Oxidative Stress In *Helicobacter pylori*-Asscoaited Gastroduodenal Disease

Hidekazu Suzuki* and Toshifumi Hibi

Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Received 13 February, 2006; Accepted 10 March, 2006

Summary *Helicobacter pylori* (*H. pylori*) is a spiral-shaped, Gram-negative rod, which induces the infiltration to the gastric mucosa by neutrophils, macrophages, and T and B lymphocytes; however, this immune and inflammatory response cannot completely clear the bacterial infection, and leaves the host prone to complications resulting from persistent inflammation. Resultantly, *H. pylori* infection causes chronic inflammation, accumulation of reactive oxygen species, and oxidative DNA damage in the gastric mucosa. Recent studies reveal that *H. pylori* injects bacterial proteins into the cytosol of the gastric host cell via the type IV injection system and regulates the intracellular signal transduction [1, 2]. This mechanism provides a novel means of resolving how *H. pylori* survives in the acidic environment of the human stomach. During persistent gastric infections, chronic gastritis may remain asymptomatic or may evolve into more severe diseases, such as peptic ulcer disease or atrophic gastritis. In addition, infection with *H. pylori* increases the risk of developing gastric cancer and mucosa-associated lymphoid tissue lymphoma. Various gastric diseases, such as gastritis, ulcers, intestinal metaplasia, and gastric cancer, can be appropriately developed by inoculating this bacteria into Mongolian gerbils [3]. In addition, the successful eradication of *H. pylori* is useful as a preventive approach against gastric cancer [4]. This review focuses on the aspects of the oxidative mechanism in the *H. pylori*-associated gastric mucosal lesion formation.

Key Words: oxidative stress, *Helicobacter pylori*, gastric cell injury, monochloramine, ammonia

Bacterial Side of Oxidative Stress

Although successful and persistent colonization of the gastric mucosa depends on the ability to respond to changing environmental conditions and co-ordinate the expression of virulence factors during the course of infection, *H. pylori* possesses relatively few transcriptional regulators. According to the report by Barnard et al. [5], CsrA was necessary for full motility and survival of *H. pylori* under conditions of oxidative stress. Loss of csrA expression was reported to be deregulated the oxidant-induced transcriptional responses of napA and ahpC (encoding alkyl hydroperoxide reductase), the acid induction of napA, cagA, vacA, the urease operon, and fur, as well as the heat shock responses of napA, groESL and hspR [5]. Although the level of napA transcript was higher in the csrA mutant, its stability was similar in the wild-type and mutant strains, and less NapA protein

*To whom correspondence should be addressed.
Tel: 81-3-5363-3914   Fax: 81-3-5363-3967
E-mail: hsuzuki@sc.itc.keio.ac.jp
was produced in the mutant strain [5]. They concluded that CsrA has a broad role in regulating the physiology of *H. pylori* in response to environmental stimuli, and may be important in facilitating adaptation to the different environments encountered during colonization to the gastric mucosa [5].

Loughlin *et al.* [6] generated isogenic, nonpolar *H. pylori ruvC* mutants to investigate the function of RuvC, a Holliday junction endonuclease that resolves recombinant joints into nicked duplex products. The *H. pylori ruvC* mutants were more susceptible to oxidative stress and exhibited reduced survival within macrophages [6]. O’Rourke *et al.* [7] addressed the question as to whether the pathogen DNA is subject to lethal or mutational damage by the host-generated oxidative response. *H. pylori* strains lacking a functional endonuclease III showed elevated spontaneous and induced mutation rates and were more sensitive than the parental strain to killing by exposure to oxidative agents or activated macrophages. In the mouse infection with *H. pylori* strains lacking a functional endonuclease III, the stomach bacterial load gradually decreases while the population in the wild-type strain remains stable, showing that endonuclease III deficiency reduces the colonization capacity of the pathogen. These results show that the host effectively induces lethal and premutagenic oxidative DNA adducts on the *H. pylori* genome [7].

