Brief Report: Accelerated Atherosclerosis in Low-Density Lipoprotein Receptor–Deficient Mice Lacking the Membrane-Bound Complement Regulator CD59

Sheng Yun, Viola W.Y. Leung, Marina Botto, Joseph J. Boyle, Dorian O. Haskard

Objective—Whereas studies in humans and animal models have suggested a role for complement activation in atherosclerosis, there has been little analysis of the importance of complement regulators. We tested the hypothesis that the terminal pathway inhibitor CD59 plays an essential role in limiting the proinflammatory effects of complement activation.

Methods and Results—CD59 gene targeted mice (CD59a−/−) mice were crossed with low-density lipoprotein receptor–deficient (Ldlr−/−) mice. CD59-deficient Ldlr−/− mice had significantly more extensive en face Sudan IV staining of thoracoabdominal aorta than Ldlr−/− single knock-outs, both after a low-fat diet (6.51 ± 0.36% versus 2.63 ± 0.56%, P < 0.001) or a high-fat diet (17.05 ± 2.15% versus 7.69 ± 1.17%, P < 0.004). Accelerated lesion formation in CD59a−/−/Ldlr−/− mice on a high-fat diet was associated with increased lesional vascular smooth muscle cell (VSMC) number and fibrous cap formation.

Conclusion—Our data show that CD59 deficiency accelerates the development of lesions and increases plaque VSMC composition. Assuming that the main function of CD59 is to prevent the development of C5b-9 membrane attack complexes, our observations are consistent with the terminal complement pathway having proatherogenic potential in the Ldlr−/− mouse model, and highlight the importance of complement regulation.

Key Words: atherosclerosis ■ inflammation ■ immune system ■ complement ■ mice

Although inflammatory mechanisms are recognized as playing critical roles in atherosclerosis and its clinical complications,1 the contribution of complement to atherogenesis is still poorly defined. Previous experimental studies investigating the role of complement in atherogenesis have focused on the effects of deficiencies of individual complement pathway components.2 Whereas there is evidence that the classical pathway has protective functions, the terminal pathway has been shown in rabbits to have proatherogenic effects.3,4

Normally the complement system is controlled by the balance between complement activators and a variety of fluid-phase and membrane-bound regulatory proteins. As transport of plasma-derived inhibitors into the arterial wall may be limited, it is possible that complement regulation in atherosclerotic plaques may depend particularly on cell surface inhibitors, such as protectin (CD59), decay accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46), and, in the mouse, complement receptor 1 (CR1)-related gene y (Cryn),

CD59 is a glycoporphosphoinositol lipid-anchored glycoprotein that protects cells from complement-mediated injury by inhibiting the insertion of C9 into cell membranes and thereby preventing the development of C5b-9 membrane attack complexes.5,6 It is known to be expressed by macrophages, T lymphocytes, endothelial cells, and vascular smooth muscle cells (VSMCs) in human atherosclerosis.7 The CD59 gene in mice is duplicated, with CD59a being widely expressed and CD59b restricted to testis. CD59a−/− mice appear healthy but show exacerbated inflammation in various disease models.8–10 We report herein the effect on atherogenesis of deleting CD59a in Ldlr−/− mice.

Materials and Methods

Reagents

Oil Red O, dextrin, gelatin, Mayer Hematoxylin, L-glutamic acid, glycerol, sodium azide, calcium chloride, magnesium sulfate, and sodium phosphate were obtained from Merck/BDH. Buffered formal saline (4% w/w formaldehyde solution) was from Pioneer Research Chemicals. OCT compound was from CellPath. Other reagents were from Sigma-Aldrich.

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Mice and Diets
The mice and diets used in the study are described in the supplemental materials (please see http://atvb.ahajournals.org).

Lipoprotein, Cholesterol, and Triglyceride Analysis
Analysis for lipoprotein profiles and serum total cholesterol and triglycerides was as described.4

En Face Staining of Aorta
Methodology for en face staining of aortic lesions is in the supplemental materials.

