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Inhibition of vascular smooth muscle cell migration by serum from rats treated orally with Saiko-ka-Ryukotsu-Borei-To, a traditional Chinese formulation

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Abstract

Oral administration of Saiko-ka-Ryukotsu-Borei-To (SRB), a traditional Chinese formulation, has been found to prevent intimal thickening of the carotid artery after balloon endothelial denudation in cholesterol-fed rats. To clarify the mechanism of this effect, the present study investigated if SRB inhibits vascular smooth muscle cell (VSMC) migration, which plays an important role in the development of intimal thickening after endothelial injury. The serum (SRB serum) sampled from cholesterol-fed rats treated orally with SRB for 3 days before and 4 days after the injury dose-dependently inhibited the migration of cultured VSMCs. When added directly to cultured VSMCs, the SRB extract did not inhibit VSMC migration. It is remarkable that SRB serum, which may contain a much lower concentration of SRB ingredients compared with the SRB extract, inhibited cultured VSMC migration. The present testing system using serum obtained from animals treated orally with traditional Chinese formulations may be useful for clarifying the pharmacological efficacy of such drugs, including many non-absorbable components. Furthermore, it may be useful in the search for new active compounds in serum after oral administration of traditional Chinese formulations, the active metabolites of which have not been identified.

Introduction

When freeze-dried extracts prepared from decoctions of traditional Chinese formulations, including many non-absorbable high molecular weight components, are orally administered, some ingredients are not absorbed into the blood. Their pharmacological effects cannot be adequately evaluated by conventional in-vitro methods using direct addition of extracts to cultured cells and/or enzymes. Therefore, suitable methods for elucidating the mechanisms of the effects of traditional Chinese formulations are needed. In the present study, we examined the inhibitory effects of the serum from rats treated orally with a traditional Chinese formulation, Saiko-ka-Ryukotsu-Borei-To (SRB), on migration of cultured vascular smooth muscle cells (VSMCs).

SRB (Chaihu-jia-Longgu-Muli-tang in Chinese) has been used clinically to improve the symptoms of chest discomfort and palpitation in patients with hypertension in Japan (Katayose & Shirato 2001). We have previously reported that oral administration of SRB significantly inhibited intimal thickening and VSMC proliferation 7 days after denudation in both normal-diet-fed (Kim et al 2002) and cholesterol-fed (Chung et al 2003) rats. To clarify the mechanism of action of SRB, the effect of SRB on VSMC migration was investigated by performing both in-vivo and in-vitro experiments. VSMC migration and proliferation are considered to play important roles in the onset and development of intimal thickening after endothelial injury (Califf et al 1991). The inhibitory effects of oral administration of SRB on VSMC migration were examined by immunohistochemical staining of cross-sections of the carotid artery after denudation in cholesterol-fed rats. The inhibitory effects of the serum (SRB serum) obtained from cholesterol-fed rats treated orally with SRB on cultured VSMC migration were also examined. The results obtained using the in-vitro testing system and SRB serum were compared with those obtained by conventional in-vitro methods using direct addition of SRB extract to the VSMC culture medium.

Materials and Methods

SRB and other reagents

The freeze-dried extract of SRB (lot no. 280012010 prepared by Tsumura & Co., Tokyo, Japan) was the same as that used in our previous study (Chung et al 2003), in which the HPLC fingerprint and the content of two of its ingredients (baicalin and saikosaponin b2) were described. The SRB extract contained 10 crude drugs, conforming to the standards of the Japanese Pharmacopoeia XIV: bupleuri radix (5.0), pinellia tuber (4.0), cinnamomi cortex (3.0), hoelen (3.0), scutellariae radix (2.5), zizyphi fructus (2.5), ginseng radix (2.5), fossilia ossis mastodi (2.5), ostreae testa (2.5), and zingiberis rhizoma (1.0). The figures in parentheses represent the weight (g/day) of each drug in the formulation. The usual daily dose in humans (60 kg) is 4.5 g of extract.

Simvastatin, a hydroxymethylglutaryl coenzyme A reductase inhibitor (Banyu Pharm. Co., Ltd, Tokyo, Japan), Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Ltd, Tokyo, Japan), fetal bovine serum (JRH Biosciences, Kansas, USA), penicillin (Gibco BRL, New York, USA) and streptomycin (Gibco BRL) were all used. All other chemicals and solvents were of analytical grade.

Balloon endothelial denudation in carotid artery in cholesterol-fed rats

Balloon endothelial denudation in the left carotid artery of rats anaesthetized with pentobarbital was performed according to our previously reported method (Chung et al 2003). Briefly, male Wistar rats (13 weeks old, 340–360 g) were fed a normal diet (Powdered CE-2; CLEA Japan, Tokyo, Japan) containing 1% cholesterol and SRB extract (375, 750, 1500 mg kg^{-1} daily, n = 8) for 3 days before and 4 days after the injury. Simvastatin (0.83 mg kg^{-1} daily, n = 8) was administered orally during the same period.

