Original Article

Probucol Suppresses Macrophage Infiltration and MMP Expression in Atherosclerotic Plaques of WHHL Rabbits

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Aim: Probucol is a lipid-lowering drug that is often prescribed for the treatment of familial hypercholesterolemia. However, it is not known whether probucol can change the lesion quality of atherosclerosis.

Methods: We examined this possibility using WHHL rabbits, a model of human familial hypercholesterolemia. Three-month-old male WHHL rabbits were treated with either probucol (85 mg/kg/day) or atorvastatin (6 mg/kg/day) for 16 weeks, and their plasma lipid levels and atherosclerotic lesions were compared with those of a control group.

Results: We found that probucol treatment reduced the plasma cholesterol levels, but less remarkably than atorvastatin treatment. In spite of this, probucol treatment led to a prominent reduction of aortic *en face* lesions by 39% ($P < 0.01$), whereas atorvastatin reduced these by 16% ($P > 0.05$), compared with those in the control. Histological examinations revealed that the aortic lesions of probucol-treated rabbits were characterized by reduced macrophages and increased smooth muscle cells compared with those from both the control and atorvastatin groups. Furthermore, probucol treatment reduced the coronary artery stenosis and increased the plaque stability.

Conclusions: These results suggest that probucol treatment may have beneficial effects on the plaque stability of hypercholesterolemic patients.

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Key words: Probucol, WHHL rabbits, Stability, Coronary artery, Hypercholesterolemia

Introduction

Atherosclerotic diseases, including myocardial infarction, aortic aneurysms and stroke constitute major causes of morbidity and mortality in both developed and developing countries¹. Hypercholesterolemia is the major risk factor for atherosclerosis;

therefore, lipid-lowering therapy is the primary means for the treatment and prevention of atherosclerosis². The stabilization of coronary plaques is also critical for patients who have culprit plaques, because plaque rupture is a major cause of many cardiac events, especially in patients with acute coronary syndrome³. Therefore, it is necessary to find a new therapeutic strategy to stabilize the plaques, because no such treatment is currently available.

Probucol is a di-phenolic lipid-lowering compound that reduces the plasma LDL cholesterol level⁴. Many studies have shown that probucol protects against atherosclerosis in rabbits⁵⁻⁷ and humans⁸. It has been demonstrated that, in addition to a chole-

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terol-lowering effect⁹⁾, the potent anti-oxidative^{5, 7, 10)} and anti-inflammatory^{11, 12)} functions of probucol play major roles in its anti-atherosclerotic effects. Because of its unique mechanisms of action, probucol is often prescribed for hyperlipidemic patients who are resistant to statins^{13, 14)}. Although probucol has multiple pharmacological functions against atherosclerosis, it is not known whether it has a similar anti-atherogenic effect to statins, or whether it exerts any effects in terms of plaque stabilization. In this study, we examined these possibilities using Watanabe heritable hyperlipidemic (WHHL) rabbits, a model of human familial hypercholesterolemia.

In the present study, we treated WHHL rabbits with optimal doses of probucol or atorvastatin (Lipitor[®]), the most commonly prescribed statin for lowering plasma cholesterol. We found that probucol treatment significantly reduced both aortic and coronary atherosclerosis, and such anti-atherogenic effects were even stronger than those of atorvastatin. Furthermore, probucol treatment led to a remarkable reduction of the lipid core size, accompanied by increased fibrotic lesions. These results suggest that probucol stabilizes atherosclerotic plaques.

Methods

Animals

Myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits were used for the current study¹⁵⁾. Sixteen WHHLMI rabbits (male, three months old) were randomly divided into three groups. The rabbits were fed either a chow diet (a restricted diet of 100 g/rabbit/day) for 16 weeks as a control, or a chow diet supplemented with 0.3% probucol (approximately 300 mg/day or 85 mg/kg/day) supplied by Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) or a chow diet supplemented with 0.02% atorvastatin calcium (approximately 20 mg/day or 6 mg/kg/day) purchased from Sequoia Research Products (Pangbourne, UK). The doses of probucol and atorvastatin used for the rabbit experiments were screened and optimized on the basis of pilot studies. All animal experiments were performed with the approval of the Animal Care Committee of the University of Yamanashi and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011).

