Table 1. The change of JOA and Harris scores.

Date	Mar. 17 20xx	Apr. 17 20xx	July. 10 20xx	Apr. 9 20xx+1
JOA hip score				
R	86	85	82	85
L	68	74	76	88
Harris score				
R	81	89	87	97
L	60.8	75	77	93

Abbreviation: JOA, The Japanese Orthopaedic Association.[7]

changes progress. It is considered that degeneration and wear of cartilage occur when mechanical loads, such as labor, exercise, and trauma, are added to background factors, such as race, gender, aging, obesity, and heredity. The metabolic disorder of chondrocytes occurs and cartilage destruction progresses, which induces inflammation of the synovial membrane and causes swelling of the joint and bone destruction. It progresses from pre-coxarthrosis → early stage → progressive stage → end stage in the general natural course, and various clinical symptoms appear in each disease stage.

There is 'primary' and 'secondary' osteoarthritis of the hip, as described above, and the presented case involved acetabular dysplasia-associated secondary coxarthrosis. In Japan, most patients with this disease are female, as was this patient. In treatment, conservative treatment is prioritized, such as instruction in daily living and physical and drug therapies, but some physicians consider that surgery in the early stage is desirable, even though symptoms are mild, when progression is apparently predicted. Various surgical methods have been proposed corresponding to the condition and stage of acetabular dysplasia and coxarthrosis (various osteotomy procedures, shelf operation, and total hip replacement).

The patient showed end-stage coxarthrosis on X-ray radiography, and felt pain not only while going up and down stairs but also when walking, limiting ADL. However, pain was rapidly alleviated after the initiation of Kampo treatment (Keishikaryojutsubuto and Boiougito) and ADL improved. Methods to judge therapeutic effects on osteoarthritis of the hip are roughly divided into comprehensive health scales[8,9] widely covering physical functions through mental health and disease-specific scales specialized for specific diseases including coxarthrosis. Of the latter, the Harris hip score[6] is the most internationally common criteria. It is comprised of pain (44 points), function (47 points), deformation (4 points), and range of motion (5 points). Pain is divided into 6 categories, and function is comprised of the walking ability (33 points) including claudication, support for walking, walking distance, and daily living activities (14 points) including going up and down stairs, wearing shoes and socks, sitting, and the use of public transportation. The clinical evaluation criteria of osteoarthritis of the hip most commonly used in Japan are the hip joint function assessment criteria established by the Japanese Orthopedic Association (JOA hip score).[7] The current JOA hip score was prepared in 1995 based on the old JOA hip score established in 1971, and it is comprised of the following 4 items: pain (40 points), range of motion (20 points), walking ability (20 points), and daily living activities (20 points). Categories proposed by Charnley, such as unilateral, bilateral, and multiple joint developments, were adopted. The maximum score of the range of motion is 20 points, accounting for a large proportion of the overall score, compared to that in the Harris hip score. In this patient, the JOA and Harris hip scores improved over the about one-year course, and so we consider that the objective effect of Kampo treatment was exhibited.

In Japan, Kampo drugs are covered by national health insurance and clinically applied for various diseases. Arthralgia has been treated with Kampo for centuries, and many Kampo formulations are administered for osteoarthritis,[10] rheumatoid arthritis,[11] and psoriatic arthritis.[12] This kind of remedy is often used with several joints such as a knee joint[13] in addition to hip joint caused by some pathogenesis such as degeneration, metabolism or inflammation. Although there has been no report on administrations of Boiogito and Keishikaryojutsubuto for osteoarthritis of the hip, an effect on osteoarthritis of the knee comparable to that of NSAIDs and synergistic effect with NSAIDs have been reported.[14]

The mechanism of the effect of Kampo on osteoarthritis has not been clarified. Pathologically, cracks and erosion occur in the cartilage surface in excessively loaded regions, loosing joint cartilage, in osteoarthritis of the hip. In the deep layer of joint cartilage, blood vessels invade calcified cartilage, and subchondral bone comes to exhibit a bone effect. On the other hand, osteophytes are formed in non-loaded regions. For rheumatoid arthritis,

an immunomodulatory effect of Kampo has been shown,[15] and the inhibition of bone destruction in some cases has been reported.[16] The inhibition of bone turnover in osteoarthritis is assumed.

Additionally, there is another problem in this kind of treatment. Namely, Kampo Medicine has the important feature that differ from Western Medicine; the diagnostic system in Kampo medicine is different from that in Western medicine. Therefore, it is surely thought that Kampo diagnosis may not be easy for readers to understand. When we treat RA patients with Kampo Medicine, it is necessary to make a Kampo diagnosis as well as a diagnosis by Western medicine. This issue makes it difficult to perform controlled clinical trials. In this case, we selected the keishikaryojutsubuto and boiogito according to the traditional diagnosis system. The target group for keishikaryojutsubuto and boiogito is follows: easy fatigability, coldness, obesity, sweating with swollen joints.[5,17]

Furthermore, Kampo formulae are generally composed of several herbal components, but not purified chemical compound. Therefore it is considered that these remedies are safe. However, pseudoaldosteronism by licorice root is well known to be an adverse effects of herbal medicine, and there are also allergic effects, such as skin eruptions and liver injury, that can be induced by crude drugs.[18–20]

In summary, we reported a patient with end-stage osteoarthritis of the hip accompanied by acetabular dysplasia in whom the QOL was improved by Kampo treatment. Although the clinical use of this remedy should be with careful deliberation due to the absence of evidence, the course of this disease varies depending on the lifestyle among patients and Kampo formulations may offer safe, potent supplemental treatment. The clinical course of a present patient may open the way to the achievement of randomized controlled trials and the further basic analysis of this remedy in the future.

Disclosures

Author(s) have provided signed confirmations to the publisher of their compliance with all applicable legal and ethical obligations in respect to declaration of conflicts of interest, funding, authorship and contributorship, and compliance with ethical requirements in respect to treatment of human and animal test subjects. If this article contains identifiable human subject(s) author(s) were required to supply signed patient consent prior to publication. Author(s) have confirmed that the published article is unique and not under consideration nor published by any other publication and that they have consent to reproduce any copyrighted material. The peer reviewers declared no conflicts of interest.

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ORIGINAL RESEARCH

TRADITIONAL HERBAL MEDICINES (KAMPO) FOR PATIENTS WITH RHEUMATOID ARTHRITIS RECEIVING CONCOMITANT METHOTREXATE: A PRELIMINARY STUDY

Toshiaki Kogure, MD, PhD; Takeshi Tatsumi, MD, PhD; Hiroko Sato, MD, PhD; Yuko Oku, MD, PhD; Daijiro Kishi, MD; Tomoyuki Ito, MD

Objective • To assess the clinical effectiveness and safety of traditional herbal medicines (THM: Kampo) used in combination with oral methotrexate (MTX) in order to control the disease activity of rheumatoid arthritis (RA) in patients whose disease remains active despite treatment with MTX.

Methods • Patients (n=13; male:female=1:12) with RA who achieved only a suboptimal response to MTX therapy (≥6mg/week and >6 months) were enrolled in this assessment. All patients were treated with Keishinieppiittokaryojutsubu (KER; decoction) according to the traditional diagnostic system. Every 3 months, joint symptoms were examined, and routine blood analysis and general serological tests including anticyclic citrullinated peptide antibody (aCCP) were performed, and then we calculated the disease activity score of 28 joints (DAS28).

Results • One patient withdrew from the study after 4 weeks and discontinued consultations with our department for unknown reasons. Five (41.7%) of the twelve patients were defined as responders, and seven patients (58.3%) were classified as nonresponders based on DAS28-CRP findings. On comparison between responders and nonresponders, there was no significant difference with regard to age or disease duration and the dosages of concomitant prednisolone at baseline. KER responders had lower levels of aCCP at baseline than nonresponders (mean±standard deviation: 329.2±113.9 U/mL vs 623.8±242.8 U/mL, respectively) (P=.046, Mann-Whitney test). Furthermore, responders to KER showed a significant decrease in the serum levels of aCCP. The annual cost for KER treatment is much less than that for other new drugs.

Conclusion • In patients whose active RA persists despite treatment with MTX, KER in combination with MTX is safe and well tolerated and provides clinical and economic benefits. Furthermore, pretreatment serum levels of aCCP are a useful predictor of a good response to KER treatment, and a decrease in serum levels of aCCP may be an adjunctive indicator in predicting the efficacy of this kind of treatment. (*Altern Ther Health Med*. 2010;16(1):46-51.)

Toshiaki Kogure, MD, PhD, is a professor, Takeshi Tatsumi, MD, PhD, is a lectural professor, Hiroko Sato, MD, PhD, is an assistant professor, and Daijiro Kishi, MD, is a clinical fellow, all in the Department of Integrated Japanese Oriental Medicine, School of Medicine, Gunma University, Maebashi City, Japan. Yuko Oku, MD, PhD, is a chief doctor in the Department of Internal Medicine, Gunma Central General Hospital, Maebashi City, Tomoyuki Ito, MD, is a clinical fellow in the Department of Internal Medicine (Division of Rheumatology), Nagaoka Red-cross Hospital, Nagaoka City, Japan.

