Effect of Helicobacter pylori-mediated inflammation on nonsteroidal anti-inflammatory drugs-induced gastric mucosal injury

Norimasa Yoshida and Toshikazu Yoshikawa

First Department of Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan

Abstract. Helicobacter pylori (H. pylori) infection and nonsteroidal anti-inflammatory drugs (NSAIDs) are two major causes of gastric ulceration, but relation between H. pylori infection and use of these drugs in gastric mucosal injury is controversial. Neutrophils have been implicated in the pathogenesis of gastric mucosal damage induced by H. pylori or NSAIDs. H. pylori itself and H. pylori extract induce neutrophil activation, such as superoxide production, expression of adhesion molecule (CD11b/CD18) and transendothelial migration, capillary plugging. Several clinical and experimental studies demonstrated that the degree of H. pylori-induced gastric mucosal injury was closely correlated with the extents of H. pylori infection and of neutrophil infiltration, suggesting implication of extravascular neutrophils for H. pylori-induced gastric mucosal injury. On the other hand, aspirin promotes neutrophil-endothelial adhesive interactions via increasing CD11b/CD18, but not neutrophil migration to extravascular space. In addition, our recent in vivo study suggests that neutrophils adhering to the blood vessels, but not neutrophils migrating to the interstitium, are implicated in aspirin-induced gastric mucosal injury. Recently, we found that administration of aspirin to gerbils three weeks after H. pylori inoculation produced severe gastric mucosal injury via marked infiltration of neutrophils. In this animal model, pretreatment with anti-neutrophil serum, elastase inhibitor or scavengers of reactive oxygen species remarkably inhibited gastric mucosal injury. These results suggest that H. pylori infection potentiates aspirin-induced gastric mucosal injury by mechanisms that include accumulation of activated neutrophils.

Key words: H. pylori, aspirin, nonsteroidal anti-inflammatory drugs, neutrophils, gastric mucosal injury

Role of Neutrophils in Aspirin-induced Gastric Mucosal Injury

It is probable that neutrophil-endothelial cell adhesion is an early event in the pathogenesis of nonsteroidal anti-inflammatory drugs (NSAIDs)-induced gastric ulceration. In vitro studies have indicated that aspirin could increase surface expression of CD11b/CD18 on neutrophils and could induce neutrophil adherence to endothelial cells followed by neutrophil-mediated endothelial cell injury. In vivo studies have demonstrated that aspirin and indomethacin also promote leukocyte rolling and adherence in rat mesenteric venules. Finally, rendering animals neutropenic or interfering with neutrophil adhesive interactions with the microvasculature largely prevents epithelial cell and microvascular injury after administration of NSAIDs. Taken together, these studies indicate that aspirin and other NSAIDs produce gastritis by promoting neutrophil-endothelial cell adhesive interactions that ultimately result in neutrophil-mediated tissue injury. Recently, we found that aspirin induced leukocyte plugging in capillaries by impairments in leukocyte rheology using the narrow microchannel which is an in vitro model of capillary (data not shown). These results suggest that aspirin induces neutrophil-mediated microvascular disturbance via eliciting both neutrophil plugging in capillary and neutrophil adhesion in postcapillary venule (Fig. 1). Actually, our recent in vivo study revealed that neutrophils adhering to the blood vessels, but not neutrophils migrating to the interstitium, are implicated in aspirin-induced gastric mucosal injury in rats.
Role of Neutrophils in \textit{H. pylori}-induced Gastric Mucosal Injury

\textit{H. pylori}-induced neutrophil infiltration

Many clinical studies have demonstrated that the degree of \textit{H. pylori}-induced gastric mucosal injury is closely correlated with the extents of \textit{H. pylori} infection and of neutrophil infiltration.\textsuperscript{9} The important first step for tissue infiltration of neutrophils is adhesion of circulating neutrophils to vascular endothelial cells, followed by emigration into the submucosa. It has recently been shown that this leukocyte-vascular endothelium interaction is regulated by various cell adhesion molecules. Among these adhesion molecules, CD11/CD18 glycoprotein complex expressed on activated neutrophils has been shown to be an important mediator of neutrophil-endothelial cell interactions in a variety of \textit{in vivo} and \textit{in vitro} models of inflammation.\textsuperscript{10} The CD11/CD18 consists of three heterodimers and CD11a/CD18 and CD11b/CD18 are generally considered the most important heterodimers involved in both neutrophil adhesion to endothelial cells and neutrophil transendothelial migration.

