and tissue injury. On the basis of the position of cysteine residues in their amino terminal domain, chemokines can be divided into four groups: the C, C-C, C-X-C, and CXC families. These four groups act on different types of leukocytes. The C chemokines are principally chemotactic for CD8+ T lymphocytes (9), the C-C chemokines, such as monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α, mediate the chemotaxis of monocytes/macrophages but not neutrophils (11), whereas C-X-C chemokines such as MIP-2 (4) and cytokine-induced neutrophil chemoattractant (CINC)-2α (15) act as potent chemoattractants for neutrophils but not mononuclear cells. The CXC chemokines mediate leukocyte migration and adhesion to endothelial cells (8).

We have previously reported (31) that systemic injection of IL-1β, a proinflammatory cytokine, causes neutrophil accumulation via induction of adhesion molecules on both neutrophils and endothelial cells and that ~90% of healed ulcers recurred at scarred mucosal sites within 48 h after IL-1β injection. In our model of gastric ulcer recurrence using IL-1β, neutrophils accumulated in scarred mucosa by 24 h, especially where the surface epithelium was disrupted. Neutrophils were also numerous in both the margins and the bed of recurrent ulcers. An antibody against neutrophils completely prevented ulcer recurrence, suggesting that this model is neutrophil dependent.

In this model of ulcer recurrence, macrophages were also abundant in scarred mucosa, and IL-1β further increased the number of macrophages in scarred mucosa but did not do so in normal mucosa. Theses findings suggest that overexpression of chemokines in scarred mucosa may occur in response to IL-1β stimulation, leading to the promotion of neutrophil and macrophage infiltration into scarred mucosa during gastric ulcer recurrence. However, at present, the dynamics and roles of chemokines in ulcer recurrence remain unclear. Similar to IL-1β, TNF-α is a proinflammatory cytokine and possesses many biological effects including the induction of expression of other cytokines, chemokines (24), and adhesion molecules (17), as well as activation of neutrophils (10) and is also involved in the pathogenesis of a variety of gastric injuries such as those caused by NSAIDs (21), Helicobacter pylori infection (3), and stress (7). These three ulcerogenic factors are major causes of peptic ulcer recurrence, indicating a possible role for TNF-α as a potent inducer of this process.

Increased production of TNF-α is closely associated with NSAIDs-induced gastric injury (1, 14, 21). Administration of indomethacin to rats resulted in elevation of plasma TNF-α.

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levels within 30 min, and pretreatment with anti-TNF-α antibody or pentoxifylline (PTX), an inhibitor of TNF-α synthesis, reduced gastric injury caused by indomethacin (1). Santucci et al. (20) demonstrated that pretreatment with IL-2 and lipopolysaccharide, which increased TNF-α release, enhanced neutrophil margination and gastric mucosal injury but that administration of dexamethasone, PTX, and granulocyte colony-stimulating factor, which inhibited TNF-α release, provided almost total protection against such injury. Thus TNF-α may trigger mucosal inflammation in NSAIDs-induced gastric injury, but the roles played by chemokines in maintenance and/or enhancement of inflammation, including leukocyte infiltration during development of this injury, have not been fully studied.

In the present study, we examined whether TNF-α can induce gastric ulcer recurrence in rats, and we also evaluated the dynamics of leukocyte infiltration and expression of chemokines (MCP-1, MIP-1α, MIP-2, and CINC-2α) in gastric ulceration including gastric ulcer recurrence and indomethacin-induced gastric injury. We also investigated the effects of immunoneutralization of MCP-1 by an anti-MCP-1 antibody on the gastric mucosal inflammatory responses in such injuries.

MATERIALS AND METHODS

Animals and ulcer induction. Specific pathogen-free male Wistar rats weighing ~200 g were obtained from Japan SLC (Hamamatsu, Japan). Chronic gastric ulcers were induced by injection of 0.02 ml of 20% acetic acid into the submucosal layer of the antral oxyntic border of the anterior wall (25). All experimental procedures were approved by the Animal Care Committee of Osaka City University Graduate School of Medicine.

