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

Ayano ITOH,[a] Katsuhiro ISODA,[a] Masuo KONDOH,[a] Masaya KAWASE,[a] Akihiro WATARI,[a] Masakazu KOBAYASHI,[b] Makoto TAMESADA,[b] and Kiyohito YAGI*,[a]

[a] *Graduate School of Pharmaceutical Sciences, Osaka University; 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan: and* [b] *Research and Development Center, Kobayashi Pharmaceutical Co., Ltd.; 1–30–3 Toyokawa, Ibaraki, Osaka 567–0057, Japan.* Received December 22, 2009; accepted March 16, 2010; published online March 19, 2010

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

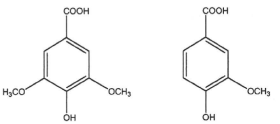
The edible mushroom *Lentinula edodes* (shiitake) contains bioactive compounds that have immune-modulating, antitumor, antibacterial, antiviral, and antiparasitic effects.[1—4] The mycelia of *L. edodes* can be cultured in solid medium, and the hot-water extract (L.E.M.) is commercially available as 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



Syringic acid Vanillic acid

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

* To whom correspondence should be addressed. e-mail: yagi@phs.osaka-u.ac.jp

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

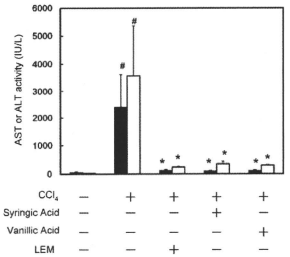


Fig. 2. Effect of Syringic Acid and Vanillic Acid on CCl_4-Induced Chronic Hepatic Injury

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).

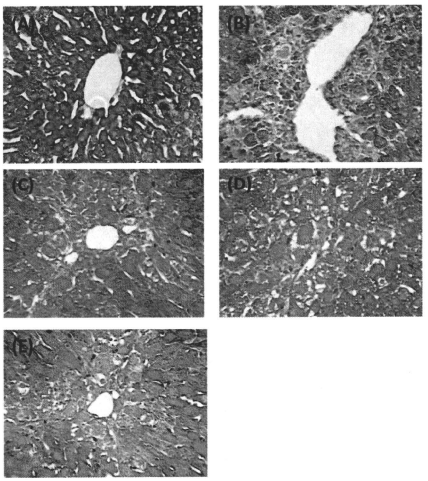


Fig. 3. Azan Staining of Liver Sections

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.

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

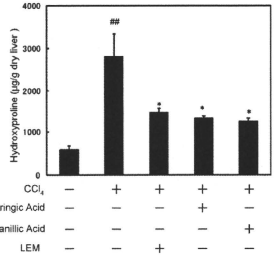


Fig. 4. Effect of Syringic Acid and Vanillic Acid on the Hydroxyproline Content of the Liver

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).

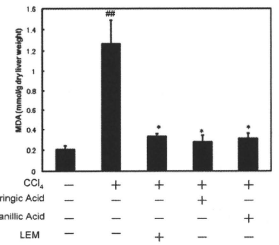


Fig. 5. Effect of Syringic Acid and Vanillic Acid on the Suppression of Oxidative Stress

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).

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

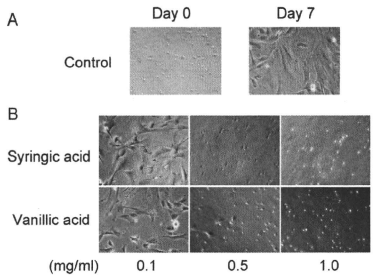


Fig. 6. Phase-Contrast Micrographs of Cultured HSCs

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.

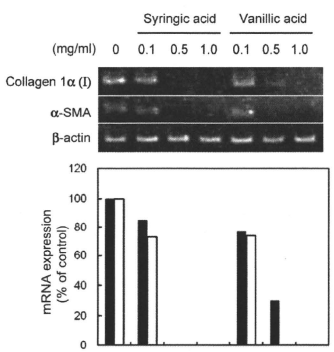


Fig. 7. RT-PCR Analysis of Gene Expression Relating to HSC Activation

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

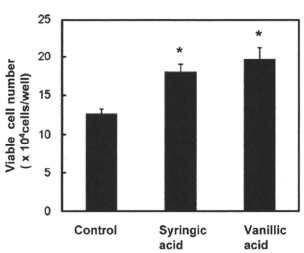


Fig. 8. Effect of Syringic Acid and Vanillic Acid on Viability of Cultured Hepatocytes

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 Iα 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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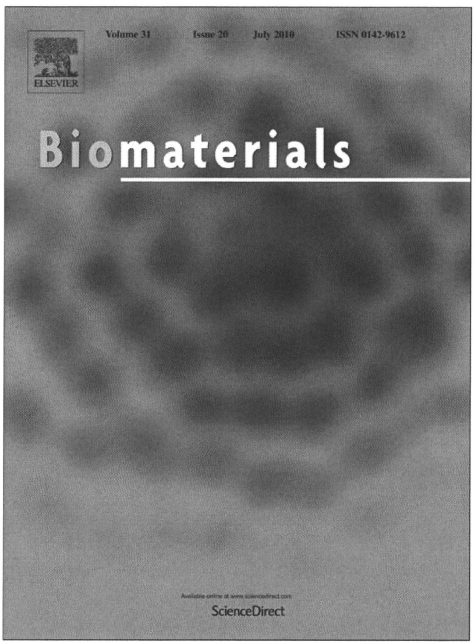

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Mucosal vaccination using claudin-4-targeting

Hideki Kakutani [a], Masuo Kondoh [a,*], Masahiro Fukasaka [a], Hidehiko Suzuki [a], Takao Hamakubo [b], Kiyohito Yagi [a,**]

[a] Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan
[b] Department of Molecular Biology and Medicine, Research Center for Advanced Science and Technology, The University of Tokyo, Meguro, Tokyo 153-8904, Japan

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ABSTRACT

Mucosa-associated lymphoid tissue (MALT) plays pivotal roles in mucosal immune responses. Efficient delivery of antigens to MALT is a critical issue for the development of mucosal vaccines. Although claudin-4 is preferentially expressed in MALT in the gut, a claudin-4-targeting approach for mucosal vaccination has never been developed. In the present study, we found that claudin-4 is expressed in nasal MALT, and we prepared a fusion protein of ovalbumin (OVA) as a model antigen with a claudin-4-binder, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) (OVA-C-CPE). Nasal immunization with OVA-C-CPE, but not a mixture of OVA and C-CPE, induced the production of OVA-specific serum IgG and nasal, vaginal and fecal IgA. Deletion of the claudin-4-binding region in OVA-C-CPE attenuated the induction of the immune responses. OVA-C-CPE immunization activated both Th1 and Th2 responses, and nasal immunization with OVA-C-CPE showed anti-tumor activity in mice inoculated with OVA-expressing thymoma cells. These results indicate that the claudin-4-targeting may be a potent strategy for nasal vaccination.

1. Introduction

Each year, 17 million people die from infectious diseases worldwide, and 7 million people die from cancers worldwide (http://www.globalhealth.org/infectious_diseases/; http://www.reuters.com/article/healthNews/idUSN1633064920071217). Thus, the development of methods to prevent and treat infectious diseases and cancers is an important issue for healthcare worldwide. Vaccination against these diseases is a promising approach because of its low frequency of side effects and its great preventative and therapeutic effects. Vaccination strategies are classified as parenteral or mucosal. Parenteral vaccination is effective for the elimination of infectious cells and cancer cells by the induction of systemic immune responses. Parenteral vaccines are administered by injections, which are invasive, painful, and have low levels of patient compliance; moreover, mucosal immunological defense is not induced. In contrast, mucosal vaccine elicits both mucosal and systemic immune responses, resulting in the prevention of infection on the mucosal surfaces and the elimination of pathological cells [1–3]. Mucosal administration is needle-free, less painful, and has improved patience compliance. Thus, mucosal vaccination appears to be an ideal vaccination strategy, although mucosally administered protein antigens are poorly immunogenic. Various approaches for the mucosal delivery of antigens have been investigated [4–6]. Mucosa-associated lymphoid tissues (MALTs) play pivotal roles in mucosal immunological responses [7,8]. MALTs comprise gut-associated lymphoid tissues (GALT), nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT). MALT contains lymphocytes, M cells, T cells, B cells and antigen-presenting cells (APCs), and the efficient delivery of antigens into MALT is essential for mucosal vaccinations [9]. Indeed, there have been several attempts to deliver antigens to MALT using microparticles, liposomes, saponins or chitosans [4–6].

