Probucol Attenuates Inflammation and Increases Stability of Vulnerable Atherosclerotic Plaques in Rabbits

Tingting Li,¹ Wenqiang Chen,¹ Fengshuang An,¹ Hongbo Tian,² Jianning Zhang,³ Jie Peng,¹ Yun Zhang¹ and Yuan Guo¹

¹The Key Laboratory of Cardiovascular Remodeling and Function Research, Chinese Ministry of Education and Chinese Ministry of Health, Jinan, P.R. China
²Department of Cardiology, Shandong Provincial Hospital affiliated to Shandong University, Jinan, P.R. China
³Department of Critical Care Medicine, Qilu Hospital of Shandong University, Jinan, P.R. China

Probucol, a lipid-lowering agent with anti-oxidant properties, has been implicated in protection against atherogenesis, whereas its effect on plaques stability remains to be fully elucidated. The present study was aimed to test the hypothesis that probucol may attenuate inflammation and increase stability of vulnerable atherosclerotic plaques using a rabbit model. After abdominal aortic balloon injury, 45 rabbits were fed a 1% cholesterol diet for 24 weeks. From week 12 to week 24, the animals were treated with probucol (1% by weight in the diet), simvastatin (5 mg·kg⁻¹, positive control) or no drugs (control), respectively. At the end of week 22, recombinant-p53 adenovirus was injected into the abdominal aortic plaques. Two weeks later, plaque disruption was induced by injection of Chinese Russell’s viper venom and histamine. The results showed that the incidence of plaque disruption in probucol or simvastatin groups was significantly lower than that in the control group (7.15% or 14.29% vs. 71.43% respectively, both P < 0.01). Probucol significantly increased the thickness of fibrous caps and decreased plaque vulnerability index. Serum concentrations of inflammatory cytokines and matrix metalloproteinases, and expression levels of Toll-like receptor (TLR)-2, TLR-4, monocyte chemoattractant protein-1, intercellular adhesion molecule 1, scavenger receptor A, CD36 and oxidized low-density lipoprotein receptor 1 within the lesions were markedly lower in both treatment groups than in the control group. We conclude that probucol increases the stability of vulnerable plaques, possibly through its lipid lowering, anti-inflammation and scavenger receptors suppression effects, suggesting probucol as a promising pharmacologic approach to stabilize vulnerable plaques.

Keywords: Atherosclerosis; inflammation; probucol; ultrasonography; vulnerable plaque


It is generally established that disruption of the vulnerable atherosclerotic plaque with superimposed thrombus formation is the main cause of acute coronary syndromes (ACS). Accordingly, treatment aimed at stabilization of vulnerable plaques is of great clinical importance. Although statins have been recognized as the most potent drugs for stabilizing plaques, the Prove-It trial found that 22.4% of enrolled patients suffered an acute coronary event despite an intensive statin therapy for 2 years (Ridker et al. 2005). Moreover, statins are not universally tolerable with some adverse effects such as elevated liverenzymes, rhabdomyolysis and cancer (Alsheikh-Ali et al. 2007). Therefore, it is of high priority to develop safer and more effective drugs to stabilize vulnerable plaques.

Current evidence supports strongly a central role for inflammation, involving both innate and adaptive arms of immunity, in all stages of atherosclerosis including the transition of stable atherosclerotic plaques to vulnerable plaques (Libby 2002; Hansson et al. 2002). Consequently, drugs targeting inflammation and immunoreactions may have a great potential to stabilize atherosclerotic plaques. Probucol, a bisphenol compound, was synthesized as an anti-oxidant and was found with a cholesterol-lowering potential (Barnhart et al. 1970; Yamashita and Matsuzawa 2009). As an anti-oxidant and lipid-lowering drug, probucol has been in clinical use during the past few decades for the prevention and treatment of cardiovascular diseases. Probucol has recently been elucidated with the capacity to inhibit the expression of oxidation-sensitive inflammatory factors, such as vascular cell adhesion molecule-1 (Wu
al. 2007), monocyte chemotactic protein-1 (MCP-1) (Chang et al. 1995) and interleukin (IL)-1 (Ku et al. 1990). In experimental and clinical studies, probucol has been reported to reduce intimal proliferation after balloon injury in animals (Schneider et al. 1993; Lau et al. 2003) and inhibited restenosis after coronary angioplasty with (Tardif et al. 2003) and without (Tardif et al. 1997) stent in humans. Furthermore, it dramatically retarded atherosclerosis in hypercholesterolemic animals (Sasahara et al. 1994; Bräsen et al. 2002; Braun et al. 2003). Since most preclinical probucol treatments have been done in the early stage of atherosclerosis, the exact effect of probucol on the final steps of atherosclerosis, namely plaque destabilization and eventually plaque rupture, and the underlying mechanisms have not been well explored.