When neutrophil infiltration associated with \textit{H. pylori} infection was studied in relation to the expression of adhesion molecules, interesting findings were obtained.\textsuperscript{11} When human neutrophils were activated by a water extract of \textit{H. pylori} (HPE), CD11b/CD18 (Mac-1) was expressed on the cell membrane within several minutes, and neutrophils strongly adhered to human endothelial cells. Because this adhesion is inhibited by monoclonal antibody directed against CD11b, CD18, or ICAM-1, it would seem that the adhesion takes place through interaction of CD11b/CD18 on neutrophils and ICAM-1 on endothelial cells. Furthermore, when the HPE was applied dropwise onto rat mesenteric venules, adhesion of circulating leukocytes to endothelium and their extravascular migration were observed. The substance that cause neutrophils to express adhesion molecules is resistant to pepsin and acid, and studies on its molecular weight suggested that it contains at least 3 activating substances. One of such substances is a protein with a molecular weight of 150 kDa, and its gene has been identified and termed neutrophil-activating protein (NAP) gene.\textsuperscript{12} HP-NAP is highly conserved among \textit{H. pylori} strains, though its level of expression varies. Hansen \textit{et al.}\textsuperscript{13} showed that the protein in \textit{H. pylori} sonicates stimulates neutrophil via a mechanism insensitive to pertussin toxin, while HP-NAP binds to a specific receptor, which is coupled to a pertussin toxin-sensitive trimetric G protein, and activates NADPH oxidase of neutrophils.\textsuperscript{14} Our recent study demonstrated that HPE also induced the expression of endothelial adhesion molecules as well as CD11b/CD18 on neutrophils.\textsuperscript{15}

Cytokine-induced neutrophil infiltration

In the gastric mucosa infected with \textit{H. pylori}, the gastric epithelial and endothelial cells are considered to produce IL-8. IL-8 has potent chemotactic activity for neutrophils like PAF and LTB\textsubscript{4}, and it also induces expression of adhesion molecules, CD11b/CD18, and the production of active oxygen species. Aihara \textit{et al.}\textsuperscript{16} reported that IL-8 production required a direct contact between the cells and live \textit{H. pylori} and involved tyrosine phosphorylation and NFkB activation. However, we found that a nonprotein, acid resistant substance of low molecular weight in HPE, as well as bacteria itself, activated gastric epithelial cells (MKN-45) and human umbilical vein endothelial cells (HUVEC), and caused the expression of IL-8 mRNA and protein.\textsuperscript{17} In addition, our results showed that IL-8 production by HPE is mainly dependent on the activation of PKC but also partially dependent on PKA or PTK.

Endothelial cell injury by neutrophils

Adhesion of neutrophils to target cells is a key to neutrophil cytotoxicity. When neutrophils adherent to cultured endothelial cells by HPE were observed at appropriate intervals, endothelial cell injury (detachment of endothelial cells) was observed after 3 to 6 hours.\textsuperscript{18} Since endothelial cell injury was inhibited by monoclonal antibody directed against CD18 or ICAM-1, it was considered that adhesion of neutrophils to endothelium through CD11/CD18-ICAM-1 was im-
involved in the induction of severer cytotoxicity. It has been proposed that leukocytes, as well as red blood cells or platelets, can plug narrow capillaries and cause microcirculatory disturbance. Recently, we reported that HPE induced prolongation of passing time and obstruction of the narrow microchannel which is an in vitro model of capillary. This flow disturbance is attributed to plugging of microchannels by neutrophils and platelets. These results indicate that H. pylori infection may induce capillary plugging by neutrophils and microvascular disturbance in the gastric mucosa.

