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The Role of Neutrophils and Inflammation in Gastric Mucosal Injury

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Gastric inflammation is a highly complex biochemical protective response to cellular/tissue injury. When this process occurs in an uncontrolled manner, the result is excessive cellular/tissue damage that results chronic inflammation and destruction of normal tissue. Current evidence suggests that *Helicobacter pylori* (*H. pylori*) infection and nonsteroidal anti-inflammatory drug (NSAID) ingestion are major causative factors in the pathogenesis of gastric mucosal injury in humans. In response to *H. pylori* infection or NSAID, neutrophils are recruited to the site of inflammation and generate reactive oxygen and nitrogen species and proteases. However, neutrophils are not able to kill the bacteria that live in the gastric mucus, and compounds produced by activated neutrophils themselves may be potentially harmful for normal tissue. It has been shown that leukocyte-vascular endothelial cell interaction is regulated by various cell adhesion molecules, and that this interaction is directly or indirectly modified by many factors, the origin of which is *H. pylori* and NSAIDs. This review describes the potential role of neutrophils and neutrophil-associated inflammation for gastric oxidative stress and injury induced by *H. pylori* and/or NSAID.

Keywords: Inflammation, neutrophil, Helicobacter pylori, NSAID, adhesion molecule

INTRODUCTION

Current evidence suggests that *Helicobacter pylori* (*H. pylori*) infections and nonsteroidal anti-inflammatory drug (NSAID) ingestion are major causative factors in the pathogenesis of duodenal and gastric mucosal injury in humans. Especially in Japan, more than half of the total population has been infected with *H. pylori*, and thus chronic atrophic gastritis, peptic ulcer, and gastric carcinoma, which are mainly the result of the *H. pylori*-positive population, are the most popular diseases (Figure 1). Further studies have demonstrated that reactive oxygen species, including superoxide anions, are involved in the pathogenesis of gastric mucosal damage which is induced by *H. pylori* or NSAID. The importance of reactive oxygen species in the mechanism of gastric mucosal injury has been demonstrated by the fact that several scavengers, allopurinol, or antineutrophilic antibody reduced these injuries induced by indomethacin[1,2] ethanol[3,4], ischemia-reperfusion[5,6], or stress[7]. The production of these reactive oxygen

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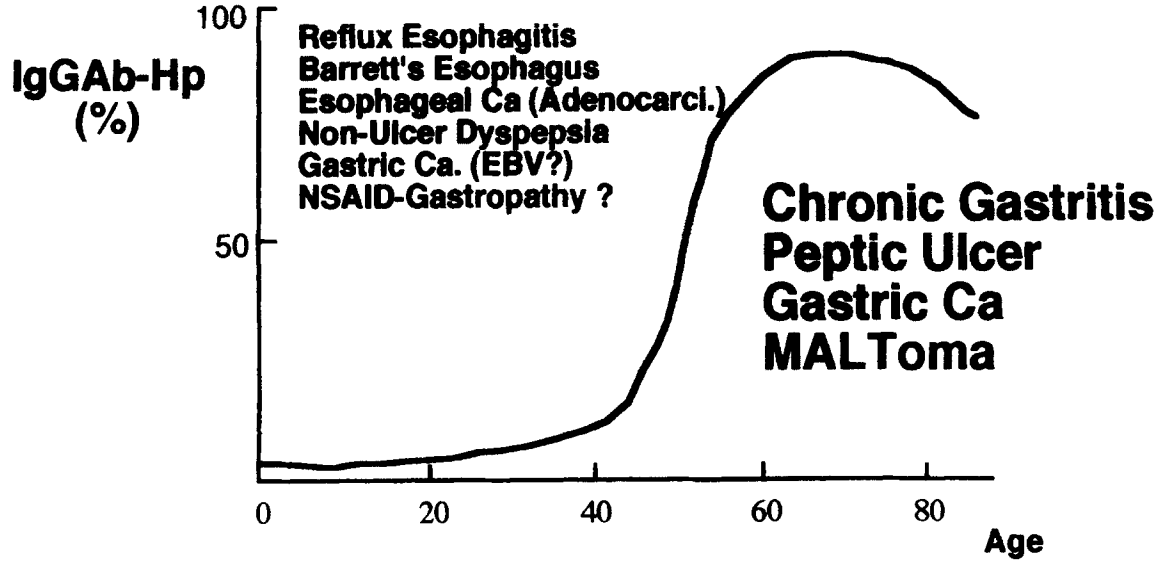

FIGURE 1 *H. pylori*-infection and upper gastro-intestinal diseases in Japan

species, mainly by inflammatory cells, especially polymorphonuclear neutrophils, is modulated by several mediators such as cytokines and prostglandins. Infiltration of inflammatory cells into tissue occurs via the adhesion of leukocytes to endothelial cells, and thus leukocytes and endothelial cells play an important role in inflammatory reactions. We will review the significance of neutrophil-associated inflammation in the pathogenesis of gastric mucosal injury.

