Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E

JONATHAN D. SMITH*, EUGENE TROGAN, MICHAEL GINSBERG, CLAIRE GRIGAUX, JASON TIAN, AND MASAHI MIYATA

Laboratory of Biochemical Genetics and Metabolism, The Rockefeller University, 1230 York Avenue, New York, NY 10021

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ABSTRACT To develop a murine model system to test the role of monocyte-derived macrophages in atherosclerosis, the osteopetrotic (op) mutation in the macrophage colony-stimulating factor gene was bred onto the apolipoprotein E (apoE)-deficient background. The doubly mutant (op/apoE-deficient) mice fed a low-fat chow diet had significantly smaller proximal aortic lesions at an earlier stage of progression than their apoE-deficient control littermates. These lesions in the doubly mutant mice were composed of macrophage foam cells. The op/apoE-deficient mice also had decreased body weights, decreased blood monocyte differentials, and increased mean cholesterol levels of ~1300 mg/dl. Statistical analysis determined that atherosclerosis lesion area was significantly affected by the op genotype and gender. The confounding variables of body weight, plasma cholesterol, and monocyte differential, which were all affected by op genotype, had no significant additional effect on lesion area once they were adjusted for the effects of op genotype and gender. Unexpectedly, there was a significant inverse correlation between plasma cholesterol and lesion area, implying that each may be result of a common effect of macrophage colony-stimulating factor levels. The data support the hypothesis that macrophage colony-stimulating factor and its effects on macrophage development and function play a key role in atherogenesis.

The arterial fatty streak is an early and reversible precursor to advanced fibroproliferative atherosclerotic lesions (1). These early lesions contain mostly monocyte-derived macrophages that have taken up modified lipoproteins to become lipid-enriched foam cells. Monocytes adhere to and migrate through the arterial endothelium via cell adhesion molecules, such as VCAM-1, which is focally expressed by endothelial cells over sites where atherosclerotic lesions occur (2). In addition to macrophage foam cells, human lesions also contain T cells and smooth muscle-derived foam cells (1), and the essential role of monocyte-derived macrophages in atherosclerosis remains unproven. Apolipoprotein E (ApoE)-deficient mice are hypercholesterolemic, and unlike other rodent models of atherosclerosis, they develop atherosclerosis spontaneously on a low-fat chow diet (3). The hypercholesterolemia and atherosclerosis in these mice are diet responsive, in that they are increased by feeding a high-fat Western-type diet (3). Remarkably, these lesions are excellent models for human atherosclerosis in that they progress with age from early fatty streaks to complex fibrous lesions with necrotic cores and are found at the same sites of predilection as human lesions (4). Similar to human and rabbit lesions, oxidized lipoprotein epitopes are found in the lesions of apoE-deficient mice, and sera from these mice have high titers of autoantibodies that recognize oxidized lipoproteins and can stain lesions in rabbits (5). The apoE-deficient mice are therefore an attractive model system in which to elicit factors that mediate or modulate the pathogenesis of atherosclerosis.

The osteopetrotic (op) mouse has a spontaneously derived mutation in the gene encoding macrophage colony-stimulating factor (MCSF) and the phenotype has been well characterized in many previous studies (6–15). The op defect in the MCSF gene is a frame-shift mutation leading to the complete absence of MCSF activity in the serum and tissues; further, partial reversal of the op phenotype has been achieved by chronic MCSF injection (6, 13–16). op mice have decreased body weight and an almost complete deficiency in osteoclasts, resulting in bone remodeling defects and skeletal deformities, the most severe of which is the lack of incisors (7, 8). The hematological parameters of op mice have been previously studied; they have normal hematocrits and granulocyte counts but have decreased total leukocyte and lymphocyte counts and severely reduced blood monocytes and peritoneal macrophages (8, 12). In systematic morphometric studies op mice have moderately decreased tissue macrophages in several tissues, including liver, yet other macrophage populations, such as dendritic cells, are unaffected (7, 9, 15). op mice have decreased fertility, and females have lactational defects (10, 11).

