Salvia miltiorrhiza Inhibits Intimal Hyperplasia and Monocyte Chemotactic Protein-1 Expression After Balloon Injury in Cholesterol-Fed Rabbits

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Abstract

Antioxidants that prevent low density lipoproteins (LDL) from oxidation may inhibit atherosclerosis and post-angioplasty restenosis. Salvia miltiorrhiza (SM) has been shown to inhibit LDL oxidation and reduce atherosclerosis in cholesterol-fed rabbits. The effects of SM on neointimal hyperplasia and monocyte chemotactic protein-1 (MCP-1) expression after balloon injury were studied. Male New Zealand white rabbits were fed a 2% cholesterol diet together with daily SM (4.8 gm/kg body wt.) treatment (SM; n=10) or without SM as a control (C; n=9) for 6 weeks. Probucol-treated (0.6 gm/kg body wt.) rabbits (P; n=9) were used as a positive control group. A balloon injury of the abdominal aorta was performed at the end of the third week. Aortas were harvested at the end of 6 weeks. The plasma cholesterol levels were lowered in SM group. The neointimal hyperplasia in abdominal aortas was significantly inhibited in SM group [neointima/media area ratio: 0.63±0.05 (SM) versus 0.78±0.05 (C); P<0.05] and in P group [0.45±0.02 (P) versus 0.78±0.05 (C); P<0.05] when compared with C group. SM treatment significantly reduced MCP-1 mRNA and protein expression in balloon-injured abdominal aorta. These inhibitory effects on intimal response after balloon injury might be attributed to antioxidant capacity and cholesterol lowering effect of SM. SM treatment may offer some protection against post-angioplasty restenosis. J. Cell. Biochem. 83: 484–493, 2001. © 2001 Wiley-Liss, Inc.

Key words: angioplasty; atherosclerosis; cholesterol; cytokines; restenosis; Salvia miltiorrhiza

Restenosis after successful coronary intervention remains a major limitation to the long-term outcome of this procedure [Leimgruber et al., 1986]. Intimal hyperplasia, elastic recoil, and vessel wall remodeling are some major mechanisms responsible for post-angioplasty restenosis [Post et al., 1994]. The formation of intimal hyperplasia is mainly due to the phenotypic conversion and proliferation of smooth muscle cells as well as the accumulation of activated macrophages and foam cells [Hanke et al., 1994; Chen et al., 1997]. Activated macrophages and smooth muscle cells may express a number of cytokines, growth factors, and chemoattractant proteins, which involved in the vessel wall remodeling, and could further exacerbate the progression of restenotic lesions [Schwartz et al., 1995]. The monocyte chemotactic protein-1 (MCP-1) is an important mediator of monocyte recruitment into the vascular wall at sites of active inflammation or injury. Increased levels of MCP-1 have been demonstrated in human atherosclerotic arteries [Yla-Herttuala et al., 1991], experimental atherosclerotic lesions [Yla-Herttuala et al., 1991; Yu et al., 1992; Wysocki et al., 1996], and some animal models of post-angioplasty restenosis [Wysocki et al., 1996].

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These findings suggested that MCP-1 may play an important role in the pathogenesis of vascular lesion formation.

An evolving concept in recent years is the role of oxygen free radicals and oxidative stress in the development of atherosclerosis and post-angioplasty restenosis [Halliwell, 1989]. There is considerable evidence supporting the notion that oxidized low density lipoprotein (oxLDL) contributes to foam cell formation and subsequently, the progression of atherosclerotic and restenotic lesions [Steinberg et al., 1989]. Products of low density lipoprotein (LDL) oxidation have been reported to be chemotactic for vascular smooth muscle cells [Autio et al., 1990] and monocytes [Berliner et al., 1990]. It is plausible that antioxidants, which prevent LDL from oxidative modification, may interrupt the progression of atherosclerosis and restenosis [Lusis and Navab, 1993]. Probucol, a potent lipophilic antioxidant, has been shown to inhibit atherosclerosis [Daugherty et al., 1991; Sashihara et al., 1994] and reduce restenosis after angioplasty [Ferns et al., 1992; Schneider et al., 1993; Tardif et al., 1997]. Several studies also suggest that water-soluble antioxidants, such as vitamin C [Jialal and Grundy, 1991], galbordin [Fuhrman et al., 1997], and flavonoids [Hertog et al., 1993], are effective in inhibiting LDL oxidation and reducing the risk of coronary heart disease [Hertog et al., 1993]. Our previous study also showed that red wine with antioxidant capacity inhibited MCP-1 expression and reduced intimal hyperplasia in a hyperlipidemic rabbit restenosis model [Feng et al., 1999].

