発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Akimoto M, Kozako T, Sawada T, Matsushita K, Ozaki A, Hamada H, Kawada H,Yoshimitsu M, Tokunaga M, Haraguchi H, Uozumi K, **Arima N**, and Tei C.	Anti-HTLV-1 Tax Antibody and Tax-specific Cytotoxic T Lymphocyte are Associated with a Reduction in HTLV-1 Proviral Load in Asymptomatic Carriers.	*Journal of Medical Virology*	79	977-986	2007
Arimura K, **Arima N**, Matsushita K, Akimoto M, Park C-Y, Uozumi K, and Tei C.	High incidence of morphological myelodysplasia and aototic bone marrow cells in Behcet's disease.	*Journal of Clinical Immunology*	27	145-151	2007
Owatari S, Uozumi K, Haraguchi K, Ohno N, Tokunaga M, Tokunaga M, Suzuki S, Hanada S, **Arima N.**	A new cytogenetic abnormality, t(2;7)(33;q36), in acute promyelocytic leukemia.	*Cancer Genetics and Cytogenetics*	174	71-74	2007
Akimoto M, Matushita K, Suruga Y, Aoki N, Oaki A, Uozumi K, Tei C, **Arima N.**	Clinical manifestations of human T lymphotrophic virus type I-infected patients with systemic lupus erythematosus.	*Journal of Rheuatology*	34	1841-1848	2007
Ozaki A, **Arima N**, Matsushita K, Uozumi K, Akimoto M, Hamada H, Kawada H, Hourai S, Tanaka Y, and Tei C.	Cyclosporin A inhibits HTLV-1 Tax expression and shows anti-tumor effects in combination with VP-16.	*Journal of Medical Virology*	79	1906-1913	2007

研究成果の刊行物・別刷

International Immunopharmacology (2008) 8, 12–19

www.elsevier.com/locate/intimp

Enhancement of antitumor activity of OK-432 (Picibanil) by Triton X-114 phase partitioning

Masahito Hashimoto[a], Katsuhiro Takashige[a,b], Maiko Furuyashiki[a], Keitaro Yoshidome[a], Ryoko Sano[a], Yutaka Kawamura[c], Shinji Ijichi[c], Hirofumi Morioka[c], Hiroyuki Koide[d], Naoto Oku[d], Yoichiro Moriya[e], Shoich Kusumoto[f], Yasuo Suda[a,*]

[a] *Department of Nanostructure and Advanced Materials, Graduate School of Science and Engineering, Kagoshima University, 1-21-40 Korimoto, Kagoshima 890-0065, Japan*
[b] *Bio-product Technology Research Department, Chugai Pharmaceutical Co., Ltd., Kita, Tokyo 115-8543, Japan*
[c] *Health Service Center, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan*
[d] *Department of Medical Biochemistry, Graduate School of Pharmaceutical Science, University of Shizuoka, Suruga, Shizuoka, 422-8526, Japan*
[e] *Product Research Department, Chugai Pharmaceutical Co., Ltd., Kamakura, Kanagawa 247-8530, Japan*
[f] *Suntory Institute for Bioorganic Research, Shimamoto, Mishima, Osaka 618-8503, Japan*

Received 21 August 2007; received in revised form 27 September 2007; accepted 27 September 2007

KEYWORDS
OK-432;
Picibanil;
Antitumor;
Triton X-114;
Cytokine

Abstract

OK-432 (Picibanil), a Streptococcal immunotherapeutic agent, has been used for immunotherapy of various cancers as a biological response modifier (BRM). However, OK-432 contains multiple components consisting of immunotherapeutic ones and contaminants which may weaken the effects or exert side-effects. In this study, we investigated extraction of contaminants from OK-432 using Triton X-114 (TX-114)–water phase partitioning and examined an antitumor effect of the resulting preparation. OK-432 was subjected to TX-114 partitioning to give residual precipitate designated as OK-TX-ppt. OK-TX-ppt exerted no TLR2-mediated activity, but induced interleukin (IL)-6 in human PBMC. OK-TX-ppt also induced tumor necrosis factor (TNF)-α, IL-10, IL-12, and interferon (IFN)-γ in PBMC. Moreover, IFN-γ-inducing activity of OK-TX-ppt was significantly higher and IL-10 production was lower than that of OK-432. In tumor-bearing mice model, administration of OK-TX-ppt i.p. extended the survival time of Meth-A-bearing mice compared to OK-432. OK-TX-ppt also increased the levels of IL-12 and IFN-γ in mouse spleen cells in vitro. These results indicated that TX-114 partitioning removed some contaminants, which attenuates the antitumor effect, from OK-432 and increase the immunotherapeutic effects of OK-432.

* Corresponding author. Tel./fax: +81 99 285 8369.
E-mail addresses: hassy@eng.kagoshima-u.ac.jp (M. Hashimoto), ysuda@eng.kagoshima-u.ac.jp (Y. Suda).

1567-5769/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.intimp.2007.09.021

1. Introduction

OK-432 (Picibanil) is a lyophilized preparation of low virulent *Streptococcus pyogenes* strain Su treated with H_2O_2 and penicillin and was developed by Okamoto et al. [1]. OK-432 has been used for immunotherapeutic agent for various kind of malignant tumor including head and neck cancer and is reported to be clinically effective [2–7]. OK-432 activates the host immune system and the activities are considered to be responsible for the antitumor effects. OK-432 stimulates immune cells, including macrophages, T cells and natural killer (NK) cells, and induces various cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-12, and interferon (IFN)-γ [8–11]. Since some of the cytokines may induce apoptosis of tumor cells and IL-12 induces development of Th1 cells and IFN-γ production which enhances killer cell activities, the cytokines appear to be necessary to exhibit antitumor activity for OK-432.

Since OK-432 is prepared from whole bacteria, it contains multiple components consisting of immunotherapeutic ones and contaminants which may weaken the effects or exert side-effects. Thus identification of the active components is important to clarify the mechanism of antitumor effects and enhance the therapeutic effects. Okamoto et al. demonstrated that OK-PSA purified from OK-432 with affinity chromatography using monoclonal antibody TS-2, which recognizes an IFN-γ-inducing components in OK-432, is a potent inducer of Th1-type cytokines and shows marked antitumor activity, whereas OK-PTF, a TS-2-unbinding fraction, induces Th2-type cytokines [12,13]. They also reported that OK-DNA, a DNA preparation derived from OK-432, induces Th1-type cytokines and augments killer cell activities [14].

Toll-like receptors (TLRs) are transmembrane proteins and act as receptors for the innate immune system [15]. An individual TLR recognizes its own specific pathogen-associated molecular patterns (PAMP) and activates signal transduction cascades to express cytokines. TLR4 in combination with an adapter molecule MD-2 is characterized as a receptor of a lipopolysaccharide (LPS) in bacterial cell wall [16], TLR2 is used for a recognition of bacterial lipoprotein [17,18], and TLR9 senses a bacterial DNA containing unmethylated CpG motif (CpG DNA) [19].

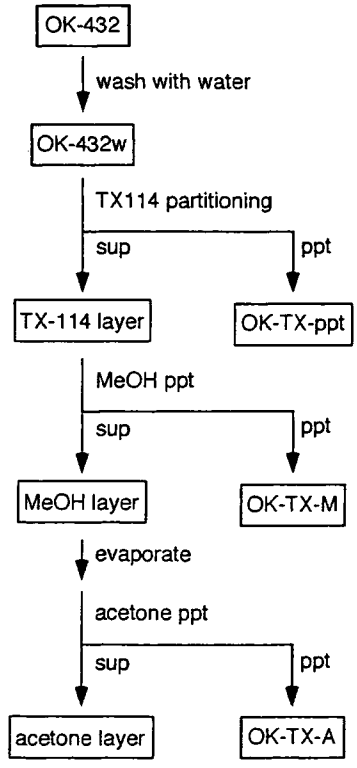
It is well known that activation of TLR9 is responsible for induction of Th1-type response and anticancer immunity [20]. In contrast, mechanisms of Th1/Th2 regulation via TLR2 and TLR4 have been controversial. For TLR4, it was shown that low-level LPS induces Th2 response, whereas high-level LPS results in Th1 response [21]. The TLR signaling pathway was also shown to be crucial [22]. For TLR2, synthetic ligands, Pam_3CSK_4 and FSL-1, were reported to induce Th2 cytokines in mice models of antigen sensitization [23,24], whereas TLR2 was shown to trigger Th1 effecter function [25]. Further, Nucleotide-binding oligomerization domain-2 (NOD2), another receptor for innate immune system, was shown to modulate TLR-mediated Th1/Th2 responses [26,27].

It was reported that some of TLRs are involved in the immune reactions induced by OK-432. OK-PSA-induced anticancer activity was regulated by TLR4, OK-PTF-induced signaling was mediated by both TLR2 and TLR4 [13], and OK-DNA stimulated immune cells via TLR9 [14]. Since OK-432 triggers immune activation via multiple receptors, such as TLR2, TLR4, TLR9 and probably NOD2, mechanisms of Th1/Th2 regulation may be complicated. Among them, TLR9 and probably TLR4 are responsible for the Th1-type response in OK-432. However, participation of TLR2 in Th1/Th2 regulation by OK-432 is unclear. Thus we decided to investigate the effect of TLR2 ligands in OK-432 for the anticancer activity. Recently we separated TLR2-activating fractions from *Staphylococcus aureus* cells by Triton X-114 (TX-114)–water phase partitioning [28]. In the present study, we, thus, depleted TLR2 agonists from OK-432 by TX-114 phase partitioning and the residual components were subjected to immunobiological assay in vitro and in vivo.

2. Materials and methods

2.1. Stimuli

OK-432 (Picibanil) was supplied by Chugai Pharmaceutical Company (Tokyo, Japan) in a frozen (non-lyophilyzed) form. Since OK-432 contained salts and water soluble stabilizers and the amount of these are over 95% of total dry weight, it was washed with water three times to remove such materials to prepare bacterial cells designated as OK-432w (Scheme 1). OK-432w was subjected to TX-114–water phase partitioning [29]. Briefly, OK-432w was suspended in phosphate buffered saline (PBS) and combined with 1/10 volume of 10% aqueous TX-114. The mixture was rotated at 4 °C for 1 h and centrifuged to give supernatant and precipitated cell debris. The debris was washed with methanol twice to obtain residual precipitate designated as OK-TX-ppt. The supernatant was centrifuged at 37 °C to separate TX-114 phase from aqueous one. The upper aqueous phase was treated again with TX-114. TX-114 phase was combined and subjected to 75% methanol precipitation to



Scheme 1.

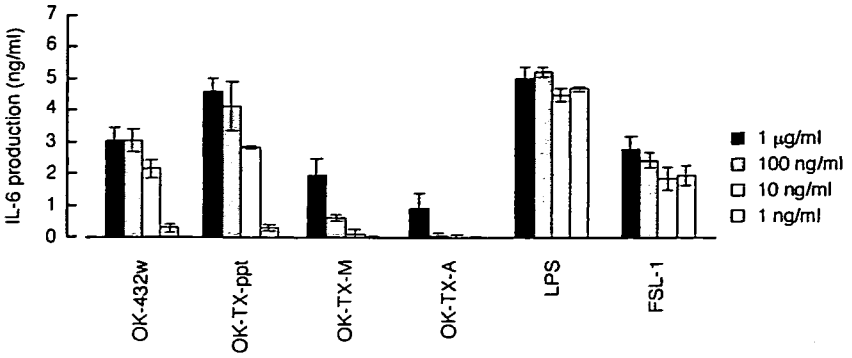


Figure 1 IL-6-inducing activity of the preparations extracted from OK-432 in human PBMC. PBMC were obtained from healthy volunteer (MF). PBMC were incubated with indicated concentrations of stimuli for 24 h. The levels of IL-6 in the culture supernatants were measured by ELISA. The data represent the mean and standard deviation obtained from independent three experiments.

remove TX-114 and give precipitate designated as OK-TX-M. The methanolic phase was evaporated and then subjected to 90% acetone precipitation to give a precipitate designated as OK-TX-A. LPS derived from *Escherichia coli* O55:B5 were purchased from Sigma-Aldrich (St. Louis, MO) and further purified by deoxycholate method to deplete contaminating lipoproteins as described [30]. Synthetic FSL-1 was obtained from EMC Microcollection GmbH (Tübingen, Germany).

