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2014 년 2 월

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February 2014

Seoul National University

College of Medicine

Sung Wook Hwang

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Probiotic suppression of the *H. pylori*-induced responses by conjugated linoleic acids in a gastric epithelial cell line

by

Sung Wook Hwang, M.D.

A Thesis Submitted to the Department of Medicine in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medicine (Internal Medicine) at the Seoul National University College of Medicine

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Approved by thesis committee:

Professor	Joo Sung Kim	Chairman
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Professor	Jin Soo Moon	

학위논문 원문제공 서비스에 대한 동의서

본인은 본인의 연구결과인 학위논문이 앞으로 우리나라의 학문발전에 조금이나마 기여할 수 있도록, 서울대학교 중앙도서관을 통한 “학위논문 원문제공 서비스”에 다음과 같은 방법 및 조건하에 논문을 제공함에 동의합니다.

1. 인터넷을 통한 온라인 서비스와 보전을 위하여 저작물의 내용을 변경하지 않는 범위 내에서의 복제를 허용함.
2. 저작물을 이미지DB(PDF)로 구축하여 인터넷을 포함한 정보통신망에서 공개하여 논문 일부 또는 전부의 복제·배포 및 전송에 관한 일체를 무료로 제공함을 동의함.
3. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판허락을 하였을 경우 1개월 이내에 서울대학교 중앙도서관에 알림.
4. 배포, 전송된 학위논문은 이용자가 다시 복제 및 전송할 수 없으며 이용자가 연구목적이 아닌 상업적 용도로 사용하는 것을 금함에 동의함.

논문제목 : Probiotic suppression of the H. pylori-induced responses by conjugated linoleic acids in a gastric epithelial cell line

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서울대학교총장 귀하 □

ABSTRACT

Introduction: Conjugated linoleic acids (CLA) produced by *Lactobacillus acidophilus* was reported to decrease the activation of nuclear factor-kappa B. CLA was suggested as one of the anti-inflammatory molecular mechanisms of probiotics. In the present study, the effects of CLA on *H. pylori*-induced multiple responses were evaluated.

Methods: IL-8, TNF-α and iNOS were measured in mRNA and/or protein levels in AGS cells after pretreatment with CLA or CLA-containing conditioned medium (CM) produced by *Lactobacillus acidophilus* or *Lactobacillus plantarum.*

Results: The increased expressions of IL-8 mRNA/protein and TNF-α mRNA were significantly suppressed by pretreatment with CM or CLA. The levels of IL-8 protein and TNF-α mRNA were suppressed by CM pretreatment better than CLA. The expression of iNOS mRNA was also significantly inhibited by CM pretreatment.

Conclusions: These results suggest that the suppression of multiple mediators by CLA-containing CM plays a key role in the anti-inflammatory and anti-carcinogenic effects of probiotics on *H. pylori* infection.

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Keywords: *Helicobacter pylori*; Probiotics; Conjugated linoleic acids; Interleukin-8; Tumor necrosis factor-alpha; Inducible nitric oxide synthase.

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LIST OF ABBREVIATIONS

Helicobacter pylori, *H. pylori*; Conjugated linoleic acids, CLA; Linoleic acids, LA; Conditioned medium, CM; Nuclear factor-kappa B, NF-κB; IκB kinase, IKK; Interleukin-8, IL-8; Tumor necrosis factor-alpha, TNF-α; inducible nitric oxide synthase, iNOS; Enzyme-linked immunosorbent assay, ELISA

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative, microaerophilic bacterium that colonizes on the surface of the mucosa in the stomach.(1) The host immune response is provoked during *H. pylori* infection, and both innate and adaptive immunity, especially T-helper cell 1 type, are induced and enhanced by various pathways.(2) However, the immune response is insufficient to eradicate the microorganism. The subsequent inflammatory process as well as the bacterial infection persists for decades; this results in mucosal damage, gastritis, and finally gastric neoplasm.(2,3) Recently, the decreased eradication rates of the standard triple therapy and the high antimicrobial resistance of *H. pylori* have become important issues.(4) Several treatment modifications, such as sequential therapy, have been suggested as alternatives to the standard therapy,(4-6) and among the alternative treatments probiotics have been proposed as a useful adjunct.(7-9) Augmentation of the eradication rate by probiotics was usually the focus of earlier studies, and the immunomodulatory effects of probiotics have been widely explored.(10,11)

Conjugated linoleic acids (CLA) refer to a group of positional and geometric isomers of linoleic acids (LA) with conjugated double bonds.(12) CLA is mainly found in dairy products and meat, and several strains of probiotic bacteria, such as *Lactobacillus* species, are capable of converting LA to CLA.(12,13) CLA has been known to have multiple biological effects attracting considerable attention, such as anti-carcinogenic, anti-atherogenic and anti-diabetogenic properties,(12) and there have been several reports

regarding the immunomodulatory effect of CLA in various areas.(14-16) However, the effects of CLA on *H. pylori* infection in gastric pathogenesis have been rarely investigated.

