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Heterozygous Osteopetrotic (op) Mutation Reduces Atherosclerosis in LDL Receptor–deficient Mice

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Abstract

Previous studies of osteopetrotic (op) mice lacking macrophage colony-stimulating factor (M-CSF) have revealed an inhibition of atherosclerosis development in the apolipoprotein E (apo E)-deficient model and in a diet-induced model. Using LDL receptor–deficient mice, we now show that atheroma development depends on M-CSF concentration, as not only did homozygous osteopetrotic (op/op) mice have dramatically reduced lesions (~0.3% of control lesion size) but heterozygous (op+/+) mice had lesions <1% of controls. Mice heterozygous for the op mutation (op+/+) had plasma levels of M-CSF about half those in controls (+/+). The finding that an ~2-fold reduction in M-CSF expression reduced lesion size ~100-fold suggests the requirement for a threshold level of M-CSF. The effect of M-CSF on atherosclerosis did not appear to be mediated either by changes in plasma lipoprotein levels or alterations in the number of circulating monocytes, since both op/op and op/+ mice exhibited higher levels of atherogenic lipoprotein particles and (op+/+) mice showed a near normal number of circulating monocytes. LDL receptor–null littermates of genotypes from op/op, op/+, to +/+ showed monocyte differentials of ~4.5, 8, and 10%, respectively. Taken together, these results suggest that the effects of M-CSF on atherogenesis may not be mediated by expression of M-CSF systemically or by modulation of the number of circulating monocytes. These studies support the conclusion that M-CSF participates critically in fatty streak formation and progression to a complex fibrous lesion. (J. Clin. Invest. 1998, 101:2702–2710.) Key words: hypercholesterolemia • atherogenesis • osteopetrosis • monocytes • macrophages

Introduction

Recruitment, activation, survival, and proliferation of mononuclear phagocytes in the vessel wall contribute importantly to atherosclerosis (1–3). Several adhesion molecules, chemotactic cytokines, and growth factors may orchestrate these events in atherosclerosis (2, 3). Accumulating evidence suggests that macrophage-colony stimulating factor (M-CSF) can play a key role in this process. M-CSF selectively regulates the growth and survival of mononuclear phagocytes by binding to a specific cell surface receptor, c-fms (4–8). In addition, M-CSF functions as a chemotactic factor for monocytes, regulates the effector functions of mature monocytes and macrophages, and modulates inflammatory responses by stimulating the production of other cytokines and growth factors (4, 9, 10). Atherosclerotic lesions derived from humans and rabbits contain elevated levels of M-CSF mRNA and immunoreactive protein (11–13). Atherogenic oxidized LDLs induce aortic endothelial and smooth muscle cell expression of M-CSF, in part through activation of nuclear factor-κB (11, 14, 15). Macrophages proliferate within atherosclerotic lesions (16, 17). M-CSF stimulates proliferation of monocyte precursors and is important for the survival of macrophages in culture and in vivo (4–8). Thus, the localized expression of M-CSF within the vessel wall may be critical in promoting the survival of lipid-laden foam cells observed in early and advanced stages of atherosclerosis. M-CSF also regulates systemic lipoprotein levels by enhancing the clearance of LDL through both LDL receptor (LDLR)-dependent and -independent pathways (18–21). M-CSF stimulates cholesterol esterification in human monocyte-derived macrophages and modulates lipoprotein lipase secretion in macrophages (22, 23). The ability of M-CSF to enhance the uptake and degradation of modified lipoproteins by upregulating scavenger receptor may contribute to the removal of oxidized lipoproteins from extracellular spaces and the generation of foam cells (12, 24).

Osteopetrotic (op/op) mice that lack M-CSF due to a point mutation in the M-CSF gene have proven to be useful in examining the role of M-CSF in the growth and function of mononuclear cells in vivo (25–29). Lack of M-CSF in op/op mice results in impaired growth and differentiation of monocytes and their precursors in bone marrow, causing a deficiency of blood monocytes and peritoneal and tissue macrophages (26, 27). These mice also show severe deficiency of osteoclasts, resulting in impaired bone remodeling and skeletal deformities (25–

1. Abbreviations used in this paper: LDLR, LDL receptor; M-CSF, macrophage colony-stimulating factor; op, osteopetrotic.
Homozygous (op/op) mice have domed skulls and lack teeth, impairing consumption of solid food. Chronic injection of recombinant M-CSF partially corrects the phenotypic defects in op/op mice (8).

