Inhibitory Action of Indomethacin on Neutrophil Infiltration in Monosodium Urate-Induced Pleurisy in Rats

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ABSTRACT—The effects of indomethacin on the production of cytokines at inflammatory sites were investigated in the monosodium urate (MSU) pleurisy model characterized by both cellular influx and edema. Indomethacin (10 mg/kg) orally administered 0.5 hr prior to MSU injection into the pleural cavity significantly inhibited MSU-induced neutrophil accumulation in the cavity. In addition, the drug slightly enhanced the level of MSU-induced tumor necrosis factor production without affecting interleukin-1 production. Furthermore, indomethacin inhibited both the levels of MSU-induced rat cytokine-induced neutrophil chemoattractant (CINC/gro) and interleukin-6 (IL-6) production by 78.3% at 3 hr and 45.8% at 4 hr post-injection, respectively. Although intrapleural injection of CINC/gro induced neutrophil infiltration in a dose-dependent manner, IL-6 did not affect the action of CINC/gro on neutrophil influx. These findings suggest that the inhibitory action of indomethacin on neutrophil infiltration is, at least, partly mediated by a decrease in the MSU-induced CINC/gro content in this model.

Keywords: Indomethacin, Monosodium urate, Inflammation, Rat cytokine-induced neutrophil chemoattractant (CINC/gro), Cytokine

It is well known that inflammation induces leukocyte infiltration and fluid accumulation accompanied by primary signs such as heat, edema, erythema and pain. To investigate these inflammatory processes, many experimental models using animals have been used. Pleurisy induced by microcrystals of monosodium urate (MSU) in the rat is one such model.

Releases of interleukin (IL)-1 (1), IL-6 (2) and tumor necrosis factor α (TNF-α) (3) from monocytes and synovial lining cells are induced by MSU. IL-1 or TNF-α promotes neutrophil infiltration via the induction of adhesion protein expression by the endothelial cells (4). IL-1 and TNF-α are also known to induce the release of the neutrophil chemotactic/activating cytokine IL-8 (5). In vitro studies indicate that IL-8 is the major neutrophil chemotactic factor released from MSU-activated human monocytes (6). Although IL-8 in rats has yet to be identified, rat cytokine-induced chemoattractant (CINC/gro, a member of the human IL-8 family) seems to function as a chemoattractant for neutrophils in lipopolysaccharide (LPS)-induced inflammation of rats (7). However, studies of the production kinetics of cytokines in MSU-induced pleurisy of rats have yet to be investigated.

Indomethacin has been known to inhibit prostaglandins (PGs) synthesis. However, suppression of inflammation requires doses of non-steroid anti-inflammatory drugs (NSAIDs) higher than those required to inhibit PGs synthesis (8). This suggests that the effects of NSAIDs on neutrophils are not directly related to the inhibition of PGs production.

As the mediation of inhibitory action of indomethacin on neutrophil accumulation by these cytokines or lipid mediators in vivo is unclear, we investigated whether the inhibitory effect of indomethacin on leukocyte accumulation was related to the modulation effects of cytokines. The time-related production of PGE₂, leukotriene (LT) B₄ and cytokines (IL-1, IL-6, CINC/gro and TNF) in the inflamed pleural cavity of MSU-induced pleurisy was therefore examined.

MATERIALS AND METHODS

Animals
Male Sprague-Dawley rats (Charles River, Shizuoka), weighing 200–250 g, were used in the experiments. Animals were caged in groups of five and were allowed free access to food and water in a room maintained at 22±2°C with 55±10% humidity under an alternating 12-
hr light/dark cycle (lights on at 07:00 hr).

**MSU-induced pleurisy in rats**

MSU crystals was prepared from uric acid (Nacalai Tesque, Kyoto) as previously described (9). Under ether anesthesia, sterile saline solution (1 ml) containing 1% MSU was injected into the right pleural cavity (10). At various times after MSU injection, rats were sacrificed by exsanguination under ether anesthesia. The thoracic cavity was surgically exposed, and the pleural exudate was harvested after rinsing the pleural cavity with sterile saline solution (1 ml) containing 10 µg indomethacin (Sigma Chem. Co., St. Louis, MO, USA).