Salvia miltiorrhiza (SM) has been used as a blood-quickening, stasis-dispelling agent in traditional Chinese medicine [Yagi et al., 1989; Wu et al., 1998]. Salvianolic acid B (Sal B), a potent water-soluble polyphenolic antioxidant extracted from the roots of SM, has been shown to scavenge 1,1-diphenyl-2-picrylhydrazyl radicals and inhibit LDL oxidation more effectively than probucol [Wu et al., 1998]. In a recent study, we demonstrated an increase of vitamin E content in LDL and reduction of atherosclerosis in cholesterol-fed rabbits by a water-soluble antioxidant-rich fraction of SM [Wu et al., 1998]. The purposes of the present study were to examine the effects of SM treatment on intimal response and MCP-1 expression in the aorta after balloon injury in cholesterol-fed rabbits.

**MATERIALS AND METHODS**

**Agents**

Probucol was obtained from Marion Merrell Dow, USA. Sudan IV and hematoxylin were purchased from Sigma Chemical Co., USA. Sal B was a gift from the Institute of Materia Medica, Chinese Academy of Medical Sciences at Beijing, China.

**Preparation of SM and Determination of Sal B Content**

The dry roots of SM were extracted with a mixture of water and ethanol (4:1, v/v) at room temperature for 24 h. After evaporation of solvent under reduced pressure, the SM extract was stored under nitrogen at 4°C before use. The content of Sal B in SM was determined by reversed-phase HPLC.

**Animal Experiments**

Twenty-eight male New Zealand white rabbits, 3 months of age and weighing 2.5–3.0 kg, were housed in the animal center of the Taipei Veterans General Hospital, Taiwan. After 1 week on a commercial rabbit chow diet (Scientific Diet Services, Essex, UK), the animals were then fed a 2% high cholesterol diet (Purina Mills, Inc., USA) and randomly divided into three groups: (1) without drug treatment as the control group (group C; n=9), (2) probucol treatment with oral ingestion of probucol (0.6 gm/kg body wt. per day) as the positive control group (group P; n=9), and (3) SM treatment with oral ingestion of SM extract (0.6 gm/kg body wt. per day) as the experimental group (group SM; n=10). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The protocol was approved by the Animal Care and Use Committee of National Yang-Ming University, Taipei, Taiwan. The choice of SM dose was based on its antioxidant activity comparable to that of probucol in Cu²⁺-induced LDL oxidation [Wu et al., 1998]. Water was allowed ad libitum. Animals were bled periodically for measurement of plasma cholesterol, liver function, and renal function. At the end of the third week of high cholesterol diet feeding with or without drug treatment, the animals in each group were fasted for 12 h and anesthetized with an intramuscular injection of xylazine (5 mg/kg).
and ketamine hydrochloride (35 mg/kg). The surgery was performed under sterile conditions as previously described [Feng et al., 1999]. The right femoral artery was exposed and a 3 Fr. (1 French = 0.33 mm) Fogarty arterial embolectomy balloon catheter (Baxter Healthcare, Buckinghamshire, U.K.) was introduced retrogradely into the femoral artery and the lower abdominal aorta. Denudation was then performed by inflating the balloon with normal saline. The surgical wound was closed and the animals were continued on the same diets and treatments. At the end of the sixth week of the experiment, the rabbits were sacrificed. The abdominal aorta and right iliac artery were harvested for 16 cm by measuring from the previous arteriotomy site. The abdominal aorta was cut into six segments. A small part of each arterial segment was taken, immersion-fixed with 4% buffered paraformaldehyde and paraffin-embedded, then cross-sectioned for morphometry, in situ hybridization, and immunohistochemistry. The remaining larger portion of each arterial segment was immediately frozen in liquid nitrogen for RNA isolation.