Induction of ulcer recurrence. Endoscopy was performed on day 90 after the induction of gastric ulcers. The method used has been described in detail elsewhere (31). With the use of endoscopic techniques, healed ulcers could be identified by a white coating on the ulcer bed in the anterior wall of the stomach, whereas healed ulcers exhibited a scarred mucosa with an irregular surface but were without white coating. Rats with healed ulcers were subjected to experiments examining the induction of ulcer recurrence by TNF-α. Animals received either an intraperitoneal injection of recombinant human TNF-α (Genzyme, Boston, MA) at a dose of 0.1–10 μg/kg or an intraperitoneal or intravenous injection of recombinant rat MCP-1 at a dose of 10 μg/kg (PeproTech EC, London, UK) diluted to 1 ml with PBS or vehicle (PBS) alone and were killed 0, 4, 24, or 48 h later. Ulcers found on endoscopy to have healed that regained a white coating after cytokine injection were considered to be recurrent ulcers. Some rats received a single intraperitoneal injection of 2 ml rabbit anti-rat neutrophil serum (ANS; Inter-Cell Technologies, Hopewell, NJ) at the same time as 1 μg/kg TNF-α. In a preliminary study, this volume of ANS decreased circulating neutrophils by ~90% and 80% at 24 and 48 h, respectively, without affecting the numbers of other types of leukocytes in circulation. In addition, to determine the specific role of MCP-1 in TNF-α-induced gastric mucosal inflammation, the other rats received a single intraperitoneal injection of rabbit anti-rat MCP-1 antibody (250 μg/kg; Chemicon International, Temecula, CA) or normal rabbit IgG (Sigma) at the same time as 1 μg/kg TNF-α and were killed 24 or 48 h later. This dose of anti-MCP-1 neutralizing antibody has been reported to inhibit infiltration of macrophages into the rat jejunal muscularis by 75% in endotoxemia induced by an intraperitoneal injection of 15 mg/kg lipopolysaccharide (27). We also evaluated the role played by gastric acid in ulcer recurrence induced by TNF-α. Rats were intraperitoneally administered 30 mg/kg rabeprazole (a proton pump inhibitor), a gift from Eisai (Tokyo, Japan), for 3 days, and received 1 μg/kg TNF-α 24 h after the first administration of rabeprazole.

After death, the stomachs of the rats were removed and scarred tissues were subjected to measurement of MPO activity (a marker of neutrophil infiltration), an assay of mRNA levels for MCP-1, MIP-1α, and CINC-2α, and ICAM-1 by RT-PCR, and immunohistochemical staining for chemokines and monocytes/macrophages.

Indomethacin-induced gastric injury. Gastric injury was induced by oral administration of indomethacin at a dose of 20 mg/kg. Indomethacin was suspended in 0.5% methyl cellulose. To determine the role of cytokines/chemokines in indomethacin-induced gastric injury, we gave PTX (100 mg/kg) intraperitoneally with or without recombinant 1 μg/kg TNF-α, 250 μg/kg anti-MCP-1 antibody, or vehicle. These agents were given intraperitoneally 30 min before indomethacin administration. The rats were killed 30 min and 4 h after administration of indomethacin. The extent of gastric damage was evaluated by calculating the total length of the macroscopically visible erosions.

Measurement of MPO activity. MPO activity of the gastric tissue was assayed by the method of Bradley et al. (2). One unit of MPO activity was defined as that degrading 1 μmol peroxide/min at 25°C. Proteins were measured with a modified bichinchoninic acid method with a BCA protein assay reagent kit (Pierce, Rockford, IL). Results were expressed as units per gram of protein.