We and others have used these mice fed an atherogenic diet or crossed with hypercholesterolemic apo E–null mice as a model to examine the role of M-CSF deficiency on atherogenesis, arterial calcification, and atherosclerotic lesion histology (28, 29). Mice lacking either apo E or LDLR exhibit hypercholesterolemia and develop advanced atherosclerotic lesions similar to those observed in humans (30–33). Our previous studies have provided evidence that M-CSF deficiency in mice fed an atherogenic diet or having an apo E–null genotype results in reduced fatty streak development and atherosclerosis but increased arterial calcification (28, 29). This report describes findings regarding the effects of M-CSF deficiency on atherosclerosis in the LDLR-null mouse, an animal model for human familial hypercholesterolemia. By crossing LDLR-null mice with op/op mice, we have developed mice strains highly susceptible to atherosclerosis in which the role of M-CSF deficiency could be assessed. Our present results indicate that complete M-CSF deficiency in LDLR-null mice also significantly reduces atherosclerosis but, most remarkably, they demonstrate concentration-dependent effects of M-CSF expression on atherogenesis as mice heterozygous for the op mutation exhibited an ~100-fold decrease in lesion size. The LDLR-null mice heterozygous for the op mutation exhibited an ~20% reduction in the number of circulating monocytes as compared with controls, suggesting that the number of monocytes in circulation may not explain the effect of M-CSF on lesion development.

**Methods**

**Mice.** Breeding pairs of homozygous LDLR (−/−) mice on a C57BL/6J background and heterozygous (op+/+) mice on a C57BL/6J × C3HeB/FeJ hybrid genetic background were purchased from The Jackson Laboratory (Bar Harbor, ME). Heterozygous (op+/+) mice were subsequently backcrossed for four generations to inbred strain C57BL/6J mice (28) to produce a homogeneous genetic background. Mice were housed in a virus antibody-free environment, maintained in a temperature-controlled room with a light/dark cycle and fed a chow diet (Purina). Mice lacking M-CSF (op/op) were fed powdered diet in a liquid suspension using a glass feeding tube to optimize food intake and growth during the period after weaning (28). At the age of 3 mo, control C57BL/6J mice, heterozygous (op+/+) mice, and homozygous (op/op) mice on the C57BL/6J background were fed with the atherogenic diet for 16 wk. The atherogenic diet (TD 90221; Food-Tek, Inc., Morris Plains, NJ) contained (wt/wt) 15% fat, 1.25% cholesterol, and 0.5% cholic acid (28).

**Genotyping.** Tail tips were digested in solution containing SDS and proteinase K, and DNA was extracted by phenol-chloroform, precipitated, and stored at −20°C. Tail DNA was amplified by PCR in a total reaction volume of 0.5 ml and was subjected to restriction enzyme digestion with BglII and NcoI (12). Each PCR reaction consisted of 0.5 ml of PCR reaction mix (2X PCR buffer, 200 μM dNTPs, 200 μM MgCl2, 1 units of Taq polymerase) and 5–10 μg of DNA template. The PCR products were separated on a 12% polyacrylamide-urea DNA sequencing gel. The bands were visualized by autoradiography. PCR products for wild-type and mutant LDLR alleles were visualized by agarose gel electrophoresis in the presence of ethidium bromide. In certain circumstances, we also used the Southern blotting method for genotyping. Briefly, 5–10 μg samples of each DNA preparation from normal and mutant mice were subjected to restriction enzyme digestion, agarose gel electrophoresis, and transfer to nylon filters followed by cross-linking by ultraviolet irradiation. Under standard conditions, filters were hybridized with specific probes, washed, and autoradiographed.