LEUKOCYTE AND VASCULAR ENDOTHELIAL CELL INTERACTIONS

The sequence of events in the extravasation of leukocytes from the vascular lumen to the extravascular space is divided into (i) margination and rolling, (ii) adhesion and transmigration between endothelial cells, and (iii) migration in interstitial mucosal tissues towards a chemotactic stimulants (Figure 2). As vascular permeability increases in the early stages of inflammation, fluid exits the vascular lumen and blood flow slows. As a result, the leukocytes settle out the central column, migrating to the vessel periphery. Subsequently, the leukocytes tumble onto the endothelial surface, transiently sticking along the way (rolling). The relatively loose and transient adhesion involved in rolling can be accounted for by the selectin family of molecules. Selectin molecules have at least two functions in the inflammatory process: capturing and rolling of blood leukocytes; and activation of captured leukocytes and endothelial cells. P-selectin is expressed by both endothelial cells and platelets and is stored intracellularly in the so-called Weibel-Palade bodies, which fuse with the luminal cell membrane on stimulation with cytokines, lipopolysaccharide (LPS), histamine, thrombin, and anoxia/reoxygenation. E-selectin is found on cytokine- or LPS-stimulated endothelial cells. Stimulation for 1–4 hours up-regulates E-selectin, but continuous stimulation results in decreasing concentrations. Because of its intracellular storage, P-selectin is mobilized much faster than E-selectin and might therefore be involved in the initial marginaliza-