Quantification of mRNA expression for chemokines and ICAM-1. Total RNA from gastric tissue was extracted with ISOGEN (Nippon Gene, Tokyo, Japan) and purified according to the manufacturer’s protocol. The expression of mRNAs for chemokines and ICAM-1 was quantified by real-time RT-PCR (TaqMan PCR). The PCR primers and TaqMan probes were designed using the software program Primer Express (Applied Biosystems Japan, Tokyo, Japan; Table 1). TaqMan probes were labeled with the fluorescent reporter dye 6-carboxyfluorescein at the 5’-end and the fluorescent quencher 6-carboxy-tetramethyl-rhodamine at the 3’-end. Real-time quantitative RT-PCR analyses were performed using an ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems Japan). The reaction mixture was prepared according to the manufacturer’s protocols using the PLATINUM quantitative RT-PCR THERMOSCRIPT One-Step System (Invitrogen, Carlsbad, CA). The thermal cycling conditions were 50°C for 30 min and 95°C for 5 min, followed by 45 cycles of amplification at 95°C for 15 s and 60°C for 1 min. The expression levels of mRNAs encoding cytokines/chemokines and ICAM-1 in injured gastric tissue and normal gastric tissue were standardized to GAPDH mRNA, and the mRNA levels of TNF-α, IL-1β, MCP-1, MIP-1α, MIP-2, CINC-2α, and ICAM-1 in injured gastric tissue were expressed as ratios to the mean value for normal gastric tissue.

Immunohistochemical analysis. Serially cut cryostat sections of 6-μm thickness were mounted on silanized slides (DAKO Japan, Kyoto, Japan) and stained with mouse monoclonal antibodies against rat monocytes/macrophages (mouse anti-rat ED-1, Serotec, Oxford, England) and ICAM-1 (Seikagakukogyo, Tokyo, Japan), rabbit polyclonal antibodies against rat MCP-1, MIP-1α, and MIP-2 (all from Cedarlane Laboratories, Ontario, Canada), and a goat polyclonal antibody against rat CINC-2α (Genzyme). Immunohistochemical staining was performed using a streptavidin-biotin peroxidase method according to the manufacturer’s instructions (LSAB2 kit, DAKO Japan) for monocytes/macrophages, ICAM-1, MCP-1, MIP-1α, and MIP-2, and with the goat ImmunoCruz Staining System (Santa Cruz Biotechnology, Santa Cruz, CA) for CINC-2α. Counterstaining was performed with methyl green (DAKO Japan). As a negative control, the primary antibody was replaced by either isotype-matched mouse IgG, normal rabbit serum (both from DAKO Japan), or normal goat IgG (Santa Cruz Biotechnology). Staining for morphological observations or neutrophil identification was achieved using hematoxylin and eosin.

After monocytes/macrophages and chemokines were stained as described above, the monocytes/macrophages and cells positive for each chemokine were counted within four randomly chosen areas of
scarring. The width inspected was 0.5 mm, whereas the depth of inspection depended on the height (from base to top) of the mucosa in the area of observation. The ×200 objective of a light microscope was used, and regions to be inspected were measured with reference to the eyepiece of the microscope. Results are expressed as the number of cells per square millimeter. Counting was performed without knowledge of the treatment groups to which specimens belonged.

**Results**

**Analysis of gastric acid secretion.** Gastric acid secretion was measured in rats given 1 μg/kg TNF-α, 10 μg/kg MCP-1, and 30 mg/kg rabeprazole. The pylorus was ligated 30 min after the administration of these drugs, and the rats were killed 4 h later. The volume was measured, and the acid concentration was determined using a titration system (AT-500N, Kyoto Electronics Manufacturing, Kyoto, Japan).

**Statistical analysis.** Results other than the rate of recurrence are expressed as means ± SE. One-way analysis of variance was used to test for significant differences between treatment group means, and results were analyzed using Fisher’s protected least-significant difference test. Differences in the rate of recurrence were evaluated by the chi-square test. Differences with P values <0.05 were considered significant.

**Results**

**TNF-α-induced gastric ulcer recurrence.** Rats found to have healed ulcers after endoscopic examination received an intraperitoneal injection of TNF-α at a dose of 0.1–10 μg/kg to induce gastric ulcer recurrence. At no dose did TNF-α induce ulcer recurrence within 24 h. By 48 h, seven of the eight healed ulcers in the group given 10 μg/kg TNF-α had recurred within the scarred mucosa (Table 2) as had seven of the nine healed ulcers in the group given 1 μg/kg TNF-α (Fig. 1). TNF-α at a dose of 0.1 μg/kg, both intraperitoneal and intravenous MCP-1 at a dose of 10 μg/kg, and vehicle each failed to induce gastric ulcer recurrence. TNF-α did not cause injury to normal mucosa located some distance from the scarring. The ulcer recurrence induced by 1 μg/kg TNF-α was completely prevented by treatment with rabeprazole and ANS.

