REVIEW

Gastric Mucosal Injury: Microcirculation and Helicobacter Pylori

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Abstract. The integrity of gastric mucosa is well-balanced by an array of defensive mechanisms which protect the mucosa against external aggressive factors. When excessive stimulation of autonomic nervous system (irritation) is induced, microcirculatory disturbances easily lead to the gastric mucosal damage due to the formation of vasoactive mediators and oxygen radicals. In this review, our discussion has been focused on the co-ordinating function of the autonomic nervous system as well as the microcirculation as an important defense bastion. In this context, *Helicobacter pylori* represents an important pathogenic factor. In particular, we have discussed the contribution of monochloramine, and active oxidant, which is formed by neutrophils in the presence of ammonia derived from *H. pylori* to the gastric mucosal injury. Microcirculatory disturbances may be also involved in the pathogenesis of *H. pylori*-induced mucosal injury. On the basis of these considerations, we should not depend solely on the use of anti-acid secretory drugs for the treatment of gastric mucosal injury, but also should be aware of beneficial effect of mucosal protective drugs which may act on microcirculation and the autonomic nervous system. (Keio J Med 43 (1): 1–8, March 1994)

Key words: autonomic nervous system, irritation, oxygen radicals, gastritis, gastric ulcer

Introduction

In 1937, Hans Selye pointed out that stress can affect not only the adrenal glands, thymus, and lymphatic system but also the gastric mucosa.¹ It should be noted, however, that he was not the first person who found gastric mucosal lesions as a result of stress. His teacher, Dr James Reilly described in early 1930's the concept of an irritation syndrome characterized by nonspecific hemorrhagic lesions in multiple organs due to excessive stimulation of autonomic nervous system.²

A variety of factors is known to contribute to the pathogenesis of the gastric mucosal lesions. As might be anticipated, aggressive factors from the luminal side are important, especially the role of acid, a feature referred to as hydrogen back diffusion theory.³ Davenport postulated in 1964 that when acid penetrates the mucosal barrier into lamina propria, mast cells are activated and release histamine. Acid and histamine induce micro-

vascular, neural and epithelial damage in the gastric mucosa. The precise contribution of microcirculatory and autonomic nervous system factors remains, however, largely undocumented.³ In addition, it has recently been reported that a particular strain of bacteria, *Helicobacter pylori* (*H. pylori*), was one of pathogenic factors of gastric mucosal injury.⁴ In the present review, we have summarized the pathogenesis of gastric mucosal injury from a microcirculatory viewpoint (Fig 1).

Morphological Findings of Gastric Mucosa

Abundant vascular networks are revealed in the lamina propria of the gastric mucosa by scanning electron microscopic observations. These capillary sized vessels are shown to reach the surface of the mucosa. Collecting venules which drain blood from the capillaries, descend directly into the submucosa. Under higher magnification of gastric mucosal surface, fine nerve fibers can be detected

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Fig 1 Summary of a major factor in the pathogenesis of gastric mucosal injury. The three important factors for gastric mucosal homeostasis are autonomic nervous factor, microcirculatory factor and mucus factor. Not only do acid, alcohol, drugs and *Helicobacter pylori* appear to be involved, but the aforementioned three factors could represent aggressive factors during the course of irritation.

along side of the true capillary networks which surround the epithelial cells. The cholinergic innervation of the stomach is made up of an intrinsic and extrinsic set of fibers from the vagal nerve. The vagal input to the enteric nervous system originates in the dorsal motor nucleus of the medulla oblongata.⁵ The vagi at the level of the diaphragm contain an average of 56,138 afferent fibers and only 1736 efferent fibers.⁶ Most of the cholinergic nerves in the gastric mucosa belong to the enteric nerves. The histochemical localization of such cholinergic nerves was demonstrated by acetylcholinesterase (AChE) activity⁷⁻⁹ and choline acetyltransferase (ChAc) immunoreactivity.¹⁰ With this method, cholinergic nerve fibers in the lamina propria were stained a brown color and could be seen to be distributed along the true capillaries up to the surface of mucosa (Fig 2). This relationship suggested a close functional interaction among true capillaries, the autonomic nervous system and epithelial cells. Sympathetic pathways transmit command signals from the central nervous system via spinal nerves terminating in celiac ganglia and function to reduce gastrointestinal blood flow and motility. From these ganglia, the postganglionic adrenergic fibers reach the stomach either as discrete nerves, or as nerves closely associated with vagal nerves or the nerves that accompany the arterial vessels. Nerve fibers showing specific noradrenaline fluorescence are located mainly in the perivascular plexuses of the arterioles and to a lesser extent in the myenteric and submucosal nerve plexuses by the Falck-Hillarp's method and its modification (Fig 3). It has also been reported that adrenergic nerves were located in close contact with arterioles and venules