Gastric mucosal injury by neutrophils

H. pylori colonize mainly at the gastric mucous layer, and infiltrating neutrophils accumulate mainly in the lamina propria and the submucosal layer, and accordingly, at H. pylori infection, neutrophils cannot exhibit a full bactericidal effect against H. pylori; rather they may become cytotoxic. In fact, when tissue with H. pylori-related gastric mucosal injury was observed, neutrophils were found to infiltrate between gastric epithelial cells. Interestingly, recent report has shown that intraepithelial neutrophilic infiltration was predominantly localized to the proliferative zone of the gastric mucosa (zone 2) where the density of H. pylori was considerably lower than the surface epithelium. In addition, Kim et al. reported that HPE inhibited neutrophil apoptosis via suppression of caspase-3 activation and upregulation of Bel-XL expression, suggesting prolongation of neutrophil life-span in H. pylori infected gastric mucosa.

In activated neutrophils, NADPH oxidase in cell membranes becomes activated, and an electron transfer takes place from NADPH in cells to oxygen inside and outside cells, and the oxygen that received electrons becomes superoxide radicals (O$_2^-$). From superoxide, hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (•OH) are considered to be generated. Furthermore, hypochlorite (HOCl) is generated from hydrogen peroxide due to myeloperoxidase (MPO). Actually, in gastric mucosa infected with H. pylori, lipid peroxidation, which is considered to be induced by active oxygen species, significantly increases, compared with normal tissues.

Davies et al. also found a positive association between reactive oxygen species production and the infective load of H. pylori. In addition, it has been reported that neutrophils enhance apoptosis of H. pylori-infected gastric epithelial cells via TNF-α and soluble Fas ligands.

When neutrophils and H. pylori were added to cultured gastric mucous cells, the mucous cells were injured, and monochloramine (NH$_2$Cl), formed by hypochlorite and ammonia, produced by H. pylori, is considered an important injury factor. Also, it has been reported that monochloramine induces apoptosis of gastric mucosal cells and generates cytotoxicity. We recently found that HPE caused neutrophils to adhere to MKN-45 cells derived from human gastric mucosal cells, and that this adhesion took place through CD11b/CD18 on neutrophils and ICAM-1 on MKN-45 cells. These findings suggest that H. pylori infection induces the adhesion of neutrophils to gastric mucosal cells in the same mechanism as neutrophil-endothelial cell interactions, followed by oxidants-mediated gastric mucosal injury (Fig. 1).

Interactions between H. pylori and NSAID in Gastric Mucosal Injury

Clinical trials

Helicobacter pylori infection and nonsteroidal anti-inflammatory drug (NSAID) use are well-established risk factors for gastrointestinal mucosal injury. Many clinical studies have explored relationships between these two factors.

Among the clinical studies, Publig et al. have reported that the prevalence of H. pylori infection in patients taking NSAIDs who developed gastric ulcers was 83%, significantly greater than in patients without gastric ulcers (45%). Recently, Huang (28) et al. reported a meta-analysis data of 25 studies. They showed that both H. pylori infection and NSAIDs use independently and significantly increased the risk of peptic ulcer and ulcer bleeding and that there was synergy for the development of peptic ulcer and ulcer bleeding between H. pylori infection and NSIDs use. In addition, Chan et al. reported that eradication of H. pylori reduced the risk of ulcers for patients starting long-term NSAID treatment.