The addition of *Lactobacillus* species to the triple therapy has been reported to increase the *H. pylori* eradication rate.(9-11) Recently, Kim et al. demonstrated that CLA-containing conditioned medium (CM) produced by *Lactobacillusacidophilus* has anti-inflammatory effects on *H. pylori* infection.(17) CM significantly inhibited the activation of the core inflammatory gene signal, nuclear factor-kappa B (NF-κB), in gastric epithelial cells by dissociation of the complex between heat shock protein 90 (Hsp90) and IκB kinase (IKK)-γ subunit. CLA-containing CM also inhibited the upregulated expression of IL-8, and it was suggested that CLA produced by probiotics has anti-inflammatory activity on *H. pylori* infection.(17) From this background, the aim of the present study was to evaluate and compare the immunomodulatory effects of CLA contained in CM, which was produced by two different CLA-producing *Lactobacillus* species, and commercially purified CLA mixture/isomers. In addition, *H. pylori*-induced multiple mediators, such as TNF- α and iNOS, were employed to determine the various immunomodulatory effects of CLA on *H. pylori* infection.

MATERIALS AND METHODS

Cell culture

Human gastric adenocarcinoma AGS cells (ATCC CRL 1739; obtained from American type culture collection, Bethesda, MD, USA) were cultured in RPMI 1640 media (GIBCO BRL, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum, 100 unit/ml penicillin and 100 μg/ml streptomycin in a humidified environment at 37 °C in 5% CO_2.

H. pylori strain

H. pylori strain G69a ($CagA^+$, $VacA^+$) expressing green fluorescence protein (a gift from Dr. Reiner Haas, Munich, Germany) was used in this study. Bacteria were cultured at 37 °C under micro-aerobic conditions (5% O_2, 10% CO_2 and 85% N_2) at 37 °C on Chocolate agar plates for 3 days. Bacteria were harvested and resuspended in RPMI-1640 medium for co-culture with the AGS cells.

Preparation of CM, measurement of CLA contained in CM and cell treatment

L. acidophilus HY2177, *L. plantarum* KY1032 and *L. zeae* HY RM1-5 were provided by KOREA YAKULT Co., Ltd. (Seoul, Korea). These probiotics were incubated at 37 °C in MRS broth (Becton Dickinson Microbiology System, Franklin Lakes, NJ, USA) under anaerobic conditions.

For the preparation of CM, each *Lactobacillus* species was separately incubated for 48 h in RPMI 1640 media with 0.5 g/l of LA (Sigma Chemical Co., St Louis, MO, USA). The concentration of 1×10^8 CFU/ml was used for the preparation of CM (CM produced by *L. acidophilus*, CM-A8; CM produced by *L. plantarum*, CM-P8). After centrifugation, live bacteria were removed by filtration through 0.2 μm syringe filter. CM was diluted to 20% with RPMI media and this CM was placed onto the AGS cells for pretreatment.

The amounts of CLA in CM were measured as described previously.(18) After the incubation of *L. acidophilus*, *L. plantarum* or *L. zeae* for 48 h in RPMI 1640 with 0.5 g/l of LA, samples were centrifuged at 6000g for 20 min. Supernatants were obtained, and lipids were extracted with chloroform:methanol (2:1). After vortex–sonification, samples were centrifuged at 6000g for 20 min, and the lower layer of the mixture was obtained. The fatty acids methylated by in-situ transesterification (19) were injected into Hewlett-Packard 7890A gas chromatography (Hewlett Packard Co., Palo Alto, CA, USA).

The AGS cells were seeded into six-well plates and serum-starved (0.5%) at 37 °C for approximately 16 h before experiments. The cells were separately pretreated with each CM, commercially purified CLA mixture (Sigma Chemical Co., St Louis, MO, USA) or commercially purified *cis*-9, *trans*-11 (*c*9,*t*11)/*trans*-10, *cis*-12 (*t*10,*c*12) isomer (Matreya, St College, PA, USA) 1 h prior to the addition of *H. pylori* at a multiplicity of infection of 100.

RNA extraction, reverse transcription and real-time PCR

After 6 h of co-culture, the medium was removed and the cells were washed with phosphate buffered saline (PBS). Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer, and the collected RNA was purified using RNeasy mini kits (Qiagen, Valencia, CA, USA). RNA samples were diluted to a final concentration of 0.5 mg/ml in RNase-free water and stored at −80 °C until use. Synthesis of the cDNA was performed with 1 mg of total RNA with M-MLV reverse transcription reagents (Invitrogen, Carlsbad, CA, USA). Real-time PCR reaction was carried out on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in 20 μL SYBR Premix Ex TaqTM (Takara Bio, Shiga, Japan) using 200 ng cDNA. The following primers were used (20): Interleukin-8 (IL-8) forward, CTTGGCAGCCTTCCTGATTT; IL-8 reverse, TGCACCCAGTTTTCCTTGG; Tumor necrosis factor-alpha (TNF-α) forward, AAGACCACCACTTCGAAACC; TNF-α reverse, GGCCTAAGGTCCACTTGTGT; inducible nitric oxide synthase (iNOS) forward, CTGCATGGATAAGTACAGGCTGAGC; iNOS reverse, AGCTTCTGATCAATGTCATGAGCAA. The thermal cycler conditions were 10 s holding at 95 °C, followed by 40 cycles of 5 s at 95 °C and 33 s at 60 °C. The human ACTB (beta-actin gene, NM_001101) was used as an endogenous reference to control for expression independent sample-to-sample variability. Relative gene expression was determined from the obtained Ct values using the $2^{-\Delta\Delta Ct}$ method.(21,22) All equipments and reagents were purchased from

Applied Biosystems and used according to their recommended protocols.

