

Non-steroidal anti-inflammatory drugs are not involved in ICAM-1 expression of endothelial cells but in that of gastric fibroblasts in vitro

Hiroki TAKEYAMA^{1,2)}, Taku TSUKUI²⁾, Atsushi TATSUGUCHI²⁾,
Ken WADA²⁾, Kazumasa MIYAKE²⁾, Kei SHINOKI²⁾, Yoko SHINJI²⁾,
Tadasu IIZUMI²⁾, Tetsuro HIRATSUKA²⁾, Katya GUDIES²⁾,
Seiji FUTAGAMI²⁾, Shuhei MIURA¹⁾, Yuji MIZOKAMI¹⁾,
Takeshi MATSUOKA¹⁾, Choitsu SAKAMOTO²⁾

¹⁾Fifth Department of Internal Medicine, Tokyo Medical University

²⁾Third Department of Internal Medicine, Nippon Medical School

Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) are known to cause gastric mucosal injury by upregulating ICAM-1 expression, and thus activating neutrophils in the gastric mucosa in vivo. However, studies differ on just how NSAIDs affect ICAM-1 expression in vitro. We therefore examined the effects of the NSAIDs indomethacin and NS-398, a non-selective and a selective cyclooxygenase-2 (COX-2) inhibitor, respectively, on ICAM-1 expression in human umbilical vein endothelial cells (HUVEC) and gastric fibroblasts in vitro. IL-1 β at 10 ng/ml strongly stimulated increases in ICAM-1 expression in HUVEC and gastric fibroblasts. Indomethacin and NS-398 did not affect ICAM-1 expression in endothelial cells, while both indomethacin and NS-398 significantly inhibited IL-1 β -stimulated ICAM-1 expression in gastric fibroblasts without any influence on COX-2 expression. To our interest, the addition of prostaglandin E2 not only reversed the suppression, but synergistically down-regulated ICAM-1 expression with indomethacin in gastric fibroblasts. MG-132 pretreatment strongly suppressed IL- β -stimulated ICAM-1 expression in both cell types.

The results suggest that NSAIDs-stimulated ICAM-1 expression is not likely to directly affect the endothelial cells in the gastric mucosa, and that COX-2 plays no role in NSAID-stimulated increases in ICAM-1 expression in endothelial cells and gastric fibroblasts.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to induce gastrointestinal mucosal injury by inhibiting prostaglandin (PG) production, and thereby affecting the mechanisms protecting the mucosa^{1,2)}. Serious complications, such as mucosal bleeding and perforation, have also been reported in

long-term NSAID users^{3,4)}. One of the most significant mechanisms suggested relates NSAID-caused mucosal injury to NSAID-induced neutrophil activation, apparently triggered by the inhibition of PG production^{5,6)}. It is well known that the adhesion of leucocytes to intercellular adhesion molecules 1 (ICAM-1) in endothelial cells is the first step initiating neutrophil activation^{7,8)}; in turn leading to transendothelial neutrophils,

Received October 18, 2002, Accepted January 31, 2003

Key word : Indomethacin, Intercellular adhesion molecules 1, Cyclooxygenase, Gastric fibroblasts, Interleukin-1

Reprint requests to : Hiroki Takeyama. Fifth Department of Internal Medicine, Tokyo Medical University, 3-20-1 Chuo, Amimachi, Inashiki-Gun, Ibaraki 300-0385, JAPAN

which appear to be involved in mucosal injury. A number of reports show high plasma and tissue TNF- α levels, and marked up-regulation of endothelial ICAM-1 levels in the gastric mucosa of animals given NSAIDs^{9,10}. Thus it may be that NSAIDs play an important role in the up-regulation of ICAM-1 expression in endothelial and other interstitial cells of the gastric mucosa at least indirectly through the increase in proinflammatory cytokine levels in plasma and tissue.

In contrast to these *in vivo* studies, *in vitro* studies have shown that NSAIDs inhibit not only ICAM-1, but also the vascular cell adhesion molecule-1 (VCAM-1) in human umbilical vein endothelial cells (HUVEC)^{11,12}, while one report indicates NSAIDs up-regulate ICAM-1 levels by inhibiting cellular cyclooxygenase-2 (COX-2) in vascular smooth muscle cells¹³. Thus, reports conflict regarding the effect of NSAIDs on ICAM-1 expression in endothelial and interstitial cells *in vitro*. Since Vane et al¹⁴ reported that NSAIDs combine and inhibit cyclooxygenase, which is the rate-limiting enzyme of arachidonate cascade, the enzyme is well known as a molecular target of NSAIDs. Recent papers¹⁵, however, reported other functions of NSAIDs on NF κ B, which is one of the transcription factors and which affects ICAM-1 expression. One showed that NSAIDs enhance TNF- α production by macrophages, the cytokines facilitate translocation of NF κ B into the nucleus, and NF κ B, in turn, enhances ICAM-1 expression.

But another paper¹⁶ showed that NSAIDs directly combine and inactivate I κ B kinase β , leading to the inhibition of the translocation of NF κ B. Thus, reports also conflict on the effects of NSAIDs on NF κ B.

Therefore, the aims of this study are to clarify the effects of indomethacin and a specific COX-2 inhibitor: NS-398 on ICAM-1 expression levels of HUVEC and gastric fibroblasts, common tools in ICAM-1 expression studies *in vitro*, under the presence or absence of a proinflammatory cytokine such as IL-1 β ^{17,18}. We also looked at the effects of a specific NF κ B inhibitor on ICAM-1 expression to determine which factor is predominant in both cell types.

Materials and Method

Reagents

Indomethacin and prostaglandin (PG) E2 were purchased from SIGMA Chemical Corporation (St Louis, MO, USA). NS-398 (N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide) was gift from Taisho Pharmaceutical Co. (Tokyo, Japan). Recombinant tumor necrosis factor (TNF)- α and interleukin(IL)-1 β were obtained from Genzyme techne (Minneapolis, MN, USA). MG-132 was purchased from Peptide Institute Inc. (Minoo, Japan). Horseradish peroxidase (HRP)-

conjugated goat anti-mouse IgG and anti-rabbit IgG were purchased from Promega (Madison, WI, USA). Leupeptin, pepstatin A, aprotinin, phenylmethylsulfonyl fluoride (PMSF), and 3-[(3, cholamidopropyl)-dimethylammonio]-l-propane-sulfonate (CHAPS) were purchased from SIGMA.