**Effects of TNF-α, MCP-1, and Rabeprazole on Gastric Acid Secretion.** Intraperitoneal injection of 1 μg/kg TNF-α decreased gastric acid secretion by 39% but did not affect volume (Table 3). Rabeprazole at a dose of 30 mg/kg decreased acid secretion and volume by 82 and 55%, respectively. Intravenous injection of 10 μg/kg MCP-1 affected neither acid secretion nor volume.

### Table 1. The PCR primers and TaqMan probes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (forward)</th>
<th>Primer (reverse)</th>
<th>Probe</th>
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</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>5′-CCAGGAGAAGAGGTCAGGCTCTCT-3′</td>
<td>5′-TCATACCAAGGGCTTGTAGTCA-3′</td>
<td>5′-FAM-AGAGCCCGTCTCGCAAGACAGCCT-TAMRA-3′</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5′-CACGCTCTCAAGGCAAGGACAGCAG-3′</td>
<td>5′-GCTTTCTGATGCAAAGGTCAAAC-3′</td>
<td>5′-FAM-GTGCCGACGATTTCTCAGGCTGAGA-TAMRA-3′</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5′-GCTGCTACTATGCTAGCTGGCAA-3′</td>
<td>5′-TGCTGCTGATTCTCTTGTTGTA-3′</td>
<td>5′-FAM-TGGCCGACGATTTCTCAGGCTGAGA-TAMRA-3′</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>5′-TGTTTCTCTCTGGACATGGC-3′</td>
<td>5′-ATAGGAGAAGGAGGAGGGTC-3′</td>
<td>5′-FAM-CTTTGCAACCCCTGAAAGCCCT-TAMRA-3′</td>
</tr>
<tr>
<td>MIP-1β</td>
<td>5′-GCCACTCTTAAGGATGTCTAG-3′</td>
<td>5′-TTTTTGAGACGTCCTCTGAAA-3′</td>
<td>5′-FAM-GTGTGGTCGCTACTGCTCACCCAT-3′</td>
</tr>
<tr>
<td>CINC-2α</td>
<td>5′-AACATCCAGAGGCTGAGCTTG-3′</td>
<td>5′-CTTTTGGACATCTCTGCTGAGA-3′</td>
<td>5′-FAM-AGTTTGTCTCAACCCTGAAAGCCCT-TAMRA-3′</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>5′-TGCTGCTGGTGATTCTCTTGTA-3′</td>
<td>5′-ATAGGAGAAGGAGGAGGGTC-3′</td>
<td>5′-FAM-AGTTTGTCTCAACCCTGAAAGCCCT-TAMRA-3′</td>
</tr>
</tbody>
</table>

MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; CINC, cytokine-induced protein neutrophil.

**Table 2. Effect of cytokines on gastric ulcer recurrence in rats**

<table>
<thead>
<tr>
<th>Treatment, μg/kg</th>
<th>Numbers, recurred/treated, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle, ip</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>TNF-α (0.1), ip</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>TNF-α (1), ip</td>
<td>7/9 (78)*</td>
</tr>
<tr>
<td>TNF-α (10), ip</td>
<td>7/8 (88)*</td>
</tr>
<tr>
<td>TNF-α (1), ip + ANS</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>TNF-α (1), ip + anti-MCP</td>
<td>1/6 (17)</td>
</tr>
<tr>
<td>TNF-α (1), ip + RPZ</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>MCP-1 (10), ip</td>
<td>0/8 (0)</td>
</tr>
<tr>
<td>MCP-1 (10), iv</td>
<td>0/6 (0)</td>
</tr>
</tbody>
</table>

Rats found to have healed ulcers received an injection of TNF-α (0.1–10 μg/kg), MCP-1 (10 μg/kg), or vehicle alone. Some rats were given anti-rat neutrophil serum (ANS) or anti-MCP antibody together with 1 μg/kg TNF-α. The rats were killed 48 h after injection of the cytokines. *P < 0.05 compared with controls (vehicle-treated group), ip, intraperitoneal; iv, intravenous; RPZ, rabeprazole.
Effects of TNF-α and MCP-1 on leukocytic infiltration in scarred mucosa. The number of monocytes/macrophages in scarred mucosa was significantly increased at 4 h after treatment with 1 μg/kg TNF-α. It reached a maximal level at 24 h and remained similarly elevated at 48 h (Fig. 2). The MPO activity in scarred tissue increased with time after treatment with 1 μg/kg TNF-α; a significant increase in MPO activity was observed from 24 h onward (Fig. 2). TNF-α at a dose of 0.1 μg/kg increased neither the number of monocytes/macrophages nor MPO activity at any time point (data not shown).

