

의학 박사학위 논문

**Protective Mechanism of  
Epigallocatechin-3-gallate against  
*Helicobacter pylori*- induced Gastric  
Epithelial Cytotoxicity *via* the Blockage  
of TLR-4 Signaling**

아주대학교 대학원

의학과

이기명

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against *Helicobacter pylori*- induced Gastric  
Epithelial Cytotoxicity via the Blockage of TLR-4  
Signaling**

by

**Kee Myung Lee**

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Supervised by

**Sung Won Cho, M.D., Ph.D.**

**Department of Medical Sciences  
The Graduate School, Ajou University**

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이기명의 의학 박사학위 논문을  
인준함.

심사위원장 함 기 백 인

심사위원 조 성 원 인

심사위원 김 진 흥 인

심사위원 조 용 관 인

심사위원 김 대 용 인

아 주 대 학 교 대 학 원

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-ABSTRACT-

**Protective Mechanism of Epigallocatechin-3-gallate against *Helicobacter pylori*- induced Gastric Epithelial Cytotoxicity via Blockage of TLR-4 Signaling**

*Helicobacter pylori* infection leads to gastric mucosal damage by several mechanisms including the direct effect of virulence factors produced by *H. pylori*, propagation of inflammation, oxidative stress, DNA damage, and induction of apoptosis. (-)-Epigallocatechin-3-gallate (EGCG), one of green tea catechins, is known to suppress *H. pylori*-induced gastritis through its antioxidative and anti-bacterial actions. In this study, we evaluated protective mechanism of EGCG against *H. pylori*-induced cytotoxicity in gastric epithelial cells. For analyzing EGCG effect on viability of gastric epithelial cells, MTT assay and dye exclusion assay were performed. The degree of DNA damage was evaluated by Comet assay and apoptotic DNA fragmentation assay. To investigate EGCG effect on *H. pylori*-induced the toll-like receptors 4 (TLR-4) signaling, RT-PCR and western blot analysis corresponding to glycosylated TLR-4 was done. LOX metabolites were measured with RP-HPLC. EGCG pretreatment effectively rescued gastric mucosal cells from the *H. pylori*-induced apoptotic cell death and DNA damage, and administration of this catechin enhanced gastric epithelial cell proliferation. *H. pylori* infection stimulated the glycosylation of TLR-4 which initiates intracellular signaling of infected host cell, and then pretreatment of EGCG completely blocked

its glycosylation. The blockage of TLR-4 activation by EGCG resulted in inactivation of ERK1/2 and NF- $\kappa$ B as downstream molecules of TLR-4 signaling induced by *H. pylori*. This disturbance of *H. pylori*-induced host cell signaling by EGCG attenuated the synthesis of proinflammatory mediators, HETEs. EGCG pretreatment showed significant cytoprotective effects against *H. pylori*-induced gastric cytotoxicity via interference of TLR-4 signaling induced by *H. pylori*. Thus, our result implies that continuous intakes of green tea could prevent the deleterious consequences of *H. pylori* infection.

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Key words: EGCG, *Helicobacter pylori*, Toll-like receptor-4, Apoptosis, ERK1/2, NF- $\kappa$ B.

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## ABBREVIATION

EGCG; epigallocatechin-3-gallate, *H. pylori*; *Helicobacter pylori*, IL-8; interleukin 8, MAPK; mitogen-activated protein kinase, ERK; extracellular signal response kinase, NF- $\kappa$ B; nuclear factor-kappa B, I $\kappa$ B; inhibitor protein kappa B, TLR; Toll-like receptor, HETE; hydroxyeicosatetraneoci acid, LPS; lipopolysaccharide

## I. INTRODUCTION

*H. pylori* infection was found to be an important casual factor in the pathogenesis of gastritis and peptic ulcer disease and World Health Organization defined *H. pylori* as class I carcinogen for gastric cancer in 1994 (Uemura etc, 2001). Besides of remarkable induction of gastric inflammation and oxidative stress (Hahm etc, 1998), *H. pylori* infection gives rise to significant DNA damage (O'Rourke etc, 2003; Bagchi etc, 2002), apoptosis of epithelial cells (Moss etc, 1996) and induce cell cycle dysregulation (Ahmed etc, 2000), all of which are closely associated with significant oncogenic insults on infected mucosa (Mannick etc, 1996; Fan etc, 1998). Toll-like receptors (TLRs) play a crucial role in host innate and adaptive immune responses to microbial pathogens and their products (da Silva etc, 2002). Among several kinds of TLR, TLR-4 is activated by the lipopolysaccharide (LPS) of gram-negative bacteria and is required for LPS-induced NF- $\kappa$ B and AP-1 activation and chemokine expression. Glycosylation of the protein after translation is required for moving to cell membrane after synthesis and the biological effects of TLR-4 are intensified by its receptor glycosylation. Previously, Su B *et al* (Su etc, 2003) suggested that *H. pylori* activated TLR-4 expression in epithelial cells and that TLR-4 can serve as a receptor for *H. pylori* binding.

Several phytochemical or drugs had been tried to decrease *H. pylori*-associated gastric inflammation (Qasim etc, 2002) and as the general therapeutics for *H. pylori* eradication, triple therapy with a proton pump inhibitor and two antimicrobials,

amoxicillin, and clarithromycin are usually recommended (Iovene etc, 1999). However, the occurrence of resistant strains to these antimicrobial drugs, the persistence of gastric inflammation even after the eradication of *H. pylori*, the association between the severity of gastric inflammation and higher risk of complications like gastric atrophy and malignancy, high prevalence of infected populations at earlier onset of age, and some negativism about the eradication of *H. pylori* urged us to search for agents enabling to considerably attenuate gastric inflammation or impose cytoprotection against *H. pylori*-induced cytotoxicity.

Green tea could be one of candidates to attenuate of *H. pylori*-induced gastric epithelial injury, which has long history of human consumption (Graham etc, 1992). (-)-epigallocatechin-3-gallate (EGCG), a major ingredient of green tea, has been known to inhibit LPS-induced expression of the proinflammatory cytokines and TNF- $\alpha$  (Yang etc, 1998; Katiyar etc, 1999). It also has been revealed that administration of EGCG significantly reduced IL-1 $\beta$ , IL-8, and IFN- $\gamma$ -induced NO and COX 2 production through the inhibition of the NF- $\kappa$ B signal pathway (Katiyar etc, 1999; Trompezinski etc, 2003; Ahmed etc, 2002), all of which are also pathogenically implicated in perpetuated gastric inflammations of *H. pylori*. Besides of these anti-inflammatory actions of EGCG, it has antibacterial activity against various food-borne pathogenic bacteria (Ikigai etc, 1993) and anti-urease against *H. pylori* (Mabe etc, 1999; Yee etc, 2000). The antibacterial effect of amoxicillin in subclinical concentration was significantly enhanced by the presence of EGCG (Yanagawa etc, 2003) and Matsubara *et al* (Matsubara etc, 2003) reported that

EGCG suppressed *H. pylori*-induced gastritis in Mongolian gerbil model.