The present study was performed to evaluate the effect of probucol on the atherosclerotic plaque stability and its possible mechanism in a rabbit model of vulnerable atherosclerotic plaques we previously verified (Chen et al. 2007; Zhang et al. 2008; Zhong et al. 2008). Since recent clinical trials demonstrated a better outcome in patients with ACS under intensive statin treatment (Grundy et al. 2004; Chan et al. 2007), a high-dose of simvastatin was chosen as a positive control therapy to compare with probucol.

Materials and Methods

Animal model

All procedures and care of animals were approved by the Animal Management Rules of the Chinese Ministry of Health (document no. 55, 2001), and the study was approved by the Animal Care Committee of Shandong University. The rabbit vulnerability plaque model was established as we previously described (Chen et al. 2007). Forty-five male New Zealand White rabbits (2.0 to 2.5 kg) were fed with an atherogenic diet containing 1% cholesterol for 24 weeks after balloon-induced aortic endothelial injury as described. From week 12 to week 24, rabbits were randomized into 3 groups (n = 15 per group) and were fed the atherogenic diet supplemented with probucol (1% wt/wt), simvastatin (5 mg/kg of body weight), or no drugs respectively. Pure probucol and simvastatin were added to the diet as diethyl ether solution (Carew et al. 1987). The control diet was treated with the same amount of pure solvent. All diets were dried of the solvent before use. The doses used were based on previous literature reports (Carew et al. 1987) and the ratio of the highest clinical dose of simvastatin in patients to the highest experimental dose of simvastin in rabbits (Hernández-Presa et al. 2003; Wiviott et al. 2006). The amount of diet for each animal was restricted to 120 g/day during the study period. Water was supplied ad libitum. Dietary consumption and weight gain did not differ among the three groups of animals.

To induce vulnerable plaques, at the end of week 22, all animals were injected with adenoviral vector containing recombinant p53 as described previously (Chen et al. 2007). In brief, with the rabbits under sufficient anesthesia, a midline incision along the abdomen was made and the abdominal aorta was exposed. Under the guidance of intravascular ultrasound, a 50 μl suspension of adenovirus containing p53 (8 × 10^9 pfu/ml) was injected into the abdominal aortic segments with the largest plaque, which were mainly located between the right renal and the common iliac arteries. The suspension was left in situ for 10 minutes after temporary ligation of the artery. The injected position was marked with nesis, the abdominal cavity was closed, and antibiotics were injected intravenously to prevent infection. At the end of week 24, plaque rupture was induced by pharmacological triggering: 0.15 mg·kg⁻¹ of Chinese Russell’s viper venom was injected intraperitoneally followed by intravenous injections of 0.02 mg·kg⁻¹ histamine 30 min later.

Biochemical studies

Blood was drawn from a marginal ear vein of all rabbits fasting overnight at baseline and at the end of week 12 and 24, respectively. Serum levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured by enzymatic assays using an automated biochemical analyzer (Roche Hitachi 917, Japan). Serum levels of high sensitive C-reactive protein (hs-CRP), IL-18, IL-6, matrix metalloproteinases-9 (MMP-9) and tumor necrosis factor-α (TNF-α) were assayed by use of ELISA kits (R&D Systems, Chicago, IL, USA).

Ultrasoundographic study

At the end of week 24, all rabbits under sufficient anesthesia underwent abdominal aortic ultrasound examination by use of a high-resolution ultrasound scanner HP Sonos 7500 equipped with a linear array 7.5 MHz transducer. The aortic longitudinal axis views were scanned to visualize the plaque length from the cartilago ensiformis to the branch spot of common iliac artery on B-mode. End-diastolic luminal diameters (Dd) and the intima-media thickness (IMT) were measured by two-dimensional echocardiography. The IMT was measured from the leading edge of the adventitia to the far edge of the intima and the largest value was used for analysis. The scan head was subsequently regulated to Doppler and the aortic peak velocity (Vp), mean velocity (Vm) and velocity-time integral (VTI) were recorded.

Integrated backscatter analysis

The ultrasonic integrated backscatters from the aortic wall and the atherosclerotic plaques were analyzed by use of acoustic densitometry technique. The acoustic intensities (All) of the aortic intima-media (All-I) and adventitia (All-A) in the atherosclerotic plaques were averaged, and the corrected All (Allc%) was calculated as: Allc% = All-I / All-A × 100%.