Cell culture

HUVEC were purchased from Asahi Technoglass Corporation (Tokyo, Japan). The cells were maintained in an EBM culture medium kit (Asahi Technoglass Co., Tokyo, Japan) containing human epidermal growth factor (EGF), hydrocortisone, bovine brain extracts (BBE), and fetal bovine serum (FBS, Biosciences Pty. Ltd., Lorne, Victoria, Australia). All supplements were eliminated 12 hours before initiating experiments.

Gastric fibroblasts, derived from gastric ulcer patients (Hs 262. St), were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in RPMI-1640 (Nikken Chemicals Co. Tokyo, Japan) supplemented with 10% FBS. FBS was eliminated 12 hours before initiating experiments.

For cell ELISA assay, fifty thousand cells were seeded into 96-well type-1 collagen coated plates (Beckton Dickinson Labware, Franklin Lakes, NJ, USA) and incubated for 12 hours at 37°C in 5% CO₂. The cells were then washed twice with phosphate buffered saline (PBS, pH 7.4) and cultured in serum-free medium for another 12 hours. After being washing with PBS, the cells were stimulated with TNF- α (10 ng/ml) and IL-1 β (10 ng/ml) in the presence or absence of a non-selective NSAID indomethacin, a selective COX-2 inhibitor NS-398, PGE2 and a proteasome inhibitor MG-132 for 12 hours.

Cell ELISA

The cells were fixed with 1% paraformaldehyde in PBS for 20 min. Non-specific binding was blocked by 0.5% bovine serum albumin (BSA, Serologicals, Kankakee, IL, USA) for 1 hour. ICAM-1 antibody (Chemicon Co., Temecula, CA, USA) and HRP-conjugated anti-mouse IgG antibody were used as the first and the second antibodies, followed by the addition of tetramethyl benzidine (TMB) peroxidase EIA substrate (Bio-Rad Laboratories, Hercules, CA, USA) as indicated¹⁹ in the manufacturer's instructions. The optical density (OD) of each well was determined with a microplate reader (Bio-Rad) at 450 nm. Relative ICAM-1 expression was calculated by the following formula: (sample (OD)-negative control (OD))/positive control (OD)-negative control (OD)) \times 100. Data are expressed as means \pm SE.

Western blotting

The cells (5×10^5 /ml) were cultured in 10 cm culture-dishes as described above. After being washed with

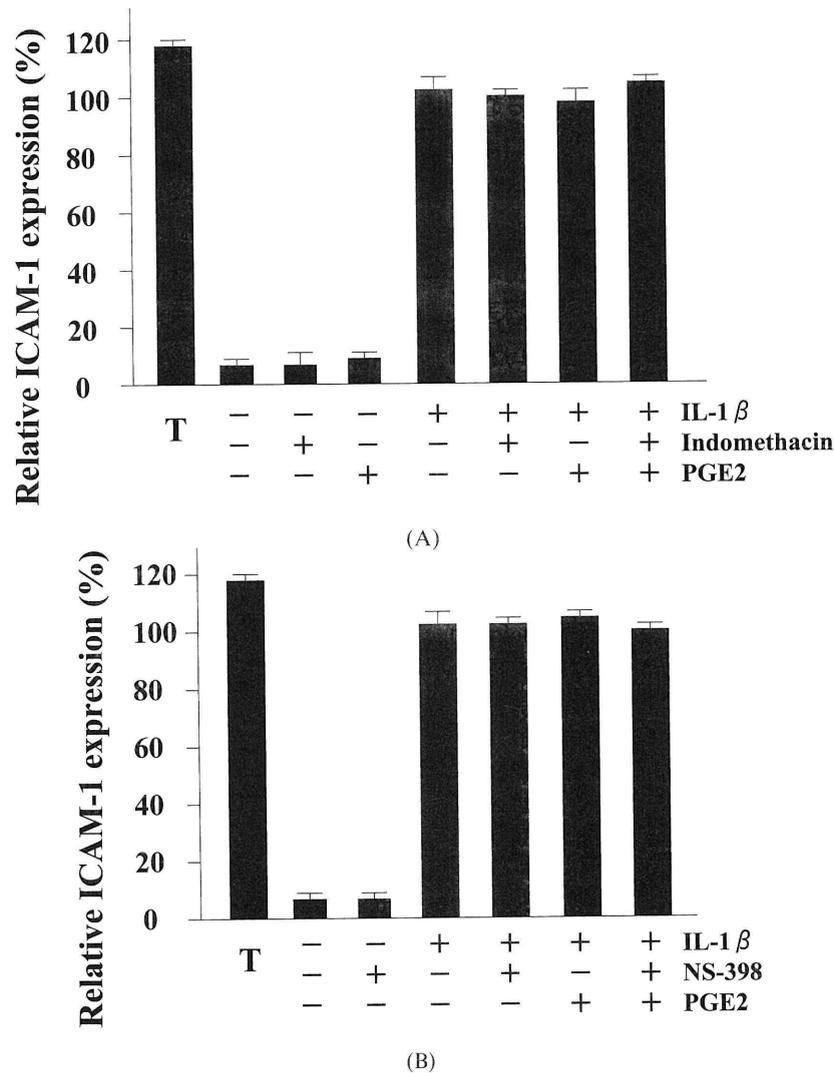


Fig. 1 Effects of PGE2, Indomethacin and NS-398 on ICAM-1 expression of HUVEC. HUVEC were cultured with and without IL-1β(10 ng/ml) or TNF-α(10 ng/ml) for 12 h and detected ICAM-1 expression with cell ELISA methods. Relative expression of ICAM-1 was measured by cell ELISA and calculated as described in Materials and Methods. Values are means ±SE of a representative of 7 experiments. (A) HUVEC were pretreated with indomethacin (10 μM) 1 h before addition of IL-1β and/or PGE2 (1 μM). Lane T indicates control reactions incubated with TNF-α. (B) HUVEC were pretreated with NS-398 (10 μM) 1 h before addition of IL-1β and PGE2 (1 μM).

PBS, the cells were scraped and harvested in buffer. The pellets were collected by centrifugation at 10,000 g, and then lysed in 150 μl extraction buffer consisting of 50 mM Tris-HCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 M sucrose, 1 mM pepstatin A, and 1 mM PMSF and 1% CHAPS. Samples containing 50 μg of protein were separated on 10% acrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Then, the proteins were electrophoretically transferred to a nitrocellulose membrane, and probed with anti-COX-2 antibody specific for human COX-2 protein (Immuno-Biological Laboratories, Fujioka, Japan). Bound antibodies were detected with HRP conjugated anti-rabbit IgG antibody using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech Ltd.).