Corresponding Author: Toshiaki Kogure
E-mail: tkogure@showa.gunma-u.ac.jp

Rheumatoid arthritis (RA) is a chronic, progressive disease that requires early diagnosis and aggressive treatment to minimize morbidity.[1] Disease-modifying antirheumatic drugs (DMARDs) are used to reduce inflammation and decrease the progression of articular damage. A prominent feature of several newer DMARDs is their immunosuppressive properties.[1] Methotrexate (MTX) is the most widely used DMARD because of its favorable efficacy and safety profile, its record of safety, and its ability to maintain a prolonged response.[2-4] However, use of MTX as monotherapy most often induces a partial response, and therefore patients are commonly given a combination of MTX and other DMARDs.[5-8] Combination therapy with MTX is beneficial for patients in whom the maximally tolerated dose of MTX does not provide adequate disease control.[5,8]

Traditional herbal medicines (THM: Kampo), which are covered by the national health insurance in Japan, are often prescribed for primary care and also applied as an alternative remedy for serious diseases such as RA. Generally, RA treatment with THM has been performed as adjunctive therapy for RA.[9] Several investigators have shown clinical effects of THM for RA,[9-11] and its immunomodulatory actions have been demonstrated in a collagen-induced arthritis mice model. Although the main target molecule remains unclear, we have demonstrated that the Kampo formula suppresses the production of interleukin-6 from macrophages and fibroblasts and that serum levels of anti-type II collagen antibody titer

are decreased in a collagen-induced mouse model.[12] These phenomena suggest that this Kampo formula controls polyclonal B cell activation; however, the effects of THM on RA patients with a suboptimal response to MTX have not yet been assessed. It is useful to evaluate those effects because the ultimate goals in managing RA are to prevent or control joint damage, prevent loss of function, and decrease pain. In the guidelines for management of RA, the indications for combination therapy, other monotherapy, or biologics should be considered in patients with suboptimal MTX response.[13]

Keishinieppiittokaryojutsubu (KER; decoction), one of the Kampo formulae, is often used in the adjunctive treatments for RA. KER is usually administered following traditional diagnosis, in addition to diagnosis by Western medicine. The traditional target group for KER comprises patients with thirst, sweating, coldness in the extremities, and swollen joints, as well as polyarthralgia in patients lacking physical strength.[14] If RA patients are outside this target group, other Kampo formulae such as Daibofuto or Bologito are prescribed. We previously demonstrated that KER decreased the serum levels of IgM-rheumatoid factor (RF), as well as the Lansbury articular index.[15] There have not been any reports of toxic effects, although pseudoaldosteronism induced by licorice root is possible. Therefore, KER is considered safe.

In the present study, we determine whether KER, a THM formula, combined with a therapeutic dose of MTX, is safe and effective for relief of the signs and symptoms of disease in patients who attained only a partial clinical response to MTX therapy.

PATIENTS AND METHODS

Patients

All patients treated between 2007 and 2008 who fulfilled the American College of Rheumatology (ACR) criteria[16] for the classification of RA and attained only a suboptimal response to MTX therapy (≥6mg/week and >6 months) were enrolled in this assessment. A suboptimal response to MTX was defined as a combination of moderate or high disease activity and moderate or no response despite MTX treatment by Disease Activity Score, including a 28-joint count, c-reactive protein (DAS28-CRP). All were outpatients of our department at Gunma University Hospital and were followed for at least 6 months. Patients who had previously been treated with biologics targeting tumor necrosis factor and tacrolimus (TAC) were excluded. This investigation was approved under the comprehensive agreement provided by Gunma University Hospital.

Study Design

The study design was a self-control trial for 6 months. All patients were treated with Keishinieppiittokaryojutsubu (KER; decoction) according to the traditional diagnostic system,[14] which differs from that of Western medicine. Although it is difficult to explain that system, in brief, we selected the RA patients with sweating, thirst, swollen joints, and coldness of the extremities. Some patients were also being treated with nonsteroidal antiinflammatory drugs (NSAIDs), bucillamine (BC), salazosulphapyridine (SASP), or prednisolone (PSL) at the start of treatment. All patients had taken NSAIDs, eight patients had taken PSL, seven had taken SASP, and four had taken BC. These drugs were administered 12 months before the start of MTX treatment. These concomitant drugs were continued without changing either the drugs or dosages during the 4 months before or during the observation period of this study. Every 3 months, joint symptoms were examined, and routine blood analysis and general serological tests were performed.

After the self-controlled trial, we observed patients for another 6 months to assess their outcome.

KER (THM) Therapy

The herbs comprising KER are shown in Table 1 and Figure 1. These herbs are covered by the national health insurance in Japan. Twelve herbs were mixed with 600 mL of water and boiled down to 300 mL, then the aqueous extract was filtered through a sieve. The extract, called a decoction, was administered twice a day before meals in the morning and evening.

Clinical Evaluation

DAS28-CRP was calculated according to the established formula available at http://www.das-score.nl/. Since the values obtained by DAS28-CRP are reported to be less than those obtained by the original DAS28 using the erythrocyte sedimentation rate, we used a threshold of 4.1 instead of the original 5.1 as the cutoff for high activity and 2.7 instead of 3.2 as the cutoff for low activity. Thus, we defined a value of DAS28-CRP >4.1 as high activity, 2.7 to 4.1 as moderate activity, <2.7 as low activity, and <2.3 as remission.[17] The response to KER therapy was evaluated every 3 months by the European League Against Rheumatism

TABLE 1 The Herb Composed of Keishinieppiittokaryojutsubu (KER)

Component (herb)	Weight (g)
Atractylodis lanceae rhizoma	10.0
Hoelen	5.0
Gypsum fibrosum	5.0
Zizyphi fructus	4.0
Cinnamomi cortex	3.0
Ephedrae herba	3.0
Paeoniae radix	3.0
Glycyrrhizae radix	3.0
Zingiberis rhizoma	1.0
Aconiti tuber	1.5
Sinomeni caulis et rhizoma	5.0
Astragali radix	5.0

Twelve herbs were mixed with 600 mL of water and boiled down to 300 mL; then the aqueous extract was filtered through a sieve. The extract, called a decoction, was administered twice a day before meals. These herbs were supplied by Uchida Wakanyaku Co Ltd, Tokyo, Japan.

FIGURE 1 Twelve dried traditional herbs (a mixture) were used as Keishinieppiittokaryojutsubu (KER). These herbs are covered by national health insurance in Japan.

(EULAR) response criteria using 4.1 and 2.7 as the thresholds for high and low disease activities, respectively. Consequently, patients who showed low activity (DAS28-CRP <2.7) after treatment with KER and whose DAS28 decreased to >1.2 were defined as showing a good response, and patients whose DAS28 decreased to >0.6 were defined as showing a moderate response. We recognized patients showing a good or moderate response as responders to KER, and patients showing no response as nonresponders.

Annual Drug Cost

We calculated the annual drug cost (Japanese Yen) for each drug. KER 300 mL decoction/day (Table 1), TAC 1.5 mg/day, and infliximab 150 mg (3 mg/kg) IV/8 week were recognized as maintenance dosages.

Measurement of Serum Levels of aCCP

To assess the association between the serum level of anticyclic citrullinated peptide antibody (aCCP) and the response to KER, we monitored the serum levels of aCCP at baseline and after 6 months.

Serum IgG aCCP was measured using enzyme-linked immunosorbent assay kits (Axis-Shield UK, Cambridgeshire, England) according to the manufacturer's instructions. Each assay was carried out in duplicate. Antibody titers of more than 5 arbitrary units per milliliter were considered positive.

Statistical Analysis

Data are expressed as mean (SD) values. All data were collected in a computer database. Mann-Whitney test or repeated measures analysis of variance was used to analyze each set of data to determine changes in aCCP titers. For all statistical tests, differences were regarded as significant at $P<.05$.

RESULTS

Baseline Demographic and Clinical Characteristics

Of 13 patients enrolled in this study, 12 patients completed 6 months of KER treatment. One patient withdrew from the study after 4 weeks and discontinued consultations with our department for unknown reasons. Baseline demographic and clinical characteristics of 12 patients receiving KER therapy are summarized in Table 2. None of the patients withdrew because of adverse effects.

Classification by Clinical Response

Five (41.7%) of 12 patients were classified in the responder group, and seven patients (58.3%) were classified in the nonresponder group based on DAS28-CRP findings. Of five patients in the responder group, three patients showed a good response, and two patients showed a moderate response. On comparison of the responder group and nonresponder group, there were no significant differences with regard to age or disease duration. Furthermore, the dosages of concomitant PSL at baseline did not differ between the two groups.

Outcomes of Patients in the Nonresponder Group

Seven patients showed high or moderate activity without a decrease >0.6 in DAS28-CRP 3 or 6 months after treatment with KER was initiated. These patients were defined as nonresponders to KER. Other treatments such as TAC or biologics were administered to these patients.