Intravascular ultrasound (IVUS) studies

IVUS studies involved use of a commercially available system (Galaxy, Boston Scientific Corporation, USA) at the end of week 24 as described (Chen et al. 2007), with the rabbits under sufficient anesthesia. The 40-MHz catheter was advanced into the aortic arch through the left femoral artery and the ultrasonic images were recorded by a motor-driven progressive withdrawal of the catheter to the iliofemoral artery at a speed of 0.5 mm/sec. Images from the abdominal aortic cross-sectional images were analyzed subsequently by two independent observers and the values were averaged for data analysis. Quantitative and qualitative analysis were performed in accordance with the guidelines for acquisition and analysis of IVUS images issued by the American College of Cardiology and European Society of Cardiology (Mintz et al. 2001). The external elastic membrane area (EEMA) and lumen area (LA) are assessed by manually tracing the leading edge of the adventitia and the intimal leading edge.
Histological and immunohistochemical analysis

All rabbits were euthanized by intravenous overdose of pentobarbital, and the aortas were dissected from aortic arch to iliac bifurcation. Tissue samples marked described as before were taken from the abdominal aorta and fixed in 4% paraformaldehyde overnight, and then cut into two equal segments. One segment was embedded in paraffin and then cut into serial 5-μm cross-sections for general histological staining with hematoxylin & eosin (H&E), picrosirius red, and specific immunohistochemical staining. The other segment was used for cryosections which were cut into 6-μm thick sections and stained with oil red O. Additional portions were snap frozen in liquid nitrogen and stored at −80°C for molecular biological studies.

Immunohistochemical staining involved standard techniques. In brief, after deparaffinization and hydration, sections were incubated with 3% H₂O₂ in distilled water for 10 min to quench the endogenous peroxidase activity. After incubation in a goat serum blocking solution for 30 min, they were incubated with antibodies to α-smooth muscle cell actin (Sigma Chemical, USA), RAM-11 (Dako Corp, USA), Toll-like receptor 2 (TLR2) and TLR4 (Gene Tex Corp, USA), MCP-1, intercellular adhesion molecule 1 (ICAM-1), CD36, scavenger receptor A (SRA) and oxidized low-density lipoprotein (ox-LDL) receptor 1 (LOX-1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After a PBS wash, the sections were incubated with the biotinylated secondary antibody for 30 min and then counterstained with hematoxylin.

Histopathological staining slides were scanned by microscope (Olympus BX51) and were analyzed by use of a computer-assisted morphometric analysis system (Image-Pro Plus 5.0, Media Cybernetics). Five cross-sections from each aortic section were used for analysis of the fibrous cap thickness and IMT and the values averaged. Lipid, collagen, and immunostaining positive areas were quantitated and the percentage of positive-stained area relative to the plaque area in each cross-section was calculated. The vulnerability index was calculated as (macrophage staining% + lipid staining%) / (vascular smooth muscle cells (VSMCs)% + collagen fiber%) (Shiomi et al. 2001). Disrupted plaques were defined as those plaques with an overlying premortem thrombus (Johnstone et al. 2001).

Quantitative Real-Time RT-PCR (Q-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) from aorta samples. Total RNA was quantified by spectrophotometry and reverse-transcribed (Revert Aid M-MuLV Reverse Transcriptase) with oligo-dT primers. Q-PCR was performed with the use of Applied Lightcycler 2.0 detection system (Roche Applied Science, Germany) and TaqMan probes following the manufacturer’s instructions. The primer sequences (Shanghai Biological Engineering, China); TLR-2: 5′-TTTTCCAGCAGCAGGCCA (sense), 5′-TTAGCACCAAGTGAACAGAAACA (antisense); TLR-4: 5′-CCA CAGAGCCGGGAAGGTATT (sense), 5′-GCCAGGTCT GACCAATCTCATAT (antisense); ICAM-1: 5′-AG TC CC TC GTCCATCGTG (sense), 5′-GAAAGGGCTGTTAGGTCC (antisense); MCP-1: 5′-CAGCCAGATGCGGTGAA(sense), 5′-TTGGTGTTGAAATAAGAGGT (antisense); GAPDH: 5′-GAAACGG GAAA CTCAGTCGCA (sense), 5′-CCTCCTGT AGT TC AT ACTTAGC (antisense). The threshold cycles (Ct) were used to quantify the mRNA expression of target genes in plaque. The transcript amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was quantitated as an internal control. Relative mRNA was calculated as 2ΔΔCt, where ACT is the difference in threshold cycles for the target genes and GAPDH.

