Effect of Saireitoh on Rabbit Smooth Muscle Cell Proliferation and Experimental Atherosclerosis

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Saireitoh is a traditional Chinese medicine that is often given to patients with nephrotic syndrome or glomerulonephritis. Studies have reported that Saireitoh stimulates intrinsic steroid secretion in rats and suppresses the proliferation of fibroblasts in vitro. We examined the effects of Saireitoh on vascular smooth muscle cell proliferation and migration in vitro and experimental atherosclerosis in vivo. Saireitoh rabbit serum obtained from New Zealand White rabbits which were given a diet containing 2% Saireitoh for 3 days significantly inhibited [³H]-thymidine incorporation by smooth muscle cells, which were isolated from thoracic aorta explants of rabbits. The addition of 10% Saireitoh rabbit serum to a culture medium containing smooth muscle cells inhibited DNA synthesis by 50% as compared with a control culture to which 10% normal rabbit serum was added. We also found that the number of smooth muscle cells in the culture containing Saireitoh rabbit serum was decreased. When PDGF was used as a chemoattractant, we demonstrated that Saireitoh rabbit serum slightly inhibits the migration of smooth muscle cells. In in vivo experiments, Saireitoh did not suppress the development of atherosclerosis but tended to reduce the damage. We concluded that although Saireitoh inhibited the proliferation of smooth muscle cells, the effect of prevention on the development of atherosclerosis is weak in the in vivo condition. J Atheroscler Thromb, 2000; 6: 33-41.

Key words: Chinese medicine, Smooth muscle cells, Atherosclerosis

Introduction

Atherosclerosis is caused by a complicated and altered interaction between cells such as endothelial cells, smooth muscle cells (SMC), macrophages or lymphocytes and other factors such as cytokines, growth factors and the extra cellular matrix. Examination of the atherosclerotic lesion reveals diffuse changes in the structure of the SMCs and the extra cellular matrix in the intima and media (1). The extra cellular matrix has also been shown to play an important role in the progression of atherosclerosis (2). Hypercholesterolemia impairs endothelial function and promotes the interaction between various cells and the vessel wall (3). The accumulation of cholesterol in the arterial vessel wall is a prominent feature of atherosclerosis. Arterial intimal thickening induced after endothelial denudation by means of a balloon catheter is one of the most commonly used experimental models for a developing atheromatous plaque (4-8). Intimal thickening is mainly due to the migration of SMC into the intima and the proliferation of these cells mediated by various inflammatory cytokines and growth factors that have been released by inflammatory cells or synthesized within the arterial wall (9,10). Studies have shown that immunological and inflammatory mechanisms play an important role in proliferative arterial disease (11-13).

Saireitoh is a popular Chinese medicine composed of twelve different natural herbs. This preparation, in combination with Goreisan and Shosaikotoh, has been used clinically for nephritis, nephrotic syndrome or hepatic disease (14). Previous studies have reported that Saireitoh stimulates intrinsic steroid secretion in rats and
suppresses the proliferation of fibroblasts in vitro. Saireitoh has also been shown to have cardio-protective properties (15, 16). Therefore, we investigated the effects of Saireitoh on rabbit SMC proliferation and experimental atherosclerosis in New Zealand White rabbits using the cholesterol-fed model and de-endothelial model.

Materials and Methods

Materials

Saireitoh (TJ-114 Tsumura) was obtained from Tsumura Inc. (Tokyo, Japan). Rabbit chow. Tissue culture plates were obtained from Falcon Labware (Becton & Dickinson, Lincoln Park, NJ, USA). Dulbecco’s modified Eagle’s Medium (DMEM) and fetal calf serum (FCS) were obtained from Gibco (Grand Island Biological, Grand Island, NY, USA). [methyl-3H]Thymidine was obtained from Amer sham (Arlington Heights, IL, USA). Trypsin, PDGF and other chemicals were from Sigma (St. Louis, MO, USA). Rabbit sera were prepared from blood collected from overnight fasted New Zealand White rabbits that were fed normal chow (normal rabbit serum) or normal chow supplemented with 2% Saireitoh for 3 days (Saireitoh rabbit serum). The rabbit sera and FCS were heat-inactivated at 56°C for 30 min before use. The animals used in this study were 15-week-old male New Zealand White rabbits (n = 40) weighing 2.8 kg to 3.0 kg and were purchased from Saitama Animal Laboratory (Saitama, Japan).

