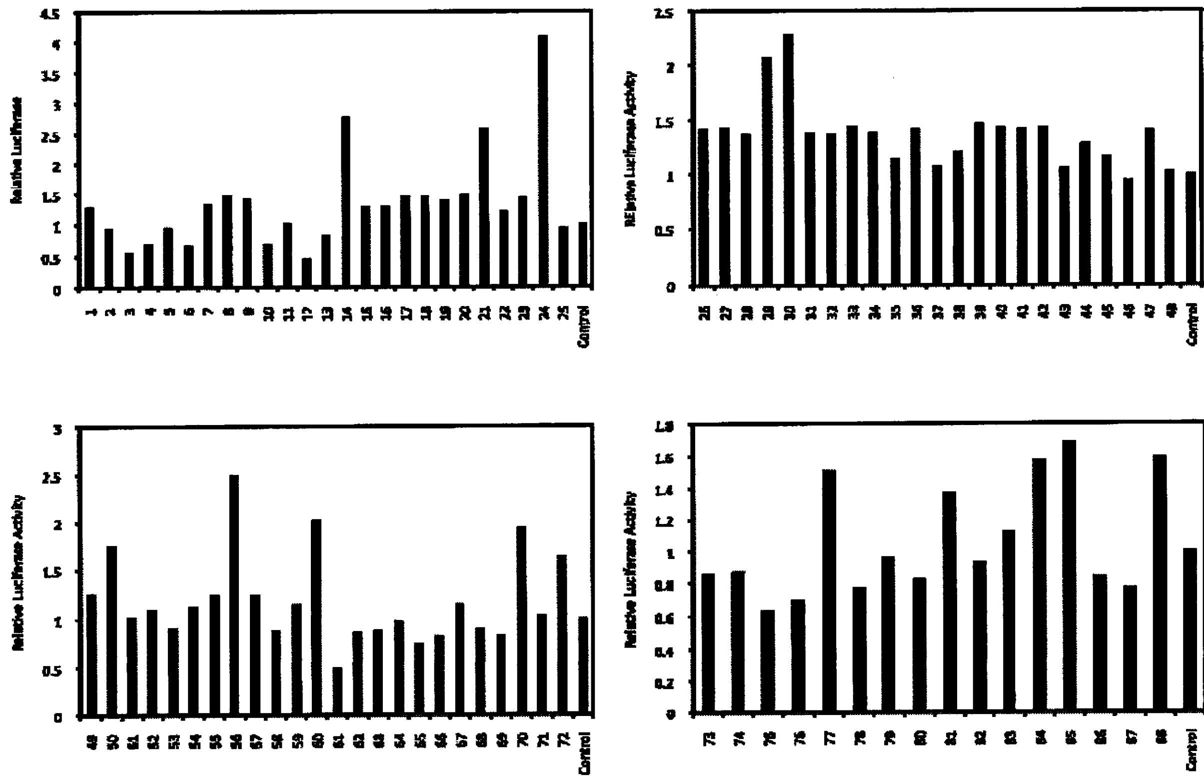
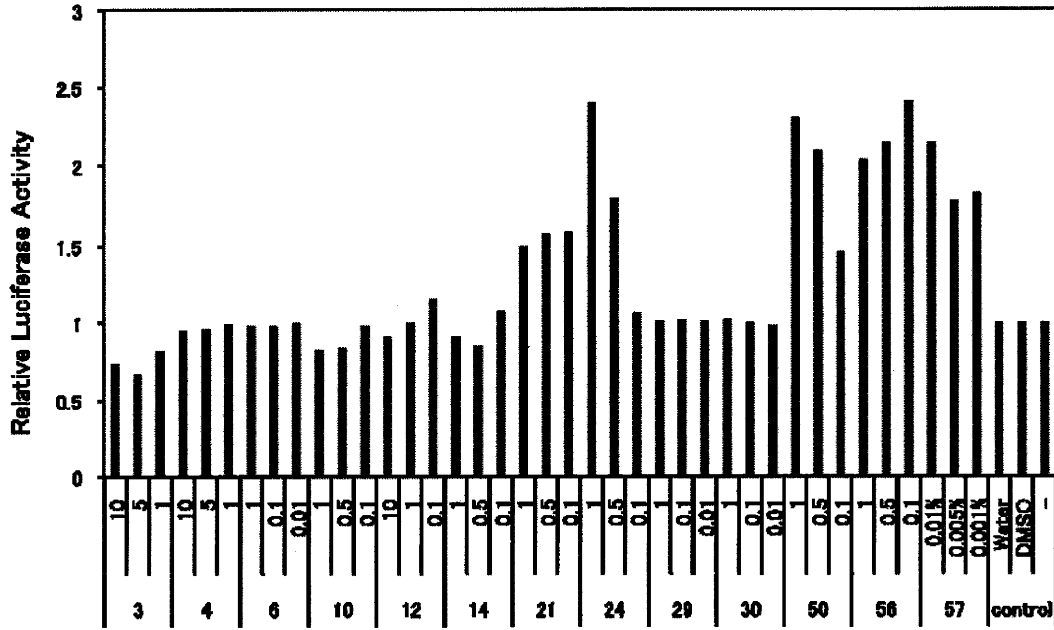


Fig. 4 Luciferase activity of claudin-4 reporter cells treated with
88 food additives.
Claudin-4 reporter gene-expressing MCF7 cells (MPCP #35) treated with
88 food additives for 24 hour were lysed and the luciferase activity was measured.



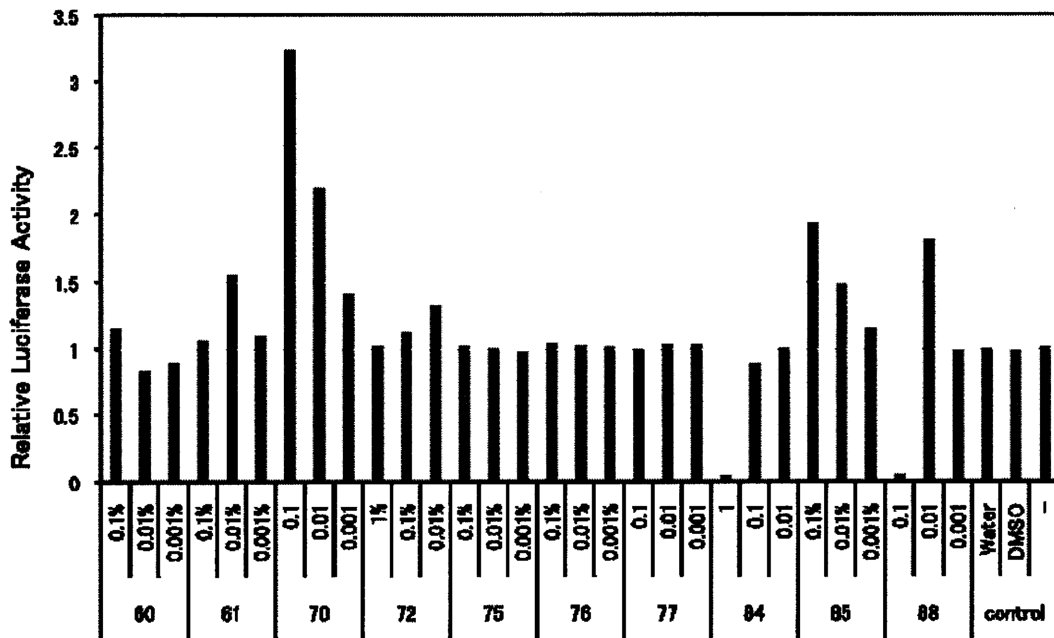
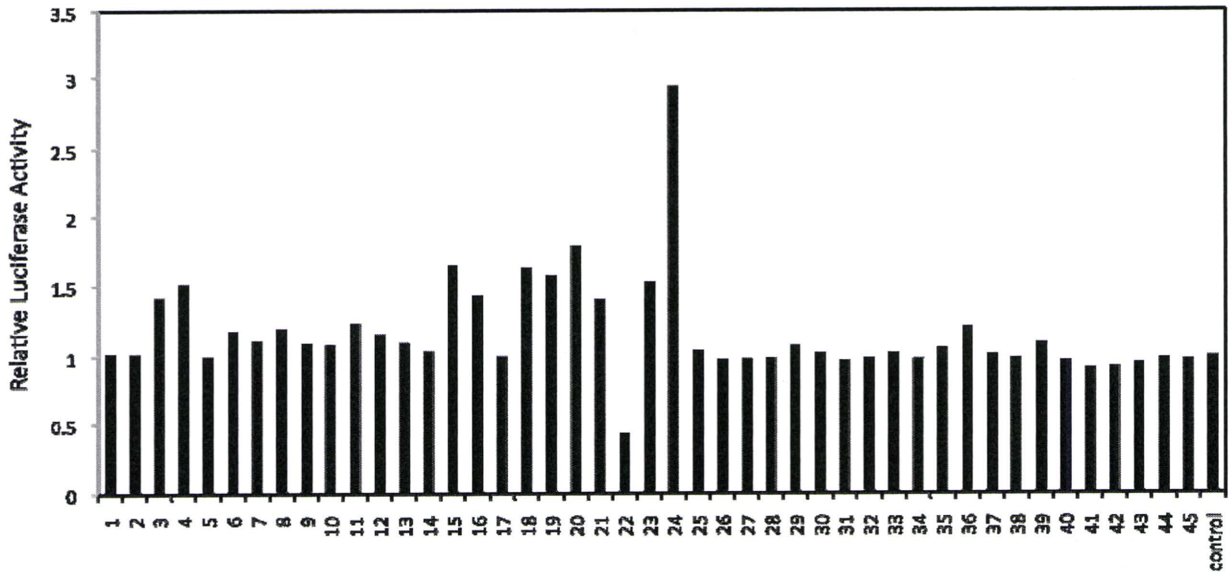


Fig. 5 Luciferase activity of claudin-4 reporter cells treated with 23 food additives .

