Modification of Macrophage Functions by Shosaikoto (Kampo Medicine) Leads to Enhancement of Immune Response

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The effects of Shosaikoto, one of the Kampo medicines, on macrophage functions were studied in mice. Oral administration of Shosaikoto (1.2 g/kg of body weight) increased the change of the membrane fluidity of macrophages and diminished prostaglandin E_2 production. Moreover, macrophages from mice orally given Shosaikoto phagocytized antigen more efficiently than control macrophages, resulting in presentation of much more antigen to lymphocytes. These results suggest that Shosaikoto enhances the immune response through at least two different routes, that is, through eliminating the inhibition of lymphocyte functions by prostaglandin E_2 and through presenting antigen more efficiently.

Keywords macrophage; prostaglandin E_2; phagocytosis; antigen presentation; immune response

Introduction

Kampo-hozai (traditional herbal medicine in Japan) is a system of drug therapy which has been developed from clinical experience accumulated over some thousands of years in China. Recently, chemical, physiological and pharamacological investigations on Kampo-hozai have been started to evaluate it scientifically. We have already shown that some Kampo-hozai prescriptions modulate immune responses in several ways.[1-3] Shosaikoto, one such Kampo-hozai prescription, was suggested to facilitate the development of immunity by stimulating macrophages.[3] Although glycyrrhizin and saikosaponins, which are ingredients of Shosaikoto, are known to modify prostaglandin (PG) production by macrophages *in vitro*,[4,5] the mechanism by which Shosaikoto stimulates macrophages has not yet been investigated sufficiently. Immunoregulatory roles of macrophages have extensively described for a variety of lymphocyte functions. Macrophages stimulate many lymphocyte activities, including proliferation and lymphokine production, acting mainly through the production of interleukin 1.[6] Conversely, macrophages can reduce lymphocyte functions, by releasing suppressive molecules, principally prostaglandins of the E series.[7,8] In the present report, we demonstrate that Shosaikoto modulates the function of macrophages to enhance immunity.

Materials and Methods

Animals Male ICR mice and female CBA mice (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were used at 6 and 8 weeks of age, respectively. They were kept in an air-conditioned room (24 °C) and given commercial diet and water *ad libitum*.

Reagents RPMI-1640 was purchased from the Research Foundation for Microbiological Diseases of Osaka University, Osaka, Japan. Sheep red blood cells (SRBC) and fetal bovine serum (FBS) were obtained from Nippon Bio. Supp. Center Co., Ltd., Tokyo, Japan. Lipopolysaccharide (*Escherichia coli* 0111 : B4) was from Difco Laboratories.

1-[4-(Trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) was from Dojin Chem. Co., Ltd. Latex beads (1.09 μm in diameter) was from Sigma Chem. Co. Casein was purchased from Nakarai Chem. Co., Ltd. ^{125}I-PGE_2 radioimmunoassay kit was obtained from New England Nuclear. Crude drugs were provided by Tsumura Co., Ltd.

Macrophage Isolation Peritoneal macrophages, which we used throughout this experiment, were harvested from ICR male mice 3 d after i.p. injection of 5% casein solution and then purified by adherence for 2 h to coverslips (18 × 18 mm, 1.5 × 10^6 cells/cover slip). Nonadherent cells were removed by washing with phosphate buffered saline (PBS), and the remaining cell were incubated at 37 °C in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum.

Preparation of Shosaikoto Shosaikoto (dose per person per day) was prepared as follows. Bupleuri Radix (7 g), Pinelliae Tuber (5 g), Scutellarae Radix (3 g), Ginseng Radix (3 g), Zingbers Rhizoma (4 g), Zizyphi Fructus (3 g) and Glycyrrhizae Radix (2 g) were added to 700 ml of water, boiled for 1 h and concentrated to 300 ml. This decoction was lyophilized to give 7.2 g of powdered extract.

Determination of Prostaglandin E_2 (PGE_2) After the treatment of cultured macrophages (1.5 × 10^6 cells) with zymosan (0.1 mg/ml), the supernatants were collected and PGE_2 was extracted according to the reported method of Salmon's.[9] PGE_2 released from macrophages was determined by radioimmunoassay.

Determination of ^{3}H-Arachidonic Acid Metabolites Macrophages (1.5 × 10^6 cells) were labeled with ^{3}H-arachidonic acid (2 μCi) for 16 h. After extensive washing of the macrophages with PBS, macrophages were incubated in 1.5 ml of RPMI-1640. Zymosan (0.1 mg/ml) was added as a stimulant and 0.1 ml aliquots of culture medium were taken at the times indicated. The radioactivity of the aliquots was counted with a liquid scintillation counter.