Statistical Analysis

Statistical analyses were performed using SPSS, v13.0 (SPSS Inc., Chicago, IL, USA). All data are expressed as mean ± s.d. The ANOVA test, followed by LSD post hoc test was used for comparison of continuous variables among multiple groups and paired t test for within animal comparisons at different time points. Comparison of the incidence of plaque rupture was made by Fisher’s exact test. A P < 0.05 was considered statistically significant.

Results

Rabbit parameters

Three rabbits (one in each of the three groups) died of excessive anesthesia, urine retention and cerebral or myocardial infarction during the experiment and the remaining rabbits completed the entire study. The weight of rabbits was 2.01 ± 0.15 kg at baseline but increased to 3.45 ± 0.31 kg at week 12 (P < 0.01). Body weight did not change significantly from the end of week 12 to 24. There was no significant difference in body weight among the three groups at baseline, week 12 or week 24.

Effect of probucol on serum lipid profile and inflammatory markers

The atherogenic diet caused a significant increase in plasma cholesterol levels by week 12. At baseline and at the end of week 12, plasma cholesterol levels among the three groups of rabbits did not differ significantly. As shown in Table 1, compared with control group, plasma TC

<table>
<thead>
<tr>
<th>Groups</th>
<th>TC (mmol·L⁻¹)</th>
<th>TG (mmol·L⁻¹)</th>
<th>HDL-C (mmol·L⁻¹)</th>
<th>LDL-C (mmol·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probucol</td>
<td>28.68 ± 6.76**</td>
<td>3.15 ± 0.53</td>
<td>1.31 ± 0.54***</td>
<td>25.25 ± 6.88**</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>22.69 ± 5.65**</td>
<td>3.01 ± 0.64</td>
<td>1.99 ± 0.45</td>
<td>18.71 ± 5.37**</td>
</tr>
<tr>
<td>Control</td>
<td>35.49 ± 8.66</td>
<td>3.41 ± 0.78</td>
<td>1.86 ± 0.39</td>
<td>30.97 ± 4.20</td>
</tr>
</tbody>
</table>

Data are means ± s.d. for 14 animals of each group. *P < 0.05, **P < 0.01 versus the control group. *P < 0.05, **P < 0.01, versus the simvastatin group. TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein.
concentration fell 36% in response to simvastatin treatment. In contrast, probucol lowered cholesterol only by 19%. Besides, simvastatin was extremely effective in lowering LDL levels, producing an average decrease of 40% in these hypercholesterolemic rabbits. However, probucol was not nearly as effective, producing an average decrease of only 18%. Simvastatin therapy produced a small increase in HDL cholesterol levels that averaged 7%. In striking contrast, probucol therapy caused a 30% decrease in HDL cholesterol levels. Triglyceride levels showed no significant changes throughout the course of the study. The serum levels of hs-CRP, IL-6, IL-18, MMP-9 and TNF-α were significantly lower in the simvastatin- or probucol-treated group than in the control group, with no significant difference between the two drug-treated groups (Table 2).

### Table 2. Serum inflammatory markers as measured by enzymelinked immunosorbent assay in rabbits of the three groups after 12-week intervention.

<table>
<thead>
<tr>
<th>Groups</th>
<th>hs-CRP (μg·ml⁻¹)</th>
<th>IL-6 (pg·ml⁻¹)</th>
<th>TNF-α (ng·ml⁻¹)</th>
<th>MMP-9 (ng·ml⁻¹)</th>
<th>IL-18 (pg·ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probucol</td>
<td>2.1 ± 0.3**</td>
<td>144.7 ± 17.9**</td>
<td>2.1 ± 0.3**</td>
<td>35.5 ± 6.0**</td>
<td>43.1 ± 7.1**</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>2.0 ± 0.3**</td>
<td>132.9 ± 27.9**</td>
<td>2.3 ± 0.4**</td>
<td>40.1 ± 7.1**</td>
<td>37.9 ± 6.2**</td>
</tr>
<tr>
<td>Control</td>
<td>3.2 ± 0.4</td>
<td>213.4 ± 23.7</td>
<td>3.4 ± 0.5</td>
<td>98.9 ± 19.4</td>
<td>104.1 ± 23.4</td>
</tr>
</tbody>
</table>

Data are means ± s.d. for 14 animals of each group. **p < 0.01 versus the control group. hs-CRP, high sensitive C-reactive protein; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IL-18, interleukin-18; MMP-9, matrix metalloproteinases-9.