Claudin-4 reporter gene-expressing MCF7 cells (MPCP #35) treated with different dose of 23 food additives for 24 hour were lysed and the luciferase activity was measured.



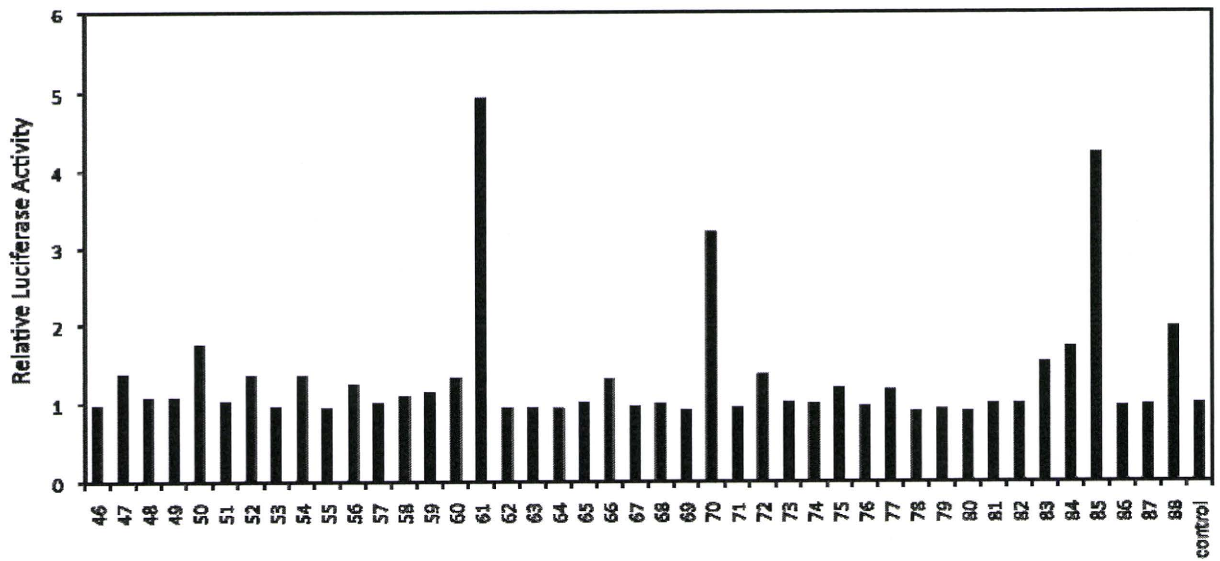


Fig. 6 Luciferase activity of claudin-4 reporter cells treated with 88 food additives for 12 hour.

Claudin-4 reporter gene-expressing MCF7 cells (MPCP #35) treated with 88 food additives for 12 hour were lysed and the luciferase activity was measured.

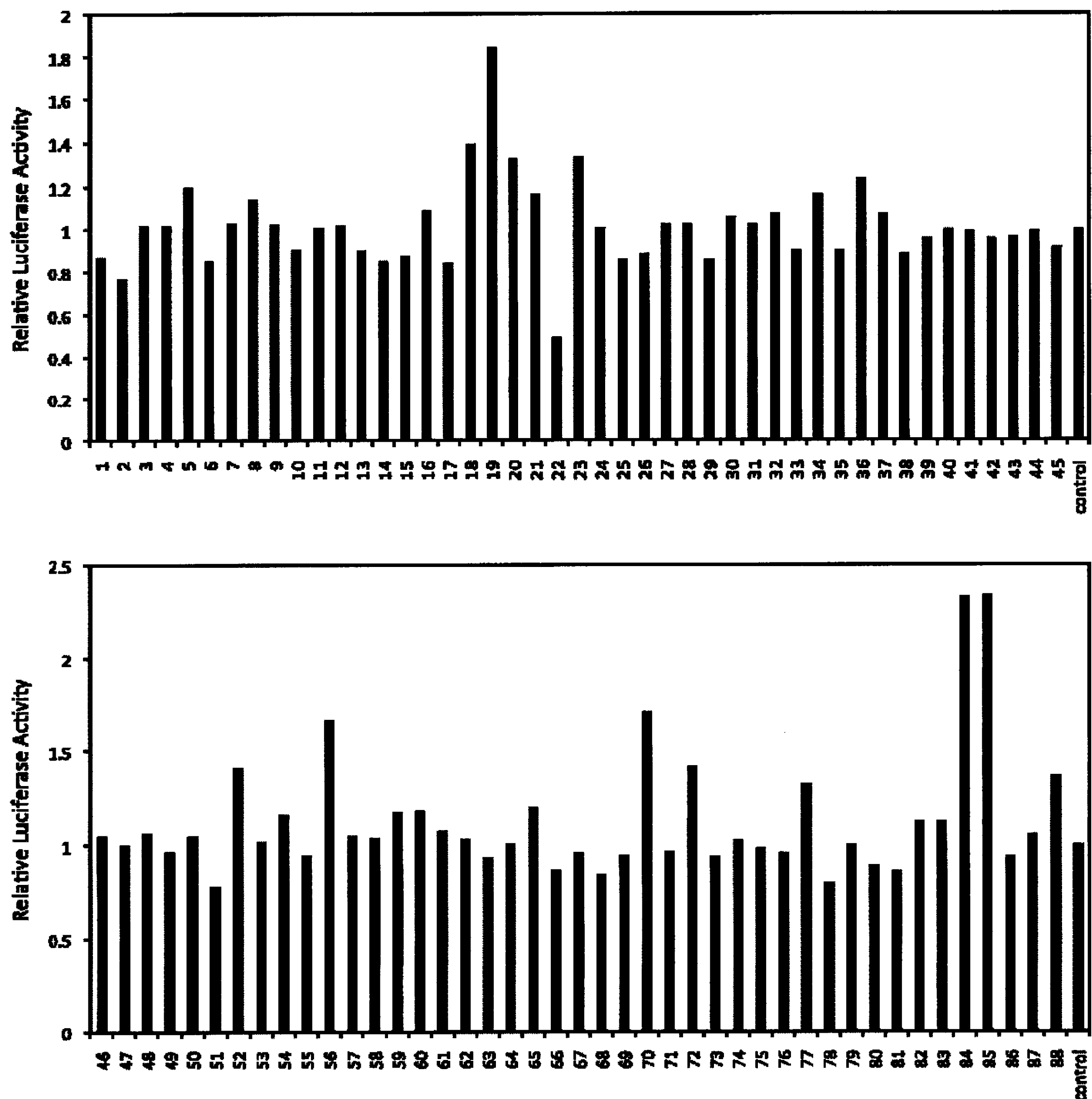


Fig. 7 Luciferase activity of claudin-4 reporter cells treated with
88 food additives for 48 hour.
Claudin-4 reporter gene-expressing MCF7 cells (MPCP #35) treated with
88 food additives for 48 hour were lysed and the luciferase activity was measured.

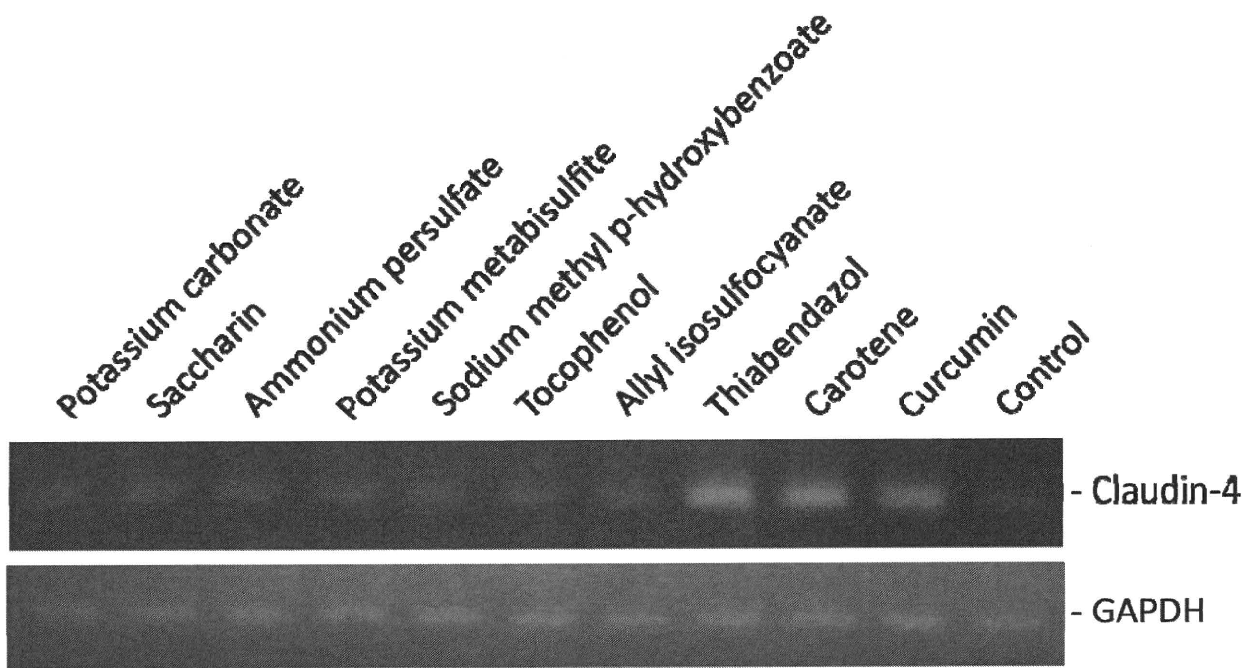


Fig. 8 Expression of claudin-4 mRNA in MPCP #35 cells treated with food additives modulating claudin-4 reporter activity.
Claudin-4 mRNA in claudin-4 reporter cells treated with potassium carbonate, saccharin, ammonium persulfate, potassium metabisulfite, sodium methyl p-hydroxybenzoate, tocopherol, allyl isosulfocyanate, thiabendazole, carotene and curcumin for 24 hour were analyzed by RT-PCR. GAPDH was used as a loading control.