**Blood Sampling**

Blood samples were collected from each animal before the start of experiment, and at the times of balloon denudation (third week), and sacrifice (sixth week). Samples were separated by centrifugation, and plasma was stored at −20°C prior to analysis. The levels of total cholesterol and triglycerides were measured with Kodak Ektachem DT 60 and DTSC analyzer.

**Analysis of Extent of Atherosclerosis**

The thoracic aortas (from aortic arch to the diaphragm) were also collected and cleaned of adhering tissue when animals were sacrificed. Thoracic aortas were stained with a solution of Sudan VI to visualize the atherosclerotic lesion area. Sudanophilic areas were quantitatively measured by computer-assisted planimetry. The extent of the lesions was expressed as the proportion of the total area with sudanophilic areas (the surface area of lesions/the surface area of the whole thoracic aorta).

**Hematoxylin-Eosin Stain and Morphometry**

One paraffin-embedded cross-section, at a thickness of 5 μm, was obtained from each segment of the abdominal aorta and stained with hematoxylin and eosin. Morphometric analysis of the six arterial sections per animal was performed by the use of a LV-2 Image Analyzer (Winhow Instruments, Taipei, Taiwan). For each arterial cross-section specimen, the intimal and medial thickness were measured at eight separate points, the intima/media thickness ratio was calculated for each point, and the overall intima/media ratio for each section was expressed as a mean of these eight values. The intimal and medial areas for each arterial cross-section specimen were measured and the intima/media area ratio was determined.

**RNA Isolation and Northern Blot Analysis**

The MCP-1 expression in the arterial specimen was examined at the RNA level by using Northern blot analysis. Total RNA was obtained as described previously [Chirgwin et al., 1979; Feng et al., 1999]. RNA was transferred onto a nylon membrane (Nytran 0.45 μm, Schleicher & Schuell, Inc., USA) and fixed by ultraviolet irradiation. After hybridizing with the 32P-labeled MCP-1 cDNA, the membrane was washed with 1X standard saline citrate containing 1% SDS at room temperature for 15 min and then exposed to X-ray films (X-OMAT, Kodak, Rochester, NY) at −70°C for autoradiography. The intensity of each hybridized band on XAR film was determined using optical densitometry (Molecular Dynamics, USA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels served as the internal standard to normalize the MCP-1 signals [Alwine et al., 1977]. The following DNA fragments were used as probes: a 0.7 kb EcoRI and BamHI fragment containing full-length MCP-1 cDNA and a 1.3 kb PstI fragment containing GAPDH cDNA (received from American Type Culture Collection, Rockville, MD).

**In Situ Hybridization and Immunohistochemistry Analysis**

To examine the cellular expression and localization of the MCP-1 gene and protein, in situ hybridization and immunohistochemistry were performed on serial sections of the aorta. The first tissue section was hybridized with digoxigenin-MCP-1 cDNA. The second and the third sections were incubated with smooth muscle cell-specific and macrophage-specific antibodies to identify smooth muscle cells and macrophages, respectively. The last section was used to detect the expression of MCP-1 protein.
In situ hybridization. MCP-1 cDNA was labeled with digoxigenin (DIG)-dUTP according to the manufacturer's instructions (DIG labeling and detection kit, Boehringer Mannheim Biochemica, Mannheim, Germany) and was used as a probe for in situ hybridization, which was performed according to a previously published method [Pang et al., 1996]. Paraffin-embedded arterial sections (5-μm thickness) were placed on poly-L-lysine-coated slides, deparaffinized, treated with proteinase K (1 mg/ml) for 15 min at 37°C, and acetylated (0.25% acetic anhydride in 0.1 M triethanolamine and 0.9% NaCl) for 10 min. Sections were then washed with 2X SSC, dehydrated, air dried for 30 min and stored at −70°C before use. Before hybridization, each section was prehybridized in a humid chamber with 100 ml prehybridization solution (5X SSC, 5X Denhardt's solution, 50% deionized formamide, 250 μg/ml yeast t-RNA, 250 μg denatured salmon sperm DNA and 4 mmol/L EDTA) for 3 h. Hybridization was performed at 50°C for 16–24 h in a humid chamber with a 25 μl/section prehybridization solution containing 10 ng/μl DNA probe. Following the hybridization, sections were washed with SSC. Sections were then blocked for 30 min, incubated with alkaline phosphatase-conjugated anti-DIG antibody (30 min) and detected with a color solution containing 337.5 mg/ml nitroblue tetrazolium salt and 175 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (10–30 min) according to the manufacturer's instructions. In some experiments, tissue sections were hybridized with DIG-labeled probes plus 50-fold unlabeled cDNA, which abolished the signal as controls.