Aortic Root Histology and Quantification
Cryosections of the aortic root were stained with Oil Red O and Mayer hematoxylin and analyzed blind, as previously described.4

Immunohistochemistry
Immunohistochemistry and confocal microscopy techniques are described in the supplemental materials.

Statistics
Data handling is described in the supplemental materials.

Results
There was strong immunohistochemical staining of CD59 in the aortic root of Ldlr<sup>−/−</sup> but not in CD59<sup>a<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice (supplemental Figures I and II). Lesions were barely detectable in the en face preparations of aortae of low-fat diet-fed Ldlr<sup>−/−</sup> mice but were significantly increased in the CD59<sup>a<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice on this diet (CD59<sup>a<sup>−/−</sup>/Ldlr<sup>−/−</sup> 6.51±0.36% versus Ldlr<sup>−/−</sup> 2.63±0.56%, mean±SEM, P<0.001). Similarly, lesions in the aortic root were more than 3-fold greater in CD59<sup>a<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice, either when expressed as absolute lesion area (P<0.006) or as an area fraction (P<0.001). High-fat diet feeding enhanced en face aortic lesion area in Ldlr<sup>−/−</sup> mice, and again CD59<sup>a<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice had significantly larger lesions (CD59<sup>a<sup>−/−</sup>/Ldlr<sup>−/−</sup> 17.05±2.15% versus Ldlr<sup>−/−</sup> 7.69±1.17%, P<0.004). Aortic root lesion areas in high-fat-fed mice were not different between groups (Figure 1 and supplemental Figure III).

Lesions in low-fat–fed mice consisted almost exclusively of macrophages and extracellular debris. In contrast, aortic root lesions of high-fat–fed CD59<sup>a<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice were more complex than those in Ldlr<sup>−/−</sup> mice, despite the similarity in size. Thus there was a reduction in the proportion of lesional cells staining with the macrophage marker and a 3-fold increased presence of alpha actin–positive VSMCs (47.7±3.7% versus 16.0±2.8% in Ldlr<sup>−/−</sup>, P<0.0001; Figure 2). Furthermore, fibrous caps covered all lesions in high-fat–fed CD59<sup>a<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice, compared with ∼25% of lesions in Ldlr<sup>−/−</sup> mice. Further details of immunocytochemical staining, body weights and lipid profiles are given in the supplemental materials.

Discussion
To our knowledge this is the first experimental study addressing the importance of an endogenous complement regulator in atherosclerosis. Our data show that CD59 deficiency accelerates the development of lesions and increases plaque acceleration of atherosclerosis in

VSMC composition. While we interpret this as evidence of accelerated plaque progression, the question arises as to whether the effects of CD59 deficiency might be to promote a relatively stable plaque phenotype characterized by a robust fibrous cap containing matrix and VSMC.11,12

The simplest explanation for our observations is that CD59 inhibits the development of MAC in the arterial wall, but this remains to be established. Whereas the assembly and insertion of C5b-9 into cell membranes may lyse nonnucleated cells, sublytic levels can activate proliferation or proinflammatory gene expression.11 It should be noted however that our data do not exclude the contribution of other mechanisms, such as an effect on the innate immune system of the mild hemolysis that has been reported in CD59<sup>a<sup>−/−</sup> mice.8

Our results need to be viewed alongside those showing an acceleration of atherosclerosis in Ldlr<sup>−/−</sup> mice that are defi-
cient in the classical complement pathway activator C1q.4

Taken together with previous reports,14,15 a paradigm is emerging in which the controlled activation of the classical and possibly other upstream complement pathways is protective through facilitation of the clearance of apoptotic cells and probably also enzymatically-modified LDL and other debris, whereas complement regulators such as CD59 help prevent this upstream complement activation translating into the elaboration of downstream proinflammatory effects.

In summary, our data show that CD59 retards atherosclerosis. The relative roles of other fluid phase and membrane-bound complement regulators in atherosclerotic lesion development and in shaping plaque phenotype now deserve further investigation.