2.2. Cytokine assay

Human blood was obtained from healthy volunteers and subjected to Histopaque (Sigma-Aldrich) density gradient separation to prepare human peripheral blood mononuclear cells (PBMC). Cells were placed in 96-well plate at 2×10^5 cells/well and stimulated as above.

Eight-week-old male BALB/c mice were obtained from Kyudo (Kumamoto, Japan). The animals received humane care in accordance

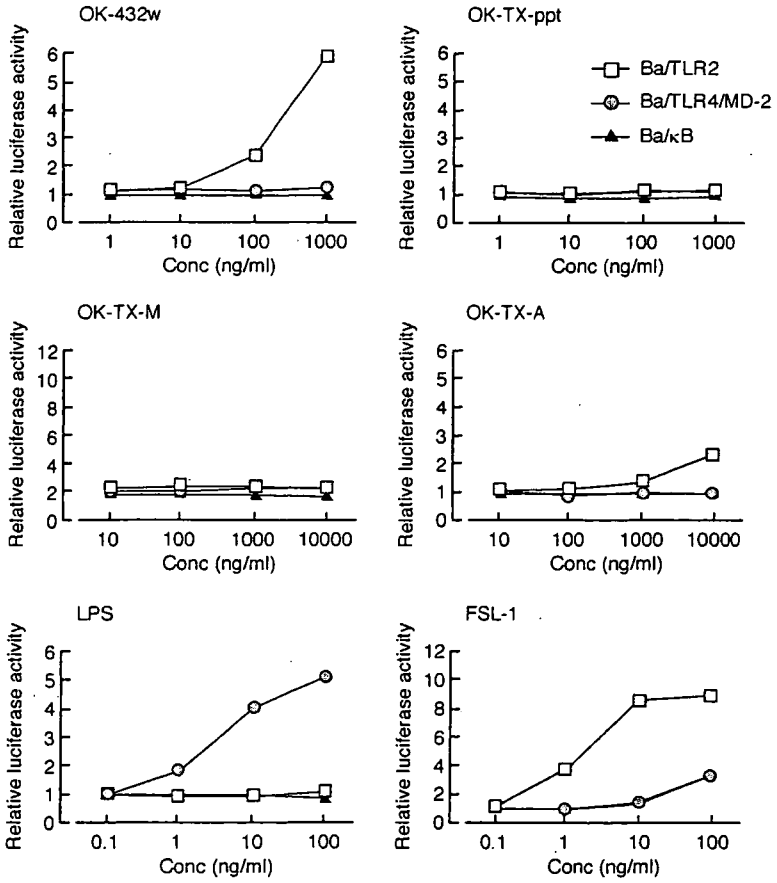


Figure 2 NF-κB activation in Ba/κB, Ba/mTLR2, or Ba/mTLR4/mMD-2 cells induced by the preparations extracted from OK-432. The cells were incubated with indicated concentrations of stimuli for 4 h. NF-κB activation was measured with a luciferase assay. Results are shown as relative luciferase activity, which was determined as the ratio of stimulated to non-stimulated activity.

with our institutional guidelines and the legal requirements of Japan. Mice spleen cells were obtained from healthy BALB/c, suspended in RPMI1640 supplemented with 10% fetal bovine serum (FBS) (Medical & Biological Laboratories, Nagoya, Japan), and distributed in 96-well plate at 1×10^6 cells/well. Cells were stimulated with the indicated concentrations of the test specimens in culture medium supplemented with 10% FBS at 37 °C in humidified air containing 5% CO_2. After incubation, the culture supernatants were collected.

Mice were injected with 500 μg of OK-432w in 500 μl PBS i.p., 500 μg of OK-TX-ppt in 500 μl PBS i.p., or 500 μl of PBS i.p. every other day from day 0 to day 10. On day 11, the mice were sacrificed and sera were collected.

The cell supernatants or sera were subjected to the cytokine assay using an ELISA kit for secreted cytokines (R&D systems, Minneapolis, MN). The concentration of secreted cytokines from cells were determined using a standard curve of recombinant cytokines prepared in each assay. Cytokine concentrations in different experimental groups were analyzed for statistical significance by using Welch's *t* test.

2.3. Luciferase assays

Ba/F3 cells stably expressing p55IgκLuc, an NF-κB/DNA binding activity-dependent luciferase reporter construct (Ba/κB), murine TLR2 and the p55IgκLuc reporter construct (Ba/mTLR2), and murine TLR4/MD-2 and the p55IgκLuc reporter construct (Ba/mTLR4/mMD-2) were kindly provided by Prof. K. Miyake (Institute of Medical Science, University of Tokyo, Japan). NF-κB-dependent luciferase activity in these cells was determined as described previously [31]. Briefly, cells were inoculated into each well of a 96-well flat bottomed plate at 1×10^5 cells in 80 μl of RPMI1640 supplemented with 10% FBS, and stimulated with the indicated concentrations of the test specimens. After 4 h incubation at 37 °C in humidified air containing 5% CO_2, 80 μl of Bright-Glo™ luciferase assay reagent (Promega, Madison, WI) was added to each well and luminescence was quantified with a luminometer ARVO SX multilabel counter (Perkin Elmer, Wellesley, MA). Results are shown as relative luciferase activity, which was the ratio of stimulated activity to non-stimulated activity, in each cell line.

2.4. Animal study using BALB/c mice bearing Meth-A tumors

BALB/c mice were inoculated with 1×10^5 Meth-A cells i.p. The animals were injected with 100 μg of OK-432w in 200 μl PBS i.p., 100 μg of OK-TX-ppt in 200 μl PBS i.p., or 200 μl of PBS i.p. everyday from day 1 to day 10 after the inoculation of Meth-A. Mice were observed until 42 days after inoculation.

3. Results

3.1. Extraction of active components from OK-432

An extraction of OK-432 was performed using TX-114–water phase partitioning (Scheme 1). The yields of OK-TX-ppt, OK-TX-M, and OK-TX-A were 90%, 0.1%, and 0.1% based on OK-432w, respectively. IL-6-inducing activity of the preparations in PBMC were shown in Fig. 1. The activities of OK-TX-ppt were slightly higher than that of OK-432w. The preparations OK-TX-M and OK-TX-A were

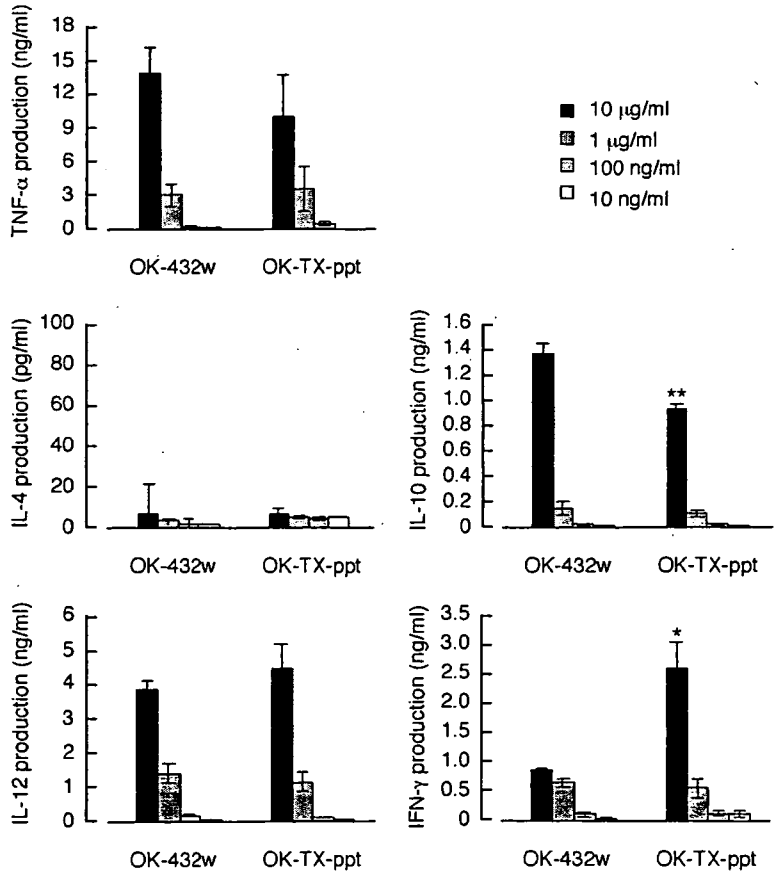


Figure 3 Induction of cytokines by OK-432w or OK-TX-ppt in human PBMC. PBMC were obtained from healthy volunteer (MF). PBMC were incubated with indicated concentrations of stimuli for 24 h. The levels of cytokines in the culture supernatants were measured by ELISA. The data represent the mean and standard deviation obtained from independent three experiments.

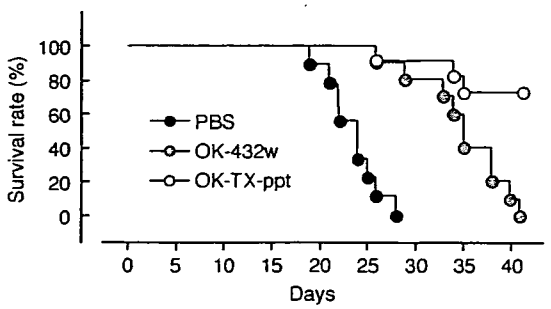


Figure 4 Antitumor effect of OK-432w or OK-TX-ppt in tumor-bearing mice. Meth-A tumor-bearing mice were administrated i.p. with OK-432w (100 μg, gray circle) or OK-TX-ppt (100 μg, open circle) or PBS (filled circle) everyday from day 1 to day 10 after inoculation of Meth-A cells.

significantly active but quite lower than that of OK-432w. TLR-mediated NF-κB activation of the preparations were demonstrated using Ba/mTLR2, Ba/mTLR4/mMD-2, and negative control Ba/κB cells (Fig. 2). OK-432w exerted the TLR2-mediated activities. The activities of OK-TX-M were negligible. The activity of OK-TX-A was low but significantly mediated by TLR2. In contrast, no TLR2-mediated activity was observed in Ba/mTLR2 cells stimulated with OK-TX-ppt. All the preparations had no TLR4-mediated activity.

3.2. Cytokine production in human PBMC stimulated with OK-TX-ppt and OK-432w

Cytokine-inducing activities of OK-TX-ppt were investigated using human PBMC and compared with that of OK-432w (Fig. 3). OK-TX-ppt induced similar amount of TNF-α as OK-432w. The level of IL-12 production stimulated by OK-TX-ppt was also comparable to that by OK-432w. Both exerted no IL-4-inducing activity. IFN-γ-inducing activity of OK-TX-ppt was significantly higher than that of OK-432w ($p<0.05$) and IL-10 production by OK-TX-ppt was lower than that of OK-432w ($p<0.01$).