**Bacillary Form vs Coccoid Form of *H. pylori***

Most *H. pylori* cultured under an optimum O$_2$ concentration (7%) were the bacillary form, whereas about 80% of cells cultured under aerobic or anaerobic conditions were the coccoid form. Park *et al.* [8] described the effect of oxygen tension on the transformation and ROS metabolism of this pathogen. They observed that the bacillary form of *H. pylori* generated predominantly O$_2^−$, whereas the coccoid form generated preferentially OH$^−$. Specific activities of cellular respiration, urease, and superoxide dismutase (SOD) decrease markedly after transformation of the bacillary form to the coccoid form, with concomitant generation of protein carbonyls and 8-OHdG. The frequency of mutation of cells increased significantly during culture under nonoptimum O$_2$ conditions. These results indicate that ROS generated by *H. pylori* catalyze the oxidative modification of cellular DNA, thereby enhancing the transformation from the bacillary to the coccoid form. The enhanced generation of premutagenic OH$^−$ in the coccoid form might accelerate mutation and increase the genetic diversity of *H. pylori*. Peroxiredoxins, the enzymes that catalyze the reduction of H$_2$O$_2$ and organic hydroperoxides, are ubiquitous proteins that protect organisms from damage by ROS. *H. pylori* contains three members of the peroxiredoxin family: AhpC (alkyl hydroperoxide reductase), Tpx (thiol-specific peroxidase), and bacterioferritin comigratory protein (BCP). Wang *et al.* indicates that *H. pylori* BCP plays a significant role in efficient host colonization [9]. Exposure of *H. pylori* cells to air for 2 h elevated the level of free iron by about 4-fold as measured by electron paramagnetic resonance (EPR) spectroscopy [10]. The *tpx* (encoding thiolperoxidase) strain of *H. pylori* was clearly more sensitive than the parent strain to both oxygen and cumene hydroperoxide, and colonized only 5% of the inoculated mice [11]. Two different classes of oxygen-sensitive *ahpC* mutants were recently described [12]. Neither of these mutants was able to colonize mouse stomachs, whereas 78% of the mice inoculated with the parent strain became *H. pylori* positive [11].

**Bacterial DNA Mutation***

*H. pylori* cells accumulated more free iron as they approached stationary phase growth, and they concomitantly suffered more DNA damage. Mutant cells defective in either catalase (KatA), in SOD (SodB) or in alkyl hydroperoxide reductase (AhpC) were more sensitive to oxidative stress conditions; and they accumulated more free (toxic) iron; and they suffered more DNA fragmentation compared to the wild type. A significant proportion of cells of *sodB, ahpC*, or *katA* mutant strains developed into the stress-induced coccoid form or lysed; they also contained significantly higher amounts of 8-oxo-guanine associated with their DNA, compared to the wild type [10].

Enhanced expression of NapA and other oxidative stress proteins (catalase or MdaB; an NADPH quinone reductase etc.) is a compensatory response to loss of major *H. pylori* stress resistance factors [10].

*H. pylori* mutS mutants are more sensitive than the wild-type to oxidative stress induced by agents such as H$_2$O$_2$. Exposure of mutS cells to oxidative stress results in a significant elevation of mutagenesis [13]. Under oxidative stress conditions, mutS cells accumulate higher levels of 8-oxoG DNA lesions than the wild-type and have reduced colonization capacity in comparison to the wild-type mouse [13].

The iron-responsive regulator fur controls iron metabolism in many bacteria, including *H. pylori*, and thus is directly or indirectly involved in regulation of oxidative stress defense. Ernst *et al.* recently showed that *H. pylori* fur was a versatile regulator involved in many pathways essential for gastric colonization, including superoxide stress defense [14]. The activity of SOD in the gastric mucosa decreased significantly following the successful *H. pylori* eradication, whereas in the corpus activity did not change significantly [15], suggesting that while bacterial SOD reduces after the eradication, gastric mucosal cellular SOD restores by the inflammatory remission.

On the other hand, sialic acid-binding SabA adhesin is a prerequisite for the nonopsonic activation of human neutrophils and, thus, is a virulence factor important for the
pathogenesis of \textit{H. pylori} infection \cite{16}.