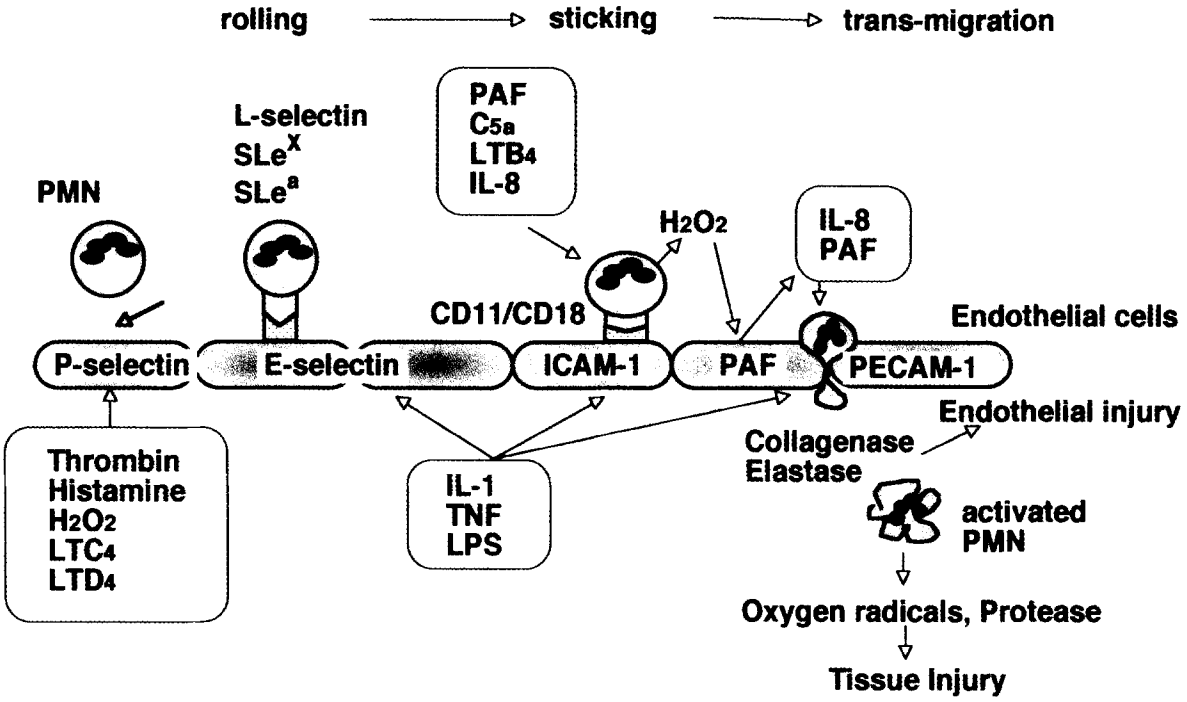

FIGURE 2 Adhesion molecules, cytokine, and chemical mediators in leukocyte-endothelial cell interactions
Leukocyte-endothelial cell interactions are regulated by various cell adhesion molecules. With stimuli, such as various cytokines and inflammatory mediators, leukocytes roll slowly on endothelial cells through interactions between L-selectin and carbohydrate antigen on leukocytes, and P- and E-selectin on endothelial cells. They strongly adhere to endothelial cells via CD11/CD18 glycoproteins and intercellular adhesion molecule-1 (ICAM-1). The leukocytes then migrate into the endothelial space and destroy the basement membrane in order to infiltrate into tissue

tion and rolling of the circulating leukocytes that have L-selectin expression.

Eventually, the leukocytes adhere strongly to endothelial cells via CD11/CD18 glycoproteins and endothelial adhesion molecules of immunoglobulin superfamily, which are active in the inflammatory process by arresting and firmly binding rolling leukocytes, flattening the arrested leukocytes, thus mediating their transendothelial migration. The endothelial adhesion molecules include the intercellular adhesion molecule 1 (ICAM-1) and the vascular cell adhesion molecule 1 (VCAM-1). ICAM-1 is expressed by activated and non-activated endothelial cells and by lymphocytes, monocytes, and epithelial cells including gastric epithelial cells. Its expression is stimulated by molecules such as IL-1, IL-17, TNF-α, IFN-γ, and LPS. ICAM-1 binds β2 integrins lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18) and Mac-1 (CD11b/CD18), which are stored in the intracellular granules of leukocytes. VCAM-1 is found in a membrane-bound form on various cells, including endothelial cells and monocytes/macrophages and also exist in a soluble form. The expression of VCAM-1 is stimulated by TNF-α, IL-1β, IL-4, IFN-γ, and LPS.

After firmly binding to the endothelial surface (primarily CD11/CD18 binding to ICAM-1), the leukocytes transmigrate between cells along the intercellular junction. Platelet endothelial cell adhesion molecule 1 (PECAM-1), a cell-cell adhesion molecule, is a likely candidate for mediating this process. After passing the