### Table 3. Abdominal aorta ultrasonographic measurements in rabbits of the three groups after 12-week intervention.

<table>
<thead>
<tr>
<th>Groups</th>
<th>IMT (mm)</th>
<th>Dd (mm)</th>
<th>Vp (cm·s⁻¹)</th>
<th>Vm (cm·s⁻¹)</th>
<th>VTI (cm·s⁻¹)</th>
<th>AIIc%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probucol</td>
<td>0.85 ± 0.20**</td>
<td>3.14 ± 0.46</td>
<td>96.1 ± 21.7</td>
<td>75.4 ± 18.1</td>
<td>9.46 ± 1.99</td>
<td>85.5 ± 8.7**</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>0.76 ± 0.22**</td>
<td>3.28 ± 0.36</td>
<td>90.3 ± 19.2</td>
<td>71.3 ± 21.0</td>
<td>8.89 ± 2.13</td>
<td>81.4 ± 9.2**</td>
</tr>
<tr>
<td>Control</td>
<td>1.26 ± 0.30</td>
<td>3.41 ± 0.33</td>
<td>84.5 ± 24.3</td>
<td>65.2 ± 16.6</td>
<td>8.42 ± 1.55</td>
<td>68.2 ± 11.7</td>
</tr>
</tbody>
</table>

Data are means ± s.d. for 14 animals of each group. **p < 0.01 versus the control group. IMT, intima-media thickness; Dd, end-diastolic diameters; Vp, peak velocity; Vm, mean velocity; VTI, velocity-time integral; AIIc%, corrected averaged ultrasonic intensity (AII).

![Fig. 1. Intravascular Ultrasound Imaging.](image)

Yellow dotted lines show the lumen intimal borders; Red dotted lines indicate the external elastic membrane (EEM). The external elastic membrane area (EEMA), plaque area (PA) and plaque burden (PB% = PA/EEMA × 100%) in the abdominal aorta of probucol group (A) and simvastatin group (B) were remarkably lower than those in the control group (C).

**High-frequency ultrasonography**

As disclosed in Table 3, the two drug-treated groups exhibited remarkably lower IMT (both P < 0.01) but higher AIIc% than the control group (both P < 0.01), with no significant difference in IMT or AIIc% between the two drug-treated groups. However, these groups did not differ in Dd, Vp, Vm or VTI.

**IVUS measurements**

Quantitative IVUS analysis indicated that the values of EEMA, PA and PB% in the abdominal aorta of both drug-treated groups were remarkably lower than those in the control group, with no difference between the drug-treated groups in these parameters. The three groups did not differ in LA. (Fig. 1, Table 4).
Effect of probucol on plaque morphology and composition

When compared with those of the control group, fibrous cap thickness of the abdominal aortic plaque was significantly increased in probucol- or simvastatin-treated group (275 ± 46 µm or 259 ± 53 µm, respectively, vs. 139 ± 30 µm, both \( P < 0.01 \)), whereas the abdominal aortic IMT was decreased (601 ± 139 µm or 573 ± 150 µm, respectively, vs. 932 ± 168 µm, both \( P < 0.01 \)). After pharmacological triggering, plaque disruption and thrombosis occurred in 10 rabbits involving a total of 12 lesions in the control group. There were 1 rabbit involving 1 lesion in the probucol group and 2 rabbits involving 2 lesions in the simvastatin group developing disruptions and thrombi. The rabbit with more than one disrupted plaques was considered as one when calculated the plaque disruption rate. The plaque disruption rate of probucol group or simvastatin group was considerably lower than that in the control group (1/14, 7.15% or 2/14, 14.29% vs. 10/14, 71.43%, both \( P < 0.01 \)), with no significant difference between the two drug-treated groups. An example of these disrupted lesions is shown in Fig. 2.