3.3. Comparison of the antitumor effects of OK-TX-ppt and OK-432w in Meth-A-bearing mice

The antitumor effects of i.p. administration of OK-TX-ppt and OK-432w were investigated using Meth-A-bearing mice (Fig. 4). All mice (9/9) administered with PBS as a negative control died

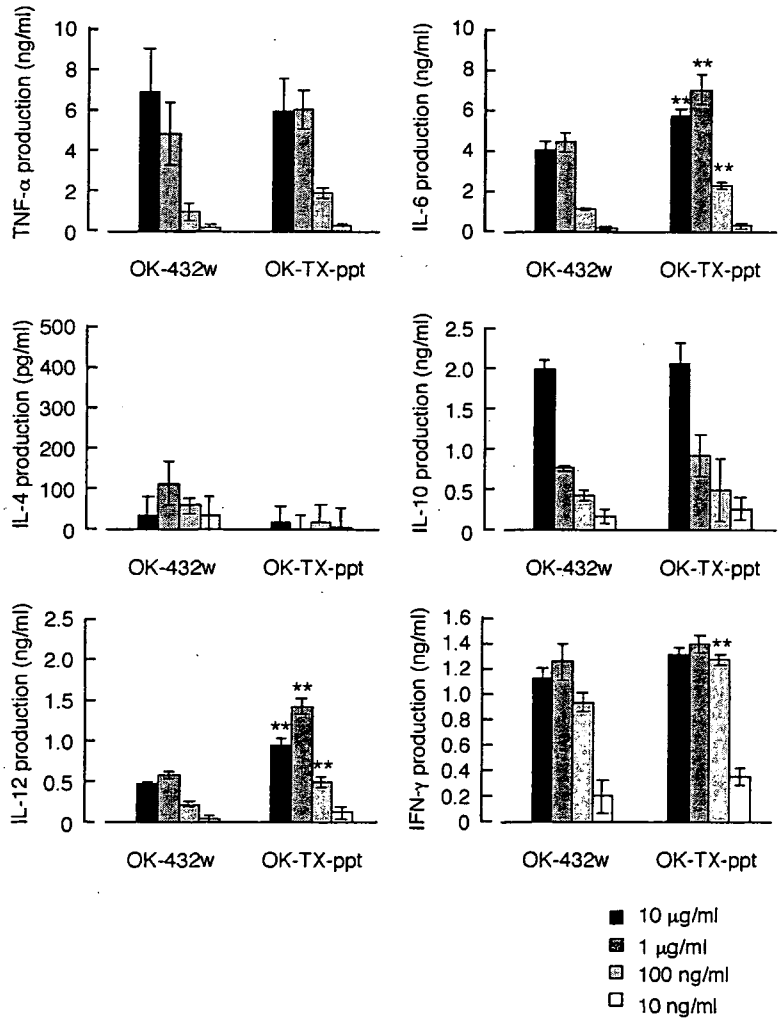


Figure 5 Induction of cytokines by OK-432w or OK-TX-ppt in mice spleen cells. Spleen cells were obtained from BALB/c. Cells were incubated with indicated concentrations of stimuli for 24 h. The levels of cytokines in the culture supernatants were measured by ELISA. The data represent the mean and standard deviation obtained from independent three experiments.

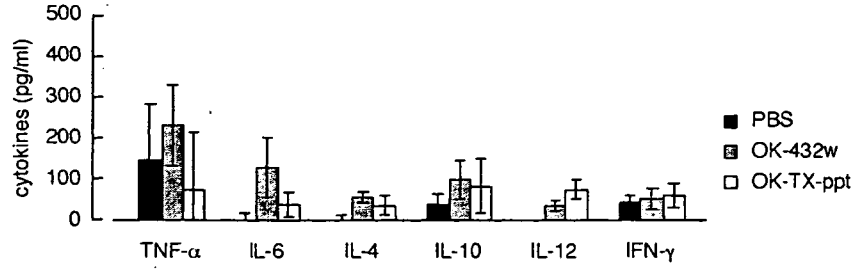


Figure 6 Cytokines in the sera obtained from mice administrated with OK-432w or OK-TX-ppt. Mice were injected i.p. with OK-432w (500 μg, gray bar) or OK-TX-ppt (500 μg, open bar) or PBS (filled bar) every other day from day 0 to day 10. On day 11, the mice were sacrificed and the levels of cytokines in the sera were measured by ELISA. The data represent the mean and standard deviation obtained from independent three experiments.

within 28 days after inoculation of Meth-A. Survival time of Meth-A-bearing mice treated with OK-432w as a positive control was extended, but all mice (10/10) died within 41 days. The treatment with OK-TX-ppt further extended their survival time compared with OK-432w, and eight of eleven mice were alive on day 42.

3.4. Cytokine production in mice stimulated with OK-TX-ppt and OK-432w

Cytokine production in mice spleen cells stimulated with OK-TX-ppt and OK-432w were shown in Fig. 5. The level of TNF-α and IL-10 production stimulated by OK-TX-ppt was equivalent to those by OK-432w. IL-6, IL-12, and IFN-γ production by OK-TX-ppt were significantly higher than those of OK-432w. IL-4-inducing activity of OK-TX-ppt was almost negative, and was slightly lower than OK-432w. Amounts of cytokines in sera of mice administered i.p. with OK-TX-ppt and OK-432w were also analyzed (Fig. 6). However, the values were very low and no significant differences were observed between the groups using each three mice except for IL-12. The amount of IL-12 induced by OK-TX-ppt appeared to be higher than that of OK-432w ($p<0.08$).

4. Discussion

To date, several active components in OK-432 have been prepared. By a standard DNA isolation method (cell lysis and then isopropanol precipitation), OK-DNA was prepared [14]. It activated cells through TLR9. Since CpG DNA is a well known potent inducer of Th1 polarization, OK-DNA may be a major active component of OK-432. OK-PSA and OK-PTF were obtained by butanol–water extraction followed by affinity chromatography and shown to stimulate cells via TLR4 and TLR2 [12,13]. Butanol–water method has been widely used for the extraction of bacterial macromolecular glycoconjugates such as LPS and LTA [32,33]. Since Okamoto et al. suggested that the structure of OK-PSA was similar to that of LTA, one of the active components in OK-432 might be such glycoconjugates [34]. On the other hand, OK-PTF was shown to be involved in Th2 polarization. However, a principal component in OK-PTF was not yet clarified. Therefore, we attempted an extraction of components involved in Th2 polarization from OK-432. Previously, we demonstrated that *S. aureus* butanol extract contained lipoprotein-like compounds which activated cells via TLR2 [35]. Since it was also reported that TLR2 was involved in Th2 polarization of Th cells [23,24], such compounds in OK-432 may affect the anticancer activities via Th1 response. OK-432 was first subjected to phenol–chloroform–petroleum ether (PCP) extraction as described by Galanos et al. [36]. PCP soluble fraction, OK-PCP, appeared to contain a Th2-polarizing components (unpublished data). However, further analysis was impossible because of its low activity and small yield. Chloroform–methanol–water (CMW) extraction according to the method of Bligh and Dyer [37] was also applied, but was not effective. In the present study, we attempted to extract a TLR2-activating lipoprotein-like compound from OK-432 by TX-114 partitioning, and found the effectiveness to remove TLR2-mediated activity from OK-432w. TX-114-treated residual precipitate, OK-TX-ppt, did not exert TLR2-dependent activity in luciferase assay (Fig. 2) at all, indicating that most of TLR2 ligands in OK-432w were extracted by the partitioning. OK-TX-ppt still has IL-6-inducing activity in PBMC (Fig. 1) and also induced TNF-α, IL-10, IL-12, IFN-γ from PBMC (Fig. 3), showing that active components other than TLR2 ligands were still retained in OK-TX-ppt. However, the level of IL-10 production by OK-TX-ppt was decreased and that of IFN-γ was increased compared with those by OK-432w (Fig. 3). These observations suggested that OK-TX-ppt is a better Th1 inducer in PBMC than OK-432w.

Oshikawa et al. reported that a Th1 inducer, OK-PSA, exerted an antitumor effect in tumor-bearing mice [13]. Thus we investigated the antitumor effect of OK-TX-ppt in a similar system and found that the activity of OK-TX-ppt was quite higher than OK-432w in vivo (Fig. 4). This effect can be considered to be caused by the increased Th1-type cytokine production in mice cells stimulated by OK-TX-ppt (Fig. 5). However, no significant difference was detected in amount of cytokines in sera of mice between administered i.p. with OK-TX-ppt and with OK-432w, except for IL-12 (Fig. 6). Since we injected both tumor and agents to the peritoneal cavity in the present in vivo experiment, it was suggested that the local concentration of cytokine at lesion was important to elicit effective anticancer activity.

Results obtained in this study suggested that removal of TLR2 ligand from OK-432w was a possible explanation for these Th1-polarizing effects. The TLR2 ligands extracted may be Th2-polarizing components. OK-PTF, which induced Th2-type cytokine, was reported to decrease antitumor effect in tumor-bearing mice compared with OK-PSA [13]. OK-PTF contained both TLR2 and TLR4 ligands. Since the TLR4 ligands would be a Th1 inducer according to the results for OK-PSA, the TLR2 ligands may be involved in inducing Th2-type cytokines and attenuating antitumor activity.

However, we did not get TLR2 ligands quantitatively even in OK-TX-A. Isolation of TLR2 ligands should be examined further. It may also be probable that other mechanisms were responsible for skewing the response to Th1 by OK-TX-ppt. Treatment of OK-432 may result in a potential increase of a content of OK-DNA-type stimuli in OK-TX-ppt, or enhancement of accessibility of the stimuli to immune cells. TLR knockout mice studies may reveal a detailed mechanism.

Although we were not able to determine the effect TLR2 ligand itself in OK-432, it was found that the removal of TLR2 ligand by TX-114 partitioning was effective for enhancement of the anticancer function of OK-432. Since OK-432 contains both Th1- and Th2-type cytokine inducer, separation of the components should be important to suppress side-effects or enhancement of anticancer activity. Our separation methods, readily applicable for the production, may help with improvement of immunotherapeutic effects of OK-432.

Acknowledgements

This work was supported in part by Grants-in-Aid for Encouragement of Young Scientists (B) (16710160) from the Ministry of Education, Culture, Sports, Science, Technology and for Scientific Research (C) (17510179) from the Japanese Society for the Promotion of Science, SUNBOR grant from Suntory Institute for Bioorganic Research, and the collaborative research grant from Chugai Pharmaceutical Co., Ltd. We thank Professor Kazuhisa Sugimura at Kagoshima University for measuring luciferase activities.

References

[1] Okamoto H, Shoin S, Koshimura S, Shimizu R. Studies on the anticancer and streptolysin S-forming abilities of hemolytic streptococci. Jpn J Microbiol 1967;11:323–36.

[2] Torisu M, Katano M, Kimura Y, Itoh H, Takesue M. New approach to management of malignant ascites with a streptococcus preparation, OK-432. I. Improvement of host immunity and prolongation of survival. Surgery 1983;93:357–64.

[3] Uchida A, Micksche M, Hoshino T. Intrapleural administration of OK-432 in cancer patients: augmentation of autologous tumor killing activity of tumor-associated large granular lymphocytes. Cancer Immunol Immunother 1984;18:5–12.

[4] Watanabe Y, Iwa T. Clinical value of immunotherapy with the streptococcal preparation OK-432 in non-small cell lung cancer. J Biol Response Modif 1987;6:169–80.

[5] Kikkawa F, Kawai M, Oguchi H, Kojima M, Ishikawa H, Iwata M, et al. Randomised study of immunotherapy with OK-432 in uterine cervical carcinoma. Eur J Cancer 1993;29A:1542–6.

[6] Sato M, Yoshida H, Yanagawa T, Yura Y, Urata M, Atsumi M, et al. Effect of intradermal administration of streptococcal preparation OK-432 on interferon and natural killer cell activities in patients with oral cancer. Int J Oral Surg 1984;13:7–15.

[7] Sato M, Harada K, Yoshida H, Yura Y, Azuma M, Iga H, et al. Therapy for oral squamous cell carcinoma by tegafur and streptococcal agent OK-432 in combination with radiotherapy: Association of the therapeutic effect with differentiation and apoptosis in the cancer cells. Apoptosis 1997;2:227–38.

[8] Oshimi K, Kano S, Takaku F, Okumura K. Augmentation of mouse natural killer cell activity by a streptococcal preparation, OK-432. J Natl Cancer Inst 1980;65:1265–9.

[9] Saito M, Ebina T, Koi M, Yamaguchi T, Kawade Y, Ishida N. Induction of interferon-γ in mouse spleen cells by OK-432, a preparation of *Streptococcus pyogenes*. Cell Immunol 1982;68:187–92.

[10] Misaki T, Watanabe Y, Iida Y, Hidaka A, Kasagi K, Fukushima H, et al. Recruitment of T lymphocytes and induction of tumor necrosis factor in thyroid cancer by a local immunotherapy. Cancer Immunol Immunother 1992;35:92–6.

[11] Fujimoto T, Duda RB, Szilvasi A, Chen X, Mai M, O'Donnell MA. Streptococcal preparation OK-432 is a potent inducer of IL-12 and a T helper cell 1 dominant state. J Immunol 1997;158:5619–26.

[12] Okamoto M, Kaji R, Kasetani H, Yoshida H, Moriya Y, Saito M, et al. Purification and characterization of interferon-γ-inducing molecule of OK-432, a penicillin-killed streptococcal preparation, by monoclonal antibody neutralizing interferon-gamma-inducing activity of OK-432. J Immunother 1993;13:232–42.