Immunohistochemical analysis

HUVEC and gastric fibroblasts were cultured on a SlideFlask (Nunc, Roskilde, Denmark) as described above. After fixation with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at 4°C overnight, the slides were washed with PBS once and were incubated with 10% normal horse serum for 20 min to block nonspecific binding of secondary antibody, and then incubated overnight again with primary antibody at 4°C. Mouse monoclonal antibody against ICAM-1 (Novocastrol Laboratories, Burlingame, CA, USA; dilution, 1 : 150) was used as the primary antibody. The antibody was allowed to react with a secondary antibody (horse anti-mouse IgG; Vector; dilution, 1 : 100) labeled with FITC. Antibody binding was detected by the

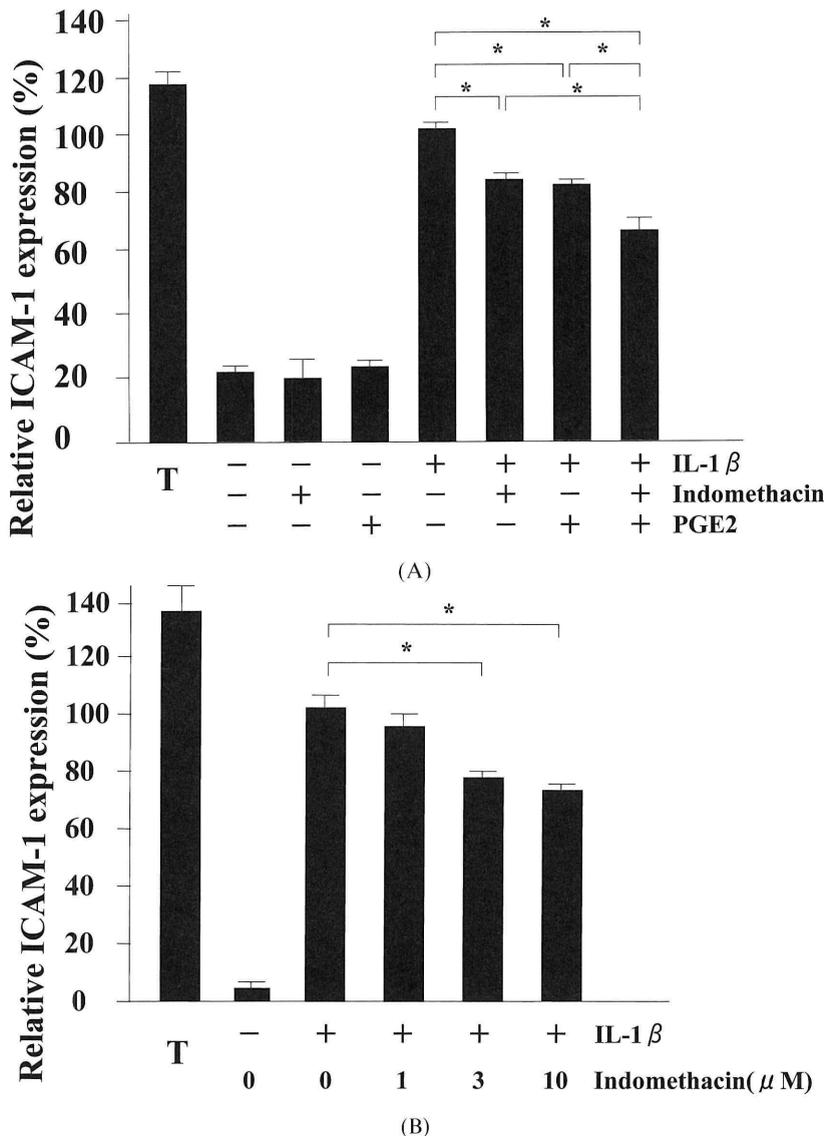


Fig. 2 Effects of PGE2, Indomethacin and IL-1β on ICAM-1 expression of gastric fibroblasts. Gastric fibroblasts were cultured with and without IL-1β(10 ng/ml) or TNF-α (10 ng/ml) for 12 h and detected ICAM-1 expression with cell ELISA methods. as described in Materials and Methods. Relative expression of ICAM-1 was measured by cell ELISA and calculated as described. Values are means ±SE of a representative of 3-7 experiments. * indicates P<0.05 (A) Effects of indomethacin and PGE2 were tested in the presence of IL-1β. Fibroblasts were pretreated with indomethacin (10 μM) 1 h before addition of IL-1β and PGE2 (1 μM). Lane T shows control reactions incubated with TNF-α. (B) Dose related effects of indomethacin were tested in the presence of IL-1β.

immunofluorescence methods and confocal laser scanning microscopy (model TCS4D/DMIRBE; Leica, Heidelberg, Germany) equipped with argon and argon-krypton laser sources. The nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI; Sigma) for 15 min to facilitate identification of morphological features. Negative control immunohistochemical procedures included replacement of the primary antibody with normal mouse IgG.

Statistics

All data are expressed as means±SE. Statistical significance was determined with an analysis of variance (ANOVA) followed by Fisher’s PLSD test; a P value

of less than 0.05 was considered to indicate a statistically significant difference.

Results

Effects of Indomethacin and NS-398 on ICAM-1 Expression in HUVEC First of all, we tested the effects of indomethacin on ICAM-1 expression without any cytokine stimulation in HUVEC. Fig. 1A shows the relative expression of ICAM-1 against that of IL-1β stimulation with the cell ELISA method. Ten micromolar indomethacin, which has been reported to be a sufficient concentration for inhibiting COX activities, had no influence on the basal level of ICAM-1 expres-