Measurement of IL-8 and TNF-α protein by enzyme-linked immunosorbent assay

After co-culture with *H. pylori* for 24 h, IL-8 in the culture supernatant was assayed by enzyme-linked immunosorbent assay (ELISA). The supernatant was taken after centrifugation at 13,000 rpm for 10 min to remove any contaminants. IL-8 and TNF-α were quantified using commercially available kits (R&D systems, Minneapolis, MN, USA) according to the instructions provided by the manufacturers.

Statistical analyses

The level of mRNA was expressed as fold changes (mean±SEM) relative to the uninfected control group. The level of protein was expressed as pg/ml (mean±SEM). Mann–Whitney U-test was used for the comparison between two groups. A *P* value of less than 0.05 was considered significant.

RESULTS

The amounts of CLA in culture supernatants

After the co-incubation of each *Lactobacillus* species with LA, the concentration of CLA in CM was measured by gas chromatography (see Table 1). Both concentrations of CLA in CM produced by *L. acidophilus* and *L. plantarum* were similar. CLA was not detected in CM produced by *L. zeae*, which was used as a negative control.

Table 1. The amount of conjugated linoleic acids in conditioned medium produced by *Lactobacillus* species.

	Concentration
Lactobacillus acidophilus	3.537 μg/ml (12.65 μM)[a]
Lactobacillus plantarum	3.584 μg/ml (12.80 μM)[a]
Lactobacillus zeae	not detected[b]

[a] The molar concentration was calculated using the molecular weight of CLA. For the pretreatment, CM was diluted to 20% with RPMI media (*L. acidophilus*, 2.53 μM; *L. plantarum*, 2.56 μM).

[b] Used as negative control.

The effect of CLA on IL-8 mRNA expression in AGS cells infected with *H. pylori*

To determine the appropriate concentration of CLA inducing suppression

of IL-8 mRNA expression, different CLA concentrations (10, 20 and 40 μM; CLA10, CLA20 and CLA40, respectively) were employed. 20 μM pure isomers *c*9,*t*11 and *t*10,*c*12 were also employed to evaluate isomeric effects of CLA. When IL-8 mRNA expression in the *H. pylori*-stimulated AGS cells was measured by real-time PCR, it was found to be increased up to 91.5-fold (SEM=22.7) relative to the uninfected control (Fig. 1). Pretreatment with CLA mixture significantly downregulated the increased IL-8 mRNA expression of *H. pylori*-stimulated AGS cells, compared to the *H. pylori*-stimulated AGS cells without CLA pretreatment. (CLA10, 28.3±10.0; CLA20, 24.7±5.4; CLA40, 34.8±8.2; mean fold increase±SEM, n=5; CLA10 and CLA40, $P<0.05$; CLA20, $P<0.01$). CLA20 yielded better suppression than the other CLA concentration groups, but there was no statistical significance between CLA20 and the other CLA groups. Between CLA isomers, *t*10,*c*12 group demonstrated better result than *c*9,*t*11; *t*10,*c*12 showed a statistical significance compared to the *H. pylori*-stimulated AGS cells without CLA pretreatment (*c*9,*t*11, 45.8±15.0; *t*10,*c*12, 29.3±9.2; n=5; *t*10,*c*12, $P<0.05$) (Fig. 1). To appropriately compare the suppressive effects of CLA and CM, CLA20 was chosen for the next experiments.

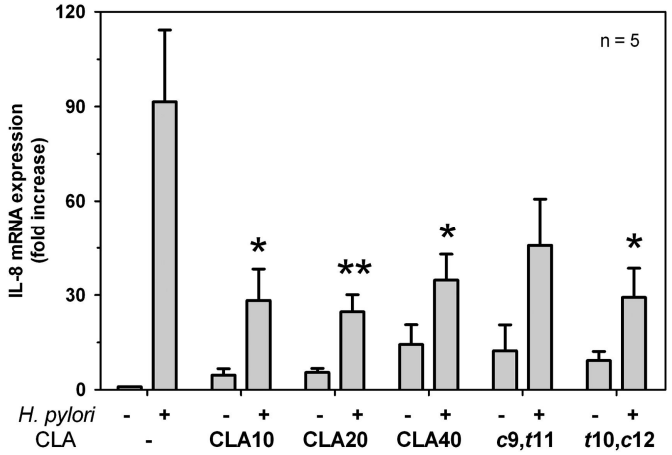

Fig. 1. Effect of CLA on IL-8 mRNA expression in AGS cells infected with *H. pylori*. AGS cells were pretreated with commercially purified CLA mixtures for 1 h, followed by co-culture with *H. pylori* for 6 h (10, 20 and 40 μM; CLA10, CLA20 and CLA40). 20 μM isomers, *c*9,*t*11 and *t*10,*c*12, were also employed. The levels of IL-8 mRNA expression were assessed by real-time PCR and expressed as fold increase compared to the uninfected control group (mean±SEM) (n=5). An asterisk means a statistically significant difference based on Mann–Whitney U-test compared with the *H. pylori*-stimulated AGS cells without pretreatment (*P<0.05, **P<0.01).