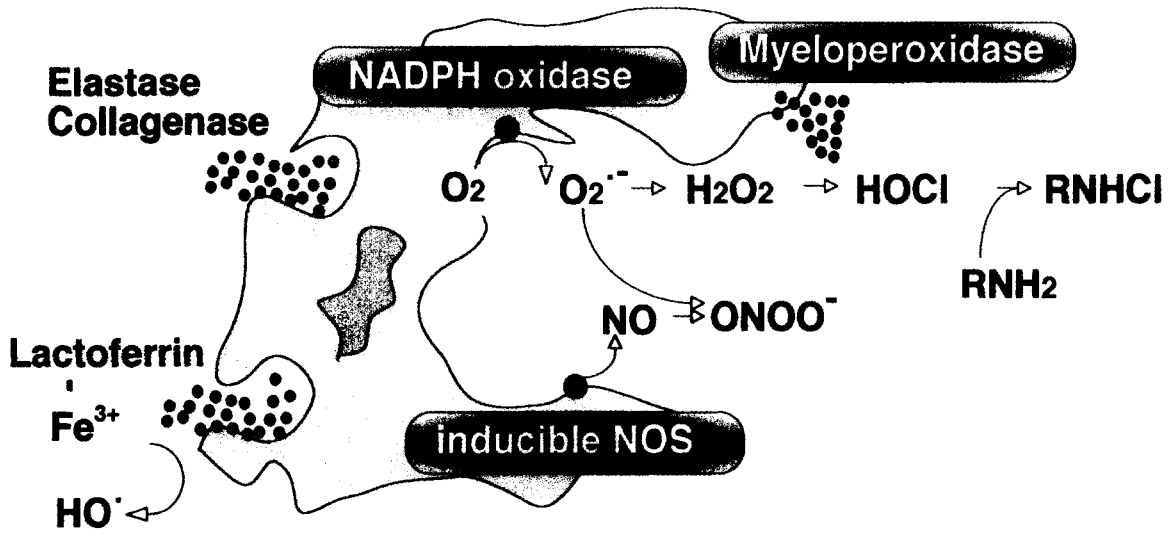

FIGURE 3 Cytotoxic factors produced by neutrophils
Neutrophils produce and release proteases, such as elastase and collagenase, as well as various cytotoxic factors including reactive oxygen and nitrogen species

endothelial junctions, leukocytes are able to cross the basement membrane by focally degrading it with secreted collagenases.

After extravasation, leukocytes emigrate towards the site of the mucosal injury along a chemical gradient (chemotaxis). Both exogenous and endogenous substances are able to act as chemotactic agents for leukocytes including 1) soluble bacterial products, particularly peptides with N-formyl-methionine termini, 2) components of the complement system, particularly C5a, 3) products of the lipoxygenase pathway of arachidonic acid metabolism, particularly LTB4, and 4) cytokines especially those of the chemokine family (IL-8).

LEUKOCYTE-DEPENDENT GASTRIC MUCOSAL INJURY

Extravascularly migrated neutrophils infiltrate the region around bacteria and target cells, depending on the concentration of the chemoattractants, and take actions advantageous to the body, such as killing bacteria and cancer cells, while they may also injure normal cells and tissue. 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 ($O_2^{\cdot-}$), which is rapidly converted to hydrogen peroxides (H_2O_2) by spontaneous dismutation or enzymatic superoxide dismutase (SOD), and hydroxyl radicals ($^{\cdot}OH$), which are formed nonenzymatically in the presence of Fe^{2+} as a secondary reaction (Figure 3). In neutrophils, myeloperoxidase also results in the formation of the potent oxidant hypochlorous acid (HOCl) from H_2O_2 in the presence of chloride ions. Reactive oxygen species may also stimulate inflammatory cells directly, thereby amplifying inflammatory and oxidant events.

Reactive oxygen species are highly reactive. When they are generated close to cell membranes, possibly by gastric mucosal cells, as has

been shown in rats, they induce oxidative stress and oxidize membrane phospholipids (lipid peroxidation), which may continue in a form of a chain reaction. The most-studied free radical chain reaction in living systems is lipid peroxidation, which is mediated by oxygen free radicals and is believed to be an important cause of cell membrane destruction and cell damage. Biomembranes contain large amounts of polyunsaturated fatty acids (PUFAs) in their phospholipids. PUFAs contain two or more carbon-double bonds within their structure. This makes them susceptible to oxidative damage by free radical attack. Niki et al.[8–10] have made major contributions to the concept of lipid peroxidation and their inhibition by antioxidants including α-tocopherol.

Experimental evidence suggests that lipid peroxidation reactions on cell membranes may play an important role in free radical-mediated cell injury. In vitro studies with purified membrane preparations have shown that lipid peroxidation of biological membranes will cause both structural alterations and abnormal membrane functions. The most obvious consequence of membrane lipid peroxidation is the perturbation of various cellular and organellar membrane functions, including transport processes, maintenance of ion and metabolite gradients, receptor-mediated signal transduction, etc. Lipid peroxides, because of their long life, migrate from one site to another in the body and thus propagate injuries. To further support this toxicity of lipid peroxides, it is reported that lipid peroxide increases in blood provokes injury to the endothelial cells of the artery, and that lipid peroxides produced by burnt skin, can injure the gastric mucosa by subsequent lipid peroxidation [11].

Reactive oxygen species, especially ·OH, may severely disrupt cell membrane function and may lead to cell death, or the damage of DNA in gastric mucosal cells. They also oxidize certain amino acids in proteins, such as methionine and cysteine, and profoundly alter the function of the modified proteins. Many of the effects of reactive oxygen species in gastric mucosa may be mediated by the secondary release of inflammatory lipid mediators such as 4-hydroxy-2-nonenal, which is known to induce various cellular events such as proliferation and activation of signaling pathway [12].

