Complement C6 Deficiency Protects Against Diet-Induced Atherosclerosis in Rabbits

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Abstract—Low-density lipoprotein (LDL) can be transformed to an atherogenic moiety by nonoxidative, enzymatic degradation. Enzymatically degraded LDL induces macrophage foam cell formation, provokes release of cytokines, and also activates complement. To determine whether complement activation may contribute to atherogenesis, 6 pairs of homozygous C6-deficient rabbits and their non-C6-deficient heterozygous siblings were fed a cholesterol-rich diet for 14 weeks. Cholesterol levels and plasma lipoprotein profiles of the animals in the C6-competent and C6-deficient groups did not significantly differ, and the high density lipoprotein and LDL cholesterol ratios at the end of the experiment were \(0.07 \pm 0.01\) and \(0.08 \pm 0.01\) (SEM), respectively. However, differences in atherosclerotic plaque formation were discernible macroscopically, with extensive aortic lesions being visible in all C6-competent animals and absent in all C6-deficient animals. Aortas were sectioned from thorax to abdomen, and 10 sections were stained from each aorta. Quantification of atherosclerotic lesions and lumen stenosis with the use of computer-based morphometry documented a dramatic protective effect of C6 deficiency on the development of diet-induced atherosclerosis. We conclude that the terminal complement sequence is centrally involved in atherosclerotic lesion progression. (Arterioscler Thromb Vasc Biol. 1998;18:1790-1795.)

Key Words: complement activation ■ atherosclerosis

The possible relevance of complement activation in atherogenesis has not received much attention to date, and only a few reviews are available on the topic.1,2 The first immunohistochemical studies on complement deposition in atherosclerotic lesions appeared in 1985 to 1987,3–5 and C5b-9 complexes were subsequently quantified by ELISA in detergent extracts of lesion homogenates.6 In those studies, the stages of lesion development were not defined, so it could not be excluded that complement activation might have occurred subsequent to tissue damage. An experimental study was then performed in rabbits, leading to the clear demonstration that diet-induced deposition of lipids in the subendothelium was temporally associated with complement activation, which occurred before lesion infiltration by monocytes.7 A directed search led to tentative identification of the complement-activating entity. Heterogeneously sized lipid droplets containing high amounts of free cholesterol were isolated from early lesions and were shown to be capable of spontaneously activating the alternative complement pathway.8 The origin of this lipid, termed the lesion complement activator (LCA), was unknown, but the possibility that it represented an LDL derivative was obvious. To corroborate this assumption, attempts were undertaken to transform LDL in vitro into a complement-activating moiety. This was found to be possible by combined treatment of the lipoprotein with a protease, cholesterol-esterase, and neuraminidase,9 enzymes that occur ubiquitously in lysosomes of mammalian cells and that are required for liberation of cholesterol after LDL is endocytosed via the physiological LDL receptor pathway.10,11 Enzymatically transformed LDL (E-LDL) was not oxidized. Its complement-activating properties were possibly due to the high content of free cholesterol, as also found in LCA.8,9 It has been known for 20 years that nonesterified cholesterol spontaneously activates the alternative complement pathway,12,13 and that neuraminic acid restricts such activation.14 Hence, a simple model for the creation of a complement-activating lipid via enzymatic degradation of LDL emerged.9 E-LDL exhibits additional atherogenic properties: it is rapidly taken up via a scavenger receptor–dependent pathway, thus inducing macrophage foam cell formation,9 and this is accompanied by massive release of MCP-1 from the cells.14a

The question of whether E-LDL might be identical to LCA, and whether this LDL derivative might be detectable in atherosclerotic lesions, was addressed using specific monoclonal antibodies. These antibodies reacted with E-LDL and LCA, but not with native or oxidized LDL, and it became
possible to demonstrate extensive extracellular deposition of E-LDL in all early human atherosclerotic lesions.\textsuperscript{15} Significantly, C5b-9 almost invariably colocalized with E-LDL, corroborating the hypothesis that LDL is enzymatically transformed to a complement-activating moiety at an early stage in atherogenesis. In contrast to E-LDL, oxidized LDL is not endowed with complement-activating properties.\textsuperscript{9}

The possibility now arose that unhalted complement activation may play a deleterious role during lesion progression. A literature search revealed that 1 study had been published on this topic. In 1977, Geertinger and Soerensen\textsuperscript{16} reported that C6-deficient rabbits developed significantly fewer atherosclerotic lesions than did C6-competent animals. This study is rarely cited, possibly because the method used for quantifying lesion development was subjective and because the experimental animals were not clearly characterized. In particular, pairs of homozygous and heterozygous siblings were not used, and in some cases it was stated that C6 deficiency was partial, which is not understandable. In the present work, we therefore reinvestigated this issue under carefully controlled experimental conditions. We report that C6 deficiency is, indeed, highly protective, and the terminal complement sequence now emerges as an effector mechanism that is critically involved in the pathogenesis of atherosclerosis.

