Atherosclerosis in the Apolipoprotein E–Deficient Mouse
A Decade of Progress

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Abstract—Arguably the most critical advancement in the elucidation of factors affecting atherogenesis has been the development of mouse models of atherosclerosis. Among available models, the apolipoprotein E–deficient (apoE−/−) mouse is particularly popular because of its propensity to spontaneously develop atherosclerotic lesions on a standard chow diet. A Medline search reveals over 645 articles dedicated to studies using this reliable and convenient “super” animal model since its inception (Piedrahita JA et al, Proc Natl Acad Sci USA 1992;89:4471–4475; Plump AS et al, Cell 1992;71:343–353) with a more or less steady increase from year to year. This review will examine our present understanding of the pathology and progression of plaques in this animal and highlight some of the nutritional, pharmacological, and genetic studies that have enhanced this understanding. (Arterioscler Thromb Vasc Biol. 2004; 24:1006-1014.)

Key Words: apolipoprotein E  ■  atherosclerosis  ■  knockout  ■  mouse model

Murine Cholesterol Metabolism and Apolipoprotein E

Despite greater cholesterol ingestion and synthesis, rapid murine hepatic LDL clearance results in a total serum cholesterol level of approximately 85 mg/dL, mostly carried as high-density lipoproteins (HDL), a lipid profile significantly different from that of humans. Because normal mice do not develop atherosclerosis unless challenged for long periods with Western type diets,1 developing useful mouse models of atherosclerosis required the disruption of a critical antiatherogenic gene product involved in cholesterol metabolism.

Apolipoprotein E (apoE), a glycoprotein synthesized mainly in the liver and brain, is a constituent of all lipoproteins except low-density lipoproteins (LDL). It functions as a ligand for receptors that clear chylomicrons and very low-density lipoprotein (VLDL) remnants. ApoE is also synthesized by monocytes and macrophages in vessels, and is thought to have local effects on cholesterol homeostasis and on inflammatory reactions in atherosclerotic vessels.2 It may also function in dietary absorption and biliary excretion of cholesterol.3

The apoE–deficient (apoE−/−) mouse was created practically simultaneously in two separate laboratories, through gene inactivation by targeting. On a chow diet, the mice demonstrated a total cholesterol level >500 mg/dL, mostly in the VLDL and chylomicron remnant fractions. A Western diet quadrupled these fractions.4,5 The apoE−/− mice available today through The Jackson Laboratories are descendants of the original apoE−/− mouse created by the Maeda group (t002052 B6.129P2-Apoetm1Unc).

Plaque Pathology in the ApoE−/− Mouse

Fatty streaks were first observed in the proximal aorta of a chow-fed, 3-month-old mouse.4 On this diet, as early as 10 weeks of age, foam cell lesions were observed by light microscopy. Intermediate lesions containing foam cells and smooth muscle cells were seen at 15 weeks, and fibrous plaques appeared at 20 weeks of age. A Western diet accelerated the process. Histological and morphometric analyses of plaque progression revealed an increase in complexity as well as in lesion size with age (Figure 1).6 Although molecular studies of vascular remodeling in these mice had not yet begun, fragmentation of the elastic lamina was well documented, as were calcification and wall thinning in most mice aged at least 32 weeks.

Once the presence of plaques resembling human lesions was confirmed, there ensued explosive use of the model in the study of factors affecting plaque size and composition. Two quantitation methods are used extensively to measure plaques. The first measures plaque cross sectional area in slices taken at the level of the aortic sinus,1 and is useful for mild or early stage disease. The second (increasingly popular) “en face” method involves pinning out the aorta and quantifying lesion area as a percentage of total surface area.5,7 This method requires older mice, as it appears to be generally agreed on that plaques first appear proximally and only later appear distally.6,8 Because there is no map of lesion appear-
ance and accumulation, nor “standard curves” for lesion quantitation, laboratories may establish their own standard curves for lesion size, based on gender, diet, and age (Figure 2 demonstrates our results). Interestingly, despite inbreeding, the mouse-to-mouse variability in lesion area is tremendous, and appears to increase with high-fat diets.

Although extrapolation from murine to human studies is problematic for several reasons, including differences in cholesterol metabolism and lipid profile, differences in cardiovascular physiology, differences in plaque pathology, and a relative lack of lesion progression leading to thrombotic occlusion and clinical events, it has recently been shown that in older apoE−/− mice, brachiocephalic arterial plaques demonstrate features likely to be the murine parallel of those in vulnerable human plaques, including the formation of an acellular necrotic core, erosion of the necrotic mass through to the lumen, and intraplaque hemorrhage.9 This site will likely become more popular with time.

