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Emodin Promotes Atherosclerotic Plaque Stability in Fat-Fed Apolipoprotein E-Deficient Mice

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Increasing evidence indicated that plaque stabilization is attributed to the composition of the atherosclerotic plaque, and inflammation plays an important role in the formation and progress of vulnerable atherosclerotic plaque (VAP), which is prone to rupture. Emodin, an important component of traditional Chinese herb rhubarb, has obvious anti-inflammatory effect, although its effect on atherosclerotic plaque stabilization is unknown. Apolipoprotein E (ApoE) is an important component of plasma lipoprotein with anti-atherosclerosis function, and the plaque in the aorta of *ApoE*-deficient mice has been demonstrated with characteristics of VAP. Therefore, this study was designed to determine whether emodin can stabilize the VAP in the *ApoE*-deficient mice and explain the possible mechanism. After fat-fed for 13 weeks, mice were randomized into three groups (11 animals/group) and intragastrically administrated with emodin, simvastatin or distilled water for 13 weeks, respectively. The plaque stability was evaluated by the morphology and composition of atherosclerotic plaques. Additionally, the expression of peroxisomal proliferator-activated receptor-γ (PPAR-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), and matrix metalloproteinase 9 (MMP-9) in plaques was determined by the immunohistochemistry method. We showed that emodin could decrease the lipid core area and the ratio of lipid to collagen content in plaques. In addition, emodin significantly inhibited the expression of GM-CSF and MMP-9, whereas it induced the expression of PPAR-γ in plaques. In conclusion, these results suggest that emodin can stabilize the VAP in the aortic root of *ApoE*-knockout mice, which is probably due to its anti-inflammatory effect. ———— emodin; vulnerable atherosclerotic plaque; inflammatory reaction.
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In order to reduce the acute coronary events, new measures have been taken to stabilize the atherosclerotic plaque rather than to enlarge the blood lumen (Peter 2002). Increasing evidence indicated that plaque stabilization is attributed to the composition of the atherosclerotic plaque (John and Eulogio 2002). The unstable atherosclerotic plaques which are prone to rupture and trigger occlusive thrombus formation have been defined as vulnerable atherosclerotic plaque (VAP)

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(Naghavi et al. 2003). The VAP is generally composed of an atrophic fibrous cap, a lipid-rich necrotic core and accumulated inflammatory cells (Hansson 2001; Schmermund and Erbel 2001).

Apolipoprotein E (ApoE) is an important component of plasma lipoprotein with anti-atherosclerosis function. *ApoE*-deficient mice can rapidly develop atherosclerotic lesions that are very similar to the advanced lesions in humans (Plump et al. 1992; Zhang et al. 1992), and this model has been widely used to evaluate the morphological changes of plaque instability (Florian et al. 2002).

Inflammation plays an important role both in the progress of atherosclerosis and in the formation of VAP (Emiliano et al. 2003; Naghavi et al. 2003). Inflammatory cells can produce different inflammatory mediators and ensue with the disruption of vulnerable atheroma, which triggers the thrombosis and the onset of the acute coronary syndromes (Peter 2002).

Emodin, an important component of rhubarb, a traditional Chinese herb, exerts an obvious anti-inflammatory (Chang et al. 1996; Kuo et al. 2001) and anti-oxidative effects (Huang et al. 1995; Yuan and Gao 1997; Yen et al. 2000), and inhibits the platelet accumulation and improves microcirculation. Recent reports revealed that emodin could prevent the formation and progress of atherosclerosis by inhibiting the proliferation of human vascular smooth muscle cells (Pan et al. 2004) and reducing the plasma concentration of malondialdehyde (MDA) and oxidized low density lipoprotein (OxLDL) (Hei et al. 2006). However, whether emodin can stabilize the VAP is still unclear. Therefore, the effect of emodin on the atherosclerotic plaque stability and its possible mechanism were intensively investigated in this study using *ApoE*-deficient mice.