Immunohistochemistry. For immunohistochemistry, the second, the third, and the last serial paraffin-embedded sections were used. The tissue sections were deparaffinized, rehydrated, washed with PBS, and treated with 5mg/ml serum albumin in PBS for 1 h to block non-specific binding. The last section was incubated with goat anti-human MCP-1 primary antibody (1:15 dilutions, R&D systems, USA). Antibodies were localized by an indirect immunoperoxidase technique (avidine-biotin-horseradish peroxidase complex) employing diaminobenzidine as a chromogen (Vector Lab, USA). The other two sections were incubated with mouse anti-α-smooth muscle actin (IA4, Sigma Chemical Co.) and mouse anti-rabbit macrophage (Ram II, Dako Corp., USA) to detect smooth muscle cells and macrophages, respectively. These two sections were then incubated with FITC-conjugated goat anti-mouse secondary antibody (Sigma Chemical Co.) and observed by fluorescent microscopy. Negative control was performed by omitting the incubation of the primary antibodies in the tissue sections.

Statistics

Values were expressed as mean±SEM. Data for the three groups of animals were compared using one-way ANOVA. When ANOVA indicated a significant difference between groups, data for individual groups were compared by the Dunnett test. A probability of P < 0.05 was considered statistically significant.

RESULTS

The content of Sal B in SM extract was 8.61%. Each group of animals was matched for age and weight at the start of the experiment. There was no difference in body weight at the end of the 6-week feeding period.

Blood Biochemistry

The plasma cholesterol concentrations in the C group were apparently increased after 3 weeks of feeding a 2% high cholesterol diet, and cholesterol levels increased further after 6 weeks of feeding, up to 53.92±4.10 mmole/L (Table I). The plasma cholesterol concentrations in the SM group at 3-week and 6-week

| TABLE I. Plasma Cholesterol Levels at Baseline and at 3, 6 Weeks After High-Cholesterol Feeding in Different Groups of Rabbits |
|-----------------|--------------|-------------|----------|
|                 | C            | P           | SM       |
| Baseline        | 1.27±0.14    | 0.84±0.20   | 1.21±0.17|
| 3 weeks         | 39.33±7.22   | 30.04±7.00  | 20.16±3.56|
| 6 weeks         | 53.92±4.10   | 38.94±6.19  | 35.30±3.79* |

Values are means±SEM. *: P<0.05 compared with the C group at 6 weeks. C: control, P: probucol, SM: Salvia Miltiorrhiza, Cholesterol: mmole/L.
were 20.16±3.56 mmole/L and 35.30±3.79 mmole/L, respectively. The SM group showed significantly lower cholesterol levels than the control group at the 6-week point \((P<0.05)\). Probiocul treatment showed a trend of lower cholesterol levels but was not significant when compared to that of the control group. Plasma triglyceride levels were not increased significantly during the experimental period in these three groups.

**SM and Probiocul Treatment Inhibit the Formation of Atherosclerotic Lesions**

The extent of atherosclerotic lesions, expressed as atherosclerotic area/total surface area ratio, in the thoracic aortas of the control group was 0.232±0.040. In the probiocul and SM groups, the corresponding values were 0.038±0.023 and 0.106±0.041, respectively. Probiocul treatment resulted in an 84% decrease and SM treatment caused a 47% decrease in atherosclerotic areas. Both decreases were statistically significant compared with the control group \((P<0.05)\).