The effect of CM on IL-8 mRNA expression in AGS cells infected with *H. pylori*

The effects of CLA-containing CM produced by *L. acidophilus* (CM-A8) or *L. plantarum* (CM-P8) on IL-8 mRNA expression were evaluated. Without CM pretreatment, IL-8 mRNA expression increased up to 84.5-fold (SEM=20.8) relative to the uninfected control after *H. pylori* stimulation (Fig.

2). The increased expression of IL-8 mRNA was significantly suppressed by CM pretreatment compared to the *H. pylori*-stimulated AGS cells without CM pretreatment (CM-A8, 27.4±7.7; CM-P8 33.5±8.8; mean fold increase±SEM, n=5; CM-A8, $P<0.05$). Significant suppression of IL-8 mRNA expression was also found with CLA20 pretreatment (20.7±3.8; $P<0.05$), and there was no significant difference between CM groups and CLA20 after the stimulation of *H. pylori* (Fig. 2).

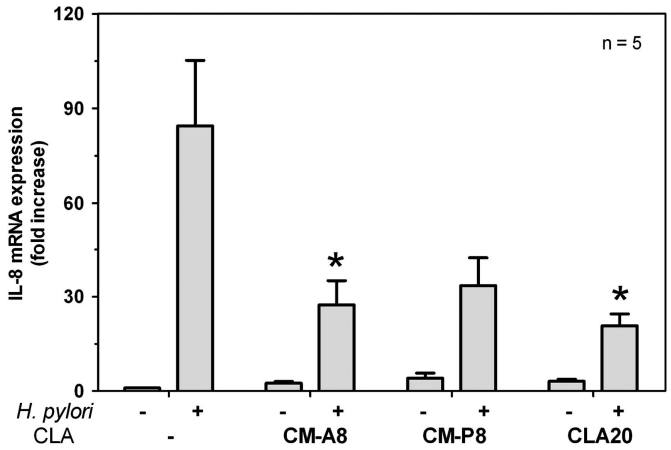

Fig. 2. Effect of CLA-containing CM on IL-8 mRNA expression in AGS cells infected with *H. pylori*. AGS cells were pretreated with CLA-containing CM and 20 μM purified CLA mixture (CLA20) for 1 h, followed by co-culture with *H. pylori* for 6 h. For the preparation of CM, the *Lactobacillus*concentration of 1×10^8 CFU/ml was used (*L. acidophilus*, CM-A8; *L. plantarum*, CM-P8). The levels of IL-8 mRNA expression were assessed by real-time PCR and expressed as fold increase compared to the uninfected control group (mean±SEM) (n=5). An asterisk means a statistically significant difference based on Mann–Whitney U-test compared with the *H.*

pylori-stimulated AGS cells without pretreatment (*P<0.05).

The effects of CLA and CM on IL-8 protein production in AGS cells infected with *H. pylori*

When IL-8 protein levels were measured by ELISA, *H. pylori* infection induced an increase of IL-8 protein production from 49.8 (SEM=21.2) to 582.8 (SEM=88.5) (Fig. 3). CM-A8 and CM-P8 significantly suppressed the protein production when compared to the *H. pylori*-stimulated AGS cells without pretreatment (CM-A8, 101.3±18.4; CM-P8, 102.1±23.3; mean±SEM, pg/ml, n=6; P<0.01). CLA20 also inhibited IL-8 protein production with statistical significance (196.7±62.4; P=0.01). Although two CM groups showed better suppression than CLA20, there was no statistical significance (Fig. 3).

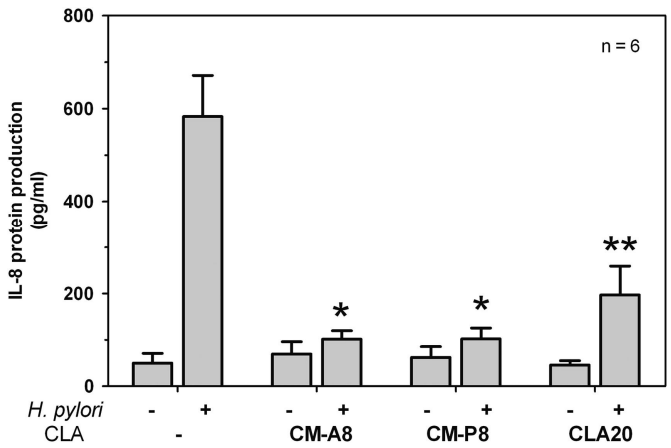

Fig. 3. Effects of CLA and CM on IL-8 protein production in AGS cells infected with *H. pylori*. AGS cells were pretreated with CLA-containing CM

and 20 μM CLA mixture (CLA20) for 1 h, and then co-cultured with *H. pylori* for 24 h. For the preparation of CM, the *Lactobacillus* concentration of 1×10^8 CFU/ml was used (*L. acidophilus*, CM-A8; *L. plantarum*, CM-P8). The levels of IL-8 protein expression were determined by ELISA and expressed as pg/ml (mean±SEM) (n=6). An asterisk means a statistically significant difference based on Mann–Whitney U-test compared with the *H. pylori*-stimulated AGS cells without pretreatment (*P<0.01, **P=0.01).

