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Preventive effects of a traditional Chinese formulation, Chaihu-jia-Longgu-Muli-tang, on intimal thickening of carotid artery injured by balloon endothelial denudation in rats

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Abstract

We report here that the traditional Chinese formulation, Chaihu-jia-Longgu-Muli-tang (CLM), significantly inhibited the increase in intimal thickening in rat carotid artery injured by balloon endothelial denudation, which mimics many aspects of restenosis after percutaneous coronary intervention (PCI) in humans. CLM, Saiko-ka-Ryukotsu-Borei-to in Japanese, is commonly prescribed for symptoms accompanying hypertension and atherosclerosis in Japanese Kampo medical care. CLM administered orally 1 week before and 1, 4 and 8 weeks after balloon injury inhibited the increase in intimal area, intimal/medial ratio and stenosis ratio. To our knowledge, this is the first report demonstrating inhibitory effects of a traditional Chinese formulation on intimal thickening of carotid artery after balloon injury. It is worth noting that CLM maintained its inhibitory effect up to 8 weeks after balloon injury. The reduction in intimal thickening by CLM could have resulted from inhibition of intimal smooth muscle cell proliferation, which was assessed by immuno-histochemical analysis using monoclonal antibody against proliferating cell nuclear antigen. Therefore, CLM may be a favourable candidate for prevention of restenosis after PCI. Moreover CLM may have a therapeutic value in the prevention of atherosclerosis, because restenosis after PCI is considered to be an accelerated atherosclerosis.

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Introduction

Percutaneous coronary intervention (PCI) is an important therapeutic device in the treatment of patients with coronary artery disease. However, a problem with this balloon catheter method remains the restenosis of the artery occurring within the first 6 months after the procedure in 57% of patients (Topol et al 1993). Restenosis appears to be the result of a wound-healing response to severe intimal and medial damage by balloon endothelial denudation (Califf et al 1991). Although a number of pharmacological approaches have been attempted to prevent restenosis after PCI, including antiplatelet drugs and cholesterol-lowering agents (Lefkovits & Topol 1997), adequate clinical results have not been obtained. Since the preventive effect of natural medicinal resources has currently attracted interest (Elisabetta et al 2000), we examined the effects of traditional Chinese formulations. Among many traditional Chinese formulations, we selected Chaihu-jia-Longgu-Muli-tang (CLM), which is used in Japanese Kampo medical care for patients who have an

oppressive feeling over the chest, insomnia and palpitations (Yamagiwa 1996). These are similar to symptoms accompanying hypertension and atherosclerosis.

This paper deals with evaluation of the effect of oral administration of CLM on intimal thickening in rat carotid artery induced by balloon endothelial denudation, an animal model of restenosis after PCI. To clarify the mechanism of CLM, the inhibitory effect on intimal smooth muscle cell proliferation in rat carotid artery extirpated 7 days after denudation was examined by an immuno-histochemical method using monoclonal antibody against proliferating cell nuclear antigen (PCNA). The data of CLM obtained here are compared with those of tranilast, which has been shown to inhibit intimal thickening in the balloon injury model (Fukuyama et al 1996).

Materials and Methods

Chaihu-jia-Longgu-Muli-tang (CLM) and other chemicals

The mixture of 11 crude drugs contained in CLM (human dose 29.5 g/day; Figure 1) was boiled in tap water (600 mL) for 40 min to obtain the freeze-dried extract of CLM. The yield of extract (4.85 g) is a common clinical human (60 kg) daily dose. To ensure the homogeneity of the CLM extract batches, its HPLC-profile was analysed (Figure 1). The 11 crude drugs purchased from Tochimoto-tenkaido (Osaka) were of the Japanese Pharmacopoeia XIII standard. The voucher specimens were deposited in the Institute of Natural Medicine, Toyama Medical and Pharmaceutical University.