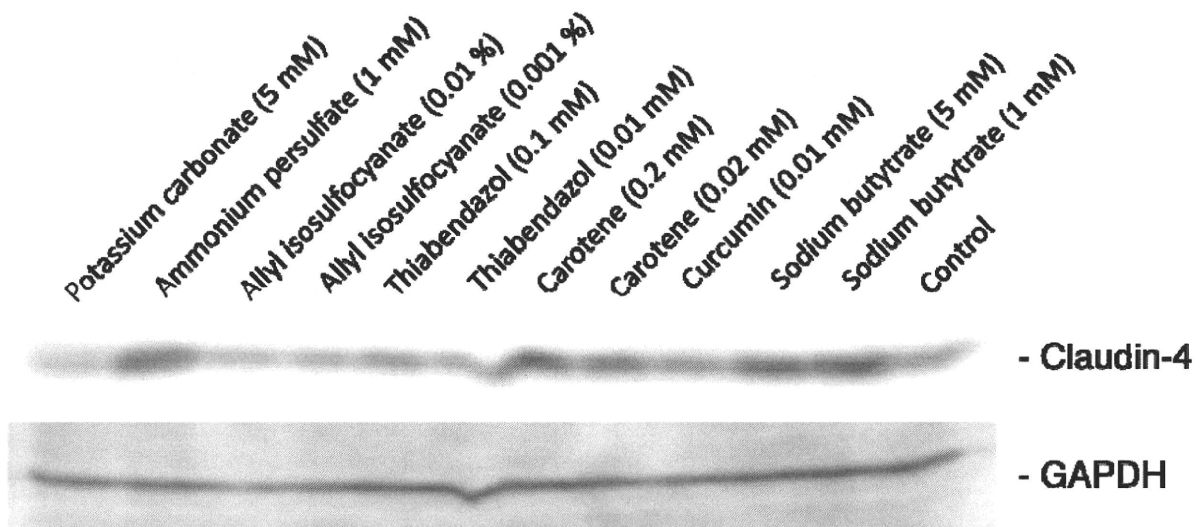


Fig. 9 Expression of claudin-4 protein in MPCP #35 cells treated with food additives modulating claudin-4 reporter activity and mRNA expression.
Claudin-4 protein in claudin-4 reporter cells treated with potassium carbonate, ammonium persulfate, allyl isosulfocyanate, thiabendazole, carotene, curcumin and sodium butyrate for 48 hour were analyzed by western blotting. GAPDH was used as a loading control.

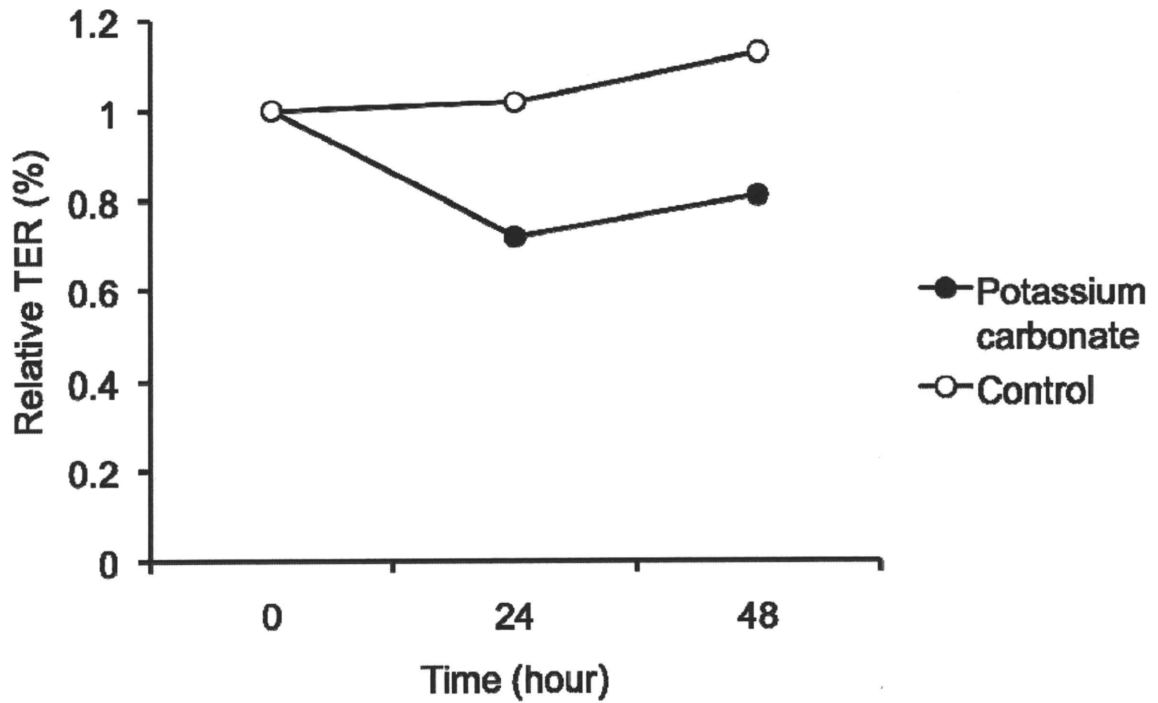


Fig. 10 Effect of potassium carbonate on TJ barrier in Caco-2 cells. Caco-2 cells were cultured on transwell chambers. When TER values reach plateau, the cells were treated with potassium carbonate (10mM). After culture for 24 and 48 hours, TER values were measured.

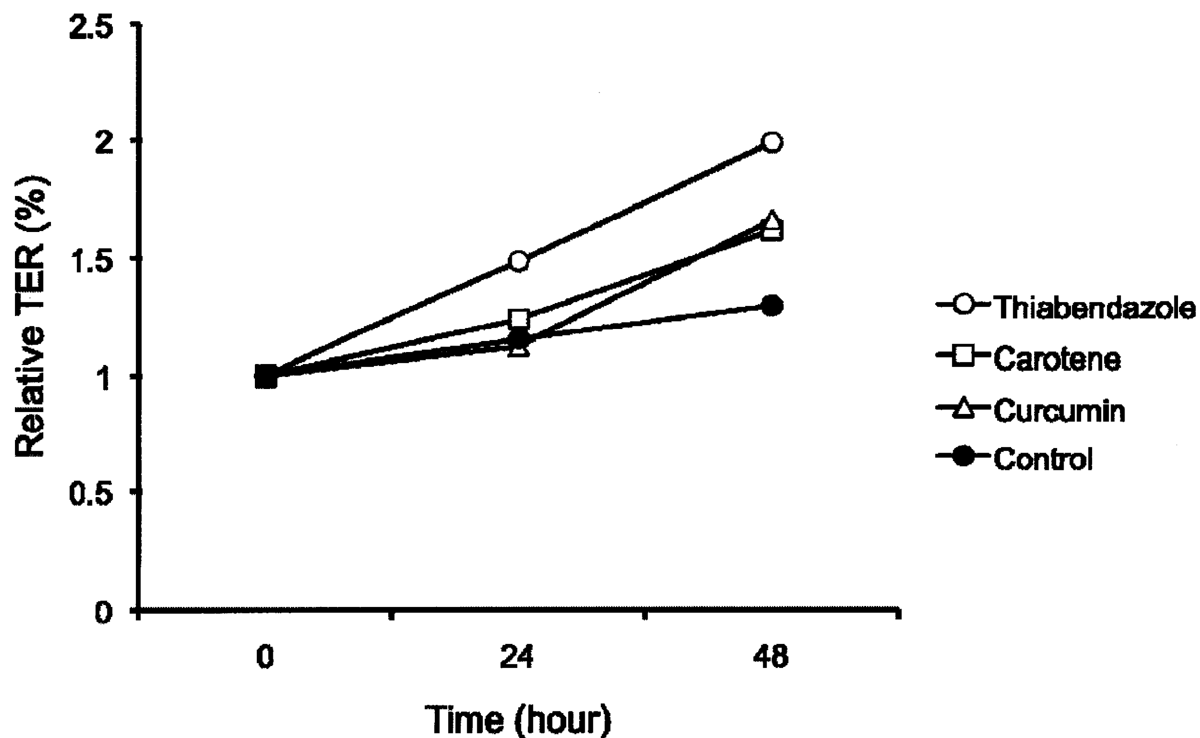


Fig. 11 Effect of food additives increased claudin-4 reporter activity on TJ barrier in Caco-2 cells.
Caco-2 cells were cultured on transwell chambers. After 7 days culture, the cells were treated with thiabendazol (0.05 mM), carotene (0.2 mM), curcumin (10μM). After culture for 24 and 48 hours, TER values were measured.