However some other studies found no evidence of potentiated injury when H. pylori infection coexisted with NSAID use. Among patients with rheumatoid arthritis taking NSAIDs Graham et al. reported, gastric erosions in 34% and bleeding in 32% of the patients who also had H. pylori infection, representing lower incidences than in patients without H. pylori infection (57% and 61%, respectively). Recently, Labenz et al. reported that omeprazole or triple therapy reduced the occurrence of NSAID associated mucosal injury in H. pylori positive patients. In addition, Hawkey et al. revealed that H. pylori infection did not affect relapse of lesions in patients taking NSAID.

These disagreements may be related to differences in severity of gastric mucosal atrophy between patients with and without H. pylori infection as well as differences in the basal level of mucosal inflammation.
Animal models using Mongolian gerbils

**Inflammatory responses in gastric mucosa exposed to *H. pylori* and aspirin**

We investigated the influences on and consequences of neutrophil-associated inflammatory reactions in gastric mucosa exposed to both experimental *H. pylori* infection and NSAIDs using Mongolian gerbils. Three weeks after inoculation with *H. pylori* (ATCC43504), aspirin (400 mg/kg) and 0.1 N HCl were administered orally to gerbils fasted for 18 hours. At 3 hours after aspirin administration, macroscopic gastric damage was examined under a dissecting microscope using a square grid, and was quantified as the total area of haemorrhagic erosions (erosion index). The mucosa was scraped off and homogenised to measure the concentration of thiobarbituric acid-reactive substances (TBARS, an index of lipid peroxidation), myeloperoxidase (MPO) activity (an index of neutrophil infiltration) and content of KC/GRO (a chemoattractive cytokine). Macroscopic findings at three weeks revealed that mild edema and erythema were observed macroscopically in the gastric mucosa of gerbils inoculated with *H. pylori*. Oral administration of aspirin produced mild haemorrhagic erosions except in the *H. pylori* infected gerbils, where severe haemorrhagic erosions resulted. Figure 2 shows the index of gastric erosion for gerbils exposed to *H. pylori* and/or aspirin. The erosion index in gerbils receiving aspirin was significantly greater than untreated control gerbils. In addition, oral administration of aspirin to *H. pylori*-infected gerbils resulted in a mean erosion index 2.5 times greater than in aspirin-treated gerbils without *H. pylori* infection. Microscopic examination revealed neutrophils and mononuclear cells in the gastric lamina propria in gerbils infected with *H. pylori*. In gerbils receiving only aspirin, occasional neutrophils and mild erosions were observed. In *H. pylori*-infected gerbils, administration of aspirin resulted in intense infiltration by neutrophils and severe erosions of the gastric mucosa (Fig. 3). MPO activity and KC/GRO content in the gastric mucosa of *H. pylori*-infected gerbils was significantly greater than in controls. However, mucosal MPO activity and KC/GRO content in gerbils treated with aspirin alone was unchanged. MPO activity and KC/GRO content in gerbils exposed to both *H. pylori* and aspirin was significantly greater than in gerbils receiving either *H. pylori* or aspirin alone. The TBARS content in the gastric mucosa of gerbils treated with either *H. pylori* or aspirin alone was not increased relative to TBARS in untreated control gerbils. However, TBARS content in gastric mucosa of gerbils treated with both *H. pylori* and aspirin was significantly greater than in gerbils treated with either *H. pylori* or aspirin alone.

In the next experimental series, to investigate the role of neutrophils, rabbit antiserum against gerbil neutrophils was administered to gerbils. Anti-neutrophil serum (ANS) was obtained by immunising rabbits with gerbil neutrophils in Freund's complete adjuvant. The circulating neutrophil count showed a 77.2% decrease 18 hours after administration of ANS, 1660 ± 260/mm³ vs. 7300 ± 840/mm³ in gerbils treated with normal rabbit serum. Platelet count was not influenced by ANS administration. Erosion index, MPO activity, and TBARS were significantly less in such neutrophil-depleted gerbils exposed to *H. pylori* plus aspirin than in similarly exposed animals treated with normal rabbit serum. However, administration of ANS had no influence on the increase in KC content. On the other hand, aspirin-induced mucosal injury in uninfected animals was also reduced after administration of ANS. Inhibitory effect in mucosal damage by ANS administration was much greater in *H. pylori*-infected gerbils (65%) than uninfected gerbils (31%). In addition, elastase...
inhibitor and a combination of SOD and catalase inhibited the increase in gastric erosions and mucosal TBARS induced by administration of aspirin to H. pylori-infected gerbils.

