ICAM-1 and P-selectin expression in a model of NSAID-induced gastropathy

Z. Morise, S. Komatsu, J. W. Fuseler, D. N. Granger, M. Perry, A. C. Issekutz, and M. B. Grisham. ICAM-1 and P-selectin expression in a model of NSAID-induced gastropathy. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G246-G252, 1998. — A growing body of experimental evidence suggests that neutrophilic polymorphonuclear leukocyte (PMN)-endothelial cell interactions play a critical role in the pathophysiology of nonsteroidal anti-inflammatory drug (NSAID)-induced gastropathy. The objective of this study was to determine whether the expression of endothelial cell adhesion molecules is enhanced in a model of NSAID-induced gastropathy. Gastropathy was induced in male Sprague-Dawley rats via oral administration of indomethacin (Indo, 20 mg/kg). Lesion scores, blood-to-lumen clearance of radiolabeled monoclonal antibody (MAb) technique. For some experiments, blocking MAb directed at either ICAM-1 (1A29) or P-selectin (RMP-1) or their isotype-matched controls was injected intravenously 10 min before Indo administration. We found that P-selectin expression was significantly increased at 1 h but not 3 h after Indo administration, whereas ICAM-1 expression was significantly increased at both 1 and 3 h after Indo administration. The blocking ICAM-1 and P-selectin MBAs both inhibited Indo-induced increases in lesion score, mucosal permeability, and epithelial cell necrosis. However, the Indo-induced gastropathy was not associated with significant PMN infiltration into the gastric mucosa.

METHODS

Indo-induced gastropathy. Male Sprague-Dawley rats weighing 225–275 g were obtained from Harlan Laboratories (Frederick, MD) and were administered Indo (20 mg/kg, dissolved in 5% sodium bicarbonate at the concentration of 10 mg/ml) orally after the deprivation of food, but not water, for 18–22 h. All procedures involving the use of animals were approved by the Louisiana State University Medical Center Animal Care and Use Resources Committee.

Measurements of mucosal permeability. Before and at 1, 2, and 4 h after Indo administration, rats were anesthetized with pentobarbital. Mucosal clearance of radiolabeled monoclonal antibody (MAb) technique. For some experiments, blocking MAb directed at either ICAM-1 (1A29) or P-selectin (RMP-1) or their isotype-matched controls was injected intravenously 10 min before Indo administration. We found that P-selectin expression was significantly increased at 1 h but not 3 h after Indo administration, whereas ICAM-1 expression was significantly increased at both 1 and 3 h after Indo administration. The blocking ICAM-1 and P-selectin MBAs both inhibited Indo-induced increases in lesion score, mucosal permeability, and epithelial cell necrosis. However, the Indo-induced gastropathy was not associated with significant PMN infiltration into the gastric mucosa.

neutrophils; endothelial cells; inflammation; nonsteroidal anti-inflammatory drug (NSAID)-induced gastric mucosal injury significantly limits the use of these drugs for the treatment of chronic inflammatory disorders such as rheumatoid arthritis. Although it has been proposed that the mechanism by which NSAIDs induce this gastric mucosal injury is via their ability to inhibit cyclooxygenase (COX)-mediated production of “protective” prostaglandins, several lines of evidence suggest that the mechanism may be more complex than originally thought. For example, Ligumsky et al. (14) have shown that inhibition of prostaglandin production by >95% via rectal administration of certain NSAIDs did not induce gastric ulcers. In addition, recent studies by Langenbach et al. (12) demonstrate that homologous recombination to disrupt the Ptgs 1 gene encoding for COX-1 in mice does not result in spontaneous gastric ulcers. In fact, these COX-1-deficient animals are less sensitive to NSAID-induced gastropathy than their age-matched wild-type controls.