Animal care and all animal experiments were conducted in a manner conforming to the Guidelines of the Animal Care and Use Committee of Toyama Medical and Pharmaceutical University approved by the Japanese Association of Laboratory Animal Care.

VSMC migration into intima assessed in-vivo by immunohistochemistry

Four days after denudation, VSMC migration in the intima was examined immunohistochemically (Muranaka et al 1998). Left carotid artery sections were incubated overnight with a monoclonal antibody against human nuclei and chromosomes (MAB 1276, 1:100; Chemicon International Inc., California, USA). The number of immunoreactive cells (brown/red labelled nuclei) was counted to evaluate the migration labelling index (%): (number of migrated VSMCs in intimal area) × 100/(total number of VSMCs in intimal area).

Cultured VSMC migration assessed by in-vitro methods

Blood samples collected from the abdominal aorta 7 days after denudation were separated by centrifugation and serum was stored at −80°C before analysis. Three samples of SRB serum, obtained from cholesterol-fed rats pre-treated orally with SRB for 10 days (7 days after denudation), were diluted in DMEM free to give a final concentration of 10% SRB serum. Simvastatin serum was prepared in a similar way.

VSMCs (rat thoracic aorta SMCs: A7r5; Dainippon Pharmaceutical Co., Ltd, Tokyo, Japan) were grown in DMEM supplemented with antibiotics (100 units mL^{-1} penicillin G and 100 μg mL^{-1} streptomycin) and 10% fetal bovine serum, and incubated at 37°C in a humidified atmosphere with 5% CO_2.

VSMC migration was assayed as reported by Bilato et al (1995) using a microchemotaxis chamber (Neuro Probe Inc., New York, USA) and polycarbonate filters (8 μm in diameter; Nucleopore Corp., New Jersey, USA) (see Figure 1). Briefly, the VSMC suspension (1.5×10^5 cells mL^{-1}) was placed in the upper compartment. Two types of samples were placed in the lower compartment (Experiments 1 and 2). Experiment 1 (using serum from treated rats): three samples of SRB serum or simvastatin serum in DMEM free (600 mL). Experiment 2 (conventional in-vitro method using direct addition of samples): SRB extract in DMEM (600 mL) containing 10% rat serum (as a migration stimulator) so that the concentration of rat serum in the medium was the same as that in Experiment 1. After 4 h of incubation in the microchemotaxis chamber, non-migrated VSMCs were scraped from the upper surface of the filter. The filter was fixed in MeOH and stained with hematoxylin–eosin. The number of VSMCs that had migrated to the lower surface of the filter was determined microscopically. Migration activity was calculated as the mean number of migrated cells observed (five measurements).

Statistical analysis

The results are presented as means ± s.d. of the number (n) of experiments. One-way analysis of variation and multiple comparison test analysis of variation were used

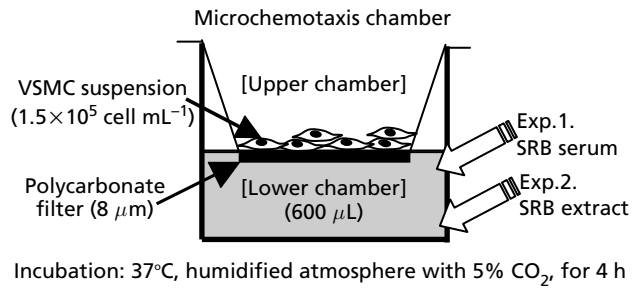

Figure 1 Assay of cultured vascular smooth muscle cell (VSMC) migration using a microchemotaxis chamber.