The plaque composition, including lipids, collagen, macrophages and VSMCs, was demonstrated by oil red O staining, sirius red staining, RAM-11 and \( \alpha \)-SM actin immunohistochemical staining, respectively (Fig. 3). The relative content of macrophages in plaques was lower in probucol group or simvastatin group than that in the control group (13.7 ± 3.8% or 11.1 ± 3.1% vs. 32.2 ± 6.2% respectively, both \( P < 0.01 \)). Similarly, lipid deposition was less pronounced in the probucol- or simvastatin-treated group compared with the control group (13.9 ± 4.5% or 20.3 ± 3.2% vs. 35.6 ± 6.2% respectively, both \( P < 0.01 \)). In contrast, rabbits in probucol- or simvastatin-treated group had significantly higher VSMC content (29.9 ± 5.8% or 28.4 ± 4.5% vs. 18.1 ± 6.2% respectively, both \( P < 0.01 \)) and higher levels of interstitial collagen (24.8 ± 4.5% or 20.3 ± 3.2% vs. 15.5 ± 4.1% respectively, both \( P < 0.01 \)) than controls. As a result, the vulnerability index, a parameter calculated to determine the destabilization of vulnerable plaques, was significantly lower in probucol- or simvastatin-treated group than that in the control group (0.51 ± 0.16 or 0.55 ± 0.15 vs. 2.12 ± 0.57 respectively, both \( P < \))
Effect of probucol on the atherosclerotic inflammatory mediators and scavenger receptors

The percentage of positive stained cells for TLR2, TLR4, MCP-1, ICAM-1, SRA, CD36 and LOX-1 of the abdominal aortic sections was noticeably lower in probucol- or simvastatin-treated group than that in the control group (all \( P < 0.01 \)), but no difference was observed between the two drug-treated groups. (Fig. 4 and Fig. 5)

The expression of mRNAs for TLR2, TLR4, MCP-1 and ICAM-1 in the abdominal aortic plaques was remarkably lower in probucol- or simvastatin-treated group than that in the control group (all \( P < 0.01 \)), but did not differ between the two drug-treated groups. (Fig. 6)

Discussion

The results of the present study suggest that oral administration of probucol reduced atherosclerotic plaque burden, attenuated plaque inflammation and vulnerability in rabbits. These molecular and cellular effects translated into the protection of plaque from disruption. To the best of our knowledge, this is the first study to show that oral treatment of probucol had effects comparable to those of simvastatin in attenuating local and systemic inflammation and promoting stability of atherosclerotic plaques, although probucol was less effective on lowering serum lipid levels.

Probucol is a potent anti-oxidative drug and has a long history of clinical application for the treatment of xanthomas in familial hypercholesterolemia (FH) patients (Yamamoto et al. 1983). In 1987, Kita et al. and Carew et al. found that oral administration of probucol significantly
Fig. 4. Effect of probucol on inflammatory mediators and scavenger receptors expression in abdominal atherosclerotic lesions of the three groups after 12 week intervention.

Immunohistochemical staining displaying less positive TLR2, TLR4, MCP-1, ICAM-1, SRA, CD36 and LOX-1 staining in probucol group (A,D,G,J,M,P,S) and simvastatin group (B,E,H,K,N,Q,T) than the control group (C,F,I,L,O,R,U) (arrowheads, bar = 50 μm), TLR2, toll-like receptor-2; TLR4, toll-like receptor-4; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule 1; SRA, scavenger receptor A; LOX-1, oxidized low-density lipoprotein (ox-LDL) receptor 1.
Fig. 5. Relative protein expression by immunohistochemical staining.
Relative expression of TLR2, TLR4, MCP-1, ICAM-1, SRA, CD36 and LOX-1 proteins in immunohistochemical staining was presented as the percentage of positive stained areas within the whole intima regions by computer analysis. Pro, probucol group; Sim, simvastatin group; Con, control group. **P < 0.01 significantly different from the control group; abbreviations are as described in the legend to Fig. 4.

Fig. 6. Expression of TLR2, TLR4, MCP-1 and ICAM-1 mRNAs.
TLR2, TLR4, MCP-1, ICAM-1 mRNA expression were analyzed by quantitative real-time PCR. The n-fold differential mRNA expression of target genes in experimental sample related to the control counterpart was calculated as 2^{ΔΔCt}.
**P < 0.01 significantly different from the control group; abbreviations are as described in the legend to Fig. 4.
reduced the extent of fatty-streak lesions in Watanabe heritable hyperlipidaemic (WHHL) rabbits. From then on, probucol continued to reveal its anti-atherogenic properties and unique mechanisms of action in various basic researches (Sasahara et al. 1994; Bräsen et al. 2002; Braun et al. 2003). Recently, Yamashita et al. (2008) reported that long-term probucol treatment may prevent secondary cardiovascular attack in a higher cardiovascular risk population of heterozygous FH in Japan without severe adverse effect. Earlier studies attributed the anti-atherosclerotic effects of probucol to its antioxidative activities (Kita et al. 1987; Carew et al. 1987; Schneider et al. 1993; Sasahara et al. 1994). Subsequent studies associated these protective effects with functional reendothelialization (Lau et al. 2003) and inhibition of VSMCs proliferation (Sekiya et al. 1998). Most recently, Wu et al. (2006) demonstrated that probucol prevented atherogenesis by induction of the anti-inflammatory enzyme heme oxygenase-1 independent of lipid oxidation or cholesterol reduction. In spite of its success in inhibiting early lesion development, data on modeling the stability of already established lesions and the eventual plaque rupture are lacking.