The MCSF deficiency in op mice may affect atherosclerosis susceptibility in several ways. MCSF injections reduce cholesterol levels in rabbits, primates, and humans (17–19). MCSF increases uptake of low density lipoprotein (LDL) and acetylated LDL by macrophages (20) and induces scavenger receptor activity and mRNA levels (21, 22). MCSF can also reduce atherosclerosis and carrageenan-induced granuloma lipid accumulation in Watanabe hyperlipidemic rabbits (19, 23). In an effort to determine the effect of decreasing monocytes on atherosclerosis, we bred the op mutation onto the apoE-deficient background. We found that the doubly mutant apoE-deficient–op mice had an almost 3-fold increase in plasma cholesterol compared with their apoE-deficient controls. Despite this severe hypercholesterolemia, these double mutant mice had significantly less atherosclerosis in the proximal aorta.

MATERIALS AND METHODS

Mice. The creation of the apoE-deficient mice used in this study has been described. The C57BL/6 × 129 apoE-deficient female mice [E0; we use the convention of the gene name (E for apoE) followed by the number of wild-type alleles to describe the mouse genotypes] were bred to op heterozygous (op1) male mice (The Jackson Laboratory) on the C57BL/6 × C3H background. The heterozygous E1 progeny were screened for the op mutation by a PCR assay (described below), and the op1/E1 among them were interbred to generate op1/E0 mice that served as the parental genotype for all animals in the study. Offspring of the parental op1/E0 were

Abbreviations: apoE, apolipoprotein E; op, osteopetrotic; MCSF, macrophage colony-stimulating factor; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

*To whom reprint requests should be addressed.
op0/E0, op1/E0, and op2/E0 littersmates, which served as the subjects of our study. These offspring were obtained at an \( \approx 1:2:1 \) ratio, as predicted, although due to increased mortality, fewer op0/E0 mice survived to the end of the study period. Mice were fed ad libitum with powdered chow diet. Genotype analysis was done on tail-tip-derived DNA samples. The \( op \) genotype PCR assay used 50 pmol of the primers (designed by Jonathan Pollard, Albert Einstein College of Medicine) GTTGCTAACCTAAGAGGCG and GCATGCTCTCATCTTATAT; \( 5 \mu \)l of genomic DNA; 1 \( \mu \)l of adenine 5'-[\( 35 \)S]dithiophosphate; 2 units of Taq polymerase; and 44 \( \mu \)l of a buffer containing 0.23 mM (each) of dCTP, dGTP, and dTTP; 0.05 mM dATP; 18.87 mM ammonium sulfate; 76 mM Tris (pH 8.8); 12 mM dithiothreitol; 10% (vol/vol) dimethyl sulfoxide; and bovine serum albumin at 20 mg/ml. The reaction was done at 94°C for 40 sec, 55°C for 2 min, and 72°C for 2 min for 40 cycles. The products were run on a 12% (vol/vol) acrylamide-urea gel, and an autoradiograph of the dried gel was used to distinguish the 59-bp wild-type product from the 60-bp \( op \) product. The \( apo \) genotype assay used three primers: a second exon sense primer, CTCTGGGCGGTGCTGTGGTCCATCTGCTGAC; a third exon antisense primer, CTCAGCTGTATCCTCCTCCTGTCCTCCC; and a neo gene primer, GCGCGTCCCGATTGCGACGCGCATCGC. The reaction contained 3 \( \mu \)l of genomic DNA, 0.5 \( \mu \)l of the three primers pooled at 50 pmol/\( \mu \)l each, and 20 \( \mu \)l of PCR buffer, similar to the buffer above but containing 0.23 mM dATP. The reaction was preheated to 99°C for 5 min, cooled to 70°C, and run for 30 cycles at 94°C for 1 min, 64°C for 1 min, and 72°C for 3 min. The products were run on 1.2% agarose gels to distinguish the 660-bp wild-type \( apo \) allele product from the 350-bp knockout allele product.

**Blood Monocyte and Plasma Cholesterol Analysis.** Mice at 16 weeks of age were bled by ventricular puncture into heparinized syringes, and for some, blood smears on glass slides were prepared immediately. The dried smears were stained with Diff-Quik (Baxter), and monocytes were determined from a minimum of 60 white blood cells. Total plasma cholesterol was determined enzymatically (Boehringer Mannheim), and very low density lipoprotein (VLDL) cholesterol was separated from LDL + high density lipoprotein (HDL) cholesterol by equilibrium density centrifugation at a density (\( \rho \)) of 1.006; LDL and HDL were determined on the \( \rho > 1.006 \) fraction after dextran sulfate and magnesium ion precipitation of apoB-containing particles (Sigma). For fast protein liquid chromatography analysis 100 \( \mu \)l of pooled plasma was injected onto two Superose 6 columns (Pharmacia) in series, as described (3).