Treatment with ANS inhibited the TNF-α-induced increase in MPO activity but did not inhibit the number of monocytes/macrophages present in scarred mucosa (Table 4). Intravenous MCP-1 affected neither MPO activity nor the number of monocytes/macrophages in scarred mucosa.

Effects of TNF-α on expression of chemokine mRNAs. MCP-1 mRNA expression in scarred mucosa was significantly increased at 4 h after treatment with 1 μg/kg TNF-α and reached a maximum at 24 h (Fig. 3). The expression of mRNAs encoding MIP-2 and CINC-2α in scarred mucosa was not increased 4 h after TNF-α treatment. However, expression of both these chemokines exhibited marked induction thereafter, peaking at 24 h and remaining high at 48 h (Fig. 3). The levels of MIP-1α mRNA did not change within 24 h but were significantly increased by 48 h. Treatment with 1 μg/kg TNF-α did not affect the expression of chemokines in normal mucosa (data not shown). In addition, TNF-α at a dose of 0.1 μg/kg did not affect expression of the mRNAs for these chemokines in either scarred tissue or normal gastric tissue (data not shown).

Histological and immunohistochemical results. Injection of TNF-α at doses of 1 and 10 μg/kg caused marked leukocyte infiltration into scarred mucosa at 24 h. The majority of these leukocytes was monocytes/macrophages. Neutrophils also infiltrated into scarred mucosa, especially the superficial portion, at this time. By 48 h, a white coating was visible macroscopically on the antral oxyntic border of the anterior wall where the acetic acid-induced ulcer was produced. Ulcers with a loss of epithelium were observed in this area. These ulcers extended to the submucosal layer, accompanied by marked leukocyte infiltration. The mucosa of ulcer margins were thin with increased connective tissue and displayed dilatation of gastric vessels.

Table 3. Effect of TNF-α, MCP-1, and RPZ on gastric acid secretion

<table>
<thead>
<tr>
<th>Drug</th>
<th>Acid Secretion, μEq/4 h</th>
<th>Volume, ml/4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>334.7±46.8</td>
<td>4.4±0.3</td>
</tr>
<tr>
<td>RPZ</td>
<td>61.5±13.9†</td>
<td>2.0±0.1†</td>
</tr>
<tr>
<td>TNF-α</td>
<td>203.7±52.2*</td>
<td>3.6±0.5</td>
</tr>
<tr>
<td>MCP-1</td>
<td>280.3±28</td>
<td>4.0±0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 6. Rats received an intraperitoneal injection of 30 mg/kg RPZ or 1 μg/kg TNF-α or received an intravenous injection of 10 μg/kg MCP-1, and the pylorus was ligated for 4 h. *P < 0.05, †P < 0.01 compared with control.
mucosa, indicating that these glands had regenerated after ulceration by acetic acid and that the ulcer had recurred at the site of the scar (Fig. 4A). Macrophages were abundant in both ulcer margins and beds (Fig. 4C), whereas neutrophils were mainly present in ulcer beds (Fig. 4B).

At 0 h (before treatment with TNF-α), a few inflammatory cells stained for chemokines within the scarred mucosa. The dynamics of the number of cells stained for chemokines (MCP-1, MIP-1α, MIP-2, or CINC-2α) in scarred mucosa after treatment with 1 μg/kg TNF-α were similar to those of the levels of chemokine mRNA (Fig. 5); the number of MCP-1-positive cells increased from 4 h after treatment with TNF-α, whereas the number of MIP-2- or CINC-2α-positive cells increased from 24 h. A significant increase in the number of MIP-1α-positive cells was only observed after 48 h. The cells staining for the chemokines were inflammatory cells infiltrating scarred mucosa. The majority of these cells was monocytes/macrophages, and some neutrophils also expressed chemokines (Fig. 6).