[13] Oshikawa T, Okamoto M, Ohe G, Furuichi S, Nishikawa H, Uddin Ahmed S, et al. Anti-tumor immune response induced by the fractions derived from OK-432, a streptococcal preparation, by using a monoclonal antibody TS-2 that neutralizes the interferon-γ-inducing activity of OK-432: comparison between the TS-2-binding and TS-2-unbinding fraction. Int Immunopharmacol 2003;3:643–55.

[14] Oshikawa T, Okamoto M, Tano T, Sasai A, Kan S, Moriya Y, et al. Antitumor effect of OK-432-derived DNA: one of the active constituents of OK-432, a streptococcal immunotherapeutic agent. J Immunother 2006;29:143–50.

[15] Underhill DM, Ozinsky A. Toll-like receptors: key mediators of microbe detection. Curr Opin Immunol 2002;14:103–10.

[16] Shimazu R, Akashi S, Ogata H, Nagai Y, Fukudome K, Miyake K, et al. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. J Exp Med 1999;189:1777–82.

[17] Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int Immunol 2001;13:933–40.

[18] Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol 2002;169:10–4.

[19] Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408:740–5.

[20] Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 2002;20:709–60.

[21] Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. J Exp Med 2002;196:1645–51.

[22] Kaisho T, Hoshino K, Iwabe T, Takeuchi O, Yasui T, Akira S. Endotoxin can induce MyD88-deficient dendritic cells to support T(h)2 cell differentiation. Int Immunol 2002;14:695–700.

[23] Redecke V, Hacker H, Datta SK, Fermin A, Pitha PM, Broide DH, et al. Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. J Immunol 2004;172:2739–2743.

[24] Kiura K, Kataoka H, Yasuda M, Inoue N, Shibata K. The diacylated lipopeptide FSL-1 induces TLR2-mediated Th2 responses. FEMS Immunol Med Microbiol 2006;48:44–55.

[25] Imanishi T, Hara H, Suzuki S, Suzuki N, Akira S, Saito T. Cutting edge: TLR2 directly triggers Th1 effector functions. J Immunol 2007;178:6715–9.

[26] Watanabe T, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. Nat Immunol 2004;5:800–8.

[27] Tada H, Aiba S, Shibata K, Ohteki T, Takada H. Synergistic effect of Nod1 and Nod2 agonists with toll-like receptor agonists on human dendritic cells to generate interleukin-12 and T helper type 1 cells. Infect Immun 2005;73:7967–76.

[28] Hashimoto M, Tawaratsumida K, Kariya H, Aoyama K, Tamura T, Suda Y. Lipoprotein is a predominant Toll-like receptor 2 ligand in *Staphylococcus aureus* cell wall components. Int Immunol 2006;18:355–62.

[29] Shibata K, Hasebe A, Sasaki T, Watanabe T. *Mycoplasma salivarium* induces interleukin-6 and interleukin-8 in human gingival fibroblasts. FEMS Immunol Med Microbiol 1997;19:275–83.
[30] Hirschfeld M, Ma Y, Weis JH, Vogel SN, Weis JJ. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2. J Immunol 2000;165:618–22.
[31] Hashimoto M, Asai Y, Ogawa T. Separation and structural analysis of lipoprotein in a lipopolysaccharide preparation from *Porphyromonas gingivalis*. Int Immunol 2004;16:1431–7.
[32] Morrison DC, Leive L. Fractions of lipopolysaccharide from *Escherichia coli* O111:B4 prepared by two extraction procedures. J Biol Chem 1975;250:2911–9.
[33] Morath S, Geyer A, Hartung T. Structure–function relationship of cytokine induction by lipoteichoic acid from *Staphylococcus aureus*. J Exp Med 2001;193:393–7.
[34] Okamoto M, Ohe G, Oshikawa T, Nishikawa H, Furuichi S, Yoshida H, et al. Induction of Th1-type cytokines by lipoteichoic acid-related preparation isolated from OK-432, a penicillin-killed streptococcal agent. Immunopharmacology 2000;49:363–76.
[35] Hashimoto M, Tawaratsumida K, Kariya H, Kiyohara A, Suda Y, Krikae F, et al. Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in *Staphylococcus aureus*. J Immunol 2006;177:3162–9.
[36] Galanos C, Luderitz O, Westphal O. A new method for the extraction of R lipopolysaccharides. Eur J Biochem 1969;9:245–9.
[37] Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Biophysiol 1959;37:911–7.

Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron Letters
ELSEVIER
Tetrahedron Letters 48 (2007) 6577–6581

Synthesis of immunoregulatory *Helicobacter pylori* lipopolysaccharide partial structures

Yukari Fujimoto,[a] Masato Iwata,[a] Noriko Imakita,[a] Atsushi Shimoyama,[a] Yasuo Suda,[b] Shoichi Kusumoto[c] and Koichi Fukase[a,*]

[a]*Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan*
[b]*Department of Nanostructure and Advanced Materials, Graduate School of Science and Engineering Kagoshima University, Kagoshima 890-0065, Japan*
[c]*Suntory Institute for Bioorganic Research, Shimamoto, Osaka 618-8503, Japan*

Received 8 June 2007; revised 30 June 2007; accepted 3 July 2007
Available online 2 August 2007

This Letter is dedicated to the memory of the late Professor Yoshihiko Ito

Abstract—The synthesis of immunoregulatory glycoconjugates, namely the active entity of lipopolysaccharide (LPS) from *Helicobacter pylori* was achieved. The results of biological activities of the LPS partial structures provide the structural basis for the immunobiological activity, especially for the immune inhibitory activity of *H. pylori* LPS.

Helicobacter pylori is a Gram-negative bacterium and an etiological agent of gastroduodenal diseases such as chronic gastritis, gastroduodenal ulcers and gastric cancer.[1,2] Gram-negative bacteria are distinguished by the presence of lipopolysaccharide (LPS) in their outer membrane. LPS, also called an endotoxin, is a potent immunostimulator, often causing sepsis in severe infections. However, *H. pylori* lipopolysaccharide (LPS) shows very low endotoxic activity compared to other enterobacterial preparations such as *Escherichia coli* LPS.[3–6] The *H. pylori* LPS has the characteristic lipid A component, the structure of which is distinct from those of the enterobacterial ones as in the following ways: (1) the presence of fewer, but longer fatty acid residues, (2) the absence of the 4′-phosphate group and (3) the occasional presence of an ethanolamine group linked to the glycosyl phosphate functionality.[7,8] We have previously synthesized *H. pylori* strain 206-1 lipid A, which has tri-acyl groups and ethanolamine at the anomeric phosphate (**1b**). This lipid A induced lower levels of cytokines such as IL-18 and TNF-α upon activation of the LPS receptor, a Toll-like receptor 4 (TLR4)/MD-2 complex,[9,10] as it was known that the activation leads to induction of proinflammatory cytokines such as IL-6, IL-18 and TNF-α. On the other hand, it has been recently reported that *H. pylori* LPS from other strains has inhibitory effects on TLR4 activation.[11] This discrepancy in biological activities may come from the structural heterogeneity of LPS, especially in the acyl group distribution and in the phosphate part in lipid A (Fig. 1, **1a–d**). Synthetic study is therefore required for elucidation of biological activity of *H. pylori* LPS, since chemical synthesis can provide homogeneous preparations.

It has been reported that the number of acyl groups is crucial for the biological activity of lipid A.[12,13] *E. coli* type lipid A having hexaacyl groups has strong endotoxic activity, whereas a biosynthetic precursor Ia having tetraacyl groups shows antagonistic activity in humans.[12] Triacyl lipid As also showed antagonistic activity though the potency was low. There have been known some bacteria, LPS of which have significant inhibitory activity against *E. coli* LPS, such as *Rhodobacter sphaeroides*,[14,15] and *Rhodobacter capsulatus*,[16] which have an unsaturated acyl group, and also *Rhizobium sin*-1[17,18] which has pentaacyl groups of relatively longer fatty acids and lactone structure at the anomeric position. However, in the case of *H. pylori*, the LPS shows weaker antagonistic activity, and at the same time, shows weak proinflammatory activity. Chain

* Corresponding author. Tel.: +81 6 6850 5388; fax: +81 6 6850 5419; e-mail: koichi@chem.sci.osaka-u.ac.jp

0040-4039/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2007.07.036

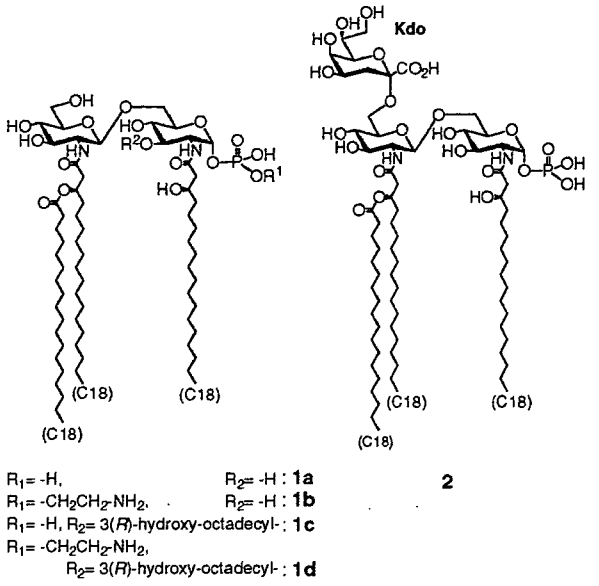


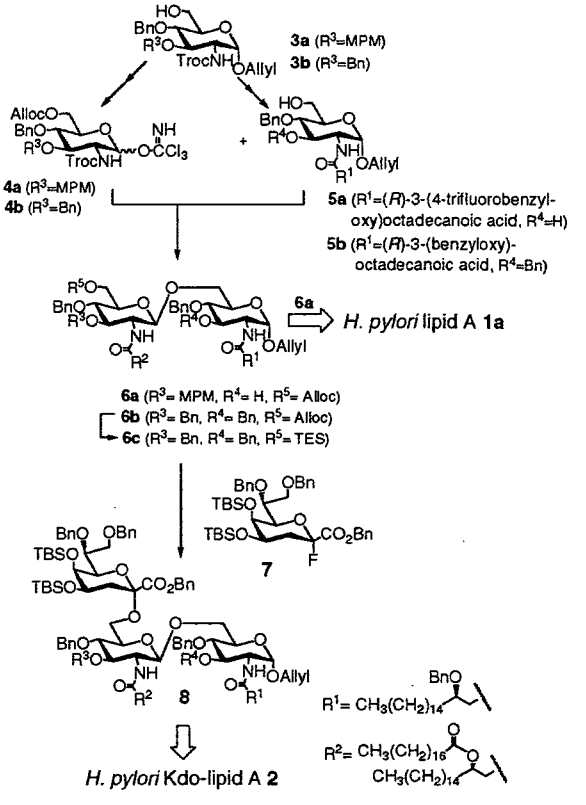
Figure 1. Chemical structures of *Helicobacter pylori* lipid A and Kdo-lipid A.

length of fatty acids in lipid A also influences the biological activity. A biosynthetic precursor Ia analog having shorter lipids (two C14 and two C10 fatty acids) showed much weaker antagonistic activity, whereas that having four C10 fatty acids did not show the activity.[19] On the other hand, an *E. coli* lipid A analogue having shorter lipids (two C14 and four C12 fatty acids) were ca. 100 times more active than *E. coli* lipid A.[20]

The acidic functional groups of lipid A are also crucial for the biological activity.[12,13] Monophosphoryl lipid A analogues show much weaker activity than the corresponding diphosphates and the analogues lacking phosphoryl group did not show the activity. The phosphate groups in lipid A can be replaced with carboxymethyl groups without loss of the activity in the case of *E. coli* lipid A and the tetraacyl biosynthetic precursor.[21 24] However, in our recent studies with lipid A analogues containing acidic amino acid residues, immunostimulating or antagonistic activity was observed depending on the anionic charges (phosphoric acid vs carboxylic acid).[25,26]

As for another aspect, it has been reported that *H. pylori* LPS activates TLR2.[11,27,28] Some of them also indicated that *H. pylori* LPS antagonizes TLR4.[11,28] Another parasitic bacteria, *Porphyromonas gingivalis*, which has similar acylated patterns of lipid A to the one of *H. pylori* though the acyl groups have branched terminus,[29,30] also showed TLR2 stimulatory and TLR4 antagonizing activities.[28] These biological activities are considered to be associated with atherosclerosis, which is a chronic inflammatory vascular disease and leads to cerebral stroke and cardiac affection—most major causes of death in industrial countries. The heterogeneity of the lipid A structures in *H. pylori* LPS, however, complexifies understanding the molecular basis of these biological activities. There is also a potential issue on the contamination of immunoreactive compounds in preparations from natural sources. We thus planned to synthesize the series of the lipid A and LPS partial structures to clarify the structural basis of the immunoregulatory activities.