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書 籍 名	出版社名	出版地	出版年	ページ
	該当事項なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Suzuki H Kakutani H Kondoh M Watari A Yagi K	The safety of a mucosal vaccine using the C-terminal fragment of *Clostridium perfringens* enterotoxin	*Pharmazie*	10	766-769	2010
Itoh A Isoda K Kondoh M Kawase M Watari A Kobayashi M Tamesada M Yagi K	Hepatoprotective effect of syringic acid and vanillic acid on CCl_4-induced liver injury	*Biol Pharm Bull*	33	983-987	2010
Kakutani H Kondoh M Fukasaka M Suzuki H Hamakubo T Yagi K	Mucosal vaccination using claudin-4 targeting	*Biomaterials*	31	5463-5471	2010
Yagi K Kawase M Isoda K Kondoh M	Development of novel culture system for regulation of hepatocyte function	*YAKUGAKU ZASSHI,*	130	537-543	2010
Kakutani H Kondoh M Saeki R Fujii M Watanabe Y Mizuguchi H Yagi K	Claudin-4-targeting of diphtheria toxin fragment A using a C-terminal fragment of *Clostridium perfringens* enterotoxin	*Eur J Pharm Biopharm*	75	213-217	2010
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Ushitora M Sakurai F Yamaguchi T Nakamura S Kondoh M Yagi K Kawabata K Mizuguchi H	Prevention of hepatic ischemia-reperfusion injury by pre-administration of catalase-expressing adenovirus vector	*J Control Rel*	142	4331-4337	2010
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Kakutani H Takahashi A Kondoh M Saito Y Yamaura T Sakihama T Hamakubo T Yagi K	A novel screening system for claudin binder using baculoviral display	*PLoS ONE*	6	e16611	2011
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Takahashi A Kondoh M Kodaka M Yagi K	Peptides as tight junction modulators	*Curr Pharm Design*			accepted

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The safety of a mucosal vaccine using the C-terminal fragment of *Clostridium perfringens* enterotoxin

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The C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) is a claudin-4 binder. Very recently, we found that nasal immunization of mice with C-CPE-fused antigen activated antigen-specific humoral and mucosal immune responses and that the deletion of the claudin-4-binding domain attenuated the immune responses. C-CPE-fusion strategy may be useful for mucosal vaccination. C-CPE is a fragment of enterotoxin, and the safety of C-CPE-fused protein is very important for its future application. In the present study, we investigated whether C-CPE-fused antigen induces immune responses without mucosal injury by using ovalbumin (OVA) as a model antigen. Immunohistochemical analysis showed that claudin-4 was expressed in epithelial cell sheets bordering the nasal cavity. Nasal immunization with C-CPE-fused OVA dose-dependently elevated the OVA-specific serum IgG titer, which was 1000-fold greater than the titer achieved by immunization with OVA or a mixture of OVA and C-CPE at 5 μg of OVA. Nasal immunization with C-CPE-fused OVA (5 μg of OVA) activated Th1 and Th2 responses. Histological analysis showed no mucosal injury in the nasal cavity or nasal passage. C-CPE-fused OVA exhibited mucosal vaccination without mucosal injury. These findings indicate that claudin-4-targeting using C-CPE can be a potent strategy for mucosal vaccination.

1. Introduction

Vaccination is the most potent therapeutic method to overcome infectious diseases. Vaccines are classified as parenteral or mucosal. Parenteral immunizations activate systemic immune responses, while mucosal immunizations activate both systemic and mucosal immune responses. Parenteral vaccination can activate immune responses against the invaded pathogenic microorganisms and infected cells; in contrast, mucosal vaccination prevents entry of the pathogenic microorganisms and activates immune responses against the infected cells (Kunisawa et al. 2008; Neutra and Kozlowski 2006). Although mucosal immunization is promising, an immune response is not activated by the mucosal administration of antigen alone. Efficient antigen delivery into mucosal associated lymphoid tissue (MALT) is the key technology needed for the development of mucosal vaccines (Kunisawa et al. 2008; Neutra and Kozlowski 2006).
The mucosa is covered by epithelial cell sheets, which separate the outside of the body from the inside of the body. Tight junctions (TJs) are located between adjacent epithelial cells and seal intercellular junctions, preventing the free movement of solutes across epithelial cell sheets (Scheneeberger and Lynch 1992). Claudin, a tetra-transmembrane protein family consisting of 24 members, plays a pivotal role in the mucosal TJ barrier (Furuse and Tsukita 2006; Tsukita et al. 2001). In 2003, Tamagawa et al. reported that claudin-4 is expressed in the epithelium of intestinal MALT. These findings indicate that claudin-4-targeting may be a novel strategy for the development of mucosal vaccines; however, the claudin-4-targeting vaccine had never been developed because of a delay in the preparation of claudin-4 binder. *Clostridium perfringens* enterotoxin (CPE) causes food poisoning in humans (McClane and Chakrabarti 2004). The CPE receptor is claudin-4, and a 14-kDa polypeptide, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), is a claudin-4 binder (Katahira et al. 1997; Sonoda et al. 1999). We previously found that C-CPE enhanced jejunal, nasal and pulmonary absorption of drugs through its interaction with claudin-4 (Kondoh et al. 2005; Uchida et al. 2010). C-CPE is used as a claudin-4 ligand molecule for proteins (Ebihara et al. 2006; Saeki et al. 2009). These findings strongly indicate that C-CPE may be a potent ligand for MALT. Very recently, we have found that intranasal administration of C-CPE-fused ovalbumin (OVA) increased OVA-specific immune-responses in serum, nasal, vaginal and intestinal mucosa (Kakutani et al. 2010). However, C-CPE is a fragment of enterotoxin, and the safety of C-CPE-fused vaccine has never been investigated.
In the present study, we investigated whether nasal immunization with C-CPE-fused OVA activated immune responses without mucosal injury, and we found that mucosal vaccine using C-CPE activated Th1 and Th2 immune responses without nasal mucosa injury.

2. Investigations and results

C-CPE is a binder of claudin-4, and we recently found that claudin-4-targeting using C-CPE might be a potent strategy for mucosal vaccine (Kakutani et al. 2010). C-CPE is a fragment

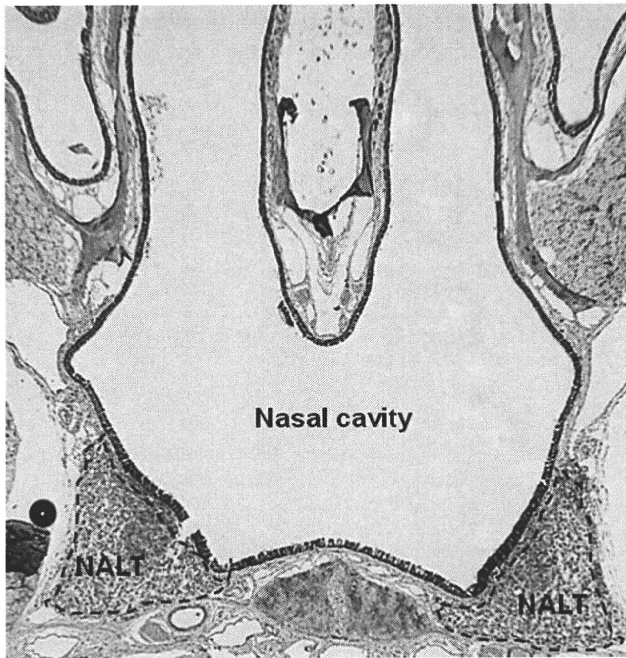

Fig. 1: Immunohistochemical analysis of claudin-4 in NALT Specimens were cryosectioned (4 μm) and stained with anti-claudin-4 Ab, followed by horseradish peroxidase-labeled secondary Ab. The immunoreactive regions were stained with a commercially available staining kit. The regions surrounded by dotted lines are NALT

of CPE (Katahira et al. 1997). CPE is a 35-kDa single polypeptide toxin produced by *Clostridium perfringens*. Experimental and epidemiologic evidence indicates that CPE causes foodborne disease and non-foodborne diarrheal illnesses (McClane 2001; McClane et al. 2000). CPE forms a CPE-containing complex in the plasma membrane that creates massive alterations in plasma membrane permeability that lead to cell death and histological damage to the intestine (McClane and Chakrabarti 2004). The safety of C-CPE is a critical issue for pharmaceutical applications of C-CPE. First, we investigated the expression of claudin-4 in nasal mucosa. Immunohistochemical analysis reveals that claudin-4 is expressed in the epithelium bordering the nasal cavity (Fig. 1). The epithelium that covers nasal MALT, nasopharynx-associated lymphoid tissue (NALT), is rich in claudin-4. These data correspond to our previous data on the expression of claudin-4 mRNA and protein in NALT (Kakutani et al. 2010).

To investigate the dose dependency of OVA-C-CPE in mucosal vaccination, mice were nasally immunized with OVA-C-CPE at 0.5, 1.0 or 5.0 μg of OVA. As shown in Fig. 2A, OVA-specific serum IgG levels were elevated in a dose-dependent manner and reached a level that was 1000-fold greater than the OVA values at 5 μg of OVA, which is the maximal dose of OVA-C-CPE due to its solubility. A mixture of OVA and C-CPE did not increase OVA-specific serum IgG levels (Fig. 2B), and OVA-C-CPE (5.0 μg of OVA) immunization activated IgG2a (a Th1 immune response) and IgG1 (a Th2 immune response) responses (Fig. 2C). We performed a histopathological analysis of mice immunized with OVA-C-CPE at 5.0 μg of OVA. Hematoxylin and eosin (HE) staining revealed no apparent mucosal injury in the nasal squamous cavity, the respiratory cavity, and the nasal passage (Fig. 3A). There was also no inflammatory cell infiltration in the nasal mucosa (Fig. 3B). These findings indicate that nasal immunization with OVA-C-CPE activated immune responses without histological injury in nasal mucosa.