**Table 4. Effect of ANS and MCP-1 on leukocyte infiltration into scarred mucosa**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MPO activity, U/g protein</th>
<th>Macrophages, cells/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Control</td>
<td>0.61±0.12</td>
<td>0.67±0.24</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.87±0.17</td>
<td>0.78±0.11</td>
</tr>
<tr>
<td>ANS + MCP-1</td>
<td>0.58±0.17</td>
<td>0.66±0.09</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.67±0.24</td>
<td>0.66±0.09</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 6. Neutrophil infiltration was assessed by measurement of MPO, whereas macrophage infiltration was assessed by counting ED1-positive cells in scarred mucosa. *P < 0.05, †P < 0.01 compared with 0-h group (control group).

Effects of TNF-α on ICAM-1 expression. ICAM-1 was mainly detected in endothelial cells and some infiltrating cells in the deep portions of scarred mucosa at 0 h (before TNF-α treatment). Treatment with 1 μg/kg TNF-α caused marked induction of ICAM-1 expression in scarred mucosa from 4 h.

Treatment with 1 μg/kg TNF-α caused a 2.1-fold increase in ICAM-1 mRNA expression in scarred tissue by 4 h (P < 0.05), and a 3.6 (P < 0.01)- and 1.5-fold (P = 0.36) increase by 24 and 48 h, respectively.

Effects of MCP-1 immunoneutralization on TNF-α-induced ulcer recurrence and gastric mucosal inflammation. Treatment with an anti-MCP-1 antibody significantly reduced gastric ulcer recurrence by 48 h; only one of the six healed ulcers had recurred in this group, whereas 9 of the 10 healed ulcers in the
group given nonspecific rabbit IgG had recurred by 48 h after TNF-α treatment. Anti-MCP-1 antibody inhibited increases in MPO activity and decreased the numbers of monocytes/macrophages infiltrating scarred mucosa by 24 h after treatment with 1 μg/kg TNF-α, the numbers of monocytes/macrophages in anti-MCP-1 antibody- and nonspecific rabbit IgG-treated groups being 594.7 ± 36.2 and 911.5 ± 112.0 cells/mm², respectively (P < 0.01) and MPO activity in anti-MCP-1 antibody- and nonspecific rabbit IgG-treated groups being 4.0 ± 1.3 and 12.0 ± 0.3 U/g protein, respectively (P < 0.01).

Anti-MCP-1 antibody also prevented an increase in the levels of mRNAs for MCP-1, MIP-2, and CINC-2α in scarred mucosa (Fig. 7A) and the numbers of cells stained for these chemokines (MCP-1, MIP-2, and CINC-2α) infiltrating scarred mucosa (Fig. 7B) by 24 h, whereas it affected neither the level of MIP-1α mRNA nor the number of MIP-1α-positive cells in scarred mucosa. Immunohistochemical staining revealed that the TNF-α-induced increase in ICAM-1 expression in scarred mucosa was markedly reduced by the anti-MCP antibody. This antibody also inhibited the increase in expression of ICAM mRNA in scarred mucosa by 34% at 24 h after treatment with TNF-α.

A nonspecific rabbit antibody failed to inhibit increases in MPO activity, monocyte/macrophage infiltration, and expression of chemokines and ICAM-1 and failed to prevent ulcer recurrence.

**Roles of TNF-α and MCP-1 in indomethacin-induced gastric injury.** Indomethacin treatment caused macroscopic erosion after 4 h accompanied by an increase in MPO activity by 2.5- and 8.9-fold at 30 min and 4 h later, respectively. Anti-MCP-1 antibody and PTX inhibited the indomethacin-induced gastric injury by 51 and 70%, respectively. The inhibitory effect of PTX on gastric injury was abolished by exogenous TNF-α (Fig. 8).

MPO activity and mRNA expression for cytokines/chemokines were determined at the early phase of injury (30 min after treatment with indomethacin) when no macroscopic lesion was observed. The increase in MPO activity was significantly prevented by PTX and anti-MCP-1 antibody by 55 and 46%, respectively. The inhibitory effect of PTX was abolished by treatment with exogenous TNF-α.