Immunization at one mucosal surface can generate secretory IgA responses at other mucosal sites. Ideally, vaccination at a single site would provide both humoral and cell-mediated protection, not only

Abbreviations: MALT, mucosa-associated lymphoid tissue; OVA, ovalbumin; C-CPE, C-terminal fragment of *Clostridium perfringens* enterotoxin; OVA-C-CPE, fusion proteins of OVA and C-CPE; GALT, gut-associated lymphoid tissue; NALT, nasopharynx-associated lymphoid tissue; BALT, bronchus-associated lymphoid tissue; APC, antigen-presenting cell; FAE, follicle-associated epithelium; TJ, tight junction; CPE, *Clostridium perfringens* enterotoxin; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BV, budded baculovirus; FBS, fetal bovine serum; TBS, tris-buffered saline; IFN, interferon; IL, interleukin.

* Corresponding author. Tel.: +81 6 6879 8196; fax: +81 6 6879 8199.
** Corresponding author. Tel./fax: +81 6 6879 8195.
E-mail addresses: masuo@phs.osaka-u.ac.jp (M. Kondoh), yagi@phs.osaka-u.ac.jp (K. Yagi).

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at the relevant mucosal surface, but also throughout the body [4]. In this regard, nasal vaccination has shown particular potential. Nasally administered vaccines induced mucosal IgA antibody responses in the salivary glands, respiratory tracts, genital tracts, and intestines [10–12]. The nasal route can also induce cytotoxic T lymphocytes in distant mucosal tissues including the female genital tract [13]. Additionally, nasal immunization produced greater systemic antibody responses than other mucosal immunization routes [12,14]. However, despite these encouraging characteristics, free antigens are usually unable to stimulate immune responses following intranasal administration due to their ineffective delivery to immune response-inducing sites [15]. Thus, the effective delivery of antigens to NALT is needed for the development of a potent nasal vaccine.

A single layer of epithelial cell sheet follicle-associated epithelium (FAE) covers NALT. FAE contains M cells, which are key antigen-sampling cells for the delivery of mucosally encountered antigens to the underlying APCs, and FAE plays a pivotal role in the mucosal immunological response [16–18]. Antigen delivery using a ligand for the FAE that covers NALT would be a potent strategy for the development of a mucosal vaccine. Epithelium has well-developed tight junctions (TJs) that seal the intercellular space on the epithelial cell sheets [19,20]. Occludin, claudin and junctional adhesion molecule are components of TJs [21]. Among these components, claudin-4 was preferentially expressed on the dome region of FAE in GALT [22]. We found that claudin-4 was also expressed in NALT (Fig. 1). These findings strongly indicate that claudin-4-targeting may be useful for mucosal vaccines; however, a mucosal vaccine that uses a claudin-4-binder has never been developed.

Clostridium perfringens enterotoxin (CPE) causes food poisoning in humans [23]. A receptor for CPE is claudin-4, and the C-terminal fragment of CPE (C-CPE) is a claudin-4-binder [24–26]. We previously prepared a claudin-4-targeting cytotoxic molecule by genetically fusing a cytotoxin with C-CPE [27,28]. In the present study, we investigated whether claudin-4-targeting is a potent strategy for mucosal vaccine using C-CPE-fused antigen protein.

2. Materials and methods

2.1. Animals

Female BALB/c mice and C57BL/6 mice (6–8 weeks old) were purchased from SLC, Inc. (Shizuoka, Japan). The mice were housed at 23 ± 1.5 °C with a 12-h light/dark cycle and were allowed free access to standard rodent chow and water. After their arrival, the mice were allowed to adapt to their environment for at least 1 week before the experiments. The animal experiments were performed according to the guidelines of Osaka University.

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total mRNA was extracted from NALT using Isogen (Nippongene, Toyama, Japan), and the mRNA was reverse-transcribed using an RNA PCR kit (AMV, Ver.3.0) according to the manufacturer's instructions (Takara, Kyoto, Japan). The polymerase chain reaction (PCR) amplification from the resultant cDNA was performed using primer pairs for claudin-4 (forward, 5′-tggatgaactgcgtggtg-3′; reverse, 5′-ggttgtagaagtcgcggatg-3′) for 35 reaction cycles (94 °C, 45 s; 52 °C, 60 s; 72 °C, 30 s) or β-actin (forward, 5′-tagatgggcacagtgtggg-3′; reverse, 5′-ggcgtgatggtgggcatgg-3′) for 30 reaction cycles (94 °C, 30 s; 58 °C, 60 s; 72 °C, 30 s). The amplified products were separated by electrophoresis on a 2% agarose gel and visualized with ethidium bromide.

2.3. Immunoblotting for claudin-4

NALT was lysed in a lysis buffer (50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.1% Triton X-100, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride). The lysates (10 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting with anti-claudin-4 (Zymed Laboratory, South San Francisco, CA) or anti-β-actin antibodies (Sigma–Aldrich, St. Louis, MO). The immunoreactive bands were detected with a peroxidase-labeled secondary antibody followed by visualization with a chemiluminescence reagent (Amersham Bioscience, Piscataway, NJ).

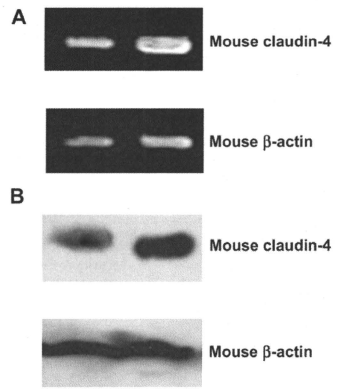

Fig. 1. Expression of claudin-4 in NALT. A) RT-PCR analysis. mRNA was isolated from NALT of mice, and expression of claudin-4 was assayed by RT-PCR. B) Immunoblot analysis. The lysate of NALT was subjected to SDS-PAGE, followed by western blotting with anti-claudin-4 Ab. β-actin was used as an internal control.

2.4. Preparation of OVA-C-CPE fusion proteins

We prepared expression plasmids encoding fusion proteins of OVA with C-CPE or C-CPE303, in which the claudin-4-binding C-terminal 16 amino acids of C-CPE were deleted [29]. Oligonucleotides containing a G4S linker and multiple cloning sites, including KpnI, SpeI, SmaI and PacI sites, were subcloned into NdeI-digested pET16b (Novagen, Darmstadt, Germany), pET-C-CPE and pET-C-CPE303 [30], resulting in pET-MCS and pET-MCS-C-CPEs. OVA cDNA was PCR amplified using pCMV Script/OVA (Kindly provided from Dr. S. Nakagawa, Osaka University, Japan) as a template, a forward primer (5′-gcggtaccatgggctccatcggcgcagc-3′, KpnI site is underlined), and a reverse primer (5′-ccttaattaaaggggaaacacatctgccaa-3′, PacI site is underlined). The resulting OVA fragment was inserted into pET-MCS and pET-MCS-C-CPEs at the KpnI/PacI site, resulting in pET-OVA, pET-OVA-C-CPE and pET-OVA-C-CPE303. The OVA-fusion protein plasmids were transduced into *Escherichia coli* strain BL21 (DE3), and the production of OVA and OVA-C-CPEs was induced by the addition of 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 PMSF, 1 mM 2-mercaptoethanol, and 10% glycerol) supplemented with 8 M urea when necessary. The lysates were applied to HiTrap™ HP (GE Healthcare, Buckinghamshire, UK), and the fusion proteins were eluted with buffer A containing 100–500 mM imidazole. The solvent was exchanged with phosphate-buffered saline (PBS) using a PD-10 column (GE Healthcare), and the purified protein was stored at −80 °C until use. Purification of the fusion proteins was confirmed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue and by immunoblotting with anti-his-tag antibody. Protein assays were performed using a BCA protein assay kit (Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Budded baculovirus (BV) displaying mouse claudin-1 or -4 was prepared as described previously [28]. Briefly, the DNA fragments of claudin-1 or -4 were subcloned into the baculoviral transfer vector pFastBac1 (Invitrogen, Gaithersburg, MD). Recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen). Sf9 cells maintained in Grace's Insect medium containing 10% fetal bovine serum (FBS) at 27 °C were infected with the recombinant baculoviruses. After 70 h, the conditioned medium was recovered and centrifuged. The resultant pellets of the BV fraction were suspended in Tris-buffered saline (TBS) containing protease inhibitor cocktail and then stored at 4 °C until use.