It is becoming increasingly apparent that neutrophilic polymorphonuclear leukocytes (PMNs) may play an important role in the pathogenesis of NSAID-induced gastropathy. Wallace and colleagues (27–29) have demonstrated that NSAID-induced gastric ulcerations may be attenuated by rendering animals neutropenic or by infusing blocking antibodies directed against CD18, intercellular adhesion molecule 1 (ICAM-1), P-selectin, and to a lesser extent E-selectin. The latter findings suggest that NSAIDs may enhance the expression of cell adhesion molecules on the surface of endothelial cells. Qualitative data that support this possibility were provided by immunohistological experiments that demonstrated an increased staining of gastric vessels for ICAM-1 30 min after oral administration of indomethacin (Indo) (2). The mechanisms by which adhesion of PMNs to postcapillary venules induces gastric epithelial cell injury are not at all clear. There has been some suggestion that leukocyte adhesion and/or aggregation occludes the microvasculature, resulting in ischemic mucosal injury (2, 27–29). The recent development of a method to quantify surface expression of endothelial cell adhesion molecules in vivo (19) has prompted us to determine the temporal effects of Indo on gastric mucosal surface expression of P-selectin and ICAM-1 in vivo in an established model of NSAID-induced gastropathy. Furthermore, we compared these Indo-induced changes in adhesion molecule expression to PMN extravasation and blood flow in the gastric mucosa.
with an intraperitoneal injection of 120 mg/kg sodium 5-ethyl-1-\((1\text{-methyl-propyl})\)-2-thiobarbituric acid (lactin; Byk-Gulden, Konstanz, Germany). Body temperature was maintained at 37°C, with a thermost-controlled water pad (Aquamatic K-Modules K-20; Baxter, Valencia, CA). The animals underwent tracheotomy, and the right femoral artery was cannulated for arterial pressure recording and blood sampling. The right femoral vein was also cannulated for injection of the isotope marker. A laparotomy was performed using a midline abdominal incision. Both renal vessels were ligated to prevent rapid excretion of the radioisotope marker into the urine. The stomach was cannulated orally using Silastic tubing (Dow Corning, Arlington, TN; ID 0.025 mm) for infusion of saline (pH 3.5). The stomach was also cannulated from the proximal portion of the duodenum into the proximal region of the gastric pylorus, using Silastic tubing (ID 0.25 mm) to collect the solution. The perfused tubing was ligated to prevent rapid excretion of the radiolabeled microsphere-reference organ technique (24). Blood flow was calculated using the equation

\[ \text{blood flow (ml/min)} = \frac{Q \times C_{pl}}{C_{per} \times W} \]

where \( Q \) is the luminal perfusion rate, \( C_{per} \) and \( C_{pl} \) are counts per minute in the tissue and the reference blood sample, respectively, and \( W \) is the weight of the stomach. Mucosal permeability was determined from the mean of the four clearance values.

Lesion scoring, tissue preparation, and biochemical analysis. After measurement of mucosal permeability, the animals were killed with an overdose of pentobarbital sodium (Butler, Columbus, OH), and the perfused stomach was excised. The stomach was opened along the greater curvature and examined. Because Indo produced linear ulcers, the lesion score of each animal was expressed as the sum of the length of lesions (mm) (29).

After being weighed, each stomach was sectioned for histology and myeloperoxidase (MPO) determination. MPO activity was determined as described previously (32). MPO activity was expressed as units per gram wet weight of the stomach.

For histological analysis, a tissue sample was obtained from each animal, fixed, dehydrated, and embedded in JB-4 (Polysciences, Warrington, PA). Sections (2.5 µm) were cut with glass knives and stained with hematoxylin and eosin. Measurement of blood flow. Blood flow was quantified using the radiolabeled microsphere-reference organ technique (24). Immediately before, 5 min after, and 1, 2, and 4 h after Indo administration, rats were anesthetized via an intraperitoneal injection of 120 mg/kg lactic acid. Body temperature was maintained at 37°C with a previously described thermistor-controlled water pad. The animals underwent tracheotomy to facilitate breathing. Cannulas placed in the right femoral artery and the right carotid artery both connected to the pressure transducers. The carotid artery cannula was advanced into the left ventricle; the position of the cannula tip was confirmed by a ventricular pressure tracing.