Therefore, we hypothesized whether EGCG pretreatment can enhance the rescue of cytotoxicity developed by the propagation of inflammatory signaling after *H. pylori* infection. Here, we performed several cell and molecular biologic experiments to document the cytoprotective actions of EGCG against *H. pylori*-induced gastric epithelial cell damages and found that intakes of green tea can prevent the deleterious consequence of *H. pylori* infection.

## II. MATERIALS AND METHODS

### A. MATERIALS

#### 1. Chemicals and Reagents

(-)-Epigallocatechin-3-gallate (EGCG) was obtained from Wako chemical company (Japan), dissolved in PBS (0.01 M NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> 145 mM NaCl, pH 7.2) and employed appropriate concentrations for the treatment of human gastric epithelial cells. MTT (3-[4, 5-demethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and trypan blue dye was purchased from Sigma chemical Co.

#### 2. Cells, Bacterial strains, and Growth conditions

The human gastric cancer cells, AGS cells, were purchased from American Type Culture Collection (ATCC strain, Rockville, MD), which was established from gastric adenocarcinoma. They were cultured in RPMI 1640 medium (Gibco BRL, Gathersburg, MD) supplemented with 10% fetal calf serum (FCS, HyClone, Logan, UT) in humidified environment at 37°C in 5% CO<sub>2</sub>. A *CagA*- and *VacA*-positive strain of *Helicobacter pylori* (ATCC43504) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). *H. pylori* were recovered from frozen stock by seeding on a blood agar plate including 7% sheep blood at 37°C for 5 days under microaerophilic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>) generated with campy pouch (Becton Dickinson Microbiology Systems, Sparks, Maryland). For inoculation of the bacteria, *H. pylori* were resuspended in PBS to an A<sub>450</sub> of 1.2 units, which

corresponds to a bacterial concentration of  $5 \times 10^8$  CFU/ml and co-cultured with AGS cells at the concentration of  $2 \times 10^6$  CFU/ml (MOI 20).

## **B. METHODS**

### **1. MTT assay and cell growth**

MTT (3-[4, 5-demethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay was performed according to the method of Alley *et al* (Alley etc, 1988). In brief, AGS cells were seeded into 96 well plate at  $1 \times 10^5$  cells/ml in triplicate, treated with different concentrations of *H. pylori* ( $0.5 \times 10^6$  to  $8.0 \times 10^6$  CFU/ml) or EGCG (5 to 1000  $\mu$ M) for 24 h, respectively. MTT was added to each well and followed by incubation at 37°C for 4 h. After brief centrifugation, the formazan grains formed by viable cells were dissolved in DMSO, the color intensity was measured at 540 nm.

To determine the cell growth rate, AGS cells were seeded into 24 well plate at  $2 \times 10^5$  cells/ml in triplicate and pretreated with EGCG for 24 h. To remove the direct toxicity of EGCG against *H. pylori*, we washed AGS cells with serum-free culture media three times and then employed with *H. pylori* inoculation ( $2 \times 10^6$  CFU/ml) for 24 h or 48 h. Cell numbers and their viability were determined by trypan blue exclusion assay.

## **2. Comet assay**

Cells were washed with PBS twice and mixed with 1% low melting point (LMP) agarose in a 1:1 ratio to a density of  $2.5 \times 10^4$ /ml. A 300  $\mu$ l volume of cells in agarose was applied to slides and laid on top to form a thin layer. After 1 h incubation in lysis buffer (60 mM NaOH, 1 M NaCl, 0.5% (w/v) N-lauryl sarcosine, pH 12.5), and a further 1 h in DNA unwinding solution (40 mM NaOH, 2 mM EDTA, pH 13), the slides were electrophoresed at 25 V for 30 min. After staining with Ethidium bromide, comets were analyzed using fluorescence microscopy.

## **3. DNA fragmentation assay**

Both floating and adherent cells were lysed for 15 min in 10 mM Tris Cl (pH 7.4), 5 mM EDTA, and 1% Triton X-100 and centrifuged at 12000 rpm for 15 min. The supernatant was incubated with 0.1 mg/ml proteinase K at 37°C for 1 h and extracted with an equal volume of phenol/chloroform, and the cellular DNA was precipitated with 0.3 M sodium acetate and 2 volumes of absolute ethanol overnight at -70°C. The precipitate was dissolved in 20  $\mu$ l TE buffer containing 200  $\mu$ g/ml RNase and incubated for 1 h at 37°C. The extracted DNA was resolved on 1.8% agarose gel and stained with ethidium bromide.

## **4. Western blot assay**

Total proteins were extracted from *H. pylori* or EGCG-treated cells, electrophoresed on 12% SDS-PAGE gels, and transferred to PVDF membranes using a semidry

transfer system (Hoeffer Phamacia Biotech, San Francisco, CA). Membranes were blocked in 5% nonfat dry milk and probed with specific antibodies corresponding to caspase-3 (Cell Signaling Technology, Beverly, MA), PARP (Zymed, San Francisco, CA) and TLR-4 (Santa Cruz, San Francisco, CA), respectively. The membranes were washed three times and incubated with a horseradish peroxidase-conjugated secondary antibody, developed using a commercial enhanced chemiluminescence system (ECL), and exposed to films.

## **5. RT-PCR**

Total RNA was extracted by using TRIzol reagent (Life technologies, Milan, Italy) and two microgram of total RNA was reverse transcribed according to the manufacturers' instructions of M-MLV Reverse transcriptase (Promega). The PCR was performed by using the Premix Ex Taq kit (Takara, Japan) with specific primers as follows: 5'-GAAATGGAGGCACCCCTTC-3' and 5'-TGGATACGTTTCCTTATAAG-3' for TLR-4; 5'-TGTTGCCATCAATGACCCC-3' and 5'-TGACAAAGTGGTCGTTGAGG-3' for GAPDH. The PCR reaction was carried out 28 thermal cycles of 94°C for 1 min, 55°C (TLR-4) or 50°C (GAPDH) for 1 min, and 72°C for 1 min 30 sec. The product was resolved on 1% agarose gel and stained with ethidium bromide.