Determination of the Plasma Lipid Levels

Blood samples were collected after a 16-h fast. The plasma levels of total cholesterol, HDL choles-

terol (HDL-C) and triglycerides were measured using Wako assay kits (Wako Pure Chemical Industries, Ltd., Osaka) every two weeks. Serum biochemical parameters were analyzed using an autoanalyzer (JCA-BM2250, JEOL, Tokyo).

Analysis of Aortic and Coronary Atherosclerosis

At the end of the experiments, all rabbits were sacrificed by an injection of an overdose of sodium pentobarbital solution (100 mg/kg). Aortas and hearts were fixed in 10% neutral buffered formalin. The aortas were *en face* stained with Sudan IV to evaluate the gross atherosclerotic lesions, as described previously¹⁶⁾. For the microscopic quantification of the lesion areas and lesion features, aortas were divided into arch, thoracic and abdominal segments, and each segment was further cut into cross sections (eight for the aortic arch and 20 each for the thoracic and abdominal aorta). All sections were cut into 3 μ m-thick specimens after dehydration, and were embedded in paraffin. All specimens were stained with hematoxylin and eosin (HE) and elastica van Gieson (EVG) stain. For the microscopic evaluation of the cellular components of the lesions, serial paraffin-embedded sections of the aorta were immunohistochemically stained with monoclonal antibodies (mAbs) against rabbit macrophages (RAM11), α -smooth muscle actin (HHF35), matrix metalloproteinase-1 (MMP-1) and matrix metalloproteinase-9 (MMP-9)¹⁷⁾.

To assess the extent of coronary atherosclerosis, rabbit hearts were sectioned into five blocks. Three sections were cut from each block at 500 μ m intervals, and a total of 15 sections were stained with HE and EVG for the analysis. The lesions of the left and right coronary arteries were quantified as the stenosis (%) of the lumen area (lesion area / total lumen area $\times$ 100) using the image analysis system. For this purpose, all sections for the microscopic quantification were captured under an Olympus BX51 light microscope equipped with a DP70 digital camera (Olympus, Tokyo) and were assessed with the Lumina Vision V2.04 image analysis software program (Mitani Co., Tokyo).

To analyze whether probucol had any effects on the lesion quality, we divided the lesions into three categories with reference to the American Heart Association classification for human atherosclerosis¹⁸⁾: (1) fatty streaks, referring to lesions that consist of a few layers of macrophages or macrophage-derived foam cells with few smooth muscle cells (SMCs); (2) fibrotic lesions, referring to lesions that are almost totally composed of extracellular matrix intermingled with SMCs or fibroblasts but few macrophages and (3)

advanced lesions, referring to those lesions that contain a fibrotic cap and a lipid or necrotic core with or without calcification. To quantify each lesion, the total length of each lesion of the aortic arch (eight specimens) was calculated for comparison. The lipid core number and size (expressed as the total length of the cores on the section) were also measured.

***In Vitro* Experiments**

Monocyte Chemotaxis Assay

To evaluate whether probucol affects the monocyte migration, we performed a chemotaxis assay using a Boyden chamber (Kurabo, Osaka). Probucol was dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO was the same in all experiments. Human monocytic cells, U937 cells (5×10^5 cells) in 200 μ L of 1640 medium, were loaded in the upper wells in the presence of probucol (30 μ M, 3 μ M and 0.3 μ M). The lower wells were loaded with chemoattractants, human MCP-1 (10 ng/mL, Pepro-Tech, Inc., Rocky Hill, NJ) or 4% zymosan-activated human serum. After a 2-h incubation (37°C, 5% CO₂), the number of monocytes that had moved across the membrane was calculated by counting 10 randomly-selected high-power fields.