Cell culture

Primary cultures of SMC were obtained from rabbit aortae by the explant method. Aortae were obtained from 3-month-old male New Zealand White rabbits that had been fed a normal chow diet. The tissue was cut into 2 mm × 2 mm square pieces for explants and placed individually into a 24-well plate with each medium as follows: condition N) DMEM with 10% FCS; condition A) DMEM contained with 10% FCS and 10% serum from a control diet fed rabbit; condition B) DMEM with 10% FCS, 5% serum from a control diet fed rabbit and 5% serum from a Saireitoh diet fed rabbit; condition C) DMEM with 10% FCS and 10% serum from a Saireitoh diet fed rabbit. The plate was then incubated at 37°C in a humidified atmosphere with 95% air / 5% CO2.

Condition of the serum in the culture

In the following experiments to determine SMC proliferation, the proportions of Saireitoh rabbit serum and normal rabbit serum added in the medium were 0, 2.5, 5.0, 7.5 and 10.0% and 10.0, 7.5, 5.0, 2.5 and 0%, respectively.

Measurement of SMC proliferation-outgrowth

Explants were observed every day by phase contrast microscopy and the time to the commencement of outgrowth of one or more cells from the explants was measured. This was referred to as the lag phase before the commencement of outgrowth.

Measurement of SMC proliferation-cell number

After explanting, the culture medium was changed every 48 hrs and the cells were passaged at about 75% confluence. At the 8th passage, the cells were incubated with DMEM containing 0.4% FCS for 48 hrs and the medium was changed to the medium containing each concentration of Saireitoh rabbit serum and normal rabbit serum as previously mentioned. We established the control as the medium containing 10% normal rabbit serum and 0% Saireitoh rabbit serum. Seventy-two hours later, the number of cells was counted by a Coulter counter, and we studied the dose effect of the Saireitoh rabbit serum on SMC proliferation. We then determined the time course of the cell proliferation in 10% Saireitoh rabbit serum and in 10% normal rabbit serum.

DNA synthesis

The SMC reached quiescence after they were cultured in 96-well plates with DMEM containing 0.4% FCS for 48 hrs. Then, fresh medium together with 0-10% Saireitoh was added to the culture. Incorporation of [3H] thymidine (NEN, specific activity 20 Ci/mmol) into cells was measured by the addition of 2 μCi/ml[3H] thymidine during the 24 hrs incubation time.

Migration of SMC

SMC migration was assayed in a 48 well micro chemotaxis chamber fitted with a polycarbonate membrane filter with pores 5 mm in diameter. PDGF (10 ng/ml) or Saireitoh rabbit serum was introduced into the lower well of the chamber and beneath the filter. Then, a freshly prepared suspension of SMCs (5 × 10^5 per assay) in 10% FBS/DMEM was introduced into the upper well. The chambers were incubated for 6 hrs at 37°C under 5% CO2 in air. The filter was then removed, fixed, stained with hematoxylin, and the number of cells in the filter was determined by counting the number of nuclei in 10 high-power fields (HPF) (×400). All assays were performed in triplicate, and the experiments were repeated at least three times. The magnitude of SMC migration was expressed as the average number of cells/10 HPF in three separate experiments.

Measurement of plasma lipids and body weight

Blood samples were obtained from the marginal ear veins of fasting rabbits and put into plastic tubes with heparin. After the plasma was separated by centrifugation at 4°C and 1,700 g for 15 min, plasma total cholesterol (TC), triglyceride (TG) and phospholipid (PL) were measured by the enzymatic method. The body weights were measured every week in each study.

Materials

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Experimental Protocol

Cholesterol fed model

Fifteen-week-old male New Zealand White rabbits (n = 20) were fed a standard pellet diet before the study period. From the beginning of the study, rabbits were given 1.5% cholesterol diet for 2 weeks, and they were divided into the Saireitoh group (n = 10) and the control group (n = 10) by matching body weights and plasma TC levels. The pellet that contained 2.0% Saireitoh and 1.5% cholesterol was administered orally to the experimental rabbits for 12 weeks in the Saireitoh group, the pellet that contained 1.5% cholesterol was administered orally to the control rabbits for 12 weeks.

