Impact of age and sex on the development of atherosclerosis and expression of the related genes in apoE deficient mice

Mengyang Liu^{1,¶}, Wenwen Zhang^{1,¶}, Xiaojun Li¹, Jihong Han^{1,3}, Yuanli Chen^{2,3}, Yajun Duan^{1,3}

¹College of Life Sciences and ²School of Medicine, ³State Key Laboratory of Medicinal Chemical Biology and Collaborative Innovation Center of Biotherapy, Nankai University, Tianjin, China

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Correspondence should be addressed to:

Yuanli Chen, PhD, School of Medicine; Yajun Duan, PhD, College of Life Sciences

Nankai University, 94 Weijin Road, Tianjin 300071, China

Tel: +86 22 23501776; Fax: +86 22 23501776

Email: yuanli2014@nankai.edu.cn; yajunduan@nankai.edu.cn

Abstract

Development of atherosclerosis is a chronic pathological process. ApoE deficient (apoE^{-/-}) mice spontaneously develop atherosclerotic lesions. However, the impact of age and sex on lesions and expression of the related genes have not been fully elucidated. In this study, we collected blood and tissue samples from normal chow fed male and female apoE^{-/-} mice at different ages, and determined serum lipids, PCSK9 levels, *en face* aortic lesions and expression of some pro- or anti-atherogenic genes. We determined that lesion development was clearly associated with age, and more lesions in males than females (12.6±1.7% vs. 8.9±1.1% at 8 months old, P<0.05). Associated with age, serum total, LDL- and HDL-cholesterol and PCSK9 levels increased with more PCSK9 in females than males (313±31 ng/mL vs. 239±28 ng/mL at 8 months old, P<0.05); expression of liver LDLR and ABCA1 decreased while of SR-BI increased; expression of macrophage ABCA1 and SR-BI decreased but of CD36 increased. Estrogen and tamoxifen induced ABCA1 and SR-BI expression, respectively, in macrophages isolated from female mice at the different age. Taken together, our study suggests that aging facilitates lesion development in apoE^{-/-} mice with greater effect on male mice. The lesion development is also related to expression of pro- or anti-atherogenic genes in tissues, particularly in macrophages.

Key words: atherosclerosis; age; sex; ABCA1; CD36

Abbreviations used: ABCA1, ATP-binding cassette transporter A1; CHD, coronary heart disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LDLR, LDL receptor; PCSK9, proprotein convertase subtilisin kexin type 9; SR-BI, scavenger receptor type BI.

Introduction

Atherosclerosis is one of the major causes for cardiovascular diseases including coronary heart disease (CHD), the leading cause of morbidity and mortality worldwide[1]. Epidemiological studies have demonstrated that multi-factors, such as hypertension, serum total and low-density lipoprotein (LDL) cholesterol levels, diabetes and smoking influence the development of atherosclerosis[2]. The accumulating evidence also suggests that aging is a major risk factor for atherosclerosis[3]. In addition, females have a much lower rate of CHD than males before the menopause[4].

Formation of lipid-laden macrophage/foam cells, the prominent part of lesions, is the initial and critical step for lesion development. Besides the disorder of cholesterol/lipid metabolism, other molecules can regulate foam cell formation. ATP-binding cassette transporter A1 (ABCA1) enhances macrophage free cholesterol efflux to extracellular cholesterol acceptor, apoA1/II[5]. Scavenger receptor type BI (SR-BI) functions as HDL receptor and mediates bi-directional cholesterol flux across plasma membrane[6]. Expression of ABCA1 and SR-BI, particularly in macrophages, has demonstrated anti-atherogenic properties[7,8]. In contrast, CD36, another member of scavenger receptor type B family, binds and internalizes oxidized LDL (oxLDL), thereby facilitating lesion development[9,10].