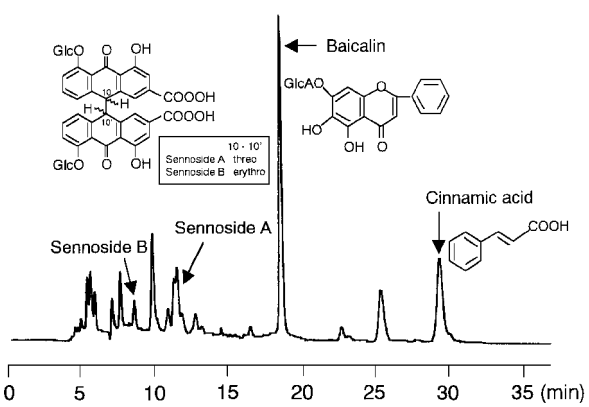

Figure 1 HPLC profile of CLM extract used in this experiment. CLM contains 11 crude drugs: Bupleuri Radix (5.0), Pinellia Tuber (4.0), Cinnamomi Cortex (3.0), Hoelen (3.0), Scutellariae Radix (2.5), Zizyphi Fructus (2.5), Ginseng Radix (2.5), Fossilia Ossis Mastodi (2.5), Ostreae Testa (2.5), Zingiberis Rhizoma (1.0) and Rhei Rhizoma (1.0). Each figure in parenthesis represents the ratio in the formulation (g/day, human dose). In the HPLC profile, sennosides A and B (from Rhei Rhizoma), baicalin (from Scutellariae Radix) and cinnamic acid (from Cinnnamomi Cortex) were identified on the basis of co-chromatography using authentic compounds. HPLC analysis: the methanol-soluble portion of the freeze-dried extract of CLM was analysed by HPLC with ODS-AQ 312 column (YMC Co. 6.0 mm $\times$150 mm) and UV spectrometer (detection at 270 nm) using mobile phase, H_2O–CH_3CN–MeOH–AcOH (70/25/5/1). Flow rate: 0.5 mL min^{-1} (0–14 min), 1.3 mL min^{-1} (15–40 min).

Tranilast was purchased from Kissei Pharmaceutical Company (Matsumoto, Japan). Monoclonal anti-PCNA antibody (PC-10) and biotinylated anti-mouse second antibody streptavidine-conjugated peroxidase were purchased from Dako Co., Ltd (Kyoto). All other chemicals and solvents were of analytical and HPLC grade.

Balloon endothelial denudation in rat carotid artery

Male Wistar rats (13 week olds, weighing 340–360 g) purchased from Sankyo Lab. Service (Tokyo) were maintained under controlled conditions with a 12-h light–dark cycle in the Laboratory for Animal Experiments, Toyama Medical and Pharmaceutical University.

Balloon catheter denudation of the carotid artery was performed according to the method of Clowes et al (1983). Briefly, the right iliac artery of rats anaesthetized by pentobarbital (50 mg kg^{-1}, i.p.) was cannulated with a balloon catheter (2F, Baxter Healthcare Co.) and the balloon was inflated with saline (0.02 mL) and rotated while pulling it back through the common carotid to denude the vessel of endothelium. All animal experiments were carried out in accordance with the Guidelines of the Animal Care and Use Committee of Toyama Medical and Pharmaceutical University approved by the Japanese Association of Laboratory Animal Care.

Histological analysis of intimal thickening to clarify intimal/medial (I/M) and stenosis ratio

Basal diet (Powdered CE-2, CLEA Japan, Tokyo) containing CLM extract (400, 800, 1200 mg kg^{-1} daily, n = 8) was administered 1 week before and 1, 4 and 8 weeks after denudation. Tranilast (300 mg kg^{-1} daily, n = 8) was suspended in 0.5% carboxymethylcellulose sodium salt (Sigma) and orally administered though a gastric sonde for the same period as above.