## **6. Electrophoretic mobility shift assay (EMSA)**

The nuclear fractions for EMSA were prepared using NE-PER Nuclear and



Cytoplasmic Extraction Kit (Pierce, Rockford, IL) following the manufacturer's protocol. Sequences of double stranded oligonucleotides used for EMSA of NF- $\kappa$ B as follows; 5'-AGT TGA GGG GAC TTT CCC AGG C-3' and the oligonucleotides were labeled with Biotin 3' End DNA Labeling Kit (Pierce, Rockford, IL). EMSA reaction was performed with LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL) according to the manufacturers' instructions.

### **7. RP-HPLC for identification of LOX metabolites**

After appropriate treatment, AGS cell monolayer was incubated in culture medium supplemented with 2.0  $\mu$  M calcium ionophore A23187 in DMSO at 37°C. The eicosanoids extraction from the cells was performed by the method of Wescott *et al* (Wescott etc, 1986) using a slight modification. To identify 15S-HETE, 12S-HETE and 5S-HETE, extracts were eluted from the column with methanol and H<sub>2</sub>O (76:24, v/v) adjusted to pH 3.0 with acetic acid, isocratically at a flow rate of 1.4 ml/min for 30 min and monitored at 234 nm with a Hewlet Packard spectrophotometry.

### **8. Statistical Analysis**

Results are expressed as the means  $\pm$  S.E.M. The data were analyzed by the one-way analysis of variance (ANOVA) and statistical significance between groups was determined by Duncan's multiple range test. Statistical significance was accepted within  $p < 0.05$ .

### III. RESULTS

#### 1. Prevention of *H. pylori* –induced gastric mucosal cytotoxicity with EGCG pretreatment

AGS cells were co-cultured with diverse CFUs of *H. pylori* for 24 h and cell viability was measured by MTT assay. As shown in Fig. 1A, a, viability of AGS cells was reduced dose-dependently. From this experiment, we determined the optimal inoculation dose of *H. pylori* for the following experiment, which was  $2 \times 10^6$  CFU/ml (MOI 20). Since EGCG has been known to have biphasic effects depending on their concentrations and cell kinds, we repeated MTT assay to evaluate whether EGCG has cytotoxicity on cultured gastric epithelial cells with different concentrations. While high dose over 250  $\mu$ M of EGCG showed significant cytotoxic effects against gastric cancer cells, presented with marked attenuation of AGS cell viability by 23%, the low dose of EGCG, especially lesser 10  $\mu$ M, have no effect on cell survival or cell death (Fig. 1A, B). All these findings signified that EGCG has a biphasic effect on survivals of gastric epithelial cells according to its doses. When we evaluated the effect of pretreatment of low doses of EGCG like 0.05  $\mu$ M, 0.5  $\mu$ M, and 5  $\mu$ M on *H. pylori* of  $2 \times 10^6$  CFU/ml-induced cytotoxicity, as shown in Fig 1B, pretreatment of low dose EGCG significantly rescued gastric epithelial cells from the *H. pylori*-induced cytotoxicity in a dose dependent manner at 24 h. Following 48 h of treatment, cell growth enhanced over 2-folds in all concentrations tested compared to *H. pylori* alone group. Like stated

already in methods, there was no possibility of the influence of EGCG on either *H. pylori* viability or cell survival since three times of cell washing were done before *H. pylori* infection. These data suggested that pretreatment of EGCG effectively prevented gastric mucosal cytotoxicity during *H. pylori* infection.

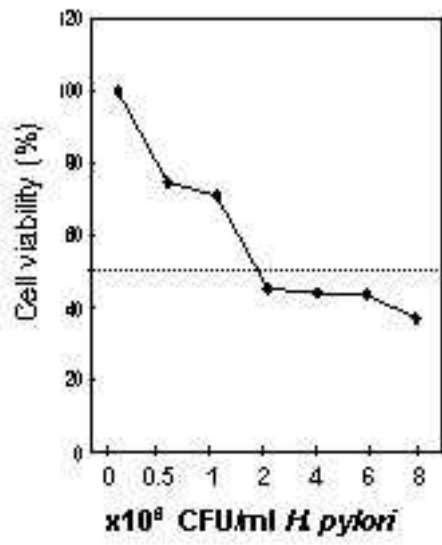
## **2. DNA damage by *H. pylori* infection, and its attenuation by EGCG**

Since *H. pylori* infection is well known to induce apoptotic cell death in infected gastric epithelium, subsequently leading to loss of gastric epithelial cells and these apoptotic cell death are accompanied with irreversible genomic DNA damage characterized as DNA fragmentation, we investigated apoptotic genomic DNA fragmentation and DNA damages provoked by *H. pylori* infection and the influence of EGCG treatment on these events. Remarkable apoptotic genomic DNA fragmentation was observed in *H. pylori* infected gastric epithelial cells, but pretreatment of EGCG decreased *H. pylori*-induced DNA fragmentation reflected with the findings that attenuated DNA laddering after the administration of EGCG of 5  $\mu\text{M}$  (Fig. 2A). Concomitant with the changes of genomic DNA fragmentation, the expressions of active form of caspase-3, most executive enzyme involved in apoptosis, and cleavage of PARP, proteolysis of its substrate, were significantly reduced after EGCG treatment more than 0.5  $\mu\text{M}$  EGCG. These findings suggested that low dose of EGCG pretreatment around 5  $\mu\text{M}$  could protect from *H. pylori*-induced apoptotic cell death and apoptotic DNA damages.