**Methods**

**Experimental Animals**

C6-deficient rabbits were originally a gift of Drs K. and U. Rother (Institute for Immunology, University of Heidelberg, Germany), and a breeding colony was established at the Institute of Medical Microbiology and Hygiene, University of Mainz. The animals used in the experiments were 6 pairs of homozygous and heterozygous siblings. C6 deficiency arises from a single gene defect and is not known to be associated with other genetic abnormalities.\textsuperscript{17} Homozygous animals are completely deficient in C6, and their serum is devoid of hemolytic activity. Heterozygous animals have C6 and C6 homologous activity (CH50 titer).\textsuperscript{17} Serum was collected from each animal and only a slightly reduced total hemolytic complement activity (CH50 U/mL of hemolytic complement activity in their sera, which is comparable to that of commercial New Zealand White rabbits.

The rabbits, aged 3 to 4 months, were fed a 0.5% cholesterol-supplemented diet for a total of 14 weeks to produce a model for the evolution of early atherosclerotic lesion development.\textsuperscript{18} After 8 and 12 weeks, serum samples were obtained and total cholesterol was determined. Before euthanization, blood samples were drawn in 10 mmol/L EDTA for determination of plasma lipoproteins.

**Plasma Lipoproteins and Biochemical Determinations**

Total plasma cholesterol was determined conventionally at the Laboratory for Clinical Chemistry, University of Mainz. LDL and HDL were isolated from plasma containing EDTA (1 mmol/L) by sequential ultracentrifugation in a fixed-angle rotor in a Beckman ultracentrifuge as described.\textsuperscript{19} The protein concentration of the lipoprotein fractions (LDL and HDL) was determined by a modified Lowry procedure.\textsuperscript{20}

**Preparation of Tissue**

After euthanization, the thoracic aortas of the rabbits were rapidly removed and dissected free of adventitial tissue. Aortas were cut into 1-cm segments, each of which was divided into 2 parts: 1 part was methacrylate-embedded (for morphometry), and the other part was shock-frozen in LN\textsubscript{2}-cooled isopentane (for immunohistochemistry) and stored at −80°C. Ten segments were obtained from each aorta.

**Morphometry of Aortic Parameters**

After staining of 4-\mu m aortic cross sections with hematoxylin and eosin, they were recorded by a 3 chip CCD video camera (Sony DXC-750) mounted on a microscope (Olympus BH-2). The following parameters were quantified with the use of a computer-based morphometry system\textsuperscript{21,22} (VIABAM 0.0–VFG1 frame grabber); outer and inner circumference, area of the media and of the plaque, and lumen stenosis (percentage of lumen occupied by the lesions).

**Statistical Analysis**

The results were calculated as mean ± SEM. All statistical procedures were performed using a personal computer version of the SIMSTAT program (Provalis Research). Statistical significance was determined by the Mann-Whitney U–Wilcoxon rank sum W test or the unpaired Student’s \( t \) test.

**Immunohistochemistry**

Six-micron cross-sections were cut in a Microm Microtome, placed on silan-precoated microscope slides, and exposed to absolute acetone (10 minutes, −20°C), followed by air-drying (10 minutes). Nonspecific sites were blocked with 2% swine serum (Life Technologies) in PBS (10 minutes). Endogenous peroxidase activity was suppressed by using 0.3% H\textsubscript{2}O\textsubscript{2} in PBS (10 minutes). Then mouse anti-human C5b-9 monoclonal antibody cross-reacting with rabbit C5b-9 (clone 978/394) from our laboratory was added (final concentration: 2 \( \mu \)g/mL, 90 minutes, 37°C). Control sections were incubated with irrelevant isotype-matched monoclonal antibodies. Immunoenzymatic streptavidin-biotinylated horseradish peroxidase complex procedure was used, and reactions were developed with 3,3’-diaminobenzidine solution (Pierce). Nuclei were counterstained with hematoxylin.

**Results**

Total serum cholesterol levels did not significantly differ in the 2 groups of experimental animals (Figure 1). Plasma lipoprotein determinations undertaken at the end of the experiments also revealed no significant differences. LDL protein levels were 1.13 ± 0.11 mg/mL in the C6-competent
group and 0.99 ± 0.1 mg/mL (SEM) in the C6-deficient group, and the LDL cholesterol to protein ratios were 4.12 ± 0.51 and 3.69 ± 0.75 in the 2 groups, respectively. HDL protein concentrations were 0.58 ± 0.07 mg/mL and 0.46 ± 0.13 mg/mL (SEM), and the HDL to LDL cholesterol ratios were 0.07 ± 0.01 and 0.08 ± 0.01 (SEM) in the C6-competent versus C6-deficient animals, respectively. There were no statistical differences between any pair of these values.

Differences in the extent of atherosclerosis were discernible by eye. All C6-competent animals had extensive aortic lesions that were most pronounced at side branches of the aorta. In contrast, atherosclerosis in all C6-deficient siblings was scarce and discrete. The typical macroscopic appearances of aortas from a pair of siblings are shown in Figure 2.