The effect of CLA on mRNA expression and protein production of TNF-α in the *H. pylori*-infected AGS cells

To confirm the suppressive effect of CLA on other inflammatory cytokines, mRNA expression and protein production of TNF-α were considered. When the expression of TNF-α mRNA was measured in AGS cells, it was found to be increased up to 10.2-fold (SEM=3.2) by *H. pylori*stimulation. The increased expression was significantly decreased with CLA20 pretreatment in *H. pylori*-stimulated AGS cells, when compared with the *H. pylori*-stimulated AGS cells without CLA pretreatment (CLA10, 4.5±1.5; CLA20, 3.1±1.1; CLA40, 3.7±0.9; mean fold increase±SEM, n=6; CLA20, P<0.05) (Fig. 4A). The expression was also significantly suppressed by *t*10,*c*12 isomer (*c*9,*t*11, 5.0±1.7; *t*10,*c*12, 3.7±1.7; n=6; *t*10,*c*12, P<0.05). There was no statistical significance between CLA mixture and isomeric groups. After pretreatment with CLA-containing CM, the increased expression of TNF-α mRNA (12.0±1.8) was significantly suppressed (Fig.

4B), and CM groups provided better result than CLA20 (CM-A8, 3.1±0.4; CM-P8, 2.1±0.6; CLA20, 6.3±2.2; n=5; CM-A8 and CM-P8, $P<0.01$; CLA20, $P<0.05$), although there was no statistical significance between CM and CLA20.In the TNF-α protein measurement by ELISA, the levels of TNF-α protein were very low, and there was no significant increase of TNF-α protein even after *H. pylori*stimulation of the AGS cells (data not shown).

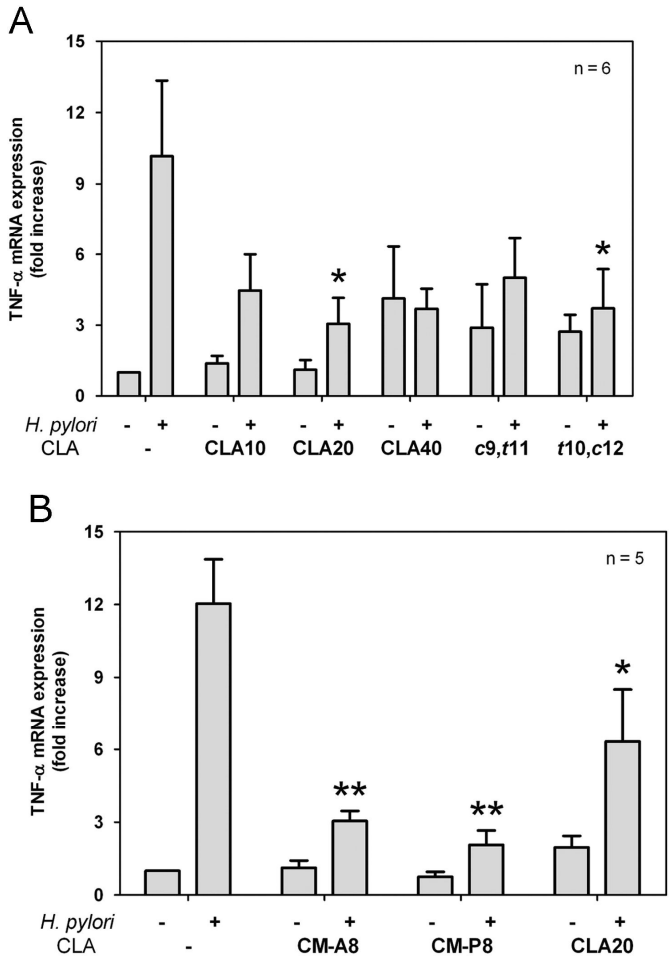

Fig. 4. Effects of CLA and CM on TNF-α mRNA expression in the AGS cells infected with *H. pylori*. (A) AGS cells were pretreated with commercially

purified CLA mixture for 1 h, followed by co-culture with *H. pylori* for 6 h (10, 20 and 40 μM; CLA10, CLA20 and CLA40). 20 μM isomers, *c*9,*t*11 and *t*10,*c*12, were also employed. (B) AGS cells were pretreated with CLA-containing CM and 20 μM purified CLA mixture (CLA20) for 1 h, followed by co-culture with *H. pylori* for 6 h. For the preparation of CM, the *Lactobacillus* concentration of 1×10^8 CFU/ml was used (*L. acidophilus*, CM-A8; *L. plantarum*, CM-P8). The levels of IL-8 mRNA expression were assessed by real-time PCR and expressed as fold increase compared to the uninfected control group (mean±SEM) ((A) n=6, (B) n=5). An asterisk means a statistically significant difference based on Mann–Whitney U-test compared with the *H. pylori*-stimulated AGS cells without pretreatment (**P*<0.05, ***P*<0.01).