Sources of Funding

This study was funded by a Programme Grant from the British Heart Foundation.

Disclosures

None.

References

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Online Supplementary Material

MATERIAL AND METHODS

Reagents.

Oil Red O, dextrin, gelatin, Mayer’s Haematoxylin, L-glutamic acid, glycerol, sodium azide, calcium chloride, magnesium sulphate and sodium phosphate were obtained from Merck/BDH, Poole, UK. Buffered formal saline (4% w/w formaldehyde solution) was from Pioneer Research Chemicals, Colchester, Essex. OCT compound was from CellPath, Newtown, Powys, UK. Other reagents were from Sigma-Aldrich, Poole, UK.

Mice and diets

CD59 gene-targeted mice (CD59a−/−) were generated in-house ¹. Ldlr−/− mice were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). Both CD59a−/− and Ldlr−/− mice were back-crossed for 10 generations on to the C57BL/6 background prior to intercrossing to form CD59a−/−/Ldlr−/− double knockout mice. Mice genotypes were determined by polymerase chain reaction. All mice in the study were female and were studied at 22 weeks of age. Animals were housed in a specific pathogen-free environment and studied according to UK Home Office regulations. The experimental groups were gradually transferred onto high or low fat diets at 10 weeks of age. The high fat diet (Arieblok Diet W, cat. 4021.06, Hope Farms, Woerden, The Netherlands) consisted of 15% cocoa butter, 1% corn oil, 0.25% cholesterol, 40.5% sucrose, 10% cornstarch, 20% casein, free of cholate, total fat content 16%. In contrast, the low fat diet from the same supplier (Arieblok Reference Diet, cat. 4068.02) consisted of
54.3% glucose, 10% cornstarch, 5% soya oil, 20% casein, total fat content 5.2% and no added cholesterol.

**Lipoprotein, cholesterol and triglyceride analysis**

Analysis for lipoprotein profiles and serum total cholesterol and triglycerides was as described \(^2\).

**En face staining of aorta**

Mice were killed by excess inhalation of carbon dioxide. Hearts and aortae were then perfused *in situ* with oxygenated Krebs-Henseleit buffer at 37\(^\circ\)C under a pressure of \(~110\) cm water via a cannula inserted in the left ventricle and an outlet created by incision of the right atrium. After 10 min, the buffer was replaced with buffered 4% formal saline at 37\(^\circ\)C for 10 min, followed by a lipid staining solution containing 0.5% Sudan IV, 35% ethanol and 50% acetone for a further 10 min. The heart and aorta were then removed and placed on ice cold PBS. For each specimen, the aorta was cut at the arch start site. By using 85mm (0.025x 0.015 mm superfine tips) microdissectiong scissors and 110mm Dumont, non-serrated dissection forceps, the extraneous fatty and connective tissue around the aorta were carefully trimmed off until a clear and transparent aorta was obtained. All small arteries were excised from each aorta specimen, and the remaining intact aorta was removed and transferred into a culture dish containing PBS. The entire aorta was then cut longitudinally from the heart near the innominate artery to the iliac bifurcation. The dissected aorta was destained with 80% ethanol for one minute to remove non-specific lipid staining, and was then washed in PBS and allowed to lay flat onto a Superfrost slide. Quantification was performed by taking images with a macroscopic CCD camera, drawing around
the *en face* aortic lesions and the entire aorta profile using the *Image ProPlus*™ software (version 4.5, Media Cybernetics, USA). Red segmentation was used to assist this procedure and make it more objective. The lesion area fraction was calculated by dividing the mean lesion area by the mean area of the aorta and expressed as a percentage.