LDL receptor (LDLR) mediates binding and endocytosis of plasma LDL-cholesterol in liver cells where the free cholesterol can be further metabolized into bile acids and eventually secreted in feces[11]. Deficiency of LDLR expression

results in hypercholesterolemia and atherosclerosis in animal models, and CHD in humans[12]. LDLR expression can be reduced by proprotein convertase subtilisin kexin type 9 (PCSK9) mediated degradation. Thus, PCSK9 increases LDL-cholesterol levels and lesions[13-15].

A much lower rate of CHD in premenopausal women compared to men at the same age indicating that sex may impact the disease. However, after menopause, associated with decreased estrogen levels, women are also susceptible to atherosclerotic disease as men[16]. Both clinical and basic studies demonstrate that female sex hormones, particularly estrogen, may have cardiovascular protective effects[17,18]. Tamoxifen, a selective estrogen receptor modulator, has also shown to reduce atherosclerosis in animal models[19,20].

ApoE deficient ($\text{apoE}^{-/-}$) mice can spontaneously develop atherosclerosis which covers the whole spectrum of human lesions[21]. Several studies have reported that older animals tend to develop more lesions when they are fed high fat diet[3,22]. However, the association between the development of lesions in detail and ages or/and sex have not been fully elucidated when the animals are fed normal chow. In addition, if the expression of pro- and anti-atherogenic genes, particularly in macrophages, is changed with ages or sex, also remains unknown. In this study, we examined lesions in the normal chow fed $\text{apoE}^{-/-}$ mice (males and females) at different ages. We also determined expression of several atherosclerosis related genes in macrophages and the liver.

Materials and methods

Reagents

Estradiol was purchased from LKT Laboratories (St. Paul, MN). Rabbit anti-SR-BI and ABCA1 polyclonal antibodies were purchased from Novus Biologicals (Littleton, CO). Rabbit anti-CD36 and LDLR polyclonal antibodies were purchased from Proteintech Group (Chicago, IL). Rabbit anti-PCSK9 polyclonal antibody was purchased from Cayman Chemical (Ann Arbor, MI). Tamoxifen and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except where indicated.

Animals

The protocol for *in vivo* study with mice was granted by the Ethics Committee of Nankai University and conforms to the Guide for the Care and Use of Laboratory Animals published by NIH. ApoE^{-/-} mice (males and females with C57BL/6J background) were fed normal chow after weaning (~3 weeks old) with free to access to drinking water.

Determination of serum cholesterol and PCSK9 levels

ApoE^{-/-} mice (≥10/group) at the indicated ages were anesthetized and euthanized in a CO₂ chamber. Blood was collected and kept for 2 h at room temperature (RT). After centrifugation for 20 min at 2,000 g, serum was transferred into a new test tube and kept at -20°C. To determine cholesterol levels, serum (~150 µL/sample) was diluted with PBS (1:1), and then loaded onto an automatic biochemical analyzer

(Model 7020, Hitachi, Tokyo, Japan) to complete the assay. Serum PCSK9 levels were determined using an ELISA kit (R&D systems, Minneapolis, MN).

Extraction of total protein from macrophages and the liver, and determination of protein expression by Western blot

To collect peritoneal macrophages, mice were i.p. injected with 3 mL 4% thioglycollate solution and maintained on normal chow plus water for 5 days. After euthanasia, peritoneal macrophages were collected from mouse abdomen by lavage with cold PBS. The cells were cultured in complete RPMI medium for 1 h followed by removal of the floating cells. The adhesive cells (macrophages) were used to extract total cellular protein, or to be cultured with complete RPMI medium for another two days before the treatment in serum-free medium.

Macrophages or a piece of liver (~30 mg) was lysed or homogenized in a cold protein lysis buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mmol/L PMSF, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 50 µg/mL aprotinin/leupeptin). The cellular lysate or homogenates were spun for 20 min at 13,000 rpm at 4°C, and the supernatant was collected as total protein extract. Expression of ABCA1, SR-BI and CD36 in macrophages, and PCSK9, LDLR, ABCA1 and SR-BI in the liver was determined by Western blot as described[23].

Collection of aortas and determination of en face aortic lesions

After euthanasia, the entire aorta was collected and used to determine *en face* aortic lesions using Oil Red O staining as described[24].