IL-8 and TNF-α mRNA expressions after pretreatment with medium not containing CLA in the *H. pylori*-infected AGS cells

The expressions of IL-8 and TNF-α mRNA were evaluated after pretreatment with CM produced by *L. zeae*, which did not produce CLA from LA. In addition, the measurements were also performed with CM produced by the three *Lactobacillus* species without co-incubation with LA (see Table 2). After *H. pylori* stimulation, the increase of IL-8 mRNA expression was not suppressed by pretreatment with CM which had been produced by each *Lactobacillus* species but was not co-incubated with LA. In case of *L. zeae*,

the expression was significantly higher than that of control even before *H. pylori* stimulation (n=7, $P<0.01$). Furthermore, IL-8 mRNA expression after *H. pylori* stimulation was significantly increased compared to that of control, with pretreatment with CM produced by *L. zeae* co-incubated with LA ($P<0.05$). Similarly, all the CM groups not containing CLA did not significantly inhibit the increased expressions of TNF-α mRNA in the *H. pylori*-stimulated AGS cells (see Table 2).

Table 2. IL-8 and TNF-α mRNA expressions after pretreatment with conditioned medium not containing CLA in the *H. pylori*-stimulated AGS cells.

	IL-8 mRNA		TNF-α mRNA	
	H. pylori infection		*H. pylori* infection	
	-	+	-	+
Control	1.0±0.0	19.9±6.7	1.0±0.0	4.4±1.0
L. zeae with LA	17.8±6.5*	32.3±4.6**	1.5±0.6	3.8±1.0
L. zeae without LA	1.6±0.6	33.9±10.6	2.4±1.4	8.3±2.9
L. acidophilus without LA	1.5±0.4	29.5±9.7	3.1±1.9	8.1±3.5
L. plantarum without LA	1.7±0.2	31.9±10.9	0.6±0.3	3.3±1.8

Data are shown as mean fold increase±SEM. n=7.

* Significantly different from the uninfected control group ($P<0.01$).

** Significantly different from the *H. pylori*-stimulated control group ($P<0.05$).

Effect of CLA on iNOS mRNA expression in the *H. pylori*-infected AGS cells

To evaluate the effect of CLA on other mediators involved in the process of inflammation and carcinogenesis,(2,23) expression of iNOS mRNA was measured in the *H. pylori*-stimulated AGS cells (Fig. 5). With purified CLA, there was no significant suppression in the expression of iNOS mRNA in the *H. pylori*-infected AGS cells, whereas the expression was increased by purified CLA mixture/isomers before *H. pylori* stimulation (CLA10, 1.72±0.57; CLA20, 2.71±0.70; CLA40, 1.89±0.55; mean fold increase±SEM, n=5). On the contrary, the increase of iNOS mRNA expression after *H. pylori* stimulation was suppressed by CM pretreatment (Fig. 5). CM-A8 demonstrated a statistical difference when compared to the *H. pylori*-stimulated AGS cells without pretreatment (control, 1.83±0.33, CM-A8, 0.48±0.28; $P<0.05$). This statistical difference was not apparent in the case of CM-P8 (0.92±0.54).

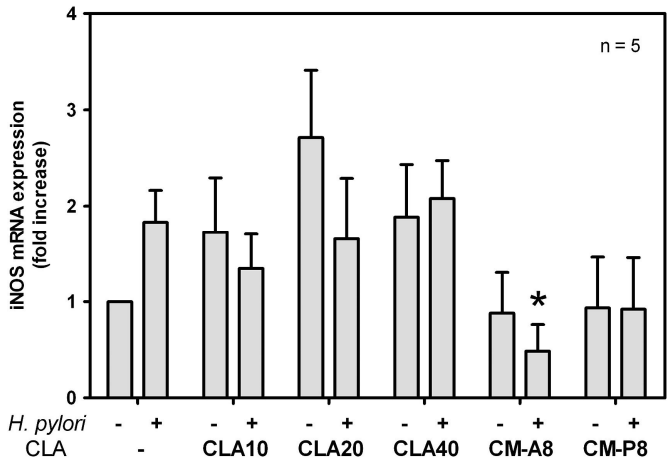

Fig. 5. Effect of CLA on iNOS mRNA expression in AGS cells infected with *H. pylori*. AGS cells were pretreated with commercially purified CLA mixture (10, 20 and 40 μM; CLA10, CLA20 and CLA40) and CLA-containing CM for 1 h, followed by co-culture with *H. pylori* for 6 h. For the preparation of CM, the *Lactobacillus* concentration of 1×10^8 CFU/ml was used (*L. acidophilus*, CM-A8; *L. plantarum*, CM-P8). The levels of iNOS mRNA expression were assessed by real-time PCR and expressed as fold increases compared to the uninfected control group (mean±SEM) (n=5). An asterisk means a statistically significant difference based on Mann–Whitney U-test compared with the *H. pylori*-stimulated AGS cells without the pretreatment with CLA (*P<0.05).