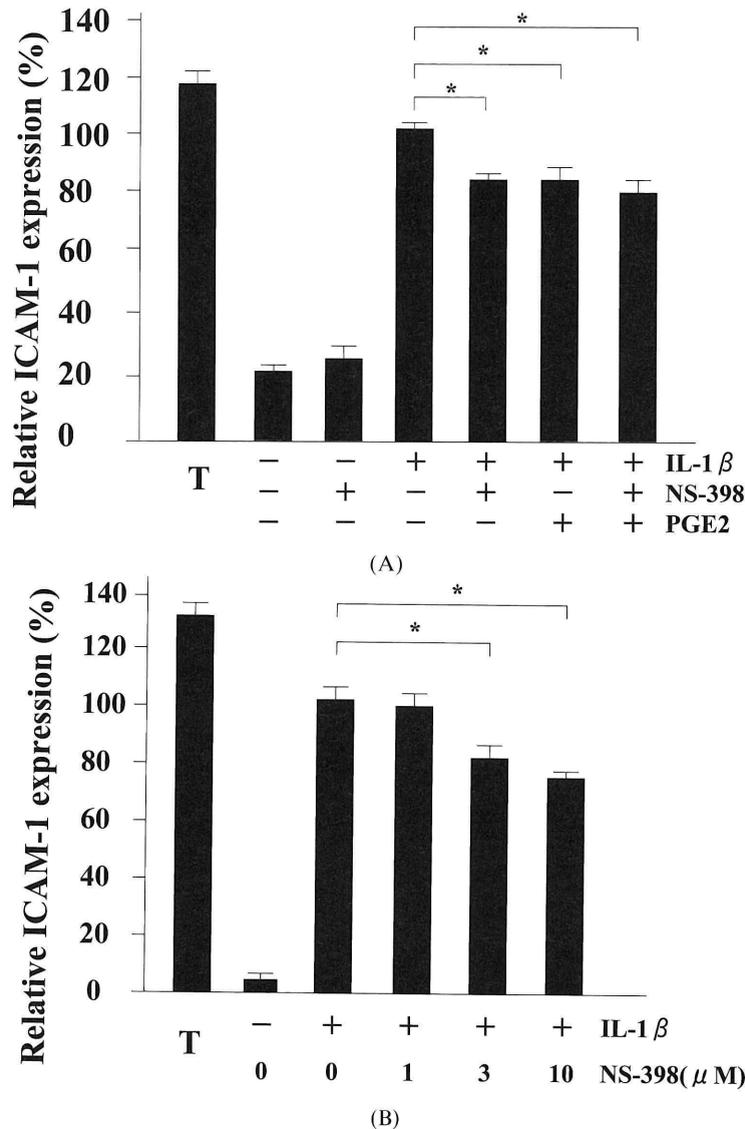


Fig. 3 Effects of PGE2, NS-398 and IL-1β on ICAM-1 expression of gastric fibroblasts. Gastric fibroblasts were cultured with and without IL-1β(10 ng/ml) or TNF-α(10 ng/ml) for 12 h and detected ICAM-1 expression with cell ELISA methods as described. Relative expression of ICAM-1 was measured by cell ELISA and calculated as described. Values are means ±SE of a representative of 3-7 experiments in Materials and Methods. * indicates P<0.05. (A) Effects of PGE2 and NS-398 were tested in the presence of IL-1β. Fibroblasts were pretreated with NS-398 (10 μM) 1 h before addition of IL-1βand PGE2 (1 μM). Lane T shows control reactions incubated with TNF-α. (B) Dose-response suppression of NS-398 were tested in the presence of IL-1β.

sion. Since previous reports²⁰⁾ have shown that indomethacin inhibits COX activities at concentrations of under 14 μM, we also tested the effects of indomethacin at concentrations of 1, 3 and 30 micro molar, and none of them were significantly different from that of the medium culture. Because TNF-α and IL-1β strongly induced increases in ICAM-1 expression in HUVEC, we also investigated the effects of indomethacin on cytokine-stimulated ICAM-1 expression (Fig. 1A). Ten micro molar indomethacin did not affect the IL-1β-stimulated ICAM-1 expression level at all. The effects of indomethacin on HUVEC were also observed within

concentrations of 1 to 30 μM (data not shown).

Since selective COX-2 inhibitor : NS-398 was able to inhibit COX activities at 10 μM²¹⁾, we examined the effect of NS-398 (10 μM) (Fig. 1B). In the same way as indomethacin, NS-398 had no influence on the basal or cytokine-stimulated ICAM-1 levels in HUVEC at concentrations up to 30 μM at all (Fig. 1B).

These results indicate that indomethacin and NS-398 neither enhanced ICAM-1 expression in HUVEC, nor modified IL-1β-stimulated ICAM-1 expression of the cells in vitro.