Smooth Muscle Cell Culture

The aortas of Japanese white rabbits were collected under sterile conditions, and the aorta was cleaned of the intima and adventitia under a dissecting microscope. The aortic medial parts were sectioned into approximately 1 mm² pieces, and then were incubated with PBS containing collagenase I (1 mg/mL) and elastase (0.25 mg/mL, Worthington Biochemical Corp., Lakewood, NJ) for 60 minutes at 37°C. Smooth muscle cell (SMC) suspensions were centrifuged for five minutes at 1,500 rpm, and the cell pellet was resuspended in 5 mL of DMEM-F12 (Nacalai Tesque, Inc., Kyoto) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco/Invitrogen, Carlsbad, CA). The SMCs were subcultured and used for experiments within three passages.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

To induce macrophage differentiation, U937 monocytes were incubated with phorbol myristate acetate (PMA, 50ng/mL) for 72 h. Macrophages were pretreated with probucol (30 μ M, 3 μ M or 0.3 μ M) for 12 h, 24 h, 48 h and 72 h, followed by TNF- α stimulation (100 ng/mL PeproTech, Inc., Rocky Hill, NJ) for 24 h to investigate whether probucol had any

Supplemental Table 1. Primers used for real-time RT-PCR

Gene		Primers
Rabbit		
Collagen Type I	Forward	CGGTGGTTACGACTTTGGTT
	Reverse	TGAGGAGGGTCTCAATCTGG
Collagen Type III	Forward	TCAACCAGTACAAGTGACCAAC
	Reverse	ATGTGTTTGGTGGAACAGCA
GAPDH	Forward	ATCACTGCCACCCAGAAGAC
	Reverse	GTGAGTTTCCCGTTCAGCTC
Human		
MMP-1	Forward	CATGCTTTTCAACCAGGCCC
	Reverse	CGATGGGCTGGACAGGATTT
MMP-9	Forward	TTGACAGCGACAAGAAGTGG
	Reverse	CCCTCAGTGAAGCGGTACAT
TIMP-1	Forward	ACATCCGGTTCGTCTACACC
	Reverse	TGATGTGCAAGAGTCCATCC
TNF- α	Forward	CCCGAGTGACAAGCCTGTAG
	Reverse	TGAGGTACAGGCCCTCTGAT
IL-6	Forward	AGCCAGCTATGAACCTCCTTC
	Reverse	GCGGCTACATCTTTGGAATC
IL- β	Forward	ACCTGTCTCGTGTGTGAAA
	Reverse	AGACGGGCATGTTTCTGCT
GAPDH	Forward	CAATGACCCCTTCATTGACCTC
	Reverse	AGCATCGCCCCACTTGATT

effects on the macrophage MMP expression¹⁹). Rabbit aortic SMCs were also treated with probucol (30 μ M, 3 μ M or 0.3 μ M) along with transforming growth factor- β (TGF- β , 10ng/mL, as a positive control) for 48 h to investigate whether probucol had any effect on the collagen expression.

The total RNA was extracted using the Trizol reagent, and the cDNA was reverse-transcribed from total RNA using a QuantiTec Reverse Transcription kit (Qiagen, Inc. Dusseldorf, Germany). The expression levels of collagen I and collagen III by SMCs and those of MMP-1, MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) by macrophages were analyzed by real-time RT-PCR (CFX Connect™ Real-Time PCR Detection System, Hercules, CA).

Furthermore, we investigated whether probucol had any anti-inflammatory effects *in vitro* using human U937 differentiated macrophages as described above. The macrophages were pretreated with probucol (30 μ M, 3 μ M or 0.3 μ M) for 12 h, 24 h, 48 h and 72 h, followed by lipopolysaccharide stimulation (LPS, 10 μ g/mL) (Sigma-Aldrich, Inc., St. Louis, MO, USA) for 12 h. The expression levels of TNF- α , IL-6 and IL-1 β were analyzed by real-time RT-PCR as described above. The panel of primers used to analyze the gene expression is shown in **Supplemental-Table 1**.

