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Effect of Pregnancy Associated Plasma Protein- A Overexpression on Atherosclerotic Plaque Morphology in Mice

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Abstract

Objective: A novel metalloproteinase, Pregnancy Associated Plasma Protein-A (PAPP-A), has been implicated in the development of atherosclerosis, and is under consideration as a novel biomarker for acute coronary syndrome and unstable plaque. The aim of this study was to determine if PAPP-A overexpression directly contributes to plaque vulnerability.

Methods: Apolipoprotein E knock-out (ApoE KO) mice, a model of atherosclerotic development, and ApoE KO mice overexpressing PAPP-A transgene in arterial smooth muscle (ApoE KO/Tg) were fed a high fat diet for 20 or 40 weeks starting at seven weeks of age. At harvest, the brachiocephalic artery (BCA) was fixed, embedded in paraffin, sectioned and stained for morphologic analyses.

Results: After 20 weeks on high fat diet, significantly more ApoE KO/Tg mice had BCA lesions with a necrotic core and fibrous cap than did ApoE KO mice. After 40 weeks on high fat diet all mice had BCA plaques with necrotic cores, but plaque progression with healed ruptures (i.e., buried fibrous caps), and plaque inflammation were greater in BCA of ApoE KO/Tg mice than in ApoE KO mice.

Conclusion: Overexpression of PAPP-A in arterial smooth muscle of ApoE KO mice is associated with accelerated plaque progression and development of vulnerable and ruptured plaque.

Keywords: Pregnancy associated plasma protein-A; Brachiocephalic artery; Plaque stability; Vulnerable plaque; Apolipoprotein E knock-out mice

Introduction

Atherosclerosis, one of the major diseases of industrialized nations, represents a complex response to chronic vascular injury that involves several cell types and associated cytokines, growth factors and enzyme systems [1-3]. Among the spectrum of events, injurious agents promote the infiltration of monocytes from the circulation, and these in turn become lipid-laden macrophages (foam cells) forming fatty streaks in the intima of the vessel. Transition from relatively simple fatty streaks to more advanced lesions is associated with accumulation of smooth muscle cells in the luminal space that proliferate, take up modified lipoproteins and synthesize extracellular matrix. Complex lesions are characterized by a necrotic lipid-rich core covered by a fibrous cap of smooth muscle cells or fibroblasts.

In humans, lipid-laden plaques with thin or uneven fibrous cap are the most prone to rupture ('vulnerable' plaque), especially at the shoulder region of eccentric plaques [4, 5]. Unfortunately, identification of culprit and vulnerable plaques usually comes after the fact, (i.e., at autopsy or coronary atherectomy), since imaging systems generally evaluate luminal narrowing but not plaque composition. Thus, the quest for treatment options to prevent plaque progression would benefit from better understanding of the pathobiology of atherosclerosis and the use of animal models that produce vulnerable plaques "at risk" for rupture [6].

Pregnancy associated plasma protein-A (PAPP-A), a newly recognized metalloproteinase in the Insulin-like Growth Factor (IGF) system, has been implicated in vascular repair processes *in vitro* and *in vivo* [7], and as a circulating biomarker for acute coronary syndrome in humans [8,9]. Furthermore, there is strong PAPP-A immunostaining in human autopsy samples of vulnerable atherosclerotic plaque that is associated with activated macrophages and smooth muscle

cells, especially in the structurally-weakened shoulder region of an eccentric culprit plaque [8].

To determine if PAPP-A contributes directly to atherosclerotic plaque development, we crossed PAPP-A knock-out (KO) mice with apolipoprotein E (ApoE) KO mice, the latter being an established murine model of atherosclerosis [10]. Compared to ApoE KO mice, the ApoE/PAPP-A double KO mice had significantly reduced aortic plaque burden and delayed progression from fatty streaks to complex lesions [11]. Conversely, ApoE KO mice overexpressing PAPP-A in arterial smooth muscle had significantly increased aortic lesion area [12]. However, the effect of PAPP-A overexpression on plaque vulnerability has not been evaluated. Thus, this study was designed to test the hypothesis that targeted overexpression of PAPP-A in arterial smooth muscle of ApoE KO mice accelerates the development of atherosclerotic lesions with morphometric characteristics of vulnerable plaque and plaque rupture.