3. Discussion

Only a mucosal vaccine can prevent the entry of pathological viruses into the mucosal membrane; however, injectable vaccines are currently used in both developing and industrialized countries. Very recently, we found that claudin-4-targeting using C-CPE can be a novel strategy for mucosal vaccination (Kakutani et al. 2010). In the present study, we showed that nasal immunization with C-CPE-fused antigen activated Th1 and Th2 immune responses without histological injury in nasal mucosa. The efficient delivery of C-CPE-fused antigen to immunocompetent cells is critical for mucosal vaccination. A mixture of OVA and C-CPE did not activate immune responses, and therefore OVA may be delivered to the immunocompetent cells as the C-CPE-fused protein. The uptake of nasally administered antigens is achieved through a unique set of antigen-sampling cells, the M cells, located in follicle-associated epithelium. After the uptake of antigens by M cells, the antigens are immediately processed and presented to the underlying dendritic cells (Neutra and Kozlowski 2006). A recent report indicates that claudin-4 is expressed in M cells (Rajapaksa et al. 2010). Claudin-4 contains sorting signal sequences to endosomes, an ALGVLL motif at amino acids 92 to 97 and a YVGW motif at amino acids 165 to 168 (Ivanov et al. 2004). These findings suggest that OVA-C-CPE may be taken up by clathrin-mediated endocytosis in

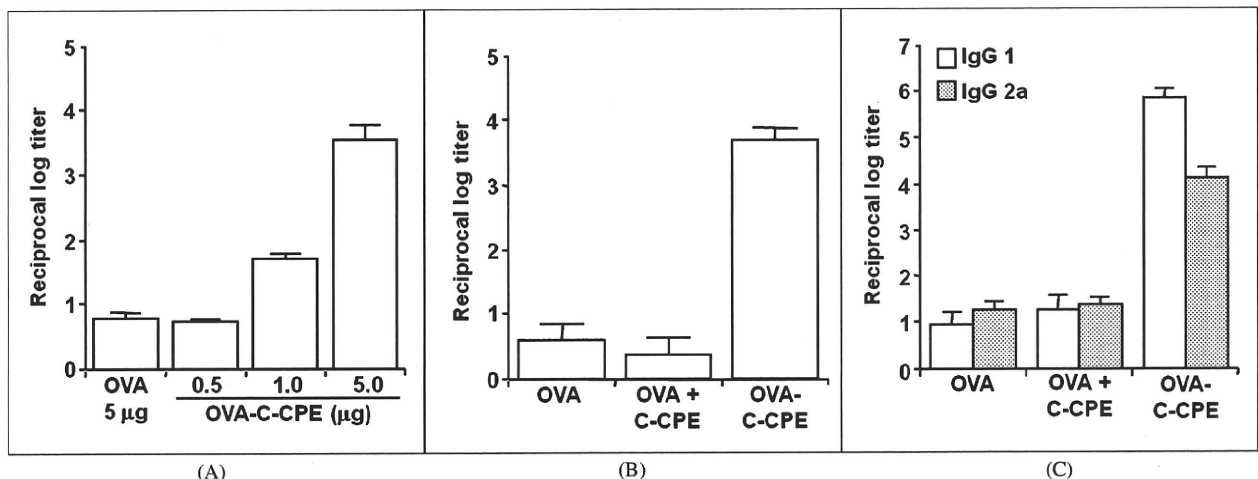

Fig. 2: Production of OVA-specific serum IgG by nasal immunization with OVA-C-CPE Mice were nasally immunized with OVA (5 μg), a mixture of OVA (5 μg) with C-CPE or OVA-C-CPE at the indicated dose of OVA once a week for 3 weeks. Seven days after the last immunization, the serum IgG level was determined by ELISA (A). Mice were nasally immunized with OVA, a mixture of OVA with C-CPE or OVA-C-CPE at 5 μg of OVA once a week for 3 weeks. Seven days after the last immunization, the serum IgG (B), IgG1 and IgG2a (C) levels were determined by ELISA. Data are means ± SEM (n = 4)

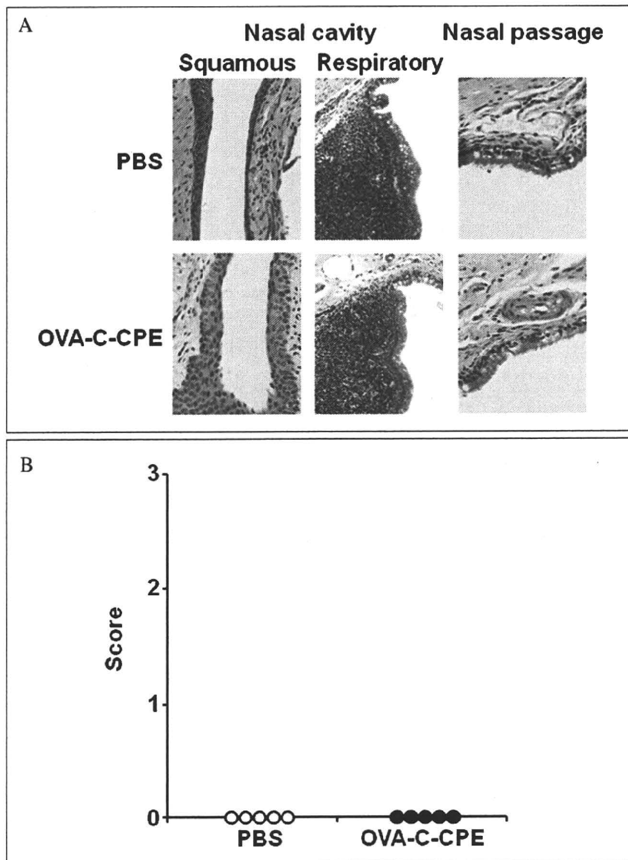

Fig. 3: A lack of histological injury caused by OVA-C-CPE Mice were nasally immunized with PBS or OVA-C-CPE (5 µg OVA) once a week for 3 weeks. Seven days after the last immunization, NALT was collected and fixed with formalin. Thin tissue-sections were stained with hematoxylin (A), and inflammation was scored according to the severity of the inflammatory cell infiltration (B): 0, none; 1, weak; 2, moderate; 3, severe. Scoring was performed blindly to avoid bias

M cells. The underlying mechanism for immunopotentiation by OVA-C-CPE must be clarified.

Nasal mucosa is adjacent to the central nervous system, and intranasal drug delivery to the brain has been developed (Dhuria et al. 2010). An influx of a vaccine protein into the central nervous system can be a risk factor in its clinical application. C-CPE is a modulator of the epithelial barrier. Treatment of nasal mucosa with C-CPE increased the nasal absorption of a peptide drug. OVA-C-CPE also modulated the epithelial barrier *in vitro* (data not shown). Claudin-deficient mice showed a size-dependent leak of solutes smaller than 1,000 Da in the epithelium and endothelium (Furuse et al. 2002; Nitta et al. 2003). C-CPE enhanced the mucosal absorption of dextran with a molecular mass of ~20 kDa (Kondoh et al. 2005). Because OVA-C-CPE has a molecular mass of 65 kDa, and the infiltration of inflammatory lymphocytes was not observed in nasal mucosa (Fig. 3B), OVA-C-CPE might not cause an influx of solutes across the nasal epithelium. The claudin family comprises at least 24 members. Interestingly, the barrier-function and expression profiles of claudin family members differ among tissues. Claudin is believed to form homophilic and heterophilic adhesions in TJ strands, and various combinations of the 24 family members are thought to create diversity in the structure and functions of TJ barriers (Furuse and Tsukita 2006; Morita et al. 1999; Tsukita et al. 2001). Targeting the type of claudin specifically expressed in NALT may reduce the risk of delivering solutes to the central nervous system.

In summary, we showed that nasal immunization with C-CPE-fused antigen activated Th1 and Th2 immune responses without mucosal injury. This is the first report to indicate the safety of a claudin-4-targeting mucosal vaccine using C-CPE. Future improvement of the claudin specificity may lead to clinical applications of this type of vaccine.

4. Experimental

4.1. Animals

Female BALB/c mice were purchased from SLC, Inc. (Shizuoka, Japan). The mice were housed at 23 ± 1.5 °C with a 12-h light/dark cycle and had free access to standard rodent chow and water. The protocol of this study was approved by the Animal Care and Use Committee for Graduate School of Pharmaceutical Sciences, Osaka University.

4.2. Immunohistochemical analysis

Immunohistochemical staining for claudin-4 was performed with an autostainer (Dako, Glostrup, Denmark). Slide-mounted, fixed cryosections (4-µm thick) of nasal MALT were incubated in Dako target retrieval solution (pH 9) at 125 °C for 30 min and then 90 °C for 10 min. The slides were blocked with peroxidase-blocking reagent (Dako) for 5 min and then with 10% bovine serum albumin for 30 min. The slides were further incubated with anti-claudin-4 antibody followed by horseradish peroxidase-labeled secondary antibody. The immunoreactive proteins were stained with DAB substrate (Dako). The slides were also stained with hematoxylin solution. Tissue sections were observed under a microscope.

4.3. Preparation of C-CPE-fused OVA

C-CPE-fused OVA (OVA-C-CPE) was prepared as described previously (Kakutani et al. 2010). Briefly, the plasmid pET-OVA-C-CPE was transduced into *Escherichia coli* BL21 (DE3), and the production of OVA-C-CPE was stimulated with isopropyl-D-thiogalactopyranoside. The harvested cells were lysed in buffer A (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol). The lysates were applied to HiTrap™ Chelating HP (GE Healthcare UK Ltd., Buckinghamshire, UK), and OVA-C-CPEs were eluted with buffer A containing 100-400 mM imidazole. The buffer was exchanged with phosphate-buffered saline (PBS) by using a PD-10 column (GE Healthcare UK Ltd.), and the purified protein was stored at −80 °C before use. Purification of the OVA-C-CPEs was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant Blue. Protein was quantified by using a BCA protein assay kit (Pierce Chemical, Rockford, IL) with BSA as a standard.