The BV displaying claudins was diluted with TBS and adsorbed to the wells of 96-well ELISA plates (Greiner Bio-One, Tokyo, Japan) overnight at 4 °C. The wells were blocked with TBS containing 1.6% BlockAce (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 2 h at room temperature and the C-CPE, OVA-C-CPE or OVA-C-CPE303 was added. After 2-h incubation, the wells were washed and incubated with anti-his-tag antibody followed by a horseradish peroxidase-conjugated secondary antibody. The immunoreactive proteins were detected using TMB peroxidase substrate at an absorbance of 450 nm.

2.6. Nasal immunization

Mice were nasally immunized with 10-μl aliquots of OVA, a mixture of OVA and C-CPE, OVA-C-CPE or OVA-C-CPE303 at the indicated schedules. The doses of the proteins were equal to 5 μg of OVA and 1.89 μg of C-CPE.

2.7. OVA-specific antibody production

Seven days after the last immunization, serum and mucosal secretions (nasal washes, vaginal washes, and fecal extracts) were collected. Fecal pellets (100 mg) were suspended in 1 ml of PBS and extracted by vortexing for 10 min. The samples were centrifuged at 3000×*g* for 10 min, and the resultant supernatants were used as fecal extracts. Vaginal and nasal mucosa were washed with 100 or 200 μl of PBS, respectively.

The titers of OVA-specific antibody in serum, extracts and mucosal washes were determined by 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 anti-mouse IgG, IgG1, IgG2a or IgA. The OVA-specific antibodies were detected using TMB peroxide substrate. End-point titers were expressed as the dilution ratio, which gave 0.1 above control values obtained for serum of naïve mice at an absorbance of 450 nm.

2.8. Cytokine ELISA

Serum interferon γ (IFN-γ) and Interleukin-13 (IL-13) were measured with an ELISA kit according to the manufacturer's protocol (R&D Systems, Inc., MN).

2.9. Cell cultures

A murine thymoma cell line EL4 (H-2^b) was cultured in RPMI 1640 supplemented with 10% FBS. EG7-OVA cells (OVA-transfected EL4 cells) were maintained in RPMI 1640 containing 10% FBS in the presence of 400 μg/ml of G418.

2.10. Anti-tumor activity

In an anti-tumor assay, female C57BL/6 mice (6–8 weeks) were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, OVA-C-CPE or OVA-C-CPE303 once a week for 3 weeks. All non-vehicle immunizations contained equivalent amounts of OVA (5 μg). Seven days after the last immunization, the mice were subcutaneously inoculated with 1×10^6 EG7-OVA cells. Tumor growth was monitored by measuring two diameters, and the tumor volume was calculated as $a \times b \times b/2$, where a is the maximum diameter of the tumor and b is the minimum diameter of the tumor.

2.11. Statistical analysis

Results were analyzed by an analysis of variance (ANOVA) followed by the Dunnett multiple comparison test, and statistical significance was assigned at $p < 0.05$.

3. Results

3.1. Expression of claudin-4 in NALT

Nasal vaccine is a potent therapy for infectious diseases and cancers since nasal vaccination potentiates humoral and cellular immune responses throughout the body. NALT is the nasal lymphoid tissue, and effective delivery of antigens to NALT is critical for the development of mucosal vaccinations. A previous report showed that claudin-4 is expressed in GALT [22], whereas it is unclear whether claudin-4 is expressed in NALT. To investigate the expression of claudin-4 in NALT, NALT was isolated from mice, and the NALT lysate was subjected to RT-PCR and immunoblotting analyses. As shown in Fig. 1A and B, claudin-4 mRNA and protein were detected in NALT. These data indicate that claudin-4-binder may be a targeting molecule for NALT.

3.2. Preparation of claudin-4-targeting OVA

Claudin has low antigenicity, and there has been little success in the preparation of antibodies against the extracellular region of claudin. C-CPE corresponding to aa 184–319 at the C-terminal of CPE is a claudin-4-binder [24,25]. We previously prepared a claudin-4-targeting cytotoxic molecule genetically fused with C-CPE [27]. To evaluate whether a claudin-4-targeting strategy is an effective method for mucosal vaccination, we genetically fused C-CPE with OVA, a popular model antigen for vaccination, to yield OVA-C-CPE (Fig. 2A). OVA-C-CPE was produced by *E. coli* and purified by affinity chromatography. Purification of the protein was confirmed by SDS-PAGE and immunoblotting (Fig. 2B). The molecular size was identical to the predicted size of 62 kDa for OVA-C-CPE. To evaluate the binding of OVA-C-CPE to claudin-4, we performed ELISA with a claudin-displaying BV-coated immunoplate. OVA-C-CPE or C-CPE was added to wells coated with wild-type BV, claudin-1-BV or claudin-4-BV. The bound proteins were detected using anti-his-tag antibody. Like C-CPE, OVA-C-CPE bound to claudin-4-BV but not wild-type BV or claudin-1-BV (Fig. 2C).

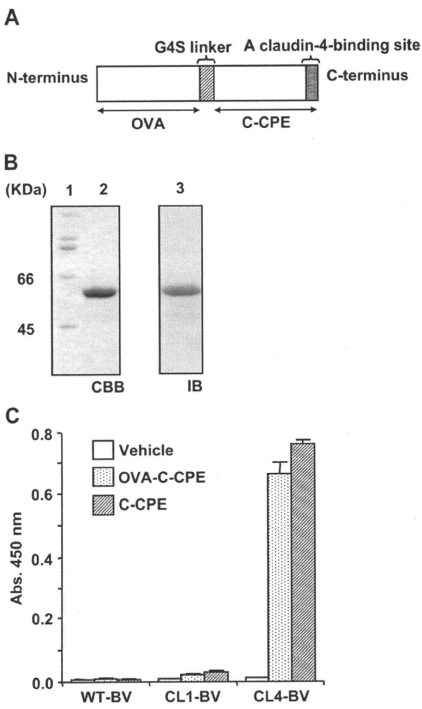

Fig. 2. Preparation of OVA-C-CPE. A) Schematic illustration of OVA-C-CPE. The claudin-4-binding site of C-CPE is located in the C-terminal 16 amino acids [29]. OVA was fused with C-CPE at the N-terminal of C-CPE, resulting in OVA-C-CPE. B) Purification of OVA-C-CPE. OVA-C-CPE was expressed in *E. Coli* as a his-tagged protein and isolated by Ni-affinity chromatography. The purification of OVA-C-CPE was confirmed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB, left panel) and by immunoblotting with an anti-his-tag antibody (IB, right panel). Lane 1: molecular weight marker; lane 2, 3: OVA-C-CPE. The putative molecular mass of OVA-C-CPE is 62 kDa. C) Binding of OVA-C-CPE to claudin-4. Wild-type BV (WT-BV), BV displaying claudin-1 (CL1-BV) or -4 (CL4-BV) was absorbed onto a 96-well immunoplate, and then vehicle, OVA-C-CPE or C-CPE was added to the well. OVA-C-CPE or C-CPE bound to BV was detected by an anti-his-tag Ab followed by horseradish peroxidase-labeled secondary Ab. C-CPE was used as a positive control for a claudin-4-binding. Data are means ± SD ($n = 4$).