Materials and Methods

Transgenic PAPP-A overexpression and atherosclerosis. PAPP-A Tg mice (FVB genetic background) were crossed with ApoE KO mice (C57BL/6 and 129 genetic background), as previously described [12]. It is of note that transgene expression is driven by the minimal SM22α promoter that had been modified by deletion of the repressor elements, which are triggered by vessel injury [13]. The highest PAPP-A transgene expresser, Tg6' [12], was used for this study. Offspring from this mating, heterozygous for the ApoE gene and positive for high level PAPP-A transgene expression, were then intercrossed to produce ApoE KO mice and ApoE KO mice expressing the PAPP-A transgene, (ApoE KO/Tg). These littermates, males and females housed separately up to five per cage, were fed a high-fat, Western-style diet [21% by weight (42% of calories) fat and 0.15% by weight cholesterol (Harland Tekland, South Eaton, MA)] for 20 and 40 week starting at 7 weeks of age. This protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Mayo Clinic.

Morphology

At harvest, the Brachiocephalic Artery (BCA) was fixed *in situ* by perfusion with Phosphate Buffered Saline (PBS)-formalin at physiological pressure. Individual arteries were removed, placed in PBS-buffered formalin, and fixed for 24 hours before paraffin embedding. Cross-sections (5.0 μm thick) were collected over the length of the BCA. Each end and middle sections were stained with hematoxylin and eosin. Adjacent sections were stained with Verhoeff-Van Gieson (Accustain; Sigma-Aldrich, St. Louis, MO). Microscopic analysis was performed by an expert cardiovascular pathologist (WDE) [14], blinded to genotype, and according to criteria used in anatomic pathology and modified for mice (Table 1). Fisher's exact test was used for statistical comparisons between ApoE KO and ApoE KO/Tg mice.

Immunohistochemistry. De-paraffinized sections of BCA were stained for macrophages using F4/80 as primary antibody, as described previously [15].

Results

Body weights of ApoE KO and ApoE KO/Tg mice are shown in Table 2. There were no differences between the two strains of mice prior to or 20 and 40 weeks after high fat diet. Summarized data for the histopathology review of BCA plaque morphology are presented in Table 3 for mice 20 weeks on high fat diet and in Table 4 for mice 40 weeks on high fat diet. There was no evidence of luminal thrombus, surface erosion, or plaque hemorrhage in any of the sections, and all plaques were eccentric.

After 20 weeks on high fat diet, significantly ($P = 0.002$) more ApoE KO mice that were overexpressing PAPP-A had a necrotic core in BCA plaques than did ApoE KO mice negative for the transgene (Table 3). There was no significant difference between the two groups of mice in terms of plaque grade and internal elastic membrane (IEM) disruption, and there was no calcification or inflammation in either group. Figure 1 presents

Lumin
Thrombus (0 = no, 1 = yes) Thrombus (0 = not applicable, 1 = platelet/fibrin, 2 = red cell, 3 = both 1 & 2)
Intima
Plaque (0 = absent, 1 = eccentric, 2 = concentric, 3 = both 1 & 2) Plaque grade (1 = 1-25%, 2 = 26-50%, 3 = 51-75%, 4 >75% of luminal obstruction) Surface erosion/fissure (0 = absent, 1 = present) Fibrous cap (1 = thick, 2 = thin, 3 = absent) Foam cells without fibrous cap (0 = absent, 1 = present) Foam cells within the fibrous cap (0 = absent, 1 = present) Foam cells beneath cap (0 = absent, 1 = present) Other plaque cells (0 = absent, 1 = chondrocyte-like) Necrotic core of plaque (0 = absent, 1 = present) Calcification (0 = absent, 1 = present) Plaque inflammation (0 = absent, 1 = chronic, 2 = acute, 3 = both 1 and 2) Plaque hemorrhage (0 = absent, 1 = present) Plaque progression (0 = absent, 1 = present)
Internal Elastic Membrane (IEM)
Fragmentation/disruption (0 = absent, 1 = present)
Media
Thinning under the plaque (0 = absent, 1 = present) Thickening under the plaque (0 = absent, 1 = present) Hypertrophy opposite the plaque (0 = absent, 1 = present)
Adventitia
Inflammation (0 = absent, 1 = chronic, 2 = acute, 3 = both 1 and 2)