Nutritional Intervention Studies in ApoE−/− Mice

Nutritional intervention studies range broadly from dietary restriction to alterations in macro- and micronutrients (Table 1).10–24 Of note are the meticulous fatty acid studies that evaluated the effect of diets isocaloric to chow, supplemented with various fatty acids. Interestingly, the effects of different oils differ between males and females.11

Among the antioxidant vitamins, while vitamin E deficiency increases lesion size,17 vitamin C deficiency appears to cause a shift in plaque morphology without affecting plaque size.18

Ferrous iron ions induce oxidation of cellular polyunsaturated fatty acids in intact macrophages, which can in turn induce LDL oxidation.19 Although these effects might be expected to aggravate atherogenesis, both iron deficiency20 and iron overload21 appear to decrease lesion area.

Recent interest has turned to subfractions of foods with antioxidant or antiinflammatory properties. Examples of these are the black rice pigment fraction22 and the tannin fraction of pomegranate juice,23 both of which have been shown to reduce atherosclerosis.

Among the amino acids, supplementation with methionine and homocysteine increases lesion area, but does not appear to cause plaque instability.24

Pharmacological Studies

Perhaps not surprisingly, drugs known to reduce serum lipids, such as the new and potent cholesterol absorption inhibitors, dramatically reduce atherosclerotic lesion area in apoE−/− mice (Table 2).25 Pharmacological agents that affect cardiovascular risk factors other than serum lipids have been the subject of several murine studies. Among antihypertensive drugs, the angiotensin converting enzyme inhibitors and angiotensin II receptor blockers have been extensively studied, and have been shown to effectively reduce lesion area and inhibit lipid peroxidation.26–28 Other agents that reduce cardiovascular risk include anti-diabetic drugs. The thiazolidinediones enhance insulin sensitivity by activating peroxisome proliferator-activated receptor γ (PPAR-γ). PPAR transcription factors are activated by their natural ligands, fatty acid derivatives and eicosanoids.29 Thiazolidinedione (TZD)-
activated PPARγ/Retinoid X Receptors increase hepatic scavenger receptor class B, type I (SR-BI) levels, which may lead to increased hepatic cholesterol uptake and less peripheral tissue lipid accumulation. Thiazolidinediones rosiglitazone and troglitazone have been shown to prevent hyperglycemia and hyperinsulinemia in apoE−/− mice and to reduce neointima formation after carotid arterial injury. In a combined hypercholesterolemic/ diabetic mouse model (streptozotocin-treated apoE−/− mice), rosiglitazone reduced lesion area without affecting plasma glucose.

In addition to agents targeted to cardiovascular risk factors, the interest in atherosclerosis as an inflammatory disease has led to atherosclerosis studies of antiinflammatory drugs in apoE−/− and other mouse models. A salutary effect to no effect, to a deleterious effect on plaque size and progression has been demonstrated. Studies are needed to elucidate any clinical role these drugs may eventually play in human atherosclerosis.

**Drug Development Studies**

Beyond currently available agents, the apoE−/− mouse has lent itself to the examination of experimental agents such as PPARα/γ coagonists and leukocyte adhesion inhibitors, with promising results.

The nuclear receptors known as Liver X Receptors (LXRs) LXRα and LXRβ are emerging as central regulators of lipid homeostasis. In the macrophage, LXR’s appear to coordinate a physiological response to cellular cholesterol loading. Several genes involved in the cholesterol efflux pathway are under transcriptional control of LXRs, such as ATP-binding cassette (ABC) A1, ABCG1, and apoE. The promotion of reverse cholesterol transport (RCT) and inhibition of intestinal cholesterol absorption would be expected to reduce atherosclerotic risk. Indeed, male apoE−/− mice receiving a synthetic LXR agonist exhibited reduced lesion area compared with control mice, as well as upregulated aortic ABCA1 and ABCG1 expression.

Also localized to the periphery, Acyl-coenzyme A: cholesterol acyltransferase (ACAT) converts cholesterol to cholesteryl esters. ACAT1, the isoform found in macrophages, is under transcriptional control of LXRs, such as ATP-binding cassette (ABC) A1, ABCG1, and apoE. The promotion of reverse cholesterol transport (RCT) and inhibition of intestinal cholesterol absorption would be expected to reduce atherosclerotic risk. Indeed, male apoE−/− mice receiving a synthetic LXR agonist exhibited reduced lesion area compared with control mice, as well as upregulated aortic ABCA1 and ABCG1 expression.