Measurement of the Membrane Fluidity The fluidity of plasma membrane of macrophages was measured according to the methods described elsewhere.[10,11] Macrophages adhering to the fluorescence-free cover slips (1.0 × 10^6 cells/cover slip) were equilibrated with 1.0 × 10^{-5} M TMA-DPH in 0.15 M PBS, pH 7.4 at 37 °C. The sample was monitored at 430 nm, with a Shimadzu RF-540 spectrofluorometer. Fluorescence was monitored continuously, and the change that occurred following the addition of the stimulus was reported as a relative change in fluorescence (F/F_0), which was calculated from the ratio of the fluorescence change measured after the addition of lipopolysaccharide (LPS) (F) to the resting fluorescence of the cell suspension measured after equilibration with TMA-DPH but before the addition of the stimulant (F_0).

Determination of Phagocytic Ability Macrophages were isolated as described above and were incubated in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum for 2 h at 37 °C in 5% CO_2/95% air. After the addition of 1.09 μm latex beads at 0.02% final concentration, macrophages were incubated for 30 min. The number of macrophages that phagocytized more than one latex bead was counted through a microscope. The phagocytic ability was represented as follows.

$$T(\%) = (\text{number of macrophages phagocytizing latex beads}) / (\text{total number of macrophages}) \times 100$$

Determination of Antigen Presentation Peritoneal macrophages were hervested from the peritoneal cavity of CBA female mice (8 weeks old) given Shosaikoto (1.2 g/kg) or water orally for 3 d. Macrophages were exposed to SRBC (4 times more than the number of macrophages) in culture dishes at 37 °C. After a 30 min incubation, the excess SRBC were washed off with PBS and macrophages were scraped off with a rubber policeman. A constant number of macrophages (3 × 10^5 cells) was transferred to the peritoneal cavity of the same strains of mice. After 7 d, the mice were decapitated and the serum obtained was inactivated at 56 °C for 60 min. Hemagglutinin (HA) titer was assayed by the methods described by Ceglowski and Friedman.[12] Two serial dilutions were made with 0.15 M PBS, pH 7.2, followed by mixing and standing at 10 °C for 18 h, then the HA titer of 25 μl aliquots was assayed.

Results and Discussion

Effect of Shosaikoto Administration of PGE_2 Production in Macrophages Macrophages are known to release much more prostaglandins than lymphocytes.[13] Therefore, the macrophage is considered to play an important role in regulating immune responses by releasing prostaglandins. Prostaglandins released from peritoneal macrophages were analyzed by high performance liquid chromatography. PGE_2 and PGI_2 were mainly produced together with a small amount of thromboxane A_2 (TXA_2)(data not shown). Therefore, PGE_2 released from macrophages after the addition of zymosan (0.1 mg/ml) was determined by radioimmunoassay (RIA). As shown in Fig. 1, macrophages prepared from mice treated with Shosaikoto (1.2 g/kg) produced less PGE_2 than control macrophages during the course of the incubation. In the following experiment, arachidonic acid metabolites released from macrophages labeled with ^{3}H-arachidonic acid after stimulation with zymosan (0.1 mg/ml) were investigated by counting the released metabolites with a liquid scintillation counter. Oral administration of Shosaikoto (1.2 g/kg) for 3 d reduced the amount of ^{3}H-arachidonic acid metabolites released (Fig. 2). Therefore, in order to determine whether the reduction of PGE_2 is due to the modification of phospholipase A_2, which is a key enzyme in the release of arachidonic acid from membrane phospholipids, we prepared microsome and cytosol fractions from macrophages, and measured phospholipase A_2 activity in the microsome fraction and phospholipase A_2-inhibitory activity in the cytosol fraction. However, no difference in these activities between the two groups was observed (data not shown).

Effect of Shosaikoto Administration on Membrane Fluidity There is a possibility that the reduction of arachidonic acid metabolites or PGE_2 released resulted from the reduced access of phospholipase A_2 to phospholipids as a substrate owing to membrane perturbation. Therefore, the change of the membrane fluidity of macrophages was measured after addition of LPS as a stimulant. As shown in Fig. 3, the fluorescence intensity significantly increased in macrophages prepared from mice given Shosaikoto as

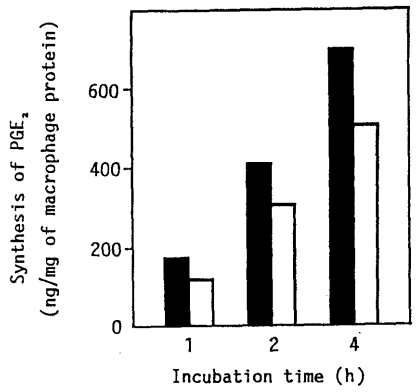

Fig. 1. Effect of Shosaikoto on PGE_2 Synthesis in Macrophages

Closed column, control macrophages; open column, macrophages prepared from 6 mice treated with Shosaikoto (1.2 g/kg of body weight) orally for 3 consecutive days. Values show the average of two experiments carried out in duplicate. ■, control; □, Shosaikoto.