De-endothelial model

Fifteen-week-old male New Zealand White rabbits (n = 20) were fed on a standard pellet diet before balloon injury. Rabbits were anesthetized with pentobarbital (Nembutal sodium, Abbott Laboratories, North Chicago, IL, USA). Experimental intimal thickening was induced in the right carotid arteries of rabbits by removal of the endothelium using an inflated Fogarty balloon catheter (2F). The balloon was inflated, drawn and pulled back three times to denude the endothelium. After treatment, pellets containing 2.0% Saireitoh was administered orally to the experimental rabbits (n = 10) for 4 weeks, and standard pellets were administered orally to the control rabbits (n = 10) for 4 weeks.

Morphological evaluation

Rabbits were sacrificed after 12 weeks of administration, and the aortae were removed and analyzed in the cholesterol fed study. Tissue samples were obtained from the aorta, rinsed and fixed with 4% paraformaldehyde. The aortae were stained by Sudan IV staining. The aortae were stained by Sudan IV staining. The intima/media ratio of each area of tissue was calculated using a color image analyzer (OLYMPUS SP500). The Atherosclerotic Index (A.I.) was also determined by the point counting method and represented the severity of the atherosclerotic area. The lesions were macroscopically classified into four groups as follows: no lesion (N); fatty streak (a); fibrous plaque (b), and complicated lesion (c). The area of each lesion was then measured, and S.I. and A.I. were calculated, as shown in Table 1. After the tissues were analyzed, three parts of the damaged aorta were selected, stained with haematoxylin and eosin (H.E.) and Elastica Van Gieson (E.V.G.), and analyzed microscopically. The area of tissue containing atherogenic changes to the intima/media ratio was determined (Fig. 1). The percentage of outgrowth in the explants containing 10% Saireitoh medium was lower than that containing control medium. When the Saireitoh rabbit serum was added to the culture medium, a significant dose-dependent reduction in SMC proliferation was observed (Fig. 2). A significant time-dependent inhibition of SMC proliferation by Saireitoh rabbit sera was observed (Fig. 3). A maximum inhibitory effect was seen when 10% Saireitoh rabbit sera was cultured with SMC and incubated for 72 h. In order to determine the cause of inhibition of SMC proliferation, we investigated the level of DNA synthesis in SMC. When 5.0–10.0% Saireitoh rabbit sera was present in the medium, they significantly inhibited DNA synthesis in SMC in a dose-dependent manner to the control that included only 10% normal rabbit serum (p < 0.05) (Fig. 4).

In another de-endothelial study, the rabbits were sacrificed under pentobarbital anesthesia after 4 weeks of Saireitoh administration, and the carotid arteries were removed and analyzed. The balloon injured segments were confirmed by Evans blue staining. The injured right and uninjured left common carotid arteries were excised and fixed in 4% paraformaldehyde. Removed rinsed samples were stained by H.E. staining and E.V.G. staining and microscopically analyzed. The intima/media ratio was calculated by a color image analyzer.

Statistics

All results are given as mean (SEM) values. For comparison between unpaired groups, statistical significance was assessed using the Mann-Whitney U test, and P < 0.05 was considered significant.

Results

The influence of Saireitoh on SMC proliferation in vitro

The effect of different concentrations of Saireitoh rabbit serum on the rate of SMC outgrowth from the explants was determined (Fig. 1). The percentage of outgrowth in the explants containing 10% Saireitoh medium was lower than that containing control medium. When the Saireitoh rabbit serum was added to the culture medium, a significant dose-dependent reduction in SMC proliferation was observed (Fig. 2). A significant time-dependent inhibition of SMC proliferation by Saireitoh rabbit sera was also observed (Fig. 3). A maximum inhibitory effect was seen when 10% Saireitoh rabbit sera was cultured with SMC and incubated for 72 h. In order to determine the cause of inhibition of SMC proliferation, we investigated the level of DNA synthesis in SMC. When 5.0–10.0% Saireitoh rabbit sera was present in the medium, they significantly inhibited DNA synthesis in SMC in a dose-dependent manner to the control that included only 10% normal rabbit serum (p < 0.05) (Fig. 4).

Table 1. Surface Involvement (S.I.) and Atherosclerotic Index (A.I.)