**Quantitative and Qualitative Atherosclerosis Analysis.** The mean lesion area per section of aortic root was measured as described (24). For qualitative analysis, the freshly removed hearts were snap-frozen in OCT embedding medium (Miles). Six-ten-micrometer frozen sections were stained with oil red O and hematoxylin or were processed for immunohistochemistry as follows. Slides were treated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity, followed by blocking in normal goat serum. Rabbit polyclonal anti-mouse macrophage (Accurate Chemicals) was used at 1:100 dilution. Biotinylated goat anti-rabbit antibody, avidin-horseradish peroxidase complex (ABC Elite kit), and diaminobenzidine peroxidase substrate were used according to manufacturer’s specifications (Vector Laboratories). Slides were counterstained with hematoxylin.

**Statistical Analysis.** Atherosclerosis lesion areas were not normally distributed and were logarithmically transformed before statistical analysis. The genotype and gender effects on lesion area and other variables were determined by the two-way ANOVA model. Individual differences among the three genotypes were determined by Duncan’s multiple-range test and Student’s \( t \) test. The effects of confounding variables on lesion area, adjusted for genotype and gender effects, were determined by the analysis of covariance (ANCOVA) model.

**RESULTS**

Male and female mice of three genotypes were analyzed at 16 weeks of age: op0/E0, homozygous op–apoE deficient; op1/E0, heterozygous op–apoE deficient; and op2/E0, wild-type op–apoE deficient. The atherosclerosis lesion area in the aortic root was determined on 71 mice, and individual data points are plotted by genotype and sex in Fig. 1. There were two clear trends; the first was toward smaller lesions in the op0/E0 mice, and the second was toward larger lesions in females in general. This assay displayed considerable inter-animal variation, as observed in many previous studies that measured proximal aortic lesion area in mice (24–30). Some individual variation in lesion area is probably stochastic, and some variation might be from the mixed genetic background of these mice. However, the lesion area differences between genotypes cannot be attributed to genetic background, as the study animals were all littermate progeny from op1/E0 parents. Statistical analysis of this data appears in Table 1. op0/E0 mice had lesions averaging \( \approx 10,000 \mu m^2 \) per section, whereas lesions in the op2/E0 males and females were \( \approx 4 \)-fold and 10-fold larger, respectively. Using an ANOVA model, both \( op \) genotype and gender had highly significant and independent effects on mean lesion area. Using both Duncan’s multiple-range test and two-way ANOVA.

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<th>Table 1. Atherosclerosis lesions in 16-week-old mice by sex and genotype</th>
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\( ^{+} \) n values are in parentheses. \( P = 0.004 \), for genotype effect on lesion area by two-way ANOVA in males; \( P < 0.005 \), for genotype effect on lesion area by two-way ANOVA in females alone and both sexes combined; \( P = 0.0015 \) for gender effect on lesion area by two-way ANOVA.

\( ^{\#} \) Different from op1/E0 and op2/E0 by Duncan’s multiple range test, \( \alpha = 0.01 \).

\( ^{*} \) Different from op1/E0 and op2/E0 by two-way, two-tailed \( t \) test, \( P < 0.04 \).

\( ^{\#} \) Different from op1/E0 and op2/E0 by two-way, two-tailed \( t \) test, \( P < 0.0005 \).

\( ^{\#} \) Different from op1/E0 and op2/E0 by two-way, two-tailed \( t \) test, \( P < 0.001 \).

\( ^{\#} \) Different from op2/E0 by two-tailed \( t \) test, \( P < 0.05 \).
Student's $t$ test, mean lesion areas were significantly less in op0/E0 mice compared with their op1/E0 and op2/E0 littermates for males, females, and both genders combined (Table 1). In addition, the two-way $t$ test revealed that op1/E0 females had significantly smaller lesions than op2/E0 females, showing an MCSF gene dosage effect on lesion area in females. Although this trend was also seen in males and in the combined data, it was not statistically significant.

