Inhibition of Collagen Production by Traditional Chinese Herbal Medicine in Scleroderma Fibroblast Cultures

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The in vitro effect of one traditional Chinese herbal medicine (Japanese name: “Keishi-bukuryo-gan”), which has been empirically used in scleroderma patients in China and Japan, on collagen production in fibroblast cultures was studied. Fibroblasts from 3 scleroderma patients and 2 normal controls were incubated with various concentrations of “Keishi-bukuryo-gan” and collagen production was then determined by a radiochemical method. “Keishi-bukuryo-gan” significantly and selectively inhibited collagen synthesis in a dose-dependent manner, with a tendency of a stronger effect on scleroderma fibroblasts than control cells. The results may explain the clinical usefulness of this medicine, and it may become a promising new agent for the treatment of scleroderma.

(Internal Medicine 33: 466-471, 1994)

Keywords: Kampo medicine, fibrosis, therapy

Introduction

Scleroderma is a generalized connective tissue disorder characterized by a vascular change, an inflammatory and fibrotic change of the skin and internal organs, and various immunological abnormalities (1, 2). In particular, the fibrosis of the lungs and heart as well as renal involvement are the major causes of morbidity and mortality in scleroderma patients. The fibrotic change is considered to result mainly from enhanced production of collagen (3), which is associated with elevated levels of the procollagen messenger RNA (mRNA) (4). Thus, inhibition of excessive collagen production in scleroderma cells by pharmacologic means is thought to result in reduced collagen accumulation in tissues, with subsequent clinical improvement. To date, a number of pharmacologic agents are known to inhibit production and accumulation of collagen at various levels (5–7), and some of them such as D-penicillamine (8), interferon-γ (9), and retinoids (10) are already used for the treatment of scleroderma. However, these agents do not always provide satisfactory effects for the patients or the attending physicians. In addition and more importantly, they frequently have considerable adverse effects, and the patients may not tolerate their long-term use. Therefore, scleroderma still remains one of the most refractory autoimmune diseases.

A traditional Chinese herbal medicine (Japanese generic name: Kampo medicine) is a natural drug extracted from one or more of various medicinal plants. Thus, Kampo medicine is generally composed of several kinds of herbal extracts, and each extract further seems to have numerous functional components which may interact with each other. Because of this complicated composition, and because each constituent has not been chemically identified in most of the Kampo medicines, scientific analyses of their beneficial effects have been difficult, and such studies have rarely been published in English literature, except for a few studies (11). However, the Kampo medicines are used empirically in China and also in Japan in various chronic diseases including autoimmune connective tissue disease, since their long-term use usually shows less toxic effects than ordinary chemical agents. There have been several reports indicating the clinical usefulness of some Kampo medicines in patients with scleroderma and mixed connective tissue disease (MCTD) in these countries (12–16), although scientific endorsement of their usefulness is essentially lacking. According to these reports, the patients treated with Kampo medicines showed significant improvement in various clinical manifestations including Raynaud’s phenomenon, swollen hands, sclerotic skin, and interstitial pneumonitis (12–16).

In the present study, we have evaluated the in vitro effect of one of the herbal medicines (Japanese name: “Keishi-bukuryo-gan”) which has been reported to be useful for the therapy of scleroderma, on enhanced collagen synthesis by scleroderma fibroblasts. As a result, this Kampo medicine showed selective
inhibition of collagen production. Along with the evidence of clinical usefulness in some scleroderma patients, the Kampo medicine may become a new promising therapeutic modality in the treatment of this intractable disease.

**Materials and Methods**

**Patients**

All of the human investigations were performed in accordance with the precepts of the Helsinki Declaration. Patients studied were 3 scleroderma patients (SSC-1, 2, 3) who fulfilled the 1980 criteria for the classification of scleroderma (17). All scleroderma patients were female, and had progressive generalized disease with proximal skin sclerosis and various internal organ involvement. Anti-topoisomerase I antibody was positive in all patients. The SSC-1 patient was 57 years old, the SSC-2 patient 43, and the SSC-3 patient 40; their disease duration was 4, 1.5, and 4 years, respectively. Skin biopsy from all patients revealed typical scleroderma changes. As a control, 2 healthy women were entered in the study; Control-1 was 46 years old and Control-2 32.

**Fibroblast cultures**

Fibroblast cultures were derived from the biopsy sample of the 3 scleroderma patients, and also from the normal skin of the 2 control subjects. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in a humidified incubator under 5% CO₂ and 95% air. The cultures were studied in passages 6–10. Scleroderma fibroblasts and control fibroblasts were simultaneously cultured in the subsequent experiments.