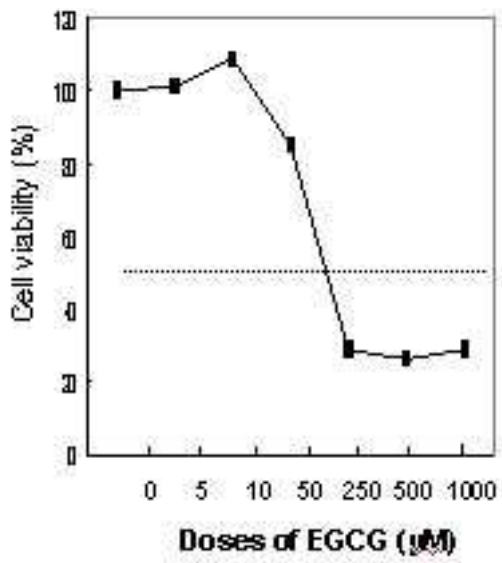
Using the Comet assay, we confirmed whether DNA damages induced by *H. pylori*

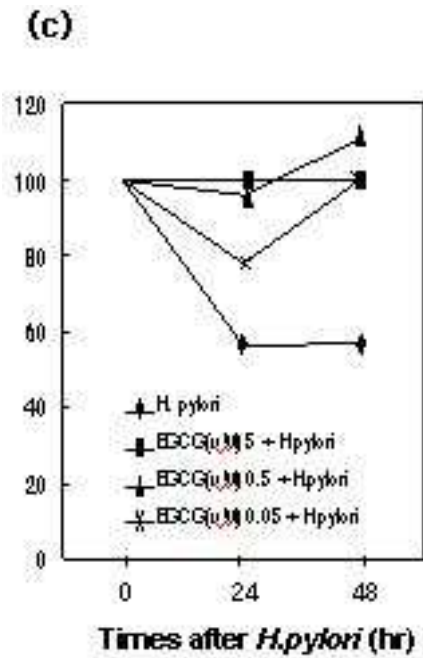
infection could be attenuated by EGCG pretreatment. Single cell DNA damage was evaluated by Comet assay and graded the score of DNA damage from 0 to 4 according to comet tail length, the degree of DNA migration away from the nucleus as shown in Fig. 2B, a. *H. pylori* infection caused significant single cell DNA breaks (Fig. 2B, c), but EGCG pretreatment attenuated the DNA damages of epithelial cell (Fig. 2B, d and e). When we summed and presented as a mean  $\pm$  SE of all groups (Fig. 2C), *H. pylori* infection provoked significant increment of Comet cells by 161% compared to control cells ( $p < 0.05$ ) and the pretreatment of EGCG showed statistically significant decrease by 109.2% Comet at 0.5  $\mu$ M ( $p < 0.05$ ).

(a)



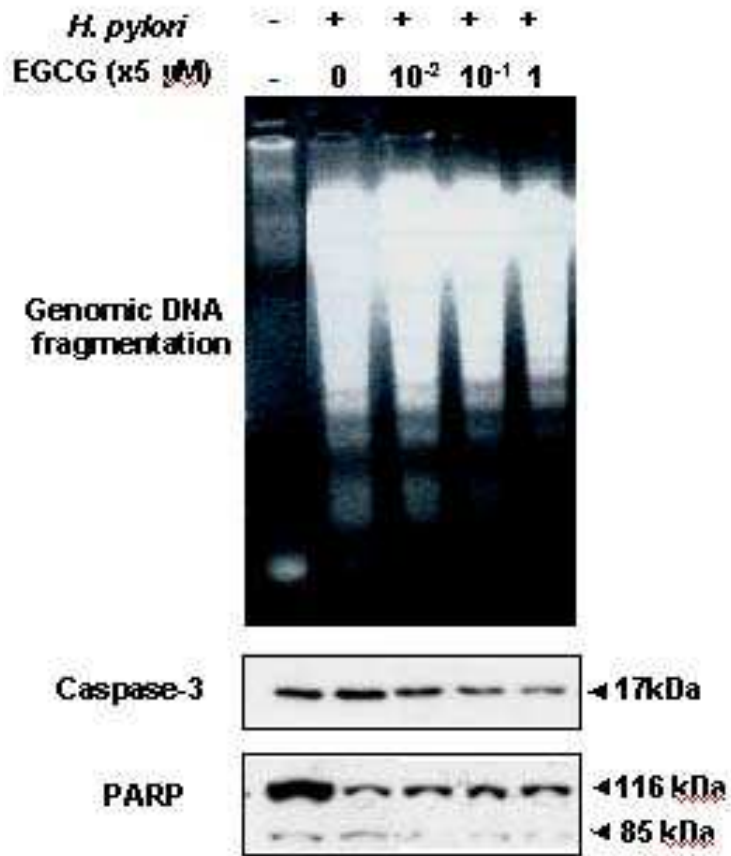
(b)

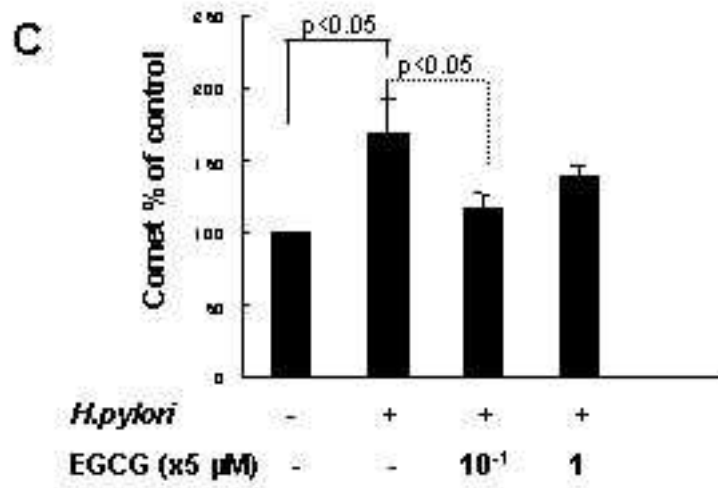
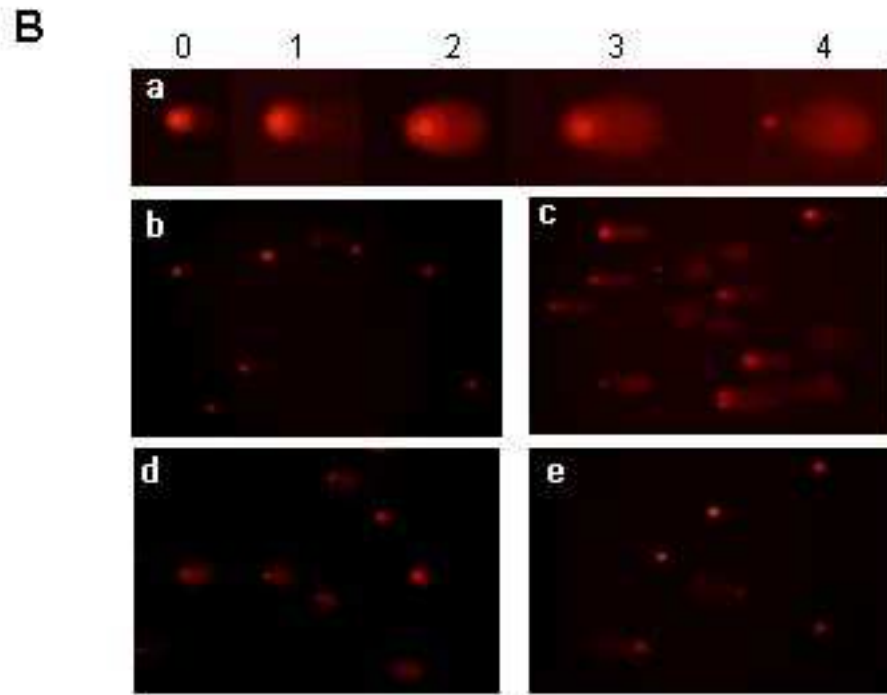