**SM and Probiocul Treatment Reduce Neointimal Formation After Balloon Injury**

Histopathological sections of abdominal aortas following balloon injury were shown in Figure 1. The control group showed markedly thickened neointima with foam cell accumulation (Fig. 1A). Both probiocul (Fig. 1B) and SM (Fig. 1C) treatment significantly reduced the neointimal hyperplasia. The thickness ratios of the neointima to the media in the probiocul (0.61±0.03) and the SM (0.75±0.06) groups were significantly lower than that in the control group (1.07±0.06), as shown in Figure 2A. Similarly, the area ratios of the neointima to the media in the probiocul (0.45±0.02) and the SM (0.63±0.05) groups were significantly less when compared to the control group (0.78±0.05), as shown in Figure 2B.

**MCP-1 mRNA Expression by Northern Blot Analysis**

The cholesterol-fed control group exhibited a significant expression of MCP-1 mRNA in the abdominal aorta after balloon injury (Fig. 3). The expression of MCP-1 mRNA was significantly inhibited in the probiocul treatment group (0.69-fold lower) and in the SM treatment group (0.23-fold lower) than in the control group. The SM group showed significantly the lower MCP-1 expression than the P group.
In Situ Hybridization Combined With Immunohistochemistry for the Expression of MCP-1 mRNA and Protein

The cellular localization and specific cell type associated with the MCP-1 gene and protein expression were examined in the abdominal aorta after balloon injury (Figs. 4–6). In the cholesterol-fed control group, the MCP-1 mRNA was strongly expressed in the thickened neointima (Fig. 4A) where abundant smooth muscle cells (Fig. 4B) and a few macrophages (Fig. 4C) were present. The MCP-1 protein was strongly expressed in the thickened intima (Fig. 4D) in this group of rabbits. The probucol-treated group also showed the expression of MCP-1 mRNA (Fig. 5A) and protein (Fig. 5D) in the intima. Significantly less MCP-1 mRNA was detected in the aortic wall of the SM-treated (Fig. 6A) group. A weaker MCP-1 protein reaction was seen in the SM (Fig. 6D) groups. In the probucol and the SM-treated groups the less thickened intima was composed of smooth muscle cells mainly (Figs. 5B, 6B) and few macrophages (Figs. 5C, 6C).

**DISCUSSION**

In the present investigation, a moderate dietary-induced hypercholesterolemia combining balloon injury of abdominal aortas was used as an experimental restenosis animal model. The major findings of this study were that a water-soluble fraction of SM, which contained potent antioxidants including Sal B, limited the intimal response to balloon injury in cholesterol-fed rabbits by inhibiting intimal hyperplasia and reducing MCP-1 expression in abdominal aortas. In addition, SM treatment also markedly decreased atherosclerotic lesion areas in thoracic aortas without endothelial denudation. In this study, plasma cholesterol levels were significantly lowered after six weeks of daily SM treatment when compared with cholesterol-fed control rabbits. This cholesterol-lowering effect may partly explain the less atherosclerotic lesion formation in thoracic aorta, and a decreased intimal response in balloon-injured abdominal aorta of SM-treated rabbits.