**Aortic root histology and quantification.**

Cryosections of the aortic root were stained with Oil Red O and Mayer’s haematoxylin and analysed blind, as previously described.²

**Immunohistochemistry**

Immunohistochemistry was performed by standard procedures on residual sections not required for analysis of lesion size. Primary antibodies, which were diluted as appropriate in PBS, were rat mAb Moma-2 and rat anti-CD68 (both from Serotec, Oxford, UK), alkaline phosphatase conjugated mouse anti-alpha actin (clone α1A4) (Sigma-Aldrich, Poole, UK), rat monoclonal anti-mouse CD59 (clone MEL-4, a kind gift from Prof BP Morgan, Cardiff, UK), rabbit anti-cleaved (activated) caspase-3 (Pharmingen, Oxford, UK), rat anti-CD19, (Pharmingen, Oxford, UK), hamster anti-CD3 (Pharmingen, Oxford, UK), goat anti-mouse IgM (Abcam, Cambridge, UK), biotinylated goat anti-mouse IgG (Dako, Ely, UK) and goat anti-mouse C3 (ICN Cappel, Irvine, CA). Primary antibodies were followed by biotinylated rabbit or goat anti-rat immunoglobulin (Ig) secondary (Dako) and ABC-peroxidase system (Dako) using 3,3’-diaminobenzidine tetrahydrochloride (DAB) as substrate. Results of immunocytochemistry are presented as a percentage area fraction of the aortic root or as the percentage of lesional cells, as analysed by *Image ProPlus*™ software above.
**Confocal microscopy imaging**

Confocal staining was performed on cryosections using the following reagents: biotinylated Griffonia simplicifolia I lectin (Vector Laboratories, Cambridge, UK), AlexaFluor 488-conjugated ant-CD68 (Serotec), FITC-labelled anti-alpha actin (clone α1A4, Sigma-Aldrich, Poole, UK), alkaline-phosphatase-conjugated goat ant-rat Ig (Serotec), goat-anti-rat IgG (Abcam), biotinylated goat ant-rabbit IgG (Dako), AlexaFluor 488-conjugated streptavidin (Molecular Probes, Invitrogen, Paisley, UK), and alkaline phosphatase-conjugated streptavidin (Dako). Alkaline phosphatase was developed with Vector Red (Vector Laboratories), the fluorescence of which was visualized at Alexa568 settings (Ex 543nm, Em 560-615nm). Nuclei in fluorescence sections were counterstained with TOPRO-3 before mounting in 80% glycerol 20% PBS. Sections were examined by confocal or by standard fluorescence microscopy (for enumerating % actin-positive cells and fibrous caps). The confocal used was a Zeiss LSM510 Meta using a standard trichannel set up using the Ar 488nm line, the HeNe 543nm line and the HeNe 633nm line, a 1 Airy Unit pinhole (adjusted for light wavelength) and 3 photomultipliers fed via green (505-530nm BP), orange-red (560-615nm BP) and far red (LP638nm) filters. As advised by Zeiss, photomultiplier (PMT) voltages and amplifier offset were adjusted online using fast scan to maximise image clarity without saturation, and gain was left at the manufacturer’s default. PMT voltages were typically green emission 600V, orange-red emission 400V, far red emission 200V.
Statistics

Typically values for a given aortic root were the mean of five sections. Data were expressed as mean ± SEM and tested by two-tailed Student’s t-test (Excel and SigmaStat), with significance assumed at p < 0.05.
RESULTS

Immunocytochemistry

CD59 deficiency did not increase the number of apoptotic cells detectable with anti-cleaved caspase 3. Very few T or B lymphocytes were detectable in lesions of either strain, regardless of diet. We also observed diffuse lesional immunostaining of IgM, IgG and C3, which was similar in Ldlr<sup>−/−</sup> and CD59a<sup>−/−</sup>/Ldlr<sup>−/−</sup> strains (not shown).