DISCUSSION

The interaction of *H. pylori* with epithelial cells is inevitable in the inflammatory process of *H. pylori* infection.(24,25) This contact leads to the induction of an immediate early response, such as an increase in the transcription factor NF-κB and activator protein 1 (AP-1),(26) and these transcription factors activate the production of pro-inflammatory IL-8 and other chemokines that recruit neutrophils toward colonized epithelium.(24) IL-1 and TNF-α are also important cytokines during the acute phase of gastric inflammation,(3,25) and the cytokines are increased in the gastric mucosa infected with *H. pylori.*(27-29) Recently, the concept that inflammation is a crucial component of carcinogenesis has been established; the physical contact between *H. pylori* and gastric epithelial cells and subsequent inflammatory cascades are believed to be important in gastric carcinogenesis.(24) It was reported that *H. pylori* obtained from gastric cancer patients secreted significantly higher amounts of TNF-α inducing protein than that from chronic gastritis.(30) The induction of iNOS caused by *H. pylori* infection is also considered as an important factor associated with *H. pylori*-induced inflammation and has been reported to be associated with carcinogenesis.(2,23) iNOS is expressed in various solid tumor tissues (31) and can generate mutagenic concentration of nitric oxide in mice.(32)*H. pylori* infection leads to an upregulation of iNOS in gastric epithelial cells and an over-production of nitric oxide.(33) Moreover, the binding sites for NF-κB and AP-1 are present in the promoter regions of iNOS, indicating that the

expression of iNOS is influenced by NF-κB.(20,34,35)

In clinical trials investigating the first-line eradication regimens with adjunctive probiotics, the results were optimistic.(9-11) Canducci et al. showed that the administration of *L. acidophilus* LB was associated with a significant increase in the eradication of *H. pylori* in intention-to-treat and per-protocol analysis.(36) Our group also reported that the eradication rate was significantly increased compared to the control group by adding *Lactobacillus*- and *Bifidobacterium*-containing yogurt to the triple therapy.(9) Even in cases not treated with antibiotics, probiotics alone lowered the results of 13C-urea breath test, the severity and activity of gastritis and the density of *H. pylori* colonization.(11) In the literatures, immunomodulation by probiotics has been suggested as one cause of this anti-*H. pylori* effects,(10,11) and it might weaken the development of gastroduodenal pathology associated with *H. pylori* infection. In addition, culture supernatants produced by *Lactobacillus* species were used in several studies exploring the immunomodulatory interference of probiotics with *H. pylori.*(37,38) These findings may lead to the possibility of novel molecular mechanism beyond probiotic bacteria itself, although the acid production by *Lactobacillus* seems to be the most relevant mechanism of anti-*H. pylori* effects.(10)

CLA, a natural product of probiotics, has been widely researched in the literatures (17,39-41) and is well known to be produced by *Lactobacillus* species.(17,42) The most abundant isomers in nature are *c*9,*t*11 and *t*10,*c*12.(41,43) *c*9,*t*11 is mainly responsible for anti-carcinogenic effects, while *t*10,*c*12 is associated with decrease in body fat and has anti-

atherosclerotic properties.(40) With regard to the effect on immune system, CLA has been reported to decrease the release of pro-inflammatory cytokines, particularly TNF-α, in animals.(39,41) The isomeric composition of synthetically prepared CLA is different from that of naturally found CLA and varied with the method of preparation.(12,17) Because of the high cost and/or lack of availability, highly purified isomers or naturally extracted CLA is rarely used, and the synthetic mixture of isomers, predominantly *c*9,*t*11 and *t*10,*c*12, have been widely employed.(12)

Regarding *H. pylori* infection, the immunomodulatory effects of CLA produced by probiotics have been rarely researched.(40,41,43) Recently, CLA-containing CM produced by *L. acidophilus* (ATCC 832) was reported to significantly reduce the activation of NF-κB and the IL-8 production in *H. pylori*-infected MKN-45 cells.(17) CM containing CLA was found to dissociate the complex of Hsp90 and IKK-γ subunits and attenuate the *Helicobacter*-induced IKK activity which is known to release NF- κB dimers into the nucleus. In the report, CLA was suggested as one of the molecular mechanisms associated with probiotics-induced anti-inflammatory effects in *H. pylori* infection.(17) Based on the result, this present study was performed to validate and extend the anti-inflammatory concept of CLA under various conditions: (1) the multiple mediators associated with inflammatory process and carcinogenesis, such as TNF-α and iNOS, were measured as well as IL-8; (2) another species of CLA-producing probiotics, *Lactobacillus plantarum*, and CLA-nonproducing species, *Lactobacillus zeae*, were employed in addition to *Lactobacillus acidophilus*.

When the effects of CM produced by CLA-producing *Lactobacillus* species were compared with that of purified CLA mixture/isomers, CM provided better suppression of IL-8 protein release and TNF-α/iNOS mRNA expression (Figs. 3, 4B and 5), although the CLA amount in CM was lower than that of commercially purified CLA mixtures. This result is consistent with the previous report.(17) The better suppression of CM could be due to the different mixture ratio of CLA isomers in CM and/or unidentified molecule obtained from *Lactobacilli*. As a result of this experiment, it could be suggested that oral intake of probiotics itself or contained in dairy products could induce better anti-inflammatory effects than ingestion of purified CLA, considering together the long persistence of dietary *Lactobacilli* in the stomach relative to other bacteria.(44)

The CLA amounts in CM produced by *Lactobacilli* were lower than that reported in the previous literatures (45-48) (see Table 1). The difference of CLA production could be from different experimental conditions, such as different strain, smaller concentration of *Lactobacillus*, different incubation time and/or different culture media. Among them, the media used for the preparation of CM seemed to be the most critical. With MRS media, which had been used in the previous studies,(45,47) the active proliferation of *Lactobacilli* was also observed in our experiment. The previous reports have focused on the best production of CLA by *Lactobacilli*,(46-48) but the cytokine measurements and the influence of CLA on the AGS cells were the most important issues in the present study. Initially, when MRS media was tried for the preparation of CM in the present experiment, the AGS cells'

viability substantially decreased and the cytokines were not measured properly. The high concentration of *Lactobacilli* and the produced acidity might be one of the main reasons. When RPMI media was used instead of MRS, the AGS cells remained stable. Thus following experiments were performed in this media, even though the concentration of *Lactobacilli* and the amount of CLA production were lower than those in MRS media.

