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


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Morise, Z., 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 directly 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 51Cr-EDTA (mucosal permeability), and histological analysis (epithelial necrosis) were used as indexes of gastric mucosal injury. Gastric mucosal vascular expression of intercellular adhesion molecule 1 (ICAM-1) or P-selectin were determined at 1 and 3 h after Indo administration using the dual radiolabeled monoclonal antibody (MAb) technique. For some experiments, a 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 treatment. The blocking ICAM-1 and P-selectin MABs 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, nor did Indo reduce gastric mucosal blood flow. We propose that NSAID-induced gastric mucosal injury may be related to the expression of P-selectin and ICAM-1; however, this mucosal injury does not appear to be dependent on the extravasation of inflammatory cells or mucosal ischemia.

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 immunohistochemical experiments that demonstrate an increased staining of gastric venules 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.

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 and in accordance with the guidelines of 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 an intraperitoneal injection of 120 mg/kg sodium 5-ethyl-1-(1'-methyl-propyl)-2-thiobarbituric acid (I nactin; Byk-Gulden, Konstanz, Germany). Body temperature was maintained at 37°C, with a thermistor-controlled water pad (Aquamatic K-Modules K-20; Baxter, Valencia, CA). The animals underwent tracheostomy, 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 0.25 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 stomach was returned to the abdominal cavity, and the abdominal wall was closed to minimize dehydration of the organs during the experiment. The luminal contents of the stomach were removed by preperfusion with warm (37°C) saline (pH 3.5) for 30 min.

Mucosal permeability was determined using the blood-tolumen clearance of 51Cr-labeled EDTA as described previously (32). One hundred microcuries of 51Cr-EDTA (Du Pont de Nemours, Birmingham, DE) were injected via the femoral vein catheter. After a 15-min equilibration period, the perfusate was collected every 10 min for 40 min for the appearance of 51Cr-EDTA. Plasma samples were obtained at 40 min for use as reference counts. Radioactivity in each sample was determined using a multichannel gamma counter (Wallace 1282 Compugamma). Blood-tolumen clearance of 51Cr-EDTA was calculated using the equation

\[ \text{Clearance (µl min}^{-1} \text{ g wt}^{-1}) = \frac{(C_{\text{per}} \times Q)}{(C_{\text{pl}} \times W)} \times 1,000 \]

where \( C_{\text{per}} \) and \( C_{\text{pl}} \) are counts per minute per milliliter of 51Cr-EDTA in the lumen perfusate and plasma, respectively, \( Q \) is the luminal perfusion rate (400 µl/min), 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 I nactin. Body temperature was maintained at 37°C with a previously described thermistor-controlled water pad. The animals underwent tracheostomy 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 (Du Pont 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 excised. Radioactivity in each sample was determined using a multichannel gamma counter (Wallace 1282 Compugamma). Blood flow was calculated using the equation

\[ \text{Blood flow (ml min}^{-1} \text{ g}^{-1}) = \frac{RWR \times C_T}{C_R} \times 100 \]

where \( C_T \) and \( C_R \) are counts per minute in the tissue and the reference blood sample, respectively, and 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 studies revealed that Indo-induced gastric mucosal injury was initially observed at 1 h after Indo administration and reached a maximum at 3 h. Therefore, ICAM-1 and P-selectin expression were quantified at 1 and 3 h after Indo administration.

The binding MABs 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. Radiodination of the MABs was performed by the iodogen method (7). Briefly, 250 µg of protein were incubated with 250 µCi of Na125I and 125 µg of iodogen at 4°C for 12 min. After the radiiodination procedure, the radiolabeled MABs were separated from free125I 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 free125I 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 mobilities of expected molecular weight. Labeled MABs 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 thermistor-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

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

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 increase in P-selectin and ICAM-1 expression in the stomach at 1 h after Indo administration was in some respects unexpected. In vitro studies using human umbilical vein endothelial cells have demonstrated that P-selectin expression induced by histamine is very rapid because of its translocation from Weibel-Palade bodies to the endothelial cell surface. In vivo studies have shown that P-selectin expression is enhanced from 10 to 60 min after intravenous histamine injection in mice (6). These data are in fact consistent with our observations and intravital observations demonstrating increased and persistent rolling of leukocytes in venules exposed to histamine (11). On the other hand, transcriptional upregulation 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.

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
neutrophils to endothelial cells (1). This increase of ICAM-1 expression was observed at \( \sim 3 \) h and was maintained over 24 h. We observed significant increases in ICAM-1 expression at 1 and 3 h in vivo. Most 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-\( \alpha \)) 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 \( \sim 11,500 \) 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 directed against CD18 adhesion molecules attenuated the development of NSAID-induced gastropathy. There is evidence to suggest that CD11/CD18 is expressed on leukocytes in both functional and nonfunctional conformational and activation of leukocytes leads to a change in the conformation of CD11/CD18, thereby promoting adhesion (23, 31). This proadhesive alteration in CD11/CD18 conformation may be elicited by leukotriene B\(_4\) (26) as well as platelet-activating factor and TNF-\( \alpha \) (23).

The enhanced expression of P-selectin and ICAM-1 induced by Indo appears to be an important step in the development of gastric mucosal injury. We, as well as others, have demonstrated that immunoneutralizing antibodies to P-selectin or ICAM-1 inhibit the development of gastric lesions as assessed by macroscopic, histological, and physiological determinations (Figs. 5 and 6). The mechanisms by which Indo-induced PMN-endothelial interaction promotes gastric mucosal injury are not at all clear. Previous studies have suggested that Indo-induced leuko-aggregation or ischemia may promote mucosal injury (9). However, we, as well as others, have been unable to observe an ischemic event in response to NSAIDs with the use of the microsphere-reference organ technique (5, 13, 17). Indeed, when we quantify mucosal blood flow in the actual lesion area, we see a modest but significant hyperemia and not an ischemia beginning in the very early stages of ulcer development (Fig. 2). Thus the role of blood flow in NSAID-induced gastropathy remains controversial. It is conceivable that leukocyte plugging in a small percentage of capillaries within the gastric mucosa does occur; however, this would not be detected by the method employed in this study. Another interesting yet perplexing observation is the lack of any significant PMN infiltration into mucosal interstitium (Fig. 1). Exactly how adhesion of PMNs to the postcapillary venules induces epithelial cell necrosis in the absence of an inflammatory infiltrate or ischemic event remains speculative. One possible mechanism could involve receptor/ligand interaction in the form of CD18/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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