Cells were counted with a hemocytometer (CC-108; Toa Medical Electric Co., Osaka), and leukocytes were differentially counted after Wright-Giemsa staining. After measuring the volume, the exudate was centrifuged at 2,000 x g for 10 min at 4°C. The supernatant was stored at -80°C until use.

**MH60/BSF2 hybridoma proliferation assay for IL-6**

Bioactivities of IL-6 were assayed with the IL-6-sensitive MH60/BSF2 hybridoma cell line (11). Briefly, cell-free supernatants (from pleural fluid) in serially two-fold diluted aliquots were added to cultures of MH60/BSF2 cells (5 x 10^4 cells/ml in 100 µl volume) before the cells were incubated at 37°C for 48 hr. The proliferation of MH60/BSF2 cells was measured by a colorimetric assay using the sodium salt of 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate (WST-1; Dojin Chemical Co., Kumamoto). The proliferation was indexed by the reading of optical density at 450 nm minus the background at 650 nm in each well using an auto-plate reader (THERMO max; Wako, Osaka). All test samples were assayed at least twice. Standard curves with human recombinant IL-6 (hrIL-6; 1 Unit =5 pg; Genzyme Co.). After a 48-hr incubation at 37°C, in 5% CO₂ atmosphere, the supernatans were added to the CTL-2 cultures (8 x 10^3 cells/100 µl/well). CTL-2 proliferation, proportional to IL-2 content, was assayed by WST-1 reduction. The proliferation was indexed in a manner similar to the procedure described in the IL-6 assay. After compensating for inter-assay variations, data on hrIL-1 were analyzed and calculated as described in the IL-6 assay. Samples were devoid of IL-2-like activities when assessed with CTL-2 cells (data not shown).

**Rat CINC/gro assay**

Rat CINC/gro (Peptide Institute, Inc., Osaka) in pleural fluid was measured using the rat CINC/gro enzyme-immunoassay (EIA system, Amersham Co.).

**TNF assay**

TNF levels in the samples were measured with the WEHI-164 cell line as previously described (14). These cells are extremely sensitive to the lytic effects of TNF, detecting concentrations as low as 4.88 pg/ml. Briefly, the samples were serially diluted in 96-well plates. Samples were diluted in RPMI 1640 medium (Nikken Biomedical Laboratories, Osaka) containing 10% FCS, 1 mM L-glutamine, 10 mM HEPES, 50 µM 2-mercaptopoethanol (2-ME; Wako Pure Chemicals, Osaka), 100 µg/ml streptomycin and 100 Units/ml penicillin to a final volume of 100 µl. A standard curve of serially diluted human recombinant TNF-α (hrTNF-α, 1 Unit =500 pg; Genzyme Co.) was included in each assay. WEHI cells suspended in the RPMI-1640 medium (4 x 10^4 cells/100 µl/well) with 1 µg/ml actinomycin D (Sigma Chem. Co.) were then added to each well containing 100 µl sample solution (final volume =200 µl). The plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 20 hr. WST-1 was added to each well prior to further incubation for 4 hr. The absorbance was read at 450–600 nm on a ELISA reader, and TNF concentrations were calculated based on the hrTNF-α standard plot.

**Statistical analyses**

The significant difference between groups was assessed by the unpaired Student's t-test and Tukey's test for single and multiple comparisons, respectively. Differences in P value of less than 0.05 were considered significant.
RESULTS

Infiltrating cells in MSU-induced inflammatory exudate
Administration (1 ml) of MSU (10 mg/ml) suspended in saline into the pleural cavity produced an inflammatory reaction. MSU induced severe infiltration of leukocytes into the cavity. Almost all of the infiltrating leukocytes were neutrophils (Fig. 1). Neutrophils accumulated in the pleural fluid after a time-lag of 2 hr. Indomethacin inhibited leukocyte accumulation in the pleural cavity 6 hr after MSU injection in a dose-dependent manner (Fig. 2). Indomethacin (10 mg/kg, p.o.) inhibited MSU-induced leukocytes influx significantly at 6 hr and suppressed neutrophil accumulation at 3–6 hr post-MSU injection (Figs. 1 and 2).