Body weights and lipid profiles

No differences were observed between Ldr<sup>−/−</sup> and CD59a<sup>−/−</sup>/Ldr<sup>−/−</sup> strains in final body weight, or in total serum cholesterol and triglyceride levels (Table I: [http://atvb.ahajournals.org](http://atvb.ahajournals.org)). There was no difference in the lipoprotein profile of Ldlr<sup>−/−</sup> and CD59a<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice, as determined by FPLC (not shown).
REFERENCES


FIGURE LEGENDS

**On line supplementary Figure I: CD59 expression in the aortic root:** Aortic root sections of 22 week old mice fed a low fat diet were stained with anti-CD59 (brown) and counterstained with haematoxylin, (A) strong immunohistochemical staining of macrophages, VSMC and endothelial cells in Ldlr-/- mice, with less intense cellular staining within lesions; (B) no specific staining of CD59 in a CD59a/-/Ldlr/-/ mouse. Scale bars represent 100 μm. The suggestion in (A) that endothelium stained positively for CD59 was confirmed by double-staining with Griffonia simplicifolia I lectin (Online Supplementary Figure II). Expression of CD59 could also readily be detected on endothelial cells and VSMC in wild-type mouse arteries (not shown).

**On line supplementary Figure II: Colocalisation of CD59 with endothelium**
Lesions from low-fat fed Ldlr-/- mice were stained and examined by confocal microscopy to confirm the presence of CD59 on endothelium: (A) biotinylated Griffonia simplicifolia I lectin which reacts specifically with endothelial cells, followed by AlexaFluor 488-conjugated streptavidin (green), (B) Interference contrast, (C) Rat anti-mouse CD59 followed by alkaline phosphatase-conjugated goat anti-rat IgG and development with Vector Red (red), (D) TOPRO-3 nuclear dye (purple pseudo-coloured, far red original), (E) merged image showing endothelial CD59 in yellow (red / green combined), (F) Interference contrast with TOPRO-3.

**On line supplementary Figure III: CD59 deficiency accelerates aortic lipid deposition and atherosclerosis in the aortic root:** (A-D) 22 weeks old mice fed a low fat (A,B) or a high fat (C,D) diet from the age of 12 weeks were perfused in vivo with Sudan IV, after which aortae were excised and processed for en face staining as described in Methods. (A,C) Ldlr-/-, (B,D) CD59a-/-/Ldlr-/-/. Scale bar represents 1cm;
(E-H) photomicrographs of aortic roots in 22 weeks old mice following (E,F) low fat diet or (G,H) high fat diet from aged 12 weeks. (E,G) Ldlr<sup>−/−</sup>, (F,H) CD59a<sup>−/−</sup>/Ldlr<sup>−/−</sup>. Scale bars represent 1 mm.
ONLINE SUPPLEMENTARY TABLE I

Body weights and total serum cholesterol and triglycerides in 22 week old \( Ldlr^{-/-} \) and \( CD59a^{-/-}/Ldlr^{-/-} \) mice

<table>
<thead>
<tr>
<th></th>
<th>( Ldlr^{-/-} )</th>
<th>( CD59a^{-/-}/Ldlr^{-/-} )</th>
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<tbody>
<tr>
<td></td>
<td>mean ± SEM (n)</td>
<td>mean ±SEM (n)</td>
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<tr>
<td><strong>Low fat diet</strong></td>
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<tr>
<td>Final body weight (g)</td>
<td>25.13 ± 0.68 (23)</td>
<td>25.14 ± 0.58 (20)</td>
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<td>Total cholesterol (mmol/l)</td>
<td>9.43 ± 1.58 (23)</td>
<td>8.20 ± 2.04 (20)</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>1.76 ± 0.54 (22)</td>
<td>1.76 ± 0.35 (20)</td>
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<tr>
<td><strong>High fat diet</strong></td>
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<tr>
<td>Final body weight (g)</td>
<td>25.22 ± 0.73 (18)</td>
<td>27.53 ± 1.11 (18)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>23.04 ± 3.07 (12)</td>
<td>23.27 ± 4.02 (15)</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>3.266 ± 0.73 (18)</td>
<td>4.12 ± 1.78 (18)</td>
</tr>
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</table>
Online Supplementary Figure I
Online Supplementary Figure II