Fig 2 The acetylcholinesterase-positive nerve fibers are recognized aligned linearly between the gastric epithelial cell column. Bar indicates 20 μ m.

Fig 3 The noradrenaline-specific fluorescence is seen largely surrounding the arteriole and to a lesser degrees in the lamina propria mucosae. Bar indicates 10 μ m.

in the basal portion of mucosa and that adrenergic and cholinergic nerves sometimes coexist in the same Schwann

cell aligned close to the true capillary.¹¹⁻¹³ The contraction of the appropriate smooth muscle cells is also shown in pharmacological and histochemical studies to be influenced by dopamine.¹⁴ Such dopaminergic nerves are identified histochemically as dopamine-beta-hydroxylase negative and tyrosine hydroxylase positive nerve fibers. On the basis of this criterion, it has been possible to identify many dopaminergic nerves near the arterioles in the muscularis mucosae and propriae in addition to their colocalization with the adrenergic nerves in perivascular plexuses.¹⁵ Various types of peptidergic nerves are recognized in the stomach. Vasoactive intestinal peptide (VIP), gastrin releasing peptide (GRP), neuropeptide Y (NPY), and calcitonin gene-related peptide (CGRP) are especially abundant in the stomach.¹⁶ While VIP and CGRP act as a vasodilator, GRP and NPY act as a vasoconstrictor in the stomach. It has been reported that VIP increases a gastric mucosal blood flow.¹⁷ The VIPergic nerves and the GRP-immunoreactive nerves are found to have the same distribution as a cholinergic nerves.¹⁸ Although GRP was identified as a brain-gut peptide at first and thought to be mainly concerned with the secretion of gastrin from the G cell in the antral region of the stomach, recent histochemical studies have revealed that GRP is principally localized in the muscular layer.¹⁹ On the other hand, NPY is thought to be one of the typical brain-gut peptides which coexists with noradrenaline in the adrenergic nerves.²⁰ Recently, CGRP has attracted attention in the context of its relationship to the afferent nervous regulation of the gastric circulation, because acute capsaicin treatment was found to suppress the formation of gastric mucosal lesions and capsaicin has been found to have a strong effect on the afferent nervous activity mediated by CGRP.^{21,22}

Experimental Model of Irritation-induced Gastric Mucosal Injury

Male Wistar rats were used for the study of gastric mucosal injury. Experimental gastric mucosal injury was induced by repeated electrical stimuli (30V, 5msec, 50Hz) on the left arterial wall (Fig 4). Thirty minutes later, apparent gastric hemorrhagic lesions developed in the glandular stomach, and at that time gastric mucosa and regional blood from the gastric vein were taken for the assay of various biochemical parameters.²³ Endothelin is an endothelium-derived constrictive factor which was found in 1988.²⁴ On the other hand, another mediator, endothelium-derived relaxing factor (EDRF), has been identified as nitric oxide (NO) or a closely related molecule synthesized from the guanido group of L-arginine by nitric oxide synthase (NOS).²⁵ The concentration of endothelin appeared to be increased in the gastric

Fig 4 The protocol for experimental gastric mucosal injury induced by repeated electrical stimuli. The small gastric artery in the lesser curvature of fasted Male Wistar rats (250g) was exposed in order to apply repeated electrical stimulation (irritation: 30 V, 5 msec, 50 Hz) for 30 sec, three times with 10 sec intervals, using a bipolar platinum electrode. This irritation was then repeated three times with 3min interval. Prior to and after the irritation, samples of venous blood (200 μ l) were collected from the gastric vein for analysis of tissue type plasminogen activator (t-PA), plasminogen activator inhibitor (PAI) and chemiluminescence value. The area covered by ulcers and erosions was determined 30min after the completion of irritation.

regional blood immediately after the irritation, whereas there was no increase at the systemic level. In contrast, the activity of NOS was significantly decreased after the irritation, suggesting an imbalance of substances which regulate vascular tonus (unpublished observation).