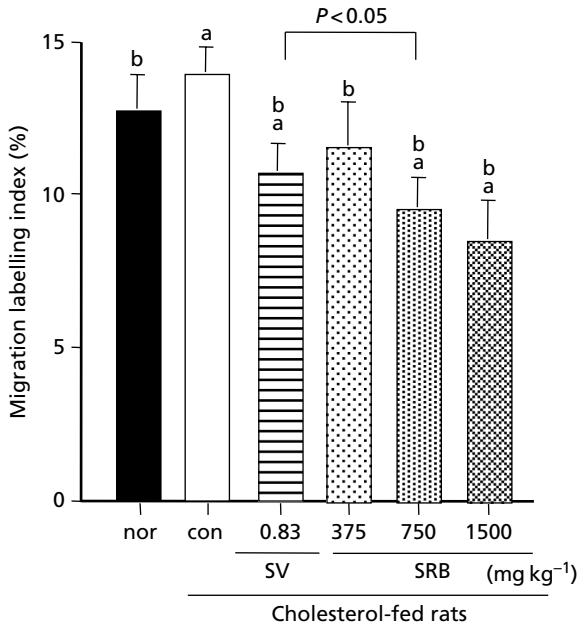

Figure 2 Inhibition of vascular smooth muscle cell (VSMC) migration by Saiko-ka-Ryukotsu-Borei-To (SRB) and simvastatin (SV): in-vivo immunohistochemical analysis. The migration labelling index (%) (number of migrated VSMCs in intimal area) × 100/(total number of VSMCs in intimal area) was estimated to be 11.6 ± 1.5% at 375 mg kg^{-1} SRB, 9.6 ± 1.0% at 750 mg kg^{-1} SRB, and 8.5 ± 1.4% at 1500 mg kg^{-1} SRB. Each value represents the mean ± s.d. (n = 8). [a]Significantly different compared with denuded normal diet-fed rats (nor) ($P < 0.05$). [b]Significantly different compared with control denuded cholesterol-fed rats (con) ($P < 0.05$).

for comparisons among groups. P values less than 0.05 were considered significant.

Results and Discussion

VSMC migration into intima assessed in-vivo by immunohistochemistry

Figure 2 shows that the VSMC migration index of the cholesterol-fed control group (13.9 ± 0.9%) was significantly greater than that of the normal-diet-fed control group (12.7 ± 1.2%). Oral administration of SRB for 7 days significantly and dose-dependently decreased the VSMC migration index. When both drugs were used at 10-times the usual human daily dose, the inhibitory effect of SRB was much greater ($P < 0.05$) than that of simvastatin, a hydroxymethylglutaryl coenzyme A reductase inhibitor that has been proven to inhibit VSMC migration in similar experiments (Hidaka et al 1992). The intimal thickening model used in the present study has similar pathological processes to atherosclerosis in humans (Clowes et al 1983) and is considered to be an accelerated atherosclerosis model (Chen et al 1994).

Cultured VSMC migration assessed by in-vitro methods

Figure 3A shows that when SRB serum (obtained from rats treated orally with SRB at 375, 750 and 1500 mg kg^{-1})

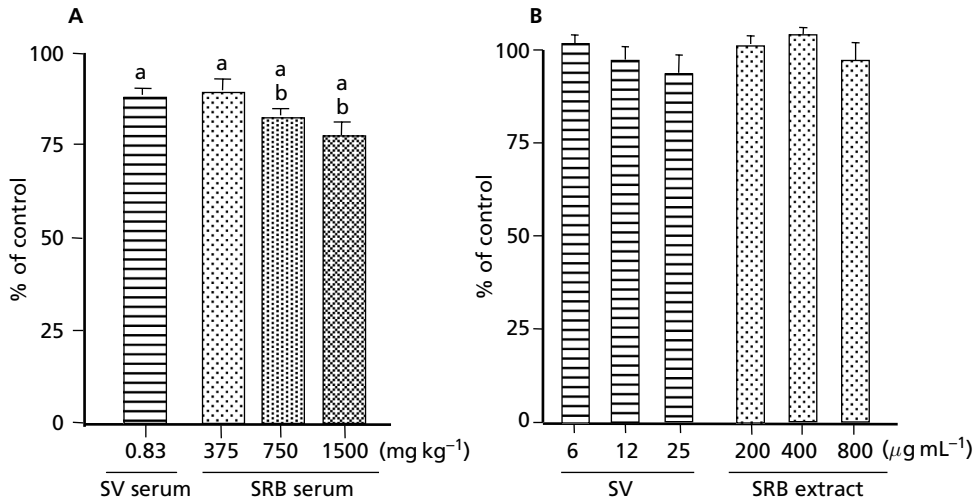

Figure 3 Inhibition of migration of cultured vascular smooth muscle cells (VSMCs) by Saiko-ka-Ryukotsu-Borei-To (SRB). Each value (% of control) represents the mean ± s.d. (n = 8). VSMC migration was stimulated by 10% rat serum. A. Results of Experiment 1. SRB serum (or simvastatin (SV) serum) was placed in the lower compartment of the microchemotaxis chamber and then the cultures were incubated for 4 h. SRB serum and simvastatin serum were obtained from cholesterol-fed rats treated orally with SRB (375, 750, 1500 mg kg^{-1}) and simvastatin (0.83 mg kg^{-1}) for 10 days (7 days after denudation), respectively. B. Results of Experiment 2 (conventional in-vitro method). SRB extract (200–800 μg mL^{-1}) or simvastatin (SV; 6–25 μg mL^{-1}) was placed directly in the lower compartment of the microchemotaxis chamber and then the cultures were incubated for 4 h. SRB (800 μg mL^{-1}: 95.1 ± 6.1%) and simvastatin (25 μg mL^{-1}: 93.3 ± 3.2%) slightly inhibited VSMC migration ($P < 0.1$; not significant). [a]Significantly different compared with control ($P < 0.05$). [b]Significantly different compared with the simvastatin group ($P < 0.05$).