Materials and Methods

Animals

Male *ApoE*-deficient mice (n = 33, aged 8 weeks, 18 to 20 g) on a C57BL/6J background, were introduced from Jackson Laboratory and bred by the Animal Unit of Peking University Health Science Center. The housing and care of the animals and all the procedures used in these studies were performed in accordance with the guidelines and regulations of the University of Bristol and the United Kingdom Home Office.

Husbandry

ApoE-deficient mice were fed with a high-fat diet containing 21% (wt/wt) fat from lard supplemented with 0.15% (wt/wt) cholesterol (Johnson et al. 2005) (Special Diets Services) for 26 weeks. In addition, 10 C57BL mice were fed a chow diet as normal control. All mice were inspected at least once every 24 hrs.

Drug treatment

After 13 weeks of the high-fat diet, the *ApoE*-deficient mice were randomized (11 mice in each group) and treated by intragastric administration with emodin (901 mg/kg per day), simvastatin (9.01 mg/kg per day), or distilled water (control group) for additional 13 weeks accompanied by high-diet feeding. The medical doses in mice were calculated according to the conversion coefficient (9.01) between human and mice.

Histology

After 13 weeks of drug therapy, the heart from each mouse was removed and embedded in paraffin. Six sections (5 μm) were serially cut every 50 μm from cardiac base's cross section until the ascending aorta appeared. Approximately six serial 5-μm sections per mouse were used for morphometric and immunohistochemical analysis. Collagen and foam cells in plaques were stained with a modified Movat pentachrome stain.

Plaque composition and morphometry

To quantitatively evaluate atherosclerotic lesions, four sections of 5 μm in thickness were selected and quantified according to ref. 15 (Suzuki et al. 1997) (Fig. 1). The average of the measured four sections per sample was recorded. Morphometry was performed with a computerized image-analysis program (Image Pro Plus, Media Cybernetics). Plaque composition mainly consists of foam cells, extracellular lipids, and collagen. The percentage of each area in the whole plaque was also counted. Lipid core mainly consists of cholesterol crystal and cholesterol ester (extracellular lipids).

Determination of plasma lipid concentration

Blood samples were drawn from the left ventricle of all the *ApoE*-deficient mice that had received high-fat diet for 26 weeks. Total cholesterol and triglycerides

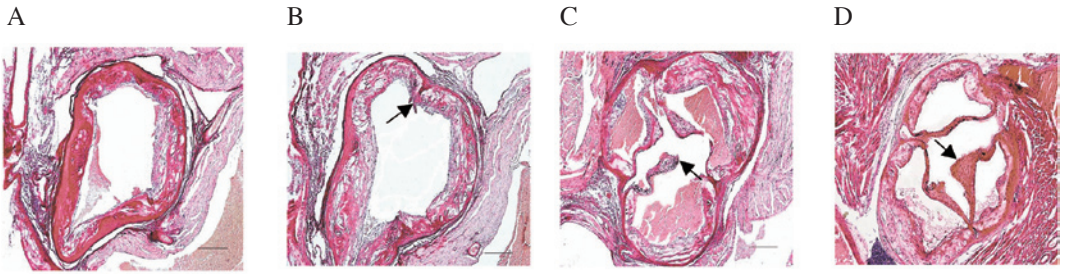

Fig. 1. Evaluation of plaque composition in *ApoE*-deficient mice. The four sections shown were used for quantitative evaluation of atherosclerotic lesions; A: The most proximal part of the ascending aorta having a round cross-section. B: The valve leaflets appearing as small nodules as indicated by black arrowhead. C: The valve attachment sites indicated by black arrowhead. D: The valves appear complete and are joined to their attachment sites as indicated by black arrowhead (H & E stain, Scale bar, 500 μm).

were determined enzymatically in serum. Low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol were determined by immunoturbidimetry. Finally, all the indices were determined by automatic biochemistry radiometer (RX-2000, American Technicon company).