**Kampo medicines**

All of the Kampo medicines prepared for the experiments were a spray-dried powder of hot water extracts with no conservatives and were kind donations from Tsumura Juntendo Co. Ltd., Tokyo, Japan. One gram of “Keishi-bukuryo-gan” water-soluble powder was extracted from each 1.71 g of Cinnamomi Cortex, Paeoniae Radix, Persicae Semen, Hoelen, and Moutan Cortex. “Keishi-bukuryo-gan” was dissolved in water and used in fibroblast cultures at a final concentration of 0.05% after filtration with Millex-HV 0.45 μm filter unit (Millipore Corp., Bedford, MA, USA). The radioactivity on the filters was determined by a liquid scintillation counter.

**Collagen assays**

The assays were performed according to those reported previously (7, 18), Briefly, after 4×10⁴ cells/well were plated in 24-well tissue culture plates and incubated for 2 days, the cultures were rinsed, and 1 ml of DMEM supplemented with 1% dialyzed FCS, 50 μg/ml of ascorbic acid, and 25 μg/ml of β-aminopropionitrile with or without the Kampo medicine tested was added to the cultures. After appropriate incubation time (mostly 12 hours), 370 kBq (10 μCi) of L-[2,3,4,5-³H]-proline (specific activity 103 Ci/m mole; Amersham International, Buckinghamshire, UK) was added, and the incubations were continued for 6 hours. At the end of incubation, the medium was removed, and the solution of proteinase inhibitors containing N-ethyl-maleimide, phenylmethylsulfonyl fluoride, and ethylenediamine tetraacetic acid was added. The cells on the plate were sonicated in Tris-buffer containing proteinase inhibitors. Aliquots of the media and cell homogenates were dialyzed, and then hydrolyzed with 12 N HCl for 18 hours at 120°C. Finally, the ³H-hydroxyproline content was determined by radiochemical methods and the synthesis of radioactive hydroxyproline in the non-dialyzed fraction was taken as an index of collagen production. Determinations were done usually in triplicates. Aliquots of the cell homogenates were also used for protein determination in a dye binding assay (19).

**Non-collagenous protein assays**

The fibroblast cultures were prepared as described in the methods for collagen assays, but the cultures were labeled with 185 kBq (5 μCi) of L-[5-³H]-tryptophan (specific activity 25 Ci/m mole; Amersham International). At the end of incubation, proteinase inhibitors as described above were added to the medium. Non-collagenous protein synthesis was determined by precipitation of the mixture of culture supernatants and sonicated cell homogenates with an equal volume of cold 20% trichloroacetic acid (TCA). The precipitates were collected on glass-fiber filters using a vacuum filtration manifold, washed twice with 10% TCA, air-dried, and then counted by a liquid scintillation counter.

**Fibroblast proliferation assays**

To determine the effect of the Kampo medicine on fibroblast proliferation, 4×10⁴ cells/well were plated in a 96-well tissue culture plate, and preincubated for 12 hours in DMEM supplemented with 1% FCS with or without 0.05% “Keishi-bukuryo-gan”. After labeled with 74 kBq (2 μCi) of [methyl-³H]-thymidine (specific activity 80 Ci/m mole; Amersham International) for 6 hours, the cells were detached from the wells by brief trypsinization, and the ³H-thymidine-containing macromolecules were collected on glass-fiber filters using a cell harvester (PHD, Cambridge Medical Technology Inc., Billerica, MA, USA). The radioactivity on the filters was determined by a liquid scintillation counter.

In addition, the effect of the Kampo medicine on the fibroblast viability was determined by trypan blue dye exclusion test.

**Effect of serum of persons who took the Kampo medicine**

To examine whether there was an inhibitory effect in the serum of the persons who took the Kampo medicine, an ordinary dose (7.5 g/day) of “Keishi-bukuryo-gan” which was equivalent to 1.75 g of extract powder used for the in vitro experiment was administered to 3 healthy individuals, 22- and 24-year-old men and a 32-year-old woman, for 4 weeks, after having obtained their consent. Peripheral blood was obtained before and 1 and 4 weeks after starting administration of the.
drug. Blood aspiration was done 2-3 hours after the last administration. Each serum was dialyzed against DMEM and heat-inactivated after filtration. DMEM containing 20% of each serum was added to the cultured scleroderma fibroblasts and collagen production was assayed as described above.

Statistical analysis
The statistical significance of the differences was analyzed using Student’s t-test.