3.3. Induction of OVA-specific humoral responses

To clarify whether claudin-4-targeting activates an immune response, we investigated antigen-specific humoral responses at both systemic and mucosal sites in mice that received nasally administered OVA-C-CPE. Mice received an intranasal administration of OVA, a mixture of OVA and C-CPE, or OVA-C-CPE fusion protein once a week for 3 weeks. Seven days after the last administration, we measured the OVA-specific serum IgG, nasal IgA, vaginal IgA and fecal IgA levels. As shown in Fig. 3A, the OVA-specific serum IgG responses were increased in mice immunized with OVA-C-CPE as compared to the mice immunized with OVA or a mixture of OVA and C-CPE. The OVA-specific IgA responses in nasal washes were greater from mice immunized with OVA-C-CPE than from mice immunized with OVA or a mixture of OVA and C-CPE (Fig. 3B). It is a superior character of mucosal vaccination that antigen-specific IgA responses were induced not only at the immunized site but also at remote mucosal surfaces [4]. As shown in Fig. 3C and D, nasal immunization with OVA-C-CPE activated vaginal and fecal OVA-specific IgA responses. The OVA-specific IgA responses did not occur in mice immunized with a mixture of OVA and C-CPE. These data suggest that fusion of OVA with C-CPE is critical for successful nasal vaccination.

We previously found that the C-terminal 16 amino acids of C-CPE are essential for claudin-4-binding [29]. To investigate the involvement of claudin-4 in OVA-specific humoral responses in mice nasally immunized with OVA-C-CPE, we prepared OVA-C-CPE303, in which the claudin-4-binding region was deleted (Fig. 4A). Deletion of the 16 amino acid region attenuated the claudin-4-binding of OVA-C-CPE (Fig. 4B). OVA-specific serum IgG and nasal, vaginal and fecal mucosal IgA responses were also attenuated in mice immunized with OVA-C-CPE303 (Fig. 4C and D, 4E and F, respectively). No histological mucosal injury was found after nasal immunization with OVA-C-CPE (data not shown). These findings indicate that claudin-4-targeting may be involved in nasal vaccination by OVA-C-CPE.

3.4. Induction of Th1 and Th2 responses by OVA-C-CPE

Nasal immunization of antigen induced antigen-specific immune responses including Th1- and Th2-type responses [31,32]. We next investigated whether nasal immunization with OVA-C-CPE evoked Th1- or Th2-type responses. The OVA-specific IgG1 (a Th2 response) and IgG2a (a Th1 response) responses in the serum of mice nasally immunized with OVA-C-CPE were significantly enhanced compared to those of mice immunized with OVA alone or a mixture of OVA and C-CPE (Fig. 5A). Measurement of Th1 (IFN-γ) and Th2 (IL-13)-specific cytokines in splenocytes isolated from mice nasally immunized with OVA, a mixture of OVA and C-CPE, or OVA-C-CPE showed that nasal immunization with OVA-C-CPE increased both Th1 and Th2 cytokine production (Fig. 5B). Th1 and

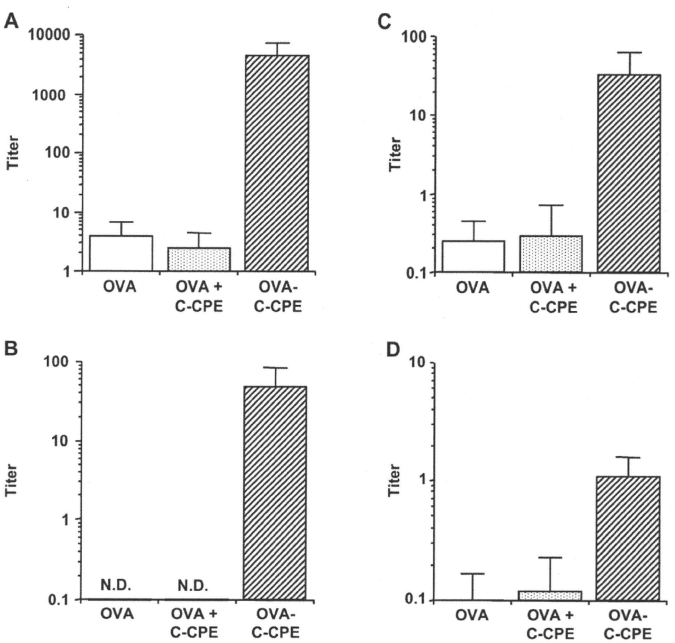

Fig. 3. Production of OVA-specific IgG and IgA by OVA-C-CPE. Mice were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, or OVA-C-CPE (5 μg OVA) once a week for 3 weeks. Seven days after the last immunization, the levels of serum IgG (A), nasal IgA (B), vaginal IgA (C) and fecal IgA (D) were determined by ELISA. Data are means ± SD ($n = 4$). The results are representative of three independent experiments. N.D., not detected.