Vasomotor derangements induce fibrinolytic activation and further exacerbate the endothelial cell damage. Subsequent to the irritation, the ratio of prostacyclin (PGI₂) and thromboxane A₂ (TxA₂) was decreased, a feature which might trigger platelet aggregation. In the gastric mucosa, the content of PAF (platelet-activating factor) and t-PA (tissue-type plasminogen activator) levels were elevated suggesting the development of endothelial damage in gastric mucosa. The t-PA activity²⁶ in the regional blood of the stomach was significantly elevated as early as 5 min after the irritation. Immunohistochemical study using anti-t-PA monoclonal antibody revealed that t-PA was detectable in the endothelial cells of capillaries and collecting venules, suggesting the involvement of endothelium-mediated fibrinolytic activity in the irritation-induced ulcer formation. Pretreatment with SOD or allopurinol significantly attenuated the irritation-induced t-PA activation, suggesting that the t-PA activity was modulated by xanthine oxidase-associated superoxide anions. CV-6209, a selective antagonist of PAF, also prevented the activation of t-PA as well as ulcer formation, leading to the concept that PAF may be associated with the local

fibrinolytic activation which in turn may cause hemorrhagic changes in the gastric mucosal microvasculature.²⁷

Inflammatory mediators including PAF induce increased vascular permeability, leukocyte accumulation and enhanced oxygen radical generation in the gastric mucosa. PAF, synthesized by activated endothelial cells, is rapidly and transiently expressed on the cell surface and signals to neutrophilic leukocytes to bind.²⁸ PAF associated with endothelial cells activates neutrophilic leukocytes by binding to a cell surface receptor. Occupation of this receptor by PAF induces upregulation of integrins which mediate the adhesive interaction.^{28,29} Neither a microvascular adherent change of leukocytes nor an increased mucosal activity of myeloperoxidase (MPO) was observed during the earliest period after the completion of the irritation, suggesting that PAF induced integrin upregulation has not been completed in such a short period. Thirty minutes after irritation, there was an increase in myeloperoxidase(MPO) activity in the gastric mucosa, a good indicator of neutrophil accumulation. Luminol-dependent chemiluminescence values from regional neutrophils was also enhanced, suggesting an important role of neutrophil-mediated oxidative stress in the process of gastric mucosal injury.³⁰ The contributory role for oxygen radicals has been reported in hemorrhagic shock-induced gastric lesions in the rat.³¹

***Helicobacter Pylori* and Gastric Mucosal Lesions**

Another important defense factor is the gastric mucus. Gastric mucus covering the mucosa was stained by PAS and gastric mucous secretory cells were clearly stained blue by alcian blue. Ruthenium red block stain was applied to show by electron microscopically the glycocalyx of mucin. Not only the surface glycocalyx but also secretory granules on the surface mucous cells were recognized as electron dense materials. Surprisingly, some types of bacteria appeared on the surface of mucosa. Such observations had already been made 1940, Doenges³² and Freedberg³³ who pointed out the presence of Spirochaeta-like organism in human gastric mucosa. However in 1954, Palmer³⁴ concluded that there was no evidence of such bacteria from observation on the basis of hematoxylin-eosin stained specimens, and there followed a thirty year silence on this debate. Then in 1983, Warren and Marshall^{4,35} reported that campylobacter-like bacilli could be found in the gastric biopsy specimens. This organism has been referred to as *Helicobacter pylori*, since in 1989, Goodwin was able to characterize this bacteria from gene and lipid analysis.³⁶ Although this organism is rarely visible after the ordinary hematoxylin-eosin staining, is clearly demonstrated by Wartin-Starry staining. The natural habitat of the bacterium appears to be the gastric mucus layer.³⁷ In line