**Blood monocyte, M-CSF ELISA, and lipid analysis.** Blood from overnight fasted mice was collected from retroorbital plexus into a tube containing EDTA. Blood smears on glass slides were prepared immediately for monocyte counting. The dried smears were stained with Diff-Quik (Baxter Healthcare Corp., Deerfield, IL), and monocytes were determined from a minimum of 100 white blood cells as described previously (29). Plasma was collected by centrifugation at 4°C. Concentration of M-CSF in mice serum was determined using an ELISA developed in our laboratory with a polyclonal rabbit anti-M-CSF antibody (35). Plasma concentrations of total and HDL cholesterol were measured in duplicate or triplicate by an enzymatic assay (28, 33). Mouse plasma lipoproteins were fractionated by FPLC as described previously (36). Plasma fractions were pooled for each genotype in equal proportion from all mice within each group and 0.2 ml was applied to the column. Cholesterol content was determined in 100-μl aliquots of each 0.5-ml FPLC fraction (36).

**Qualitative and quantitative analyses of atherosclerotic lesions.** Methods for the qualitative and quantitative analyses of atheromatous lesions in the aorta have been reported previously (28, 33). Briefly, mice were killed by cervical dislocation at 7 mo of age. The heart and proximal aorta (including ascending aorta, aortic arch, and a portion of descending aorta) were excised and washed in PBS to remove blood. The basal portion of the heart and proximal aorta were embedded in OCT compound (Tissue-Tek, Elkhart, IN), frozen on dry ice, and then stored at −70°C until sectioning. Serial 10-μm-thick cryosections (every fifth section from the lower portion of the ventricles to the appearance of aortic valves, every other section in the region of the aortic sinus, and every fifth section from the disappearance of the aortic valves to the aortic arch) were collected on poly-D-lysine–coated slides. Sections were stained with oil red O and hematoxylin and counterstained with fast green for the identification of atheromatous lesions, arterial wall calcification, and cartilaginous metaplasia (28, 33, 37). The presence of calcium deposits was confirmed by the alizarin red S and von Kossa techniques using representative sections.

**Statistical analysis.** Data analysis was performed using Statview (Student’s t test and ANOVA) software for the Macintosh. The threshold of significance for multiple comparisons among various groups of mice was adjusted according to the Bonferroni method using the formula for the adjusted level of significance: 0.1/(2 × number of comparisons).

**Results**

**Breeding and genotyping.** We intercrossed heterozygous (op+/+) males with homozygous LDLR (−/−) females following the experimental strategy described previously (28). Half of the offspring of this cross were heterozygous for both M-CSF and LDLR mutations. The first generation was screened for the op and LDLR mutations by using PCR on the genomic DNA isolated from the tail tips. Identified double heterozygotes for both LDLR and M-CSF were interbred by brother–sister mating to obtain homozygous (op/op) mice on an LDLR-null genotype. To enhance the yield of double nulls (op/op, LDLR-null), heterozygous op+/+ brothers and sisters on LDLR-null background were interbred.

Animal husbandry of the mice that lacked either M-CSF or M-CSF and LDLR was very critical. These mice were initially...
raised for 12 wk on a semipurified cholesterol-free AIN76A diet (28, 34). The diet was modified to allow feeding as a liquid suspension in order to optimize growth and survival in these mice with poor dentition after weaning. M-CSF–deficient op/op mice do not grow as well as op/1 or 1/1 mice even before weaning. We suspect that this is due to their inability to compete with normal littermates for milk from the dam. Our studies show that the op/op mice have the capacity to grow at a rate equivalent to normal after weaning if the mice are isolated from normal littermates and fed a nutritionally complete and readily consumable diet. As described previously, there were compensatory weight gains in the op/op mice during 0–4 wk of feeding after weaning as compared with normal littermates (28).

**M-CSF deficiency augments hypercholesterolemia and reduces monocyte numbers in LDLR-null mice.** When LDLR-null mice of op/op genotype consumed an atherogenic cholesterol- and cholate-supplemented diet, they exhibited enhanced hypercholesterolemia compared with littermates having either +/+ or op/+ genotypes (Fig. 1, A and B). Total plasma choles-