Immunocytochemistry

Serial 5-μm paraffin sections were dewaxed and rehydrated. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide. After blocking sections with 20% (v/v) goat serum in phosphate-buffered saline, sections were incubated overnight at 4°C with peroxisomal proliferator-activated receptor-γ (PPAR-γ) antibody (Santacruze,1 : 200), matrix metalloproteinase-9 (MMP-9) anti-body (Santacruze, 1 : 100), and anti-mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) antibody (Biolegend, 1 : 200). Sections were then incubated with the appropriate secondary antibodies. Positive areas were counted and expressed as a percentage of the whole plaque area. A negative control, where the primary antibody was replaced with either mouse or rat IgG at the same dilution, was always included. Blinded analysis of positive immunostained sections was performed with the image-analysis program (Image Pro Plus, Media Cybernetics).

Evaluation of plaque stability

The effect of emodin on plaque stability was generally evaluated with the percentage of lipid core in plaque and the ratio of lipid to collagen content (Naghavi et al. 2003).

Statistical analysis

All data were expressed as mean ± standard deviation, and all data between groups were analyzed with one-way analysis of variance. In all cases, statistical significance was concluded where $p < 0.05$.

Results

Fat-feeding provokes the formation of vulnerable plaques

Ripe plaques were observed clearly in the aortic roots of *ApoE*-deficient mice fed with high-fat diet for consecutive 13 weeks. After another 13-week high-fat diet feeding, plaques showed distinct morphologic features of vulnerable plaque, such as the large lipid core and the thin fibrous cap. The fibrous cap was usually buried in many foam cells (Fig. 2A). Moreover, many inflammatory cells infiltrated into the extima of coronary artery were observed (Fig. 2B)

Effect of emodin on plaque composition

The content of extracellular lipids in the plaque significantly decreased by 39% ($p = 0.003$ vs control) after treatment with emodin and by 51% ($p = 0.002$ vs control) after treatment with simvastatin, respectively. In addition, treatment with simvastatin, other than emodin, obviously decreased the amount of foam cells in plaque by 40.6% ($p = 0.041$ vs control) and increased the content of collagen fibers by 50% ($p = 0.031$ vs control) in whole aortic plaque. No statistical differences in the plaque composition were observed

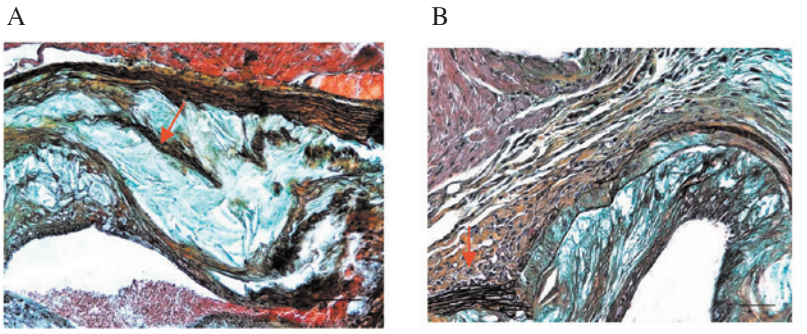

Fig. 2. The morphologic features of plaque in the aorta of *ApoE*-deficient mice in the control group. The plaque and vessel circumstance in the aortic root of the *ApoE*-deficient mice after receiving high-fat diet for 26 weeks. A: Red arrowhead points to buried fibrous cap. B: Many inflammatory cells are infiltrating adventitial coronary vessel as indicated by red arrowhead (Movat stain, Fig. A, Scale bar, 200 μm; Fig. B, Scale bar, 100 μm).