The risk of plaque disruption is associated with both intrinsic plaque vulnerability and extrinsic hemodynamic triggers. In the present study, balloon induced endothelial injury and the high-cholesterol diet resulted in large atherosclerotic plaques in the abdominal aorta. The tumour suppressor gene p53 codes for a transcription factor that activates genes involved in cell growth arrest (p21, GADD45) and apoptosis (e.g. Bax, Fas, IGF-bp3). Local transfection with recombinant p53 adenovirus induced apoptosis of VSMCs, thinning of fibrous cap and enhancement of inflammation. Chinese Russell’s viper venom (CRVV) contains proteases that activate factors V and XI. Such activation leads to thrombosis, which is most likely to occur at sites of cell injury. In addition to this procoagulant effect, CRVV is a direct endothelial toxin. However, it has less effect on normal arteries. Histamine is an arterial vasoconstrictor in rabbits and can regulate the release of norepinephrine, which raises the arterial pressure and stress on the plaques (Chen et al. 2004, 2007; Zhong et al. 2008). In this study, the atherosclerotic lesions formed in rabbits possess features resemble those found in human coronary arteries, including inflamed, thin fibrous caps, less collagen and VSMCs and abundant activated macrophages. Moreover, plaques developed disruption in the presence of local stress induced by pharmacological triggering, which resembles hemodynamic triggers commonly seen in patients with ACS. Disrupted plaques were defined as those with supervened platelet and fibrin-rich thrombi after pharmacological triggering. Our results showed that a high rate of plaque disruption (71.43%) was induced in the control group, which was similar to a previous report from our laboratory (Chen et al. 2007; Zhong et al. 2008; Zhang et al. 2008). Certain features of the ruptured plaques seen in this model are similar to those of lesions seen at autopsy of patients with fatal myocardial infarction, ie, a lesion with a fissured fibrous cap overlying a lipid mass. The eroded plaques were identified when the thrombus attached on a fibrous cap rich in VSMCs, with the absence of endothelial cells underneath the thrombus, which are commonly seen in sudden coronary death (Virmani et al. 2000). Using this model, we found that oral administration of probucol prevented vulnerable plaque formation and led the lesions to develop with more stable phenotype as shown by the following evidence: first of all, compared with the control group (71.43%), only 7.15% rabbits in probucol group developed plaque disruption after pharmacological triggering; second, ultrasonographic measurements of IMT, EEMA, PA and PB% were prominently decreased, whereas AIIe% was increased by probucol treatment. Pathologically, the thickness of fibrous cap was increased and the vulnerability index was remarkably lowered; third, serum levels of hs-CRP, IL-6, IL-18, MMP-9, and TNF-α were notably reduced; lastly, the expression levels of TLR2, TLR4, ICAM-1, MCP-1, CD36, SRA and LOX-1 were significantly reduced in plaques.