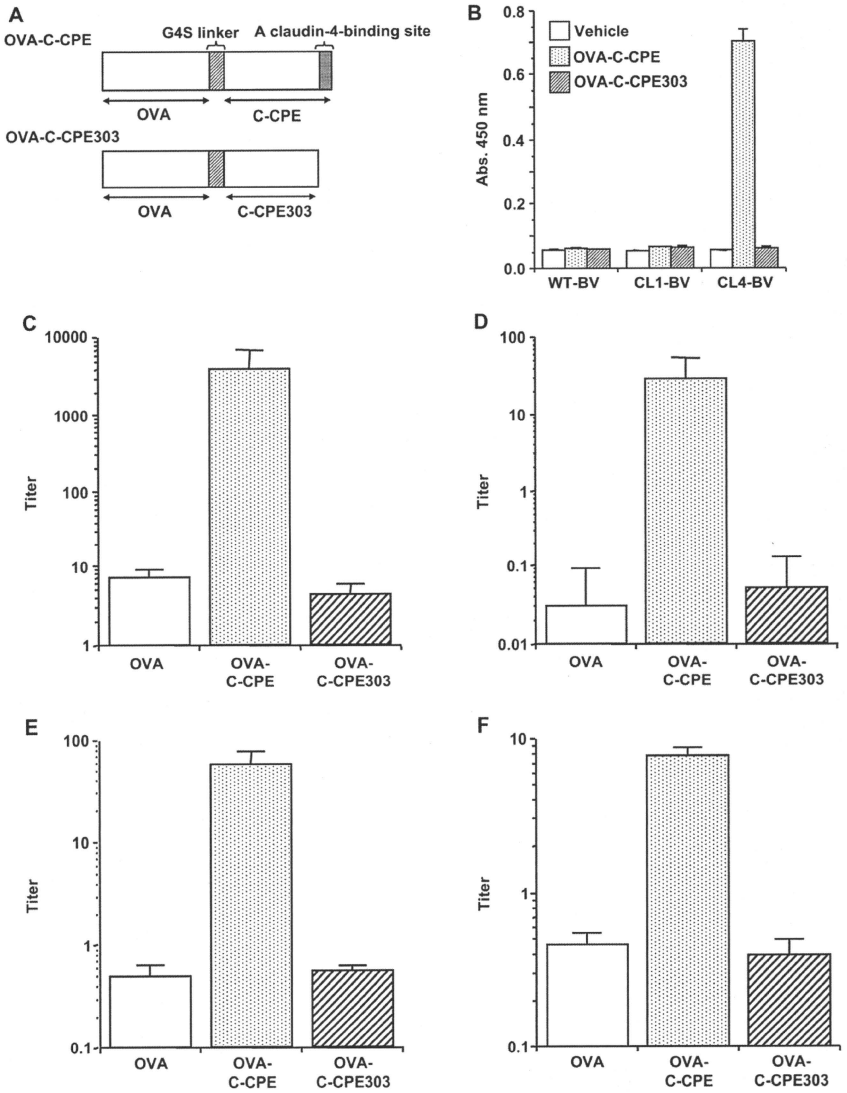

Fig. 4. Involvement of claudin-4 in the immune responses to OVA-C-CPE. A) Schematic illustration of OVA-C-CPE mutant. The C-terminal 16 amino acid-deleted C-CPE mutant (C-CPE303) did not bind to claudin-4 [29]. To clarify the involvement of claudin-4 in the immune response initiated by OVA-C-CPE, OVA was fused with C-CPE303, resulting in OVA-C-CPE303. B) Interaction of OVA-C-CPE303 with claudin-4. Binding of OVA-C-CPE303 to claudin-4 was investigated by ELISA with wild-type BV (WT-BV), claudin-1 or -4-displaying BV (CL1-BV, CL4-BV). C) Immune responses by OVA-C-CPE303. Mice were nasally immunized with OVA, OVA-C-CPE or OVA-C-CPE303 (5 μg OVA) once a week for 3 weeks. Seven days after the last immunization, the levels of serum IgG (C), nasal IgA (D), vaginal IgA (E) and fecal IgA (F) were measured by ELISA. Data are means ± SD ($n = 4$). Data are representative of three independent experiments.

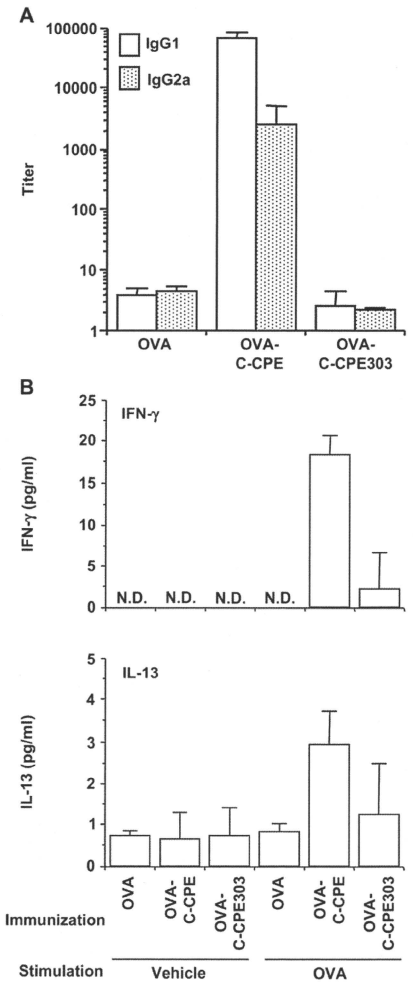

Fig. 5. Th1 and Th2 responses induced by OVA-C-CPE. Mice were nasally immunized with OVA, OVA-C-CPE or OVA-C-CPE303 (5 μg of OVA) once a week for 3 weeks. Seven days after the last immunization, serum and splenocytes were collected. Serum IgG subclass (IgG1 and IgG2a) was determined by ELISA (A). The splenocytes isolated from the immunized mice were stimulated with vehicle or OVA (1 mg/ml) for 24 h, and the cytokines (IFN-γ and IL-13) in the conditioned medium were measured by ELISA (B). Data are means ± SD ($n = 4$). N.D., not detected.

Th2 responses in IgG production and cytokines production were not observed in mice nasally immunized with OVA-C-CPE303 (Fig. 5A and B). These data indicate that claudin-4-targeting may be a potent method for mucosal vaccination.

3.5. Anti-tumor immune response induced by the claudin-4-targeting vaccine

To evaluate the immune responses induced by nasal vaccination with OVA-C-CPE, we performed an in vivo anti-tumor assay with EG7 thymoma cells, which are syngeneic tumor cells derived from OVA cDNA-transfected EL4 thymoma cells [33]. C57BL/6 mice were immunized with vehicle, OVA, a mixture of OVA and C-CPE, or OVA-C-CPE once a week for 3 weeks. Seven days after the last immunization, mice were challenged with EG7 thymoma cells. Tumor growth was monitored by calculating the average tumor volume. As shown in Fig. 6A, tumor growth was significantly suppressed in mice immunized with OVA-C-CPE, whereas the tumor growth was not suppressed in mice immunized with OVA or a mixture of OVA and C-CPE. Immunization with OVA-C-CPE303, in which the claudin-4-binding region was deleted, did not induce a protective immune response against tumor challenge. Immunization with OVA or OVA-C-CPE303 did not stimulate Th1- and Th2-immune responses including IgG1, IgG2a, IFN-γ and IL-13 production; whereas immunization with OVA-C-CPE stimulated these immune responses (Fig. 6B and C). These data indicate that nasal immunization with a claudin-4-targeting vaccine may be useful for cancer therapy.

4. Discussion

Recent progress in vaccine development has provided new insight into vaccine therapies for not only infectious diseases but also cancer, Alzheimer disease and Parkinson disease [3,34]. Mucosal vaccination, such as oral, nasal and pulmonary immunization, has greater therapeutic potential and increased patient comfort as compared to parenteral vaccination. The nasal cavity is the most promising site since it has low enzymatic activity and highly available immunoreactive sites; however, immunoresponses are not stimulated by intranasal administration of antigens [15,35–37]. Efficient delivery of antigens to NALT is critical for the development of nasal vaccines. In the present study, we found that intranasal immunization with antigen fused with a claudin-4-binder, C-CPE, stimulated humoral and mucosal immune responses and that these immune responses did not occur when the claudin-4-binding domain was deleted.

How does OVA-C-CPE activate immune responses? Claudin plays a pivotal role in the TJ-barrier in epithelium [38]. We previously found that C-CPE modulates the claudin-4 barrier and enhances mucosal absorption of dextran [30]. Activation of immune responses by OVA-C-CPE may be caused by modulation of the epithelial barrier in NALT, resulting in the uptake of OVA-C-CPE or its degradable product into NALT. OVA-C-CPE modulated the epithelial barrier in a human intestinal model of Caco-2 monolayer cells (data not shown). C-CPE enhanced jejunal absorption of dextran with a molecular mass of 4–20 kDa, and the integrity of the epithelial barrier in nasal mucosa was similar to that in jejunal mucosa [30,39]. OVA-C-CPE, which has a molecular mass of 62 kDa, may be poorly absorbed by nasal tissue. When OVA-C-CPE is degraded into fragments with a molecular mass of less than 20 kDa, the OVA fragment might be absorbed across nasal epithelium. A mixture of OVA and C-CPE did not induce an immune response, and deletion of the claudin-4-binding region in OVA-C-CPE attenuated the immune responses caused by nasal immunization with OVA-C-CPE. These findings indicate that targeting to claudin-4 rather than modulating the claudin-4 barrier by C-CPE is involved in the immune response to nasal vaccinations of OVA-C-CPE.