Each aorta was sectioned from thorax to abdomen in 1-cm segments, and a set of 5 sections from a pair of siblings is shown in Figure 3 to exemplify the remarkable differences in the extent of atherosclerotic lesion formation. Quantification of plaque formation and luminal stenosis was performed using computer-based morphometry, and the collective results are shown in Figure 4. In all sections, lesions were more pronounced in C6-competent versus C6-deficient animals, and in the majority of sections the differences were statistically significant. Staining for C5b-9 was positive in all lesions of C6-competent rabbits and negative in all C6-deficient rabbits (Figure 5). C5b-9 staining was never observed in areas outside atherosclerotic lesions.

Discussion

The present data confirm and extend the original finding, published in 1977 by Geertinger and Soerensen, that C6 complement deficiency protects against development of diet-induced atherosclerosis. In our study, pairs of homozygous and heterozygous siblings were used. Determination of serum-complement activity defined animals in both groups as either totally deficient or essentially normal with regard to serum-complement hemolytic activity. This is a demand that must be met, according to the literature. The level of plasma cholesterol and distribution of lipoproteins was shown to be the same in both groups. Atherosclerotic lesion formation was quantified using computer-based morphometry. Conventional parameters were used; ie, outer and inner circumference of the vessels, plaque area, and selective relative lumen stenosis. The latter parameter served solely as an anatomic marker of plaque formation, and no functional consequences are attached to these measurements. The values obtained both for lumen stenosis and plaque area are significant, so the conclu-
tion that the terminal complement sequence plays a pathogenic role in atherogenesis is now inescapable.

Why does complement activation occur at an early stage in lesion development? In order to offer an explanation, we depart from the conventional view that oxidative modification of LDL represents the single most important event in the development of atherosclerosis.23–31 Instead, we propose that cholesterol contained within the LDL molecule itself is endowed with proinflammatory potential, because exposure of free cholesterol to the extracellular environment will activate complement. Normally, LDL degradation and de-esterification of cholesterol occurs within cells after they take up the lipoprotein. The enzymes required for liberation of cholesterol are thus ubiquitously present in lysosomes. It appears conceivable that spontaneous, low-grade liberation of lysosomal contents occurs in vivo, so that the enzymes could be present in low concentrations in the extracellular matrix. In fact, cholesterol esterase was reported to be detectable in extracellular tissue derived from the subendothelium of human aortas.32 The endothelium of arteries is probably “leaky,” so that plasma components insudate continuously. When plasma LDL concentrations are physiological, entrapment of the lipoprotein is minimal. As LDL concentrations exceed a critical limit, however, a fraction of the lipoprotein is probably subject to degradation. In vitro, treatment of LDL with a protease plus cholesterol esterase enhances binding of the molecule to proteoglycans33,34; this presumably fosters its entrapment in the subendothelium. Evidence that LDL fuses at an early stage of lesion development to form heterogeneous lipid droplets similar to LCA or E-LDL was obtained in freeze-etch electron microscopic studies by Frank and Fogelman in 1989.35 Their finding was in accord with earlier data of Kruth,36,37 Chao et al,38 and Simionescu et al,39–41 who detected “liposomes” containing unesterified cholesterol in early lesions. All of these data are in line with our hypothesis that enzymatic transformation of LDL to the proinflammatory molecule E-LDL occurs at the earliest stages of lesion development.

The potential relevance of E-LDL is now borne out through the recognition that complement activation underlies lesion progression. Unhalted complement activation may result, first, from extensive primary formation of E-LDL. Second, it is possible that E-LDL is initially taken up by monocytes/macrophages but is then released subsequent to cell death, if the cells are unable to leave the vessel wall. Finally, complement activation may additionally be driven by other processes such as reperfusion events42–45 or liberation of intracellular complement activators from the cells.46,47

A major unanswered question relates to the problem of why the terminal complement sequence should assume such a central role in atherogenesis. It is possible that C5b-9 attack on nucleated cells occurs at some stage, accentuating proin-
flammatory events. In vitro, “bystander” damage by C5b-9 on smooth muscle cells provokes massive release of monocyte chemotactic protein-1. The possibility must also be considered that SC5b-9 complexes, the biological functions of which have not been explored in detail, contribute to lesion progression via unknown mechanisms. The terminal complement sequence may play a detrimental role in human atherosclerosis, so epidemiological analyses on the occurrence and extent of atherosclerosis in subjects deficient in terminal complement components C6-C9 are warranted. Such deficiencies occur worldwide and they may be found to confer protection against development of atherosclerosis in the human organism.

To sum, our concept views atherosclerosis as being the consequence of chronic inflammation driven by continuous activation of complement and the monocyte/macrophage system. We propose that enzymatic degradation rather than oxidation is the central event underlying transformation of LDL to a proinflammatory moiety. This would explain why oxidatively modified lipids are hardly detectable in early lesions, why subendothelially deposited LDL is morphologically altered, and why complement activation occurs at an early stage. Our concept obviates the need to seek for extraneous factors, such as oxidation or infection, to drive the pathological process. Instead, extracellular “unpackaging” of cholesterol alone is thought to be the single main event underlying the initiation and progression of the atherosclerotic lesion.

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References

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