Atherosclerosis is now recognized as a chronic and progressive inflammation of the arterial wall. It has been demonstrated that a large proportion of patients with ACS may suffer plaque disruption in coronary arteries and stable plaques may become vulnerable to disruption once they develop active inflammation. In the present study, probucol treatment decreased serum concentrations of hs-CRP, IL-6, IL-18, MMP-9, and TNF-α. It also reduced both the protein and mRNA levels of ICAM-1 and MCP-1 in plaques. All of these inflammatory factors were previously shown to be involved in inflammatory cascade, and favor the extracellular matrix degradation and the vulnerability of atherosclerotic plaques. Therefore, the downregulation of local and systemic inflammation probably is critical to the probucol-promoted plaque stability. Our data also demonstrated that treatment with probucol inhibited the expression of TLR2 and TLR4 in atherosclerotic plaques. Recent studies have identified that TLRs play a key role in innate immune signaling and initiate inflammatory responses via the nuclear factor-κB (NF-κB) pathway (Muzio et al. 1998; Faure et al. 2000). TLR2 and TLR4 are the best studied of the TLRs and have been implicated in the pathogenesis of atherosclerosis and plaque destabilization. Studies using loss-of-function approaches have demonstrated convincingly that a deficiency in TLR4 or TLR2 attenuated the development of atherosclerosis and was associated with alterations in plaque composition that indicate greater structural stability in hyperlipidemic apoE−/− mice (Michelsen et al. 2004; Liu et al. 2008). Overexpression of both TLR2 and TLR4 in the intima of carotid arteries of hyperlipidemic rabbits dramatically augmented atherosclerosis (Shinohara et al. 2007). Recently, Methe et al. (2005) established that high-dose simvastatin influenced TLR4 expression and signaling in monocytes via inhibition of protein geranylgeranlylation and farnesylation. Thus, our data demonstrating that probu-
HDL by probucol has once limited its wide use. Recent evidence (Miida et al. 2008) suggests, however, that the reduction of HDL by probucol may be due to the increased preβ1-HDL ("lipid-poor" apoA-1) participating in cellular lipid efflux, which results in a favorable metabolic sequence of cholesterol metabolism. A head-to-head comparison between simvastatin and probucol in this study revealed that probucol was less effective in lowering LDL-C compared with simvastatin. However, probucol and simvastatin have comparable anti-inflammatory and plaque stabilizing effects, as demonstrated by the similar systemic and intraplaque inflammatory marker levels, vulnerability index and incidence of plaque rupture between the two groups. These findings suggest that the specific effect on HDL metabolism by probucol might also partly contribute to its beneficial effects. Our data is an important addition to the growing literature describing the pleiotropic effects of statins and probucol, highlighting evidence that they influence TLRs and scavenger receptors expression.

In conclusion, our data established beneficial effects of probucol on the stability of atherosclerotic plaque in rabbits, which are similar to those of simvastatin. Lipid lowering, anti-inflammation and scavenger receptors suppression are the possible mechanisms underlying the beneficial effects of simvastatin and probucol. Our findings highlight the potential of probucol as a therapeutic drug for ACS, and the need for further investigation of its effect and underlying mechanism in preventing the occurrence of acute coronary events.

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Conflict of Interest
Authors have no conflict of interest to declare.

References

Consistent with previous studies, in the present study, probucol treatment reduced circulating LDL, total cholesterol and HDL levels. The prominent reduction in serum HDL by probucol has once limited its wide use. Recent results in serum HDL by probucol have once limited its wide use. Recent experiments are being performed in our laboratory to study the exact effect and the molecular mechanisms of probucol on TLR expression.

Increasing evidence indicates that the risk of atherosclerotic plaque disruption depends more on plaque composition than on plaque size and stenosis severity. Plaques susceptible to disruption are characterized by a high amount of inflammatory cells and lipids, as well as a substantial loss in VSMCs and collagen content. In the current animal model, lesions from probucol-treated animals were less mature, significantly smaller, and were predominantly VSMCs and collagen rich as compared with control lesions. To evaluate plaque vulnerability we calculated the vulnerability index, a well-accepted pathological index of the probability of plaque disruption, by dividing the area occupied with macrophages plus lipids by the area occupied with VSMCs and collagen in a given plaque (Shiomi et al. 2001). We found the vulnerability index obviously lower in probucol-treated plaques than in control plaques, indicating that probucol may stabilize atherosclerotic plaques by modulating the plaque composition. The reason why probucol treatment induces selective deletion of macrophages in plaques is not completely understood, but may be related to the decreased MCP-1 and ICAM-1 expression, as demonstrated in the present study. Moreover, the uptake of oxidized lipoproteins via scavenger receptors and the formation of foam cells play a central role in atherogenesis. As a potent anti-oxidant, probucol is known to inhibit the oxidation of LDL (Parthasarathy et al. 1986) and inhibit lipid storage in macrophages by suppressing the uptake and stimulating the release of cholesterol and other lipids into or from the macrophages (Yamamoto et al. 1986, 1988). In this study, we found that probucol treatment was associated with a reduction in lipid deposition and a decrease in the expression of principal scavenger receptors SRA, CD36, and LOX-1 in atherosclerotic plaques. Our data indicate the possibility that probucol inhibits the uptake of lipids into the macrophages and foam cell formation via reduced scavenger receptors expression. This effect may contribute to the stabilization of advanced atherosclerotic lesions, because necrotic foam cells most probably contribute to lipid core formation and enlargement of the lipid core results in plaque disruption and thrombosis formation (Ball et al. 1995). The exact effect of probucol on scavenger receptors expression and the underlying mechanisms remain to be further elucidated.

Consistent with previous studies, in the present study, probucol treatment reduced circulating LDL, total cholesterol and HDL levels. The prominent reduction in serum HDL by probucol has once limited its wide use. Recent


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