Five patients received TAC, and two patients were administered infliximab. Among five patients receiving additional TAC,

TABLE 2 Baseline Demographic and Clinical Characteristics of 12 Patients Receiving KER Therapy Among the Rheumatoid Arthritis Patients Without the Optimal Efficacy by Methotrexate Therapy*

	Male/female	Age	Duration (year)	Stage	Class	PSL (mg/day) dosage	RF	DAS28-CRP
All	1/12	55.6±11.3	6.7±5.5	2.6±0.8	2.3±0.5	3.1±3.4	127.0±83.3	4.7±0.6
Responder	0/5	58.8±5.6	5.7±5.1	2.2±0.8	2.0±0.2	5.0±4.1	130.0±97.4	4.6±0.4
Nonresponder	1/6	48.4±7.1	5.1±4.0	2.8±0.8	2.4±0.5	1.8±2.5	114.4±74.8	4.7±0.5

*PSL indicates prednisolone; RF, rheumatoid factor; DAS28-CRP, disease activity score including a 28-joint count, c-reactive protein.

two patients showed a good response, and the other three patients showed a moderate response 6 months after TAC was additionally administered. Two patients receiving infliximab showed a good response. The outcome of 12 patients with only a partial clinical response to MTX therapy alone are shown in Figure 2.

Annual Drug Cost

To compare the medical costs, we calculated the annual drug cost based on the price of each drug (Table 3). The annual cost for KER: Kampo treatment was much less than that for TAC or infliximab.

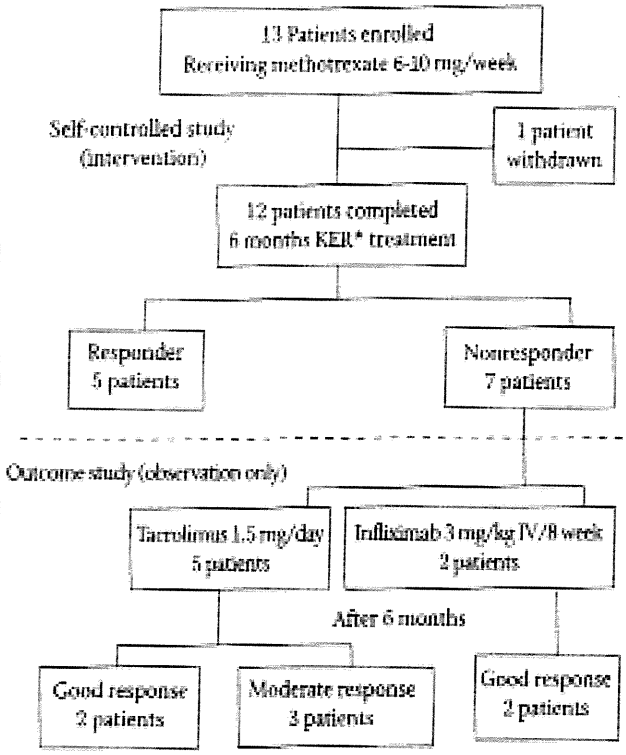

FIGURE 2 The outcomes of 12 patients who showed only a partial clinical response to MTX therapy.*

Five (41.7%) of 12 patients were classified in the responder group, and seven patients (58.3%) were classified in the nonresponder group following treatment with KER. Seven patients showed high or moderate activity without a decrease >0.6 on DAS28-CRP 3 or 6 months after treatment with KER was initiated.

Of seven nonresponders to KER, five patients were administered tacrolimus, and two patients were administered infliximab. Among five patients receiving additional TAC, two patients showed a good response and the other three patients showed a moderate response 6 months after TAC was additionally administered. Two patients receiving infliximab showed a good response.

*KER: Keishinieppitnokaryojutsubu (Herbal decoction)

TABLE 3 Dosages and Costs of Disease-modifying Antirheumatic Drugs Used in the Treatment of Rheumatoid Arthritis in This Preliminary Study

	Usual Maintenance Dose	Annual Drug Cost (Japanese Yen)
Tacrolimus	1.5 mg/day	51 000
Infliximab	3 mg/kg/8 weeks	1 053 000
KER	See Table 1	37 000

KER indicates Keishinieppitnokaryojutsubu.

Titers of Serum aCCP at Baseline

The diagnosis for THM treatment is dependent on the traditional system, not on Western medicine, and a predictor is expected for responders to KER. Therefore, we further analyzed the titers of serum levels of aCCP between the responder group and nonresponder group in patients without an optimal response to MTX therapy. KER responders had lower levels of aCCP at baseline than nonresponders (mean±SD: 329.2±113.9 U/mL vs 623.8±242.8 U/mL, respectively) (P=.046, Mann-Whitney test). Other univariate analysis did not show any significant differences in baseline clinical measures of anatomical stage, functional class, DAS28-CRP, or rheumatoid factor (RF) levels between the two groups, although patients in the responder group were slightly younger than those in the nonresponder group.

KER Effect on aCCP Levels

We are interested in aCCP changes during KER treatment because there have been recent reports focusing on aCCP changes induced by biologics targeting TNF or MTX and other DMARDs.[10]

Changes in aCCP titers were separately assessed in the responder group and the nonresponder group (Figure 3). First, in responders to KER, the serum levels of aCCP significantly decreased after 6 months of treatment compared with those at baseline. Additionally, the serum levels of RF also decreased significantly in responders (data not shown). In contrast, nonresponders did not show a decrease in aCCP levels after 6 months.

DISCUSSION

This preliminary study assessed the efficacy of KER in combination with oral MTX for the treatment of RA. All patients with persistent active disease despite treatment with the tolerated dosage of MTX were enrolled.

KER, a THM formula (Kampo), was generally safe and well tolerated by patients in this preliminary study, who also received concomitant MTX. Responders to KER comprised 41.7% of the assessed patients. Among responders to KER, KER treatment resulted in good or moderate response of DAS28-CRP, as well as low disease activity of DAS28-CRP, and serum levels of RF were significantly decreased. Thus, the clinical effects in responders to KER may be efficient as an adjunctive treatment in patients with active disease despite treatment with MTX. In a trend, biologics or combination therapy with other DMARDs should be considered for patients with suboptimal MTX response according to the Guidelines for Management of RA 2002.[10] Although there were nonresponders to KER in this series, they also showed low disease activity in response to treatment with tacrolimus or infliximab after observation for 6 months. Therefore, our pilot

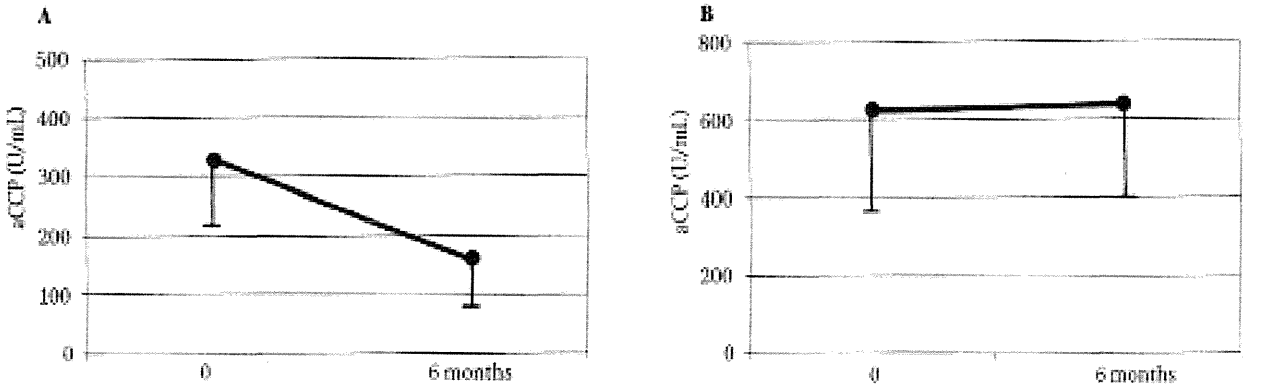

FIGURE 3 Changes in aCCP levels were assessed in each group. (A) Responder group: The levels of aCCP were significantly decreased after 6 months of treatment compared with the baseline values. (B) Nonresponder group: There was no significant decrease in aCCP levels at 6 months. Importantly, there was a significant difference in the change in aCCP levels between the responder and nonresponder groups when baseline values were compared with those after 6 months ($P<.049$, repeated measures analysis of variance).

study suggests that KER may be a useful agent for the treatment of RA patients receiving MTX. However, clinical utilization of this finding should be limited as this was a self-controlled trial rather than a randomized controlled trial and there were a limited number of patients in this series. Since there are responders to KER in the preliminary data, the efficacy of THM (Kampo) for patients with suboptimal MTX response should be further evaluated by additional clinical trials.