Every 16-week-old op2/E0 mouse analyzed had atheromatous lesions in the aortic root (Fig. 2A). These lesions included both simple raised foam cell lesions (Fig. 2C) and large, more developed fibroproliferative lesions (Fig. 2B and E). The lesions were found on the valve cusps and in the area between valve cusps. The smaller lesions in op0/E0 mice were mostly early foam cell lesions that had not progressed to more advanced stages and were found primarily on the valve cusps (Fig. 2G). Immunohistochemical staining for macrophages was done on frozen sections of proximal aortas from op0/E0 and op2/E0 mice. We observed prominent and homogeneous macrophage staining in the small raised foam cell lesions from op2/E0 mice (Fig. 2D). Macrophage staining was also seen in the larger more advanced lesions (Fig. 2F), but it was less homogenous, presumably due to the presence of smooth muscle cells and necrotic regions. In the sections from op0/E0 aortas, we observed homogeneous macrophage staining in the raised foam cell lesions (Fig. 2H), similar to the staining seen in the op2/E0 foam cell lesions (Fig. 2D).

Body weight, plasma cholesterol levels, and monocyte differentials were also determined to see whether any of these factors were affected by the op genotype and if they contributed to the observed differences in lesion area. There was a significant effect of op genotype, but not of gender, on weight, total cholesterol, and monocyte differential count (Table 2). Op0/E0 mice of both sexes weighed about three-fourths as much as, and significantly less than, their op1/E0 and op2/E0 littermates (Table 2). Op0/E0 mice on a chow diet had extremely high plasma cholesterol levels, averaging $\sim 1300$ mg/dl, close to 3-fold more than, and significantly different from, their op1/E0 and op2/E0 littermates. Thus, despite their highly elevated plasma cholesterol levels, the op0/E0 mice developed less severe atherosclerosis than their littermates. The excess cholesterol in the op0/E0 mice was primarily due to a 3.4-fold increase in the VLDL cholesterol (391 $\pm$ 108 mg/dl in 11 op2/E0 mice compared with 1341 $\pm$ 453 mg/dl in 12 op0/E0 mice, $P < 0.0001$), which is also the density primarily responsible for the hypercholesterolemia in apoe-deficient mice. LDL levels also significantly increased from 31 $\pm$ 11 mg/dl in the op2/E0 mice to 95 $\pm$ 86 mg/dl in the op0/E0 mice; however, HDL was not significantly altered. Comparison of the size distribution of lipoprotein cholesterol from op0/E0 and op2/E0 plasmas by fast protein liquid chromatography analysis confirms that the increased cholesterol resides primarily in the larger (VLDL) size class (Fig. 3). The monocyte differential count was determined on some mice and showed a clear and significant gene dosage effect with op0/E0, op1/E0, and op2/E0 mice, having $\sim 4\%$, 7%, and 9% monocytes, respectively (Table 2).

Using an analysis of covariance (ANCOVA) model, body weight, total cholesterol, and monocyte differential count each
had no significant independent effect on lesion area when adjusted for op genotype and gender (P = 0.067, 0.34, and 0.36, respectively). Even so, we looked for correlations between the logaritically transformed lesion size and these three independent variables within the females that developed larger lesions and had the larger sample size (Fig. 4). Combining all the genotypes, there was no significant relationship between body weight and lesion size (r = -0.21, P = 0.19, n = 40). However, there was a marginally significant inverse correlation between weight and lesion areas for the op/E0 females (r = -0.52, P = 0.085, n = 12), as the mice with the largest lesions tended to be smaller. In the op/E0/E0 mice there was a trend toward larger lesions in the larger animals, but this relationship failed to reach statistical significance (r = 0.36, P = 0.39, n = 8). There was also a significant inverse correlation between plasma cholesterol and lesion area in all genotypes combined (r = -0.74, P < 0.001, n = 40), which was, in part, due to the op/E0 mice with their high cholesterol and small lesions. Within any genotype alone, the only significant cholesterol–lesion area association was in the op/E0 mice, where there was an inverse correlation (r = -0.69, P < 0.001, n = 20). HDL cholesterol was not associated with lesion area (r = 0.15, P = 0.68, n = 10, data not shown). The only positive trend associated significantly with the logarithmically transformed lesion area was the monocyte differential count (r = 0.59, P = 0.01, n = 18).