<table>
<thead>
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<th>Control group</th>
<th>Saireitoh group</th>
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<tr>
<td>S.I. (%)</td>
<td>5.56±23.8</td>
<td>48.4±25.0</td>
</tr>
<tr>
<td>A.I.</td>
<td>5.8±2.3</td>
<td>5.2±2.6</td>
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were microscopically analyzed.
was only slightly inhibited by Saireitoh rabbit sera (not statistically significant) (Fig. 5). These results indicate that Saireitoh inhibits the proliferation of SMC rather than the migration. The sera used in these studies were collected from rabbits fed normal chow containing 2% Saireitoh; therefore, we administered 2% Saireitoh with the 1.5% cholesterol diet to rabbits and investigated the effect of this diet on the development of atherosclerosis.

The influence of Saireitoh on experimental atherosclerosis

Body weight levels and plasma lipids

At the beginning of the cholesterol fed study, the mean body weight of the rabbits was 3.1±0.3 kg, and this gradually increased during the experiment (Fig. 6a). In the control and Saireitoh group, the mean body weights at the end of the study were 3.4±0.4 kg and 3.2±0.4 kg, respectively. There were no significant differences in body weights between the two groups in the cholesterol fed study. The plasma TC, TG and PL levels were measured at 0 week, 4 weeks, 8 weeks and 12 weeks (Fig. 6b). After administration of the 1.5% cholesterol diet for 2 weeks, which was also the starting point of Saireitoh administration, the mean plasma TC in both groups was 1,390 mg/dl (36.0 mM). No significant differences were found in the plasma lipid levels between the two groups during the study.

In the other de-endothelial study, the mean body weight was 3.3±0.2 kg at the beginning of the experiment (Fig. 7a). The mean body weight gradually increased during the course of the experiment. We also measured the plasma TC, TG and PL levels at 0 week, 2 weeks and 4 weeks (Figs. 7b, c). The plasma TC level was 34.9±13.7 mg/dl (0.9±0.4 mM) in the Saireitoh group and 34.7±13.0 mg/dl (0.9±0.3 mM) in the control group at the time of balloon injury. After 4 weeks, the levels decreased to 5.7±5.7 mg/dl (0.1±0.1 mM) in the Saireitoh group and 6.5±7.0 mg/dl (0.2±0.2 mM) in the control group. No significant differences were found in the plasma lipid levels between the two groups during the study. Saireitoh did not seem to have an effect on the plasma lipid levels.

Evaluation of atherosclerosis

The effect of a 1.5% cholesterol diet on the atherosclerotic lesions of rabbit aorta was determined. In the
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cholesterol fed model, we determined the effect of Saireitoh on atherosclerotic lesions by Sudan IV staining. Using a color image analyzer, the S.I. was calculated to investigate the ratio of the lesion area to the entire sample area. The A.I. was determined by the point counting method and was a measurement of the severity of the lesion. The S.I. and A.I. in the control group were 55.6±23.8% and 5.8±2.3, respectively, and the S.I. and A.I. in the Saireitoh group were 48.4±25.0% and 5.2±2.6, respectively (Table 1). Although these differences were not statistically significant, Saireitoh seemed to have a tendency to reduce the progression of experimental atherosclerosis. We also investigated the maximal and minimal parts of the intimal thickening of the aorta and the cell numbers in the intimal areas of both the Saireitoh and the control groups. The mean intimal thickening of the maximal parts was 579.6±65.9 mm in the Saireitoh group and 657.7±61.4 mm in the control group. The mean intimal thickening of the minimal parts was 51.9±29.8 mm in the Saireitoh group and 83.8±35.3 mm in the control group. Both the maximal and the minimal areas of thickening were smaller in the Saireitoh group than in the control groups. The mean intimal thickening of the maximal parts was 579.6±65.9 mm in the Saireitoh group and 657.7±61.4 mm in the control group. The mean intimal thickening of the minimal parts was 51.9±29.8 mm in the Saireitoh group and 83.8±35.3 mm in the control group. Both the maximal and the minimal areas of thickening were smaller in the Saireitoh group than in the control group (Fig. 8a). The area of thickening in the intima had a tendency to be decreased in the Saireitoh group. The mean cell number in the intimal areas of