In RA, clinical effects of THM were demonstrated by several investigators, and its immunomodulatory effects were reported in several mouse models of arthritis.[9-11] The components of KER, which is a crude drug, are shown in Table 1. Several of the 12 herbs comprising KER have components—pseudoephedrine (*Ephedrae herba*), paeoniflorin (*Paeoniae radix*), and tetrandrine (*Sinomeni Caulis et Rhizoma*)—that show antiinflammatory or immunomodulatory effects. The clinical effects of KER on RA are thought to be at least partially due to the effects of each herb. Additionally, there is probably some interaction among the components.

RA has significant economic implications for individual patients as well as for society. A recent study showed that annual medical costs for a patient with RA are approximately $9519.[18] For many years, relatively low-cost options have been available for the treatment of RA. However, the advent of COX-2 inhibitors and newer DMARDs, including biologic agents, has brought cost considerations to the forefront. THM is a lower-cost agent in comparison with tacrolimus or biologics, and so it may be considered more available in today's cost-constrained environment. THM, which is covered by national health insurance in Japan, is often prescribed in the primary care field and also applied as an alternative remedy for RA. However, annual costs rise as function based on the Health Assessment Questionnaire declines. Therefore, it is expected that the efficacy of THM will be confirmed in other interventional studies. Based on these economic benefits, we will perform further interventional studies.

At present, we cannot predict the response to THM in patients with RA before such a remedy is prescribed because THM is administered according to the traditional diagnostic system, which differs from that of Western medicine. Predictors of treatment response to THM therefore need to be identified. Recently, it has been demonstrated that serum levels of aCCP may be a useful predictor of the response to MTX[19]; therefore, we measured the serum levels of aCCP in enrolled RA patients. KER responders among RA patients without optimal MTX response showed lower levels of aCCP at baseline than nonresponders. This finding suggests that low pretreatment levels of serum aCCP are associated with a more favorable response to KER treatment for RA, whereas high levels are associated with an insufficient response. To evaluate predictive availability, we further analyzed the change in serum levels of aCCP during observation. The levels of serum aCCP showed a significant decrease in the responder group, and there was a significant difference in the change in aCCP levels between the responder and nonresponder groups. These findings suggest that a decrease in serum aCCP levels after treatment, as well as low pretreatment aCCP levels, are associated with a better response to KER treatment in RA. Thus, monitoring the levels of aCCP may provide a predictive guideline for treatment with KER.

The significant decrease in serum levels of aCCP in the responders to KER is interesting because RA patients in whom the serum level of aCCP did not decrease demonstrated severe progression of joint damage, whereas low progression of the erosive lesion was observed in RA patients with a decrease in aCCP.[20,21] These findings suggest that radiographic progression may be suppressed in responders to KER who show a decrease in their serum levels of aCCP. This suggestion should be considered tentative, however, until a long-term study is carried out as the association between aCCP titer and RA severity remains controversial.[19] Recently, it has been demonstrated that aCCP titer may be influenced by several causes such as smoking rather than simply RA severity and treatment.[22]

Finally, in patients whose active RA persists despite treatment with MTX, KER in combination with MTX is safe and well tolerated and provides clinical and economic benefits. Furthermore, pretreatment serum levels of aCCP are a useful predictor of a good

response to treatment with KER, and a decrease in serum levels of aCCP may be an adjunctive indicator in predicting the efficacy of this kind of treatment. These findings suggest that further clinical studies should be promoted to make better use of THM in the field of RA treatments.

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Integrative Medicine Insights

CASE REPORT

Four Cases of Dysthymic Disorder and General Malaise Successfully Treated with Traditional Herbal (Kampo) Medicines: Kamiuntanto

Toshiaki Kogure[1,2], Takeshi Tatsumi[1,2] and Yuko Oku[3]

[1]Department of Integrated Japanese Oriental Medicine, School of Medicine, Gunma University, Japan. [2]Department of Japanese Oriental Medicine, Gunma Central General Hospital, Gunma, Japan. [3]Department of Internal Medicine, Gunma Central General Hospital, Gunma, Japan. Email: tkogure@showa.gunma-u.ac.jp

Abstract: Traditional herbal (Kampo) medicines have been used since ancient times to treat patients with mental disorders. In the present report, we describe four patients with dysthymia successfully treated with Kampo medicines: Kamiuntanto (KUT). These four patients fulfilled the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria for dysthymic disorder with easy fatigability and sleeplessness, but did not fulfill the criteria for major depressive disorder. Treatment with KUT relieved depressive status, fatigue and sleeplessness in these patients. As a result, their QOL (quality of life) was considerably improved. KUT may be useful as an additional or alternative treatment for dysthymia, especially in the field of primary health care.

Keywords: herbal medicine, dysthymic disorder, fatigue, kamiuntanto

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Introduction

Dysthymic disorder (dysthymia) is a disabling psychiatric disorder characterized by mild but persistent depressive symptoms. In the USA, it is reported that the lifetime prevalence of dysthymia ranges from 3% to 6% in the general population,[1,2] and up to 36% in psychiatric outpatient clinics.[3] In Japan, a low lifetime prevalence (1.4%)[4] and a 12-month prevalence of 0.7%[5] for dysthymia have been reported. It has been suggested that the low rate of dysthymia in Japan is due to a lack of familiarity with operational diagnostic criteria, such as the Diagnostic and Statistical Manual of Mental Disorders (DSM). Early diagnosis is of vital important for the successful treatment of dysthymia, especially in primary health care. Additionally, about 25% of patients with dysthymia experience a chronic unchangeable status, and a subset of these patients develop major depressive disorder despite various treatments, such as antidepressants and antianxiety drugs.

In Japan, traditional herbal (Kampo) medicines (THM) are covered by national health insurance and play an important role in primary care, and several kampo formulae have been prescribed for mental disorders.[6,7] THM has two points that differ from Western Medicine, i) the Kampo formula is a crude drug, not a purified chemical product; ii) the diagnostic system in Kampo Medicine differs from that of Western medicine. A Kampo formula is generally composed of several herbal components and is generally considered safe. Pseudoaldosteronism due to licorice root is a well known adverse effect of THM. There have also been allergic effects, such as skin eruption and liver injury, induced by crude drugs. Furthermore, it is crucial to understand that the Kampo diagnostic system is constructed from a paradigm that differs from the paradigm underlying Western natural science. When we treat a patient with dysthymia using Kampo Medicine, Kampo diagnosis is required in addition to that of Western Medicine. These characteristics make it difficult to perform controlled clinical trials. Therefore, there is no evidence supporting the use of Kampo formulae for dysthymia although Kampo formulae are often applied for mental disorders in Japan. However, it is a fact that there are responders to THM among patients with dysthimia. In this regard, we prescribed Kamiuntanto (KUT), one of these Kampo formulae, for the treatment of dysthymia with several physical and mental symptoms diagnosed by DSM-4th edition (DSM-IV).

Here, we describe four patients with dysthymia who were successfully treated with KUT.

Case Reports

Treatment of each patient was approved under the comprehensive agreement of Gunma University. Informed consent was obtained from all four cases before treatment with Kampo therapy. Further, each patient was treated before 2008. The authors have received training in performing clinical trials at Gunma University.

Case 1

A 63-year-old male consulted the Department of Japanese Oriental (Kampo) Medicine (DJOM), Gunma University in October 200X requesting traditional herbal medicine (Kampo) for dysthymic disorder with sleeplessness and malaise that had persisted for about 5 years despite treatment with antidepressants (Table 1). He worked at a bakery as a full-regular employee on ordinary days. He was neither a smoker nor a drinker. At the initial examination, there were no remarkable findings in the chest or abdomen, and hepato-renal and thyroid functions appeared normal on both blood analysis and image diagnosis. Additionally, he had not complained of any clinical features indicating the dementia. He had been taking time off work several days a month due to fatigue. Treatment with Kamikihito, one of the kampo formulae, for 4 weeks failed to improve his symptoms. Kamiuntanto (KUT; Table 2) was therefore administered in addition to antidepressants. After KUT therapy for 12 weeks, sleeplessness and malaise improved. The patient was accordingly relieved from dysthymia and estazolam was discontinued, and the patient became able to commute every day. The improvement in social activity has continued for 3 years with KUT. In addition, we evaluated the improvement of depressive symptoms using global assessment of functioning (GAF) scale by DSM-4th ed. Text revision (DSM-IV TR)[8] in all cases. Her GAF scale changed from 70–61 to 90–81.

Table 1. Clinical features of four patients with dysthymia.

Patient	Age/sex	Duration of dysthymia	Concomitant drugs at the first examination	Depressive state*	Appeteite loss or bulimia	Insomnia or hypersomnia	Lack of volition or fatigue	Loss of pride	Indecisive or loss of his concentration	Despair
1	63/m	5 years	Trazodone hydrochloride 50 mg Estazolam 2 mg Sulpirid 50 mg	+	–	Insomnia	both	–	+	–
2	62/f	2 years	Zolpidem tartrate 5 mg Brotizolam 0.5 mg (occasional use) Etizolam 1.0 mg	+	Mild appeteite loss	Insomnia	both	–	+	–
3	61/f	2 years	Zolpidem tartrate 5 mg	+	–	Insomnia	+	–	+	–
4	53/f	3 years	No psychotropic drugs	+	–	Insomnia	+	–	–	–

*Mild depressive mood persisting nearly all day for more than 2 years and absence of major depressive episode for at least the first 2 years. All patients were treated with Kamiuntanto (KUT), and recovered from their dysthymic condition.