Table 1: Spreadsheet key for morphological analysis of brachiocephalic arteries in atherogenic mice.

Table 2: Body weights of mice

	Grams		
	Start	20 Weeks	40 Weeks
Males			
ApoE KO	28.7 + 0.79	48.9 + 3.39	52.1 + 4.67
ApoE KO/Tg	26.1 + 0.81	44.3 + 3.29	50.4 + 4.51
Females			
ApoE KO	22.1 + 0.55	35.9 + 3.69	41.0 + 5.72
ApoE KwO/Tg	21.0 + 0.45	32.5 + 2.05	43.6 + 5.77

Table 2: Body weights of ApoE KO and ApoE KO/PAPP-A Tg mice at 7 weeks-of-age and after 20 weeks and 40 weeks of high fat diet.

Table 3: Brachiocephalic artery plaque morphology after 20 weeks on HFD: effect of PAPP-A overexpression.

	% of Mice	
	ApoE KO/Tg (18)	ApoE KO (21)
Plaque Grade		
1	45%	12%
2	45%	25%
3	--	52%
4	9%	--
Necrotic Core	9%	75%*
IEL Disruption	55%	50%
Chondrocyte-like Cells	18%	--
Calcification	--	--
Plaque Inflammation	--	--
Plaque Progression	--	25%
Adventitial Inflammation	--	--

Table 3: Results from (N) mice are expressed as % of mice with the indicated morphology. *P = 0.002

Table 4: Brachiocephalic artery plaque morphology after 40 weeks on HFD: effect of PAPP-A overexpression.

	% of Mice	
	ApoE KO (14)	ApoE KO/Tg (14)
Plaque Grade		
1	7%	--
2	57%	36%
3	14%	64%
4	14%	--
Necrotic Core	100%	100%
IEL Disruption	57%	79%
Chondrocyte-like Cells	93%	64%
Calcification	43%	43%
Plaque Inflammation	7%	29%‡
Plaque Progression	29%	64%‡
Adventitial Inflammation	29%	29%

Table 4: Results from (N) mice are expressed as % of mice with the indicated morphology. ‡P = 0.07

a grade 2 (of 4) plaque (based on 25% increments of luminal narrowing in cross-sectional area) having a necrotic core with cholesterol clefts. There are foam cells without a fibrous cap as well as within and beneath the fibrous cap. There is also an example of disruption of the IEM with extension of the plaque into the media. Interestingly, chondrocyte-like cells were only seen in the BCA plaque of ApoE KO, and plaque progression was only seen in the BCA of ApoE KO/Tg mice. However, numbers were small.

After 40 weeks on a high fat diet, the BCA plaques of all mice had necrotic cores, and there were no differences between the ApoE KO and ApoE KO/Tg mice in terms of IEM disruption, presence of chondrocyte-like cells (Figure. 2A), or calcification (Figure. 2B). However, plaque progression and inflammation were substantially greater in BCA of the ApoE KO/Tg mice than in ApoE KO mice (Table 4). This difference did not reach statistical significance (P = 0.07) likely due to the small group sizes. Plaque progression indicated healed ruptures (i.e., multiple layers of necrotic cores interspersed by fibrous tissue). Examples of these buried plaques and associated macrophage staining are presented in Figure 3.