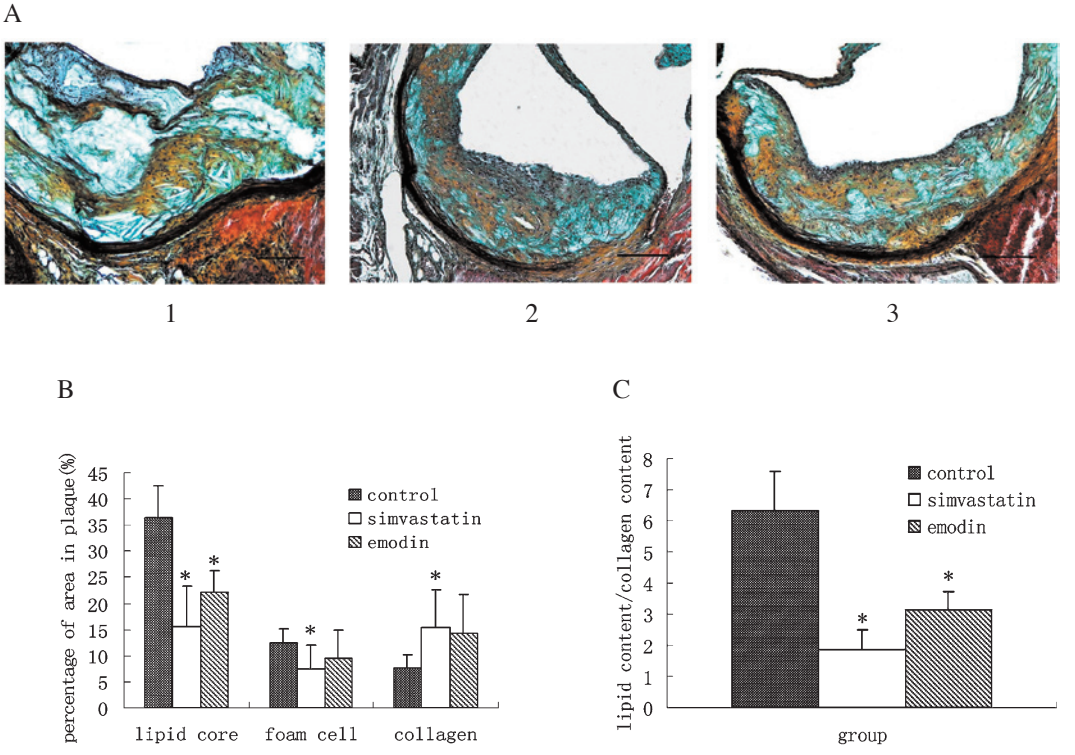

Fig. 3. Effect of emodin treatment on plaque composition in the aortic root of *ApoE*-deficient mice. A: The comparison of plaque composition in the aortic root of *ApoE*-deficient mice among groups after drug-treatment for 13 weeks. The yellow region indicates the collagen composition in the atherosclerotic plaque, and the blank region for extracellular lipid. Control group is shown in Panel 1, simvastatin group is shown in Panel 2, emodin group is shown in Panel 3 (Movat stain, Scale bar, 200 μm). B: Emodin treatment (n = 10) for 13 weeks decreased the percentage of lipids core in plaque by 39% (p = 0.003) but there was no change in foam cells and collagen compared with control mice (n = 10). C: The ratio of lipid to collagen content in plaque on emodin group (n = 10) was decreased by 50.5% (p = 0.022) compared with control group (n = 10).