While low concentrations of nitric oxide (NO) derived from constitutive NOS have been shown to protect the gastric mucosa against injury caused by various damaging stimuli, large amounts of NO released from inducible NOS may be cytotoxic. The cytotoxic effects of NO can be demonstrated in cultured gastric epithelial cells. Treatment of cultured rat gastric mucosal cells with NO donor NOC5 or NOC12 decreased cell viability [13]. NO can react with superoxide anions, resulting in the accumulation of the destructive molecule, $ONOO^-$ (peroxynitrite). Reactive nitrogen species such as $ONOO^-$ can have deleterious effects on mitochondria and damage macromolecules, such as DNA, proteins, and lipids. $ONOO^-$ modifies tyrosine residues in proteins to form nitrotyrosine, which can be detected immunohistochemically in clinical biopsy samples from patients with H. pylori infection[14] and in the gastric mucosa after ischemia-reperfusion [15]. However, the possibility of $ONOO^-$-independent nitrotyrosine formation in inflammation sites, that was reported by Eiserich et al.[16,17] should also be considered. They demonstrated that, in the presence of hypochlorous acid or MPO, nitrotyrosine could be formed from nitrite, via the formation of nitryl chloride and nitrogen dioxide [16,17].

H. PYLORI-INDUCED GASTRIC INFLAMMATION

H. pylori is a non-invasive, non-spore-forming, S-shaped Gram-negative rod bacteria measuring approximately 3.5 × 0.5 μm. The mechanism by which gastritis develops remain unclear but may relate to the combined influence of bacterial enzymes and toxins and relate to noxious chemi-

cals by the recruited neutrophils. The initial response to *H. pylori* infection in human appears to have a marked neutrophil infiltration and is associated with a transient period of achlorhydria. This initial inflammatory response is probably induced both directly by bacterial factors and indirectly via the induction of chemokines involved in the inflammatory cascade. In the clinical field, chronic gastric inflammation accompanied with neutrophil infiltration is called "chronic active gastritis", which is cured by eradication therapy against *H. pylori*.

Adhesion molecules

Yoshida et al.[18] have demonstrated that CD11b/CD18 (Mac-1) is expressed on the cell membrane within several minutes and human neutrophils adheres strongly to human endothelial cells, when neutrophils are activated by a water extract of *H. pylori*. Because this adhesion is inhibited by a monoclonal antibody directed against CD11b, CD18, or ICAM-1, it would be seem that the adhesion takes place through interaction of CD11b/CD18 on neutrophils and ICAM-1 on endothelial cells. Furthermore, when the extract was applied dropwise onto rat mesenteric venules, adhesion circulating leukocytes to the endothelium and their extravascular migration were observed. Substances that cause neutrophils to express adhesion did not contain previously known neutrophil-activating substances, such as platelet-activating factor and leukotriene B_4. The substance is resistance to pepsin and acid, and studies on its molecular weight suggested that it contains at least three activating substances. One such substance is a protein with a molecular weight of 150 kDa, and its gene has been identified and termed neutrophil-activating protein (NAP) gene[19,20]. We recently found that the *H. pylori* extract caused neurophils to adhere to MKN-45 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 oxidant-mediated gastric mucosal injury.

Interleukin-8 production

We also found that the water extract of *H. pylori* activates gastric epithelial cells (MKN-45) and human umbilical vein endothelial cells (HUVEC), and causes the expression of IL-8 mRNA and protein[21]. *H. pylori* infection is associated with elevated gastric mucosal IL-8 protein and IL-8 mRNA expression and also an increased gene expression of other C-C and C-X-C chemokines. IL-8 has potent chemotactic activity for neutrophils and induces the expression of adhesion molecules, CD11b/CD18, and the production of reactive oxygen species (Figure 4). Aihara et al.[22] reported that IL-8 production required direct contact between the cells and live bacteria. However, we confirmed the IL-8 production by gastric epithelial cells stimulated with the water extract of *H. pylori*. We have found that gel filtration localized the activity to a low-molecular-weight fraction of about 7 kDa, which was resistant to heat and trypsin digestion, and that protein kinase C (PKC) inhibitors significantly blocked *H. pylori*-induced IL-8 production by MKN 45 cells[21]. However, protein kinase A (PKA) inhibitor or protein tyrosine kinase (PTK) inhibitors showed a partial inhibitory effect. These results indicate that extracts of *H. pylori* contains a nonprotein substance of low molecular weight that is responsible for IL-8 induction in gastric epithelial cells. This induction is mainly dependent on the activation of PKC but is also partially dependent on PKA or PTK[21]. However, Beales et al.[23] reported that the secretion of epithelial chemokines induced by *H. pylori* can be blocked by PTK inhibitors such as herbimycin A and genistein but not by PKC or PKA inhibitors. Further studies have demonstrated that *H. pylori* activates the transcriptional factor NF-κB in gastric epithelial

