Lack of Complement Factor C3, but Not Factor B, Increases Hyperlipidemia and Atherosclerosis in Apolipoprotein E−/− Low-Density Lipoprotein Receptor−/− Mice

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Objective—To investigate the effect of complement deficiency on atherogenesis and lipidemia, we used mice deficient in the third complement component (C3−/−) or factor B (FB−/−).

Methods and Results—Complement-deficient mice were crossed with mice deficient in both apolipoprotein E and the low-density lipoprotein receptor (ApoE−/− LDLR−/−). The percent lesion area in the aorta at 16 weeks, determined by en face analysis, was 84% higher in C3−/− mice than in controls (11.8±0.4% versus 6.4±0.8%, mean±SEM, P<0.00005). The C3−/− mice also had 58% higher serum triglyceride levels (P<0.05) and a more proatherogenic lipoprotein profile, with significantly more low-density lipoprotein cholesterol and very-low-density lipoprotein triglycerides than control mice. The C3−/− mice weighed 13% less (P<0.01) and had a lower body fat content (3.5%±1.0% versus 13.1%±3.0%, P<0.01). There were no differences between FB−/− mice and controls.

Conclusions—Complement activation by the classical or lectin pathway exerts atheroprotective effects, possibly through the regulation of lipid metabolism. (Arterioscler Thromb Vasc Biol. 2004;24:1062-1067.)

Key Words: atherosclerosis ■ complement ■ C3 ■ factor B ■ hyperlipidemia

The complement system plays an essential role in the humoral immune response. Soluble complement components are present in the blood in precursor forms and need to be activated to fulfill their specific physiological roles. Activated complement has diverse functions, including the initiation of inflammation, recruitment of leukocytes, clearance of immune complexes, neutralization of pathogens, regulation of antibody responses, and disruption of cell membranes. The complement cascade can be activated by 3 pathways. The classical activation pathway depends on assembly of complement factors at sites of antigen–antibody complexes. The lectin pathway is initiated by mannann-binding lectin bound to pathogen surfaces. Activation of the alternative pathway is triggered by a variety of pathogen surfaces and requires the interaction of third complement component (C3), factor B (FB), and factor D. Regardless of the pathway, activation leads to the cleavage of C3. This generates the smaller, proinflammatory C3a fragment and the larger C3b fragment. C3b can act as an opsonin and triggers the terminal part of the cascade, which culminates in the assembly of the terminal complement complex (TCC) on the target surface.

C3-derived peptides have been implicated in the regulation of lipid metabolism. C3a and C3aMet,Arg, a peptide formed when the C-terminal arginine is removed from C3a by carboxypeptidase N, can act as acylation-stimulating protein (ASP).1 ASP has been implicated in adipose tissue function and maintenance of metabolic homeostasis.2 ASP and C3a increase fat storage in adipocytes through increased triglyceride synthesis3–5 and decreased intracellular lipolysis.6 Mice that are deficient in C3, and therefore unable to synthesize ASP, have delayed triglyceride clearance,6–8 which can be normalized by administration of ASP.9,10 However, Wetsel et al did not detect any impaired ability of C3−/− mice to clear triglycerides and free fatty acids from the circulation after an oral fat load.11 FB−/− mice are viable and fertile and have no overall abnormalities of the immune system.12,13 The effect of FB deficiency on adipose tissue and blood lipid profiles has thus far not been studied.

Atherosclerosis is characterized by an inflammatory response to the accumulation of subendothelial lipids, and there is evidence that the activated complement system is involved in atheroma formation.14 Complement components and activation products have been identified in the walls of diseased vessels,15–18 and mRNAs of complement components are expressed in atherosclerotic plaques. However, the role of complement inhibitors is more controversial.19,20 The degree of TCC deposition correlates with the severity of arterial...
Serum Anti-LDL Antibody Measurements

Specific antibodies to malondialdehyde (MDA)-modified low-density lipoprotein (LDL) were quantified by enzyme-linked immunosorbent assay. 35

Histological Evaluation and Immunohistochemistry

Aortic root sections were stained with hematoxylin and erythrosine or Masson trichrome (Bio-Optica, Milan, Italy) to identify foam cells, fibrosis, cholesterol clefts, acellular areas, and fibrous cap in atherosclerotic plaques. For immunohistochemistry, sections were fixed in acetone, incubated with 1% bovine serum albumin in phosphate-buffered saline--Tween, and stained with MOMA-2 (Sero- tec, Oxford, UK) and a-smooth muscle actin (EPOS; Dako A/S, Glostrup, Denmark) antibodies. The MOMA-2 antibody was detected by biotin-conjugated rabbit antiserum against rat immunoglobulins (Dako A/S) followed by ABC Vectastain Elite kit (Vector Laboratories, Burlingame, Calif) and visualized with 3',3'-diaminobenzidine tetrahydrochloride (Sigma).