Table 2. Herbal components of Kamiuntanto (KUT).

Japanese name	English name	Volume (gram)
Hange	Pinelliae Tuber	5.0
Bukuryo	Hoelen	4.0
Chinpi	Aurantii Nobilis Pericarpium	3.0
Chikujo	Banbusae Caulis	3.0
Onji	Polygalae Radix	2.0
Kanzo	Glycyrrhizae Radix	2.0
Genjin	Scrophulariae Radix	2.0
Kijitsu	Aurantii Fructus Immaturus	2.0
Sansonin	Zizyphi Semen	2.0
Jiou	Rehmanniae Radix	2.0
Taiso	Zizyphi Fructus	2.0
Ninjin	Ginseng Radix	2.0
Shokyo	Zingiberis Rhizoma	1.0

The herbs were mixed with 600 ml of water and boiled down to 300 ml, and the aqueous extract was filtered through a sieve. The extract, called a decoction, was administered twice a day before meals in the morning and evening.

Case 2

A 62-year-old female consulted DJOM requesting Kampo treatment for general malaise and lack of volition that had persisted for 2 years despite conventional Western therapy, which consisted of benzodiazepines. She was not regarded as having senile dementia. She was a housewife and barely able to perform housework. Her status was diagnosed as dysthymic disorder by operational diagnostic criteria; DSM-IV. Saikokeishikankyoto (decoction) therapy in addition to Western medicines for 4 months failed to improve her symptoms. Therefore, we changed the Kampo formula from Saikokeishikankyoto to KUT. Dysthymia, consisting of general malaise and depressive symptoms was reduced by 80% after KUT therapy for about 4 months, along with the occasional use of Kousosan (TJ-70, 2.5 g TSUMURA Co. Ltd Japan) to relieve her anxiety. Relief from dysthymia has continued for about 2 years with KUT treatment. Her GAF scale changed from 70–61 to 90–81.

Case 3

A 61-year-old female developed a feeling of heavy head and sleeplessness in April 200X. She was receiving atorvastatin for hyperlipidemia, and had also received a sleeping drug from a local hospital. However, her symptoms persisted, followed by the development of depressive symptoms and malaise,

although she continued to work as a pharmacist. She consulted our hospital requesting Kampo treatment in November 200X + 2. She was diagnosed as having dysthymic disorder based on DSM-IV. Kousosanryo (decoction) therapy for 8 weeks failed to improve dysthymia. Her symptoms relieved by 80% after 4 months of KUT administration, and thereafter she became able to concentrate on work and housekeeping. Improvement of dysthymia has continued for 18 months with KUT treatment. Her GAF scale changed from 80–71 to 100–91.

Case 4

A 53-year-old female (menopause: 51-year-old) came to DJOM requesting Kampo treatment for dysthymic disorder with sleeplessness, malaise and nervousness without vasomotor symptoms, consisting of hot flashes and sweating, which had persisted for about 5 years. She was a housewife and barely performed housework. She had not been taking antidepressant therapy, although she was taking hypotensive drugs for essential hypertension. Depressive symptoms were relieved after 4 months of KUT treatment and the improvement continued for 6 months. However, sleeplessness, easy fatigability and appeteite loss reappeared. Therefore, we changed KUT to another kampo formula (Kamikihito: decoction) and have obtained improvement by 50%. Her GAF scale changed from 70–61 to 90–81.

In all cases, there were no remarkable findings in the chest or abdomen, hepato-renal and thyroid functions appeared normal on both blood analysis and image diagnosis, and dysthymic disorder had been diagnosed by a psychiatrist based on DSM-IV criteria. During the follow-up periods, there were no adverse reaction attributable to Kampo medicines.

Discussion

Dysthymia is defined in DSM-IV as follows: mild depressive mood continued nearly all day for 2 years, and there were no major depressive episodes observed during at least the first 2 years. It has been reported that youth are susceptible to dysthymia, while elderly people demonstrate symptoms closer to major depressive disorder. Three (No. 1.2.3 in Table 1) of our cases were elderly patients, however kampo treatment with KUT resulted in an improvement of depressive mood. However, one of the patients (No. 4 in Table 1.) experienced dysthymic symptoms in the postmenopausal period, and her status was categorized as a climacteric mental disorder. It is well known that depressive symptoms in climacterium are associated with a decrease in estrogen (E2). Although an E2-like action of KUT has not been recognized, it is possible that KUT may also be effective for dysthymia in postmenopausal females.

Although dysthymia is apt to be regarded as a mild depressive disorder by non-psychiatrists, social loss due to dysthymia is serious. Cassano et al have reported that social activity shows greater reduction in patients with dysthymia than in patients with major depressive disorders.[9] The clinical features of dysthymia are characterized by low ADL despite mild depressive symptom. The etiology of low ADL remains unclear, but it is possible that it may be difficult to diagnose dysthymia early because the depressive symptoms are mild. In addition, the physical symptoms such as general malaise as well as emotional symptoms probably contribute to decreasing ADL in dysthymia. The clinical characteristics of each patient in this series are summarized in Table 1. Each patient complained of fatigue and sleeplessness. Furthermore, it is well known that dysthymia in climacterium is characterized by severe malaise. KUT treatment resulted in the improvement of depressive status, as well as easy fatigability and sleeplessness, and so ADL would probably improve. These clinical courses suggest that KUT (Kampo medicine) may be useful as an additional or alternative treatment for dysthymia, especially in the field of primary health care. During this period, we encountered 2 other patients with depressive symptoms, who did not fulfill the criteria for dysthymia because the period of mild depressive symptoms was less than 2 years. Howevere, these patients were also successfully treated with KUT (data not shown). Kampo treatment generally aims not only at improving or regaining physical health, but also taking the patient's psychic and emotional imbalance into account.[10] However, the efficacy is limited among responders to KUT treatement. To confirm this efficacy, further clinical trial such as N of 1 clinical study,[11] will be required.

In Japan, traditional herbal medicines (Kampo) are covered by national health insurance, and are generally used in primary health care. KUT (kamiuntanto) is one of the kampo formulae used for the treatment

of mental disorders, such as insomnia or dementia.[12] Kampo formula is administered following traditional diagnosis, in addition to diagnosis by Western medicine. The traditional target group for KUT comprises patients with sleeplessness, anxiety, and malaise after a serious illness as well as depressive status in patients lacking physical strength.[9] Since patients with dysthymia who complain of general malaise are close to the target group for KUT, we therefore treated 4 dysthymia patients with KUT and achieved good outcomes. Further, the traditional target group of Kamikihito (a kampo formula), which was administered in case nos. 1 and 4, comprises patients characterized by appetite loss in addition to other symptoms.

It is still not clear whether KUT improves the status of dysthymia, but several actions of KUT on the nervous system have been demonstrated. It has been reported that KUT potentiates the brain cholinergic system in an aged mouse model and its effect may be attributed to an increase in the activity of choline acetyltranseferase (ChAT).[13] Those effects have also been demonstrated in thiamine-deficient mice that demonstrate impairment of learning and memory.[14] It has been considered that the beneficial effect of KUT on Alzheimer's disease (AD) is due to the potentiation of ChAT, but not inhibition of cholinesterase (ChE).[12] Although an excess of HPA axis was observed in the patients with dysthymia, suppression of the HPA axis by KUT has not been demonstrated. However, recently it has been reported that AD and depression are significant associated in the aging population, and interestingly ChAT polymorphism is significantly associated with depression.[15] Three of our patients were elderly, and KUT might improve dysthymic status through action on ChAT as in dementia. Furthermore, Smith et al have demonstrated that cholinergic neurons were also decreased in the cerebral cortex such as the frontal lobe in postmenopausal females, and estrogen replacement therapy (ERT) suppressed the decrease in cholinergic neurons using SPECT and ^{123}I-iodobenzovesamicol.[16] Therefore, it is possible that KUT treatment may potentiate ChAT in the postmenopausal female. We consider that the dysthymic patient (Pt. no. 4 in Table 1) in climacterium was also successfully treated with KUT due to its effects on ChAT. Thus, it is considered that KUT may be useful for various patients with dysthymia.

Finally, we present 4 patients with dysthymia successfully treated with the Kampo formula: KUT. KUT may be useful and safe as an additional or alternative treatment for dysthymia. These observations encourage us to proceed further with controlled trials to confirm the efficacy of KUT.

Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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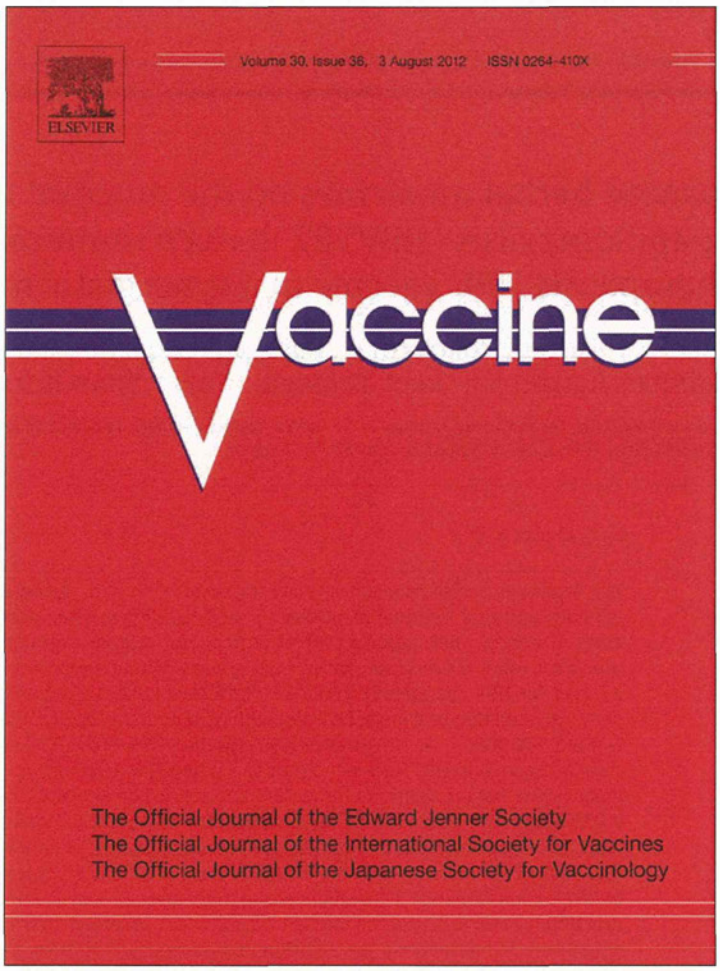

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Adjuvant effect of Japanese herbal medicines on the mucosal type 1 immune responses to human papillomavirus (HPV) E7 in mice immunized orally with *Lactobacillus*-based therapeutic HPV vaccine in a synergistic manner

Ayumi Taguchi[a], Kei Kawana[a,*], Terufumi Yokoyama[b], Katsuyuki Adachi[a], Aki Yamashita[a], Kensuke Tomio[a], Satoko Kojima[a], Katsutoshi Oda[a], Tomoyuki Fujii[a], Shiro Kozuma[a]

[a] *Department of Obstetrics and Gynecology, Faculty of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*
[b] *GENOLAC BL Corp. 503, Okinawa Industry Support Center, 1831-1, Oroku, Naha, Okinawa 901-0152, Japan*

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ABSTRACT

The Japanese herbal medicines, Juzen-taiho-to (JTT) and Hochu-ekki-to (HET), have been shown to enhance humoral immune responses to vaccine antigen when used as adjuvants for prophylactic vaccines. However, their adjuvant effect on mucosal cellular immune responses remains unstudied. The precursor lesion of cervical cancer, high-grade CIN that expresses HPV E7 oncoprotein ubiquitously is a target for HPV therapeutic vaccines that elicit mucosal E7-specific type 1 T cell responses. We have demonstrated that oral immunization with recombinant *Lactobacillus casei* expressing HPV16 E7 (LacE7) is more effective in eliciting mucosal E7-specific IFNγ-producing cells than subcutaneous or intramuscular antigen delivery. Here we report the synergistic effect of an oral *Lactobacillus*-based vaccine and Japanese herbal medicines on mucosal immune responses. Oral immunization of mice with LacE7 plus either a Japanese herbal medicine (JTT or HET) or a mucosal adjuvant, heated-labile enterotoxin T subunit (LTB), promotes systemic E7-specific type 1 T cell responses but not mucosal responses. Administration of LacE7 plus either Japanese herbal medicine and LTB enhanced mucosal E7-specific type 1 T cell response to levels approximately 3-fold higher than those after administration of LacE7 alone. Furthermore, secretion of IFNγ and IL-2 into the intestinal lumen was observed after oral administration of LacE7 and was enhanced considerably by the addition of Japanese herbal medicines and LTB. Our data indicated that Japanese herbal medicines, in synergy with *Lactobacillus* and LTB, enhance the mucosal type 1 immune responses to orally immunized antigen. Japanese herbal medicines may be excellent adjuvants for oral *Lactobacillus*-based vaccines and oral immunization of LacE7, HET and LTB may have the potential to elicit extremely high E7-specific mucosal cytotoxic immune response to HPV-associated neoplastic lesions.

1. Introduction

Human papillomavirus (HPV) infection is a major risk factor for the development of cervical cancer which is the second most common cancer among women [1]. HPV prophylactic vaccines hold promise to reduce the worldwide incidence of cervical cancer. However, limitations in current HPV vaccine strategies make the development of HPV therapeutic vaccines for the treatment of HPV-associated lesions essential. HPV E7 is an attractive target protein for HPV therapeutic vaccine strategies that are directed against a precursor lesion of cervical cancer, high-grade cervical intraepithelial neoplasia (CIN) [2]. Many therapeutic vaccines against HPV E7 have been developed and several clinical vaccination trials against high-grade CIN have been completed [3–11]. However, no therapeutic HPV vaccines are yet available. The current vaccine candidates have been shown to elicit systemic cellular immunity after intramuscular or subcutaneous injection and clinical trials have shown cellular immune responses to the vaccines in peripheral monocytes but fail to show local immunity in the cervical mucosa after vaccination. Cervical mucosal lesions may be poorly responsive to systemic cellular immunity since precursor lesions develop in the mucosal epithelium; mucosal intraepithelial lymphocytes (IELs) should be the central effector cells for the elimination of CIN. Lymphocytes involved in the mucosal immune system are found in the inductive sites of organized mucosa-associated lymphoid tissues and in a variety of effector sites such as the mucosa of the intestine, respiratory tract and genital tract [12]. The efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin $\alpha4\beta7$ [13]. Several studies have demonstrated that gut-derived integrin $\alpha4\beta7^+$ lymphocytes subsequently home to the genital mucosa [14–17].

* Corresponding author. Tel.: +81 3 3815 5411; fax: +81 3 3816 2017.
E-mail address: kkawana-tky@umin.ac.jp (K. Kawana).

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We have reported previously that the oral *Lactobacillus*-based vaccine expressing HPV16 E7 (LacE7) has substantial potential to be a novel HPV therapeutic vaccine [18]. Oral immunization with LacE7 elicited E7-specific IFNγ-producing cells (T cells with E7-type1 immune responses) among integrin $\alpha4\beta7^+$ mucosal lymphocytes collected from gut mucosa. In our previous study, oral immunization with LacE7 preferentially elicited E7-specific type1 T cell responses in mucosal lymphocytes when compared to splenocytes. Taken together with the data that gut-derived integrin $\alpha4\beta7^+$ T cells home to the cervical mucosa [19], we predicted that vaccine-induced mucosal $CD4^+$ and $CD8^+$ T cells will have antitumor effects on mucosal HPV E7-related neoplastic lesions.

Traditional Chinese herbal medicines and their Japanese counterparts, Japanese herbal medicines, are used not merely to improve weak constitutions but also to suppress many constitutional symptoms. The Japanese herbal medicines, Juzen-taiho-to (JTT) and Hochu-ekki-to (HET), have been reported to exert beneficial effects on various aspects of the immune response [20] and are thought to have great potential as adjuvants for prophylactic vaccination against a variety of microbes [21–23]. JTT's immunomodulatory actions include an enhancement of the mitogenic activity of spleen cells, a promotion of phagocytosis and anti-tumor effect [24,25]. HET activates natural killer cells and macrophages [26,27]. Orally administered HET increases antibody titers against influenza virus in mice immunized with influenza vaccines and promotes secretory IgA production after oral OVA vaccination [28,29].

Viewing the actions of JTT and HET on innate immunity within the intestinal mucosa after oral vaccination, we hypothesized that concurrent oral administration of JTT or HET and LacE7 would enhance mucosal cellular immune responses against HPV16 E7. To address the immunomodulatory effects of JTT or HET on anti-E7 immune responses, mice were given oral JTT or HET in addition to a LacE7 oral vaccine with or without the known adjuvant, a heat-labile lymphotoxin T subunit (LTB).

2. Materials and methods

2.1. Immunization protocols

LacE7 was provided from BioLeaders Corp. (Korea) and GENOLAC BL Corp. (Japan). LacE7 was generated from the recombinant *Lactobacillus casei* expressing HPV16 mutated E7 as previously described [18] and attenuated using heat. The attenuated *L. casei* were purified by washing several times with distilled water then dried to powder. LacE7 was insoluble in water-based solvents. Six-week-old female SPF C57BL/6 mice (CLEA Japan Inc., Japan) were used for immunization experiments. 1.0 mg/head of LacE7 were administered four times at weeks 1, 2, 4, and 6. All inoculums were suspended in PBS (200 μL/head) and administered once per day for five days each week via an intra-gastric tube after 3 h of fasting.