Kinetics of cytokine production in response to MSU
Post-MSU (10 mg) pleural levels of TNF, IL-1, IL-6 and CINC/gro in the rat pleural cavity were examined over a 6-hr period (Fig. 3). Pleural TNF levels detectable at 0.5 hr indicated a sharp increase and peaked at 2 hr (1.72 ± 0.28 ng/cavity) before decreasing thereafter. Oral administrations of indomethacin (10 mg/kg) 0.5 hr before MSU injection into the rat pleural cavity significantly enhanced MSU-induced TNF production at 1, 5 and 6 hr post-injection (Fig. 3A). CINC/gro contents, first detected at 1 hr post-MSU, increased markedly to 41.4 ± 9.4 ng/cavity at 2 hr and plateaued at 3 hr before showing a tendency to decrease at 5 hr. Indomethacin significantly inhibited the level of MSU-induced CINC/gro at 3 hr post-injection (Fig. 3B). IL-1, not detected until at 1 hr post-MSU, increased markedly from 2 hr to 4 hr (687.1 ± 69.3 pg/cavity) before decreasing (217.8 ± 61.3 pg/cavity at 6 hr) thereafter (Fig. 3C). Indomethacin slightly enhanced the level of MSU-induced IL-1 production at 2–3 hr post-injection. Similarly, IL-6 levels, which did not elevate until 1 hr, increased remarkably thereafter (3.75 ± 0.46 μg/cavity) before decreasing (2.63 ± 0.49 μg/cavity) at 6 hr post-injection (Fig. 3D). Indomethacin significantly inhibited the MSU-induced IL-6 increase at 4–6 hr post-injection.

Effects of indomethacin on CINC/gro and IL-6 levels
CINC/gro contents were measured with ELISA at 3 hr and IL-6 contents were bioassayed at 4 hr after MSU intra-pleural injection in rats. Oral indomethacin preparations at 5 and 10 mg/kg inhibited MSU-induced IL-6 production by 45.7 and 45.8% and CINC/gro contents by 35.8 and 78.3%, respectively (Table 1).

Effects of CINC/gro and IL-6 on neutrophil accumulation
When vehicle (0.1% FCS), CINC/gro (0.375–1.5 μg) and/or IL-6 (4 μg) were intrapleurally administered in rats (Table 2), CINC/gro dose-dependently elevated neutrophil accumulation in the pleural cavity. Although IL-6 showed a tendency to increase CINC/gro-induced neutrophil accumulation, the effect was not statistically significant.
Effects of indomethacin on PGE$_2$ and LTB$_4$ production

Plural contents of PGE$_2$ and LTB$_4$ after MSU injection (10 mg) into the rat pleural cavity were examined over a 6 hr period. Pleural PGE$_2$ levels increased markedly at 0.5 hr (1.77±0.25 ng/cavity), decreased rapidly at 2

Table 1. Inhibitory effects of indomethacin on rat cytokine-induced neutrophil chemoattractant (CINC/gro) and interleukin (IL)-6 contents induced by monosodium urate (MSU) in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>Inhibition (%)</th>
<th>Rat CINC/gro</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin</td>
<td>5</td>
<td>7</td>
<td>35.8±14.6</td>
<td>45.7±6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>78.3±9.2</td>
<td>45.8±9.1</td>
<td></td>
</tr>
</tbody>
</table>

Vehicle or indomethacin (5 and 10 mg/kg) was orally administered at 0.5 hr prior to intrapleural MSU injection in rats. CINC/gro contents were measured with ELISA at 3 hr and IL-6 contents were bioassayed at 4 hr after MSU injection. Each value represents a mean±S.E. n: number of animals used.