was placed into the lower compartment of a microchemotaxis chamber, VSMC migration stimulated with 10% rat serum was significantly and dose-dependently reduced. Simvastatin serum also inhibited VSMC migration. When rats were treated with either SRB or simvastatin at 10-times the usual human daily dose, the inhibitory effect of SRB serum obtained from rats treated orally with $750\,mg\,kg^{-1}$ SRB ($83.3 \pm 2.9\%$) was significantly ($P < 0.05$) greater than that of simvastatin serum obtained from rats treated orally with $0.83\,mg\,kg^{-1}$ simvastatin ($89.9 \pm 2.2\%$). In contrast, Figure 3B shows that direct addition of SRB extract ($200–800\,\mu g\,mL^{-1}$) or simvastatin ($6–25\,\mu g\,mL^{-1}$) did not inhibit VSMC migration.

The results obtained from in-vitro experiments using SRB serum are in accord with those obtained from the in-vivo experiments (Figure 2). In the preliminary HPLC analysis of SRB serum, there were no peaks equivalent to characteristic components of SRB (baicalin and saikosaponin b2) in the chromatogram. It is worth noting that SRB serum, which may contain only a small amount of SRB components and metabolites compared with the SRB extract, inhibited cultured VSMC migration. Further chemical studies on endogenous serum components, organic and mineral, in SRB serum are ongoing.

The present study investigated the inhibitory effects of SRB on VSMC migration, one of the important factors in the onset and progression of atherosclerosis after endothelial injury. The results demonstrate that the migration of VSMC into the intima after balloon endothelial denudation was reduced by oral administration of SRB and that of cultured VSMCs was also inhibited by SRB serum obtained from cholesterol-fed rats pre-treated orally with SRB.

References

Bilato, C., Pauly, R. R., Melillo, G., Monticone, R., Gorelick-Feldman, D., Gluzband, Y. A., Sollott, S. J., Ziman, B., Lakatta, E. G., Crow, M. T. (1995) Intracellular signaling pathways required for rat vascular smooth muscle cell migration. Interactions between basic fibroblast growth factor and platelet-derived growth factor. *J. Clin. Invest.* **96**: 1905–1915

Califf, R. M., Fortin, D. F., Frid, D. J., Harlan, W. R., Ohman, E. M., Bengtson, J. R., Nelson, C. L., Tcheng, J. E., Mark, D. B., Stack, R. S. (1991) Restenosis after coronary angioplasty: an overview. *J. Am. Coll. Cardiol.* **17**: 2B–13B

Chen, S. J., Chen, Y. F., Miller, D. M., Li, H., Oparil, S. (1994) Mithramycin inhibits myointimal proliferation after balloon injury of the rat carotid artery in vivo. *Circulation* **90**: 2468–2473

Chung, H.-J., Maruyama, I., Tani, T. (2003) Saiko-ka-Ryukotsu-Borei-To inhibits intimal thickening in carotid artery after balloon endothelial denudation in cholesterol-fed rats. *Biol. Pharm. Bull.* **26**: 56–60

Clowes, A. W., Reidy, M. A., Clowes, M. M. (1983) Kinetics of cellular proliferation after arterial injury. I: Smooth muscle growth in the absence of endothelium. *Lab. Invest.* **49**: 327–333

Hidaka, Y., Eda, T., Yonemoto, M., Kamei, T. (1992) Inhibition of cultured vascular smooth muscle cell migration by simvastatin (MK-733). *Atherosclerosis* **95**: 87–94

Katayose, D., Shirato, K. (2001) The therapeutic application of Saiko-ka-Ryukotsu-Borei-to (Chai-hu-jia-Long-gu-du-li-tang) in the cardiological clinic. *Jpn. J. Oriental Med.* **52**: 25–38

Kim, D.-W., Chung, H.-J., Nose, K., Maruyama, I., Tani, T. (2002) Preventative effects of a traditional Chinese formulation, Chaihu-jia-Longgu-Muli-tang, on intimal thickening of carotid artery injured by balloon endothelial denudation in rats. *J. Pharm. Pharmacol.* **54**: 571–575

Muranaka, Y., Yamasaki, Y., Nozawa, Y., Terakawa, H., Tanahashi, Y., Oda, N., Satoh, A., Asao, T., Miyake, H., Matsuura, N. (1998) TAS-301, an inhibitor of smooth muscle cell migration and proliferation, inhibits intimal thickening after balloon injury to rat carotid arteries. *J. Pharmacol. Exp. Ther.* **285**: 1280–1286