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**Figure 1.** (A) Effects of M-CSF deficiency on the total plasma cholesterol in LDLR-null mice maintained on a high-fat, high-cholesterol atherogenic diet. Genotypes were used as group variables. Values are expressed as mean±SEM. *P < 0.001. (B) FPLC separation of the LDLR-null mouse plasmas having genotypes op/op, op/+, or +/+ for M-CSF gene mutation. Plasma fractions were pooled for each genotype in equal proportion from all mice within each group and 0.2 ml was applied to the column. Cholesterol content was determined in 100-μl aliquots of each 0.5-ml FPLC fraction. The bars indicate the volumes in which VLDL, LDL, and HDL elute from the column.
terol levels in the double mutant mice averaged ~2,200 mg/dl in the op/op, ~50% higher than hypercholesterolemic LDLR-null littersmates. The cholesterol in each of the groups was primarily in VLDL, LDL, and HDL. The levels of HDL cholesterol in all genotypes were comparable, averaging ~84 mg/dl (Fig. 1 B). There were no significant gender influences on the total and HDL cholesterol. Monocyte differential counts in the LDLR-null mice showed that total absence of M-CSF resulted in reduced number of peripheral blood monocytes to less than half as compared with mice having full M-CSF activity. LDLR-null littersmates of genotypes from op/op, op/+, to +/- showed a gene dosage effect having monocyte differentials of ~4.5, 8, and 10%, respectively (Fig. 2). The concentration of M-CSF in the sera of op/+ mice was 65% of that in +/+ mice, and op/op mice showed a complete absence of circulating M-CSF (Table I).

Partial as well as total absence of M-CSF in hypercholesterolemic LDLR-null mice decreases atherosclerosis. The diet-induced atherosclerosis in LDLR-null mice without op mutation was severe, particularly in the aortic root, ascending aorta, and aortic arch (Figs. 3 and 4). We quantitated the aortic atheromatous lesions in mice with three different genetic combinations in the M-CSF and LDLR loci (Table II and see Fig. 3). Four double mutant mice (two males and two females) were almost free of fatty lesions in the aortic root, ascending aorta, and aortic arch (Fig. 3, a and b, and Fig. 4, a and c). Thus, the homozygous M-CSF mutation, genotype op/op, significantly reduced the development of atheromatous lesions in these regions (Table II and Figs. 3–5). The mice heterozygous for the M-CSF mutation (op+) also exhibited remarkably reduced atheromatous lesion formation (Fig. 3, c and d, and Table II). After 16 wk of the atherogenic diet, the diet-induced atheromatous lesions in the aortic root of op/+ mice were much smaller than those of LDLR-null mice (Table II and Fig. 3). There were no advanced lesions (with fibrous cap) in op/+ mice on this genetic background. Among LDLR-null mice, the frequency of atherosclerosis in the aortic arch was 0/4 in the presence of homozygous op mutation, 0/10 in the presence of heterozygous op mutation, and 10/12 in the absence of op mutation.

### Discussion

This study shows that absence of M-CSF in LDLR-null mice fed a high-fat, high-cholesterol diet augments hypercholesterolemia but significantly reduces formation of atherosclerotic lesions. These results agree with previous studies of atherosclerosis in M-CSF-deficient mice fed an atherogenic diet or on a hypercholesterolemic apo E–null background (28, 29). These results also emphasize two important additional conclusions. First, the effect of M-CSF expression is clearly concentration-dependent, since mice heterozygous for the op mutation express 35% lower than normal levels of M-CSF (26, 27, Table I) yet they exhibit <1% of the lesion development of LDLR-null mice with two normal M-CSF alleles. Second, our results suggest that the effect of the M-CSF mutation on atherogenesis is not mediated by effects on the number of circulating monocytes, since in heterozygous mice the number of circulating monocytes was decreased by only ~20%, whereas the lesion volume decreased by >100-fold. The effects of the op mutation on lesion development were more striking in this study than in previous studies using diet-induced atherosclerosis or an apo E–null model (28, 29). Presumably, this resulted from the LDLR-null background in the present study, although another confounding effect could be the noninbred genetic backgrounds used in some of the previous studies. The lesions observed in this study were also substantially larger than those previously studied. The effect of heterozygous op mutation was particularly striking on LDLR-null backgrounds; for example, whereas Smith et al. (29) observed a <40% decrease in lesions in op/+ mice as compared with +/+ mice on an apo E–null background, there was a >100-fold decrease in lesions of op/+ mice as compared with +/+ mice on an LDLR-null background. There may well be some differences in lesion development between the dietary, apo E–null, and LDLR-null models; for example, we have observed that the early lesions in LDLR-null mice are particularly lipid rich.