![Fig. 3. Effect of Saireitoh on proliferation of rabbit SMCs in the incubation time course. The cell numbers in culture containing 10% Saireitoh rabbit serum and control culture containing 10% normal rabbit serum were observed as shown in Fig. 2. The data represents mean±S.D. of three separate experiments. *P<0.05, **P<0.01 vs control.](image1)

![Fig. 4. Effect of Saireitoh on DNA synthesis of rabbit vascular SMCs. [3H]-thymidine incorporation was measured by the addition of 2 μCi/ml [3H]-thymidine during the 24 hrs incubation time in the medium containing each concentration of Saireitoh rabbit serum. The control was established when the medium containing 10% normal rabbit serum and 0% Saireitoh rabbit serum were used. The data represents mean±S.D. of three separate experiments, and expresses as % of no serum addition. *P<0.05 vs control.](image2)

![Fig. 5. Effect of Saireitoh on migration of rabbit vascular SMCs was investigated in the 48 well micro chemotaxis chamber using PDGF as a chemoattractant. The magnitude of SMC migration was expressed as the average number of cells/10 HPF in three separate experiments. The data represents mean±S.D. No significant differences were observed between the groups containing each concentration of Saireitoh rabbit serum and the only 10% normal rabbit serum.](image3)
Thickening was 1226.3 ± 629.8/\text{mm}^2 in the Saireitoh group and 1084.1 ± 392.5/\text{mm}^2 in the control group (Fig. 8b). The mean cell number in the intimal areas of thickening had a tendency to be reduced in the Saireitoh group.

In the de-endothelial study, we analyzed the balloon injured intimal areas of thickening by calculating the ratio of the intimal area to the medial area. The areas of the intima and media of the ringed sections were measured and investigated using a color image analyzer. The mean intima/media ratio was 0.27 ± 0.19 in the Saireitoh group and 0.33 ± 0.24 in the control group (Table 2).

Histological analysis

In the cholesterol fed study, there were fewer foam cells in the intima of the Saireitoh group compared with the control group (Figs. 9a, b), and there was a tendency for fewer collagen fibers in the intima (Figs. 9c, d). In the balloon injury study, a hypertrophic neo-intima was observed in both the Saireitoh and control groups (Fig. 10). The cells in the hypertrophic regions were decreased and the extra cellular matrix was more developed in the Saireitoh group as compared with the control group. The foam cells, the collagen fibers and the elastic fibers in the histological sections were not different between the two groups. These results indicate that Saireitoh suppresses the development of atherosclerosis by inhibiting SMC proliferation in both cholesterol fed and de-endothelial rabbit models.

Discussion

Atherosclerosis is characterized by a restrictive thick-
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Progression of atherosclerosis leads to severe vascular diseases such as cerebral infarction or acute coronary myocardial infarction. Foam cells are an important feature of early atherosclerotic lesions. They progress to fatty streaks and fibrous plaques which are composed of a complex of cells and extra cellular matrix (17). In the hypertrophic intima, the SMCs proliferate and the connective tissues expand, which leads to a narrowing of the arteries. This process leads to chronic hypoxia of the tissues being supplied by the damaged artery. The complex interaction between cells and cytokines, growth factors and the extra cellular matrix are important factors in the progression of atherosclerosis (18, 19).

A number of studies have investigated the various effects or pharmacological actions of Kampo medicine (14-16, 20-25). Kampo medicine is defined as a medicine that consists of many ingredients having various pharmacological actions without specificity for a disease. The same Kampo medicine is often used for different diseases. Saireitoh is one class of Kampo medicine and is composed of twelve different natural herbs (Table 3). This medicine is combined with Goryusan and Shosaikotoh and has been widely used in the management of patients with nephritis, nephrotic syndrome or hepatic disease (14). Previous studies have indicated that Saikosai such as Saireitoh or Shosaikotoh enhance the effects of corticoid hormone (20-22). Other studies also suggested that Saireitoh enhanced the effects of steroids or completely inhibited any augmentation in platelet aggregation (21). Using Saireitoh with steroid therapy reduces not only the dose of steroid needed but also reduces the side effects. Tashiro showed that Saireitoh has anti-inflammatory effects and inhibits the movement of fibrob-