Fig. 1. The plasma levels of total cholesterol (top), triglycerides (middle) and HDL-C (bottom)

** $P < 0.01$ or * $P < 0.05$ vs. the control group.

Statistical Analysis

All values are expressed as the means $\pm$ SD. A one-way ANOVA with Tukey's multiple comparisons test and the Kruskal-Wallis test was used for the parametric and nonparametric analysis. In all cases, statistical significance was set at $P < 0.05$.

Results

The Effects of ProbucoL and Atorvastatin on the Plasma Lipid Levels

There were no significant differences in the body weight among the three groups during the experiment

Fig. 2. The results of an analysis of aortic atherosclerotic lesions

Representative photographs of pinned-out aortic trees stained with Sudan IV from each group are shown (left). Quantitation of the aortic gross lesions expressed as percentages (right). Each dot represents the total aortic lesion area of an individual animal. ** $P < 0.01$ or * $P < 0.05$ vs. the control group. PB, probucoL; AT, atorvastatin

(data not shown). Atorvastatin treatment resulted in a significant reduction of the plasma total cholesterol levels starting from two weeks after treatment, while probucoL treatment reduced the plasma cholesterol levels starting from six weeks after beginning treatment, but less effectively than atorvastatin (**Fig. 1**). Neither probucoL nor atorvastatin treatment led to any changes in the plasma levels of triglycerides or HDL-C during the whole period of the experiment (**Fig. 1**).

Quantification of the Aortic and Coronary Atherosclerosis

ProbucoL treatment significantly reduced the whole aortic lesion area by 39% compared to control rabbits (**Fig. 2**). Although atorvastatin treatment also led to a 16% reduction of the lesion area, this inhibitory effect was weaker than that of probucoL, and did not reach statistical significance (**Fig. 2**). We further examined the microscopic lesions under a light microscope, and found that probucoL reduced the intimal lesion size of all of the parts of the aortas compared with those in the other groups: with a 58% reduction in the aortic arch, 76% reduction in the thoracic aorta

Fig. 3. Representative micrographs of the aortic lesions from each group (left panel)

Serial paraffin sections were stained with hematoxylin-eosin (HE) or immunohistochemically stained with mAbs against either macrophages (Mφ) or α -smooth muscle actin for smooth muscle cells (SMCs). The intimal lesion area and positively immunostained area of macrophages and SMCs were quantified using an image analysis system (right panel) as described in the Methods. ** $P < 0.01$ or * $P < 0.05$.

and 62% reduction in the abdominal aorta compared to the control rabbits (**Fig. 3**). Similar to the gross lesions, atorvastatin treatment reduced the microscopic intimal lesions compared with those in the control, but this reduction did not reach statistical significance (**Fig. 3**).

Histologically, the lesions of probucol-treated rabbits were characterized by marked fibrosis, with fewer macrophages and more SMCs. The number of macrophages was significantly reduced by 40% in the aortic arch, 65% in the thoracic aorta and 85% in the abdominal aorta versus those in the control, whereas the SMCs were increased (53% in the aortic arch, 56% in the thoracic aorta and 85% in the abdominal aorta compared to those in the control; not statistically significant) compared those in both the control and atorvastatin groups (**Fig. 3**).

We further quantified and compared the lesion types in the aortic arch. While the presence of fatty streaks was similar among the three groups, the fibrotic lesions in the probucol group were increased by 147%, while the lipid or necrotic core size

(expressed as the length) were reduced by 69%, and advanced lesions were reduced by 70%, compared with those in the control group (**Fig. 4**). The lesions of probucol-treated rabbits also contained fewer lipid or necrotic cores compared with the other groups (**Fig. 4C**). Furthermore, we performed immunohistochemical staining using Abs against rabbit MMP-1 and MMP-9, and showed that MMP-1 and 9-immunoreactive proteins in the lesions of probucol-treated rabbits were faintly stained and significantly less than those in both the control and atorvastatin groups (**Fig. 5**).