Host Side of Oxidative Stress

One of the potential toxic factors involving \textit{H. pylori}-induced gastric injury are oxygen radicals which are released from activated neutrophils, since \textit{H. pylori} exhibits chemotactic activity for neutrophils \cite{17, 18}. Neutrophil infiltration of the gastric mucosa was reportedly the initial pathological abnormality in \textit{H. pylori-associated gastritis and remains a hallmark of active infection. In response to the activation of 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 molecules that receive an electron become superoxide radicals (O\textsuperscript{2-}), which is rapidly converted to hydrogen peroxides (H\textsubscript{2}O\textsubscript{2}) by spontaneous dismutation or enzymatic superoxide dismutase (SOD), and hydroxyl radicals (OH\textsuperscript{•}), which are formed non-enzymatically in the presence of Fe\textsuperscript{2+}. In neutrophils, myeloperoxidase (MPO) also results in the formation of the potent oxidant, a hypochlorous anion (OC\textsubscript{•}-) from H\textsubscript{2}O\textsubscript{2} in the presence of Cl\textsuperscript{-}. This hypochlorous anion reacts with ammonia, derived from urea by \textit{H. pylori}-associated urease, and yield s monochloramine (NH\textsubscript{2}Cl), which enhances the mucosal cytotoxicity \cite{19} with this lipophilic property, and freely penetrates biological membranes to oxidize intracellular components \cite{20}. On the other hand, ammonia itself also induces vacuolation of gastric epithelial cells, necrosis in parietal cells and apoptosis in chief cells \cite{21}. It is suggested that \textit{H. pylori} may produce substances that degrade mucus and injure epithelial cells \cite{22}, thereby reducing the resistance of the mucosa to acid-related injury.

Production of Reactive Oxygen Species (ROS) in \textit{H. pylori}-colonized Gastric Mucosa

Reactive oxygen species (ROS) production, as determined by chemiluminescence assay, has been shown to be enhanced in endoscopic biopsied samples from the stomach of patients of gastric ulcers with \textit{H. pylori} infection \cite{23, 24}. Davies \textit{et al.} \cite{24} also found a positive association between ROS production and the mucosal load of \textit{H. pylori}. It was suggested that the sources of ROS production are probably host neutrophils, which are activated by soluble factors of \textit{H. pylori}. We previously showed that ROS production in gastric mucosa is enhanced by the infection of \textit{cagA}-positive \textit{H. pylori} species with an extensive accumulation of neutrophils in patients with gastric ulcer \cite{25}. These findings were reconfirmed by a recent study in patients with chronic gastritis in the absence of peptic ulcer \cite{26}. An \textit{in vitro} study also demonstrated that \textit{cagA}-positive strains induce an increased oxidative burst in polymorphonuclear neutrophils (PMNs) with higher ROS production \cite{27}. However, assessment of CagA status might not be useful to predict the outcome of \textit{H. pylori} infection or to discriminate patients in whom \textit{H. pylori} eradication is advisable in East Asian populations, since it has been shown that more than 90% of individuals in most East Asian populations are positive for the CagA strain. In addition, in spite of the increased production of ROS from PMNs, \textit{H. pylori} seems to be resistant to the antimicrobial action of ROS and such resistance has been attributed to bacterial productions of antioxidant species. Consequently, the excessive ROS production induces oxidative stress to the gastric mucosa, and may damage cellular components. It has been demonstrated that some of gastroprotective agents which could regulate the oxidative stress, such as rebamipide \cite{28, 29}, polaprezinc \cite{30, 31}, and ecabet sodium \cite{32}, have these properties \textit{in vitro} and \textit{in vivo}. In addition to PMNs, recent studies have demonstrated that gastric epithelial pit cells possess an isoynyme of gp91-phox, mitogen oxidase 1 (Mox1), and essential components for the phagocyte NADPH oxidase (p67\textsuperscript{-}, p47\textsuperscript{-}, p40\textsuperscript{-}, and p22-phox), and that these pit cells produce ROS via activation of nonphagocytic NADPH oxidase in response to \textit{H. pylori} \cite{33}. The same group further extended their study and found that CagA-positive \textit{H. pylori} lipid A may be a potent stimulator for innate immune responses of gastric mucosa by stimulating the toll-like receptor 4 cascade and Mox1 oxidase in gastric pit cells \cite{34}. Although further studies are necessary to clarify the physiological and pathological roles of ROS generated by Mox1, ROS may affect the cell proliferation via the activation of redox-sensitive transcription factors \cite{35}, and the excess production of ROS may cause oxidative DNA damage in the gastric mucosa.