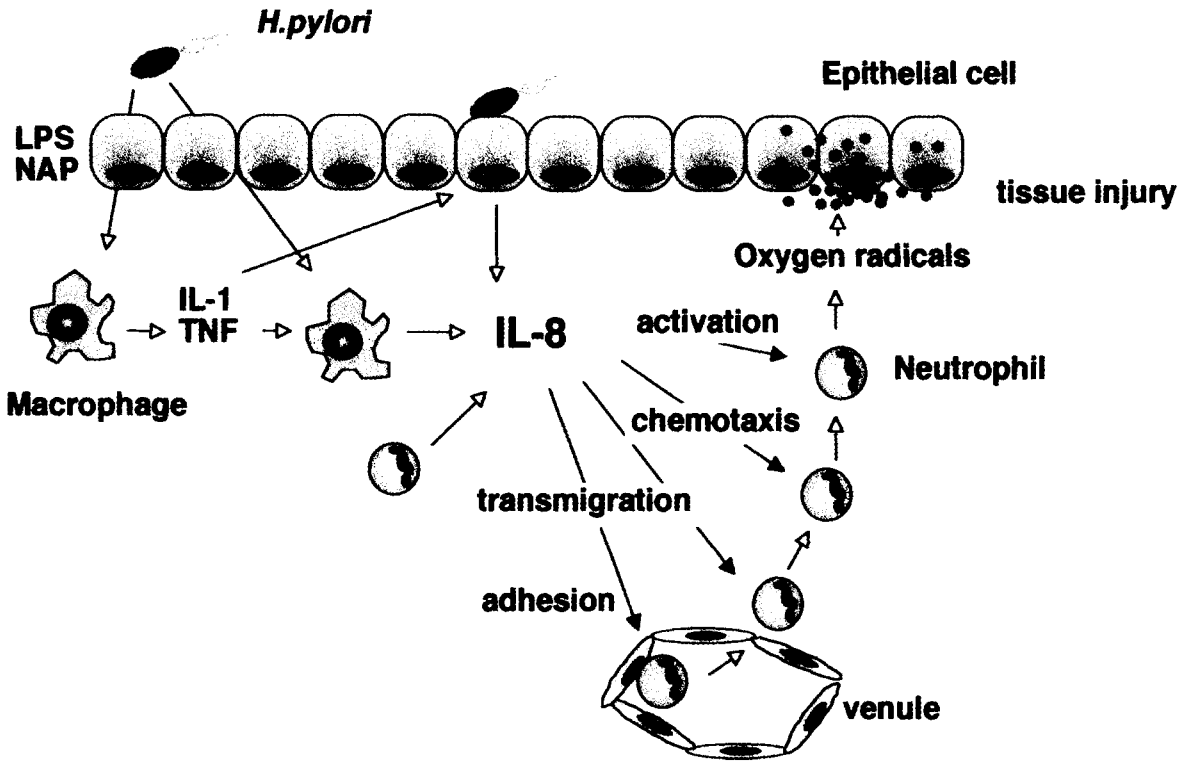

FIGURE 4 *H. pylori*-induced inflammation and inflammatory cytokine IL-8
The gastric epithelial cells and macrophages are major sources of IL-8 in the case of *H. pylori*-infected gastric mucosa. Epithelial IL-8 can be induced, not only by direct bacterial stimulation, but also following exposure to the endogenous proinflammatory mediators interleukin (IL)-1 and tumor necrosis factor (TNF)-α. Macrophages in the lamina propria are the main sources of IL-1 and TNF-α. The bacterial components inducing the proinflammatory cytokines from macrophages are likely to be multiple. *H. pylori* lipopolysaccharide (LPS) and neutrophil activating protein (NAP) will induce cytokine secretion. Neutrophils once accumulated at the site of infection may secrete chemokines, thus further amplifying the cellular response to infection

cells[24]. Following phosphorylation, ubiquitination, and degradation of inhibitory protein Iκ-B, the homo- or heterodimer NF-κB is translocated into the nucleus and leads to stimulate IL-8 transcription by binding to the promoting region of IL-8 gene.