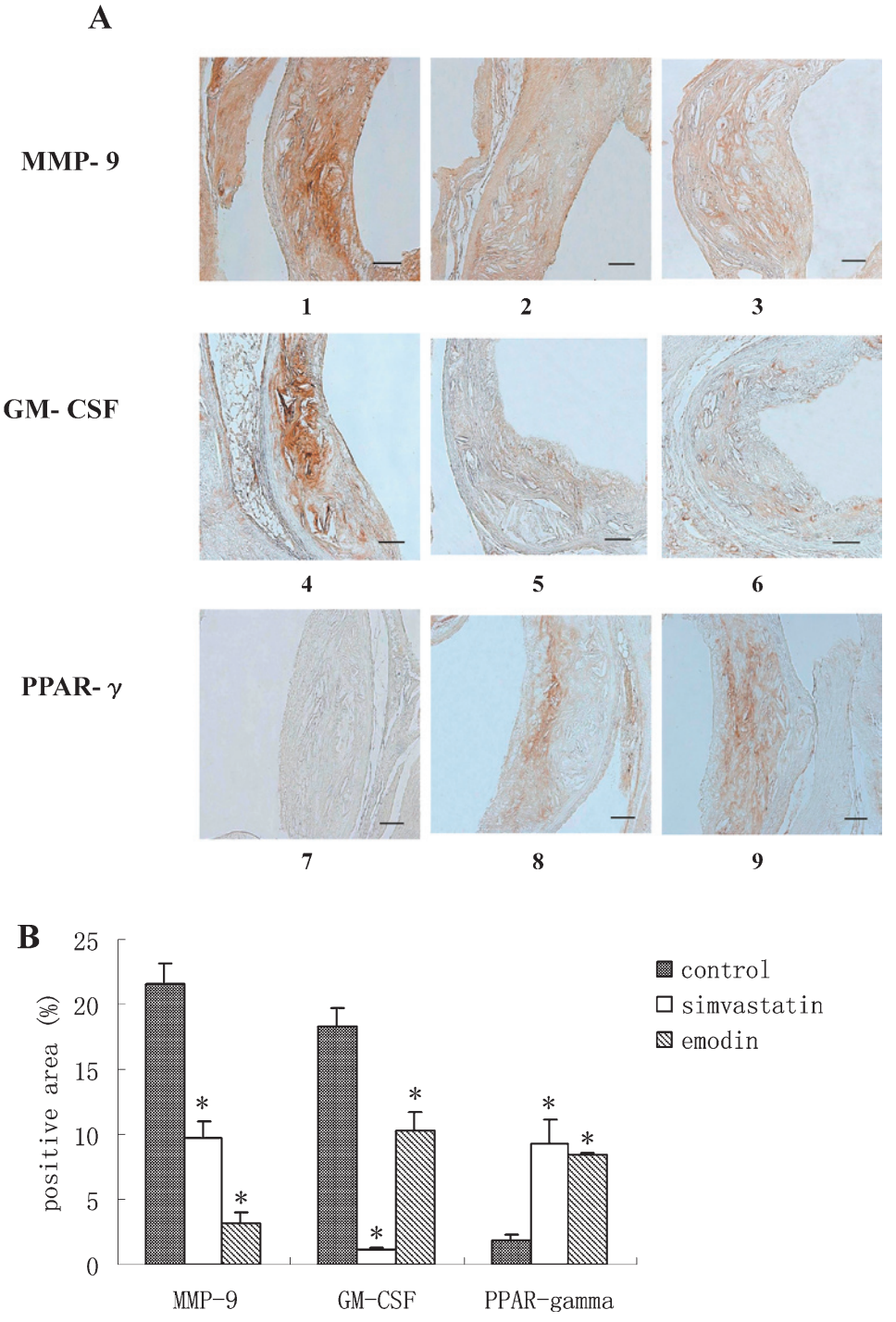

Fig. 4. Effects of emodin treatment on the expression of MMP-9, GM-CSF and PPAR-γ in plaque in the aortic root of *ApoE*-deficient mice. A: The expression (yellow-stained region) of MMP-9, GM-CSF and PPAR-γ among groups after drug-treatment for 13 weeks. Control group is shown in Panels 1, 4, and 7, simvastatin group is shown in Panels 2, 5, and 8, emodin group is shown in Panels 3, 6, and 9 (Immunohistochemistry stain, Scale bar, 200 μm). B: Emodin treatment for 13 weeks increased the expression of PPAR-γ 3.5-fold higher than that of the control in plaque ($p = 0.004$). Emodin decreased the expression of GM-CSF by 36.6% ($p = 0.002$) and MMP-9 by 90.2% in plaque ($p = 0.0003$).

in each of the two drug-treated groups compared with control ($p > 0.05$) (Fig. 3A, B).

Effect of emodin on plaque stability

Treatment with emodin and simvastatin for 13 weeks reduced the percentage of lipid core in the plaque by 39% ($p = 0.003$ vs control) and 56.9% ($p = 0.0002$ vs control), respectively. The ratio of lipid over collagen content in the plaque significantly decreased by 50.5% ($p = 0.022$ vs control) in the emodin treated group and by 70.6% ($p = 0.003$ vs control) in the simvastatin treated group (Fig. 3C).