with electron microscopical findings, *Helicobacter pylori* has several flagella. The vast majority of *H. pylori* organisms are seen in the pit mucus or in the surface mucous layer, close to the epithelial cells (Fig 5).³⁸ Ruthenium red may reveal fine filamentous strands extending between organisms and nearby epithelial cells.³⁹ Occasionally, organisms appear to congregate at and around intercellular junctions.⁴⁰ *H. pylori* may also be seen firmly attached to particular cells on the tissue surface by adherence pedestals.⁴¹ Attached bacteria are particularly striking in cases of epithelial degeneration in the presence of intraepithelial polymorph infiltration.⁴¹ *H.*

Fig 5 Scanning electron micrograph of the gastric tissues from the patients with *Helicobacter pylori* (*H. pylori*)-positive chronic gastritis. There was a long flagella and bacterial body attached with the microvilli of gastric epithelial cells. A glycocalyx (arrows) was observed between the bacterial body and microvilli and between microvilli per se. The microvilli had various forms, such as irregular shapes and thickness. The microvilli attached with the body of *H. pylori* appeared to have deteriorated and exhibited lower heights compared with the ordered microvilli observed in the upper portions of the figure. Bar indicates 0.2 μ m.

pylori appears to be a factor in the pathogenesis of both gastritis⁴²⁻⁴⁴ and gastroduodenal ulcers.^{44,45} We are now beginning to speculate whether all these *H. pylori* in the gastric mucosa have a pathogenic role or not. Inasmuch as they could detect in healthy patients, the fact that a high proportion of positive *H. pylori* infection is encountered among endoscopy patients is difficult to evaluate. Considerable differences are seen in prevalence between Japan or France and African or South American coun-

tries (Table 1).^{46,47} These considerations suggests that the presence of *H. pylori* may not be directly indicative of a pathogenic role.

Terada, who is one of the present authors, was recently able to demonstrate the distribution of *Helicobacter pylori* in the gastric mucosa by transmission electron microscopy (Fig 6).^{48,49} According to his findings, at least 66% of the bacteria was floating in the mucus layer and more than 20% was facing plasma membranes of gastric epithelial cells. Some *H. pylori* were adherent to the epithelium forming an "adhesion pedestal". Another interesting statistic was that about 3% of *H. pylori* was phagocytized by neutrophils in mucous layer.

H. pylori infection produces very high levels of urease activity, an enzyme that is produced within and on the surface of this organism.^{37,50} Urease cleaves urea to produce ammonia, which has a neutralizing or alkalinizing effect on the growth environment. A biopsy urease test relies on its urease activity to diagnose *H. pylori* infection.⁵¹⁻⁵³ A number of commercial serological kits are being developed for the diagnosis of *H. pylori* infection. The best serological tests are all enzyme-linked immunosorbent assays (ELISA), varying in the particular antigens such as urease.⁵⁴ The basis for the urea breath test is the high level of urease produced by the bacterium in vivo. When *H. pylori* is present in the stomach, swallowed ¹⁴C- or ¹³C-labelled urea is broken down and the labelled CO₂ produced is rapidly exhaled in the breath. Collected breath samples are analysed for ¹⁴CO₂ in a scintillation counter or for ¹³CO₂ by gas isotope ratio mass spectrometry.⁵⁵⁻⁵⁷ The *H. pylori*-infected gastric mucosa is characterized by a profound neutrophil infiltration. It has been reported that the elicitation of regional neutrophils is significantly elevated in patients with *H. pylori*. When luminol-dependent chemiluminescence values from neutrophil were determined, the combination of *Helicobacter pylori* and neutrophils strikingly enhanced toxic oxygen radical generation.⁵⁸