**H. pylori infection potentiates aspirin-induced gastric mucosal injury via activated neutrophils**

Haemorrhagic erosions in the gastric mucosa caused by aspirin were much more severe in gerbils with *H. pylori* infection than in uninfected gerbils. In the present study, we therefore evaluated neutrophil infiltration and neutrophil chemoattractant in aspirin- and/or *H. pylori*-induced gastric mucosal injury. While minimal neutrophil infiltration resulted when only aspirin was administered, neutrophil infiltration was more prominent when *H. pylori* infection was present additionally. In addition, aggravation of gastric mucosal lesions induced by the combination of aspirin and *H. pylori* infection was significantly inhibited in neutrophil-depleted gerbils. The reduction in mucosal injury by the administration of ANS was much greater in *H. pylori*-infected animals than uninfected animals. These findings indicate that the effects of aspirin on the gastric mucosa may be potentiated by *H. pylori* infection via neutrophil-dependent mechanisms.

Accumulation of neutrophils in *H. pylori*-infected human gastric mucosa is related to increased concentrations of interleukin (IL)-8, a potent neutrophil chemoattractant that is released by gastric epithelial cells. In the present study, we measured KC, an IL-8-like neutrophil chemoattractant in gastric mucosa. KC is considered both a potent chemoattractant and an up-regulator of CD11b/CD18 cell surface expression in rodent neutrophils. We found that gastric mucosal KC content in gerbils exposed to *H. pylori* alone was somewhat greater than in mucosa exposed to aspirin alone. Further, in gerbils treated with aspirin after inoculation with *H. pylori*, the KC content in gastric mucosa was significantly greater than in gastric mucosa of gerbils that received only aspirin. These findings suggest that increased KC content may be involved in accumulation of neutrophils in gastric mucosa. In the present study, administration of antiserum against neutrophils remarkably reduced the increase in mucosal MPO activity, but not the increase in KC content. Neutrophils, therefore, were not the main source of KC. While the source of KC was not identified in this study, KC may have been produced by gastric epithelial cells and/or macrophages. In addition, the mechanism by which KC content was particularly increased in gastric mucosa exposed to both aspirin and *H. pylori* remains undetermined. Further studies are required to investigate regulation of KC in the germil gastric mucosa. Overall, we hypothesized that administration of aspirin to *H. pylori*-infected mucosa caused adherent neutrophils to easily migrate into the extravascular space by chemoattractants such as KC.

In summary, the present study indicated that administration of aspirin to gerbils 3 weeks after *H. pylori* inoculation produced severe gastric mucosal injury via marked infiltration of neutrophils. Our present data are consistent with clinical evidence that gastric neutrophils associated with *H. pylori* infection increased the incidence of ulceration in long-term NSAID users. Eradication of *H. pylori*, then, could help to prevent NSAID-induced mucosal injury, as also indicated by a recent clinical trial where eradication of *H. pylori* before NSAID use reduced occurrence of NSAID-induced ulcers. In the present study, gerbils 3 weeks after *H. pylori* inoculation exhibited typical gastritis with neutrophil and mononuclear cell infiltration in the lamina propria and a few superficial erosions. The Mongolian gerbil model subsequently shows characteristic changes of chronic gastritis, including formation of lymphoid follicles 6 weeks after *H. pylori* infection and gastric ulcers at more than 6 months after infection. The effect of NSAID administration on chronic gastritis or ulcers, showing such mononuclear cell-mediated chronic inflammation, is of considerable importance.

**References**