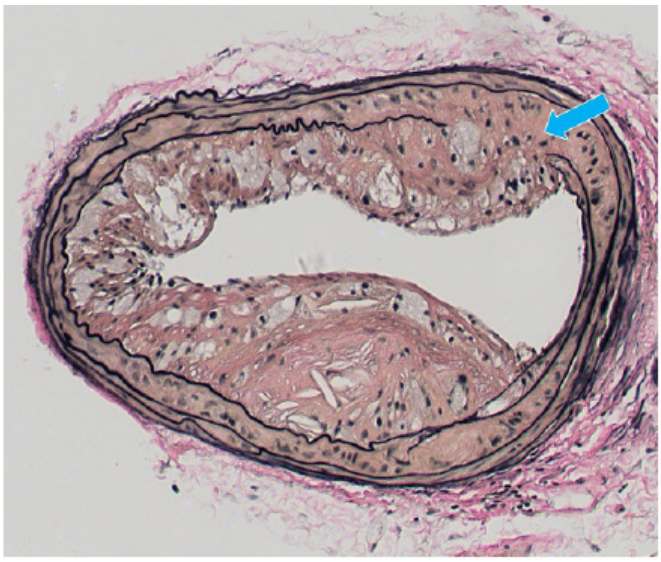

Figure 1: Brachiocephalic artery of ApoE KO/Tg mouse 20 weeks on high fat diet. Verhoeff-Van Gieson stain. Example of Grade 2 plaque with necrotic core containing cholesterol clefts. Arrow indicates IEM disruption.

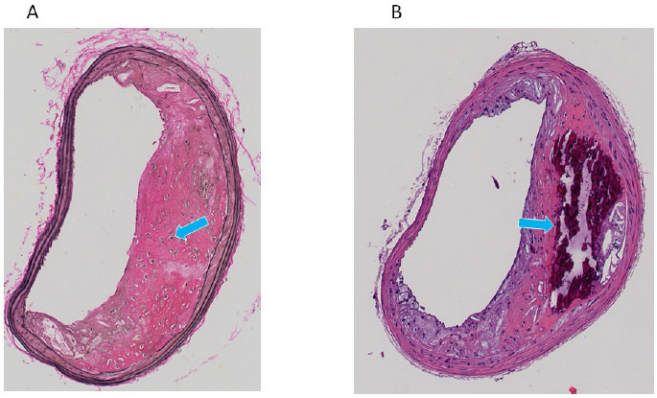

Figure 2: Brachiocephalic arteries of ApoE KO mice 40 weeks on high fat diet. Arrow indicates (A) chondrocyte-like cells or (B) calcification.

Discussion

In this study, we present morphological evidence that overexpression of PAPP-A in arterial smooth muscle of ApoE KO mice is associated with accelerated plaque progression and development of vulnerable and ruptured plaque (Figure. 4).

After 20 weeks on high fat diet, there was a significant increase in the number of ApoE KO/Tg mice bearing BCA plaque with a necrotic core. After 40 weeks on high fat diet, these BCA plaques showed numerous buried fibrous caps, which are indicative of an unstable plaque phenotype and are a surrogate marker of plaque rupture [16-19]. This layered appearance in mouse BCA plaques is similarly observed in human coronary arteries, and it has been suggested that healed plaque ruptures play a role in plaque progression and sudden coronary death in humans [20].

There has been controversy in the literature about whether plaque rupture occurs in mice. Issues arise from the definitions of plaque vulnerability and rupture and sud-