4.4. Nasal immunization and sample collection

Mice were nasally immunized once a week for 3 weeks at the indicated dose of OVA. For instance, a dose of 5 µg OVA is equal to a mixture of OVA (5 µg) and C-CPE (1.89 µg) or OVA-C-CPE (6.89 µg). Plasma was collected 7 days after the last nasal immunization.

4.5. OVA-specific IgG production

The titers of OVA-specific antibody in serum were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, an immunoplate was coated with OVA (100 µg/well in a 96-well plate). Ten-fold serial dilutions of these samples were added to the immunoplate followed by the addition of horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1 or IgG2a (Bethyl Laboratories Inc., Montgomery, TX). The OVA-specific antibodies were detected by using a TMB peroxide substrate (Thermo Fisher Scientific Inc., Rockford, IL). End-point titers were expressed as the reciprocal log of the last dilution ratio, which was 0.1 greater than the control values obtained for the serum of naïve mice at an absorbance of 450 nm.

4.6. Histological analysis of nasal mucosa in mice immunized with OVA-C-CPE

Mice from either the non-immunized group or the immunized group (6.89 µg of OVA-C-CPE) were sacrificed 7 days after immunization for histopathological analysis. Tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Sections (4 µm) were prepared for hematoxylin and eosin staining. Histopathologic examinations were performed at the Applied Medical Research Laboratory (Osaka, Japan). Inflammation was scored according to the severity of the inflammatory cell infiltration: 0, none; 1, weak; 2, moderate; and 3, severe. Scoring was performed blindly to avoid bias.

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Hepatoprotective Effect of Syringic Acid and Vanillic Acid on CCl_4-Induced Liver Injury

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The mycelia of the edible mushroom *Lentinula edodes* can be cultured in solid medium containing lignin, and the hot-water extracts (L.E.M.) is commercially available as a nutritional supplement. During the cultivation, phenolic compounds, such as syringic acid and vanillic acid, were produced by lignin-degrading peroxidase secreted from *L. edodes* mycelia. Since these compounds have radical scavenging activity, we examined their protective effect on oxidative stress in mice with CCl_4-induced liver injury. We examined the hepatoprotective effect of syringic acid and vanillic acid on CCl_4-induced chronic liver injury in mice. The injection of CCl_4 into the peritoneal cavity caused an increase in the serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. The intravenous administration of syringic acid and vanillic acid significantly decreased the levels of the transaminases. Four weeks of CCl_4 treatment caused a sufficiently excessive deposition of collagen fibrils. An examination of Azan-stained liver sections revealed that syringic acid and vanillic acid obviously suppressed collagen accumulation and significantly decreased the hepatic hydroxyproline content, which is the quantitative marker of fibrosis. Both of these compounds inhibited the activation of cultured hepatic stellate cells, which play a central role in liver fibrogenesis, and maintained hepatocyte viability. These data suggest that the administration of syringic acid and vanillic acid could suppress hepatic fibrosis in chronic liver injury.

Key words hepatoprotection; *Lentinula edodes*; syringic acid; vanillic acid; polyphenol

The edible mushroom *Lentinula edodes* (shiitake) contains bioactive compounds that have immune-modulating, antitumor, antibacterial, antiviral, and antiparasitic effects.[1—4] The mycelia of *L. edodes* can be cultured in solid medium, and the hot-water extract (L.E.M.) is commercially available as a nutritional supplement. The main components of L.E.M. are sugars, proteins, and polyphenolic compounds. Polyphenols have protective effects against cancers, cardiovascular disease, and neurodegenerative disorders.[5—7] Among polyphenols, syringic acid and vanillic acid are enriched in the solid medium of cultured *L. edodes* mycelia.[8] *L. edodes* grown in lignocellulose secretes lignin-degrading peroxidase into the culture medium.[9] The mycelia-derived enzymes degrade the lignin to produce phenolic compounds, particularly syringic acid and vanillic acid. In our previous study, we demonstrated that these phenolic compounds had a hepatoprotective effect on concanavalin A (ConA)-induced liver injury in mice.[8] We intraperitoneally injected syringic acid or vanillic acid into mice shortly before a ConA injection into the tail vein, which greatly increased the levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In addition, the inflammatory cytokines tumor necrosis factor (TNF)-α, interferon-γ (IFN-γ), and interleukin (IL)-6 in the serum increased rapidly, within 3 h of the ConA administration. The administration of syringic acid or vanillic acid significantly decreased the transaminase and inflammatory cytokine levels and suppressed the disorganization of the hepatic sinusoids. Since ConA-induced liver injury is a mouse model of immune-mediated liver injury that resembles viral and autoimmune hepatitis in humans, the phenolics appeared to have immunomodulating activity.

Polyphenols act as antioxidants by scavenging reactive oxygen species (ROS), which produce oxidative stress and can adversely affect many cellular processes. In the present study, we examined the possible hepatoprotective effects of two phenolic compounds, syringic acid and vanillic acid, on oxidative stress in chronic CCl_4-induced liver injury in mice. We found that both phenolic compounds could suppress oxidative damage, especially liver fibrosis caused by repeated administration of CCl_4.

MATERIALS AND METHODS

Reagents Syringic acid, vanillic acid, and CCl_4 were purchased from WAKO Pure Chemicals, Co., Ltd. (Osaka, Japan). The chemical structures of syringic acid and vanillic acid were shown in Fig. 1. L.E.M. was obtained from Kobayashi Pharmaceutical Co., Ltd. (Osaka, Japan). CCl_4 was dissolved in olive oil, and L.E.M., syringic acid, and vanillic acid were dissolved in phosphate buffered saline (PBS) for administration into mice. L.E.M., syringic acid, and vanillic acid were dissolved in culture medium for hepatocytes or hepatic stellate cells for *in vitro* experiments.

Animals BALB/c mice and Sprague-Dawley rats were purchased from SLC (Shizuoka, Japan). The animals were housed in an air-conditioned room at 22 °C before the experiment. The animal experiments were conducted according to the ethical guidelines of Osaka University Graduate School

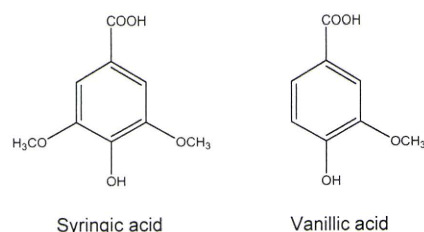

Fig. 1. Chemical Structures of Syringic Acid and Vanillic Acid

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of Pharmaceutical Sciences. The experimental protocol was submitted to the Committee on the Guidelines for Animal Experiments in Graduate School of Pharmaceutical Sciences, and the experiments were conducted after gaining the approval. Mice in the chronic liver injury model received intraperitoneal injections of CCl_4 (0.5 ml/kg body weight) and intravenously administered L.E.M., syringic acid, or vanillic acid (10 mg/kg body weight) twice a week for 4 weeks. Twenty-four hours after the L.E.M., syringic acid, or vanillic acid injection, the mice were anesthetized. Then, blood samples were collected to determine the transaminase activity, and the livers were excised for Azan staining and determination of hydroxyproline and malondialdehyde.

Assays Serum AST and ALT levels were measured by using an assay kit (Transaminase C-II, WAKO, Osaka, Japan).

Histological Analysis Liver specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut from the tissue blocks and mounted on slides. Azan staining was then performed to evaluate the extent of liver fibrosis.

Measurement of Hydroxyproline Content Hepatic hydroxyproline content was measured by using Kivirikko's method[10] with some modifications. Briefly, liver tissue (50 mg) was hydrolyzed with 6 mol/l HCl at 110 °C for 24 h in a glass test tube. After centrifugation at 3000 rpm for 10 min, 2 ml of the supernatant was neutralized with 8 N KOH. Two grams of KCl and 1 ml of 0.5 mol/l borate buffer were then added to the neutralized supernatant, followed by a 15-min incubation at room temperature and then a 15-min incubation at 0 °C. Freshly prepared chloramine-T solution was then added, and the sample was incubated at 0 °C for 1 h, followed by the addition of 2 ml of 3.6 mol/l sodium thiosulfate. The samples were incubated at 120 °C for 30 min. Then, 3 ml of toluene was added, and the samples were incubated for 20 min at room temperature. After centrifugation at 2000 rpm for 5 min, 2 ml of the supernatant was added to 0.8 ml buffer containing Ehrlich's reagent and incubated for 30 min at room temperature. The samples were then transferred to a plastic tube, and the absorbance was measured at 560 nm. The hydroxyproline content was expressed as micrograms of hydroxyproline per gram of liver.

Measurement of Malondialdehyde Lyophilized liver tissue (25 mg) was boiled for 30 min in a solution containing 250 ml of 1.15% KCl, 150 ml of 1% H_3PO_4, and 500 ml of 0.67% thiobarbituric acid. Two milliliters of *n*-butanol was added to the ice-chilled sample, and then the sample was stirred for 30 min. After centrifugation at $3000\times g$ for 10 min, the upper *n*-butanol phase was collected, and the amount of malondialdehyde was colorimetrically determined at 535 and 520 nm.

Isolation and Culture of Hepatic Stellate Cells Hepatic stellate cells (HSCs) were isolated from 10-week-old male Sprague-Dawley rats by digesting the liver with Pronase-E (Merck Darmstadt, Germany) and collagenase type I (WAKO Pure Chemicals Co., Osaka, Japan) as previously described.[11] Isolated HSCs were seeded at a density of 2×10^5 cells/cm^2 onto 24-well polystyrene culture plates (Asahi Techno Glass, Funabashi, Chiba, Japan) to observe the morphology and analyze fibrosis-related gene expression. Cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum.