Statistical Analysis

The results are presented as mean±SEM. The data were analyzed by unpaired t test. Repeated-measures analysis of variance was used to analyze the postprandial triglyceride clearance data. Differences were considered statistically significant at P<0.05.

Results

C3 Deficiency Increases Lesion Size at 16 Weeks of Age

Lesion size increased with age in all groups examined. All mice had advanced lesions composed of foam cells and abundant extracellular lipid at 16 weeks and fully developed atheromas with lipid-rich core regions covered by fibrous caps at 26 weeks. En face analysis of aortas at 16 weeks (Figure 1A and 1C) showed that the lesions were 84% larger in C3−/− than in complement-sufficient mice (11.8±0.4% versus 6.4±0.8% of surface area, P<0.00005). At 26 weeks, however, the lesions were of similar size in the 2 groups (Figure 2A). There were no differences in lesion size between FB/−/− mice and controls at any time point (Figure 2B). In serial sections of the aortic root, the extent of atherosclerosis did not significantly differ between complement-deficient mice and control Apoe−/− LDLR−/− mice (not shown).

Atherosclerotic Lesions Are Qualitatively Similar in C3−/− and Control Mice

Morphological examination of aortic root sections at 16 weeks revealed lesions in all mice, ranging from fatty streaks composed of foam cells to advanced fibrofatty lesions covered by fibrous cap. At 26 weeks, the lesions often covered the whole inner circumference of the vessel, and all were exclusively of the fibrotheromatous type, the majority with large acellular areas, a fibrous cap of varying thickness, and extensive necrosis. No difference in plaque progression was noted between groups. As shown by MOMA-2 staining, macrophages were the predominant cell type in all lesions, although lesions in the older mice were less cellular. The extent and distribution of MOMA-2 staining were similar in complement-deficient mice and controls. Fibrous caps, visualized by a-smooth muscle actin immunostaining, were observed in all sections analyzed, with no difference between the experimental groups (Figure 1, available online at http://atvb.ahajournals.org).
C3 Deficiency Increases Serum Triglycerides and Alters Plasma Lipoprotein Profiles

In C3−/− mice, serum triglyceride levels were 58% higher than in controls at 16 weeks (6.8±0.9 versus 4.3±0.4 mmol/L, *P<0.05) and 79% higher at 26 weeks (8.4±1.3 versus 4.4±0.6 mmol/L, *P<0.05) (Figure 3A). C3 deficiency did not affect serum cholesterol levels at any age (Figure 3B). Serum cholesterol and triglyceride levels were similar in F8−/− and control mice (Figure 3C and 3D). Superose 6 chromatography of pooled plasma showed a more proatherogenic lipoprotein profile in C3−/− mice, with significantly more LDL cholesterol and very-low-density lipoprotein (VLDL) triglycerides than controls at both 16 and 26 weeks (Figure 4).

C3−/− Mice Have Normal Postprandial Triglyceride Clearance

Next, we determined if the increased serum lipid levels of the C3−/− mice could be explained by an aberrant postprandial
lipid clearance. No differences in serum triglyceride levels were observed after an oral fat load in 21-week-old C3−/− (n=4) and control mice (n=5) (not shown).

C3 Deficiency Reduces Body Weight and Body Fat
Compared with controls, C3−/− mice weighed 13% less at 16 weeks (34.2±0.96 versus 38.8±1.29 g, n=17/group, P<0.01) and 22% less at 26 weeks (34.7±1.12 g, n=10, versus 42.3±1.93 g, n=11, P<0.005). There were no differences in body weight between the FB−/− and control mice at either time point. As shown by DXA analysis of body composition, C3−/− mice had approximately two-thirds less body fat than controls at 16 weeks (3.5±1.0% versus 13.1±3.0%, P<0.01) and 26 weeks (2.8±0.7% versus 9.3±1.9%, P<0.01) (Figure 5).

C3 Deficiency Does Not Alter Anti-MDA-Modified-LDL Antibody Titers
To determine if the effect of complement on atherogenesis could result from changes in B-cell–associated protective immunity, we assessed the immunoglobulin M (IgM) and immunoglobulin G (IgG) isotypes of autoantibodies against MDA-modified LDL. There were no differences in anti-MDA–LDL IgM or IgG titers between C3−/− mice and controls (not shown).

Discussion
Complement activation has been suggested to contribute to the progression of atherosclerotic lesions, and complement-derived ASP has been implicated in the regulation of lipid metabolism. To address the relative roles of the classical and
lectin pathways versus the alternative pathway of complement activation in these processes, we studied C3-/− and FB-/− mice.Remarkably, the aortic lesions were 84% larger in C3-/− mice than in complement-sufficient mice at 16 weeks of age. This finding is consistent with a recent report showing that C3 deficiency increased lipid-positive lesion size and impaired lesion maturation beyond the foam cell stage in 15-week-old LDLR-/−/− mice.30 Because serum lipoprotein/cholesterol profiles were not changed, the authors concluded that local effects of complement lead to lesion differences.