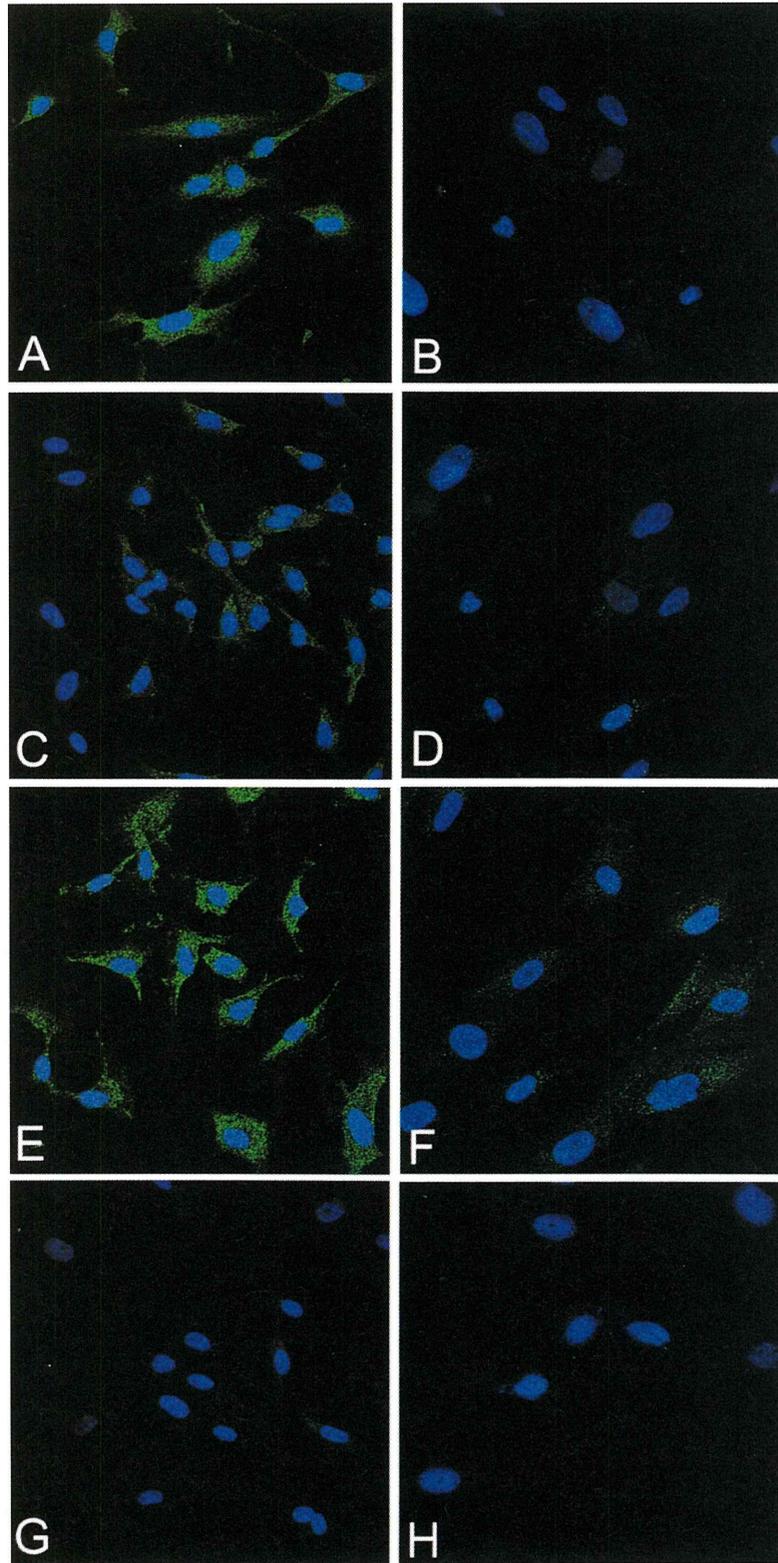


Fig. 4 Immunofluorescence staining of HUVEC and gastric fibroblasts with anti ICAM-1 antibody. A, C, E and G show the expression of ICAM-1 in HUVEC, and B, D, F and H show the expression in gastric fibroblasts. A and B: Cells were cultured in medium only. C and D: Cells were treated with indomethacin and stained with anti ICAM-1 antibody. E and F: Cells were stimulated with IL-1β and stained with anti ICAM-1 antibody. G and H: Cells were stimulated with IL-1β, but stained with control mouse IgG (original magnification; ×400).

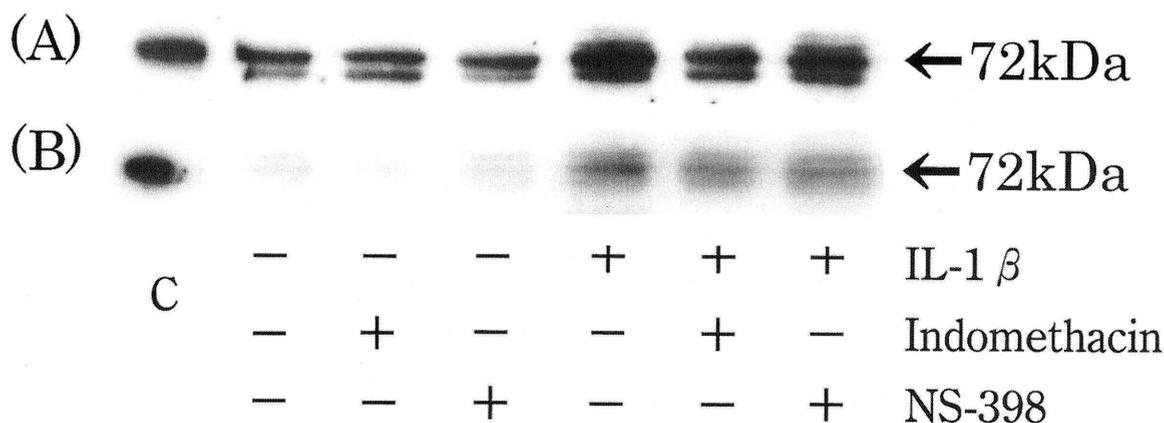


Fig. 5 Western blot analysis of HUVEC (A) and gastric fibroblasts (B) by monoclonal antibody specific for COX-2. Cells were pretreated with indomethacin (10 μ M) or NS-398 (10 μ M) 1 h before addition of IL-1 β (10 ng/ml). Lane C shows sheep COX-2 used as a positive control. Proteins (50 μ g) were loaded onto each lane. A band of 72kDa corresponded to COX-2 protein in each lane.

Effects of Indomethacin and NS-398 on ICAM-1 Expression in Gastric Fibroblasts

Since fibroblasts are known to be an important component in gastric mucosal repair, we next investigated how indomethacin and NS-398 influence the expression of ICAM-1 in cultured gastric fibroblasts, respectively. Fig 2A shows that ICAM-1 expression was not affected by the administration of 10 μ M indomethacin in culture media without cytokine stimulation. So long as indomethacin concentration was within the range of 1 to 100 μ M, ICAM-1 levels were not significantly different from the basal value. Though TNF- α and IL-1 β stimulated increases in ICAM-1 expression in gastric fibroblasts, interestingly, unlike in HUVEC, indomethacin significantly inhibited IL-1 β -stimulated ICAM-1 expression levels in gastric fibroblasts (Fig. 2A). Fig. 2B shows that indomethacin down-regulated ICAM-1 expression of gastric fibroblasts stimulated by IL-1 β to up to 74% of the maximum value in a dose-dependent manner.

In the same way as indomethacin, NS-398 had no influence on basal expression of ICAM-1 in gastric fibroblasts, and down-regulated the expression stimulated by IL-1 β to up to 73% of the maximum value in a dose-dependent fashion (Fig. 3A, B).

These results suggest that indomethacin and NS-398 did not up-regulate ICAM-1 expression of gastric fibroblasts, but significantly suppressed IL-1 β -stimulated ICAM-1 expression levels within the range of up to 10 μ M.

Effects of PGE2 on ICAM-1 Expression in HUVEC or Gastric Fibroblasts

To investigate whether the effects of indomethacin or NS-398 were involved in the inhibition of prostaglandin synthesis, we tested ICAM-1 expression in HUVEC or gastric fibroblasts under the presence of PGE2, which is

one of the most common prostaglandins in human gastric mucosa.

In the absence of cytokine stimulation, exogenously added PGE2 had no effect on ICAM-1 expression in HUVEC or gastric fibroblasts, either. (Fig. 1A, Fig. 2A). IL-1 β -stimulated ICAM-1 expression of HUVEC was not modified by PGE2 administration into the culture at all. Unlike in HUVEC, exogenously added PGE2, however, significantly inhibited ICAM-1 expression in gastric fibroblasts. Moreover, PGE2 and indomethacin suppressed IL-1 β -stimulated ICAM-1 expression levels in gastric fibroblasts synergistically (Fig. 2A). But synergistic suppression of IL-1 β -stimulated ICAM-1 expression was not observed with the addition of both PGE2 (1 μ M) and NS-398 (10 μ M) in gastric fibroblasts at these concentrations.

These data demonstrate that PGE2 did not reverse NSAIDs induced inhibition of IL-1 β -stimulated ICAM-1 expression in gastric fibroblasts.