**Fig. 1. Cytoprotective effect of low dose EGCG against *H. pylori*-induced gastric cytotoxicity.** (A) AGS cells were co-cultured with *H. pylori* (ATCC 43504 strain, *VacA*<sup>+</sup>, *CagA*<sup>+</sup>) at different concentrations for 24 h and cell viability was determined by MTT assay. (B) Influence of EGCG itself on viability of AGS cells was measured with MTT assay. (C) To evaluate effect of EGCG pretreatment on *H. pylori*-induced cytotoxicity, AGS cells were preincubated with EGCG at the concentrations indicated for 24 h and then infected by *H. pylori* ( $2 \times 10^6$  CFU/ml) for 24 h or 48 h, respectively. Cell growth and viability was determined by trypan blue exclusion assay and the results were presented mean  $\pm$  SEM from three independent experiments. Values from each treatment were expressed as a relative percent (%) to the control.

**A**







**Fig. 2. Inhibition of *H. pylori*-induced DNA damages by EGCG.** (A) Apoptotic DNA damage by *H. pylori* infection was evaluated with genomic DNA fragmentation (upper panel). Activation of caspase-3 (middle panel) and proteolysis of its substrate, PARP (middle panel), and  $\alpha$ -tubulin (lower panel) as housekeeping protein were measured by Western blotting. (B) Comet assay was performed to detect DNA damage in single cell. According to the length of comet tail and DNA migration, the degree of DNA damage was scored from 0 to 4. Representative figures were shown from control group (a), *H. pylori* alone ( $2 \times 10^6$  CFU/ml) (b), EGCG (0.5  $\mu$ M) + *H. pylori* (c), EGCG (5  $\mu$ M) + *H. pylori*, (d) ( $\times 200$  magnification). (C) Results were presented mean  $\pm$  SEM from three independent experiments in duplicate. Statistical significance was analyzed by one-way ANOVA ( $p < 0.05$ ).

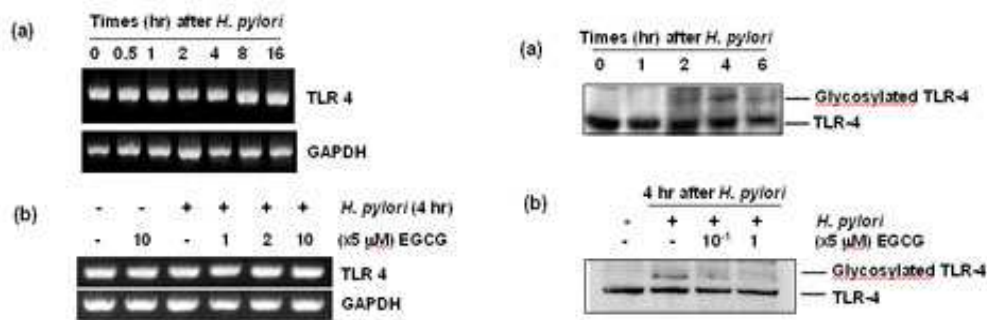
### **3. Blockage of *H. pylori*-induced TLR-4 glycosylation by EGCG**

TLR-4 is critical role in *H. pylori* induced inflammatory response and activation of the receptor by LPS of *H. pylori* or *H. pylori* initiated intracellular signaling, which bring to transactivation of some transcriptional factors such as AP-1 and NF- $\kappa$ B. These transcriptional factors stimulates expression of target genes including IL-8, IL-1 $\beta$ , INF- $\gamma$ , TNF- $\alpha$ , COX-2, and LOX which are crucial for propagation of *H. pylori*-induced inflammation. Thus, we further examined whether this cytoprotective effect of EGCG comes from interfering intracellular signaling induced by *H. pylori*.

Several studies have shown that *H. pylori* induced activation of TLR-4 signaling, subsequently resulting in activation of MAPK and NF- $\kappa$ B. However, we evaluated the expression of TLR-4 mRNA, its expression was not altered at all following *H. pylori* infection and the addition of EGCG also did not influence the expression of TLR-4 (Fig. 3A, a and b). Thus, we examined glycosylation status of TLR-4, which was evaluated by western blotting with antibodies, which can recognize both the glycosylated and total form of TLR-4.

Fig. 3B showed that there was no difference in TLR-4 expression after *H. pylori* infection, but *H. pylori* infection significantly increased the glycosylated form of TLR-4 (Fig. 3B, a), maximal activation at 4 h after *H. pylori* inoculation. EGCG pretreatment inhibited the glycosylation of TLR-4 induced by *H. pylori* infection (Fig. 3B, b).

All together, *H. pylori* initiated host cell signaling via TLR-4 glycosylation and pretreatment of EGCG significantly suppressed induction of host intracellular signaling via blocking the glycosylation of TLR-4.



**Fig. 3. Blocking of TLR-4 glycosylation during *H. pylori* infection by EGCG treatment.** (A) Expression of TLR-4 mRNA of AGS cells inoculated with *H. pylori* for different durations was measured by RT-PCR (a). Various concentrations of EGCG pretreated in AGS cells prior to *H. pylori* infection and changes of TLR-4 mRNA expression were tested (b). (B) TLR-4 protein expression after *H. pylori* infection (a) or EGCG + *H. pylori* (b) was evaluated by immunoblot with antibodies able to detect both glycosylated form and total form of TLR-4 (Santa Cruz Biotechnology, sc-10741)

#### **4. Inactivation of MAPK ERK1/2 and NF- $\kappa$ B by EGCG**

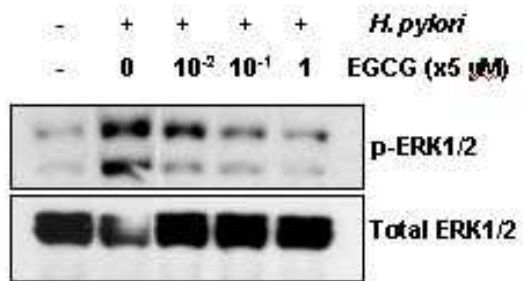
We further investigated the key downstream signaling molecules of TLR-4, ERK1/2 and its responsible transcription factor, NF- $\kappa$ B. *H. pylori* infection did not change of total ERK1/2 expression, but significantly increased phosphorylation of ERK1/2 in AGS cells (Fig. 4A). EGCG pretreatment obviously inhibited the phosphorylation of ERK1/2 of which inhibiting effect was augmented in dose dependent manner. To detect DNA binding activity of NF- $\kappa$ B, we performed EMSA with specific NF- $\kappa$ B binding oligonucleotides and found significant increase of shifting band after *H. pylori* infection. However, pretreatment of EGCG significantly inhibits NF- $\kappa$ B-DNA binding activity induced by *H. pylori* infection (Fig. 4B). These data indicated that ERK1/2 phosphorylation and NF- $\kappa$ B activity significantly increased by *H. pylori* infection and activation of these signaling enzymes could be modulated by EGCG pretreatment.