At 1, 4 and 8 weeks after denudation, the rats were anaesthetized with ether and perfused transcardially with saline, followed by 10% buffered formaldehyde. The left carotid artery was removed, and then 3-mm

thick sections (six or seven sections from each artery) were prepared and stained with haematoxylin and eosin. The cross-sectional areas were evaluated by NIH image analysis system (NIH 1.62) to evaluate the I/M ratio: (intimal area)/(medial area) and the stenosis ratio (%): (intimal area)×100/(intimal area+luminal area).

Intimal smooth muscle cell proliferation assay to clarify the PCNA labelling index

Rats were given basal diet containing CLM (400, 800, 1200 mg kg^{-1}, n = 8) for 3 days before and 7 days after denudation, and tranilast (300 mg kg^{-1} daily, n = 8) was applied through a gastric sonde. Intimal smooth muscle cell proliferation was assayed according to the method of Muranaka et al (1998). Briefly, on the 7 days after denudation, the left carotid artery sections prepared in the same way as for histological analysis were treated in a microwave for 10 min to unmask proliferating cell nuclear antigen (PCNA). After that, the arteries were rinsed in phosphate-buffered saline and then placed in 0.03% hydrogen peroxide containing NaN_3 for 15 min. The arteries were incubated with a monoclonal anti-PCNA antibody overnight. The primary antibody was localized by incubating for 4 h with a biotinylated anti-mouse second antibody, followed by streptavidine-conjugated peroxidase for 4 h, and visualization by 3, 3′-diamino-benzidine chromogen solution for 15 min. Sections were counterstained with Mayer's haematoxylin, and mounted with Eukitt. The number of brown-red labelled nuclei within each vessel layer was counted to evaluate a PCNA labelling index (%): (number of PCNA-positive smooth muscle cells)×100/(total number of smooth muscle cells).

Statistical analysis

The results are shown as the means±s.d. of independent experiments. Statistical significance of difference between the groups was determined by a one-way analysis of variation. Values of $P < 0.05$ were considered significant.

Results

Differences of food intake and body weight

There were no significant differences in food intake and body weight between the treated and control groups at 8 weeks after operation: respectively, 20.1±0.5 g/day and 456.6±6.2 g (CLM 1200 mg kg^{-1}, 15 times the human dose), 19.7±0.6 g/day and 454.6±3.1 g (tranilast 300 mg kg^{-1}, 60 times the human dose) and 19.8±0.7 g/day and 454.1±6.6 g (control group). All rats (n = 8) survived throughout the experimental period in each treated group.

Intimal thickening, I/M and stenosis ratio

The increase in intimal area of carotid artery sections harvested at 8 weeks after balloon injury seen in the control group (untreated) was reduced in the tranilast and CLM groups (Figure 2).

Figure 3 shows the inhibitory rates on I/M ratio as a percentage of the control group (denuded carotid artery; untreated) value. The inhibitory value (55.9±13.9%) of tranilast (300 mg kg^{-1}) at 1 week was almost similar to that of CLM (1200 mg kg^{-1}). The inhibitory effect of CLM (800–1200 mg kg^{-1}) on the I/M ratio was significantly greater than that of tranilast at 8 weeks after balloon injury ($P < 0.05$).

CLM (800 mg kg^{-1}: 80.9±17.7% of control) and tranilast (300 mg kg^{-1}: 80.4±5.0% of control) signifi-