Immunostaining of ICAM-1 in HUVEC and gastric fibroblasts

Since we evaluated ICAM-1 expression as % level against positive and negative controls, we next examined ICAM-1 protein expression immunohistochemically. Indomethacin did not up-regulate the immunofluorescence intensity of either cell type (Fig. 4 A-D). IL-1 β clearly stimulated increases in immunofluorescence intensity against CD54 in both HUVEC and gastric fibroblasts as compared with basal levels (Fig. 4E, F). The immunoreactivity obviously located on the cell surface in both endothelial and fibroblasts and expression levels seemed stronger in endothelial cells than in gastric fibroblasts. Suppressive effects against COX antagonists were difficult to evaluate in immunohistochemical staining (data not shown).

COX-2 Protein Expression in HUVEC and Gastric Fibroblasts

Since PGE2 did not reverse the effects of COX antagonists, we next confirmed COX-2 protein expression in HUVEC and gastric fibroblasts by western blot analysis. In HUVEC, COX-2 protein expression was detected without cytokine stimulation, and IL-1 β stimulated an increase in COX-2 expression level over the basal level (Fig. 5A). IL-1 β strongly stimulated the COX-2 expression level in gastric fibroblasts as well (Fig. 5B). Indomethacin and NS-398 had no influence on either basal level or on IL-1 β -stimulated COX expression against either cell type.

Effects of NF- κ B inhibitor on ICAM-1 expression

Since ICAM-1 expression is known to be regulated by NF- κ B, we examined the effects of MG-132, a specific NF- κ B inhibitor, to clarify the effects of the transcription factor on ICAM-1 expression in our system. IL-1 β -stimulated ICAM-1 expression levels were strongly down-regulated by pretreatment with MG-132 in both HUVEC (43%) and gastric fibroblasts (47%). The level of the suppression was greater than that of indomethacin treatment. The basal expression levels of ICAM-1 on HUVEC and gastric fibroblasts were not significantly suppressed by MG-132 pretreatment. These data imply that NSAIDs were not sufficient to inhibit IL-1 β -stimulated ICAM-1 expression through the inhibition of NF- κ B in this range of concentration (data not shown).

Discussion

In the present study, we investigated whether NSAIDs are directly involved in ICAM-1 expression in endothelial cells, for which neutrophil adherence has been suggested as the initial step in the activation of NSAID-induced gastropathy. We found that both TNF- α and IL-1 β strongly stimulated ICAM-1 expression in HUVEC, with a 10-fold increase over basal values, which is consistent with previous reports²². However, indomethacin affected neither basal ICAM-1 nor TNF- α (data not shown) and IL-1 β -stimulated increases in ICAM-1 levels in HUVEC, suggesting no direct indomethacin effect on ICAM-1 expression in HUVEC. Furthermore, exogenously added PGE2 at 1 μ M did not affect cytokine-stimulated increases in ICAM-1 expression in HUVEC. These results indicate that neither endogenous PGE2, which may be inhibited by indomethacin, nor exogenous PGE2 is involved in the regulation of ICAM-1 expression in endothelial cells. Therefore, these results further suggest that PGE2 prevention of NSAID-caused injury may not be due to its direct effect on ICAM-1 expression in endothelial cells. There is considerable evidence supporting the concept that neutrophil activation induced by its interaction with ICAM-1 in endothelial cells plays a critical role in

NSAID-induced gastropathy. First, neutropenic rats have been shown to have reduced susceptibility to NSAID-induced gastropathy^{6,23}. Second, prevention of leukocyte adherence by monoclonal antibodies against leukocyte adhesion molecules results in the reduction of NSAID-induced gastropathy^{24,25}. Third, NSAIDs have been shown to stimulate leukocyte adherence to the vascular endothelium in the gastric microcirculation^{26,27}. Fourth, NSAID administration has been shown to induce increases in plasma and gastric mucosal levels of TNF- α ^{10,28,29}, and ICAM-1^{9,30} expression in the gastric mucosa of rats. Cytokines such as TNF- α and IL-1 β have been shown to stimulate increases in ICAM-1 expression in endothelial cells *in vitro*²², as we have also found in the present study. Thus, taking into consideration the above results and those of the present study, it is safe to surmise that NSAIDs have no direct stimulatory effect on ICAM-1 expression, notwithstanding NSAID-caused neutrophil activation commonly observed *in vivo*. Rather, increased TNF- α levels in plasma and gastric mucosa, in response to NSAID administration, might be involved in ICAM-1 expression in the mucosa.

It should be noted that in addition to endothelial cells, fibroblasts and smooth muscle cells also express ICAM-1, which may directly or indirectly be regulated by NSAIDs^{13,30}. We found for the first time, in the present study, that gastric fibroblasts express ICAM-1 in response to proinflammatory cytokine stimulation. In contrast, studies that showed increases in ICAM-1 expression in the gastric mucosa by using immunohistochemistry⁹ or dual radiolabeled antibody techniques³¹ failed to show that such interstitial cells do in fact express ICAM-1. We showed that endothelial cells and gastric fibroblasts appear to express ICAM-1 protein on the surface of cells, with expression levels in endothelial cells somewhat surpassing those in fibroblasts. We also found that NSAID-regulated ICAM-1 expression differed for endothelial cells and gastric fibroblasts. In gastric fibroblasts, indomethacin significantly inhibited IL-1 β -induced increases in ICAM-1 expression levels. To date, reports have shown that NSAIDs such as diclofenac, ibuprofen, and aspirin inhibit ICAM-1 expression on various types of endothelial cells *in vitro*^{11,12,32}. It has been suggested that aspirin inhibits NF- κ B activation³³, thereby inhibiting ICAM-1 expression at transcription sites. Although it has yet to be conclusively determined how NSAIDs suppress cytokine-stimulated increases in ICAM-1 levels *in vitro*, gastric mucosal ICAM-1 expression levels *in vivo* apparently depend on indirect, as well as direct, effects of NSAIDs.

We found in the present study that neither indomethacin nor NS-398, a specific COX-2 antagonist, had any

effect on ICAM-1 expression stimulated by IL-1 β in endothelial cells. On the other hand, NS-398 slightly, but significantly, inhibited ICAM-1 expression stimulated by IL-1 β in gastric fibroblasts, as did indomethacin. Because ICAM-1 suppression levels of indomethacin are similar to those of NS-398, COX-1 inhibition is not likely to be important for ICAM-1 levels. Since we have shown IL-1 β -stimulated upregulation of COX-2 expression in those cells, the data suggest that in gastric fibroblasts both indomethacin and NS-398 act on COX-2 to suppress IL-1 β -stimulated expression of ICAM-1. On the other hand, previous results suggest that COX-2 antagonists upregulate ICAM-1 expression on smooth muscle cells *in vitro*¹³⁾ and activate neutrophil infiltration in the gastric mucosa *in vivo*³⁴⁾. It has also been shown that cercecoxib, a specific COX-2 antagonist, increases leukocyte adherence in the vascular endothelium of mesenteric venules in rats, in contrast to SC-560, a specific COX-1 antagonist³⁵⁾. Thus, it appears that the role of COX-2 in regulating ICAM-1 expression varies with cell type. In order to reach a conclusion on how COX-2 plays a role in leukocyte adherence, further work is clearly required.