In addition to these effects on the aortic lesions, probucol treatment significantly reduced the coronary stenosis (41% reduction vs the control), which was accompanied by fewer macrophages (data not shown) in comparison with those in both the control and atorvastatin groups (**Fig. 6**). Taken together, probucol treatment reduced the lesion size of the aortic coronary atherosclerosis, and the lesions contained less macrophage infiltration and fewer lipid cores.

Fig. 4. Representative micrographs of aortic lesions in WHHL rabbits (A) and quantitation of the different types of lesions (B) and lipid cores (C)

** $P < 0.01$ or * $P < 0.05$. PB, probucol; AT, atorvastatin

The Effects of Probucol on the Monocyte and Smooth Muscle Cell Functions

The finding that probucol treatment appeared to stabilize the atherosclerotic lesions prompted us to examine the possible mechanisms involved. We first investigated whether probucol had any inhibitory effects on the macrophage migration into the lesions. To examine this, we performed a chemotaxis assay using U937 monocytes. MCP-1 or zymosan-activated human serum was used as a chemoattractant to induce U937 monocyte migration across the membrane. However, even in the presence of different concentrations of probucol, we did not find any inhibitory effects on the number of migrated monocytes com-

pared with that for each chemoattractant alone (**Supplemental-Fig. 1**).

Because immunohistochemical staining showed that probucol treatment led to a reduction in the levels of MMP-1 and MMP-9 (**Fig. 5**), we further examined whether probucol had any effects on the macrophage MMP-1 and MMP-9 expression or the endogenous inhibitor, TIMP-1, because both may be involved in the plaque stability. As shown in **Supplemental-Fig. 2**, treatment with different concentrations of probucol did not affect the levels of mRNA expression of these genes in U937-derived macrophages. Furthermore, we found that probucol treatment did not lead to any changes in the TNF- α , IL-6 or IL-1 β

Fig. 5. The results of an immunohistochemical analysis of the MMP-1 and MMP-9 levels in the lesions of the aortic arch. Representative micrographs (left) and the results of a quantitative analysis of the MMP-1- and MMP-9-positive areas (right) using the image analysis system are shown. * $P < 0.05$. PB, probucol; AT, atorvastatin

Fig. 6. The results of an analysis of coronary atherosclerosis. Representative micrographs of the left coronary arteries from each group that were stained with HE and EVG are shown (top). The extent of coronary stenosis was measured, and is expressed as a percentage (bottom). LCA: left coronary artery trunk; RCA: right coronary artery trunk. ** $p < 0.01$ or * $p < 0.05$ vs. the control group. PB, probucol; AT, atorvastatin

Supplemental Fig. 1. Effects of probucol on the migration of U937 cells *in vitro*

The chemotaxis assay was performed as described in the Methods. Representative data are shown, and are expressed as the means $\pm$ SD. PB, probucol; MCP-1, monocyte chemoattractant protein-1; ZAS, zymosan activated serum; HPF, high power field.

Supplemental Fig. 2. The effects of probucol on the mRNA expression of MMP-1, MMP-9 and TIMP-1 in TNF- α -stimulated macrophages

U937-derived macrophages were incubated for 12, 24, 48 and 72 h with different concentrations of probucol in the presence of TNF- α . Representative data are shown, and are expressed as the means $\pm$ SD.

Supplemental Fig.3. The effects of probucol on the TNF- α , IL-6 and IL-1 β expression in LPS-stimulated U937-derived macrophages

U937-derived macrophages were incubated with different concentrations of probucol for 12, 24, 48 and 72 h. Representative data are shown, and are expressed as the means $\pm$ SD.

mRNA expression in U937-derived macrophages (Supplemental-Fig. 3).