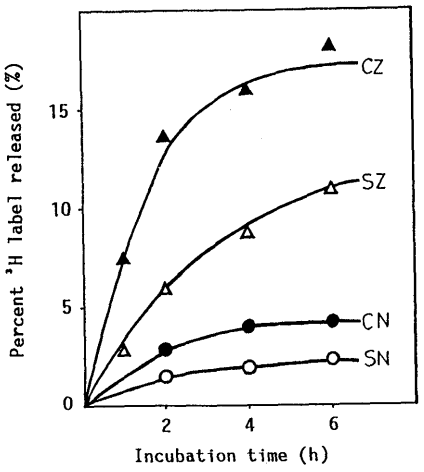

Fig. 2. Effect of Shosaikoto on Arachidonic Acid Metabolites Secretion from Macrophages

Closed symbols, control macrophages; open symbols, macrophages prepared from 6 mice treated with Shosaikoto (1.2 g/kg of body weight) orally for 3 consecutive days; triangles, +zymosan (0.1 mg/ml); circles, no stimulant. These results are from a representative experiment (from a total of four experiments each performed in duplicate).

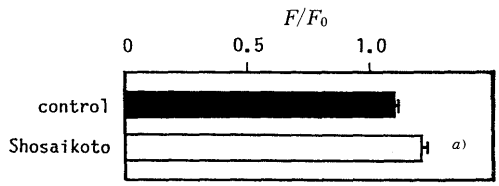

Fig. 3. Effect of Shosaikoto on the Membrane Fluidity of Macrophages

Fluorescence was expressed as the ratio of observed fluorescence (F) to fluorescence before the addition of LPS (F_0). Closed columns, control macrophages; open columns, macrophages prepared from 6 mice treated with Shosaikoto (1.2 g/kg of body weight) orally for 3 consecutive days. Data represent mean ± S.E. of quintuplicate determinants from a representative experiment that was replicated three times with similar results. *a*) $p<0.01$ *vs.* control.

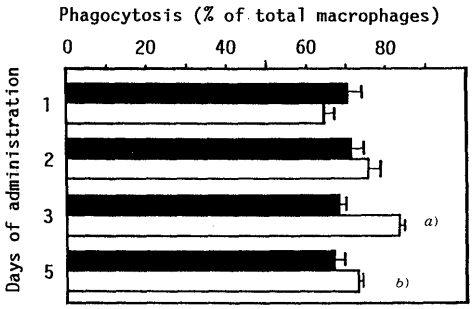

Fig. 4. Effect of Shosaikoto on Phagocytosis of Peritoneal Macrophages

Shosaikoto (1.2 g/kg of body weight) was administered orally for the number of consecutive days shown. Closed columns, control macrophages; open columns, macrophages prepared from 6 mice treated with Shosaikoto. Values are represented as mean ± S.E. of 5 cultures from a representative experiment that was replicated twice with similar results. *a*) $p<0.01$, *b*) $p<0.05$ *vs.* control.

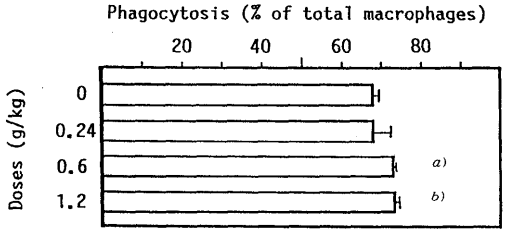

Fig. 5. Dose Dependence of the Effect of Shosaikoto on Phagocytosis

Shosaikoto (1.2 g/kg of body weight) was administered orally for 3 consecutive days. Values are represented as mean ± S.E. of 5 cultures of macrophages prepared from three mice from a representative experiment that was replicated twice with similar results. *a*) $p<0.01$, *b*) $p<0.05$ *vs.* the group without Shosaikoto.