Monochloramine

When neutrophils and *H. pylori* are added to cultured gastric mucosal cells, the mucous cells are injured, and monochloramine (NH_2Cl) is formed [25]. NH_2Cl, a product derived from the interaction of NH_3(a product by *H. pylori)* and HOCl (a product by activated neutrophil), is reported to be exceptionally reactive and toxic because of its high lipophilic property and low molecular weight [26]. NH_2Cl is bactericidal and mutagenic, inhibits the hexose-monophosphate pathway in eucaryotic cells, oxidizes erythrocyte hemoglobin and glutathione, and stimulates rat colonic secretion[27,28]. Suzuki et al.[25] demonstrated that *H. pylori*-activated neutrophils promote gastric mucosal cell injury and that NH_2Cl, at concentrations of 0.1 mM and more, plays a unique and important role in this process. Dekigai et al.[29] have also shown that NH_2Cl directly injures rat gastric mucosal cells as well as cul-

tured endothelial cells. We have demonstrated that NH_2Cl inhibits gastric mucosal cell growth and induces apoptosis in rat normal gastric mucosal cells[30]. Because NH_2Cl is a potent mediator in the oxidation of DNA and in the induction of apoptosis [31,32], it is generally considered that NH_2Cl is a target molecule in our understanding of the mechanism of *H. pylori*-associated gastric carcinogenesis, or developing a novel chemopreventive agent.

NSAIDS-INDUCED GASTRIC INFLAMMATION

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin and aspirin have enjoyed widespread clinical use as anti-inflammatory, analgestic agents, but it has been documented that gastrointestinal injury, one of the most serious adverse effects attributable to NSAIDs, may occur in patients who are treated with NSAIDs [33]. Therefore, it is a very important to develop strategies against gastrointestinal injuries, which are induced by NSAIDs. Although it has been proposed that a deficiency of endogenous prostaglandins due to inhibition of cyclooxygenase by NSAIDs is involved in these effects [34], the exact pathogenic mechanism remains to be elucidated. It has been proposed that neutrophil-dependent microvascular injures [35,36] and oxygen radical-mediated lipid peroxidation may be important prime events that lead to mucosal injury, induced by NSAIDs.

Recent reports have hypothesized that oxygen radical-mediated lipid peroxidation [1,37] and neutrophil-mediated inflammation [35,36] are involved in the development of NSAID-induced gastric mucosal injury. Five lines of evidence support this hypothesis: 1) neutrophil depletion by intraperitoneal injection of anti-neutrophil serum significantly attenuates the gastric mucosal injuries induced by indomethacin [1] or aspirin [38], 2) intravital microscopic approaches applied to the mesenteric circulation have shown that indomethacin or aspirin promotes leukocyte adherence and emigration in postcapillary venules [39,40], 3) immunoneutralization of the CD11/CD18 adherence complex on neurophils attenuates these injuries [38,41], 4) these NSAIDs directly promote neutrophil adherence to endothelium via CD11b/CD18-dependent interactions with ICAM-1 [42,43], and 5) adhesion molecules CD11a and ICAM-1 are expressed in the normal gastric mucosa and that the number of ICAM-1-stained blood vessels rapidly increase after indomethacin treatment [44]. The mechanism responsible for the up-regulation of ICAM-1 after indomethacin/aspirin administration are unclear. TNF-α is one of the candidates for a mediator involved in NSAID-induced leukocyte adherence [45], TNF-α is a proinflammatory cytokine and has recently been shown to be a crucial mediator of NSAID-induced gastric mucosal injury [45,46]. The addition of a TNF-α processing enzyme inhibitor has been shown to prevent aspirin-induced TNF-α release and protect against gastric mucosal injury in rats [47]. TNF-α augments neutrophil-derived superoxide generation [48] and up-regulates the expression of adhesion molecules on neutrophils and endothelial cells [49], leading to neutrophil accumulation and oxygen radical-mediated tissue damage. These results indicate that a crucial initial event in the pathogenesis of NSAID-induced injury appears to be adherence of neutrophils to microvascular endothelium, and that drugs targeted TNF-α will benefit development of novel strategies to treat NSAID-gastropathy.

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