Isolation and Culture of Hepatocytes Hepatocytes were isolated from male BALB/c mice by perfusing the liver with collagenase, according to the method of Seglen.[12] Cells were seeded at a density of 1×10^5 cells/cm^2 into multi-well culture plates pre-coated with collagen type I (Asahi Techno Glass, Funabashi, Chiba, Japan). The basal medium consisted of 50 U/ml penicillin G, 50 μg/ml streptomycin (ICN Biochemicals, Inc., Costa Mesa, CA, U.S.A.), 1 μM insulin, 1 μM dexamethasone (WAKO Pure Chemicals Co., Osaka, Japan), and 10% fetal bovine serum in William's medium E (MP Biomedicals, Inc., Kayserberg, France). Six hours after the cells were seeded, the basal medium was replaced with medium containing L.E.M., syringic acid, or vanillic acid at a final concentration of 1.0 mg/ml without insulin and dexamethasone. Cells were then cultured for 24—48 h, and viable cells were counted after trypan blue staining.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) The HSCs were cultured for 7 d and the total RNA was extracted using High Pure RNA Isolation Kit (Roche, Mannheim, Germany). The gene expression of collagen 1 α(I) was analyzed using the following primers: forward 5′-TGCCGTGACCTCAAGATGTG-3′ and reverse 5′-CACAAGCGTGCTGTAGGTGA-3′. The gene expression of a smooth muscle actin (α-SMA) was analyzed using the following primers: forward 5′-CCGAGATCTCACCGACTACC-3′ and reverse 5′-TCCAGAGCGACATAGCACAG-3′. The gene expression of β-actin was analyzed using the following primers: forward 5′-CCCAGAGCAAGAGAGGCATC-3′ and reverse 5′-CTCAGGAGGAGCAATGATCT-3′.

The RT-PCR was examined using RNA PDR Kit (TaKaRa, Kyoto, Japan).

Statistical Analysis The data were analyzed for statistical significance by using Student's *t*-test and Dunnett's test.

RESULTS

Effect on CCl_4-Induced Chronic Liver Injury We examined the hepatoprotective effect of syringic acid and vanillic acid on CCl_4-induced chronic liver injury in mice. As shown in Fig. 2, after 4 weeks of CCl_4 treatment, the activities of blood AST and ALT increased 30-fold and 127-fold, respectively, compared with controls. The intravenous administration of syringic acid or vanillic acid significantly decreased the activities of AST and ALT. These results suggest that syringic acid and vanillic acid suppress the hepatic inflammation caused by repeated CCl_4 treatments. We also examined the effect of syringic acid and vanillic acid on liver fibrogenesis. Figure 3 shows typical Azan staining results, in which fibrous materials are stained blue. In the controls (Fig. 3A), hardly any blue staining was observed in the pericentral area. In contrast, the livers injured by chronic CCl_4 treatment displayed a considerable accumulation of fibrous materials (Fig. 3B). CCl_4 treatment for 4 weeks caused an excessive deposition of collagen fibrils that was sufficient for the evaluation of the antifibrogenic effect of syringic acid and vanillic acid. Based on the results of Azan staining, the syringic acid and vanillic acid treatments obviously suppressed collagen accumulation (Figs. 3D, E). To quantitatively evaluate the effect of syringic acid and vanillic acid on fibrogenesis, we

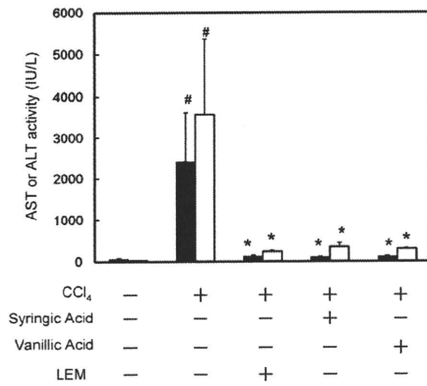

Fig. 2. Effect of Syringic Acid and Vanillic Acid on CCl_4-Induced Chronic Hepatic Injury

Mice received an intraperitoneal injection of CCl_4 and an intravenous injection of L.E.M., syringic acid, or vanillic acid twice a week for 4 weeks. The serum levels of AST (solid column) and ALT (open column) were determined. The values are mean±S.D ($n=4$). The data were analyzed by Student's t-test (# $p<0.05$, as compared to uninjured control mice) and Dunnett's method (* $p<0.05$, as compared to CCl_4-injured control mice).

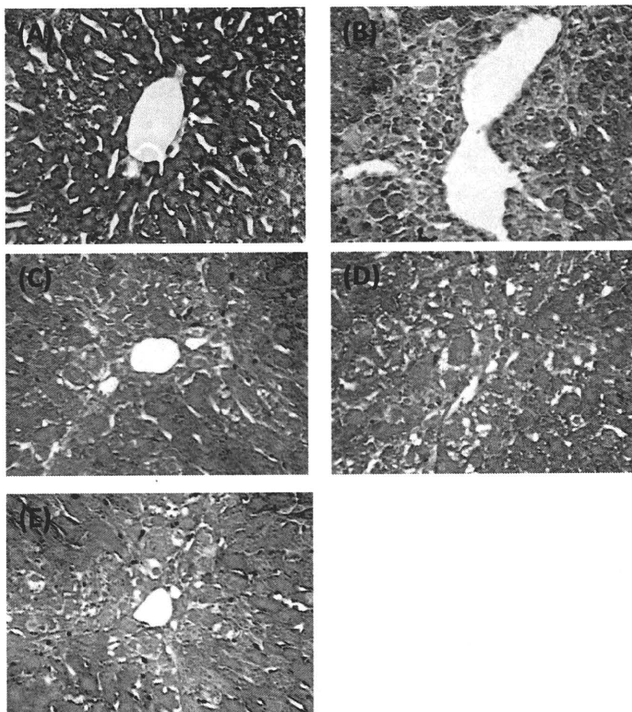

Fig. 3. Azan Staining of Liver Sections

Livers were excised from normal mice (A), CCl_4-injured control mice (B), L.E.M.-treated mice (C), syringic acid-treated mice (D), and vanillic acid-treated mice (E). Original magnification ×400.

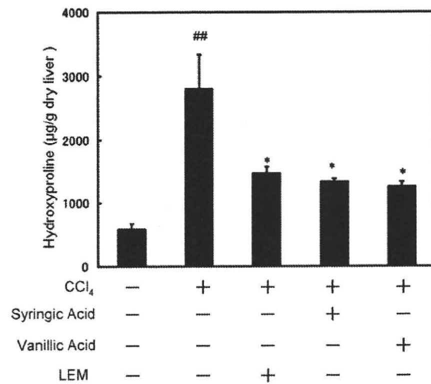

Fig. 4. Effect of Syringic Acid and Vanillic Acid on the Hydroxyproline Content of the Liver

The hydroxyproline content of the liver was measured after 4 weeks of treatments. The values are mean±S.D. ($n=4$). The data were analyzed by Student's t-test (## $p<0.01$, as compared to uninjured control mice) and Dunnett's method (* $p<0.05$, as compared to CCl_4-injured control mice).

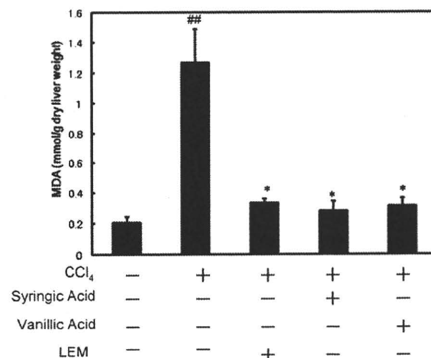

Fig. 5. Effect of Syringic Acid and Vanillic Acid on the Suppression of Oxidative Stress

The malondialdehyde content of the liver was determined after 4 weeks of experiments. The values are mean±S.D. ($n=4$). The data were analyzed by Student's t-test (## $p<0.01$, as compared to uninjured control mice) and Dunnett's method (* $p<0.05$, as compared to CCl_4-injured control mice).

measured the hepatic hydroxyproline content, which parallels the extent of fibrosis. After 4 weeks of CCl_4 treatment, the hepatic hydroxyproline content increased 4.6-fold as compared with the controls (Fig. 4). The intravenous administration of syringic acid or vanillic acid significantly decreased the hepatic hydroxyproline content. These data suggest that syringic acid and vanillic acid can suppress hepatic fibrosis in chronic liver injury. Next, we measured the amount of malondialdehyde in the liver samples as a marker of oxidative stress. The malondialdehyde content was drastically increased after 4 weeks of CCl_4 treatment, but the intravenous administration of syringic acid or vanillic acid significantly decreased the malondialdehyde content to an almost normal level (Fig. 5). The protective effects of syringic acid and vanillic acid were almost comparable to that of L.E.M. (Figs. 2—5).