Expression of mRNA for TNF-α, but not for IL-1β, was significantly increased (Fig. 9). mRNAs encoding MCP-1, MIP-2, and CINC-2α were also increased at this time. The increase in mRNA expression for MCP-1, MIP-2, and CINC-2α was inhibited by PTX, and this inhibitory effect was abolished by treatment with TNF-α. The anti-MCP-1 antibody also inhibited the increase in mRNA expression for MCP-1, MIP-2, and CINC-2α but did not affect expression of TNF-α mRNA.

**DISCUSSION**

In this study, we found that intraperitoneal injection of TNF-α induced inflammatory responses in scarred mucosa and gastric ulcer recurrence in rats in a neutrophil- and acid-dependent manner. Using this rat model of ulcer recurrence, we investigated the roles of C-C and C-X-C chemokines in ulcer recurrence. We demonstrated that these chemokines were overexpressed in scarred mucosa during gastric ulcer recurrence and that immunoneutralization of MCP-1 prevented TNF-α-induced gastric ulcer recurrence and inhibited inflammatory responses including leukocyte infiltration and chemokine expression. To the best of our knowledge, this is the first study to directly demonstrate involvement of MCP-1 in gastric injuries in vivo, although there are many reports demonstrating increased expression of chemokines such as IL-8 and MCP-1 in gastric mucosal inflammation and injuries, especially in *H. pylori*-associated gastric injuries (13, 18, 35).

It has been suggested that MCP-1 plays a crucial role in the recruitment of monocytes to sites of inflammation and injury (6). In an earlier study (26), we reported that MCP-1 mRNA expression in scarred mucosa was increased during IL-1β-induced gastric ulcer recurrence, accompanied by an increase in the number of macrophages infiltrating scarred mucosa. Furthermore, we observed that MCP-1 expression rapidly increased in scarred mucosa (4 h after TNF-α treatment) and that a single injection of anti-MCP-1, together with TNF-α, inhibited subsequent inflammatory responses such as neutrophil infiltration and overexpression of C-X-C chemokines and ulcer recurrence. Thus our results clearly demonstrate that MCP-1 plays a key role in the initiation of inflammatory responses during cytokine-induced ulcer recurrence. The main source of MCP-1 was inflammatory cells such as macrophages. In addition, TNF-α has been reported to induce MCP-1 expression in other systems (19, 23). These findings suggest that increased expression of MCP-1 may be due to an increase in the number of macrophages in response to TNF-α stimulation along with activation of resident macrophages in scarred mucosa by TNF-α.

Interestingly, MCP-1 alone did not induce gastric ulcer recurrence. MCP-1 given intraperitoneally would cause chemotaxis of monocytes/macrophages to the peritoneal cavity, resulting in a decrease in recruitment of these cells to the stomach and failure to induce ulcer recurrence. Therefore, we...
investigated the effect of intravenous injection of MCP-1 and found that MCP-1 given by this route did not induce leukocyte infiltration, gastric acid secretion, or ulcer recurrence. This finding indicates that TNF-α, MCP-1, and other mediator(s) may act in concert at an early phase of ulcer recurrence, leading to the stimulation of inflammatory responses in scarred mucosa.

MCP-1 mRNA expression in scarred mucosa was significantly increased from 4 h after treatment with 1 μg/kg TNF-α, whereas expression of mRNAs encoding MIP-2 and CINC-2α did so from 24 h. The dynamics of MCP-1 and C-X-C chemokines (MIP-2 and CINC-2α) were paralleled by increases in the infiltration of monocytes/macrophages and neutrophils (as assessed by MPO activity), respectively, suggesting that chemokines derived from inflammatory sites act as signaling proteins to coordinate selective recruitment of specific leukocyte populations in gastric ulcer recurrence, similar to other tissue injuries (4, 16, 36). The dynamics of MCP-1 and

Fig. 6. Inflammatory cell infiltration and chemokine expression in scarred mucosa at 24 h after TNF-α challenge. A: many inflammatory cells infiltrated scarred mucosa. B: immunohistochemical staining for monocytes/macrophages. The majority of the inflammatory cells was macrophages. C–H: immunohistochemical staining for chemokines. Inflammatory cells stained for MCP-1 (C and G), MIP-2 (E and H), and CINC-2α (F) were also numerous, whereas MIP-1α-positive cells were scattered throughout the scarred mucosa (D). Some neutrophils (arrows) also stained for the chemokines (G and H).
MIP-1α expression differed markedly in our model of gastric ulcer recurrence, suggesting that the two C-C chemokines play distinct roles in ulcer recurrence induced by TNF-α. Further studies are required to determine the precise role of MIP-1α in ulcer recurrence.