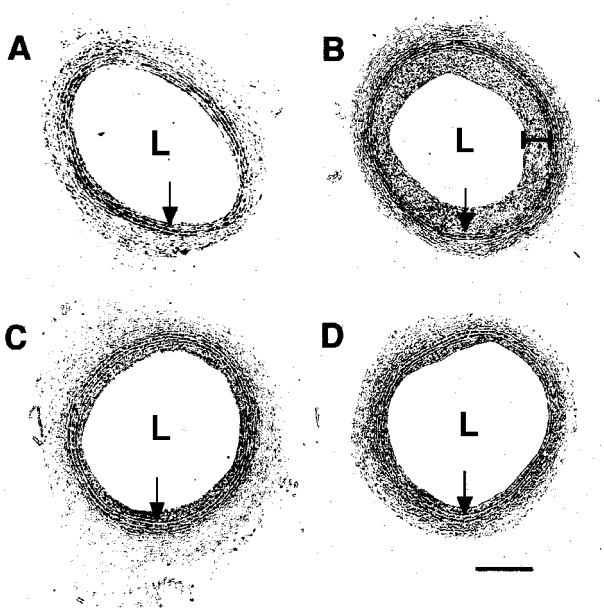

Figure 2 Photomicrographs of haematoxylin and eosin stained sections of rat carotid artery harvested 8 weeks after balloon endothelial denudation. A. normal (without denudation); B. control (denuded, untreated) (intimal area: 0.18±0.02 mm^2); C. tranilast (300 mg kg^{-1} daily)-treated rat (intimal area: 0.14±0.01 mm^2); D. CLM (800 mg kg^{-1} daily)-treated rat (intimal area: 0.15±0.01 mm^2). CLM and tranilast were orally administered for 1 week before and 8 weeks after denudation. Arrow = internal elastic lamina; L = luminal; H = intimal; bar = 200 μm.

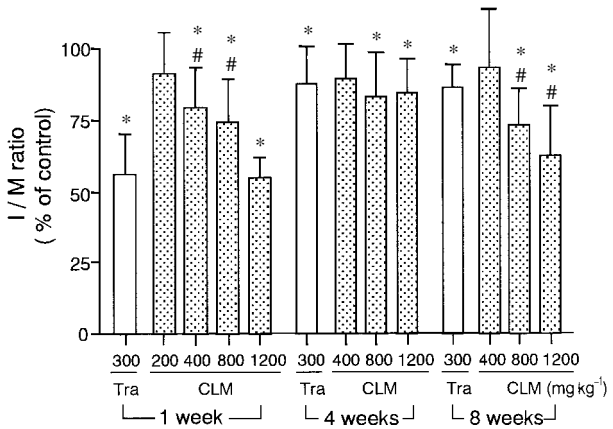

Figure 3 Effect of CLM and tranilast on the increase of I/M ratio of rat carotid artery injured by balloon endothelial denudation. Data represent means±s.d. (n = 8). *$P < 0.05$ vs control (denuded, untreated); #$P < 0.05$ vs tranilast. I/M ratio: (intimal area)/(medial area). The carotid arteries were removed at 1, 4 and 8 weeks after denudation and examined. CLM and Tra (tranilast) were orally administered for 1 week before and 1, 4 and 8 weeks after denudation. Doses of 400 mg kg^{-1} daily of CLM and 300 mg kg^{-1} daily of tranilast in the rat are equivalent to 5 and 60 times the human daily dose, respectively.