The Japanese herbal medicines, JTT or HET (40 mg/head/day, gifted from Dr. Keiichi Koizumi, University of Toyama) were mixed with powdered foods (5 g/head/day) which were taken consumed completely by five mice in a single cage. JTT or HET was administered to mice every day during each of the four rounds of LacE7 administration (weeks 0–6). Heat-labile *Escherichia coli* lymphotoxin, B subunit (LTB: 10 μg/head) was added to each LacE7 inoculum and administrated orally on the third day of each round of vaccination.

2.2. Sample collection

Lymphocytes, serum and intestinal washes were collected from immunized mice one week after the last inoculation (at week 7). After sacrifice, intestine, spleen and peripheral blood were obtained from five mice. Spleens were washed 3 times in HBSS. For intestinal specimens, the inside of intestinal tract was washed with 10 mL of HBSS with protease inhibitors after feces removal. The collected sera and intestinal washes were stored at −80 °C until use.

2.3. Preparation of murine splenocytes and intestinal mucosal lymphocytes

The intestines were opened longitudinally and shaken vigorously in RPMI1640 containing 10% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin for 30 min at 37 °C. The resulting cell suspensions were passed through a BD Falcon Cell-strainer (BD Bioscience, USA) to remove tissue debris and were subjected to discontinuous density gradient centrifugation in a 15 mL tube layered from the bottom with 70% and 40% Percoll PLUS (GE Heaithcare UK Ltd., England). The interface between the 70% and 40% layers contained lymphocytes with a cell viability of more than 95%. Splenocytes were prepared by gently teasing the spleen in PBS. Clumped debris was removed by centrifugation. Approximately $5–10 \times 10^6$ intestinal mucosal lymphocytes and 10^7 splenocytes were obtained from individual mice.

2.4. ELISPOT assays

50 μL of intestinal mucosal lymphocytes or splenocytes (5×10^6 cells/mL) were incubated for 24 h at 37 °C with antigen presenting cells comprised of 50 μL of splenocytes (5×10^6 cells/mL) treated with mitomycin C (75 μg/mL, Sigma, USA), and washed three times with PBS. 10 μL of synthetic peptide (working conc. = 1 μg/mL) corresponding to amino acids 49–57 of HPV16 E7 (a reported CTL epitope for C57BL/6 mice), mitogen (PMA 40 ng/mL + ionomycine 4 μg/mL), or medium alone (negative control) were added to a 96-well ELIIP plate (Millipore, USA) coated with anti-mouse IFNγ monoclonal antibodies from the Mouse IFNγ Kit (MABTECH AB, Sweden). IFNγ spot numbers were analyzed with a fully automated computer assisted video imaging analysis system, KS ELISPOT (Carl Zeiss Vision, Germany).

2.5. Cytokine measurements

Intestinal washes obtained from five mice were pooled and cytokine concentrations measured using the mouse Th1/Th2 ELISA Ready SET Go Kit (BD Bioscience, San Diego, CA, USA), which include IFNγ and IL-2 as representative Th1-type cytokines. The cytokine levels in each sample were normalized by total protein concentration. Measurements were repeated at least three times.

2.6. Statistical analysis

ELISPOT and ELISA data were presented as means ± standard deviations. Measurements and relative rates were compared between the immunization groups (5 mice/each group) using non-paired, two tailed Student's *t*-tests. A *p*-value of <0.05 was considered to be significant.

3. Results

3.1. The adjuvant effect of Japanese herbal medicines on E7-specific type 1 T cell responses

To examine the effect of oral administration of LacE7 vaccine plus Japanese herbal medicines on E7-specific type 1 T cell responses, the number of IFNγ-producing cells among mucosal lymphocytes or splenocytes was assessed by ELISPOT assay (Fig. 1). Each group of five mice was administered LacE7 (1.0 mg/head) orally or LacE7 plus JTT or HET (40 mg/head). JTT and HET were

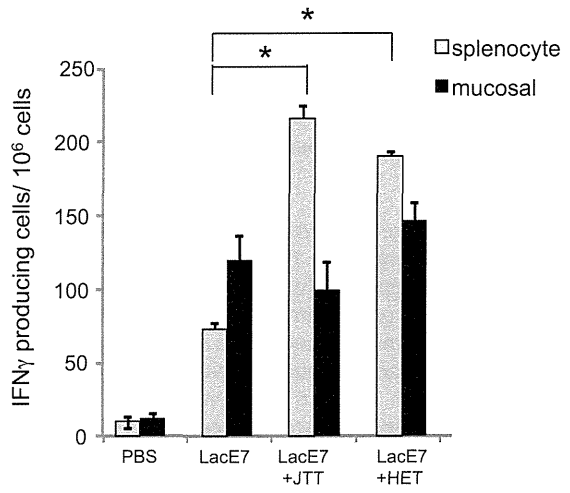

Fig. 1. Adjuvant effects of Japanese herbal medicines on type 1 T cell responses in mice orally immunized with Lac E7. The number of E7-specific IFNγ-producing cells among intestinal mucosal lymphocytes and splenocytes were assessed using ELISPOT assay. Five mice per group were immunized with LacE7 (1.0 mg/head) or PBS four times at weeks 1, 2, 4, and 6. JTT or HET was administered to mice every day during the four rounds of LacE7 administration. Mucosal lymphocyte and splenocytes were collected from immunized mice one week after last inoculation (at week 7) and approximately 10^5 of each type of lymphocyte were stimulated with the E7 peptide corresponding to HPV16E7 49–57 aa. Mean values with standard deviations are presented. Asterisks indicate those comparisons with statistical significance ($p < 0.05$) ($n = 5$).

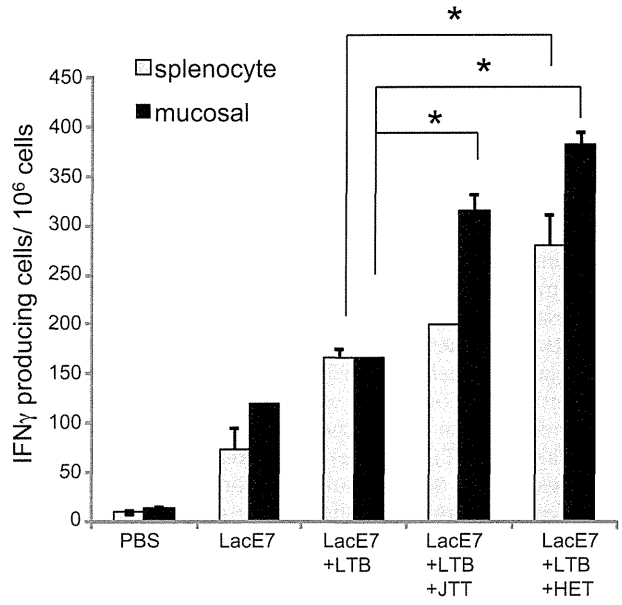

Fig. 2. Synergistic adjuvant effect of Japanese herbal medicines and LTB on type 1 T cell response. LTB (10 μg/head) was added to each LacE7 inoculum and administrated orally on the third day of each round of vaccination. This was performed in mice contemporaneously exposed to JTT, HET or control (no exposure). The number of E7-specific IFNγ producing cells among the collected intestinal mucosal lymphocytes and splenocytes was assessed using the ELISPOT assay as shown in Fig. 1. Mean values with standard deviations are presented. Asterisks indicate those comparisons with statistical significance ($p < 0.05$) ($n = 5$).

administered to mice as supplements to powdered food every day during four rounds of the LacE7 oral immunization. To detect potential adjuvant effects of the supplements on mucosal and systemic immunity, intestinal mucosal lymphocytes and splenocytes were collected from each mouse one week after the last immunization. The numbers of E7-specific IFNγ-producing cells among both mucosal lymphocytes and splenocytes increased significantly in LacE7-immunized mice but not in non-immunized (PBS) mice (Fig. 1). Oral immunization with LacE7 elicited a predominant mucosal E7-specific type 1 T cell response with E7-specific IFNγ-producing cell levels approximately 1.5–2.0-fold higher than those among splenocytes. Administration of LacE7 plus JTT or HET significantly improved systemic E7-specific type 1 T cell responses in splenocytes. However, neither JTT nor HET exhibited significant adjuvant effects on mucosal type 1 T cell responses (Fig. 1).

3.2. *Adjuvant effects of the Japanese herbal medicines when combined with LTB on mucosal immune responses*

Our initial data suggested that the use of additional adjuvants might be necessary to improve the mucosal cellular immune response to E7. We therefore repeated our investigations, adding oral LTB to LacE7 with each round of LacE7 oral immunization. Although the levels of E7-specific type 1 T cell response in mice given LacE7 plus LTB tended to increase, no significant differences were noted when comparing LacE7/LTB to LacE7 alone (Fig. 2). Mice exposed to either JTT or HET together with LTB and-LacE7 had improved mucosal E7-specific type 1 T cell response with approximately 2–2.5-fold higher levels of E7-specific mucosal IFNγ-producing cells when compared with sole exposure to LacE7 plus LTB (Fig. 2). Comparing Figs. 1 and 2, we noted that the addition of LTB to LacE7 plus either JTT or HET doubled the number of the IFNγ-producing cells among mucosal T cells, but not splenocytes. These data indicated that LTB and the Japanese herbal medicines act synergistically on the mucosal type 1 T cell response elicited by LacE7.