### Table I. M-CSF Concentration in Sera of LDLR-null Mice Having op/op, op/+, and +/- Genotypes

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<tr>
<td>op/op</td>
<td>0 (6)</td>
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<tr>
<td>op/+</td>
<td>956 ± 102 (6)</td>
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<tr>
<td>+/-</td>
<td>1455 ± 132 (6)</td>
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The number of mice sampled per group is given in parentheses. Values are represented as the means ± SD of results of duplicate assays on samples derived from individual mice. 1 unit of M-CSF is equivalent to 13 pg of protein.
Figure 3. Effect of *op* mutation on atheromatous lesions in LDLR-null mice. All sections were from mice homozygous for the LDLR-null mutation. (a) Cross-section of aortic root from *op/op* mouse showing aortic wall with no evidence of fatty lesion formation. ×12.5. (b) High-power view of *a* showing a normal valve attachment and its related aortic wall. ×50. (c) Cross-section of aortic root from *op/+* mouse showing a raised type II lesion in free aortic wall (*arrow*) and a type I lesion in valve attachment (*arrowhead*). ×12.5. (d) High-power view of *c* showing raised type II lesions in free aortic wall. ×50. (e) Cross-section of aortic root from *+/+* mouse (no *op* mutation) showing advanced type I and type II lesions. ×10. (f) High-power view of *e* showing a large advanced type II lesion with fibrous cap (*arrowhead*) and necrotic core (*N*). ×50. Mice were maintained on a high-fat, high-cholesterol diet for 16 wk. All sections were stained with oil red O, hematoxylin, and fast green.
If the effect of M-CSF on lesion formation is not attributed to levels of circulating monocytes or lipoprotein particles, it presumably results from effects on the functional properties of circulating monocytes or effects at the level of the vessel wall. It is possible that there is heterogeneity in the circulating monocytes and that classes of monocytes contributing to the lesion formation are reduced to a greater extent. We have shown previously that oxidized LDL particles substantially induce M-CSF in endothelial cells and that M-CSF expression is elevated in atherosclerotic lesions as compared with the normal artery wall (11, 12). Since monocyte migration from the arterial lumen to the subendothelial space occurs in the initial stages of atherosclerosis, it is likely that augmented expression of M-CSF in the vessel wall, in combination with the increased expression of other monocyte chemotactic and adhesion molecules such as VCAM-1 and MCP-1, results in enhanced migration of monocytes to the subendothelial space. It is noteworthy that all the cells of the vessel wall, including endothelial cells, smooth muscle cells, and monocyte-macrophages, are capable of producing M-CSF. The observation that human M-CSF stimulates the expression of MCP-1 and increases the adhesion of monocytes to endothelial monolayers further supports this conclusion. The presence of M-CSF in the vessel wall may also stimulate the differentiation of newly recruited monocytes into macrophages. Macrophages proliferate within atherosclerotic lesions and M-CSF stimulates the proliferation and survival of macrophages in culture and in vivo, suggesting that its induced expression in atherosclerotic lesion may also directly stimulate the proliferation of monocyte-macrophages. The ability of M-CSF to enhance the uptake and degradation of modified lipoproteins and cholesterol esterification, possibly by augmenting scavenger receptors, may lead to lipid loading of macro-

Figure 4. Effect of op mutation on fatty lesion formation in ascending aorta and aortic arch of LDLR-null mice. All mice were homozygous for the LDLR-null mutation. (a) Cross-section of ascending aorta from op/op mouse showing no evidence of atheromatous lesion. ×12.5. (b) Cross-section of ascending aorta from +/+ (no op mutation) mouse showing a large advanced atheromatous lesion (arrowheads). ×13. (c) Cross-section of aortic arch from op/op mouse showing no evidence of atheromatous lesion. ×25. (d) Cross-section of aortic arch from +/+ (normal M-CSF gene) showing atheromatous plaques (arrowheads). ×25. Mice were maintained on a high-fat, high-cholesterol diet for 16 wk. All sections were stained with oil red O, hematoxylin, and fast green.
Aortic lesions are expressed as mean±SEM. ANOVA among different strains was as follows: LDLR −/−, +/+ vs. LDLR −/−, op/op, P < 0.01; LDLR −/−, +/+ vs. LDLR −/−, op/+; P < 0.01; LDLR −/−, op/op vs. LDLR −/−, op/+, P < 0.01. As multiple comparisons of these data were performed, the threshold significance levels should be adjusted. Using the formula for the adjusted level of significance = 0.1/(2 × number of comparisons), the level of significance for the effect of the op mutation on lesion size in LDLR knockout mice with three groups (op/op, op/+, +/+ ) is calculated as < 0.016.