What cells are taken up OVA-C-CPE? NALT is covered by a unique epithelial layer known as FAE. Lymphocytes, T cells, B cells and APCs underlie the FAE. Antigen presentation to the immunocompetent cells by FAE is a trigger of mucosal immune responses

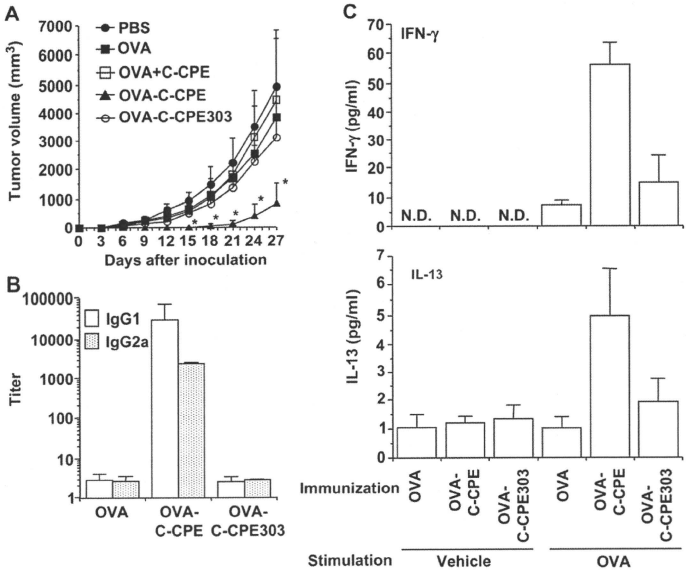

Fig. 6. Anti-tumor activity induced by immunization with OVA-C-CPE in an EG7 cancer model. A) Protective immune response against tumor challenge. C57BL/6 mice were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, OVA-C-CPE, or OVA-C-CPE303 (5 μg of OVA) once a week for 3 weeks. Seven days after the last immunization, the mice were injected s.c. on the right back with 1×10^6 EG7 cells. The tumor volumes were calculated as described in the Materials and methods. Data are means ± SD ($n = 4$). The results are representative of two independent experiments. *Significantly different from the vehicle-immunized group ($P < 0.05$). B, C) Immune responses in the cancer model. Mice were nasally immunized with vehicle, OVA, OVA-C-CPE, or OVA-C-CPE303 (5 μg of OVA) once a week for 3 weeks. Seven days after the last immunization, the serum and splenocytes were recovered. Serum IgG subclass (IgG1 and IgG2a) was determined by ELISA (B). The splenocytes were stimulated with vehicle or OVA (1 mg/ml) for 24 h, and the cytokines (IFN-γ and IL-13) in the conditioned medium were measured by ELISA (C). Data are means ± SD ($n = 4$). N.D., not detected. The results are representative of two independent experiments.

[40,41]. Claudin-4 is expressed in the FAE of MALT [22]. Claudin-4 contains clathrin-sorting signal sequences in its C-terminal intracellular region [42,43]; thus, it may be taken up by clathrin-mediated endocytosis. Indeed, Matsuda et al. (2004) showed the endocytosis of claudins during the remodeling of TJs [44], and a C-CPE-fused molecule was intracellularly taken up [27]. OVA-C-CPE may be taken up into FAE followed by the presentation of antigens to the underlying immunocompetent cells. The FAE is enriched with specialized antigen-sampling epithelial cells known as M cells. M cells form an apparent pocket at the basal membrane site, and this pocket contains T cells, B cells, macrophages and dendritic cells. M cells deliver samples of foreign material by active transepithelial vesicular transport from the lumen directly to intraepithelial lymphoid cells and to subepithelial organized lymphoid tissue [6,16,40]. An antigen delivery system to M cells has been developed, and ligands for M cells, including a lectin, a peptide or a specific antibody, have been used for mucosal vaccination [45–48]. It has not been determined if claudin-4 is expressed in M cells and if OVA-C-CPE is taken up into M cells. Further investigation to clarify the mode of action of the claudin-4-targeting vaccine is needed.

Safety is essential for clinical application of the claudin-4-targeting vaccine. Histological injury was not detected after the administration of OVA-C-CPE (data not shown). C-CPE is the receptor-binding domain of CPE without the cytotoxic domain [24,49]. Claudin functions as an epithelial barrier between the inside and the outside of the body, and modulation of the claudin-4 barrier by the claudin-4-binder may cause side effects due to the non-specific influx of xenobiotics through the loosened epithelial barrier. The claudin family contains more than 20 members, and the claudin expression and barrier-function differ among tissues [38,50]. Expression profiles of claudin in the mucosal epithelium also differ among the sites of epithelium [51,52]. To reduce the risk of solute influx, further investigation of the difference in claudin expression between MALT and the other sites is important. Preparation of a claudin binder with less modulation of the epithelial barrier is also needed.

In rodents, NALT is found on both sides of the nasopharyngeal duct dorsal to the cartilaginous soft palate. Humans do not have NALT, except at an early age [53]; but, they possess oropharyngeal lymphoid tissues, including unpaired nasopharyngeal tonsils (adenoids) and bilateral tubular palatine, and lingual tonsils (Waldeyer's ring), which seem to correspond functionally to NALT [7,54]. The expression of claudin-4 in the human MALT, such as the tonsils and adenoids, should be investigated for the development of oral mucosal vaccine.

5. Conclusions

In the present study, we prepared C-CPE-fused OVA, and we found that the intranasal administration of the fusion protein increased not only nasal IgA levels but also OVA-specific serum IgG, vaginal IgA and

fecal IgA levels. Moreover, deletion of the claudin-4-binding region in the fusion protein caused the loss of immunomodulating activities. The claudin-4-targeting antigen immunization activated both Th1 and Th2 responses and showed anti-tumor activity in mice inoculated with OVA-expressing thymoma cells. This is the first report to indicate that claudin-4-targeting may be a promising strategy for the development of mucosal vaccines.

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—Reviews—

肝細胞機能制御を目的とした新規培養システムの開発

八木清仁,* 川瀬雅也,[a] 磯田勝広,[b] 近藤昌夫

Development of Novel Culture System for Regulation of Hepatocyte Function

Kiyohito YAGI,* Masaya KAWASE,[a] Katsuhiro ISODA,[b] and Masuo KONDOH
Graduate School of Pharmaceutical Sciences, Osaka University, 1–6 Yamada-oka, Suita, Osaka 565–0871, Japan

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Cultured hepatocytes are expected to be used for drug screening and bioartificial liver. Since hepatocytes lose their functions very rapidly *in vitro*, many attempts have been made to maintain their viability and functions. First, we want to introduce the surface modification of culture substrate using a starburst dendrimer. Addition of fructose to the terminal of the dendrimer was shown to be effective in maintianing hepatocyte function. As the second topic, we will show results of the use of a three-dimensional carrier for hepatocyte cultivation. Hepatocytes and bone marrow stromal cells were cocultured in silane beads, and packed into a radial flow-type bioreactor. The perfusion culture showed the effectiveness of bone marrow stromal cells for the maintenance of hepatocyte function. The next topic will be the trial of adenoviral gene transfer into hepatocytes. Thioredoxin gene was chosen because the products play important roles in redox control and antiapoptosis. The introduction of the gene could inhibit apoptosis and maintain the hepatocyte viability. Finally, we want to introduce the results on differentiation of stem cells into hepatocytes, because it is very difficult to obtain sufficient number of human hepatocytes. Human mesenchymal stem cells were cultured in the presence of several protein factors and the hepatocyte-specific marker was expressed after 2 weeks of induction culture. The use of human stem cells could be an important strategy for the support of a drug development system.

Key words——dendrimer; hepatocyte; radial flow-type bioreactor; mesenchymal stem cell

1. はじめに

培養肝細胞は医薬品開発のスクリーニング系やバイオ人工肝臓への応用が期待されているがその機能は viability の低下に伴い急速に消失していくため，機能維持を目的とした研究が活発に行われている．本総説ではこれまで我々が検討してきた培養基材の表面修飾，3次元培養，遺伝子導入，肝細胞の分化誘導について紹介する．足場依存性の細胞は培養基材の性質によって viability が著しく変動することが知られており，機能維持の成否は優れた培養基材の開発に依存していると言っても過言ではない．そこで機能性材料として注目されている樹木状高分子デンドリマーを用いた基材表面修飾の検討において，デンドリマーの末端にフルクトースを付加すると培養肝細胞の生存性，機能が良好に維持されたことを示す．また培養用ディッシュを用いた2次元培養では応用範囲が限られるため培養工学的検討として多孔性担体を用いた3次元培養の試みを紹介する．さらに細胞を接着させた3次元担体をラジアルフロー型バイオリアクターに充填し灌流培養を行った検討において，肝細胞と骨髄間質細胞との共培養が機能維持に効果的であったことを示す．次に遺伝子工学的アプローチとしてアデノウィルスベクターを用いてチオレドキシン遺伝子を導入することにより，肝細胞のアポトーシスを制御することが可能であったことを紹介する．最後に，細胞源に関する検討として再生医工学的アプローチによりヒト由来間葉系幹細胞を肝細胞へ誘導する試みを紹介し創薬支援システムへの応用について考察したい．