Effect of emodin on the atherosclerotic inflammatory mediators

The expression of PPAR-γ in the plaque increased by about 3.5-fold upon the treatment with emodin for 13 weeks compared to controls (8.38 ± 0.24 vs 1.88 ± 0.38, $p = 0.004$, $n = 10$). In addition, after the treatment with emodin, the expression of GM-CSF decreased by 36.6% ($p = 0.002$ vs control) and MMP-9 by 90.2% in the plaque ($p = 0.0003$ vs control). As for the treatment of simvastatin, the expression of PPAR-γ in the plaque increased by about 4-fold ($p = 0.002$ vs control), and the expression of GM-CSF decreased by 82% and MMP-9 by 58% ($p = 0.0003$ and $p = 0.0002$ vs control, respectively) (Fig. 4).

Effect of emodin on plasma lipids

Emodin had no effect on total plasma cholesterol, LDL-cholesterol, VLDL-cholesterol as well as the triglyceride contents. However, male mice in the emodin treatment group showed an increase in the serum HDL-cholesterol concentration ($p = 0.0002$ vs control). Atherosclerosis index (AI), the ratio of non-HDL cholesterol to HDL-cholesterol, decreased by 36% after treatment with simvastatin ($p = 0.0003$ vs control), whereas emodin had no effect on AI (Table 1).

Discussion

Emodin ($C_{16}H_{12}O_4$, relative molecular mass 268) belongs to the anthraquinones, a group of > 170 natural compounds that make up the largest group of natural quinine (Harborne et al. 1999). It exists in many medical plants such as rhubarb, radix polygoni multiflori, etc. As an important compound in medicine, emodin has many biological activities such as anti-inflammatory, immunosuppressive and anti-cancer activities (Huang et al. 1991; Yi et al. 2004; Jing et al. 2006). Emodin can inhibit tumor necrosis factor (TNF), induced nuclear factor-kappaB (NF-κB) activation and IkappaB degradation in human vascular endothelial cells (Kumar et al. 1998), induce apoptosis and suppress proliferation in several kinds of cancer cells (Yi et al. 2004; Su et al. 2005), as well as inhibit angiogenesis induced by vascular endothelial growth factor (VEGF)-A (Kwak et al. 2006). However, whether emodin has stabilizing effects on VAP is still unclear. Our study indicated that emodin can stabilize VAP in the aortic root of fat-fed *ApoE*-deficient mice.

It is generally accepted that the stabilization of plaques, as well as the suppression of athero-

Table 1. Effects of emodin treatment on lipid concentrations.

Treatment group ($n = 10$)	Total cholesterol (mmol/l)	LDL-cholesterol (mmol/l)	VLDL-cholesterol (mmol/l)	HDL-cholesterol (mmol/l)	Triglycerides (mmol/l)	(TC-HDL)/HDL (AI)
Normal	2.71 ± 0.35	0.13 ± 0.02	0.61 ± 0.10	2.03 ± 0.31	1.31 ± 0.16	0.34 ± 0.08
Control	25.58 ± 2.28	11.1 ± 2.12	1.33 ± 0.31	3.83 ± 0.42	2.58 ± 0.31	4.53 ± 0.26
Simvastatin	13.72 ± 2.40*	11.08 ± 2.84	0.63 ± 0.13*	3.57 ± 0.29	1.31 ± 0.20*	2.90 ± 0.37*
emodin	24.84 ± 2.91	19.13 ± 2.31	1.27 ± 0.36	4.44 ± 0.25*	2.80 ± 0.20	4.59 ± 0.60

Data are mean ± S.D. Statistical analyses were performed by one-way analysis of variance (ANOVA) with (LSD/Dunn) test. *$p < 0.01$ vs control.

LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; HDL, high-density lipoprotein; (TC-HDL)/HDL, (total cholesterol minus high-density lipoprotein) / high-density lipoprotein.

sclerosis progression, is crucial to prevent the acute coronary syndromes. The advanced lesion is characterized by considerable accumulation of extracellular lipids, lipid-containing foam cells of macrophage and vascular smooth muscle cell origin, and extracellular matrices (Ross 1993). Previous study showed that the lipid components (mainly extracellular lipids and macrophage/foam cells) and collagen components within plaque were responsible for the stability of plaque (Nakamura et al. 2004). The mechanical strength in plaque and the ability to resist the mechanical strength would be decreased if the lipid content, especially cholesterol ester, increased in the plaque. But the collagen content in the plaque, especially in the fibrous cap, can resist the mechanical strength and keep the fibrous cap intact, which promotes the stability of atherosclerotic plaque. Therefore, the ratio of lipid to collagen content is an important index to evaluate the plaque stability (Naghavi et al. 2003). Our study showed that emodin can stabilize the atherosclerotic plaque in the aortic root of *ApoE*-deficient mice by decreasing the percentage of lipid core as well as the ratio of lipid to collagen content in the plaque.

Inflammation plays an important role both in the progress of atherosclerosis and in the formation of vulnerable plaque (Peter 2002; Naghavi et al. 2003). Many inflammatory mediators related to atherosclerosis such as GM-CSF, TNF-α and MMP-9, can promote the degradation of extracellular matrix in the plaque and impair the fibrous cap, and result in plaque rupture. Recently, PPAR-γ has drawn a lot of attention due to its various biological effects including inhibiting the expression of endothelial vascular cell adhesion molecule 1 and MMP, inhibiting the T-cell function and limiting the release of tissue factors in macrophages (Marx et al. 1999, 2001, 2002; Neve et al. 2001). Our study indicated that emodin treatment for 13 weeks decreased the positive expression of GM-CSF and MMP-9 in plaque. Moreover, emodin also increased the expression of PPAR-γ. A recent study of Yang et al. (2007) showed that emodin exhibited a very high binding affinity to PPAR-γ in 3T3-L1 cells (a model to study the differentiation. and physiology of adipocytes), which was consistent with our findings. Taken together, the possible mechanism by which emodin stabilizes the vulnerable plaques is through the downregulation of GM-CSF and MMP-9 in the plaque by activating PPAR-γ.

In addition, previous studies showed that PPAR-γ agonist can promote reverse cholesterol transport by upregulating scavenger receptor BI and inhibit the accumulation of cholesterol ester (Argmann et al. 2003; Malerod et al. 2003). In this study, we found that emodin can stabilize the VAP in *ApoE*-deficient mice by decreasing the lipid core, and significantly increase the expression of PPAR-γ within plaque. From this point, the up-regulation of PPAR-γ probably is critical to the emodin-promoted plaque stability.

MMPs, a series of enzymes that are responsible for the degradation of extracellular matrix, are linked to the inflammatory and fibrinolytic system as well as the lipid and homocysteine levels, and may be responsible for the vulnerability of atherosclerotic plaque (Marc et al. 2005). MMP-9 is one member of MMPs which takes gelatin, collagen (IV and V) and fibronectin as the main substrates (Marc et al. 2005). The major collagens in the atherosclerotic plaque are collagen I and III, which may explain why emodin cannot affect the total content of collagen when it decreases the expression of MMP-9 in the plaque in our study.

Furthermore, treatment of male mice with emodin for 13 weeks increased the serum HDL-cholesterol concentration, which can stimulate cholesterol efflux from macrophages (Borinick et al. 2000) and downregulate some inflammatory cytokine such as TNF-α and interleukin-1 (Codkerill et al. 1995), and thus is favorable of promoting the plaque stability. Therefore, the decrement of extracellular lipid levels within the plaque in the emodin group may be also related to the increase of serum HDL-cholesterol level.

In conclusion, we considered that emodin, a monomer extracted from natural herb rhubarb, has the beneficial effect on the stability of atherosclerotic plaque in aortic root of *ApoE*-deficient mice. The possible mechanism is related to

downregulating the expressions of GM-CSF and MMP-9 in the plaque by activating PPAR-γ. Therefore, it is necessary to further investigate the potential effect and its mechanism in preventing the occurrence of acute coronary events.

Acknowledgments

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