Results
In the preliminary experiments, various periods of incubation with the Kampo medicine and various concentrations were tested. As shown in Fig. 1, preincubation with “Keishi-bukuryo-gan” for 12 hours before adding radio-labeled proline showed a maximum inhibitory effect on collagen production by fibroblasts treated with the drug, without an apparent effect on collagen production by untreated fibroblasts. Preincubation for 24 hours caused inhibition also in untreated cells, probably due to a low concentration of FCS. Therefore, preincubation with the drug was performed for 12 hours in further experiments. Figure 2 showed a dose-dependent inhibitory effect of “Keishi-bukuryo-gan” on collagen production in the SSc-1 and Control-1 cell cultures. At all of the concentrations tested, a greater inhibitory effect was seen in the SSc-1 than the Control-1 cells; at 0.005% concentration of “Keishi-bukuryo-gan” SSc-1 cells showed 40.6% inhibition (p<0.05 vs. untreated condition) and Control-1 cells showed 27.1% inhibition (p<0.05 vs. untreated condition); at 0.01% concentration SSc-1 cells showed 47.8% inhibition and Control-1 cells showed 41.9% inhibition; and at 0.05% concentration SSc-1 cells showed 86.0% inhibition and Control-1 cells showed 78.5% inhibition. In most of the further experiments, 0.05% concentration of the drug was used.

Figure 3 showed collagen production by 3 fibroblast lines from the scleroderma patients and 2 fibroblast lines from the controls. When compared as a group, scleroderma fibroblast group spontaneously produced more collagen than the control group (p<0.01) as reported previously (3, 4, 7), although the amount varied among individuals. In the presence of 0.05% “Keishi-bukuryo-gan” in the medium, collagen production was significantly inhibited in all of the fibroblast lines (p<0.05). The rate of inhibition was 87.8% in SSc-1 cells, 71.5% in SSc-2, 76.9% in SSc-3, 75.0% in Control-1 and 54.8% in Control-2, thus indicating a tendency toward greater inhibition of collagen production by “Keishi-bukuryo-gan” in scleroderma cells than in control cells.

We also studied the effect of “Keishi-bukuryo-gan” on the production of non-collagenous proteins by analyzing 3H-tryptophan incorporation. As a result, this drug did not affect the
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Fig. 3. Effect of "Keishi-bukuryo-gan" on collagen production in fibroblast cultures from 3 scleroderma patients and 2 controls. Each fibroblast line was cultured in the absence or the presence of 0.05% "Keishi-bukuryo-gan", and collagen synthesis was assayed. Values are the mean and SD of triplicates. When compared as a group, scleroderma fibroblast group spontaneously produced more collagen than the control group (p<0.01). A significant difference (p<0.05) was also demonstrated when the result was compared between the untreated and "Keishi-bukuryo-gan"-treated condition in each fibroblast line.

Fig. 4. Effect of various concentrations of "Keishi-bukuryo-gan" on fibroblast proliferation. Various concentrations of "Keishi-bukuryo-gan" were added to SSc-1 and Control-1 fibroblasts in culture, and \( ^3\)H-thymidine uptake was measured as an index of proliferation (see Materials and Methods). Values are the mean±SD of quadruplicates. A significant difference (p<0.05) was demonstrated between untreated and each concentration-treated condition both in SSc-1 and Control-1 cells, but there were no significant differences in proliferation between SSc-1 and Control-1 cells in the absence of and at each concentration of "Keishi-bukuryo-gan".

production of non-collagenous proteins in both SSc-1 cells and Control-1 cells (data not shown), suggesting that "Keishi-bukuryo-gan" selectively inhibited collagen synthesis.

Then, the drug was tested for its influence on the proliferation of fibroblasts by analyzing the incorporation of \( ^3\)H-thymidine into the cells. Figure 4 shows an almost equal level of prolifera-

tion between the SSc-1 and Control-1 fibroblasts in the absence of the drug, and also that "Keishi-bukuryo-gan" inhibited the proliferation of scleroderma and control cells similarly in a concentration-dependent manner. However, the trypan blue dye exclusion test revealed that the viability of both cells was not affected by incubation with 0.05% concentration of this drug for 12 hours (data not shown).

In further experiments, an additional 2 drugs which have also been clinically used in scleroderma patients (12, 13, 15) were examined for their effect on collagen synthesis (data not shown). Both "Hachimi-jio-gan" and "Keishi-ka-jutsubu-to" significantly inhibited the collagen synthesis in both SSc-1 and Control-1 fibroblast cultures (p<0.05 vs. untreated condition). However, these 2 drugs had less inhibitory effect as compared with "Keishi-bukuryo-gan" (p<0.05).