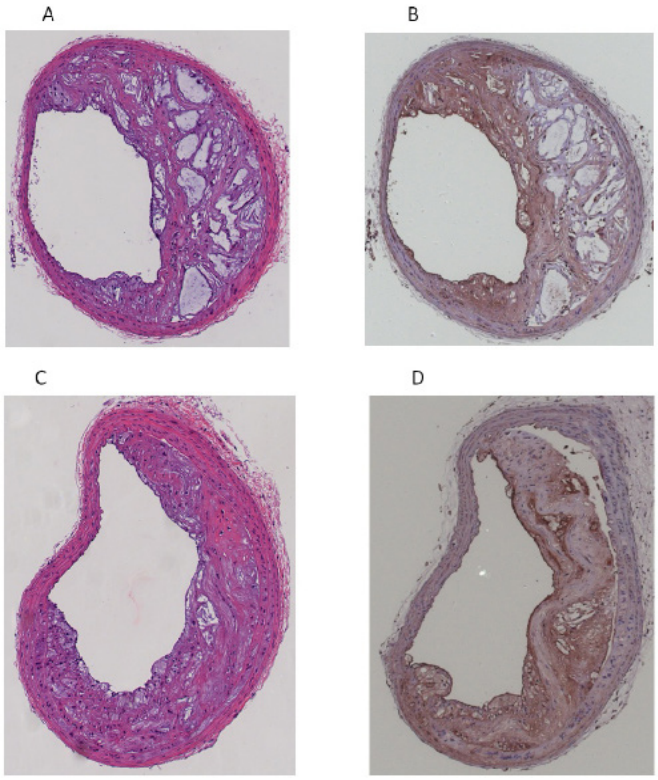

Figure 3: Brachiocephalic arteries of ApoE KO/Tg mice 40 weeks on high fat diet. (A,C) Hematoxylin & Eosin. (B,D) Immunohistochemistry for macrophages (F4/80)

Mouse Model of Vulnerable Plaque

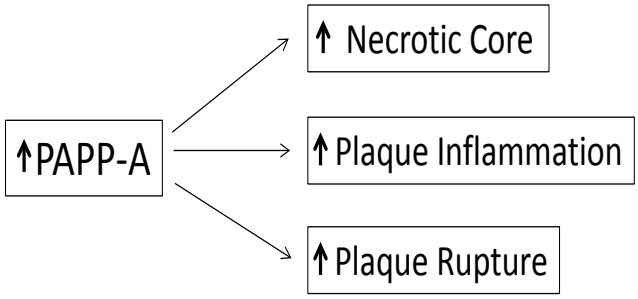

Figure 4: Summary of the effect of PAPP-A overexpression in arterial smooth muscle on atherosclerotic plaque morphology in ApoE KO mice.

den death in mice versus humans [16,21-24]. Also, several of the studies in mice have evaluated plaque stability in the aortic sinus, which is not a typical site affected by atherosclerotic disease in mice or humans. Lesions in the aortic sinus are relatively refractory to destabilization [16]. The major site of predilection for developing vulnerable plaque and plaque rupture in mice is the BCA/innominate artery connecting the aortic arch to the right common carotid and right subclavian arteries [25]. Williams et al. [17] reported a high frequency of plaque instability in the BCA and spontaneous death by myocardial infarction in ApoE KO mice after 40-60 weeks on a high fat diet. Similarly, the BCA is a site for advanced lesions in humans [22].

There were no sudden deaths in ApoE KO/Tg mice in this study, even after 40 weeks on high fat diet. As nicely discussed by Jackson et al. [16], the requirement for luminal thrombus to define plaque rupture in mice needs to be reconsidered, since occlusive thrombi are unlikely to be found in mice given their active fibrinolytic system and rapid thrombolysis. On the other hand, buried fibrous caps are indicators of repeated episodes of non-fatal rupture and repair in humans and mice [6,16-20].

In this study, we also observed an interesting pattern of lipid-laden macrophages above, within, and beneath fibrous caps, especially in BCA plaque of ApoE KO/Tg mice, and an increase in plaque inflammation in ApoE KO/Tg mice. Inflammation is known to play a critical role in the progression of atherosclerosis [1,26]. Indeed, macrophages in shoulder regions of plaque are considered to be vulnerable to rupture. It is of note that pro-inflammatory cytokines associated with atherosclerotic plaque development are potent stimulators of PAPP-A expression in human arterial smooth muscle and endothelial cells [27, 28].