Lipid A generally connects to the polysaccharide domain via an acidic sugar, 3-deoxy-D-manno-2-octurosonic acid (Kdo). Because *H. pylori* lipid A lacks a 4′-phosphate group, the acidic group of Kdo might alter the anionic charge and affect recognition by the LPS receptor. In the present study, we thus synthesized the *H. pylori* LPS partial structures, lipid A **1a** and Kdo-lipid A **2**, which do not have ethanolamine at the anomeric phosphate to observe the effect of Kdo and ethanolamine in comparison with our previously synthesized ethanolamine-type lipid A (Fig. 1).[9] Scheme 1 shows the basic strategy for lipid A synthesis. Glycosyl donors **4a/4b** and glycosyl acceptors **5a/5b** were prepared from properly protected glucosamine **3a/3b**, respectively. The acyl group of the glycosyl acceptors **5a/5b** was introduced before glycosylation. Glycosyl donor, trichloroacetimidate **5a/5b**, which possessed a 2-*N*-Troc group, was used for β-selective glycosylation to construct the lipid A backbone. *H. pylori* lipid A **1a** was synthesized from disaccharide **6a** with 1-*O*-phosphorylation and deprotection. Kdo-lipid A **2** was synthesized by glycosylation of **6b** with Kdo donor **7** and



Scheme 1. Outline for the synthesis of *Helicobacter pylori* lipid A and Kdo-lipid A. Alloc = allyloxycarbonyl, Bn = benzyl, MPM = *p*-methoxybenzyl, TBS = *t*-butyldimethylsilyl, Troc = 2,2,2-trichloroethoxycarbonyl, TMS = trimethylsilyl.

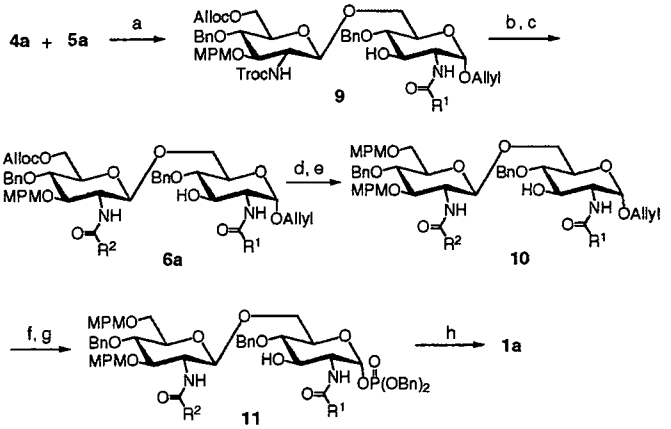
1-*O*-phosphorylation. Kdo fluoride **7** protected with TBS groups at the 4- and 5-positions was used as a donor for α-selective glycosylation, because we previously found in our *E. coli* Re-LPS synthesis that α-selectivity increased when large protective groups such as TBS were introduced at the 4- and 5-positions.[31] Benzyl-type protecting groups were used for hydroxyl, carboxy and phosphate functions, and the final deprotection of all the benzyl-type protecting groups was carried out by catalytic hydrogenolysis.

Scheme 2 shows the synthesis of lipid A **1a**. Glycosyl donor **4a** and the acceptor **5a** were synthesized in a manner similar to previously published methods.[31] The glycosylation was carried out in the presence of a catalytic amount of TMSOTf in THF solution at 0 °C to give disaccharide **9** in 85% yield. The Troc group at the 2′-position of **9** was cleaved with Zn–Cu couple in acetic acid, and (*R*)-3-octadecanoyloxyoctadecanoic acid was introduced with WSCD·HCl at 40 °C to give **6a** from compound **9** in 56% yield. The Alloc group at the 6′-position was cleaved with $Pd(PPh_3)_4$, PPh_3 and ammonium formate in THF selectively in the presence of an allyl group at 1-position, and then the MPM group was introduced at this liberated 6′-position. The allyl group at the 1-position was cleaved via isomerization to a vinyl group with an iridium complex and then with iodine and water in 87% yield, and the anomeric position was selectively phosphorylated with tetrabenzyl pyrophosphate to give **11**. Cleavage of all the benzyl-type protecting groups in **11** by hydrogenolysis gave the desired lipid A **1a**.

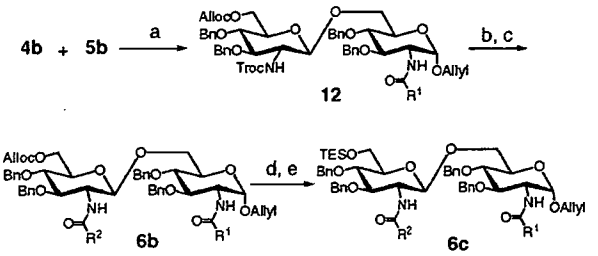
For the synthesis of Kdo-lipid A, the lipid A backbone was first prepared as shown in Scheme 3. Although we initially tried to synthesize Kdo-lipid A from **6a**, the 3′-*O*-MPM group was cleaved under the glycosylation conditions using Kdo fluoride **7** in the preliminary experiments. We, therefore, used 3-*O*-benzyl acceptor **5b** in subsequent procedures. The glycosyl donor **4b** and the acceptor **5b** were synthesized in a manner similar to compounds **4a** and **5a**, respectively.[31] The glycosylation was carried out in the presence of a catalytic amount of TMSOTf in THF solution at −78 °C to give the disaccharide **12** in 70% yield. The Troc group at the 2′-position of **12** was cleaved with Zn–Cu couple in acetic acid, and (*R*)-3-octadecanoyloctadecanoic acid was introduced with WSCD·HCl at 40 °C to give **6b** in 58% yield from compound **12**. The Alloc group at the 6′-position was cleaved with $Pd(PPh_3)_4$ in the presence of PPh_3 and ammonium formate in THF, and then the TES group was introduced at this position for subsequent glycosylation to give compound **6c**.

Scheme 4 shows the preparation of Kdo fluoride **7**, the glycosyl donor for the next glycosylation, from compound **13**.[31] In the previous study, we used (diethylamino)sulphur trifluoride (DAST) for fluorination. However, the reaction was difficult due to the high reactivity of DAST and a considerable amount of glycal was formed as a by-product. Hence, we used a new, mild fluorination reagent, *N*,*N*-diethyl-α,α-difluoro-(*m*-methylbenzyl)amine (DFMBA).[32] The anomeric stereoselectivity for fluorination was different for these two compounds: 3/1 (α/β) for DAST and 1/10 (α/β) for DFMBA. The formation of glycal **14** was suppressed when DFMBA was used at a lower temperature.

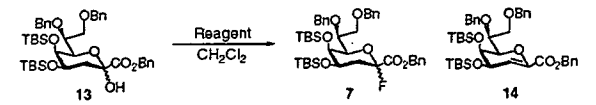
Scheme 5 shows the glycosylation between Kdo-fluoride **7** and lipid A backbone **6c**. The reaction conditions were examined using a variety of Lewis acids, solvents, and molecular sieves. The molecular sieve 5A (MS5A) was found to be effective for this reaction compared to MS4A. Because MS5A contains calcium ions, MS5A should trap fluoride anions to promote glycosylation. Thus, we successfully obtained trisaccharide **8** with the α-glycoside linkage between Kdo and lipid A, using BF_3/Et_2O and MS5A in CH_2Cl_2 at −20 °C with a high



Scheme 2. Synthesis of *Helicobacter pylori* lipid A. (a) TMSOTf (0.1 equiv), MS4A, THF, 0 °C, 1 h, 85%; (b) Zn–Cu, AcOH, rt; (c) (*R*)-3-octadecanoyloxyoctadecanoic acid, WSCD·HCl, HOAt, $CHCl_3$, 40 °C, 56% (two steps); (d) $Pd(PPh_3)_4$ (1.2 equiv), PPh_3 (3 equiv), $HCOONH_4$ (5 equiv), THF, rt, 2 h, 94%; (e) MPM imidate Sn(OTf), MS4A, THF, 88%; (f) $[Ir(H)(cod)(MePh_2P)_2]PF_6$, then I_2, H_2O, 87%; (g) tetrabenzyl pytrophosphate, $LiN(TMS)_2$, THF, −78 °C, 47%; (h) H_2 (15 kg cm^{-2}), Pd (black), THF. R^1: (*R*)-3-(4-trifluorobenzyloxy)octadecanoyl, R^2: (*R*)-3-octadecanoyloxyoctadecanoyl, HOAt = 1-hydroxy-7-azabenzotriazole, TMSOTf = trimethylsilyl trifluoromethanesulfonate, WSCD = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.



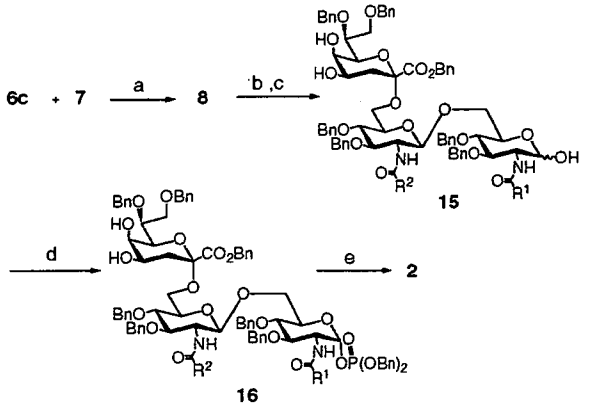
Scheme 3. Synthesis of lipid A backbone disaccharide for *Helicobacter pylori* Kdo-lipid A. (a) TMSOTf (0.2 equiv), MS4A, THF, −78 °C, 1 h, 80%; (b) Zn–Cu, AcOH, rt; (c) (*R*)-3-octadecanoyloxyoctadecanoic acid, WSCD·HCl, HOAt, $CHCl_3$, 40 °C, 58% (two steps); (d) $Pd(PPh_3)_4$, PPh_3, $HCOONH_4$, THF, 94%; (e) TESCl, imidazole, $CHCl_3$, 88%. R^1: (*R*)-3-benzyloxyoctadecanoyl, R^2: (*R*)-3-octadecanoyloxyoctadecanoyl, TES = triethyl.



Entry	Reagent	Conditions	Time	Yield[a]
1	DAST (3 equiv)	-78 °C	3 h	88% (α:β= 3:1), **14** (7%)
2	DFMBA (2 equiv)	60 °C (microwave 50 W)	10 min	68% (α:β= 1:10), **14** (12%)
3	DFMBA (4 equiv)	-14 °C	1.5 d	88% (α:β= 1:10), **14** (ND)

[a] The ratio of the αβglycoside and glycal **14** was determined by the area ratio of the $PhC\underline{H}_2OCO$ signal in ^{1}H-NMR.

Scheme 4. Fluorination of Kdo.



Scheme 5. Synthesis of *Helicobacter pylori* Kdo-lipid A. (a) $BF_3{\cdot}Et_2O$ (10 equiv), MS5A, $CHCl_3$, −20 °C, 1.5 h, 85%; (b) 47% HF:CH_2Cl_2:CH_3CN = 1:5:5, 81%; (c) $[Ir(H)(cod)(MePh_2P)_2]PF_6$, then I_2, H_2O, 78%; (d) tetrabenzyl pyrophosphate, $LiN(TMS)_2$, THF, −78 °C, 47%; (e) H_2 (20 kg cm^{-2}), Pd (black), THF, 91%. R^1: (*R*)-3-benzyloxyoctadecanoyl, R^2: (*R*)-3-octadecanoyloxyoctadecanoyl.

yield. The stereochemistry at the anomeric position was determined with the chemical shift of the protons at the 3′-position from ^{1}H NMR in comparison with the previously reported data.[31] Then, the TBS groups were removed with HF, and the allyl group at the 1-position was cleaved via isomerization to a vinyl group with iridium complex to give **15** in 78% yield. After selective phosphorylation at the anomeric position with tetrabenzyl pyrophosphate, all the benzyl-type protecting groups were removed by hydrogenolysis to give the desired compound **2**.