Finally, we examined the effects of probucol on the collagen mRNA expression of smooth muscle cells. As shown in Supplemental-Fig. 4, the mRNA expression levels of collagen-I and collagen-III were not significantly different from those of the control. Taken together, these findings indicate that probucol did not have direct effects on the expression of these genes in macrophages and SMCs *in vitro*.

Discussion

Although it has long been known that probucol has anti-atherogenic effects, it has not been clarified whether it has any beneficial effects in terms of the plaque quality or lipid core formation. In the current

Supplemental Fig.4. The effects of probucol on the collagen mRNA expression in rabbit aortic SMCs

Representative data are shown, and are expressed as the means $\pm$ SD.

study, we first demonstrated that probucol exhibited a remarkable inhibitory function on aortic atherosclerosis, consistent with the findings of previous studies⁵⁻⁷. Furthermore, we found that, at the current optimal doses, its anti-atherogenic effect was even stronger than that of atorvastatin, the most commonly used statin. Interestingly, the lipid-lowering effect of probucol was weaker than that of atorvastatin, consistent with the notion that the potent anti-atherogenic effect of probucol is basically independent of its lipid-lowering function^{7, 20}. It should be pointed out, however, that the lesions of the probucol-treated group were characterized by markedly reduced intimal macrophages, with increases in smooth muscle cells and extracellular matrix deposition, which was not evident in the atorvastatin group.

Several mechanisms may be involved in this phenomenon. For example, probucol may inhibit the initiation and progression of atherosclerosis by its strong anti-inflammatory and anti-oxidative effects²¹. The beneficial effects of probucol in this regard appear to be indirect, because probucol did not inhibit monocyte chemotaxis toward MCP-1 or induce any changes in inflammatory cytokine expression in our *in vitro* studies. The increased extracellular matrix and smooth muscle cells, along with fewer macrophages and lower MMP-1 and MMP-9 expression levels in the lesions, suggest that probucol may promote plaque stabilization. This is further supported by the fact that the lesions in the probucol-treated group were less advanced and contained fewer lipid cores.

Although it has been reported that probucol directly inhibits the production of MMPs secreted by arterial wall cells, and increases the production of collagens in balloon-injured rabbits²², we did not find any inhibitory effects of probucol on MMP-1 and MMP-9 expression by macrophages or on the collagen synthesis by smooth muscle cells in cultured cells. This finding may suggest that the probucol-mediated plaque stability may be attributable to an overall effect derived from its lipid-lowering and anti-oxidation functions, rather than a direct effect on the particular cells in the arterial wall.

Although atorvastatin treatment also led to a prominent reduction of MMP-1 and MMP-9 immunoreactive proteins in the lesions (similar to probucol treatment in the current study), the number of macrophages in the lesions were unchanged compared to the control, suggesting that atorvastatin and probucol reduce the MMP expression through the different mechanisms.

Another interesting result of our experiment is that probucol treatment reduced the coronary artery

stenosis and increased plaque stability, which was not reported in the previous studies using rabbit models⁵⁻⁷. As a lipid-lowering drug, probucol lowers the cholesterol level by increasing the rate of LDL catabolism and inhibiting cholesterol synthesis. However, for a long time, probucol was notorious for its HDL-C-lowering effect (which was considered a negative side effect), which prevented its use in the US and Europe. In the current study, we did not find a reduction of the HDL-C levels in WHHL rabbits treated with probucol, because the plasma levels of HDL-C are already low in these rabbits. Nevertheless, it has become clear that the decreased HDL-C levels induced by probucol do not affect the physiological functions of HDL, but are instead beneficial for reverse cholesterol transport²³. In Japan, probucol is used for hyperlipidemic patients who are resistant to statins²⁴. In China, however, it is widely used for the treatment of hyperlipidemia and given the same priority as statins. So-called "PAS" therapy (probucoL, aspirin and statin) in China is often prescribed for both the primary and secondary prevention of atherosclerosis²⁵.