**DISCUSSION**

The analysis of our results reveals a highly significant effect of op genotype on atherosclerosis lesion area, such that the op/E0 mice had significantly reduced atherosclerosis lesion areas and qualitatively less advanced lesions than their op/E0 and op/E0 littermates, despite cholesterol levels averaging >1300 mg/dl. There was also a significant gender effect on lesion area: females had larger lesions than males. In the females, lesion area correlated positively with monocyte differential count and correlated negatively with plasma cholesterol, although these parameters along with body weight did not affect lesion area independently when adjusted for gender and op genotype. For example, the high cholesterol seen in the op/E0/E0 mice is obviously not the cause of their smaller lesions, as we know that feeding apoE-deficient mice a high-fat diet increases both cholesterol levels and atheromatous lesion area (3). Rather, we believe that high cholesterol and small lesions are both an effect of the op genotype. Therefore, the M-CSF gene dosage is the central cause of the observed differences in lesion area, body weight, total adult body weight, and monocyte number. These data support the hypothesis that M-CSF, acting by altering either the number or some property of monocyte-derived macrophages, may profoundly influence atherosclerosis susceptibility. Macrophage number is affected by M-CSF deficiency in op mice, as there are moderately decreased tissue macrophages in many, but not all, tissues (7, 9, 15). An example of a macrophage property that may be altered by M-CSF deficiency is the level of scavenger receptors. Probably the macrophages in the aorta and other tissues of op mice express less scavenger receptors, as M-CSF has been shown to increase scavenger receptor synthesis, to increase mRNA, and to alter cellular scavenger receptor distribution in murine macrophages (22). Another possibility is that M-CSF, which is produced locally by smooth muscle cells and macrophages in atherosclerotic lesions (21), could lead to lesion macrophage proliferation.

Atherogenesis in the apoE-deficient mouse may be stimulated by factors in addition to the high levels of cholesterol-enriched VLDL. Macrophages express apoE, which is up-regulated by cholesterol loading (31), and apoE might play a protective role against foam cell formation by aiding in macrophage cholesterol efflux. The negative correlation observed here between plasma cholesterol and lesion area in female mice, combining all genotypes (Fig. 4B), may be due to a positive effect of M-CSF gene dosage on βVLDL uptake by macrophages, which could lead to both increased lesion growth and decreased plasma cholesterol. Within any one of the genotypes, this negative correlation was only statistically significant in the op/E0 females, perhaps due to more variation in M-CSF levels in mice heterozygous for the wild-type M-CSF allele.

Another striking finding in our study was the increased lesion area for the female op/E0 and op/E0 mice without higher levels of plasma cholesterol, compared with their male littermates. In a small sample of heterozygous apoE-deficient mice fed a high-fat/high-cholesterol/cholate-containing diet, females were found to have 6-fold larger lesions than males, although the females also had more marked hypercholesterolemia in response to the diet (32). Lesions were also larger in females compared with males in wild-type mice of three different strains fed a high-fat/high-cholesterol/cholate-containing diet, with no obvious correlation between sex and total or HDL cholesterol observed (25). Our data show that

![Figure 3: Fast protein liquid chromatography analysis of plasma from op/E0 and op/E2 mice.](image-url)
the gender effect on lesion area can be observed in the absence of extreme dietary stress and highlight the importance of animal males and females separately.

Results of the current study corroborate the previous results of the cholesterol-lowering effect of injected MCSF (17–19), in that the op0/E0 mice, without MCSF, have increased plasma cholesterol levels compared with op1/E0 and op2/E0 mice. However, our finding of decreased atherosclerosis in the MCSF-deficient op0/E0 mice does not corroborate the atherosclerosis-reducing effect of injected MCSF in Watanabe rabbits (23). One possible reason for this discrepancy is the comparison between two different situations, one in which MCSF is subtracted from the basal level and the other in which MCSF is added to the basal level. These models also differ in respect to their cause of hyperlipidemia (apoE deficiency vs. LDL-receptor deficiency) and the class of lipoprotein accumulating (βVLDL vs. LDL).

This study shows that MCSF deficiency leads to decreased atherosclerosis and lends support to the hypothesis that the number and/or functions of monocyte-derived macrophages play a key role in atherogenesis. In the doubly mutant op0/E0 mice, lesions were smaller and limited to early stages of progression. In the quest to develop different means to protect against and treat atherosclerosis, there are many potential sites that could be targeted for genetic or drug-mediated disruption in the processes of monocyte transendothelial migration, differentiation into macrophages, and macrophage uptake of modified lipoprotein particles. The current study supports the notion that these novel approaches may be beneficial and displays the use of the apoE-deficient mouse as an excellent rodent model system to test these potential atherosclerosis-modifying strategies.

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