Microspheres (15.5 ± 0.1 µm) labeled with 85Sr (DuPont de Nemours) were suspended in 0.9% NaCl containing 10 µl of 0.05% Tween 80. The microspheres were dispersed using an ultrasonic bath and then vigorously vortexed for 2 min before injection. A 0.3-ml suspension containing ~200,000 microspheres was injected in the left ventricle over a 15-s period, during which time a reference sample was withdrawn from the right femoral artery into a heparin-containing glass syringe at a known rate (0.68 ml/min). After the microsphere injection, the carotid cannula was attached to a syringe and flushed with 5% Ficoll solution at a rate equal to the reference withdrawal rate using a bidirectional infusion pump. The withdrawal period lasted 90 s from the time of microsphere injection.

Rats were killed by injection of saturated potassium chloride into the left ventricle. The stomach was harvested according to the method described previously (12) and separated into mucosa-submucosa and serosa-muscularis layers. Only the mucosa-submucosa layer with or without lesions was used. Radioactivity in each sample was determined using a multichannel gamma counter (Wallace 1282 Compugamma). Blood flow was calculated using the equation

\[ \text{blood flow (ml} \cdot \text{min}^{-1}) = \text{RWR} \times \frac{C_{T}}{C_{R}} \times 100/100 \]

where \( C_{T} \) and \( C_{R} \) are counts per minute in the tissue and the reference blood sample, respectively, and \( \text{RWR} \) is the reference sample withdrawal rate (0.68 ml/min).

Effects of pretreatment with anti-ICAM-1 and P-selectin antibody. Ten minutes before Indo administration, a nonbinding (vehicle) murine immunoglobulin (Ig)G1 directed against human P-selectin (P23) (16) and a blocking mouse IgG1 directed against rat ICAM-1 (1A29) (25) were injected via the penile vein. Three hours after Indo administration (a time when the severity of mucosal injury reached its peak) lesion score and mucosal permeability were quantified as previously described for each group. A group pretreated with a mouse IgG1 directed against rat P-selectin (RMP-1) (30) was also compared with the nonbinding monoclonal antibody (MAb)-treated (P23) group.

Quantification of ICAM-1 and P-selectin expression. Preliminary experiments revealed that Indo-induced gastric mucosal injury was initially observed at 1 h after Indo administration and reached a maximum of 3 h. Therefore, ICAM-1 and P-selectin expression were quantified at 1 and 3 h after Indo administration.

The binding MAb directed against either ICAM-1 (1A29) or P-selectin (RMP-1) were labeled with 125I (Du Pont-NEN, Boston, MA), whereas the nonbinding, isotype-matched MAb (P23) was labeled with 131I. Radiolabeling of the MAb was performed by the iodogen method (7). Briefly, 250 µg of protein were incubated with 250 µCi of Na2125I and 125 µg of iodogen at 4°C for 12 min. After the radiolabeling procedure, the radiolabeled MAb were separated from free 125I by gel filtration on a Sephadex PD-10 column (Pharmacia, Uppsala, Sweden). The column was equilibrated with phosphate buffer containing 1% bovine serum albumin and was eluted with the same buffer. Two fractions of 2.5 ml each were collected, the second of which contained the labeled antibody. Absence of free 125I or 131I was ensured by extensive dialysis of the protein-containing fraction. Less than 1% of the activity of the protein fraction was recovered from the dialysis fluid. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed normal heavy and light chain moieties of expected molecular weight. Labeled MAb were stored in 500-µl aliquots at 4°C and used within 3 wk after the labeling
procedure. The specific activity of labeled MAbs was 0.5 µCi/µg.

Rats were anesthetized with an intraperitoneal injection of 120 mg/kg Inactin. Body temperature was maintained at 37°C with a thermostor-controlled water pad. The animals underwent tracheostomy to facilitate breathing, and the right carotid artery and left jugular vein were cannulated. To measure ICAM-1 expression, a mixture of 5 µg of 125I-ICAM-1 MAb (1A29), 5 µg of 131I-labeled nonbinding MAb (P23), and 100 µg of unlabeled ICAM-1 MAb was administered through the jugular vein catheter. To measure P-selectin expression, a mixture of 5 µg of 125I-P-selectin MAb (RMP-1) and 5 µg of 131I-nonbinding MAb (P23) was administered through the jugular vein catheter. These doses of MAbs were determined in our previous report for ICAM-1 (19) and in preliminary experiments for P-selectin. Thereafter, the animals were heparinized (1 mg/kg sodium heparin) and rapidly exsanguinated by vascular perfusion with sodium bicarbonate buffer via the jugular vein and simultaneous blood withdrawal via the carotid artery. The inferior vena cava was then severed at the thoracic level, and the carotid artery was perfused with sodium bicarbonate buffer. After completion of the exchange transfusion, organs were harvested and weighed.