In a number of lipophilic antioxidants, such as \( \alpha \)-tocopherol, probucol, or butylated hydroxytoluene, can block the oxidation of lipoproteins in vitro [Morel et al., 1983; Steinbrecher et al., 1984] and in vivo [Morel and Chisolm, 1989; Lafont et al., 1995]. Probucol has been shown to significantly reduce the neointimal formation in response to balloon injury in the hypercholesterolemic rabbit model [Ferns et al., 1992] and in a swine model of coronary artery balloon injury [Schneider et al., 1993]. In the present study, probucol treatment was used as a positive control for comparison. We found that probucol not only significantly reduced atherosclerotic lesions in thoracic aortas but also inhibited intimal hyperplasia and MCP-1 expression in abdominal aortas after balloon injury. Several studies also suggest that water-soluble antioxidants, such as vitamin C, are effective in inhibiting LDL oxidation by the preservation of endogenous antioxidants in LDL [Jialal and Grundy, 1991]. Flavonoids, a
class of water-soluble antioxidants, are useful in reducing the risk of coronary heart disease [Hertog et al., 1993]. Glabridin, a polyphenolic compound from licorice, protects LDL against lipid peroxidation in humans and reduces atherosclerotic lesion areas in apoE-deficient mice [Fuhrman et al., 1997]. Our recent study demonstrated that red wine with abundant flavonoids inhibits MCP-1 expression and modestly reduces neointimal hyperplasia after balloon injury in cholesterol-fed rabbits [Feng et al., 1999]. In this study, SM was chosen as treatment drug because it has usually been used as a blood-quickening, stasis-dispelling agent for treatment of cardiovascular disorders in traditional Chinese medicine. Its non-polar extracts contain tanshinones, which can inhibit platelet aggregation and protect myocardium against ischemia-induced derangement [Yagi et al., 1989]. Sal B is a potent antioxidant in the water-soluble extract of SM. In a previous study, we observed the increase of vitamin E content in LDL, inhibition of LDL oxidation, protection of endothelial damage, and reduction of atherosclerosis in cholesterol-fed rabbits by a water-soluble antioxidant-rich fraction of SM [Wu et al., 1998]. In the present study, we further demonstrated that this fraction of SM was able to significantly reduce intimal hyperplasia after balloon injury in hypercholesterolemic animals.

Monocyte infiltration into the neointima after balloon injury depends on their ability to attach to the injured endothelium and their migration into the subendothelial space. The activation state of circulating monocytes and their expression of adhesion molecules, and the secretion of chemotactic factors by the vascular cells of the damaged vessels are involved in these processes, which may be inhibited by SM treatment. Monocyte chemotactic protein (MCP-1) is a chemotactic factor for monocytes with great potency [Valente et al., 1988]. MCP-1 is secreted by stimulated human lymphocytes, endothelial

Fig. 3. Northern blot analysis. The MCP-1 mRNA expression was significantly reduced in the SM and P groups compared with the control group (*P<0.05). The MCP-1 mRNA expression of the SM group was also significantly reduced compared with the P group (*P<0.05).
cells, fibroblasts, monocytes, and smooth muscle cells in vitro [Kaczmarek et al., 1985; Valente et al., 1988; Strieter et al., 1989; Yoshimura et al., 1989; Rollins et al., 1990]. Strong expression of MCP-1 mRNA has been observed in Cynomolgus monkey fed with 2% cholesterol and 10% fat diet for 6, 11 and 18 months, and in New Zealand white rabbits fed with a 2% cholesterol diet for 10 weeks [Yla-Herttuala et al., 1991; Yu et al., 1992]. In addition, the elevation of MCP-1 expression was also demonstrated in balloon-injured aortas of pigs [Wysocki et al., 1996] or rabbits [Merritt et al., 1997]. The MCP-1 mRNA expression in SM-treated or probucol-treated rabbits was lower than that in the control group in the present study. These findings implicated that antioxidants might inhibit the expression of MCP-1 and thereby reduced the intimal response after balloon injury of aortas in cholesterol-fed rabbits.

Immunostaining with specific antibody to MCP-1 revealed strong reactivity in the thickened intima of the control group. The serial sections with specific antibodies to smooth muscle cells and macrophages demonstrated
that both cells are responsible for the expression of MCP-1 during vascular response after balloon injury. The production of MCP-1 by either smooth muscle cells or macrophages in the neointima in this study may implicate a recruitment of more monocytes/macrophages to the injured vessel sites and the progression of neointimal hyperplasia. SM treatment obviously decreased MCP-1 mRNA and protein expression, thereby resulted in the reduction of intimal response in this hyperlipidemic restenosis rabbit model.

In conclusion, this study provides experimental evidence that SM treatment attenuates intimal thickening in the balloon-injured abdominal aorta of cholesterol-fed rabbits. The reduction of intimal response may be attributed to the suppression of MCP-1 release from activated macrophages and smooth muscle cells, cholesterol-lowering effect, and antioxidant capacity of SM. It is suggested that SM may be of potential benefit in preventing restenosis after coronary angioplasty, particularly in patients with hypercholesterolemia.

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