Data Analysis

All experiments were repeated at least three times, and representative results are presented. Data were presented as mean $\pm$ standard errors, and analyzed by a Student's *t*-test using Prism 5 (GraphPad Software, Inc.). The significant difference was considered if $P < 0.05$ ($n \geq 3$).

Results

Development of atherosclerotic lesions is associated with age

To determine the development of lesions in detail with age and sex, we bred mice with normal chow after weaning, and then collected aortas from both male and female mice at different age (1-8 months old). The lesions were determined by Oil Red O staining. Figure 1A shows that apoE^{-/-} mice spontaneously developed lesions in *en face* aortas with most in aortic roots. Associated with increased age, more lesions can be determined in both male and female mice (Figure 1B). When the animals are young (1 and 2 months old), the similar lesions were determined between males and females (1.8±1.0% and 2.4±0.9% in males vs. 1.6±0.3% and 2.2±1.4% in females, respectively). However, more lesions were determined in males than females when the age was ≥3 months old. Therefore, the results in Figure 1 suggest that lesions development is associated with age and males are more susceptible than females to develop lesions.

Serum cholesterol and PCSK9 levels increase with age

As shown in Table 1, serum total, LDL- and HDL-cholesterol levels increased with age in both male and female mice. However, the changes in total, LDL- and HDL-cholesterol levels are proportional. For instance, in male mice, compared to the animals at 1 month old, total, LDL- and HDL-cholesterol levels all increased about 2-fold when the animals were about 8 months old. The similar changes of serum lipids were found in female mice. The increased total and LDL-cholesterol levels may make

contribution to lesion progression.

Liver LDLR regulates cholesterol metabolism. PCSK9, a recently identified secreted protein, facilitates LDLR degradation thereby increasing LDL-cholesterol levels. We determined that serum PCSK9 levels increased with age. Interestingly, serum PCSK9 levels in female mice were higher than male mice (Figure 2A). Meanwhile, liver LDLR expression decreased with age which might be due to increased PCSK9 levels (Figure 2B).

Different regulation of hepatic ABCA1 and SR-BI expression

To investigate the effects of age and sex on ABCA1 and SR-BI expression, we collected liver samples from both male and female mice at different age, and determined their expression by Western blot. Figure 3A demonstrates that hepatic ABCA1 expression decreased while SR-BI increased in male mice with age. The similar changes of hepatic ABCA1 and SR-BI expression were determined in female mice (Figure 3B) except that ABCA1 expression was low at the age of 1 month old.

We then compared expression of ABCA1, SR-BI and PCSK9 in the liver between male and female mice at the same age. At 1 month old, expression of ABCA1 and SR-BI was higher in males than females while no difference with PCSK9 (Figure 3C). However, in the middle aged mice (4 months old), expression of ABCA1, SR-BI and PCSK9 was higher in female mice than male animals (Figure 3D). When the animals were at 8 months old, a slight higher expression of hepatic ABCA1, SR-BI and PCSK9 in female mice than males was determined (Figure 3E). Taken together, the results in

Figure 3 suggest the dynamic changes of cholesterol metabolism associated with age. Meanwhile, it might be possible that a higher activity of cholesterol metabolism in female mice than males at the middle age.

Expression of macrophage ABCA1, SR-BI and CD36 is corresponding to lesion development

To determine if macrophage ABCA1, SR-BI or CD36 expression can be linked to lesion development with age, we collected peritoneal macrophages from mice at different age, and determined that expression of macrophage ABCA1 and SR-BI in male mice decreased at 8 months old (up and middle panels, Figure 4A), the age with substantial lesion developed (Figure 1). Meanwhile, macrophage CD36 increased with age (low panel, Figure 4A). The similar changes in macrophage ABCA1, SR-BI and CD36 expression were determined in female mice (Figure 4B). Therefore, the changes of these anti- or pro-atherogenic genes expression can contribute to lesion development.