A novel approach to plaque inhibition has been examined through use of angiogenesis inhibitors. While human plaques exhibit neovascularization, one group has reported the presence of intimal vessels at the shoulders of plaques in apoE−/− mice. Treatment with either endostatin or TNP-470...
led to dramatic reductions in lesion size. The same group has more recently shown that atherosclerotic plaques contain angiogenic activity, and that inhibition of neovascularization with angiostatin not only reduces plaque neovascularization and size, but also reduces the prevalence of inflammatory cells within lesions. The purpose of angiogenesis in atherosclerosis is unknown, but it is likely part of the chronic inflammatory process associated with atherogenesis.

Genetic Studies
The apoE−/− mouse has been used extensively as a springboard for the study of other genes affecting atherogenesis. The murine products take the form of either double knockout mice or apoE−/− mice overexpressing a transgene. The additional genes fall into one of two main categories: those involved in lipid metabolism, and those involved in other mechanisms such as inflammation or hemostasis (Table 3).

Lipid Metabolism: Reverse Cholesterol Transport
A major advance in our understanding of the RCT pathway occurred with the discovery of the ATP binding cassette transporter A1 (ABCA1), the defective protein product in patients with Tangier disease. ABCA1 transporter facilitates efflux of cellular phospholipids and cholesterol to acceptors, such as apoA-I and apoE. Tangier disease is characterized by decreased plasma cholesterol, decreased low and high density lipoproteins, and increased triglycerides. Newly synthesized apolipoproteins are unable to acquire lipids which are then rapidly degraded, resulting in cholesterol accumulation in tissue macrophages, low HDL, and cardiovascular disease. Because the ABCA1 transporter is intimately involved in RCT, upregulation of ABCA1 expression might retard atherogenesis. Studies of apoE−/− mice overexpressing human ABCA1 have provided conflicting results. In one study human ABCA1 overexpressing bacterial artificial chromosome (BAC) transgenic mice were generated and crossed with ApoE−/− mice. The BAC/ApoE−/− mice demonstrated greatly reduced lesion area and increased cholesterol efflux from peritoneal macrophages. In the other study, human ABCA1 overexpressors exhibited a minimal change in the plasma lipoprotein profile, with a small increase in plasma levels of apoB-containing lipoprotein cholesterol, and surprisingly, significantly increased atherosclerosis. According to this study, in the absence of apoE, ABCA1 overexpression does not protect against plaque development, possibly because of delayed clearance of apoB-containing lipoproteins or low plasma apoA-I levels in apoE−/− mice.

An elegant set of studies using bone marrow transplantation sheds light on the above conflict. Complete absence of ABCA1 in the setting of apoE deficiency resulted in plasma lipid reductions and tissue foam cell accumulation but no increase in atherosclerosis, likely caused by a less atherogenic...
lipid profile. Selective absence of ABCA1 in macrophages, however, in the same setting (as well as in LDL-receptor deficiency), resulted in markedly increased atherosclerosis with no effect on plasma lipids. The mechanisms by which ABCA1 modulates plasma lipid levels remain unclear.62

ApoA-I, the main apolipoprotein in HDL, is a key acceptor of cholesterol from macrophages, via ABCA1-mediated transport. ApoE/H11002/H11002 mice overexpressing human apoA-I show decreased lesion size with increased HDL formation.63,64 Bone marrow transplantation of macrophage-specific human apoA-I in an apoE/H11002/H11002 background afforded nearly complete atheroprotection, which demonstrates that local apoA-I expression is as atheroprotective as apoE.65

Scavenger Receptors

The apoE−/− mouse has been used extensively to study scavenger receptor function. There are two main classes of such receptors, scavenger receptor class A (SR-A), and class B which includes CD36 and the hepatic HDL receptor, better known as the scavenger receptor class B type I (SR-BI). The scavenger receptor functions of the macrophage illustrate the centrality of this cell in both immune and metabolic aspects of atherogenesis.69 Macrophage scavenger receptors recognize ligands such as oxidized LDL and are thought to play an important role in foam cell formation and lesion initiation.70