In our study, histopathological and immunohistochemical analyses of the aortic root showed no qualitative differences in lesion morphology between C3-/− and control mice. However, we cannot exclude that the C3 deficiency may have resulted in morphological differences such as reduced collagen and smooth muscle cell content in the lesions distal to the aortic root as reported for the C3-/−/LDLR-/−/− mice.30 In addition, the strong atherogenic drive in the Apoe-/−/−LDLR-/−/− mice might have masked a milder atherogenic effect of the complement system in the vascular wall and might also explain why C3-/− and control mice had lesions of similar size at 26 weeks. Given the possible role of antibodies in atheroprotective immunity, we33,38–40 analyzed sera from C3-/− and control mice for antibodies against MDA–LDL. No differences in antibody levels were detected, indicating that the increased atherosclerosis in C3-/−/− Apoe-/−/−LDLR-/−/− mice is not likely a result of impairments in B-cell–associated protective immunity or protective antibody production.

C3-/− mice also had a more atherogenic plasma lipoprotein profile than controls, with elevated serum triglyceride levels, increased VLDL triglycerides, and increased LDL cholesterol. In addition, the C3-/− mice weighed less and had a lower body fat mass. The latter findings are in line with recent reports showing that C3-/− mice had reduced body weight and fat mass and were resistant to weight gain on a high-fat diet, despite increased food intake.8,41 These phenotypic abnormalities may reflect reduced triglyceride synthesis by adipocytes because of the absence of C3-derived ASP, as proposed by Sniderman et al.42 Thus, the absence of ASP leads to a more atherogenic lipoprotein profile and increased atherosclerosis. Cianflone et al2 suggested that discrepancies in plasma lipid levels and triglyceride clearance in C3-/−/− mice in different reports6–8,11 could be explained by the influence of genetic background, resulting in differences in insulin sensitivity, lipoprotein lipase activity, or triglyceride synthesis by adipose tissue.

In contrast to the results in C3-/− mice, the extent of atherosclerosis, serum triglyceride levels, and body weight were not significantly different in FB-/−/− Apoe-/−/−LDLR-/−/− mice and controls. Because C3, FB, and factor D are produced in adipose tissue, ASP is believed to be generated by the alternative pathway of complement activation. In support of this possibility, there is no evidence that adipocytes produce C2, an essential component of the classical and lectin pathways.5,44 Although FB-/−/− mice have normal C3 levels, they cannot generate ASP through proteolytic cleavage mediated by FB and factor D. Our findings suggest that the alternative pathway is not required for C3-mediated control of energy storage and energy use and that ASP is generated by other mechanisms, such as activation of the classical and/or lectin pathway locally in adipose tissue or in the circulation. Interestingly, genes encoding proteins of the classical pathway are expressed in human adipose tissue.45

An alternative explanation for the phenotype of the C3-/− mice is the absence of uncleaved C3. One possibility worth exploring is the capacity of C3(H2O) to bind to the C3a/ASP receptor C5L246 and to exhibit the functions ascribed to ASP. C3(H2O)—the product of spontaneous hydrolysis of the internal thioester bond of the native C3 molecule—is conformationally and functionally similar to C3b. It can bind FB and form the C3 convertase of the alternative pathway. However, it still contains the C3a fragment47–49 and therefore could, theoretically, act like ASP.

En face assessment of the whole aorta revealed a significant difference in percent lesion area between C3-/− and control mice at 16 weeks of age that were not seen by analysis of aortic root sections. Babaev et al also reported a loss of correlation between lesion areas determined by these two methods when lesions in the proximal aorta became complicated.50 Thus, for detecting effects on disease development, en face analysis of the percent lesion area appears to be a more sensitive method than measuring plaque area in sections from the aortic root, at least in the Apoe-/−/−LDLR-/−/− model.

Conclusion

In conclusion, deficiency in C3, but not FB, leads to increased hyperlipidemia, a more atherogenic lipoprotein profile, more extensive atherosclerotic lesions, and reduced body weight and body fat in Apoe-/−/−LDLR-/−/− mice. These results suggest that complement activation via the classical or lectin pathway exerts atheroprotective effects, possibly through the regulation of lipid metabolism. However, the exact mechanisms remain unclear and further investigation is warranted.

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References


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Figure I. No qualitative differences in atherosclerotic lesions between $C3^{-/-}$ mice and $Apoe^{-/-}LDLR^{-/-}$ controls at 16 or 26 weeks of age were demonstrated by Masson trichrome (MT) staining, MOMA-2 staining, or $\alpha$-smooth muscle actin (ASMA) immunostaining. Representative images are shown. Scale bar, 100 $\mu$m.