#### **5. EGCG inhibits the synthesis of 5S-HETE induced by *H. pylori* infection**

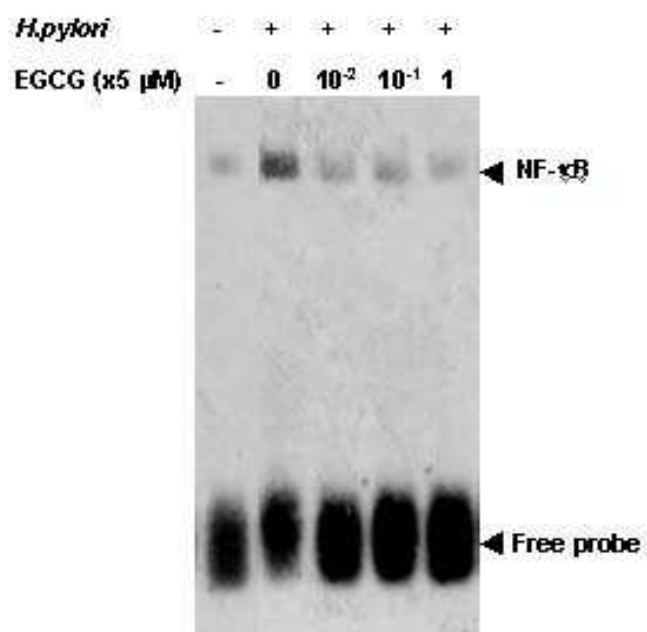
Finally we performed to know the changes of one of signaling triggered by *H. pylori* infection. In resting AGS cells, 5S-HETE was the most prevalent form among arachidonic acid metabolites synthesized by LOX and small amount of 15S-HETE was detected in chromatography of RP-HPLC (Fig. 5A). However, *H. pylori* infection triggered significant increase of 5S-HETE and 15S-HETE, no detectable change in 12S-HETE was observed. EGCG pretreatment attenuated synthesis of 5S and 15S-HETE induced by *H. pylori* infection (Fig. 5A). With higher dose of EGCG

(5 and 50  $\mu\text{M}$ ), the total and each amount of area peak was nearly decreased to the level of control cells (Fig. 5B). It means that EGCG can prevent the lipoxygenase activities induced by *H. pylori* in gastric epithelial cell.

**A**



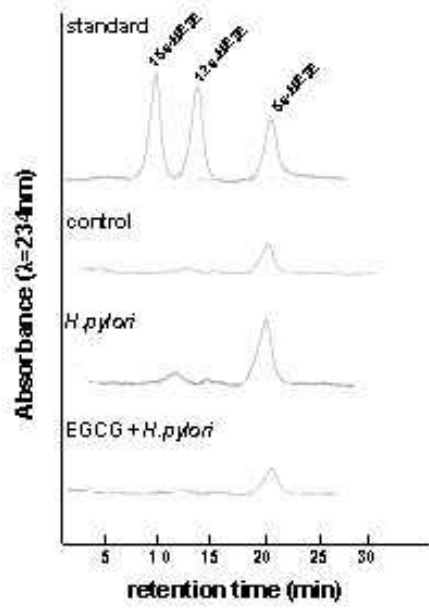
**B**



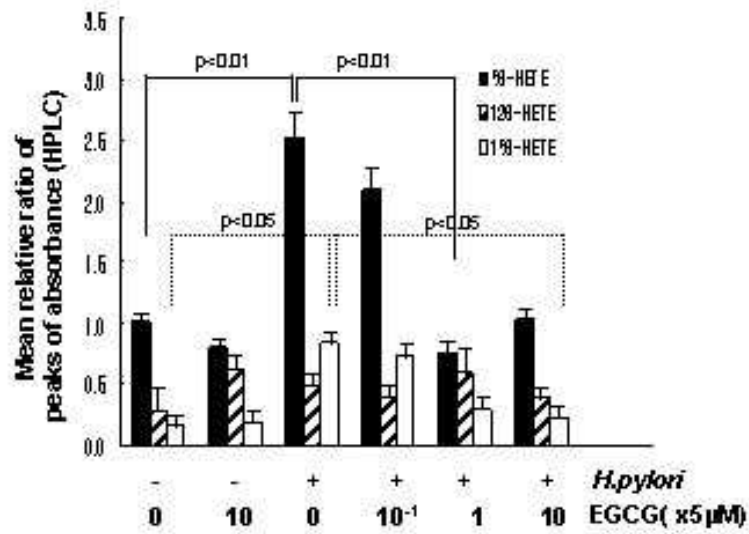
**Figure 4. Inactivation of *H. pylori*-induced MAPK and NF- $\kappa$ B signaling with EGCG.** (A) Activation of MAPK ERK1/2 was detected by Western blotting of phosphorylated ERK1/2. Phosphorylation of MAPK ERK1/2 induced by *H. pylori* inoculation was significantly inhibited by EGCG pretreatment. (B) EMSA for detecting NF- $\kappa$ B transcriptional activity was performed with nucleic proteins extracted from *H. pylori*-treated or EGCG + *H. pylori*-treated cells.