The activities of 125I (binding MAb) and 131I (nonbinding MAb) in harvested gastric mucosa and in 100-µl aliquots of cell-free plasma were counted in a 14800 Wizard 3 gamma-counter (Wallace, Turku, Finland), with automatic correction for background activity and spillover. The injected activity in each experiment was calculated by counting a 5-µl sample of the mixture containing the radiolabeled MAbs. The radioactivities remaining in the tube used to mix the MAbs, the syringe used to inject the mixture, and the jugular vein catheter were subtracted from the total calculated injected activity. The accumulated activity of each MAb in the stomach was expressed as the percent of the injected dose (%ID) per gram of tissue. The equation used to calculate ICAM-1 and P-selectin expression was as follows:

\[
\text{Expression} = \left(\frac{\% \text{ID/g for } 125\text{I}}{\% \text{ID/g for } 131\text{I}}\right) \times \left(\frac{\% \text{ID } 125\text{I in plasma}}{\% \text{ID } 131\text{I in plasma}}\right)
\]

This equation was modified from the original method (10) to correct the tissue accumulation of nonbinding MAb for the relative plasma levels of both binding and nonbinding MAbs (10). This value, expressed as %D, was converted to µg MAB/g tissue by multiplying the above value by the total injected binding MAb (µg), divided by 100.

Statistics. All values are presented as means ± SE. The data were analyzed using one-way analysis of variance followed by Student-Newman-Keuls multiple comparisons test. Statistical significance was set at \(P < 0.05\).

RESULTS

Intragastric administration of Indo induced linear hemorrhagic lesions primarily in the corpus of the
stomach that were first observed macroscopically at 1 h after administration. These hemorrhagic erosions continued to develop over the next 2–3 h and were characterized histologically by mucosal injury (edema, necrosis, and exfoliation of the mucosal epithelial cells into the gastric lumen), hemorrhage, and formation of a “mucoid cap” (a layer of mucus, fibrin, and necrotic tissue) (Fig. 1). Histological inspection of the tissue indicated that active Indo-induced gastric mucosal injury peaked between 3 and 4 h. At 4 h after Indo administration, repair of the mucosal barrier was evident, and thus gastric mucosal lesions and 51Cr-EDTA clearance were performed at 3 h after Indo administration. Interestingly, we found no histological evidence of neutrophil infiltration, nor did we observe any increase in tissue MPO activity (23.4 ± 14.2 vs. 29.7 ± 12.5 U/g tissue for control vs. 3 h after Indo). Furthermore, we found that Indo did not decrease total organ or mucosal blood flow in the stomach. In fact, we observed a significant hyperemia in the lesioned areas of the mucosa before (i.e., 1 h) and at the time of frank ulceration (Fig. 2).

We found that ICAM-1 surface expression in the gastric mucosa increased significantly (43%) at both 1 and 3 h after Indo administration, corresponding to the time of earliest mucosal lesion and peak mucosal injury, respectively (Fig. 3). P-selectin expression in the gastric mucosa increased by ~55% only at 1 h after Indo administration (Fig. 4).

The pathophysiological role of ICAM-1 and P-selectin in this model of gastropathy was assessed using monoclonal blocking antibodies directed against either ICAM-1 or P-selectin. We found that administration of anti-ICAM-1 antibody (1A29) significantly attenuated the increases in both lesion score and mucosal permeability caused by Indo, compared with the nonbinding control MAb (Fig. 5). Administration of anti-P-selectin antibody (RMP-1) also significantly attenuated the increases in both lesion score and mucosal permeability caused by Indo, compared with the nonbinding control MAb (Fig. 6).