The mechanism(s) by which PAPP-A promotes plaque progression and instability is unclear. Our working hypothesis is that activated macrophages in plaque synthesize pro-inflammatory cytokines that stimulate vascular smooth muscle and endothelial cells to synthesize and secrete PAPP-A [7]. Thus, PAPP-A can function in an autocrine/paracrine fashion as an important amplification point in plaque progression. That a key function of PAPP-A is to increase local IGF available for receptor activation [7] suggests an IGF-dependent mechanism. However, in contrast to what we found with PAPP-A overexpression, smooth muscle-specific IGF-I overexpression in ApoE KO mice was reported to have no effect on plaque burden, macrophage accumulation, oxidative stress, inflammation, or smooth muscle cell apoptosis, but it increased features of plaque stability, i.e., increased smooth muscle cell content and decreased necrotic core size in the aortic valve [29]. It remains to be determined whether these different morphologies reflect IGF-independent effects of PAPP-A or are due to analyses at different sites.

In a recent study by Zhao *et al.* [30], serum PAPP-A correlated positively with necrotic core area. Patients with unstable angina and with no-reflow after percutaneous coronary intervention had higher serum PAPP-A, coronary plaques with higher percentage necrotic core area, more thin-cap fibroatheromas, and ruptured plaque as measured by virtual histology intravascular ultrasound. These findings, in conjunction with ours, suggest PAPP-A not only as a marker of vulnerable plaque, but also as a potential therapeutic target to limit plaque progression.

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References

1) Ross R (1999) Atherosclerosis: an inflammatory disease. N Engl J Med 340:115-126.

2) Lusis AJ (2000) Atherosclerosis. Nature 407:233-241.

3) Glass CK, Witztum JL (2001) Atherosclerosis: the road ahead. Cell 104:503-516.

4) Finn AV, Nakano M, Narula J, Kolodgie FD, Virmani R (2010) Concept of vulnerable/unstable plaque. Arterioscler Thromb Vasc Biol 30:1282-1292.

5) Van der Wal AC, Becker AE (1999) Atherosclerotic plaque rupture: pathologic basis of plaque stability and instability. Cardiovasc Res 41: 334-344.

6) Cortez D, Pham S, Jin y-F, Toba H, Lindsay ML, et al.(2013) The elusive plaque: translational biologypPotential. J Cardio Vasc Med 1: 1-3.

7) Conover CA (2012) Key questions and answers about pregnancy-associated plasma protein-A. Trends Endocrinol Metab 23: 242-249.

8) Bayes-Genis A, Conover CA, Overgaard MT, Bailey KR, Christiansen M, et al. (2001) Pregnancy-associated plasma protein A as a marker of acute coronary syndromes. N Engl J Med 345: 1022-1029.

9) Schulz O, Reinicke M, Kramer J, Berghofer G, Bensch R, et al. (2011) Pregnancy-associated plasma protein A values in patients with stable cardiovascular disease: use of a new monoclonal antibody-based assay. Clin Chim Acta 412: 880-886.

10) Meir KS, Leitersdorf E (2004) Atherosclerosis in the apolipoprotein E-deficient mouse: a decade of progress. Arterioscler Thromb Vasc Biol 24: 1006-1014.

11) Harrington SC, Simari RD, Conover CA (2007) Genetic deletion of pregnancy-associated plasma protein-A is associated with resistance to atherosclerotic lesion development in apolipoprotein E-deficient mice challenged with a high-fat diet. Circ Res 100: 1696-1702.

12) Conover CA, Mason MA, Bale LK, Harrington SC, Nyegaard M, et al. (2010) Transgenic overexpression of pregnancy-associated plasma protein-A in murine arterial smooth muscle accelerates atherosclerotic lesion development. Am J Physiol Heart Circ Physiol 299: H284-H291.

13) Wamhoff BR, Hoofnagle MH, Burns A, Sinha S, McDonald OG, et al. (2004) A G/C element mediates repression of the SM22alpha promoter within phenotypically modulated smooth muscle cells in experimental atherosclerosis. Circ Res 95: 981-988.