Our above results demonstrate that female mice develop less lesions than males (Figure 1) which might be ascribed to the existence of estrogen, a sex hormone demonstrating cardioprotection. *In vitro*, we treated macrophages isolated from female mice at ages of 1 and 5 months old with estrogen, and determined that estrogen increased macrophage ABCA1 expression (Figure 4C) which was consistent with the previous study[25]. Tamoxifen has demonstrated anti-atherogenic properties. Treatment of macrophages with tamoxifen had little effect on ABCA1 expression but

substantially activated SR-BI expression (Figure 4D) which is in line with our previous study[26]. The results in Figure 4 suggest that less lesions in female mice than male mice might be due to activation of macrophage anti-atherogenic genes expression by estrogen receptor.

Discussion

Atherosclerosis develops over many years which results in that most of atherosclerotic cardiovascular diseases occur to patients with a middle age or above indicating the importance of age on the disease. Although the development of atherosclerosis is a long process with many events involved, formation of lipid-laden foam cells is the initial and critical step. High levels of circulating atherogenic lipoproteins not only supplies the source of lipids, such as oxLDL, for macrophage lipid accumulation, but also regulates expression of macrophage scavenger receptors. For instance, oxLDL induces macrophage CD36 expression in a positive forward loop while it inhibits SR-BI expression[27,28]. In addition, foam cells release multiple cytokines which can enhance the recruitment of more monocytes to form early plaques[29]. Compared to younger animals, several studies have reported that the changes which are favorable for lesion development can happen to older animals, such as reduced expression and activity of antioxidant enzymes, and elevated inflammatory molecules in the arterial wall[3,30,31]. Therefore, atherosclerosis is a pathological process with disorder of lipid metabolism, chronic inflammation and oxidative stress accumulation associated with ages.

In this study, we determined the effects of age and sex on lesions in apoE^{-/-} mice fed normal chow which can minimize the environment manipulations, such as the lipids ingested from food. We observed that lesions increased in an age dependent manner with more lesions in male mice (Figure 1). Associated with age, serum cholesterol levels increased (Table 1) which might be a contributor to lesion

development.

Macrophage ABCA1 facilitates efflux of cellular free cholesterol thereby enhancing reverse cholesterol transport (RCT), a critical process inhibiting atherosclerosis. Lack of ABCA1 expression leads to occurrence of premature CHD in humans, and development of lesions in animals[32-34]. Although SR-BI mediates bi-directional cholesterol flux, macrophage SR-BI inhibits lesions[7]. In contrast, CD36 demonstrates pro-atherogenic effects[9]. In our study, we determined that expression of macrophage ABCA1 and SR-BI decreased while of CD36 increased with age, particularly in the mice at 8 months old (Figures 4A, 4B), the age with most lesions determined (Figure 1).

Hepatic LDLR expression plays a dominant role in cholesterol metabolism although SR-BI expression can enhance the uptake of cholesteryl esters. Mutation of LDLR in humans results in hypercholesterolemia and CHD. Similarly, mice lacking LDLR expression is another animal model for atherosclerosis[11,12]. We determined that LDLR expression in the liver of apoE^{-/-} mice decreased with ages (Figure 2B). The decrease might be due to increased serum PCSK9 levels (Figure 2A). In addition, hepatic ABCA1 expression decreased (Figures 3A, 3B). Taken together, in spite of increased SR-BI expression (Figures 3A, 3B), the above results suggest that the activity of cholesterol metabolism in the liver might be attenuated with ages which might be a cause for increased serum cholesterol levels (Table 1) and a contributor for lesion development.

Compared to male mice, we determined less lesions in female mice at the same

age (Figure 1) which is consistent with epidemiological studies that women have a lower rate of CHD than men before menopause. Although serum cholesterol levels are close between male and female mice (Table 1) which indicating serum cholesterol levels are not a contributor for the difference of lesions, we determined more expression of ABCA1 and SR-BI in the liver of female mice than males. Our previous study demonstrates that SR-BI expression can be induced by activation of ER α in macrophages isolated from female but not male mice indicating the protective effect of estrogen against foam cell formation[26]. In this study, we confirmed that SR-BI is induced in macrophages isolated from female mice at different ages in response to tamoxifen treatment (Figure 4D). In addition, estrogen induced macrophage ABCA1 expression (Figure 4C). Thus, the existence of endogenous estrogen might be a potential contributor for less lesions in female mice[17,35].