Several implications can be inferred from our current investigation. First, probucol can not only inhibit the development of atherosclerosis, but also stabilize existing atherosclerotic lesions. This suggests that probucol may be useful for treating hyperlipidemia similar to statins, may be beneficial for the patients at high risk or may have protective effects for cardiac events²⁶. Although it remains to be verified clinically whether this "old drug" exhibits such a new function, it seems that probucol has stronger anti-atherogenic effects than atorvastatin, at least in WHHL rabbits.

Conflicts of Interest

None declared.

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References

- 1) Weber C, Noels H: Atherosclerosis: current pathogenesis and therapeutic options. *Nat Med*, 2011; 17: 1410-1422
- 2) LaRosa JC, Grundy SM, Waters DD, Shear C, Barter P, Fruchart JC, Gotto AM, Greten H, Kastelein JJ, Shepherd J, Wenger NK, Treating to New Targets I: Intensive lipid lowering with atorvastatin in patients with stable coronary disease. *N Engl J Med*, 2005; 352: 1425-1435

- 3) Libby P, Aikawa M: Stabilization of atherosclerotic plaques: new mechanisms and clinical targets. *Nat Med*, 2002; 8: 1257-1262
- 4) Kesaniemi YA, Grundy SM: Influence of probucol on cholesterol and lipoprotein metabolism in man. *J Lipid Res*, 1984; 25: 780-790
- 5) Kita T, Nagano Y, Yokode M, Ishii K, Kume N, Ooshima A, Yoshida H, Kawai C: Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. *Proc Natl Acad Sci U S A*, 1987; 84: 5928-5931
- 6) Daugherty A, Zweifel BS, Schonfeld G: Probucol attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Br J Pharmacol*, 1989; 98: 612-618
- 7) Carew TE, Schwenke DC, Steinberg D: Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc Natl Acad Sci U S A*, 1987; 84: 7725-7729
- 8) Kasai T, Miyauchi K, Kubota N, Kajimoto K, Amano A, Daida H: Probucol therapy improves long-term (> 10-year) survival after complete revascularization: a propensity analysis. *Atherosclerosis*, 2012; 220: 463-469
- 9) Yamashita S, Matsuzawa Y: Where are we with probucol: a new life for an old drug? *Atherosclerosis*, 2009; 207: 16-23
- 10) Siveski-Iliskovic N, Kaul N, Singal PK: Probucol promotes endogenous antioxidants and provides protection against adriamycin-induced cardiomyopathy in rats. *Circulation*, 1994; 89: 2829-2835
- 11) Brasen JH, Koenig K, Bach H, Kontush A, Heinle H, Witting PK, Yla-Herttuala S, Stocker R, Beisiegel U: Comparison of the effects of alpha-tocopherol, ubiquinol-10 and probucol at therapeutic doses on atherosclerosis in WHHL rabbits. *Atherosclerosis*, 2002; 163: 249-259
- 12) Fruebis J, Gonzalez V, Silvestre M, Palinski W: Effect of probucol treatment on gene expression of VCAM-1, MCP-1, and M-CSF in the aortic wall of LDL receptor-deficient rabbits during early atherogenesis. *Arterioscler Thromb Vasc Biol*, 1997; 17: 1289-1302
- 13) Baker SG, Joffe BI, Mendelsohn D, Seftel HC: Treatment of homozygous familial hypercholesterolaemia with probucol. *S Afr Med J*, 1982; 62: 7-11
- 14) Yamamoto A, Matsuzawa Y, Kishino B, Hayashi R, Hirobe K, Kikkawa T: Effects of probucol on homozygous cases of familial hypercholesterolemia. *Atherosclerosis*, 1983; 48: 157-166