14) Kullo IJ, Edwards WD, Schwartz RS (1998) Vulnerable plaque: pathobiology and clinical implications. Ann Intern Med 129: 1050-1060.

15) Mader JR, Resch ZT, McLean GR, Mikkelsen JH, Oxvig C, et al. (2013) Mice deficient in PAPP-A show resistance to the development of diabetic nephropathy. J Endocrinol 219: 51-58.

16) Jackson CL, Bennett MR, Biessen AL, Johnson JL, Krams R (2007) Assessment of unstable atherosclerosis in mice. Arterioscler Thromb Vasc Biol 27: 714-720.

17) Williams H, Johnson JL, Carson KGS, Jackson CL (2002) Characteristics of intact and ruptured atherosclerotic plaques in brachiocephalic arteries of apolipoprotein E knockout mice. Arterioscler Thromb Vasc Biol 22: 788-792.

18) Johnson J, Carson K, Williams H, Karanam S, Newby A, et al. (2005) Plaque rupture after short periods of fat feeding in the apolipoprotein E-knockout mouse: model characterization and effects of pravastatin treatment. Circulation 111: 1422-1430.

19) Rodgers KJ, Watkins DJ, Miller AL, Chan PY, Karanam S, et al. (2006) Destabilizing role of cathepsin S in murine atherosclerotic plaques. Arterioscler Thromb Vasc Biol 26: 851-856.

20) Burke AP, Kolodgie FD, Farb A, Weber DK, Malcom GT, et al. (2001) Healed plaque ruptures and sudden coronary death evidence that subclinical rupture has a role in plaque progression. Circulation 103: 934-940.

21) Schwartz SM, Galis ZS, Rosenfeld ME, Falk E (2007) Plaque rupture in humans and mice. Arterioscler Thromb Vasc Biol 27: 705-713.

22) Bennett MR (2002) Breaking the plaque: evidence for plaque rupture in animal models of atherosclerosis. Arterioscler Thromb Vasc Biol 22: 713-714.

23) Johnson JL, Jackson CL (2001) Atherosclerotic plaque rupture in the apolipoprotein E knockout mouse. Atherosclerosis 154: 399-406.

24) Lutgens E, van Suylen R-J, Faber GC, Gijbels MJ, Eurlings PM, et al. (2003) Atherosclerotic plaque rupture: local or systemic process? Arterioscler Thromb Vasc Biol 23: 2123-2130.

25) Rosenfeld ME, Polinski P, Virmani R, Kauser K, Rubanyi G, et al. (2000) Advanced atherosclerotic lesions in the innominate artery of the apoE knockout mouse. Arterioscler Thromb Vasc Biol 20: 2587-2592.

26) Gough PJ, Gomez IG, Wille PT, Raines EW (2006) Macrophage expression of active MMP-9 induces acute plaque disruption in apoE-deficient mice. J Clin Invest 116:59-69.

27) Conover CA, Bale LK, Harrington SC, Resch ZT, Overgaard MT, et al. (2006) Cytokine stimulation of pregnancy-associated plasma protein A expression in human coronary artery smooth muscle cells: inhibition by resveratrol. Am J Physiol Cell Physiol 290: C183-C188.

28) Conover CA, Harrington SC, Bale LK (2008) Differential regulation of pregnancy associated plasma protein-A in human coronary artery endothelial cells and smooth muscle cells. Growth Horm IGF Res 18: 213-220.

29) Shai S-Y, Sukhanov S, Higashi Y, Vaughn C, Kelly J, et al. (2010) Smooth muscle cell-specific insulin-like growth factor-1 overexpression in Apoe-/- mice does not alter atherosclerotic plaque burden but increases features of plaque stability. Arterioscler Thromb Vasc Biol 30: 1916-1924.

30) Zhao X-Y, Wang X-F, Li L, Zhang J-Y, Du Y-Y, (2013) Plaque characteristics and serum pregnancy-associated plasma protein A levels predict the no-reflow phenomenon after percutaneous coronary intervention. J Intl Med Res 41: 30-316.