The immunostimulating activities of the synthetic *H. pylori* lipid A **1a** and Kdo-lipid A **2** were examined by measuring IL-6 production from human whole blood cells (HWBC)[33] (Fig. 2). Both **1a** and **2** did not induce IL-6, but showed competitive inhibition of IL-6 induction by *E. coli* LPS (0.5 ng/mL, O111:B4). Kdo-lipid A **2** showed more potent inhibitory activity than lipid A **1a**.

These results demonstrated that the H-form tri-acylated lipid A **1a** and Kdo-lipid A **2** of *H. pylori* competitively inhibit *E. coli* LPS immunostimulation. These results indicated that **1a** and **2** are TLR4 antagonist but not TLR2 or TLR4 agonist. We also observed that introducing an acidic Kdo residue to lipid A enhanced this inhibitory activity by 10-fold. The present study provides the molecular basis for antagonistic activity of *H. pylori* LPS from certain strains.

It should be noted that the ethanolamine-form of lipid A **1b** showed weak immunostimulating activity in our previous study.[10] These results indicate that the number of anionic charges influences the biological activity of lipid A and LPS. Similar results were obtained from our studies with lipid A analogues containing acidic amino acid residues as mentioned above. Depending on the anionic charges (phosphoric acid vs carboxylic acid) in the lipid A analogues, immunostimulating or antagonistic activity was observed.[26] The present study also explains why some strains of *H. pylori* LPS show weak immunostimulating activity while others show antagonistic activity.

In conclusion, we synthesized tri-acylated lipid A **1a** and LPS partial structure **2** of *H. pylori* and found that both compounds antagonize *E. coli* LPS immunostimulation.

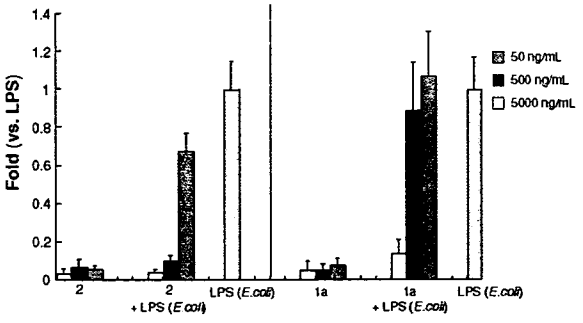


Figure 2. *Helicobacter pylori* Kdo-lipid A and lipid A competitively inhibit IL-6 induction by *E. coli* LPS. Noted concentrations of Kdo-lipid A **2a** and lipid A **1a** and 0.5 ng/mL of *E. coli* (O111:B4) LPS were added to human whole blood cells (HWBC). Amount of IL-6 produced by HWBC was determined by ELISA. Data represent averages of three repeated assays with standard deviations from individual experiments.

In future studies, we will use these *H. pylori* LPS compounds to investigate the biological activities of LPS and the factors that mediate host receptor–LPS recognition.

Acknowledgements

This work was supported in part by Grants-in Aid for Scientific research (Nos.17035050, 17310128, 18032046) from Japan Society for the Promotion of Science, grants from Suntory Institute for Bioorganic Research (SUNBOR Grant), the Houansha Foundation, and Hayashi Memorial Foundation for Female Natural Scientists (to Y.F.). DFMBA was a generous gift of Mitsubishi Gas Chemical Company, Inc.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.07.036.

References and notes

1. Cover, T. L.; Blaser, M. J. *Adv. Int. Med.* **1996**, *41*, 85.
2. Parsonnet, J. *Gut* **1998**, *43*, S6.
3. Hynes, S. O.; Ferris, J. A.; Szponar, B.; Wadstrom, T.; Fox, J. G.; O'Rourke, J.; Larsson, L.; Yaquian, E.; Ljungh, A.; Clyne, M.; Andersen, L. P.; Moran, A. P. *Helicobacter* **2004**, *9*, 313.
4. Nielsen, H.; Birkholz, S.; Andersen, L. P.; Moran, A. P. *J. Infect. Dis.* **1994**, *170*, 135.
5. Perez-Perez, G. I.; Shepherd, V. L.; Morrow, J. D.; Blaser, M. J. *Infect. Immun.* **1995**, *63*, 1183.
6. Yokota, S.; Saito, H.; Kubota, T.; Yokosawa, N.; Amano, K.; Fujii, N. *Virology* **2003**, *306*, 135.
7. Suda, Y.; Ogawa, T.; Kashihara, W.; Oikawa, M.; Shimoyama, T.; Hayashi, T.; Tamura, T.; Kusumoto, S. *J. Biochem. (Tokyo)* **1997**, *121*, 1129.
8. Moran, A. P.; Lindner, B.; Walsh, E. J. *J. Bacteriol.* **1997**, *179*, 6453.
9. Sakai, Y.; Oikawa, M.; Yoshizaki, H.; Ogawa, T.; Suda, Y.; Fukase, K.; Kusumoto, S. *Tetrahedron Lett.* **2000**, *41*, 6843.
10. Ogawa, T.; Asai, Y.; Sakai, Y.; Oikawa, M.; Fukase, K.; Suda, Y.; Kusumoto, S.; Tamura, T. *FEMS Immunol. Med. Microbiol.* **2003**, *36*, 1.
11. Lepper, P. M.; Triantafilou, M.; Schumann, C.; Schneider, E. M.; Triantafilou, K. *Cell. Microbiol.* **2005**, *7*, 519.
12. Kusumoto, S.; Fukase, K. *Chem. Rec.* **2006**, *6*, 333, and references cited therein.
13. Kusumoto, S.; Fukase, K.; Fukase, Y.; Kataoka, M.; Yoshizaki, H.; Sato, K.; Oikawa, M.; Suda, Y. *J. Endotoxin Res.* **2003**, *9*, 361.
14. Baker, P. J.; Taylor, C. E.; Stashak, P. W.; Fauntleroy, M. B.; Haslov, K.; Qureshi, N.; Takayama, K. *Infect. Immun.* **1990**, *58*, 2862.
15. Kirikae, T.; Schade, F. U.; Kirikae, F.; Qureshi, N.; Takayama, K.; Rietschel, E. T. *FEMS Immunol. Med. Microbiol.* **1994**, *9*, 237.
16. Loppnow, H.; Libby, P.; Freudenberg, M.; Krauss, J. H.; Weckesser, J.; Mayer, H. *Infect. Immun.* **1990**, *58*, 3743.
17. Vandenplas, M. L.; Carlson, R. W.; Jeyaretnam, B. S.; McNeill, B.; Barton, M. H.; Norton, N.; Murray, T. F.; Moore, J. N. *J. Biol. Chem.* **2002**, *277*, 41811.
18. Demchenko, A. V.; Wolfert, M. A.; Santhanam, B.; Moore, J. N.; Boons, G. J. *J. Am. Chem. Soc.* **2003**, *125*, 6103.
19. Fukase, K.; Fukase, Y.; Oikawa, M.; Liu, W.-C.; Suda, Y.; Kusumoto, S. *Tetrahedron* **1998**, *54*, 4033.
20. Zhang, Y.; Gaekwad, J.; Wolfert, M. A.; Boons, G. J. *J. Am. Chem. Soc.* **2007**, *129*, 5200.
21. Liu, W.-C.; Oikawa, M.; Fukase, K.; Suda, Y.; Kusumoto, S. *Bull. Chem. Soc. Jpn.* **1999**, *72*, 1377.
22. Fukase, K.; Ueno, A.; Fukase, Y.; Oikawa, M.; Suda, Y.; Kusumoto, S. *Bull. Chem. Soc. Jpn.* **2003**, *76*, 485.
23. Kataoka, M.; Hashimoto, M.; Suda, Y.; Kusumoto, S.; Fukase, K. *Heterocycles* **2006**, *69*, 395.
24. Seydel, U.; Schromm, A. B.; Brade, L.; Gronow, S.; Andrae, J.; Mueller, M.; Koch, M. H. J.; Fukase, K.; Kataoka, M.; Hashimoto, M.; Kusumoto, S.; Brandenburg, K. *FEBS J.* **2005**, *272*, 327.
25. Fujimoto, Y.; Adachi, Y.; Akamatsu, M.; Fukase, Y.; Kataoka, M.; Suda, Y.; Fukase, K.; Kusumoto, S. *J. Endotoxin Res.* **2005**, *11*, 341.
26. Akamatsu, M.; Fujimoto, Y.; Kataoka, M.; Suda, Y.; Kusumoto, S.; Fukase, K. *Bioorg. Med. Chem.* **2006**, *14*, 6759.
27. Smith, M. F., Jr.; Mitchell, A.; Li, G.; Ding, S.; Fitzmaurice, A. M.; Ryan, K.; Crowe, S.; Goldberg, J. B. *J. Biol. Chem.* **2003**, *278*, 32552.
28. Triantafilou, M.; Gamper, F. G.; Lepper, P. M.; Mouratis, M. A.; Schumann, C.; Harokopakis, E.; Schifferle, R. E.; Hajishengallis, G.; Triantafilou, K. *Cell Microbiol.*, in press.
29. Kumada, H.; Haishima, Y.; Umemoto, T.; Tanamoto, K. *J. Bacteriol.* **1995**, *177*, 2098.
30. Reife, R. A.; Coats, S. R.; Al-Qutub, M.; Dixon, D. M.; Braham, P. A.; Billharz, R. J.; Howald, W. N.; Darveau, R. P. *Cell Microbiol.* **2006**, *8*, 857.
31. Yoshizaki, H.; Fukuda, N.; Sato, K.; Oikawa, M.; Fukase, K.; Suda, Y.; Kusumoto, S. *Angew. Chem., Int. Ed.* **2001**, *40*, 1475.
32. Kobayashi, S.; Yoneda, A.; Fukuhara, T.; Hara, S. *Tetrahedron* **2004**, *60*, 6923.
33. Suda, Y.; Tochio, H.; Kawano, K.; Takada, H.; Yoshida, T.; Kotani, S.; Kusumoto, S. *FEMS Immunol. Med. Microbiol.* **1995**, *12*, 97.

Biological roles of carboxymethyl-chitin associated for the growth factor production

Hiroyuki Kariya,[1] Ai Kiyohara,[2] Shingo Masuda,[3] Yusuke Yoshihara,[3] Masaru Ueno,[3] Masahito Hashimoto,[1,2] Yasuo Suda[1,2]

[1]*Venture Business Laboratory, Kagoshima University, Kagoshima, Japan*

[2]*Department of Nanostructure and Advanced Materials, Graduate School of Science and Engineering, Kagoshima University, Kagoshima, Japan*

[3]*Japan Medical Materials Corporation, Osaka, Japan*

Received 30 January 2006; revised 31 October 2006; accepted 27 November 2006
Published online 21 March 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.31202

Abstract: Many techniques to restore cartilage defection have been tried. However, the development is still under way because of problems, including loosening of artificial joint, degenerative change of compensated tissue, risk of viral transmission via allograft/autograft, and cost of therapeutic materials for repair. In the previous research, we found that complementing cartilage defective part with carboxymethyl-chitin (CM-chitin)/β-tricalcium phosphate composite induced regeneration of cartilage in rabbits *in vivo*, and it is presumable that CM-chitin plays a key role in chondrogenesis causing the regeneration of cartilage. However, the induction mechanism of chondrogenesis with CM-chitin is still unclear. In this study, we investigated the cell responses to CM-chitin by using peritoneal exudate cell (PEC) in mice and found that CM-chitin induced the expression of inflammatory cytokines and growth factors, both of which are both considered to correlate with chondrogenesis. After intraperitoneal injection CM-chitin showed enhanced expressions of mRNA of interleukin-1β (IL-1β), interleukin-6 (IL-6), keratinocyte-derived chemokine, tumor necrosis factor-α, and transforming growth factor-β1 (TGF-β1) in PEC as observed by reverse transcriptase polymerase chain reaction. Productions of TGF-β1 protein were confirmed by enzyme linked immunosorbant assay. It was also shown that mononuclear cells in PEC were responsible for the TGF-β1 production. These results suggest that CM-chitin is an inductor of inflammatory cytokines and growth factors and may contribute to regeneration of cartilage. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 83A: 58–63, 2007

Key words: cartilage; chitin; inflammatory; mononuclear cell; TGF

INTRODUCTION

In humans, cartilage has poor capacity to repair because of its low mitotic activity and avascular nature, and it is difficult to regenerate normal cartilage at injured joint.[1,2] Cartilage is mainly composed of hyaline cartilage, which involves a rich extracellular matrix and water.[3] However, osteochondral defects that penetrate the subchondral bone usually heal with tissue that contains fibrous cartilage which lacks the durability and many of the mechanical properties of hyaline cartilage.[4] Several methods have been carried out for medical treatment of cartilage; the substitution with artificial joint, the bone marrow permeation by subchondral drilling, and abrasion arthroplasty were used for the treatments. However, these methods still have some problems, such as the loosening of artificial joint, the regeneration of fibrous cartilage from bone marrow spinal fluid, and the degenerative change of compensated tissue.[5]

In this context, tissue-engineering techniques have been developed for regeneration of cartilage at the affected part. Tissue-derived biomaterials such as cultured-autograft or allograft were used for repairs of cartilage defect: transplantation of the tissue showed well regeneration of cartilage tissue.[6] However, many problems still remain including a risk of viral transmission via allograft or a lack of tissue for transplantation, which can hardly be harvested from patient body.[7,8] Biomaterials as scaffolds constituting the organization structure and growth factors promoting cell differentiation or proliferation are also used for induction of regeneration at defective cartilage.[7,9,10] The scaffold materials such as hydroxyapatite[11,12] have been used to achieve repair small osteochondral defects. However, this may not be a widely acceptable method for complete repair of hyaline

Correspondence to: M. Hashimoto; e-mail: hassy@eng.kagoshima-u.ac.jp

cartilage.[13] Additionally, the cartilage repairing material involving the scaffolds and the growth factors associated with chondrogenesis is too expensive for practical treatment of all patients.