Table 2. Effects of rat cytokine induced neutrophil chemoattractant (CINC/gro) and interleukin (IL)-6 on neutrophil infiltration in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leukocytes (X10$^3$)</th>
<th>Neutrophils (X10$^5$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat CINC/gro dose (ug)</td>
<td>IL-6 dose (ug)</td>
<td>n</td>
</tr>
<tr>
<td>0.375</td>
<td>--</td>
<td>5</td>
</tr>
<tr>
<td>0.75</td>
<td>--</td>
<td>9</td>
</tr>
<tr>
<td>1.5</td>
<td>--</td>
<td>5</td>
</tr>
<tr>
<td>0.75</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>

Vehicle (0.1% FCS), CINC/gro (0.375-1.5 µg) and/or IL-6 (4 µg) were administered into the pleural cavity of rats. Cells in the cavity were counted 3 hr after cytokine injection. Each value represents a mean±S.E. n: number of animals used.
hr post-injection and maintained at a plateau level thereafter (Fig. 4A). Pretreatment with indomethacin (10 mg/kg, p.o.) at 0.5 hr before MSU injection completely inhibited MSU-induced PGE₂ production over a 6 hr post-injection period. The pleural LTB₄ level elevated rapidly at 1 hr post-MSU injection (3.54±0.67 ng/cavity) before decreasing rapidly to an undetectable level (Fig. 4A). Furthermore, indomethacin administered orally at 2.5, 5 and 10 mg/kg significantly suppressed the level of MSU-induced PGE₂ production by 47.5, 72.9 and 80.9%, respectively (Fig. 4B) without affecting LTB₄ contents (data not shown).

![Fig. 4](image)

**Fig. 4.** Effect of indomethacin on time-related changes in the levels of monosodium urate (MSU)-induced PGE₂ and LTB₄ production in rats. A: MSU-induced prostaglandin E₂ (PGE₂: circles) and leukotriene B₄ (LTB₄: squares) contents were measured using ELISA at 0.5, 1, 2, 3, 4, 5 and 6 hr after intrapleural MSU injection in rats (5-6 animals/group). Indomethacin (10 mg/kg; closed symbols) was orally administered at 0.5 hr prior to MSU injection, and the time-related changes in the PGE₂ content was measured. B: vehicle (open column) or indomethacin (2.5, 5 and 10 mg/kg; hatched columns) was orally administered at 0.5 hr prior to MSU injection. PGE₂ contents in the cavity of rats (5 animals/group) were measured at 1 hr post-injection. Each point plus or minus vertical bar represents a mean±S.E. Significant differences from the control with *P<0.05 were verified statistically.

**Table 3.** Effects of AA-861 on leukocyte infiltration and leukotriene (LT) B₄ levels induced by intrapleural injection of monosodium urate (MSU) in rats

<table>
<thead>
<tr>
<th>Treatments</th>
<th>n</th>
<th>Leukocytes (×10⁶ cells)</th>
<th>LTB₄ (ng/cavity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>5</td>
<td>5.76±0.55</td>
<td>5.36±2.18</td>
</tr>
<tr>
<td>AA-861</td>
<td>5</td>
<td>5.75±0.95</td>
<td>2.08±0.59</td>
</tr>
</tbody>
</table>

MSU (1 ml; 1%) containing either vehicle (0.05% ethanol) or AA-861 (5×10⁻⁵ M) was administered into the pleural cavity of rats. Leukocytes were counted 6 hr after MSU injection. LTB₄ contents were measured by ELISA 1 hr after MSU injection. Each value represents a mean±S.E. n: number of animals used, AA-861: 2-(12-hydroxydodeca-5,10-dinyl)-3,5,6-trimethyl-1,4-benzoquinone.

Effects of AA-861 (2-(12-hydroxydodeca-5,10-dinyl)-3,5,6-trimethyl-1,4-benzoquinone) on leukocyte accumulation and LTB₄ production

Leukocyte accumulation at 6 hr and LTB₄ production at 1 hr were measured after applying a combination of MSU suspension (10 mg MSU suspended in 0.05% ethanol-saline) and 5×10⁻⁵ M AA-861 (Biomol Research Laboratories, Inc., Plymouth Meeting, PA, USA) into the rat pleural cavity (Table 3). AA-861 inhibited MSU-induced LTB₄ increases by 61.2% without affecting leukocyte accumulation.