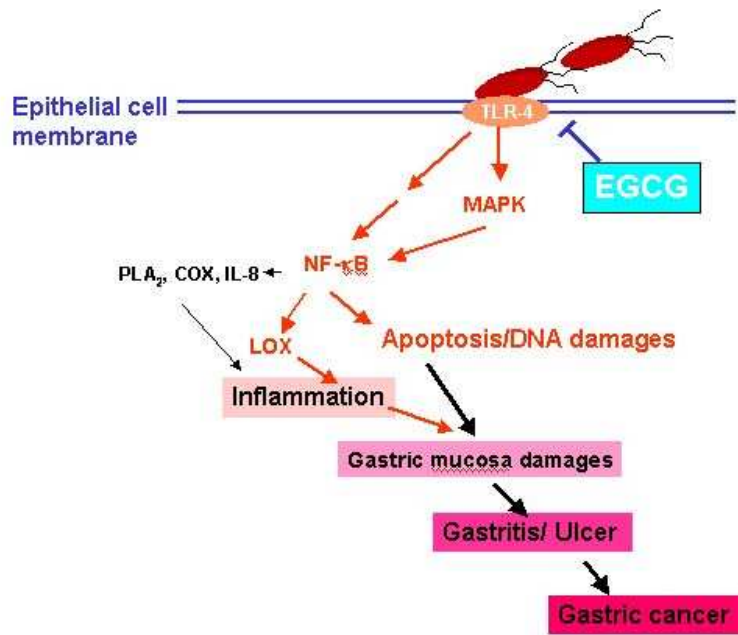
**A**



**B**



**Fig. 5. Changes of HETE synthesis pattern by *H. pylori* infection and Effect of EGCG pretreatment on its expression.** (A) For identification of LOX metabolites, the eicosanoids were extracted from AGS cells with an appropriate treatment indicated and measured by RP-HPLC according to previously described in '*Materials and Methods*'. Chromatography with authentic mono-HETE standards for 15S-HETE, 12S-HETE, and 5S-HETE were used with each sample to control for any variation in retention time and elution and the representative chromatograms of the biosynthesized samples were depicted. (B) Results were presented mean  $\pm$  SEM and statistical significance was indicated ( $p < 0.05$  or  $p < 0.01$ ).



**Fig. 6. Schematic drawings showing that the mechanistic pathway of EGCG treatment on *H. pylori*-induced cell damages.** LPS of *H. pylori* activated TLR-4, after which significant signal transduction pathways were propagated, and finally led to inductions of several cytokines or inflammatory mediators. The considerable inflammatory responses and DNA damages after these series of molecular changes after *H. pylori* infection were responsible for gastritis, peptic ulcer disease, and gastric malignancy. However, EGCG treatment, especially low dose about 5  $\mu\text{M}$ , can retard or attenuate gastric mucosal cell damages through the significant inactivation of TLR-4 signaling. As translated meaning of our findings, longer period of green tea intakes will be very beneficial in the prevention of harmful effects of *H. pylori* infection.

### III. DISCUSSION

Catechins, which constitute 5-15% of the dry weight of Chinese tea, are well-characterized isoflavonoids of green tea and EGCG is the most important and pharmacologically active form of catechins (Graham etc, 1992). The current study showed for the first time the cytoprotective actions of EGCG against *H. pylori*-associated cytotoxicity and explained its underlying cytoprotective mechanisms, including the enhancement of cell proliferation, the attenuation of DNA damages, and anti-inflammatory actions.

However, accumulating evidences suggest that certain natural flavonoids, ginsenoside, tannins or saponins have biphasic effect upon the biological activity- cytoprotection or cytotoxicity, antioxidant or pro-oxidant, and DNA damaging or DNA protection according to its concentration (Hu etc, 2001; Furukawa etc, 2003; van der Woude etc, 2003). In this study, we also found the biphasic influence of EGCG on survivals of gastric epithelial cells according to concentration. Low dose of EGCG (lesser than 50  $\mu\text{M}$ ) enhanced cell viability, but high dose of EGCG (> 100  $\mu\text{M}$ ) inhibited cell growth and decreased cell viability. Therefore, we adopted EGCG less than 50  $\mu\text{M}$  onto *H. pylori*-infected cells for evaluating the cytoprotective effect of EGCG and we found that protective action of EGCG against *H. pylori* infection might be through its prevention of DNA damages and the anti-apoptotic actions. The fact that EGCG of lesser than 50  $\mu\text{M}$  concentration exerted profound cytoprotective actions against *H. pylori* infection suggested the policy of

longstanding intakes of green tea could endow considerable disease preventing benefits in high endemic area of *H. pylori* infection.

Previous studies have already shown that *H. pylori* induce DNA damage and apoptosis with the considerable production of ROS and iNOS in several experimental backgrounds (O'Rourke etc, 2003; Bagchi etc, 2002) but EGCG have direct scavenging effect of nitric oxide and superoxide. Therefore, EGCG can prevent DNA damaging from various noxious stimulations of radiation, inflammation, and reperfusion injury (Tobi etc, 2002; Sugisawa etc, 2002). However, some studies reported contradictory results of pro-oxidative and DNA-damaging effect of putative chemopreventive antioxidants including EGCG, vitamin A, vitamin E, and N-acetylcystine (Yamashita etc, 1998; Oikawa etc, 1999). Furukawa *et al* (Furukawa etc, 2003) suggested EGCG can cause oxidative damage to double base lesions of isolated and cellular DNA in the presence of Cu(II), Fe(III) and may cause inactivation of tumor suppressor genes which may account for the potential mutagenesis and carcinogenicity.

Toll-like receptors (TLRs) play a crucial role in host innate and adaptive immune responses to microbial pathogens and their products (da Silva etc, 2002). Among several kinds of TLR, TLR-4 is activated by the lipopolysaccharide (LPS) of gram-negative bacteria and is required for LPS-induced NF- $\kappa$ B activation and chemokine expression (da Silva etc, 2002; Su etc, 2003). Glycosylation after translation is basically required for moving to cell membrane after synthesis and the biological effects of TLR-4 are intensified and activated for signal transduction by its receptor

glycosylation. *H. pylori* infection and pretreatment of EGCG alone did not change the TLR mRNA expression, but *H. pylori* infection increased glycosylated form of TLR-4. Interestingly, pretreatment of EGCG decreased the expression of glycosylated TLR-4 in cell membrane, which means that EGCG could weaken the propagation of TLR-4 activation with the hindrance of innate immune receptor after *H. pylori* infection. This important finding could be attributed to clinical aspect that the attenuation of the influences of chronic longstanding infection of *H. pylori* might be closely associated with the prevention of chronic atrophic gastritis or gastric carcinogenesis and long period consumption of green tea might be very healthy against *H. pylori* infection.

