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

Cheryl A Conover1,*, Laurie K Bale1 and William D Edwards2

1Division of Endocrinology, Metabolism, Diabetes and Nutrition, Endocrine Research Unit, USA
2Division of Anatomic Pathology, Rochester, USA

*Corresponding author: Cheryl A. Conover, Mayo Clinic, Endocrine Research Unit, 200 First Street SW, 5-194 Joseph, Rochester, MN 55905, Tel: 507-255-6415, Fax: 507-255-4828, E-mail: Conover.Cheryl@mayo.edu

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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 expression, 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 Verhoff-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...
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

Discussion

In this study, we present morphological evidence that over-expression of PAPP-A in arterial smooth muscle of ApoE KO mice is associated with accelerated plaque progression and development of vulnerable and ruptured plaque. After 20 weeks on high fat diet, the number of ApoE KO/Tg mice bearing BCA plaque with a necrotic core was significantly increased (P = 0.07). This layer of plaques and associated macrophage staining are presented in Figure 3.

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 plaques showed numerous buried fibrous caps, which are indicative of an unstable plaque phenotype and are a surrogate marker of plaque rupture. This layer of plaques and associated macrophage staining are presented in Figure 3.

Discussion

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

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 plaques showed numerous buried fibrous caps, which are indicative of an unstable plaque phenotype and are a surrogate marker of plaque rupture. This layer of plaques and associated macrophage staining are presented in Figure 3.

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-
Mouse Model of Vulnerable Plaque

- ↑ Necrotic Core
- ↑ PAPP-A
- ↑ Plaque Inflammation
- ↑ Plaque Rupture

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

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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