The suppressive effects on the expression of the multiple mediators were not different between *L. acidophilus* and *L. plantarum* and the amounts of CLA contained in CM produced by each *Lactobacillus* were similar. On the other hand, pretreatment with 20 μM of CLA mixture and *t*10,*c*12 isomer inhibited IL-8 and TNF-α mRNA expression better than CLA10/CLA40 and *c*9,*t*11, respectively (Figs. 1 and 4A). The better suppression of *t*10,*c*12 in IL-8 protein level was also shown in the previous report with the *H. pylori*-infected MKN-45 cells.(17) However, *c*9,*t*11 was reported to be better than *t*10,*c*12 in the inhibition of lipopolysaccharide-induced TNF-α production in RAW macrophage cell.(49) The isomeric effect and proper dose of CLA on inflammatory process have not been well established yet.(41,43)

Interestingly, 40 μM CLA mixture and 20 μM *c*9,*t*11/*t*10,*c*12 CLA isomers stimulated the expression of IL-8 mRNA before *H. pylori* infection, although there is no statistical significance from the control group without pretreatment (Fig. 1). This tendency was also observed in the measurement of TNF-α mRNA expression (Fig. 2). Moreover, it was revealed that all the different CLA concentration groups stimulated iNOS mRNA expression before *H. pylori* infection, in contrast to the suppressive effects of CM groups

(Fig. 5). The discordance could be explained by the pro-inflammatory property of CLA, which has been suggested in several studies.(16,41,43,50) Reynolds and Roche investigated the effects of *t*10,*c*12 CLA isomer in white adipose tissue and *t*10,*c*12-CLA treated mice had significantly increased TNF-α, IL-6 and suppressor of cytokine signaling 3 (SOCS3) mRNA expression.(50) Martinez et al. also demonstrated the effects of *t*10,*c*12 isomer on induction of IL-8, IL-6, IL-1β and cyclo-oxygenase-2 (COX-2).(16) In contrast, the pro-inflammatory tendency was not limited to *t*10,*c*12 isomer and not significantly different in the present study. The results mean that the pro-inflammatory effect of *t*10,*c*12 needs more validation in other conditions. Generally, the anti-inflammatory property of CLA is more convincing in the literatures,(16,41,43,50) but the adjustment of the concentration and/or isomeric mixture ratio of CLA could be more important for anti-inflammatory and anti-carcinogenic properties.

In conclusion, the present study demonstrated that CLA-containing CM produced by *L. plantarum* and *L. acidophilus* suppressed the multiple mediators induced by *H. pylori*. CLA provide a molecular mechanism associated with anti-inflammatory and anti-carcinogenic effects of probiotics in the gastric *H. pylori* infection. A further issue is how the effects of CLA influence the pathogenesis of *H. pylori* in human or animal model.

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국문초록

서론: 락토바실루스(*Lactobacillus*)에 의해 생산되는 포합 리놀레산(conjugated linoleic acid)는 핵인자-κB (nuclear factor-kappa B)의 활성을 감소시키는 것으로 보고되었고, 포합 리놀레산은 프로바이오틱스의 항염증 기전의 하나로 제시되었다. 본 연구에서는 위암세포주에서 헬리코박터균에 의해 유도된 다양한 반응에 대해 포합 리놀레산의 효과를 알아보고자 하였다.

방법: 위암세포주를 헬리코박터균으로 자극 후 인터루킨-8, 종양괴사인자-α, 유도산화질소합성효소의 mRNA 발현 혹은 단백질 농도를 측정하고, 유산균(*Lactobacillus acidophilus* 혹은 *Lactobacillus plantarum*)에 의해 생성된 포합 리놀레산 함유 조건배지 혹은 정제 포합 리놀레산을 전 처치 후 mRNA 발현 혹은 단백질 농도의 변화를 확인하였다.

결과: 헬리코박터균에 의해 증가된 인터루킨-8 및 종양괴사인자-α 의 mRNA 발현과 단백질 농도는 조건배지 혹은 정제 포합 리놀레산의 전 처치에 의해 유의하게 억제되었다. 인터루킨-8 단백질 농도와 종양괴사인자-α mRNA 의 억제는 정제 포합 리놀레산 전 처치보다 포합 리놀레산 함유 조건배지 전 처치에서 우월한 결과를 보였다. 유도산화질소합성효소 mRNA 의 발현 역시 조건배지 전 처치에 의해 유의하게 억제되었다.

결론: 락토바실루스에 의해 생성된 포합 리놀레산 함유 조건배지의 다양한 헬리코박터균 반응 억제가 프로바이오틱스의 항염증 및 항암 기전에 중요한 역할을 담당함을 확인하였다.

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