***In Vitro* Effect on HSC Activation and Hepatocyte Viability** We examined the direct effect of syringic acid and vanillic acid on the activation of HSCs, which play a central role in liver fibrogenesis, using the monolayer culture. HSCs are activated during the monolayer culture to transform into proliferating myofibroblast-like cells. As shown in Fig. 6A, HSCs were activated after 7 d of culture to be fibroblastic cell-type. The addition of syringic acid or vanillic acid dose-dependently suppressed the activation (Fig. 6B). HSCs maintained their quiescent state by the addition of more than 0.5 mg/ml of the respective compound. Next, the effect of syringic acid and vanillic acid on gene expression of Type I collagen and α-SMA, which are markers of activated HSCs, was examined. HSCs were cultured for 7 d in the presence or absence of syringic acid or vanillic acid, and the gene expression was analysed by RT-PCR. As shown in Fig. 7, syringic acid and vanillic acid remarkably suppressed the expression of collagen and α-SMA genes, indicating that the phenolic compounds directly act on HSCs and suppress the activation

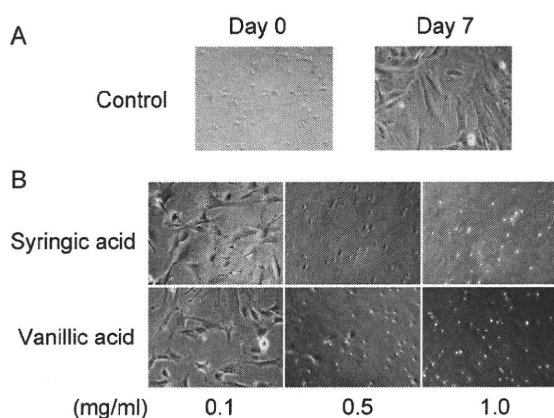

Fig. 6. Phase-Contrast Micrographs of Cultured HSCs

Freshly isolated HSCs were cultured for 7 d in the absence (A) and presence (B) of syringic acid or vanillic acid at the indicated concentration. Original magnification ×200.

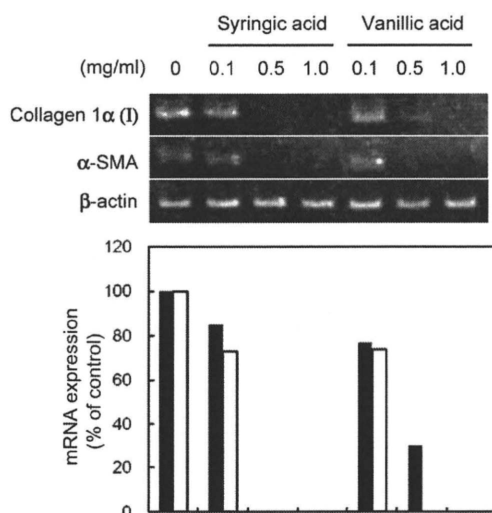

Fig. 7. RT-PCR Analysis of Gene Expression Relating to HSC Activation

Bottom figure shows the relative expression of collagen 1 α(I) (closed bar) and α-SMA (open bar) compared with the non-addition control.

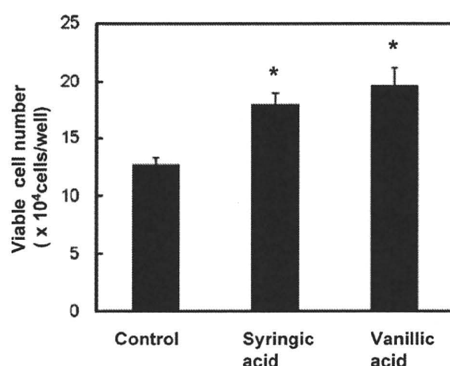

Fig. 8. Effect of Syringic Acid and Vanillic Acid on Viability of Cultured Hepatocytes

Freshly isolated hepatocytes were cultured for 24 h in the absence or presence of 1.0 mg/ml syringic acid or vanillic acid. Viability was measured by trypan blue exclusion test. The data were analyzed by Dunnett's method ($* p<0.05$, as compared to control).

to maintain the quiescent state. We then examined the effect of the compounds on liver parenchymal hepatocytes using the primary culture (Fig. 8). Hepatocytes were isolated and cultured in the presence or absence of syringic acid or vanillic acid. After 24 h of culture, viable cells were counted using trypan blue exclusion test. The addition of syringic acid or vanillic acid significantly maintained viability of cultured hepatocytes. These results suggested that syringic acid or vanillic acid might suppress liver fibrogenesis and inflammation by inhibiting HSC activation and protecting hepatocytes, respectively in chronically liver injured mice.

DISCUSSION

The physiological functions of plant-derived phenolic compounds have been extensively reported.[7,13,14] Syringic acid and vanillic acid possess antimicrobial, anti-cancer, and anti-DNA oxidation properties.[15—17] We recently found that syringic acid and vanillic acid could act as immunomodulators in mice with ConA-induced liver injury.[8] In the present study, we show that syringic acid and vanillic acid have protective effects in mice with CCl_4-induced liver injury. Both phenolic compounds dramatically suppressed liver fibrogenesis in the chronic CCl_4-treatment model. When these phenolics are orally administered to hamsters, they are adsorbed and appear in the blood within 40 min.[18] Although these compounds are intravenously administered in the present study, oral administration could also elicit the hepatoprotective effect. The syringic acid and vanillic acid contents in L.E.M. are 450 and 378 μg/g, respectively. Thus, the contents are relatively small, but these compounds are commercially available at low prices. Therefore, syringic acid and vanillic acid might be promising oral agents for the prevention of liver disease.

We evaluated the hepatoprotective effect of phenolic compounds in mice with CCl_4-induced liver injury. After intravenous administration, CCl_4 is introduced into the liver, where it is toxic to hepatocytes. Cytochrome P-450 in the endoplasmic reticulum of hepatocytes catalyzes the dehalogenation to produce an unstable complex trichloromethyl radical,[19] resulting in the extensive necrosis of hepatocytes that leads to liver inflammation. In the present study, the transaminase level in the serum was drastically increased by CCl_4 treatment. Generation of ROS degrade polyunsaturated lipids to form malondialdehyde, which is a marker of oxidative stress. The chronic CCl_4 treatment significantly increased the malondialdehyde content of the liver. Syringic acid and vanillic acid clearly suppressed the transaminase and malondialdehyde levels in CCl_4-treated mice. Since both of these compounds have 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity,[8] the suppression of ROS generation appears to be responsible for the hepatoprotective effect. Moreover, the CCl_4-induced liver fibrogenesis was suppressed by the administration of syringic acid and vanillic acid. The activation of HSCs is responsible for the development of liver fibrosis.[20,21] During liver injury with persistent inflammation, HSCs are activated to differentiate into proliferating myofibroblast-like cells and overproduce extracellular matrix, leading to fibrogenesis. Since HSCs are activated spontaneously during cell culture,[22] we examined the effect of syringic acid and vanillic acid on the activation of primary cell cultures of rat HSCs. Both of these compounds clearly inhibited the change from spherical to spindle shape and the expression of α-smooth muscle actin and collagen Type 1α genes, which are the markers of HSC activation. We also

examined the effect of syringic acid and vanillic acid on the maintenance of hepatocyte viability *in vitro*. Both of these compounds significantly maintained the viability of primary cell cultures of hepatocytes. Thus, syringic acid and vanillic acid could directly exert a physiological effect on hepatocytes and HSCs. Both phenolic compounds might affect CCl_4 metabolism to inhibit the generation of cytotoxic trichloromethyl radical in the liver. However, the direct effects of syringic acid and vanillic acid on HSCs and hepatocytes were shown in this study, and the protective effect was also shown in ConA-induced liver injured mice in our previous study.[8] Moreover, these phenolic compounds have strong radical scavenging activity. These results suggest that during the repeated treatment of CCl_4, these compounds could protect hepatocytes and HSCs from CCl_4-induced oxidative stress to suppress liver inflammation and fibrogenesis.

The hot-water extracts from cultured mycelia of *L. edodes* have versatile physiological effects and might contain promising seed compounds for pharmaceutical development. We have shown that syringic acid and vanillic acid have anti-oxidative and immunomodulating activities. In addition to these phenolics, L.E.M. could contain novel compounds with pharmaceutical potential. We are currently trying to isolate bioactive components from L.E.M.

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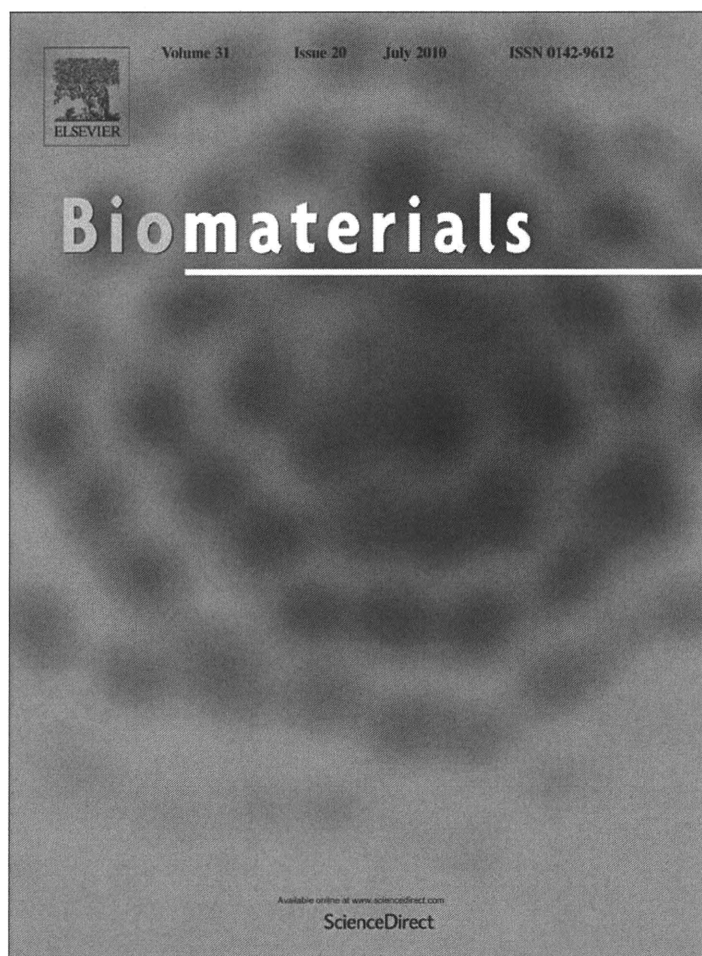

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