SR-A is a macrophage integral membrane protein whose deletion in the apoE/H11002/H11002 mouse significantly reduced atherosclerosis despite increased serum cholesterol.71 Its overexpression by bone marrow–derived macrophages reduced serum cholesterol but did not promote atherosclerosis.72 The class B scavenger receptor, CD36, also known as fatty acid translocase (FAT), is a multifunctional membrane receptor found on a wider range of cells than SR-A. On phagocytic cells it internalizes oxidatively modified lipids in the mem-

| TABLE 3. Genetic Atherosclerosis Studies in ApoE-Deficient Mice |
|-----------------|-----------------|-----------------|
| Knockout (KO)/ | Transgene (Tg)  | Effect on Atherosclerosis |
| Lipid metabolism-associated gene | | Increase/Decrease |
| Acyl CoA:cholesterol acyltransferase 2 | KO | ↓ ↓ |
| Acyl CoA:cholesterol acyltransferase 1 | KO | altered lesion composition |
| Hepatic lipase | KO | ↓ |
| Human ATP binding cassette transporter | Tg | ↓ ↓ |
| Human ATP binding cassette transporter | Tg | ↑ ↑ |
| ATP binding cassette transporter (macrophage) | KO | ↑ |
| Human apoAI | Tg | ↓ ↓ , ↑ plaque stability |
| Paraoxonase | KO | ↑ |
| Scavenger receptor class A (SR-A) | KO | ↓ |
| Scavenger receptor class A (SR-A) | Tg | — |
| Class B scavenger receptor (CD36) | KO | ↓ |
| Scavenger receptor class B, type I (SR-BI) | KO | ↑ ↑ |
| Low density lipoprotein receptor | KO | ↑ |
| Inflammatory mediator and remodeling genes | | |
| Recombination-activating gene 2 (RAG2) | KO | — |
| C-reactive protein | Tg | ↑ |
| P-selectin | KO | ↓ ↓ |
| Inducible nitric oxide synthase (iNOS) | KO | ↓ |
| Endothelial nitric oxide synthase (eNOS) | KO | ↑ |
| 12/25 lipoxigenase | KO | ↓ |
| Human matrix metalloproteinase 1 | Tg | ↓ |
| p53 | Tg | ↓ cap to intima ratio |
| Interleukin-1 beta | KO | ↓ |
| Interleukin-10 | KO | ↑ |
| Interleukin-18 | KO | ↓ |
| Fractalkine (CX3CL1) | KO | ↓ |
| Monocyte chemoattractant protein-1 | KO | ↓ |
| Hemostasis-related genes | | |
| Fibrinogen | KO | — |
| Plasminogen | KO | ↑ |
| Plasminogen activator inhibitor-1 | KO | — |
| Plasminogen activator inhibitor-1 | KO | ↑ |
branes of apoptotic cells and oxidized LDL. In apoE−/− mice, disruption of CD36 led to a dramatic decrease in atherosclerotic lesion area despite increased severity of the proatherogenic lipid profile.73

SR-BI plays a critical role in RCT, but it is also expressed in macrophages and can mediate cholesterol efflux from cells.74 By knocking out this receptor in the apoE−/− mouse, a model has at long last been created in which mice experience hypercholesterolemia, severe coronary atherosclerosis, myocardial infarctions, cardiac dysfunction, and premature death,75 pathology more striking that that observed in the apoE−/−/LDL receptor double knockout mice.76 Similarly, and more recently, reconstitution of bone marrow with SR-BI+/−apoE−/− cells in apoE−/− mice resulted in significantly increased atherosclerosis when compared with reconstitution with SR-BI+/+apoE−/− cells.77

Inflammation, Fibrinolysis, Plaque Remodeling, and Vulnerability
A great deal of evidence supports the widespread notion that atherosclerosis is a form of chronic inflammation. It is well established that T cells are often present in advanced human atherosclerotic plaques,79 but their role in atherogenesis is not yet fully understood. Although a study of double knockout ApoE−/−/RAG2−/− mice established that a complete lack of T cells does not prevent atherosclerosis,79 the hypothesis has been made that T cells are in fact required at the earliest stages of plaque development.80 The observation of enhanced atherosclerosis in apoE−/− mice overexpressing human c-reactive protein (CRP) further promotes the notion that inflammation plays a causal role in atherogenesis.81

Leukocyte–endothelial cell interaction is important in lesion formation. An elegant examination of one receptor involved in this interaction, P-selectin−/−/ApoE−/− double knockout mice, demonstrated a 3.5-fold reduction in the size of aortic sinus lesions in four-month-old mice. This reduction remained significant over time, and lesions in P-selectin−/−/apoE−/− mice had fewer macrophages and smooth muscle cells and less calcification than those in controls.82