In the previous study, we found that complementing the osteochondral defecting part with the carboxymethyl-chitin (CM-chitin)/β-tricalcium phosphate (β-TCP) composite induced the regeneration of cartilage *in vivo* of rabbits.[14] In the investigation, the regeneration of articular cartilage was confirmed with CM-chitin/β-TCP but not with β-TCP alone at 8 weeks after implantation, presuming that CM-chitin played a key role for the regeneration of cartilage. However, the induction mechanism of chondrogenesis with CM-chitin was unclear. Chondrocyte is known to be differentiated from bone marrow-derived mesenchymal stem cells, by the effect of growth factors, such as transforming growth factor (TGF) or bone morphogenetic protein (BMP).[7,10,15] Since CM-chitin contains no growth factor, this material may stimulate surrounding cells at the region of administration to induce those factors. Further, it is considered that fracture-healing process is associated with the initial inflammation caused by inflammation cytokines and with the following chondrogenesis and osteogenesis.[16,17] In the present study, we investigated the expression of inflammatory cytokines and growth factors induced by the peritoneal injection of CM-chitin in mice.

MATERIALS AND METHODS

Stimuli and cells

The CM-chitin (degree of substitution for O-carboxymethylation = 79 mol %, degree of deacethylation = 27 mol %) was prepared from chitin extracted from Queen Crab shells according to the method reported previously.[18] Soluble-type and gel-type CM-chitins were prepared as described,[14] and β-TCP granules were purchased from Taihei Chemicals (Saitama, Japan). The levels of endotoxin contamination were determined by Endospecy® test according to the manufacture's instruction.

Soluble-type CM-chitin was dissolved in PBS (10 mg/mL) and gel-type CM-chitin and β-TCP granules were suspended in PBS (10 mg/mL). Six to 8-week-old male BALB/cN sea mice were obtained from Kyudo (Saga, Japan). The animals received humane care in accordance with our institutional guidelines and the legal requirements of Japan. Mice were injected intraperitonealy with 0.5 mL of stimulus solution or suspension. Peritoneal exudate cells (PEC) were collected from respective mice in each stimulation time, at 1, 2, 4, 24, or 72 h after injection. The number of cells collected were 1–3 × 10^6 cells/mouse. The PEC from mice were subjected to the extraction of ribonucleic acid (RNA) or cultured for the detection of secreted proteins.

mRNA expression

Total cellular RNA was extracted from PEC of two mice for each treatment, using TRIzol® Reagent (Invitrogen, Carlsbad, CA) or GenElute™ Mammalian Total RNA Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's procedure. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with total RNA (≤500 ng) using RNA PCR Kit (AMV) Ver.3.0 (TaKaRa BIO, Shiga, Japan) with sense and antisence oligonucleotide primers specific for β-actin,[19] interleukin-1β (IL-1β),[20] IL-6,[20] keratinocyte-derived chemokine (KC),[21] tumor necrosis factor-α (TNF-α),[20] macrophage-colony stimulating factor (M-CSF),[22] receptor activator of NF-κ B (RANKL),[23] interferon-γ (IFN-γ),[20] inducible NO synthase (iNOS),[24] cyclooxygenase-2 (COX-2),[25] and TGF-β1.[26] The amount of RNA was corrected by β-actin expression. The amplified products were detected by electrophorsis on a 1% agarose gel.

Growth factor assay

PEC were fractionated by the density gradient method using Histopaque-1083/1119 (Sigma-Aldrich) to obtain mononuclear cells (MNC) fraction. The numbers of cells obtained were 3–7 × 10^5/mouse. Total PEC collected from one mouse or MNC from two mice were suspended in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 200 μg/mL of BSA (Nacalai Tesque, Kyoto, Japan) and plated on each well of six-well plate. The cells were incubated for 24 h at 37°C in humidified air containing 5% CO_2. After incubation, culture supernatants were collected and analyzed for secreted TGF-β1 (R&D Systems, Minneapolis, MN) using an enzyme linked immunosorbant assay (ELISA) kit according to the manufacturer's instruction. Data are the mean ± SE of three independent experiments. Serum TGF-β1 concentrations in different experimental groups were analyzed for statistical significance by using Welch's *t*-test.

RESULTS

Stimulation of murine PEC by soluble-type CM-chitin

It was hypothesized in our previous experiment[14] that CM-chitin gel in the CM-chitin/β-TCP composite stimulated cells located at the surroundings of the implanting lesion to produce cytokines and/or growth factors, and lead to promote the regeneration of cartilage. Since CM-chitin gel is a biodegradable material, which can be digested by lysozyme *in vivo*,[27,28] the resulting soluble CM-chitin fragments may act as a stimulant for the regeneration. However, it is unknown what kind of cells are involved in the stimulation by CM-chitin. Thus, we first investigated the stimulating effect of soluble-type CM-chitin on cells *in vivo*. Soluble CM-chitin was intraperitoneally injected to BALB/c mice and mRNA expression in the stimulated PEC was detected by

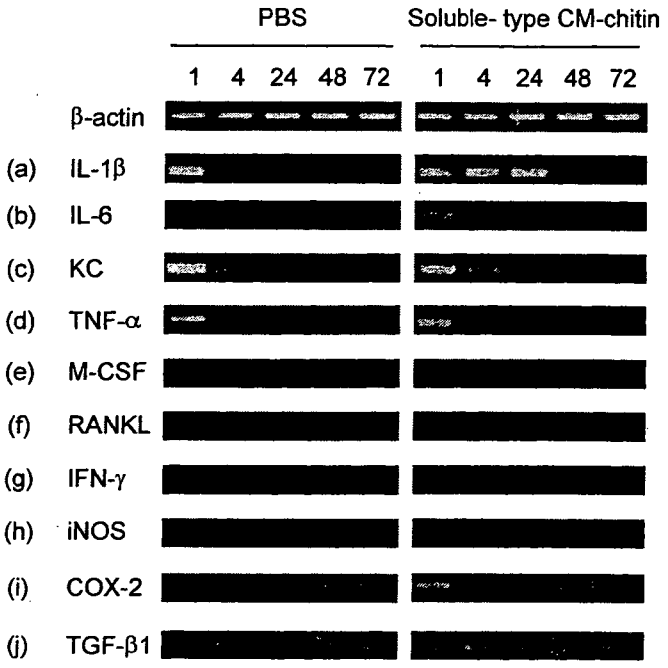


Figure 1. Time-dependent expressions of mRNA for cytokines in PEC treated with soluble-type CM-chitin. Results with soluble-type CM-chitin were compared with those with PBS. The numbers above the figures indicate elapsed times (hour) after injection of PBS or soluble-type CM-chitin in PEC in mice. IL-1β (a), IL-6 (b), KC (c), TNF-α (d), M-CSF (e), RANKL (f), IFN-γ (g), iNOS (h), COX-2 (i), and TGF-β1 (j) are surveyed by RT-PCR.

RT-PCR. Expression of mRNA for inflammatory cytokines, IL-1β, IL-6, KC, TNF-α, iNOS, and COX-2 in PEC stimulated with CM-chitin were significantly enhanced in comparison with those with PBS as a negative control [Fig. 1(a–i)]. Slight expression of these cytokines were observed in PBS injected mice at 1 h probably due to the injury of needle stick. In contrast, sustained inflammation was observed up to 24 h in CM-chitin injected mice PEC. The levels of endotoxin contamination was under the detection limit (less than 100 pg/mg). These suggested that CM-chitin was responsible for the expression of inflammation cytokines in mice.

Expression patterns of mRNA of TGF-β1 were also significantly different between two groups [Fig. 1(j)]. The expression of TGF-β1 in PBS group kept in low level throughout the period was observed, while that in CM-chitin group was enhanced. The production of TGF-β1 protein was confirmed by ELISA. The PEC harvested at 1–72 h after injection of stimuli were cultured for the secretion of protein in serum free medium and the secreted TGF-β1 was analyzed (Fig. 2). The result showed that TGF-β1 concentration from PEC stimulated with CM-chitin was significantly higher than that with PBS ($p < 0.01$ for 24 h and $p < 0.05$ for 1, 4, and 48 h). TGF-β1 level at 24 h was clearly higher than that of 1 h ($p < 0.05$), suggesting that TGF-β1 was secreted after inflammation [see Fig. 1(a–d, h, i)].

Stimulation of murine PEC by gel-type CM-chitin

In practical medical treatments, a gel-type CM-chitin/β-TCP composite material is used for the repairing of articular cartilage. Thus, we next investigated the effect of gel-type CM-chitin on PEC in mice. Expression of mRNA for inflammatory cytokines was enhanced up to 24 h after stimulation of gel-type CM-chitin in a similar manner as soluble-type CM-chitin [Fig. 3(a–e)]. TGF-β1 was also induced by CM-chitin gel [Fig. 3(e)].

β-TCP was reported to be a stimulant for cytokine induction.[29] Expression of mRNAs of inflammatory cytokines by the stimulation of β-TCP granules was observed in agreement with the report. However, the expression level by β-TCP was lower than that by CM-chitin, especially for IL-6, KC, TNF-α, and TGF-β1 (Fig. 3). The levels of TGF-β1 protein secretion was determined (Fig. 4). Gel-type CM-chitin induced high concentration of TGF-β1, while the level induced by β-TCP was similar to that by PBS. Further, TGF-β1 production stimulated by CM-chitin/β-TCP composite is slightly lower than CM-chitin only. These results suggested that CM-chitin but not β-TCP is a stimulant for TGF-β1.

Contribution of MNCs in cytokine induction by CM-chitin

It is known that macrophages are critical in the successful wound healing by participating in inflammatory and following processes.[30] Regulatory

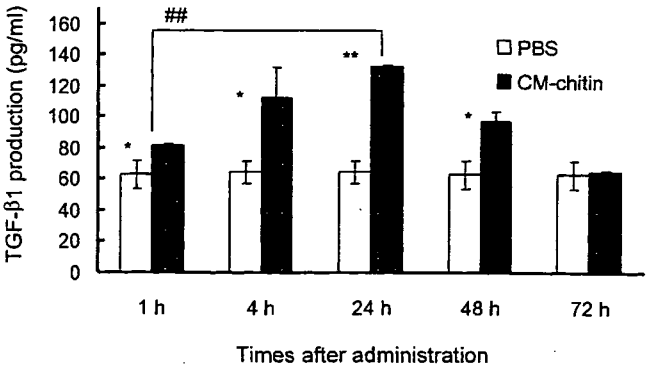


Figure 2. Effect of soluble-type CM-chitin on expression of TGF-β1 in PEC. Total PEC were harvested from mice at 1, 4, 24, 48, or 72 h after administration of CM-chitin or PBS cultured for 24 h, and their supernatants were used for ELISA to evaluate the expression of TGF-β1. Each value is the mean ± SE of three samples. * and ** indicate the significances at $p < 0.05$ and $p < 0.01$, respectively, compared with PBS treated group. ## indicates the significance at $p < 0.01$, compared with soluble-type CM-chitin treated group at 1 h.