8-OHdG (8-hydroxy-2-deoxyguanosine) is one of the most abundant markers in DNA damage induced by ROS \cite{36}. It results from attack of a singlet hydroxyl or oxygen radical on guanine. Patients with \textit{cagA}-positive \textit{H. pylori} strains had higher 8-OHdG levels than both \textit{cagA}-negative and \textit{H. pylori}-negative cases \cite{37}. The 8-OHdG levels were significantly higher in multifocal atrophic gastritis. The odds ratio for \textit{cagA}-positive patients having 8-OHdG levels were 7.12, overall, reaching 11.25 when only patients younger than 50 were considered. The \textit{cagA}-positive patients were characterized for greater oxidative DNA damage overall, at younger age and in the presence of multifocal gastric atrophy \cite{37}. Nishibayashi \textit{et al.} reported that gastric mucosal 8-OHdG levels in the body region of patients with enlarged-fold gastritis were significantly higher than in \textit{H. pylori-negative controls and H. pylori-positive patients without enlarged-fold gastritis} \cite{38}. They also reported that eradication of \textit{H. pylori} significantly decreased the 8-OHdG levels in the gastric mucosa from patients with enlarged-fold gastritis \cite{38}.

According to the report by Khanzode \textit{et al.} \cite{39}, significant increase in serum superoxide dismutase (SOD) and serum
malondialdehyde (MDA) and significant decrease in plasma ascorbic acid were observed in H. pylori gastritis and gastric cancer patients compared to control subjects. They also demonstrated that the concentration of serum SOD and MDA was significantly higher and plasma ascorbic acid was significantly lower in gastric cancer as compared to H. pylori gastritis patients. Konturek et al. [40] recently reported that vitamin C combined with acetylsalicylic acid, unlike plain acetylsalicylic acid without vitamin C, protects gastric mucosa in human probably due to the attenuation of oxidative stress and proinflammatory cytokines.

**Mongolian Gerbil Model**

Although Mongolian gerbils are considered to be a suitable animal model for understanding the development of H. pylori-associated diseases, limitations regarding the genetic information available for this animal species hamper the elucidation of underlying mechanisms. Matsubara et al. [41] identified the nucleotide sequences of cDNAs encoding Mongolian gerbil inflammatory proteins, such as interleukin-1 (IL-1β), tumor necrosis factor alpha (TNF-α), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) and then examined the mRNA expression of these genes in the glandular stomach by RT-PCR at 1–8 weeks after H. pylori infection. According to their report [41], in the pyloric region, mRNA expression levels of IL-1β, TNF-α and iNOS were increased in H. pylori-infected animals at the 2 weeks time point, while in the fundic region, expression levels of IL-1β, TNF-α and iNOS were elevated at 4 and 8 weeks. The COX-2 expression level in the fundic region was clearly elevated in infected animals compared with control animals at 4 and 8 weeks, but in the pyloric region, expression levels were similar in both infected and control animals. We also identified the nucleotide sequences of cDNAs encoding preproghrelin, a precursor of ghrelin, an appetite promoting endocrine peptide mainly produced from the A-like cells in the gastric fundus [42] and sonic hedgehog, a morphogen, which may regulate and organize the fundic gland epithelial differentiation [43]. Preproghrelin and sonic hedgehog mRNA expressions were significantly reduced in the gastric mucosa of Mongolian gerbils with persistent H. pylori colonization [42, 43].

**Other antioxidative properties**

Apurinic/apyrimidinic endonuclease-1 (APE-1)/redox factor-1 (Ref-1) repairs damaged DNA and reductively activates transcription factors, including activator protein-1 (AP-1). Considering that H. pylori generate ROS and that ROS modulate APE-1/Ref-1 in other cell types, Ding et al. [44] examined the effect of H. pylori, oxidative stress, and antioxidants on APE-1/Ref-1 expression in human gastric epithelial cells and showed that APE-1/Ref-1 protein expression was increased after stimulation with H₂O₂ or live cag pathogenicity island-bearing H. pylori, but not cag pathogenicity island-negative H. pylori or C. jejuni. They also demonstrated that H. pylori- or ROS-mediated increases in APE-1/Ref-1 expression involved de novo protein synthesis that was inhibited by antioxidants. Their data showed that H. pylori or ROS enhance APE-1/Ref-1 protein synthesis and nuclear accumulation in human gastric epithelial cells and implicate APE-1/Ref-1 in the modulation of the pathogenesis of H. pylori infection.