3.3. *Local cytokine production induced by oral immunization with LacE7, LTB and Japanese herbal medicines*

To confirm the characteristics of local cellular T cell responses stimulated by oral immunization, type 1 cytokine secretions were measured in the mucosal compartment. Levels of IFNγ and IL-2 production in intestinal washes obtained from immunized mice were measured by ELISA (Figs. 3 and 4). Both IFNγ and IL-2 levels in the mucosal fluid increased significantly in mice immunized orally with LacE7 when compared with non-immunized mice (PBS), consistent with a previous data that mucosal administration of *L. casei* alone induces Th1 cytokine production in a mucosal compartment [30]. Using comparisons mimicking those in Fig. 2, LacE7 plus either JTT or HET and LTB promoted secretion of both IFNγ and IL-2 into the intestinal lumen (Figs. 3 and 4). The secretion levels were 6–8-fold higher for IFNγ (Fig. 3) and 2–4-fold higher for IL-2 (Fig. 4) when compared with LacE7 alone. Administration of LacE7 plus LTB did stimulate increased cytokine secretion when compared with LacE7 alone. These results confirm that JTT or HET have synergistic effects when added to LacE7/LTB oral immunization protocols on local Th1 cytokine secretion, as well as the induction of E7-specific IFNγ-producing cells.

4. Discussion

The therapeutic HPV vaccines tested to date can induce enhanced cellular immune responses but none have demonstrated clinical efficacy against CIN [31–33]. We hypothesize that by using intramuscular or subcutaneous injection strategies, these approaches promote systemic cellular immunity, but not local mucosal immunity. Intraepithelial lymphocytes (IELs) residing in the cervical mucosa are most likely to represent the central effector cells for elimination of CIN and systemic vaccination with HPV E7 is not thought to elicit and retain enough E7-specific CTL within the cervical mucosa to eliminate CIN. We have previously observed

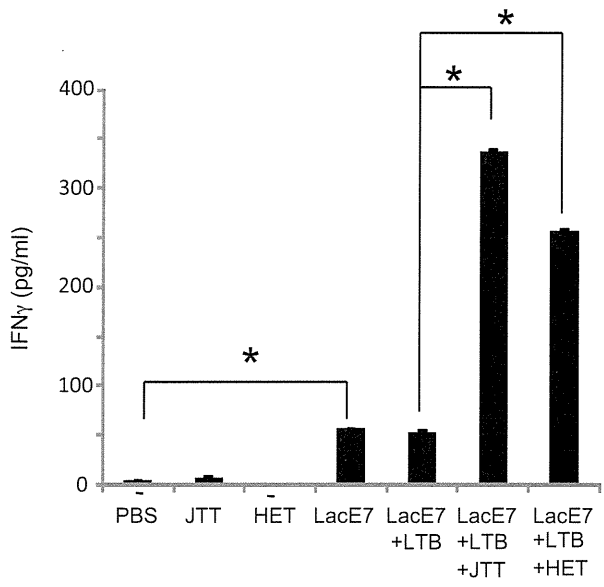

Fig. 3. IFNγ secretion into the intestinal compartment after immunization with LacE7 plus JTT or HET and LTB. IFNγ levels in the intestinal washes were measured by ELISA. The intestinal washes were collected at the same time points that were assessed in Fig. 1. Cytokine levels in each sample were normalized to corresponding total protein concentrations. Mean values with standard deviations are presented. The asterisks indicate those comparisons with statistical significance ($p < 0.05$) ($n = 5$).

and reported the induction of integrin $\alpha 4\beta 7^+$ mucosal T cells that provide E7-specific type 1 T cell responses after oral administration of LacE7 to mice [18]. We have also demonstrated that 25–30% of the $CD3^+$ cervical lymphocytes are integrin $\beta 7^+$ T cells [34]. In our previous data, the number of vaccine induced E7-specific type1 T cells peaked at exposure levels of 1.0 mg/head and decreased with doses over 3.0 mg/head when mice were orally immunized with various doses of LacE7 (0.3–100 mg/head). We believe that 1.0 mg/head may be the optimal dose of LacE7 for induction of mucosal E7-specific type 1 T cells, because high-dose antigen may induce development of E7-specific regulatory T cells. These limitations led us to consider that the addition of an effective adjuvant agent might be more effective in improving E7-specific Th1 type responses than dose-escalation of LacE7. We chose to focus on two Japanese herbal medicines that have been reported to exhibit immunomodulatory effects.

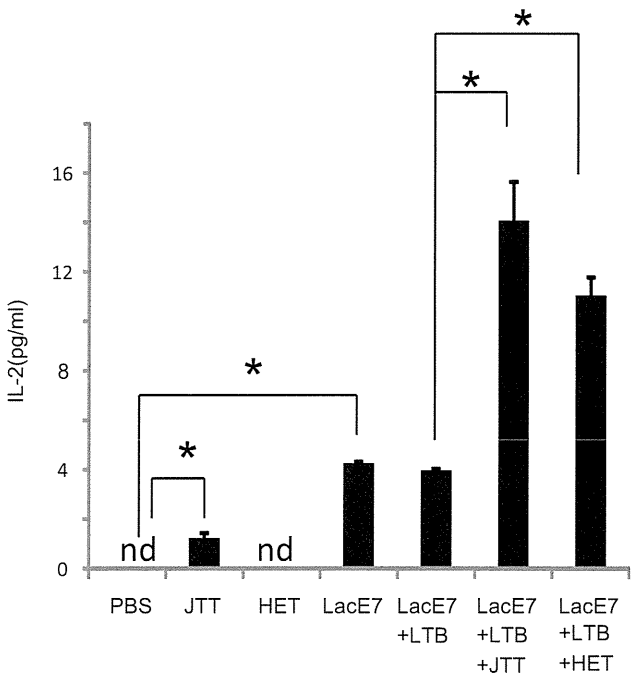

Fig. 4. IL-2 secretion into the intestinal compartment after immunization with LacE7 plus Japanese herbal medicine and LTB. IL-2 levels in the intestinal washes were measured by ELISA. The intestinal washes were collected at the same time points that were assessed in Fig. 1. Cytokine levels in each sample were normalized to corresponding total protein concentrations. Mean values with standard deviations are presented. The asterisks indicate those comparisons with statistical significance ($p < 0.05$) ($n = 5$).

Our data indicate that while JTT or HET alone exerts adjuvant effects on systemic but not mucosal type 1 T cell responses to LacE7, a combination of the mucosal adjuvant (LTB) with either Japanese herbal medicine dramatically improved the desired mucosal E7-specific type 1 T cell responses. These Japanese herbal medicine, when added to a conventional mucosal adjuvant, such as LTB, appear to act synergistically on mucosal vaccine-induced immune responses. The demonstrated adjuvant effects on mucosal immune response may be partially attributed to the strategy involving oral immunization of *L. casei*, which acts as an efficient vaccine carrier that delivers antigen across the gut to GALT but also exhibits its own vaccine adjuvant activities that promote type 1 T cell responses [4,35]. *Lactobacillus* species promote this type 1 T cell response polarization through interactions with dendritic cells (DCs) [36]. *Lactobacillus* activate DCs through TLR-2 and the activated DCs stimulate the proliferation of autologous $CD4^+$ and $CD8^+$ T cells and their secretion of IFNγ [37]. Recombinant *L. casei* alone can induce IFNγ production at mucosal sites [35]. Taken together, *L. casei* appears to be an excellent antigen delivery vehicle when mucosal type 1 T cell responses to vaccine antigen are desired. In our study, the levels of type 1 T cell responses to E7 barely increased in mice immunized with LacE7 and LTB when compared with LacE7 alone. However, the addition of Japanese herbal medicines to LacE7 and LTB resulted in two to three-fold higher levels of type 1 mucosal T cell responses when compared to LacE7 and LTB. In summary, the Japanese herbal medicines, JTT and HET act in synergy with *L. casei* and LTB in mucosal antigen delivery strategies. When Th1-type local T cell responses to vaccine antigen are desired, the combination of a Japanese herbal medicine and LTB promote efficient and mucosa-specific adjuvant activities when added to Lactobacillus delivery systems.

More specifically, the addition of specifically, the addition of specific Japanese herbal medicines and mucosal adjuvant to LacE7 may be an outstanding approach to generate E7-specific mucosal cytotoxic immune responses to HPV-associated neoplastic lesions.

Acknowledgements

We thank to Dr. Ai Kawana-Tachikawa for expert advice on ELISPOT assays. This work was supported by a grant from the Ministry of Health, Labour and Welfare of Japan for the Third-Term Comprehensive Strategy for Cancer Control and for Comprehensive Strategy for Practical Medical Technology and by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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