The striking concentration dependence of lesion development on M-CSF expression in the present studies suggests a threshold effect. The fact that op/+ mice express ∼65% of normal levels of M-CSF (Table I) yet exhibit < 1% of the lesion development of LDLR-null mice with two normal M-CSF alleles indicates that a minimal amount of M-CSF is required for rapid progression of atherogenesis. The present data show that even in the presence of hypercholesterolemia, a 35% decrease in M-CSF levels does not appreciably influence the number of circulating monocytes or the number of monocyte-macrophage progenitor cells in the bone marrow (26, 27). The substantial induction of M-CSF that occurs in atherosclerotic C57BL/6J mice, but not in resistant C3H mice, fed an atherogenic diet (15), suggests that M-CSF may contribute to genetic differences on lesion development in dietary models. Perhaps the level of M-CSF expression in the artery wall of op/+ mice is insufficient to permit proliferation or survival of vascular monocyte-macrophages.

Numerous studies have documented large differences in lesion size between male and female C57BL/6 mice. The mechanisms of this remain unclear, although it is related to sex hormones, since implanting testosterone pellets in female mice decreased lesion development (39). It should be noted that genetic differences similarly influence lesion sizes in males and females. Thus, for example, male apo E–null mice develop fewer lesions than female apo E–null mice, but in both genders lesion development is dramatically elevated as compared with wild-type mice. Previous studies with op/op mice have generally been complicated by the effects of M-CSF deficiency on nutritional status and by the mixed genetic background of commercially available mice (28, 29). A mixed genetic background may contribute to the observed results because of genetic variability. We minimized this problem in our experiments by transferring the op mutation to an inbred (C57BL/6J) genetic background through selective breeding. Poor denu-
consumption, as the animals cannot eat solid food. Reduced growth rates due to malnutrition could clearly contribute to the reduced atherosclerosis. We made efforts to eliminate this problem by developing and providing a liquid suspension of a powdered diet to maintain the body weight of the op/op mutants in the range of 30 g. However, the op/+ mice do have normal teeth, eating behavior, and growth, indicating that the problems with food intake do not contribute to reduced atherosclerosis.

Augmented hypercholesterolemia in LDLR-null mice due to total absence of M-CSF is consistent with previous findings showing that injection of M-CSF lowers plasma cholesterol levels (18–22). The induced expression of scavenger receptor by M-CSF may explain its effect on lowering of plasma cholesterol levels in humans, nonhuman primates, and hypercholesterolemic rabbits (21, 22). M-CSF enhances the clearance of LDL through both LDLR-dependent and -independent pathways in rabbits, stimulates cholesterol esterification in human monocyte-derived macrophages, and modulates lipoprotein lipase secretion in macrophages (21–23). The cholesterol-lowering effects of M-CSF may protect the vessel from developing atherosclerosis. Thus, the role of M-CSF appears to be paradoxical in the context of atherosclerosis.

In summary, our data support a crucial role for M-CSF in atherosclerosis. In particular, heterozygous mice with ~65% of normal levels of M-CSF have, like op/op mice, dramatically reduced lesion development. We hypothesize that locally produced M-CSF in the vessel wall, by influencing the recruitment, growth, survival, and function of monocyte-macrophages, may contribute to the development and progression of atherosclerosis. Additional studies are required to establish this conclusively. These results agree with the conclusion that monocyte-macrophages promote atherogenesis and are prerequisites for the development of fibrous atherosclerotic lesions. M-CSF exists in at least three isoforms and can perform requisite for the development of fibrous atherosclerotic lesions. M-CSF exists in at least three isoforms and can perform requisite for the development of fibrous atherosclerotic lesions. M-CSF exists in at least three isoforms and can perform

Acknowledgments

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References