In summary, our study demonstrates that atherosclerosis increased with ages in normal chow fed apoE^{-/-} mice. The increased lesions might be related to increased pro-atherogenic genes expression and decreased anti-atherogenic genes expression in tissues, particularly in macrophages. In addition, our study shows less lesions in female mice which might be due to higher activity of cholesterol metabolism and existence of estrogen in females. Taken together, the development of atherosclerosis is chronic process which is influenced by age and sex with involvement of pro- or anti-atherogenic genes expression.

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¶: These authors contributed equally to this work.

Figure legends

Figure 1. Development of atherosclerotic lesions is associated with age in both male and female apoE^{-/-} mice.

Aortic samples (n≥10/group) were collected from normal chow fed male and female apoE^{-/-} mice at the indicated age. Lesions in *en face* aortas were determined by Oil Red O staining, and quantified. The representative images of aortas (A) and the quantitative results of lesions (B) were presented. *: P<0.05 vs. the corresponding male mice (n≥10).

Figure 2. Serum PCSK9 levels increase while hepatic LDLR expression decreases with age.

Blood and liver samples were collected for following assay: A, serum PCSK9 levels by ELISA. *: P<0.05 vs. males (n≥10); B, expression of LDLR protein by Western blot, and the blot was scanned for density analysis. *: P<0.05 vs. mice at 1 month old (n=3).

Figure 3. The expression of hepatic ABCA1, SR-BI and PCSK9 is associated with age and sex.

A, B: a piece of liver from male or female mouse at the indicated age was collected to prepare total cellular protein. Expression of ABCA1 and SR-BI in livers of male mice (A) and female mice (B) was determined by Western blot. *: P<0.05 vs. mice at 1 month old (n=3); C-D: a piece of liver was collected from three male or

female mice at each of the indicated age for extraction of total protein. Expression of hepatic ABCA1, SR-BI and PCSK9 in livers of male and female mice at the same age was determined by Western blot. *: $P < 0.05$ vs. female mice in the corresponding group (n=3).

Figure 4. Expression of macrophage ABCA1, SR-BI and CD36 is associated with age.

A, B: peritoneal macrophages were collected from mice at the indicated age and used to determine expression of ABCA1, SR-BI and CD36 by Western blot. *: $P < 0.05$ vs. mice at one month old in the corresponding group (n=3); C, D: peritoneal macrophages were isolated from female mice at the indicated age followed by treatment with estrogen (C) and tamoxifen (D) at the indicated concentrations for 16 h, respectively. Expression of ABCA1 and SR-BI was determined by Western blot. *: $P < 0.05$ vs. control (untreated) in the corresponding group (n=3).

Table 1. Changes of serum cholesterol levels with ages

Age (month)	Total Cholesterol (mM)		HDL-C (mM)		LDL-C (mM)	
	male	female	male	female	male	female
1	7.37±1.10	8.09±2.00	1.74±0.13	1.99±0.40	3.77±0.32	4.26±0.89
3	10.16±0.85*	10.31±0.38*	2.54±0.26*	2.52±0.25	5.33±0.50*	5.39±0.44
5	10.44±2.21*	10.71±0.98*	2.60±0.49*	2.67±0.27*	5.52±1.02*	5.75±0.56*
8	13.72±1.80*	13.29±1.83*	3.44±0.49*	3.22±0.63*	7.13±0.91*	6.94±1.33*

Both male and female apoE^{-/-} mice were fed normal chow after weaning. Mice were sacrificed at the indicated age followed by collection of blood samples and determination of serum total, LDL- and HDL-cholesterol levels. *: P<0.05 (n≥6) vs. group of 1 month old.

Figure 1

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Figure 2

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Figure 4

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