TABLE I. Effect of Shosaikoto on Antigen Presentation

	Responder[a]	HA titer[b] Mean±S.E.
Control	9/11	37.6± 9.0 (n=9)
Shosaikoto	8/10	107.0±28.0[c] (n=8)

a) Numbers of responding mice per number of total mice in a group. *b*) HA titers are expressed as mean±S.E. of the number of mice shown in parentheses. *c*) $p<0.01$ *vs.* control.

compared with control macrophages. Thus, Shosaikoto administration seems to modify the macrophage membrane and reduce PGE_2 production. The modification of the membrane might be caused by the direct interaction of a component of Shosaikoto, a change of membrane phospholipids, or a change of membrane proteins.

Effect of Shosaikoto on Phagocytosis It is conceivable that the change of membrane fluidity affects phagocytosis of macrophages. Therefore, the phagocytic ability of macrophages was determined using latex beads. Oral administration of Shosaikoto for 3 and 5 consecutive days enhanced the phagocytic ability of macrophages significantly (Fig. 4). Furthermore, this enhancement showed a dose dependence (Fig. 5).

Antigen Presentation Antigen presentation is one of the most important roles of macrophages in immune response. The enhancement of the phagocytic ability led us to speculate that macrophages of treated mice should scavenge antigen more efficiently and present more antigen to lymphocytes than control macrophages. Therefore, the antigen-presenting ability was measured by the method described in Materials and Methods. Table I showed that antibody directed against SRBC was produced in mice treated with Shosaikoto more than in control mice. This result indicates that macrophages of orally Shosaikoto-treated mice can present antigen to lymphocytes more efficiently. Macrophages can elaborate a series of biochemical products with potent immunomodulatory activities. With respect to B cell function, PGE_2 inhibits both B cell proliferation and the generation of antibody-forming cells.[7,8] In addition, PGE_2 inhibits antigen- and mitogen-stimulated T cell proliferation[14] and the production of lymphokines, such as interleukin 2.[15] Furthermore, PGE_2 may also alter the function of accessory cells by down-regulating expression of Ia antigen. On the other hand, macrophages produce interleukin 1, which promotes both B and T cell growth and differentiation. In this paper, we demonstrate that the oral administration of Shosaikoto diminished prostaglandin synthesis in macrophages and augmented phagocytosis by modifying the membrane of macrophages. The decrement of prostaglandin synthesis leads to the enhancement of immune responses by reducing the inhibition of lymphocyte function. Moreover, the augmentation of phagocytosis makes macrophages present many antigens to lymphocytes and results in the enhancement of immune responses. Although the result is not shown, we confirmed that IL-1 releases was increased in macrophages prepared from mice treated with Shosaikoto. Since Shosaikoto is composed of plural ingredients, which are metabolized in the body, it is difficult to determine what component is responsible for the effect on macrophage function. However, the most important finding here is that Shosaikoto given orally does indeed enhance the immune responses through several mechanisms.

References

1) H. Iwama, S. Amagaya and Y. Ogihara, *Planta Medica*, **4**, 247 (1986).
2) H. Iwama, S. Amagaya and Y. Ogihara, *J. Ethnopharmacology*, **18**, 193 (1986).
3) H. Iwama, S. Amagaya and Y. Ogihara, *J. Pharm. Soc. Wakanyaku*, **4**, 8 (1987).
4) K. Ohuchi, Y. Kamada, L. Levine and S. Tsurufuji, *Prostaglandins Med.*, **7**, 457 (1981).
5) K. Ohuchi, M. Watanabe, T. Ozeki and S. Tsurufuji, *Planta Med.*, **1985**, 208.
6) E. A. Goldings, *J. Immunol.*, **136**, 817 (1986).
7) D. F. Jelinek, P. A. Thompson and P. E. Lipsky, *J. Clin. Invest.*, **75**, 1339 (1985).
8) P. A. Thompson, D. F. Jelinek and P. E. Lipsky, *J. Immunol.*, **133**, 2446 (1984).
9) J. A. Salmon and R. J. Flower, *Method Enzymol.*, **86**, 477 (1982).
10) J. G. Kuhry, P. Fonteneu, G. Duportail, C. Maechling and G. Laustriat, *Cell Biophys.*, **5**, 129 (1983).
11) N. E. Larsen, I. R. Enelow, E. R. Simons and R. Sullivan, *Biochim. Biophys. Acta*, **815**, 1 (1985).
12) W. S. Ceglowski and H. Friedman, *J. Immunol.*, **101**, 594 (1968).
13) M. S. Kennedy, J. D. Stobo and M. E. Goldyne, *Prostaglandins*, **20**, 135 (1980).
14) J. J. Ellner and P. J. Spagnuolo, *J. Immunol.*, **123**, 2689 (1979).
15) R. S. Rappaport and G. R. Dodge, *J. Exp. Med.*, **155**, 943 (1982).