**DISCUSSION**

There is a growing body of experimental evidence to suggest that neutrophil-endothelial cell interactions play a critical role in the pathophysiology of NSAID-induced gastropathy (2, 27–29). Evidence supporting this concept comes from studies demonstrating a reduction in NSAID-induced gastric damage in neutropenic rats (28), as well as studies demonstrating a protective effect with pretreatment with monoclonal antibodies that block certain adhesion molecules such as CD18, ICAM-1, P-selectin, and to a lesser extent E-selectin (27, 29). Furthermore, intravital microscopic studies have shown that Indo and aspirin promote leukocyte adherence in postcapillary venules of the mesentery (3, 4). Although one report suggests that certain NSAIDs may enhance gastric ICAM-1 expression using immunohistochemical localization methods, there has been no direct quantification of adhesion molecule expression in...
animals receiving NSAIDs. The dual radiolabeled antibody technique (19) allows for measurements of ICAM-1 and P-selectin expression with a resolution that is not possible with immunostaining techniques. Using this method, we found that endothelial surface expression of P-selectin and ICAM-1 is significantly increased by intragastric Indo administration. The increased adhesion molecule expression preceded the extensive mucosal injury induced by Indo. These data suggest that the enhanced surface expression of the two endothelial cell adhesion molecules may represent a cause of the mucosal injury rather than a consequence of Indo-induced gastropathy. The rapid and significant increase in ICAM-1 expression on the surface of endothelial cells was more surprising. However, work by Lo et al. (15), as well as Asako et al. (4), has shown that oxidant- or NSAID-induced increases in ICAM-1 expression or leukocyte adhesion may occur as early as 30 min to 1 h in vitro or in vivo. Stimulation of those endothelial cells with LPS, cytokines, or H2O2 led to increased expression of ICAM-1 (18), which was accompanied by increased binding of P-selectin expression induced by lipopolysaccharide (LPS) is not apparent until at least 2 h after challenge (6), suggesting that the increase of P-selectin expression we observed in our model of NSAID-induced gastropathy is due primarily to its translocation from Weibel-Palade bodies to the surface of the endothelial cell. It has also recently been reported that leukotrienes C4 and D4 induce the P-selectin translocation from Weibel-Palade bodies, suggesting a mechanism whereby Indo enhances P-selectin expression (8, 20). An interesting point to note is that a 55% increase in P-selectin expression represents an increase in ~200 molecules of P-selectin per endothelial cell, assuming a surface area of ~125 cm² of the vascular bed in 1 g stomach tissue, and there are 50,000 endothelial cells/cm² of vascular bed.

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neutrophils to endothelial cells (1). This increase of ICAM-1 expression was observed at ~3 h and was maintained over 24 h. We observed significant increases in ICAM-1 expression at 1 and 3 h in vivo. Mast cells have been considered possible effector cells in NSAID gastropathy by virtue of their ability to synthesize and release certain cytokines. Indeed, tumor necrosis factor (TNF)-α has been demonstrated to be elevated in NSAID-induced gastropathy, and this cytokine is well known to increase surface expression of ICAM-1 in vitro and in vivo (22). However, Rioux and Wallace (21) recently reported that mast cells may not play a significant role in NSAID gastropathy in that serum mast cell protease II and mast cell degranulation were not elevated after Indo administration. Furthermore, mast cell-deficient mice exhibited the same degree of gastric injury as did their wild-type controls (21). Making the same assumptions as above, an increase in ICAM-1 expression of 43% represents an increase of ~11,500 molecules of ICAM-1 per endothelial cell.

Although we have focused on endothelial cell adhesion molecules, it should be noted that adhesion molecules on the neutrophil cell surface are also important determinants for Indo-induced gastropathy. Wallace and colleagues (27, 29) have demonstrated that MAbs to P-selectin or ICAM-1 binding, which could activate specific signaling pathways within the endothelial cells, resulting in the production of certain cytokines capable of promoting epithelial cell apoptosis and/or necrosis. The precise mechanisms for epithelial cell injury remain the subject of active investigations.

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