**DISCUSSION**

The introduction of MSU into the pleural cavity provoked an inflammatory reaction, resulting in vigorous infiltration of neutrophils into the pleural cavity at 3 hr post-MSU injection. This response was accompanied by infiltration of mononuclear cells, which was a minor component in the pleural fluid at 1-4 hr after MSU injection. Indomethacin dose-dependently inhibited intrapleural leukocyte accumulation 6 hr post-MSU injection. In addition, this anti-inflammatory agent (10 mg/kg, p.o.) significantly inhibited leukocyte influx and selectively suppressed neutrophil accumulation at 6 and 3-6 hr post-MSU injection, respectively.

Effects of indomethacin on the MSU-induced production of TNF, IL-1, IL-6 and CINC/gro in the pleural cavity were investigated to define the inhibitory action of indo-
methacin on neutrophil accumulation. The sequential release of cytokines, in the order of TNF, IL-1 and IL-6, is consistent with findings in animal models of septic shock (15). After MSU injection, CINC/gro levels increased rapidly between 1 to 3 hr and peaked with 42 ng/cavity at 2 hr before decreasing to 4 ng/cavity at 5 hr. Both TNF and IL-1 have been shown to induce the expression of IL-8 in several cell types. These include the fibroblasts and epithelial cells (5, 16), which are not responsive to direct LPS stimulation. Rat CINC/gro is related to the human IL-8 family. CINC/gro levels, which increased after the release of TNF in the cavity, decreased by the time IL-1 attained the peak level. The TNF effect on CINC/gro production was more effective than that of IL-1 in this model. PGE2 has been demonstrated to down-regulate IL-6 production (17, 18) in vitro, whereas the effects of indomethacin and other NSAIDs were vice versa.

In the present study, indomethacin enhanced MSU-induced TNF production significantly and inhibited the MSU-induced CINC/gro and IL-6 contents. Neutrophil infiltration into the pleural cavity increased markedly at 3 hr post injection, while CINC/gro levels in the pleural fluid had already peaked at 2 hr after MSU injection. It has also been reported that an increase in chemotactic activity preceded neutrophil infiltration (7). Neutrophil accumulation was induced in a concentration-dependent manner at 3 hr after intrapleural CINC/gro injection in rats. In addition, IL-6 did not amplify the CINC/gro-induced neutrophil accumulation. These results suggest that the inhibitory effects of indomethacin on MSU-induced neutrophil accumulation mediate, at least in part, the inhibition of MSU-induced CINC/gro release. Further studies on the inhibitory mechanisms of indomethacin on MSU-induced CINC/gro and IL-6 production in vitro are warranted. LTB4 increases in vivo neutrophil accumulation in humans (19), rabbits (20) and guinea pigs (21). Although AA-861, which is a 5-lipoxygenase inhibitor, reduced MSU-induced LTB4 production, the leukocyte accumulation in MSU-induced pleurisy was not affected in rats. A similar lack of efficacy of LTB4 in neutrophil accumulation has been reported in rats (22). These data suggest clear species-dependent differences in the in vivo chemotactic responses to LTB4. Thus, LTB4 may not play an important role in neutrophil accumulation in MSU-induced pleurisy in rats.

Indomethacin, a cyclooxygenase inhibitor, suppressed MSU-induced neutrophil accumulation in a dose-dependent manner. Although indomethacin displays similar inhibitory potency on PGE2 at 5 and 10 mg/kg, significant suppression on MSU-induced neutrophil accumulation were elicited by the drug at 10 mg/kg. This suggests that the inhibitory effects of indomethacin on neutrophil accumulation are not due to the inhibition of PGE2 synthesis. As such, other mechanisms may be involved in this suppression of neutrophil accumulation by indomethacin.

In conclusion, the inhibitory action of indomethacin on neutrophil infiltration was, at least in part, mediated by decreasing the levels of MSU-induced CINC/gro in this model.

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