(−)-Epigallocatechin-3-gallate (EGCG), one of the green tea catechins, is known to suppress H. pylori-induced gastritis through its antioxidative and antibacterial actions. Lee et al. [45] evaluated the protective mechanism of EGCG against H. pylori-induced cytotoxicity in gastric epithelial cells. According to their report [45], EGCG pretreatment effectively rescued gastric mucosal cells from the H. pylori-induced apoptosis and DNA damage, and administration of EGCG enhanced gastric epithelial cell proliferation.

**Lipid Peroxidation in the Gastric Mucosa**

Accumulation of lipid peroxidation products in the H. pylori-infected gastric mucosa provides evidence of increased oxidative stress. Although the thiobarbituric acid (TBA) test is not specific for lipid peroxides, it is one of the oldest and most frequently used methods for measuring the peroxydation of fatty acid, membranes, and food products. It is the easiest method to use, and it can be applied to crude biological samples. It has been shown that the levels of TBA-reactive substances are higher in patients [46, 47] and Mongolian gerbils [48] with H. pylori-associated gastritis, and that these levels fall significantly in the mucosa of patients in whom H. pylori is successfully eradicated [46] and in that of Mongolian gerbils in whom H. pylori-induced inflammation is ameliorated by the antioxidant, polaprezinc, a zinc-carnosine chelate compound [31]. In the H. pylori-infected gastric mucosa, concentrations of TBA-reactive substances are also correlated with chemiluminescence levels [46], an index of ROS production, and with myeloperoxidase activity, an index of tissue neutrophil accumulation [48]. These data suggest that H. pylori infection is likely to be a cause of increased ROS generation and damage rather than merely associated with these events, and also that the correlation between chemiluminescence or myeloperoxidase activity and the levels of TBA-reactive substances is further evidence that TBA-reactive substances reflect ROS-mediated lipid peroxidation.

**Apoptosis**

H. pylori exerts much of its pathogenicity by inducing apoptosis and DNA damage in host gastric epithelial cells. We previously demonstrated the NH₄Cl induced the increase in the chromatin condensation [49] as well as in the cytoplasmic mono- and oligonucleosomes [50], one of
markers for apoptosis in the gastric cell lines. On the other hand, polyamines are abundant in epithelial cells, and when oxidized by the inducible spermine oxidase SMO(PAOh1), H₂O₂ is generated. Xu et al. [51] identified a pathway for oxidative stress-induced epithelial cell apoptosis and DNA damage due to SMO(PAOh1) activation by *H. pylori* that may contribute to the pathogenesis of the infection and development of gastric cancer.

**Nitrosative Stress**

NO is a regulator of the gastric mucosal microcirculation under resting and stimulated conditions and interacts with prostanooids and sensory neuropeptides to maintain gastric mucosal integrity. For the mucosal protective action of NO, regulation of gastric mucosal blood flow is considered to be of major importance. Other defensive mechanisms induced by NO are stimulation of mucus secretion in epithelial cells, inhibition of the aggregation and adhesion of the platelets and inhibition of the adherence of neutrophils to the endothelium and their emigration to blood vessels and scavenging of O₂·, which cause mucosal damage. However, bicarbonate secretion, one of the defensive factors in the gastroduodenal mucosa, was increased by NO synthase inhibitors, which is contradictory to the mucosal protective role of NO. On the other hand, O₂· and NO rapidly react to form peroxynitrite, which decomposes and generates strong oxidant molecules [52]. *H. pylori* reduces mucin gene expression, which may be related to low production of NO by constitutive NO synthase in gastric epithelial cells. This hypothesis should be further investigated using specific antibodies for specific isoforms of NO synthase in gastric epithelial cells. In contrast, high amounts of NO, produced by inducible NO synthase (iNOS) induced by *H. pylori* in gastric epithelial AGS cells, contribute to apoptotic cell death [53]. The studies clearly demonstrate the involvement of oxidative stress in *H. pylori*-stimulated expression of iNOS and apoptosis in gastric epithelial AGS cells. On the other hand, Miyazawa et al. [54] reported that suppressed apoptosis in the inflamed gastric mucosa of *H. pylori*-colonized iNOS-knockout mice and suggested that iNOS may play an important role in promoting apoptosis in the *H. pylori*-colonized inflamed gastric mucosa, and that persistent inflammation without apoptosis such as in iNOS-knockout mice with *H. pylori* infection may be linked to preneoplastic transformation. These contentions may reflect the biphasic aspects of the role for iNOS-derived NO in the regulation of cell turnover.