MAPKs have been documented as a central position in the signaling cascade regulating in a number of cellular processes such as cell growth, differentiation, stress responses, and apoptosis (Keates etc, 1999). Among several signaling pathways, *H. pylori* induced transactivation of activator protein-1 (AP-1) through the ERK signaling pathway in gastric cancer cells (Mitsuno etc, 2001). *H. pylori* infection significantly increased the phosphorylation of ERK1/2 among three major subfamilies of MAPKs; extracellular signal regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 (Fig. 4), which was similar to other investigators. The fact that the phosphorylation of ERK was inhibited by EGCG in a dose dependent manner suggested the cytoprotective effect of EGCG could be mediated with the inactivation of ERK pathway, but neither JNK nor p38 pathway.

NF- $\kappa$ B is one of the critical transcription factors involved in redox- and

inflammation-associated changes (Lindholm etc, 1999; Ahlstedt etc, 1999). NF- $\kappa$ B plays an essential role in immune, inflammatory, carcinogenic, and acute phase responses that rapidly activate defense genes after exposure to pathogen. Previous studies have reported that the stimulation of ERK pathways by *H. pylori* was directly responsible for the activation of the NF- $\kappa$ B and subsequent synthesis of IL-8. In present experiment, EGCG pretreatment attenuated the sequential activation of ERK signaling pathway, NF- $\kappa$ B transcription, and IL-8 expression by *H. pylori* infection (Fig. 4).

Lipoxygenase (LOX) catalyzes the oxidation of arachidonic acid to HETEs, intermediate metabolite of leukotriene and three lipoxygenases (5-LOX, 12-LOX, and 15-LOX) have been found in human tissue (Tang etc, 1996; Ghosh etc, 1997). Among these LOXs, the 5-LOX pathways are the most abundant and involved in the formation of 5S-HETE that is precursor metabolite of LTB<sub>4</sub>, a powerful chemotactic factor for neutrophils (Huseyinov etc, 1999). Using HPLC measurements, we found that *H. pylori* infection raised the production of 5S-HETE, but EGCG pretreatment attenuated the increase of 5S-HETE induced by the bacterium. Our results were consistent with the previous reports that *H. pylori* infection increased leukotriene level in gastric mucosa and EGCG inhibited LOX-dependent mechanism (Hong etc, 2001).

Detailed mechanism of anti-inflammatory effect of green tea extracts was mediated with inhibition of iNOS, IL-8 gene production, COX 2 activation, and NF- $\kappa$ B (Lin etc, 1997; Chen etc, 2002). Several kinds of phytochemicals like curcumin

and broccoli were found to inhibit the *H. pylori*-induced inflammation in gastric epithelial cells (Mahady etc, 2002) and EGCG was also proven to suppress the growth of *H. pylori* and to inhibit *VacA*, a major virulent toxin secreted from the bacterium (Yanagawa etc, 2003). Matsubara *et al* (Matsubara etc, 2003) reported that green tea extract suppressed *H. pylori*-induced gastritis in Mongolian gerbil model, but the underlying mechanisms of EGCG for prevention of *H. pylori*-induced gastritis are not clarified exactly.



#### IV. CONCLUSION

Our experiment showed that EGCG could ameliorate *H. pylori*-associated mucosal cell damages. Underlying mechanisms of EGCG against *H. pylori* infection were; 1) significant avoidance from *H. pylori*-induced DNA damages, 2) the inhibition of *H. pylori*-induced apoptotic cell death, 3) repression of transcriptional factor NF- $\kappa$ B and inactivation of ERK-1/2, 4) inhibition of 5-lipoxygenase, but all of which mechanisms were initiated with TLR-4 glycosylation and EGCG treatment effectively inhibited these receptor activation (Fig. 6). We can infer from our novel findings that long-term administration of EGCG, for instance, with the habit of drinking tea or beverages, could prevent *H. pylori*-associated chronic atrophic gastritis or gastric cancer through the rescue of longstanding gastric epithelial damages imposed by exposure to earlier infection chance in high prevalence area of *H. pylori*-associated gastric malignancy.

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## *Helicobacter* 위염에서 TLR-4 신호전달을 매개로 한 Epigallocatechin-3-gallate의 위점막 보호 효과

아주대학교 대학원 의학과

이 기 명

(지도교수: 조 성 원)

*Helicobacter pylori* (HP)가 위점막에 감염되면 염증성 사이토카인을 분비하여 염증 세포를 유인하고, 산화적 스트레스를 가하여 DNA의 손상 및 세포 사멸을 유발하여 위점막 손상을 일으킨다. 녹차 catechin의 일종인 Epigallocatechin-3-gallate (EGCG)는 과거부터 항염증 및 항균 작용이 있다고 알려져 있고 최근 HP에 의한 위점막 손상을 감소시키는 효과가 있다고 보고 되었으나 구체적으로 어떤 기전으로 위점막 손상을 감소시키는지 알려져 있지 않아 그 기전을 규명하고자 본 연구를 시작하였다. 재료 및 방법: EGCG가 HP 감염된 위상피 세포의 생존에 미치는 영향을 알기 위하여 MTT법과 dye exclusion 법을, DNA 손상에 대한 효과를 알기 위하여 Comet법과 DNA fragmentation 분석법을, toll-likers receptors 4 (TLR-4)에 대한 영향을 알기 위하여 RT-PCR법과 glycosylated TLR-4에 대한 western blot법을, lipoxygenase(LOX) 대사 산물의 변화를 알기 위하여 reverse phase-high performance liquid chromatography(RP-HPLC)법을 각각 이용하였다. 결과: EGCG를 전처리하였을 때 HP 감염에 의한 세포 사멸과 DNA 손상이 현저히 감소하였고 위상피 세포의 증식이 증가하였다. HP의 감염은 세균 감염에 대한 세포내 신호 전달을 담당하는 TLR-4의 glycosylation을 증가시키고 NF- $\kappa$ B와 ERK1/2의 발현을 증가시켰

으나 EGCG로 전처리할 경우 TLR-4의 *glycosylation*을 완전히 억제시켰고 NF- $\kappa$ B와 ERK1/2의 발현을 억제시켰다. EGCG를 전처리할 경우 *HP* 감염에 의한 염증 매개 물질 및 *hydroxyeicosatetranoic acid(HETE)*의 발현 증가를 현저히 감소시켰다. 결론: EGCG의 전처리는 TLR-4의 발현 억제를 통하여 *HP* 감염에 의한 위점막 손상을 감소시켰고, *HP*가 감염된 경우 지속적으로 녹차를 음용할 *HP*에 의한 위점막 손상을 예방할 수 있으리라 생각된다.

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핵심어: EGCG, *Helicobacter pylori*, Toll-like receptor-4, 세포사멸, ERK 1/2, NF- $\kappa$ B