ANS inhibited neutrophil infiltration and ulcer recurrence, but not macrophage infiltration, suggesting that unlike typical acute inflammation, which is characterized by neutrophil accumulation, in our model of ulcer recurrence, macrophages infiltrated into scarred mucosa at first followed by neutrophils. Marked increases in the expression of C-X-C chemokines, which are chemotactic for rat neutrophils, occurred in scarred mucosa after 24 h. Because these chemokines were mainly produced by macrophages in scarred mucosa, the macrophages may be critical for the neutrophil recruitment. This hypothesis is supported by the findings that anti-MCP-1 antibody, which inhibited macrophage infiltration, decreased expression of MIP-2 and CINC-2α, along with neutrophil infiltration. Together, our results suggest that both neutrophils and macrophages are critical for the development of gastric ulcer recurrence.

ICAM-1, which reacts with CD11a/CD18 and CD11b/CD18 on neutrophils, plays an important role in neutrophil infiltration into injured gastric tissue (30, 32). We previously found (17) that in scarred mucosa and ulcerated areas of the stomach, macrophages produce TNF-α (22, 33), a powerful inducer of ICAM-1 expression. Additionally, it has been shown that MCP-1 itself can enhance ICAM-1 expression on rat endothelial cells (34). Together with the finding that the anti-MCP-1 antibody inhibited the expression of MCP-1, MIP-1α, MIP-2, and CINC-2α in wounded areas, our results suggest that both neutrophils and macrophages are critical for the development of gastric ulcer recurrence.
antibody inhibited the increase in ICAM-1 expression seen 24 h after TNF-α challenge, accompanied by a decrease in neutrophil infiltration, these results suggest that in addition to TNF-α, MCP-1 may contribute to overexpression of ICAM-1 in scarred mucosa.

We have demonstrated that both TNF-α and MCP-1 are also critical for initiating inflammatory responses during the development of indomethacin-induced gastric injury. PTX inhibited the increase in expression of mRNA encoding MCP-1, CINC-2α, and MIP-2. Anti-MCP-1 antibody also inhibited expression of CINC-2α and MIP-2 but did not exert an inhibitory effect on TNF-α mRNA expression, suggesting that TNF-α may occur first, followed by overexpression of MCP-1 in indomethacin-induced gastric injury, leading to the induction of the C-X-C chemokines and cytokines.

We found that similar to IL-1β, TNF-α can induce gastric ulcer recurrence by enhancement of mucosal inflammation and that this ulcer recurrence is also both neutrophil- and acid-dependent. As we have previously reported (31), inflammatory cells such as macrophages were abundant in scarred mucosa. This attribute of scarred mucosa may lead to high sensitivity to cytokines, resulting in the enhancement of mucosal inflammation by both IL-1β and TNF-α and ulcer recurrence. However, the roles of IL-1β and TNF-α may differ in indomethacin-induced gastric injury; TNF-α was also involved in initiating inflammatory responses via induction of MCP-1 in this model, but expression of IL-1β was not increased at an early phase of injury. Additionally, Wallace et al. (28) have reported that intraperitoneal injection of IL-1β prevented indomethacin-induced gastric injury in rats, suggesting that increased TNF-α may be common during gastric ulceration, especially at an early phase, whereas IL-1β has dual action on gastric mucosa and may be ulcerogenic in conditions such as gastric scarring where inflammation is present.

In conclusion, MCP-1 triggered by TNF-α may pay a crucial role in mucosal inflammatory responses by regulating leukocyte recruitment and expression of C-X-C chemokines, especially at an early phase of inflammation during gastric ulceration including cytokine-induced ulcer recurrence and NSAID-induced gastric injury.

REFERENCES