Fig. 9. Photomicrographs of aortae of rabbits. The histological examination of rabbits bred for 12 weeks after cholesterol administration in the hematoxylin and eosin (H.E.) staining and Elastica Van Gieson (E.V.G.) staining. a: control (H.E. stain), b: Saireitoh (H.E. stain), c: control (E.V.G. stain), d: Saireitoh (E.V.G. stain)

Fig. 10. Photomicrographs of rabbit carotid arteries. The histological examination of rabbits for 4 weeks after balloon endothelial denudation of the internal carotid arteries in the hematoxylin and eosin (H.E.) staining and Elastica Van Gieson (E.V.G.) staining. a: control (H.E. stain), b: Saireitoh (H.E. stain), c: control (E.V.G. stain), d: Saireitoh (E.V.G. stain)
Table 3. Ingredients of Saireitoh (2.5 g).

<table>
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<th>Scientific Name</th>
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<tr>
<td>Pinelliac Tuber</td>
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<tr>
<td>Suxisariae Radix</td>
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<tr>
<td>Atractolidae Lanceae Rhizoma</td>
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<tr>
<td>Zizyphi Fructus</td>
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<td>Polyergus</td>
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<tr>
<td>Ginseng Radix</td>
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<td>Hoelen</td>
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<tr>
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</tr>
<tr>
<td>Cinnamomi Cortex</td>
<td>2.0 g</td>
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<td>Zingiber Recens Rhizoma</td>
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lasts by modifying the immune system or through its steroid-like effect (15). In one study, Saireitoh directly inhibited the growth of fibroblasts in the serum of rabbits given Saireitoh orally. This study also showed that Shosaikotoh, one of the main ingredients in Saireitoh, preserved normal macrophage function in the presence of markedly elevated serum cholesterol, which prevented the development of atherosclerosis (23, 24). Another study reported that Shosaikotoh induced the release of INF-γ by activating monocytes and T lymphocytes (25).

In this study, we investigated the influence of Saireitoh on SMC proliferation in vitro and on experimental atherosclerosis in New Zealand White rabbits using two different models: the cholesterol fed model and the de-endothelial model. In the in vitro vascular SMC proliferation and migration study, Saireitoh rabbit sera significantly inhibited the dose and time-dependent reduction of SMC proliferation and migration. In the cholesterol fed model, we evaluated the effect of Saireitoh on experimental atherosclerosis in rabbits fed a high-cholesterol diet by calculating the S.I. and A.I. and observing the histological changes microscopically. In vivo, Saireitoh did not prevent the development of atherosclerosis, but it did not exert a harmful influence on the antiatherogenic action and tended to reduce the damage. The values for S.I. and A.I. were smaller in the Saireitoh group than in the control group, and the intimal thickness, the number of foam cells and the amount of collagen fibers in the intima all tended to be reduced. In other studies, Saireitoh tends to reduce not only the intima/media ratio in the histological sections of balloon injured arteries but also the cell numbers in the intima. Histological analysis of the damaged arteries in the cholesterol fed model showed that the degree of intimal thickening formed by hypercholesterolemia tended to decrease in the Saireitoh group through the reduction of foam cells and collagen fibers. The result of these experimental vivo studies did not contradict the outcome of the vitro studies. Although various factors contribute to the progression of atherosclerosis, immunological and inflammatory mechanisms may play an important role in proliferative arterial disease (11-13). Saireitoh is thought to have immunosuppressive and anti-inflammatory effects on the arterial walls. In this study, Saireitoh inhibited SMC proliferation in vitro. Although we expected that the effects of Saireitoh on vascular SMC proliferation and migration to help prevent the progression of atherosclerosis, Saireitoh did not prevent the development of atherosclerosis in our in vivo experiment. A longer investigation may reveal an improvement in the anti-atherogenic effect.

During the course of this study, the body weights of rabbits in both the cholesterol and de-endothelial studies increased. In the de-endothelial study, the decrease in plasma TC levels did not significantly affect the atherogenicity in either groups. Although Saireitoh did not have an effect on the plasma lipid levels, the balloon treatments may have influenced these results.

This study demonstrated that Saireitoh inhibits the proliferation of SMC and tends to suppress the development of atherosclerosis in the aorta and carotid arteries of NZW rabbits independent of the plasma lipid levels. Although further studies are necessary for understanding the effect of Kampo medicine on atherosclerosis, this study showed that Saireitoh tends to have an anti-atherogenic effect.

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