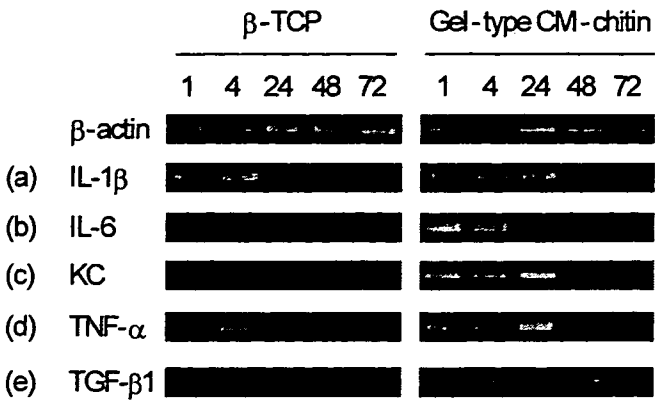


Figure 3. Time-dependent expressions of mRNA for cytokines in PEC treated with soluble-type CM-chitin. Expressions for solid gel type CM-chitin were compared with those for β-TCP, which constitute the biomaterial composite with CM-chitin. The numbers at the head of figure indicate elapsed times (hour) after injection of β-TCP or gel-type CM-chitin in PEC in mice. IL-1β (a), IL-6 (b), KC (c), TNF-α (d), and TGF-β1 (e) are surveyed by RT-PCR.

role of macrophages in bone wound healing was also reported.[31] In both cases, MNC contributed to the secretion of growth factors. Therefore, we investigated the contribution of MNC in TGF-β1 secretion. The PEC from soluble-type CM-chitin injected mice were subjected to a density gradient separation using Histopaque to fractionate MNC. As shown in Figure 5, TGF-β1 was secreted from the MNC fractionated from PEC at 24 and 48 h after injection of soluble-type CM-chitin.

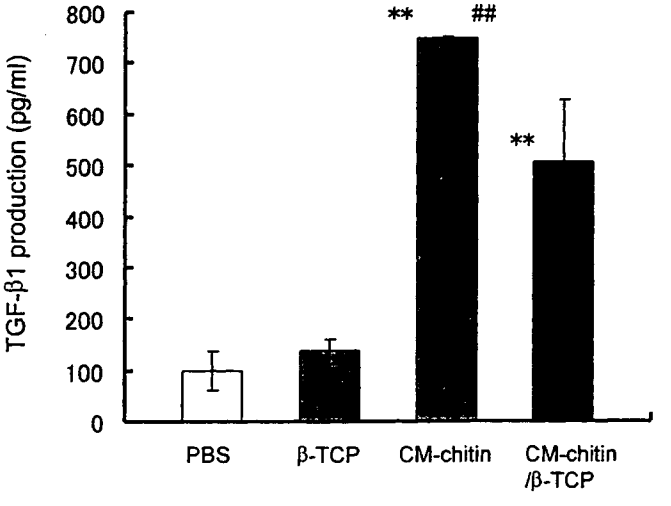


Figure 4. Effect of gel-type CM-chitin on expression of TGF-β1 in PEC. Total PEC were harvested from mice at 24 h after administration of gel-type CM-chitin, β-TCP, CM-chitin/β-TCP composite or PBS cultured for 24 h, and their supernatants were used for ELISA to evaluate the expression of TGF-β1. Each value is the mean ± SE of three samples. ** indicate the significances at $p < 0.01$ compared with β-TCP treated group. ## indicates the significance at $p < 0.01$ compared with CM-chitin/β-TCP composite treated group.

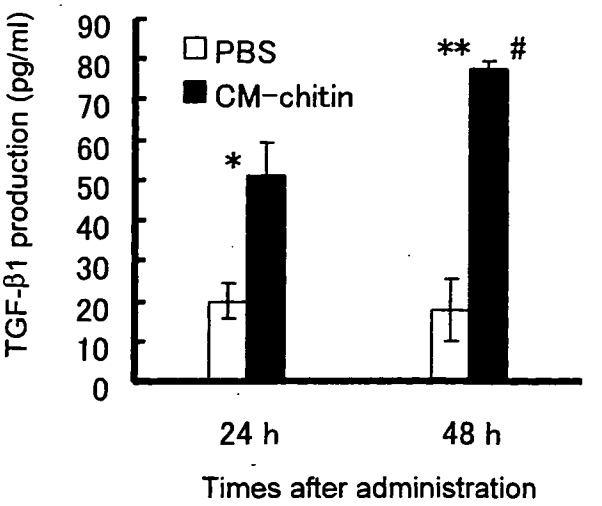


Figure 5. Effect of soluble-type CM-chitin on expression of TGF-β1 in fractionated PEC. PEC were harvested from mice at 24 or 48 h after administration of CM-chitin or PBS and fractionated by density gradient. The MNC fractions were cultured for 24 h and their supernatants were used for ELISA to evaluate the expression of TGF-β1. Each value is the mean ± SE of three samples. * and ** indicate the significances at $p < 0.05$ and $p < 0.01$, respectively, compared with PBS treated group. # indicates the significance at $p < 0.05$, compared with soluble-type CM-chitin treated group at 24 h.

DISCUSSION

In this study we found that CM-chitin stimulated cells to induce inflammatory cytokines, IL-1β, IL-6, KC, TNF-α, iNOS, and COX-2, in mice. Since CM-chitin contained no detectable endotoxin, CM-chitin is considered to be responsible for the inflammation. IL-1β and KC expression was continued to around 24 h, probably due to the paracrine interaction of early inflammatory genes.[32,33]

Some of these cytokines are considered to act as attractants of cells. KC is a mouse homologue of human chemokine CXCL1.[34] Chemokines are known as potent attractants for leukocytes such as neutrophils and monocytes.[35] Since macrophages are shown to be essential for wound and bone healing, KC might be associated with cartilage regeneration. TNF-α is reported to facilitate bone repair process by stimulating the recruitment of mesenchymal stem cells.[17] Since mesenchymal stem cells can proliferate to chondrocytes, TNF-α may also participate in the repair of cartilage.[17]

We also observed the production of TGF-β1 in PEC induced by CM-chitin. The functions of TGF-β, which primarily controls cell growth and differentiation for chondrocyte,[36] were investigated previously. It has a protective effect on the extracellular matrix of human articular chondrocyte by stimulating tissue inhibitor of metalloproteinases-1 (TIMP-1) expression,[37] or by decreasing procollagenase expression.[38]

TGF-β1 is also reported to increase the production of the chondroitin/dermatan sulfate proteoglycan, promoting the formation of the extracellular matrix, which is required for chondrogenesis,[39] and to promote the expression of several integrins, which mediate cell adhesion to other cells or to the extracellular matrix.[40–42] In the culture of mesenchymal stem cells, TGF-β1 was used to promote the chondrogenesis *in vitro* in combination with BMP-2 and/or dexamethasone.[10,43–46] Those results indicate that TGF-β1 secreted in mice with CM-chitin stimulation is a possible cause of chondrogenesis in the lesion. Interestingly, the mRNA expression and protein production of TGF-β1 were not observed by the stimulation of β-TCP stimulation. Masuda et al. reported that CM-chitin/β-TCP composite induced cartilage regeneration, but β-TCP did not.[14] These differences in cartilage repair may be associated with the cytokine expression profiles of CM-chitin and β-TCP. Further, addition of β-TCP did not promote TGF-β production by CM-chitin. These results suggested the importance of CM-chitin in cartilage regeneration.

The cytokine expression profile of CM-chitin, transient inflammation up to 24 h, followed by TGF-β1 expression (Figs. 1–3), is similar to those of wound and fracture healing.[16] In addition to that, the result shown in Figure 4 suggests that MNC were responsible for the TGF-β1 production in whole PEC. This suggestion also coincides with the previous report that TGF-β1 was secreted by activated MNC within an inflammatory tissue.[47] Thus, the mechanisms of cartilage regeneration may be expected as follows. Cells such as MNC or mesenchymal stem cells are recruited from surrounding tissues, including bone marrow and vessel, in the inflammation period, although responsible cells for inflammation evoked by CM-chitin have been clarified. The migrated MNC concluded the inflammation by producing suppression cytokines, such as TGF-β1, and then the mesenchymal stem cells proliferated to chondrocytes to regenerate cartilage tissue. Many biodegradable materials have been used for the regeneration of articular cartilage, generally with the aid of growth factor proteins.[15,48–51] The CM-chitin based β-TCP composite was demonstrated to repair articular cartilage without addition of growth factor.[14] Here, we showed that CM-chitin stimulated the induction of growth factors *in situ*. This may indicate that the composite has a dual role as a scaffold and an inducer of growth factor.

CONCLUSION

In the present study, CM-chitin was found to stimulate PEC to induce inflammatory cytokines and TGF-β1. It was also indicated that MNCs in PEC were responsible for the TGF-β1 production. Although we have not yet investigated if the cytokine expression in the composite of CM-chitin implanted lesion *in vivo*, it is assumed that similar cytokine expression is involved in regeneration of cartilage. Further researches are going on to clarify which cells are responsible to the mechanisms of chondrogenesis induced by CM-chitin.

References

1. Shapiro F, Koide S, Glimcher MJ. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. J Bone Joint Surg Am 1993;75:532–553.
2. Oka M. Biomechanics and repair of articular cartilage. J Orthop Sci 2001;6:448–456.
3. Minas T, Nehrer S. Current concepts in the treatment of articular cartilage defects. Orthopedics 1997;2:525–538.
4. Ponticiello MS, Schinagl RM, Kadiyala S, Barry FP. Gelatin-based resorbable sponge as a carrier matrix for human mesenchymal stem cells in cartilage regeneration therapy. J Biomed Mater Res 2000;52:246–255.
5. Ochi M, Adachi N, Nobuto H, Yanada S, Ito Y, Agung M. Articular cartilage repair using tissue engineering technique—Novel approach with minimally invasive procedure. Artif Organs 2004;28:28–32.
6. Britberg M, Lindahl A, Nilsson A, Ohlssion C, Isaksson O, Peterson C. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N Engl J Med 1994;331:889–895.
7. Tuan RS, Boland G, Tuli R. Adult mesenchymal stem ells and cell-based tissue engineering. Arthritis Res Ther 2003;5: 32–45.
8. Erggelet C, Steinwachs MR, Reichelt A. The operative treatment of full thickness cartilage defects in the knee joint with autologous chondrocyte transplantation. Saudi Med J 2000;21: 715–721.
9. Kim UJ, Park J, Kim HJ, Wada M, Kaplan DL. Three-dimensional aqueous-derived biomaterial scaffolds from silk fibroin. Biomaterials 2005;26:2775–2785.
10. Wang Y, Kim UJ, Blasioli DJ, Kim HJ, Kaplan DL. In vitro cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. Biomaterials 2005;26:7082–7094.
11. Chiroff RT, White RA, White EW, Weber JN, Roy D. The restoration of the articular surfaces overlying Replamineform porous biomaterials. J Biomed Mater Res 1977;11:165–178.
12. Suominen E, Aho AJ, Vedel E, Kangasniemi I, Uusipaikka E, Yli-Urpo A. Subchondral bone and cartilage repair with bioactive glasses, hydroxyapatite, and hydroxyapatite-glass composite. J Biomed Mater Res 1996;32:543–551.
13. Hasegawa M, Sudo A, Shikinami Y, Uchida A. Biological performance of a three-dimensional fabric as artificial cartilage in the repair of large osteochondral defects in rabbit. Biomaterials 1999;20:1969–1975.
14. Masuda S, Yoshihara Y, Muramatsu K, Wakebe I. Repairing of osteochondral defects in joint using β-TCP/carboxymethyl chitin composite. Key Eng Mater 2005;284–286:791–794.
15. Li WJ, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, Tuan RS. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. Biomaterials 2005;26:599–609.
16. Cho TJ, Gerstenfeld LC, Einhorn TA. Differential temporal expression of members of the transforming growth factor β superfamily during murine fracture healing. J Bone Miner Res 2002;17:513–520.