To make a rough estimate of the in vivo effect of "Keishi-bukuryo-gan", sera from 3 healthy volunteers who had taken this medicine for 4 weeks were tested for its influence on the synthesis of collagen in fibroblast cultures. No definite inhibition of collagen production was observed when sera obtained 1 and 4 weeks after beginning the administration of the drug was added in 20% concentration to the culture medium (Table 1). However, 2 of 3 subject’s sera obtained after drug administration showed a little inhibitory effect on collagen synthesis in vitro.

**Discussion**

The present study demonstrated that "Keishi-bukuryo-gan" significantly and selectively inhibited collagen synthesis in vitro, with a tendency of a stronger effect on scleroderma fibroblasts than on control cells. In addition, this drug showed an inhibition on fibroblast proliferation without affecting cell viability.

"Keishi-bukuryo-gan" originated from one of the earliest books of Chinese medicine, "Jin Gui Yao Lue" (Synopsis of prescription of the golden chamber). Initially this medicine was used in the treatment of metrorrhagia and metrostaxis due to
hysteromyoma. Because of its known action of promoting blood circulation and removing peripheral blood stasis, the clinical application of “Keishi-bukuryo-gan” was gradually extended, and recently in China and Japan it has been used also in some rheumatic diseases including scleroderma (12–14), MCTD (16), Behçet’s disease (20), and Sjögren’s syndrome (21). In addition to the beneficial effect on peripheral blood circulation, the present results showing an inhibitory effect on collagen production suggest the positive application of “Keishi-bukuryo-gan” for the therapy of not only Raynaud’s phenomenon but also sclerosis of the skin and internal organs, and thus may account for the clinical usefulness of this medicine which has been empirically used in patients with scleroderma.

Because of its nature as a mixed composition of numerous unknown functioning components, it seems impossible to identify which component(s) of the Kampo medicine contributes to the inhibitory effect on collagen synthesis observed in the present study. In three types of Kampo medicine showing an inhibition of collagen production, the only common extract was that from Cinnamomi Cortex. However, the amount of Cinnamomi Cortex extract varied in these three drugs and the amount was not parallel with the inhibitory effect, suggesting that the inhibition could not be explained by the action of this extract alone. Generally, the beneficial effect of the Kampo medicine is believed to result from an integrated action of the compounds. For identification of the extract(s) or the constituent(s) mainly responsible for the inhibitory effect, it may be necessary to study the effect of each single extract or possible single component which constitutes the drug.

It is also very difficult to determine the concentration of the Kampo medicine in the serum or tissue of an individual taking the medicine because of the nature of the drug. Therefore, it is unclear whether the 0.005%–0.05% concentration of “Keishi-bukuryo-gan” that showed an inhibition of collagen synthesis in fibroblast cultures can actually be attained within some tissues with the administration of the ordinary dose. To make an approximate estimation of in vivo efficacy of “Keishi-bukuryo-gan”, we studied the effect of sera obtained from 3 individuals who had been taking the ordinary dose of this drug for 4 weeks. There was only a tendency of an inhibitory effect on collagen production in the sera from 2 of the individuals, but we could not obtain a significant result in the present experiment. This may be partly due to the small number of volunteers studied and the short-term administration. Future experiments using a sufficient number of volunteers with prolonged administration of the drug may reveal more apparent inhibitory effect as seen in the present in vitro study.

In general, the Kampo medicine is known to have a mildly beneficial effect and less toxic side effects compared with ordinary chemical agents, and therefore is more suitable for long-term use. Scleroderma is a chronic disease and in many cases the drugs are used for years. We believe Kampo medicines such as “Keishi-bukuryo-gan” can be recommended for patients with scleroderma, especially for those with less progressive or limited scleroderma, whereas for patients with rapidly progressive disease more aggressive agents such as D-penicillamine, cytotoxic agents, and interferon-γ may well be used.

In the preliminary experiment, there was no significant difference in α₁(I) procollagen mRNA levels between untreated and “Keishi-bukuryo-gan”-treated fibroblasts, suggesting that the inhibition of collagen synthesis might occur at a posttranscriptional level (Ohta A, et al, unpublished observation). Despite limited information about the mechanism of inhibition and also despite the lack of information about the precise nature of the active component(s), the present results suggest that some Kampo medicines may become a new promising agent in the treatment of scleroderma.

References

17) Subcommittee for scleroderma criteria of the American Rheumatism
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