大阪大学大学院薬学研究科（〒565–0871 吹田市山田丘1–6）
現所属：[a]長浜バイオ大学バイオサイエンス学科（〒526–0829 長浜市田村町1266），[b]帝京平成大学薬学部（〒290–0193 市原市潤井戸2289）
*e-mail: yagi@phs.osaka-u.ac.jp
本総説は，日本薬学会第129年会シンポジウム S24 で発表したものを中心に記述したものである．

2. 機能性培養基材による肝機能制御

1985年に Tomalia らによって報告されたデンド

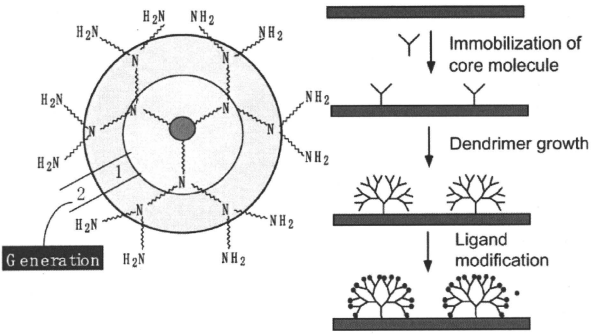

Fig. 1. Structure of Polyamidoamine Dendrimer and Application for Cell Culture

リマーは樹木状多分岐高分子であり,[1]様々な領域でその応用が検討されている．直径は約数十 nm の分子であり，Fig. 1 で示すように，中心部分のコア，骨格分子，末端アミノ基から構成され，正確な分子設計が可能である．デンドリマーは規則的な枝分かれ構造を有する分子で，中心のコア分子から，段階的に伸長反応を行うことで枝分かれ数を増加させることができる．また，伸長反応を繰り返すことにより最外部の密度が高くなり，内部の密度が低くなっている．Figure 1 に示すようなポリアミドアミンデンドリマーは末端アミノ基部分の外表面が正に帯電しており，反応性が高い特徴を有している．このような特徴を活かして，様々な分野でデンドリマーの研究が行われている.[2-5] 医療分野へのデンドリマーの応用としては，内部の密度が粗であることを利用して，デンドリマー内部に薬物や遺伝子の封入，また外部の反応性の高さを利用して外部固定を行っている．デンドリマーにアンチセンス遺伝子を導入，またデンドリマー外部に薬物を固定化することによる薬物の徐放化の検討も行われている．

このようなデンドリマーの特徴を活かし，筆者らはデンドリマーにリガンド分子を結合させ，肝細胞培養基材とする方法を考案した．Figure 2 に示すようにカリウム *tert*-ブトキシドを用い基材表面にヒドロキシル基を導入し，グルタルアルデヒドを介してデンドリマーの固定化を行った．世代増加反応はこの反応を繰り返すことにより行い，最後に末端アミノ基へリガンド分子を結合させた．

筆者らはこれまでにキトサンゲル上で肝実質細胞を培養することに成功しており，特にキトサンの分子内アミノ基をフルクトースにより修飾したフルクトースキトサン上では未修飾のキトサンゲルよりも多くの細胞が接着し，肝特異的機能を維持することを報告した．そこでフルクトースに注目し，フルクトースをリガンドとしたフルクトースデンドリマーについて検討した．細胞非接着性のポリスチレンプレートにデンドリマーを固定化し，リガンドとしてフルクトースを修飾した．その結果，デンドリマーの世代数増加に伴い，修飾されたフルクトースも増加することを確認した．フルクトースデンドリマー上で数日培養を行うと成長因子などの添加なしに，細胞が高機能化すると言われるスフェロイド（球状組織体）を形成した.[6] しかし，ここではスフェロイドの接着性が弱く，さらに接着性を上げる必要が生じた．そこで，リガンドとしてフルクトースと，肝細胞表面に存在するアシアロ糖タンパクレセプターのリガンドとなるガラクトースの混合溶液をリガンド溶液とし，共固定した F/G デンドリマーを用いたところ，スフェロイドの接着が維持された．この，F/G デンドリマー上で培養したラット初代

八木清仁

大阪大学大学院薬学研究科教授．1981 年大阪大学大学院博士後期課程修了（薬学博士）．1982–1984 年米国メリーランド大薬学部，NIH（NIEHS）で博士研究員として勤務．1983 年大阪大学薬学部助手．1992 年同助教授，2000 年 3 月より現職．現在 C 型肝炎など肝疾患を対象とした創薬研究に取り組んでいる．

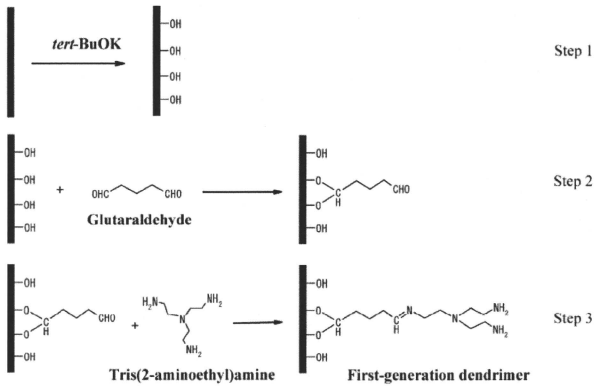

Fig. 2. Schematic Illustration of Dendrimer Immobilization onto the Surface of Polystyrene Plate

肝細胞は，リガンドがフルクトース，ガラクトース単独のものに比べ，肝特異的機能であるウレア合成能が向上し，アルブミン遺伝子の発現も維持しており，機能維持についても優れていることが確認できた．また，共焦点レーザー顕微鏡による解析から，リガンドのないデンドリマー上のスフェロイド内部の細胞はアポトーシスを起こしているのに対し，F/G デンドリマー上のスフェロイドではアポトーシスが抑制されていることを確認した.[7]

リガンド修飾デンドリマーを基材表面上に固定し，細胞培養に用いるアイデアは筆者ら独自のものである．これまでの検討から，細胞毎に異なるリガンドを用いることで，リガンド修飾デンドリマーによって最適な細胞培養表面の創出が可能であることを示してきた．大阪大学基礎工学研究科の田谷正仁教授のグループは筆者らとの共同研究においてデンドリマーの密度を変化させることにより軟骨細胞の形態，分化機能を制御することに成功した.[8,9] また D-グルコースと Epidermal growth factor をリガンドとして用いると細胞の増殖及び運動性を亢進できることを報告している.[10] さらに大阪大学医学系研究科の宮崎純一教授のグループは D-グルコースをデンドリマーにより培養表面に提示すると ES 細胞の未分化能が有意に維持されることを見い出している.[11] このようにリガンド修飾デンドリマーは，細胞毎に最適化したカスタムメイドの培養基材表面創出のツールとなり，組織工学全般の発展に大きく貢献することが期待される．

3. 3 次元培養による肝機能制御

ラジアルフロー型バイオリアクター（RFB）は一般に円筒形のリアクター内に細胞接着用の担体を充填し円筒周囲より培養液あるいは血漿が中心部に向けて流れる構造をしている．RFB は従来のリアクターに比べ，灌流液の流速による剪断力が弱いため細胞障害が少なく，酸素や栄養物の供給がより均一に行われることが知られている．これまで動物細胞が 1×10^8 cells/ml 以上の高密度で培養可能であることが示されている.[12] 筆者らは RFB をバイオ人工肝臓に応用することを目的とし多孔質ガラスビーズ（シランビーズ）を担体として肝細胞の灌流培養を行った．Figure 3 に RFB を用いた培養システム図を示した．筆者らは骨髄間質細胞を肝細胞の生存性を延長し機能を強化するための支援細胞として選択した．骨髄間質細胞は骨髄においてコラーゲン，フィブロネクチンを始めとする細胞外マトリクスや種々の増殖因子を産生し，造血幹細胞や血球系の細胞の維持に重要な役割を演じていることが知られている．プラスティックディッシュによる 2 次元培養の実験より骨髄由来の間質細胞が肝細胞の機能を維持する効果があることを既に明らかにしており，[13] その効果が 3 次元担体を用いた RFB において発現するか否かを検討した．