In the present study, the effect of NF- κ B activation on ICAM-1 expression in endothelial cells and gastric fibroblasts far surpassed that of COX inhibition. An increase in ICAM-1 gene expression in endothelial cells and gastric fibroblasts is thought to require the transcription factor NF- κ B, as we showed with MG-132 in the present study. Thus, considering the different mechanisms by which NSAIDs cause gastric mucosal injury, proinflammatory cytokine release in response to NSAIDs appears to be of greater import in neutrophil activation than the direct effect of NSAIDs on ICAM-1 expression.

Conclusion

We examined the effects of the NSAIDs indomethacin and NS-398 on ICAM-1 expression in HUVEC and gastric fibroblasts *in vitro*.

- 1) Indomethacin and NS-398 did not affect ICAM-1 expression in endothelial cells, while neither indomethacin nor NS-398 inhibited IL-1 β -stimulated ICAM-1 expression in HUVEC, but both significantly inhibited IL-1 β -stimulated ICAM-1 expression in gastric fibroblasts.
- 2) IL-1 β stimulated increases in ICAM-1 and COX-2 expression in both cell types.
- 3) Exogenously added PGE₂ neither regulated ICAM-1 expression of HUVEC, nor restored IL-1 β -stimulated ICAM-1 expression suppressed by COX antagonists in human gastric fibroblasts.
- 4) Pretreatment of NF κ B inhibitor: MG132 strongly suppressed IL-1 β -stimulated ICAM-1 expression in both cell types.

These results indicate that NSAID is not directly linked to ICAM-1 expression, and that COX-2 is not likely to be involved in NSAID-stimulated increases in ICAM-1 expression in the gastric mucosa.

Acknowledgements

We thank Ms. Hideko Yamagishi for her excellent technical help.

References

- 1) Semble EL, Wu WC: Antiinflammatory drugs and gastric mucosal damage. *Semin Arthritis Rheum* **16**: 271-286, 1987
- 2) Schoen RT, Vender RJ: Mechanisms of nonsteroidal anti-inflammatory drug-induced gastric damage. *Am J Med* **86**: 449-458, 1989
- 3) Paulus HE: FDA arthritis advisory committee meeting: serious gastrointestinal toxicity of nonsteroidal anti-inflammatory drugs, etc. *Arthritis Rheum* **31**: 1450-1451, 1988
- 4) Silverstein FE, Graham DY, Senior JR, Davies HW, Struthers BJ, Bittman RM, Geis S: Misoprostol reduces serious gastrointestinal complications in patients with rheumatoid arthritis receiving nonsteroidal anti-inflammatory drugs. A randomized, double-blind, placebo-controlled trial. *Ann Intern Med* **123**: 241-249, 1995
- 5) Asako H, Kubes P, Wallace JL, Wolf RE, Granger DN: Modulation of leukocyte adhesion in rat mesenteric venules by aspirin and salicylate. *Gastroenterology* **103**: 146-152, 1992
- 6) Lee M, Aldred K, Lee E, Feldman M: Aspirin-induced acute gastric mucosal injury is a neutrophil-dependent process in rats. *Am J physiol* **263**: G920-G926, 1992
- 7) Luscinskas FW, Brock AF, Arnauot MA, Gimbrone MA: Endothelial-leukocyte adhesion molecule-1 dependent and leukocyte(CD11/CD18)-dependent mechanisms contribute to polymorphonuclear leukocyte adhesion to cytokine-activated human vascular endothelium. *J Immunol* **142**: 2257-2263, 1989
- 8) Lawrence MB, Springer AT: Leukocyte roll on a slectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. *Cell* **65**: 859-873, 1991
- 9) Andrews FJ, Malcontenti-Wilson C, O'Brien PE: Effect of nonsteroidal anti-inflammatory drugs on LFA-1 and ICAM-1 expression in gastric mucosa. *Am J Physiol* **266**: G657-G664, 1994
- 10) Santucci L, Florucci S, Matteo FMD, Morelli A: Role of tumor necrosis factor α release and leukocyte margination in indomethacin-induced gastric injury in rats. *Gastroenterology* **108**: 393-401, 1995