By using a newly developed photobiological technique, the interaction among gastric mucosal cells, neutrophils and *H. pylori* was quantitatively analyzed.⁵⁸ A fluorescence dye named BCECF-AM(bis-carboxyethyl-carboxyfluorescein acetoxymethyl ester) served as an indicator of cellular injury. This dye easily permeates into the cultured gastric mucosal cells and is hydrolyzed to form BCECF which has fluorescence. When cells are damaged, BCECF fluorescence material is leaked from cells into the culture media (Fig 7-a). After the addition of the neutrophils and *H. pylori* to the culture media of rabbit gastric mucosal cells, significant leakage of BCECF with deformation of gastric mucosal cells was demonstrated. A scavenger of hydrogen peroxide, catalase, significantly inhibited the BCECF leakage. Taurine and methionine, scavengers of a monochloramine which is a potent cytotoxic oxidant, appeared to

Table 1 Differences in Prevalence of Positive *Helicobacter Pylori* Infection Among Endoscopy Patients

Japan	43-50%
France	48-50%
African Countries	67-97%
South American Countries	72-90%

Fig 6 Transmission electron micrograph of the gastric tissues from the patients with *Helicobacter pylori* (*H. pylori*)-positive chronic gastritis. *H. pylori* attached to or adjacent to the gastric epithelial cells were observed. As a consequence of the infections with *H. pylori*, vacuolar degeneration and aggregation of mitochondria within the epithelial cells were detected. Also, the junctions of each cell appear to have become looser than those of uninfected epithelial cells. Bar indicates 4 μ m.

Fig 7 a: BCECF-AM (2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester) which is a stable nonfluorescent compound that can diffuse into cells, is hydrolyzed to BCECF (2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein) by intracellular nonspecific esterase and is hereby trapped within the intact cells. BCECF is a fluorescent molecule. When cell damage is induced, BCECF fluorescence is leaked from cells. b: Cytotoxicity of rabbit fetal gastric cells was measured as a specific % cytotoxicity by the release of the fluorescence dye (BCECF: 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein), which is retained in intact cells. Gastric mucosal cells that were monolayered in a 48-well culture plate were incubated with BCECF-AM (2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester, 5 μ M) for 20 min at 37°C. Thereafter, the dye loaded cells were washed with PBS (phosphate buffered solution) once and incubated in 0.5 ml DMEM (Dulbecco's modified Eagle's medium). In the assay, the gastric mucosal cells were incubated with neutrophils (750,000 cells/well) and *Helicobacter pylori* (*H. pylori*) (7,500,000 colony forming unit/well) for 2 hour at 37°C. The addition of neutrophils and *H. pylori* to the culture media of gastric mucosal cells led to a significant increase in cytotoxicity. Increased cytotoxicity of gastric mucosal cells in the neutrophils plus *H. pylori* group was significantly attenuated by the pretreatment of catalase (2,000 U/ml), taurine (50 mM), methionine (50 mM) and acetohydroxamic acid (0.5 mg/ml).

* $p < 0.01$ compared with value of control for plus *H.* § $p < 0.001$, ¶ $p < 0.01$, # $p < 0.05$, compared with value of neutrophils with *H. pylori*.

express attenuating effects on cellular injury. Acetohydroxamic acid, an inhibitor of urease also appeared to have an inhibitory effect (Fig 7-b). It has been reported that ammonia which is produced by urease from *Helicobacter pylori*, could directly damage the

gastric mucosa. However, our results imply the important role of neutrophils in this regard since monochloramine which can be formed by neutrophils in the presence of ammonia may enhance gastric mucosal damage (Fig 8).

The so-called "triple therapy"⁵⁹ consisting of amoxicillin, bismuth and metronidazole is widely used, but it seems to be aggressive for gastric mucosa. On the other hand, proton pump inhibitors, such as Omeprazole⁶⁰ or Lansoprazole⁶¹ are claimed to have potential cytotoxic effect on this organism, but their *in vivo* effects are not clearly established. It would appear to be more useful to explore the usage of anti-oxidant drugs or agents which modify neutrophil activation.

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Fig 8 Scheme showing possible mechanisms involved in *Helicobacter pylori* (*H. pylori*)-induced gastric mucosal microcirculatory injury. Urease-generated ammonia may contribute to the gastric mucosal injury associated with *H. pylori*. This ammonia-dependent injury may be evoked by activated neutrophils. Participation of ammonia-derived oxidant NH₂Cl may be an important factor in the formation of *H. pylori*-induced gastric mucosal injury.

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