**Glutathione (GSH) and Iron Involvement**

Reports on the effects of *H. pylori* on GSH contents in the gastric mucosa are conflicting, with the reports in humans showing that the GSH values are significantly lower in patients infected with *H. pylori* than in patients who are *H. pylori* negative [55, 56], while a study using gerbils suggests that the contents of total GSH are significantly elevated at 12 weeks by *H. pylori*-inoculation without any change in GSH peroxidase activity [48]. This lack of agreement might be explained by differences in species or durations of infection in this stage of *H. pylori* infection. Oh et al. found that oxidative stress played a critical role in the augmented mucosal damage provoked by water immersion restraint stress (WIRS) in *H. pylori* infection and that an antioxidant, α-tocopherol, could ameliorate the aggravation of stress-associated gastric mucosal damage [57]. They showed a significantly higher oxidative stress documented by iNOS, lipid peroxides, and GSH level detected in gastric homogenates of the *H. pylori*-infected group [57]. In addition, α-tocopherol pretreatment significantly prevented the gastric mucosal damage and reduction of HSP27, caused by WIRS in the presence of *H. pylori* [57].

**Proteomics**

Back et al. [58] reported that MS analysis (matrix-assisted laser desorption/ionization-time of flight MS) of the tryptic fragment and a data search allowed the identification of the four increased proteins (78 kDa glucose-regulated protein precursor, endoplasmin precursor, aldehyde dehydrogenase 2 and L-lactate dehydrogenase B chain) and the four decreased proteins (intracellular chloride channel protein 1, glutathione S-transferase, heat-shock protein 60 and cytokeratin 8) caused by *H. pylori* infection in the gastric mucosa. These proteins are related to oxidative-stress-mediated cell damage, including cell proliferation, carcinogenesis, cytoskeletal function and cellular defence mechanism [58]. Proteomic analysis using 2-dimensional electrophoresis showed a decrease in HSP27 and other chaperone proteins in *H. pylori*-positive cohorts [57].

According to the global proteomic analysis, which includes high-resolution 2-DE followed by MALDI-TOF-MS and bioinformatic databases search/peptide-mass comparison by Chuang et al. [59], protein expression levels of urease accessory protein E (UreE, an essential metallochaperone for urease activity) and alkylhydroperoxide reductase (AhpC) with antioxidant potential were greatly decreased under stress conditions. Measurements of mRNA transcription level by performing RT-PCR on total mRNA also confirmed that gene expressions for these two proteins are consistently repressed under oxygen tension [59]. These changes form a firm basis to account for the loss of urease activity and antioxidative ability of *H. pylori* after long-term exposure to reactive oxygen. Conceivably, UreE and AhpC may be listed as potential targets for the development of therapeutic drugs against *H. pylori* [59].
Conclusions

Reduced host defense including antioxidant capacity in gastric mucosa are major pathogenic mechanism of gastric disorders associated with *H. pylori* infection. Inflammatory genes, and related mediators stimulating cell proliferation and defensive molecular chaperones are induced by *H. pylori* in gastric epithelial cells, which is believed to be mediated by oxygen free radicals. Gastric mucosal oxidative stress was established by the summation of oxidative stress in both bacterial and host sides (Table). Broad knowledge of the source of oxygen free radicals, mechanisms of injury initiation and biochemical regulation of antioxidant defense systems should provide fruitful insights into the novel therapeutic strategies for the regulation of *H. pylori*-associated gastroduodenal diseases.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research C (2) from the Japan Society for the Promotion of Science (JSPS) (17590675, to H.S.), by a grant from Sato Memorial Foundation for Cancer Research, and by a Keio Gijyuku Academic Development Fund.

References