- 15) Shiomi M, Ito T, Yamada S, Kawashima S, Fan J: Development of an animal model for spontaneous myocardial infarction (WHHLMI rabbit). *Arterioscler Thromb Vasc Biol*, 2003; 23: 1239-1244
- 16) Wang Y, Niimi M, Nishijima K, Yu Y, Koike T, Kitajima K, Inoue T, Waqar AB, Liu E, Kohashi M, Ketamura Y, Yoshikawa T, Zhang J, Ma L, Zha X, Watanabe T, Asada Y, Chen EY, Fan J: Human apolipoprotein AII protects against diet-induced atherosclerosis in transgenic rabbits. *Arterioscler Thromb Vasc Biol*, 2013; 33: 224-231
- 17) Liang J, Liu E, Yu Y, Kitajima S, Koike T, Jin Y, Morimoto M, Hatakeyama K, Asada Y, Watanabe T, Sasaguri Y, Watanabe S, Fan J: Macrophage metalloelastase accelerates the progression of atherosclerosis in transgenic rabbits. *Circulation*, 2006; 113: 1993-2001
- 18) Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W, Jr., Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW: A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation*, 1995; 92: 1355-1374
- 19) Yu Y, Koike T, Kitajima S, Liu E, Morimoto M, Shiomi M, Hatakeyama K, Asada Y, Wang KY, Sasaguri Y, Watanabe T, Fan J: Temporal and quantitative analysis of expression of metalloproteinases (MMPs) and their endogenous inhibitors in atherosclerotic lesions. *Histol Histopathol*, 2008; 23: 1503-1516
- 20) Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL: Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med*, 1989; 320: 915-924
- 21) Wu BJ, Di Girolamo N, Beck K, Hanratty CG, Choy K, Hou JY, Ward MR, Stocker R: Probucol [4,4'-[(1-methylethylidene)bis(thio)]bis-[2,6-bis(1,1-dimethylethyl)phenol]] inhibits compensatory remodeling and promotes lumen loss associated with atherosclerosis in apolipoprotein E-deficient mice. *J Pharmacol Exp Ther*, 2007; 321: 477-484
- 22) Wu TC, Chen YH, Leu HB, Chen YL, Lin FY, Lin SJ, Chen JW: Carvedilol, a pharmacological antioxidant, inhibits neointimal matrix metalloproteinase-2 and -9 in experimental atherosclerosis. *Free Radic Biol Med*, 2007; 43: 1508-1522
- 23) Yamamoto S, Tanigawa H, Li X, Komaru Y, Billheimer JT, Rader DJ: Pharmacologic suppression of hepatic ATP-binding cassette transporter 1 activity in mice reduces high-density lipoprotein cholesterol levels but promotes reverse cholesterol transport. *Circulation*, 2011; 124: 1382-1390
- 24) Teramoto T, Sasaki J, Ishibashi S, Birou S, Daida H, Dohi S, Egusa G, Hiro T, Hirobe K, Iida M, Kihara S, Kinoshita M, Maruyama C, Ohta T, Okamura T, Yamashita S, Yokode M, Yokote K, Japan Atherosclerosis S: Comprehensive risk management for the prevention of cardiovascular disease: executive summary of the Japan Atherosclerosis Society (JAS) guidelines for the diagnosis and prevention of atherosclerotic cardiovascular diseases in Japan -- 2012. *J Atheroscler Thromb*, 2013; 20: 603-615
- 25) Adhikari BK, Meng X: Challenges of PAS therapy for coronary atherosclerotic plaque reversal. *Heart*, 2012; 98: E167
- 26) Yamashita S, Hbujo H, Arai H, Harada-Shiba M, Matsui S, Fukushima M, Saito Y, Kita T, Matsuzawa Y: Long-term probucol treatment prevents secondary cardiovascular events: a cohort study of patients with heterozygous familial hypercholesterolemia in Japan. *J Atheroscler Thromb*, 2008; 15: 292-303