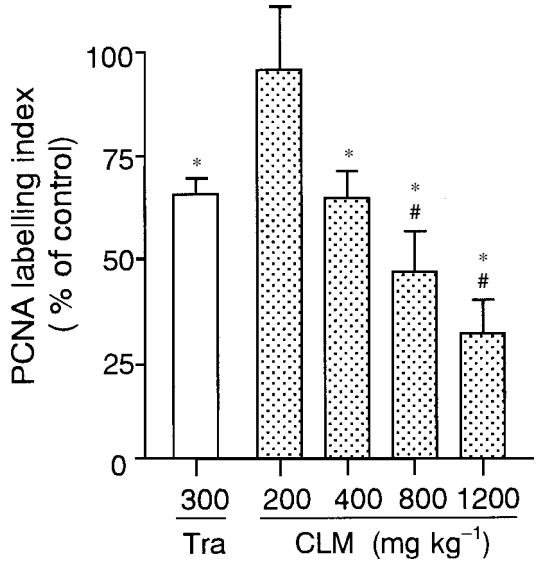

Figure 4 The effect of CLM and tranilast on the increase of PCNA labelling index of rat carotid artery injured by balloon endothelial denudation. Data represent means±s.d. (n = 8). *$P < 0.05$ vs control; #$P < 0.05$ vs tranilast. The carotid arteries were removed on the 7th day after denudation and examined immuno-histochemically. PCNA labelling index (%): (number of PCNA-positive smooth muscle cells)×100/(number of total smooth muscle cells). CLM and tranilast were orally administered for 3 days before and 7 days after denudation. The level of inhibition (63.9±6.7%) of CLM (400 mg kg^{-1}, 5 times the human dose) is almost the same as that (65.3±3.4%) of tranilast (300 mg kg^{-1}, 60 times the human dose), which is significantly lower than that of CLM (800–1200 mg kg^{-1}).

cantly inhibited the increase in stenosis ratio at 8 weeks after denudation. The time-course of inhibition was similar to that seen with the I/M ratio.

Intimal smooth muscle cell proliferation and the PCNA labelling index

The effect on proliferation of smooth muscle cells in the intimal layer after denudation was examined by PCNA labelling index. As shown in Figure 4, CLM (400–1200 mg kg^{-1} daily) significantly and dose-dependently reduced the increase PCNA labelling index. The inhibitory effect of CLM (800–1200 mg kg^{-1}) on PCNA labelling index was significantly greater than that of tranilast.

Discussion

CLM, a traditional Chinese formulation possessing vasoactive actions (Sanae et al 2000), is currently used for patients with symptoms of an oppressive feeling over the chest and palpitations in Japanese Kampo medical care (Yamagiwa 1996).

In this study, the effect of oral administration of CLM on intimal thickening of carotid artery injured by balloon endothelial denudation in rats was examined. CLM administered for 1, 4 and 8 weeks significantly and dose-dependently inhibited increased intimal area. At 8 weeks after balloon injury, the effect of CLM (800 mg kg^{-1}, 10 times the human dose) on I/M ratio was significantly greater than that of tranilast (300 mg kg^{-1}, 60 times the human dose), a positive compound possessing inhibitory effects on intimal thickening in animal models. Although a very high dose of tranilast was necessary to inhibit the increase in I/M ratio, the results obtained here were found to be similar to those of a previous report (Muranaka et al 1998).

To elucidate a mechanism for reduction of intimal thickening, the effect of CLM on intimal smooth muscle cell proliferation was examined by immuno-histochemical experiments. The inhibitory effect of CLM (800–1200 mg kg^{-1}) was significantly greater than that of tranilast (300 mg kg^{-1}). The results suggest that the reducing effects of CLM on intimal thickening could have resulted from an inhibition in smooth muscle cell proliferation, which is considered to play a central role in development of restenosis after PCI. The inhibitory

effect of CLM on smooth muscle cell proliferation in-vivo is comparable with that of in-vitro results (Yokozawa et al 1996).

In summary, our results suggest that CLM may be identified as a promising inhibitor of smooth muscle cell proliferation and may be a promising candidate as a therapeutic agent in restenosis after PCI. To our knowledge, this is the first report which demonstrates the inhibitory effects of a traditional Chinese formulation on intimal thickening of carotid artery injured by balloon endothelial denudation. It is worth noting that the inhibitory effect of CLM remained until 8 weeks after balloon injury. Furthermore, CLM (1200 mg kg^{-1}, 15 times the human dose) for 8 weeks had no remarkable toxic effects such as reduction of food intake and body weight in this study.

Since restenosis after PCI is regarded as an accelerated atherosclerosis, the effects of CLM shown in this study may imply therapeutic potential for the treatment in atherosclerosis derived from long-term inappropriate life-style. Further studies to clarify effects of CLM on intimal thickening in experimental animals fed on a high-cholesterol diet are in progress.

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