実験は SD 系雄性ラット由来の細胞を用いて行った．肝細胞は生体内においては肝再生時旺盛に増殖

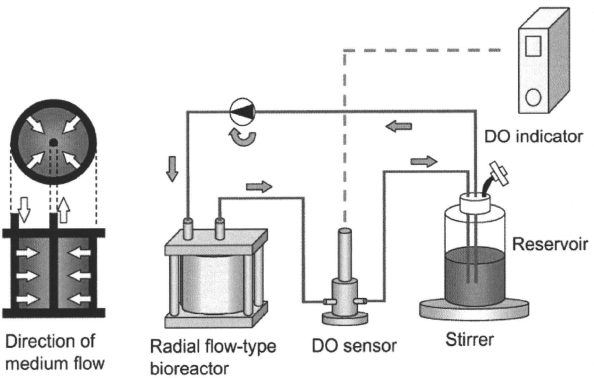

Fig. 3. Perfusion Culture of Hepatocytes by Radial Flow-type Bioreacor

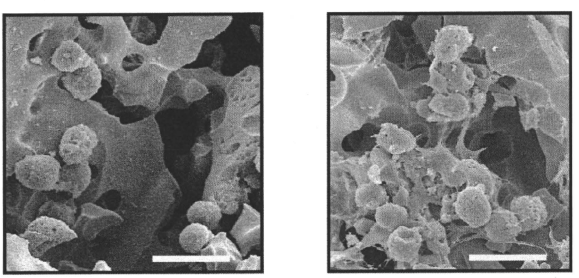

Fig. 4. Electron Micrographs of Hepatocytes Cultured in Silan Beads
White bar indicates 50 μm.

するが *in vitro* では増殖させることは困難であるため初期接着した細胞数を維持することが重要となる．一方骨髄間質細胞は *in vitro* で活発に増殖するため，共培養時には最初に骨髄間質細胞を播種し担体上でサブコンフルエントに達するまで培養した後に肝細胞を播種した．Figure 4 にシランビーズを用いた肝細胞単独培養，骨髄間質細胞との共培養を行った際の電子顕微鏡写真を示した．単独培養では直径約 15–20 ミクロンの球状をした肝細胞がシランビーズ上に接着している様子が観察される．一方共培養においてはシランビーズ上に伸展した骨髄間質細胞に肝細胞が接着していた．そしてそれぞれのシランビーズを充填した RFB を用いて 4 日間の連続潅流培養を行った．その間 24 時間おきにサンプリングを行い，肝特異機能である尿素合成能を評価した．その結果，対照として行った肝細胞単独の 2 次元培養では 4 日間で機能は約 9％に低下したが，RFB の 3 次元培養においては 29％までの低下にとどまった．したがって RFB による潅流培養の効果が示された．さらに骨髄間質細胞との共培養により 47％の機能が有意に維持され，肝細胞に対する効果が 2 次元培養のみならず RFB を用いた 3 次元培養においても発現することが明らかとなった．骨髄間質細胞は培養によって増幅することが可能でありヒト由来，あるいは患者自身の細胞を将来使用することも視野におくとバイオ人工肝臓の機能を強化し得

る細胞源として有望と考えている．さらには骨髄細胞中には肝細胞へ分化可能な間葉系幹細胞（MSC）が存在することから患者由来の細胞を利用した薬物代謝評価系の構築も可能であり個の医療への応用も期待される．

4. 遺伝子導入による肝機能制御

これまで肝がん細胞にアンモニア代謝，薬物代謝に係わる個々の遺伝子を導入する試みは国内の他グループにより報告されている．[14,15] われわれは人工肝臓が担うべき数百という肝機能を考慮し細胞全体をグローバルに活性化し，かつ細胞死に対する抵抗性を付与することを目的として遺伝子導入を試みている．

チオレドキシンと呼ばれるタンパク質はリボヌクレオチドリダクターゼの生理的還元剤として発見されたが酸化的ストレスやアポトーシスに対して抵抗性を付与するという機能が報告され注目を浴びている．[16,17] われわれはこのチオレドキシン遺伝子を肝細胞へ導入することにより生体外において引き起こされるストレス及びアポトーシスに対し抵抗性を獲得させることを試みた．肝細胞は生体外では増殖が困難であること，そして遺伝子導入効率を考慮しアデノウィルスをベクターとして用いることとした．ヒトチオレドキシン遺伝子を挿入した組換えアデノウィルスを作成しラット肝細胞へ感染させた．ヒトチオレドキシンが発現していることをウェスタンブロットで確認後，過酸化水素処理に対する抵抗性を調べた．1 mM 過酸化水素で 24 時間処理した後，アポトーシスを起こした細胞数を fluorescence activated cell sorting（FACS）により測定した．コントロールの肝細胞は約 80％がアポトーシスを起こしたのに対し，チオレドキシン遺伝子を導入した肝細胞は約 25％とアポトーシスに対して抵抗性を獲得したことが示された．また，通常のポリスチレンプレートで培養したときの寿命が延長されるか否かを調べたところ明らかな効果が観察された．尿素合成能も同時に維持されチオレドキシン遺伝子導入の有効性が示された．[18] リアクターへ充填する細胞へ当該遺伝子をウィルスベクターを用いて導入することも可能であり，またチオレドキシントランスジェニック動物を作出しその肝細胞をバイオ人工肝臓や医薬品開発のスクリーニング系に適用することも将来可能となるであろう．

5. 肝細胞源の検討

肝細胞源としてはヒトの細胞を用いることが理想的である．再生医療用の細胞源としてこれまで ES 細胞，骨髄細胞などが主に検討されてきたがわれわれは通常廃棄される組織から肝細胞へ分化可能な幹細胞を単離することができれば有用であると考えた．歯科領域では歯髄から MSC が単離されたことが報告されており，[19,20] 抜歯され廃棄される歯に着目した．虫歯の場合，病原菌が含まれ再生医療に適用することは困難であるため，歯科矯正時に抜歯される第 3 大臼歯，通称“親知らず”を用いることとした．矯正時に抜歯されるものは埋伏した状態であり，未分化な歯胚組織が維持されている可能性が高く，分化が進むと象牙質，歯髄となる歯乳頭組織には有用な MSC が存在することが予想された．そこでインフォームドコンセントを得た後，破棄された親知らずより歯乳頭組織を採取し MSC のクローン単離を試みた．

歯乳頭組織をはさみで細かく切断し，コラゲナーゼにより細胞を分散後組織培養用ディッシュに播種し α-MEM を用いて培養を行った．接着性の細胞を回収し FACS を用いて 96 穴プレートの 1 ウェルあたり 1 つの細胞が入るように播種した．単一細胞からコロニー形成したものを継代しさらに増殖させ，2×10^4 cells を分化能の評価に使用し，残りの細胞を凍結保存した．カルセインを利用した骨分化能を指標として幹細胞としての特性を有するクローンの選択を行った結果，コロニー形成能を有するものの約 30％が骨分化能を発現した．その中から特に高い骨分化能を示したクローンを用いて以下の検討を行った．

肝細胞への分化誘導には Hamazaki らの方法[21]に準じ，HGF，デキサメタゾン，ITS に加えて線維芽細胞増殖因子（FGF），オンコスタチン M（OSM）を用いた．培養初期には細長い線維芽細胞用の形態であるが分化誘導を継続するにつれ，2 週間後にはサイズの大きい多角の形態へと変化した．RT-PCR による解析の結果，分化誘導 10 日でアルブミン遺伝子の発現が観察され，逆に初期分化マーカーである AFP 遺伝子発現は減少する傾向にあった．次に肝障害ラットを用いて移植の効果を検討した．

ヒト細胞を移植するため拒絶反応を起こさない免疫不全のヌードラットを使用した．9 週齢のフィッ