- 11) Kapiotis S, Sengoelge G, Sperr WR, Baghestanian M, Quehenberger P, Bevec D, Li SR, Menzel EJ, Muhl A, Zapolska D, Virgolini I, Valent P, Speiser W : Ibuprofen inhibits pyrogen-dependent expression of VCAM-1 and ICAM-1 on human endothelial cells. *Life Sciences* **58** : 2167-2181, 1996
- 12) Sakai A : Diclofenac inhibits endothelial cell adhesion molecule expression induced with lipopolysaccharide. *Life Sciences* **58** : 2377-2387, 1996
- 13) Bishop-Bailey D, Burke-Gaffney A, Hellewell PG, Pepper JR, Mitchell JA : Cyclooxygenase-2 regulates inducible ICAM-1 and VCAM-1 expression in human vascular smooth muscle cells. *Biochem Biophys Res Commun* **249** : 44-47, 1998
- 14) Vane JR : Inhibition of prostaglandin synthesis as a mechanism of action for the aspirin-like drugs. *Nature-New Biology* **231** : 232-235, 1971
- 15) Min-Jean Yin, Yumi Yamamoto, Richard B. Gaynor : The anti-inflammatory agents aspirin and salicylate inhibit the activity of I κ B kinase- β . *Nature* **396** : 77-80, 1998
- 16) Morise Z, Grisham MB : Molecular mechanisms involved in NSAID-induced gastropathy. *J Clin Gastroenterol* **27 Suppl 1** : S87-90, 1998
- 17) Yoshida N, Takemura T, Granger N, Anderson DC, Wolf RE, McIntire LV, Kvietys PR : Molecular determinants of aspirin-induced neutrophil adherence of endothelial cells. *Gastroenterology* **105** : 715-724, 1993
- 18) Noguchi K, Iwasaki K, Ishikawa I : Prostaglandin F $_{2\alpha}$ upregulates intercellular adhesion molecule-1 expression in human gingival fibroblasts. *J Periodont Res* **34** : 277-281, 1999
- 19) Molet S, Gosset P, Vanhee I, Tillie-Leblond B, Wallaert M, Capron M, Tonnel AB : Modulation of cell adhesion molecules on human endothelial cells by eosinophil-derived mediators. *J Leukoc Biol* **63** : 351-358, 1998
- 20) Nothnick WB, Pate JL : Interleukin-1 beta is a potent stimulator of prostaglandin synthesis in bovine luteal cells. *Biology of Reproduction* **43** : 898-903, 1990
- 21) Perkins DJ, Kniss DA : Rapid and transient induction of cyclo-oxygenase 2 by epidermal growth factor in human amnion-derived WISH cells. *Biochem J* **321** : 677-681, 1997
- 22) Pober JS, Slewik MR, DeLuca LG, Ritchie AJ : Elevated cyclic AMP inhibits endothelial cell synthesis and expression of TNF-induced endothelial leukocyte adhesion molecule-1 and vascular cell adhesion molecule-1, but not intercellular adhesion molecule-1. *J Immunol* **150** : 5114-5123, 1993
- 23) Wallace JL, Keenan CM, Granger DN : Gastric ulceration induced by nonsteroidal anti-inflammatory drugs is a neutrophil-dependent process. *Am J Physiol* **259** : G462-G467, 1990
- 24) Wallace JL, Arfors KE, McKnight GW : A monoclonal antibody against the CD18 leukocyte adhesion molecule prevents indomethacin-induced gastric damage in the rabbit. *Gastroenterology* **100** : 878-883, 1991
- 25) Wallace JL, McKnight GW, Miyasaka M, Tamatani T, Paulson J, Anderson DC, Granger DN, Kubes P : Role of endothelial adhesion molecules in NSAID-induced gastric mucosal injury. *Am J Physiol* **265** : G993-G998, 1993
- 26) Kitahora T, Guth PH : Effect of aspirin plus hydrochloric acid on the gastric mucosal microcirculation. *Gastroenterology* **93** : 810-817, 1987
- 27) Asako H, Kubes P, Wallace J, Gaginella T, Wolf RE, Granger DN : Indomethacin-induced leukocyte adhesion in mesenteric venules: role of lipoxigenase products. *Am J Physiol* **262** : G903-G908, 1992
- 28) Santucci L, Fiorucci S, Giansanti M, Brunori PM, Matteo FMDi, Morelli A : Pentoxifylline prevents indomethacin induced acute gastric mucosal damage in rats: role of tumor necrosis factor alpha. *Gut* **35** : 909-915, 1994
- 29) Appleyard CB, McCafferty DM, Tigley AW, Swain MG, Wallace JL : Tumor necrosis factor mediation of NSAID-induced gastric damage : role of leukocyte adherence. *Am J Physiol* **270** : G42-G48, 1996
- 30) Noguchi K, Iwasaki K, Shitashige M, Umeda M, Izumi Y, Murota S, Ishikawa I : Down regulation of lipopolysaccharide-induced intercellular adhesion molecule-1 expression via EP2/EP3 receptors by prostaglandin E $_2$ in human fibroblasts. *Inflammation* **25** : 75-81, 2001
- 31) Morise Z, Komatus S, Fuseler JW, Granger DN, Perry M, Issekutz AC, Grisham MB : ICAM-1 and P-selectin expression in a model of NSAID-induced gastropathy. *Am J Physiol* **274** : G246-G252, 1998
- 32) Voisard R, Fisher R, Osswald MO, Voglic S, Baur R, Susa M, Koenig W, Hombach V : Aspirin (5 mmol/L) inhibits leukocyte attack and triggered reactive cell proliferation in a 3D human coronary in vitro model. *Circulation* **103** : 1688-1694, 2001
- 33) Weber C, Erl W, Pietsch A, Weber PC : Aspirin inhibits nuclear factor- κ B mobilization and monocyte adhesion in stimulated human endothelial cells. *Circulation* **91** : 1914-1917, 1995
- 34) Futagami S, Hiratsuka T, Wada K, Tatsuguchi A, Tsukui T, Miyake K, Akamatsu T, Hosone M, Sakamoto C, Kobayashi M : Inhibition of Helicobacter pylori-induced cyclooxygenase-2 aggravates NSAID-caused gastric damage in Mongolian gerbils. *Aliment Pharmacol Ther* **16** : 847-855, 2002
- 35) Wallace JL, McKnight W, Reuter BK, Vergnolle N : NSAID-induced gastric damage in rats: requirement for inhibition of both cyclooxygenase 1 and 2. *Gastroenterology* **119** : 706-714, 2000

非ステロイド抗炎症薬は内皮細胞の ICAM-1 発現に作用しないが 胃線維芽細胞には作用する

竹山裕樹^{1),2)} 津久井拓²⁾ 辰口篤志²⁾
 和田謙²⁾ 三宅一昌²⁾ 篠木啓²⁾
 進士陽子²⁾ 飯泉匡²⁾ 平塚哲郎²⁾
 カチャ・グディー²⁾ 二神生爾²⁾ 三浦崇幣¹⁾
 溝上裕士¹⁾ 松岡健¹⁾ 坂本長逸²⁾

¹⁾東京医科大学内科学第五講座

²⁾日本医科大学内科学第三講座

【要旨】 NSAIDs は *in vivo* においては胃粘膜の ICAM-1 の発現を増加させ、好中球を活性化させることにより胃粘膜障害を起こすことで知られている。しかし、*in vitro* においては NSAIDs が ICAM-1 発現にどう作用するかは結果は一定していない。今回我々は、インドメタシン、選択的 COX-2 阻害薬である NS-398、この 2 種の NSAIDs の ICAM-1 発現に対する作用を、ヒト臍帯静脈内皮細胞 (HUVEC)、胃線維芽細胞を用いて調べた。内皮細胞、胃線維芽細胞において IL-1 β 10 ng/ml 刺激で ICAM-1 発現は著増した。内皮細胞ではインドメタシン、NS-398 は ICAM-1 発現に影響しなかったが、胃線維芽細胞では両者とも有意に IL-1 β 刺激による ICAM-1 発現を抑制したが、COX-2 発現の影響はうけなかった。胃線維芽細胞では PGE₂ を作用させると NSAIDs の作用を打ち消さず、むしろ相加的に ICAM-1 発現を抑制した。NF- κ B 阻害薬 MG-132 を作用させると、IL-1 β による ICAM-1 発現は著明に抑制された。

これらの結果より、NSAIDs による好中球活性化はこれらの NSAIDs の ICAM-1 発現に対する影響と直接関係していないこと、胃粘膜において、NSAIDs による ICAM-1 発現の増加に COX-2 の発現は直接的な役割を果たしていないことが示唆される。

<Key words> インドメタシン、ICAM-1、サイクロオキシゲナーゼ、胃線維芽細胞、インターロイキン-1