Among the local vascular factors which are believed to play a role in atherogenesis, endothelial, neuronal, and inducible nitric oxide synthase (eNOS, nNOS, and iNOS, respectively) are all expressed in atherosclerotic lesions. The finding that iNOS is expressed in lesional leukocytes and smooth muscle cells (and not constitutively) is consistent with the recent demonstration of decreased atherogenesis in iNOS/apoE−/− double knockout mice, possibly caused by decreased oxidative stress in the vascular wall.83 Interestingly, the converse has been demonstrated for knockout of the constitutive eNOS.84

Arachidonic acid metabolites are among the most interesting and diverse endogenous inflammatory molecules in the immune system. Much research into their role in cardiovascular morbidity and mortality has recently been stimulated with the advent of cyclooxygenase (COX)-2 inhibitors. Although the effect of these agents on atherogenesis has been studied, that of COX-2 gene knockout in the setting of apoE-deficiency is unknown. Within the same family, however, 12/15-lipoxygenase knockout in apoE−/− mice has been shown to decrease both lipid peroxidation and atherogenesis.85

Matrix metalloproteinase-1 (MMP-1), or interstitial collagenase, has been hypothesized to contribute to the progression of human atherosclerotic lesions by digesting the fibrillar collagens of the neointimal extracellular matrix. Unexpectedly, human MMP-1 expression in macrophages in apoE−/− mice caused a significant reduction in lesion size and complexity in mice fed a Western diet, suggesting that remodeling of the neointimal extracellular matrix by MMP-1 is beneficial in the progression of lesions.86

Today, the balance of interest appears to be shifting toward lesion quality, particularly in the form of unstable plaque morphology. In one set of studies, although gene transfer of p53 to apoE−/− mice did not alter plaque size, it did cause a shift toward vulnerable plaque morphology with a decrease in cap-to-intima area ratio, cap breaks, and intraplaque hemorrhages.87

Thrombotic complications are the ultimate expression of the unstable plaque, and investigative teams have examined the relative importance of thrombosis and hemostasis in the atherogenesis. Circulating activated platelets deliver chemokines to affected arterial endothelium and increase leukocyte adhesiveness, thereby promoting atherogenesis.83 In apoE−/− mice, although fibrinogen deficiency has not been shown to be protective,94 plasminogen deficiency appears to be deleterious.95 In human epidemiologic studies, elevated plasma levels of the main physiological inhibitor of plasminogen activators, plasminogen activator inhibitor-1 (PAI-1), have been linked to increased risk of myocardial infarction. In mice, however, the PAI-1 gene likely plays only a limited, perhaps protective role in atherogenesis.96-97

Emerging Areas of Investigation
The apoE−/− mouse is proving its versatility with studies in fields unrelated to atherosclerosis, including gene therapy,98 Alzheimer’s dementia, and noninvasive imaging.

Noninvasive Imaging
In general, atherosclerosis studies in mice involve plaque examination by histologic methods, which necessitate euthanizing the animals. Patients, however, are evaluated as much as possible by noninvasive techniques. Accordingly, one team developed a noninvasive magnetic resonance (MR) microscopy technique to study lesions in live apoE−/− mice fed a chow diet, with good correlation between MR and histopathology in grading of lesion shape and type (slope=0.97, r=0.91 for lesion shape; slope=0.64, r=0.90 for lesion type).99 The technique has been applied to mice fed a Western diet with excellent results, including differentiation between various plaque components. Interestingly, the authors also generated a standard curve of wall thickness versus age, in which wall thickness increased exponentially while lumen diameter increased linearly.100 It is not beyond the realm of imagination to envision future studies involving nutritional, pharmacological, and genetic manipulations, with end points measured largely by noninvasive techniques.

Future Directions
The advent of the apoE−/− and other mouse models of atherosclerosis has revolutionized the study of mechanisms of
this disease. Although imperfect, this model is currently the most popular and convenient mouse model used by investigative teams around the globe. As we witnessed a short lag between the model’s creation and its use, we are likely witnessing a similar lag in the take-off of studies involving noninvasive imaging techniques. We also wait with anticipation further studies examining clinical event end points and their prevention. From there, it is hoped that within the next decade or two, the revolution will be felt as strongly at the bedside as it has been at the bench.

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