

In Vivo Oxidation, Platelet Activation and Simultaneous Occurrence of Natural Immunity in Atherosclerosis-Prone Mice

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ABSTRACT: **Background:** Several murine models are susceptible to atherosclerosis, such as low density-lipoprotein receptor-deficient (LDLR^{-/-}) and apolipoprotein E-deficient (apoE^{-/-}) mice, and are used for studying pathophysiological mechanisms. Atherosclerotic lesions in the aortic valve and thoracic/abdominal aorta are commonly associated with hyperlipidemia. We recently demonstrated the development of large atherosclerotic plaques in *Helicobacter pylori*-infected heterozygous LDLR^{+/-} apoE^{+/-} mice.

Objectives: To measure novel biomarkers related to atherosclerosis, blood coagulation, and oxidative stress in order to investigate their possible pathogenic roles in atherosclerosis-prone mice.

Methods: Mice were fed with a normal chow diet or high-fat diet and sacrificed at different age intervals to measure aortic plaque size. Plasma cholesterol was enzymatically measured. Enzyme-linked immunosorbent assay was used to measure oxidized LDL (oxLDL)/beta-2-glycoprotein I (β2GPI) complexes, immunoglobulin M (IgM) antibodies against native LDL, oxLDL, or oxLDL/β2GPI, and urine 11-dehydrothromboxane B2 (11-dhTxB₂) or 8-hydroxy-deoxyguanosine.

Results: There was a parallel increase in plaque size, plasma cholesterol, and urinary 11-dhTxB₂ in atherosclerosis-prone mice. In contrast to atherosclerosis-prone strains, an elevation of urinary 11-dhTxB₂ with no significant plaque generation was observed in LDLR^{+/-} apoE^{+/-} mice. The atherogenic autoantigen oxLDL/β2GPI complex was detected only in LDLR^{-/-} mice. These levels seem to depend on plaque size. IgM antibodies against oxLDL in apoE^{-/-} mice were found, accompanied by atherosclerotic progression.

Conclusions: Progression of atherosclerotic lesions was associated not only with hypercholesterolemia but also with platelet activation and natural autoimmune-mediated regulatory mechanism(s) in murine models.

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KEY WORDS: atherosclerosis, oxidized low-density lipoprotein (oxLDL), beta-2-glycoprotein I (β2GPI), natural antibodies, 11-dehydrothromboxane B2

Atherosclerosis is a chronic inflammatory disease of the arterial wall associated with dysregulation of the lipoprotein metabolism, the formation of pro-inflammatory lipid peroxidation byproducts, and abnormal host immune responses. In vitro observations suggest that oxidatively modified LDL and related metabolites are highly pro-inflammatory and pro-atherogenic [1,2]. OxLDL accumulates in macrophage-derived foam cells within atherosclerotic lesions [2]. Oxidation of LDL produces a wide variety of oxidation-specific epitopes with immunogenic properties that may lead to intense immune responses, mediated either by immunoglobulin G or M natural autoantibodies that modulate the progression of atherosclerotic lesions.

We have demonstrated that oxLDL/beta-2-glycoprotein I complexes function as major atherogenic and thrombogenic autoantigens in patients with antiphospholipid syndrome [3-7]. Antiphospholipid syndrome is a systemic autoimmune disease characterized by vascular (venous and arterial) thrombotic events. In addition to systemic autoimmune diseases, oxLDL/β2GPI complexes have been detected in patients with diabetes mellitus and chronic renal disease [8-11]. It has been shown that oxLDL/β2GPI induces β2GPI-specific autoreactive T cells [12,13].

The autoimmune response to epitopes of oxLDL in atherosclerosis-prone apolipoprotein E-deficient mice is strong. Palinski and colleagues [14] isolated a panel of B cell hybridomas with specificity for oxLDL neopeptides from the spleen of diseased apoE^{-/-} mice that had not received experimental immunization or *in vitro* stimulation. The resulting monoclonal IgM natural antibody was called EO, which bound strongly to malondialdehyde-LDL and Cu²⁺-oxLDL.

We recently studied *Helicobacter pylori* infection in heterozygous LDLR^{+/-} apoE^{+/-} mice [15]. These heterozygous mice did not exhibit significant hyperlipidemia or progression of atherosclerosis. Nevertheless, infection with *H. pylori*-SS1 strain caused the development of severe atherosclerosis.

LDL = low density lipoprotein
OxLDL = oxidized low density lipoprotein
β2GPI = beta-2-glycoprotein
apoE = apolipoprotein E

The current study is an initial quantitative comparison of novel biomarkers involved in oxidative stress, atherosclerotic autoimmune inflammation and platelet activation in LDLR^{-/-}, apoE^{-/-}, and heterozygous LDLR^{+/-} apoE^{+/-} mice.

MATERIALS AND METHODS

ANIMAL STUDIES

This study followed the Ethical Committee's Guidelines for Animal Research at our university. Male mice (6 weeks old; groups of 72–96 mice of each strain) of two atherosclerosis-prone strains (obtained from Jackson Laboratories, Bar Harbor, ME, USA) and the heterozygous LDLR^{+/-} apoE^{+/-} were used. Their genotypes were verified by polymerase chain reaction. Half of the mice were fed a normal diet and the rest with a high-fat diet (containing cholic acid). Specimens (urine, EDTA-plasma, aortic valve, and thoracic/abdominal aorta) were collected every 2 weeks beginning at age 6 weeks. Mice were sacrificed at each time point to harvest whole blood, stomach, spleen, liver, and aorta. The aortic tree was perfused with phosphate-buffered saline (pH7.4) containing butylated hydroxytoluene and EDTA, by inserting a cannula into the left ventricle and allowing free efflux from an incision in the vena cava. After removal of the surrounding adventitial fat tissue, the aorta was opened longitudinally from the aortic root to the iliac bifurcation, fixed with PBS containing 4% formaldehyde, and stained with Sudan IV. The extent of atherosclerosis was determined by imaging the aortas and the size of the lesion as percent of aorta using Scion Image analysis. Plasma cholesterol was determined enzymatically using the Cholesterol E-test kit (Wako Pure Chemical Industries, Osaka, Japan).

ELISA

The ELISA to measure plasma oxLDL/β2GPI complexes was performed with slight modifications of the human assay system [8] as follows. Briefly, anti-β2GPI antibody, WB-CAL-1 (8 μg/ml), was adsorbed onto 96-microwell plates (Maxisorp, Nunc, Denmark) by overnight incubation at 4°C. After blocking with bovine serum albumin, mouse plasma (100-fold diluted) was incubated overnight. The wells were subsequently incubated with horseradish peroxidase-labeled rabbit anti-mouse LDL antibodies for 3 hours. Color was developed by adding TMBUS substrate (Moss Inc, Pasadena, MD, USA). The reaction was terminated and the optical density at 450 nm was measured. The wells were extensively washed between each step with tris-buffered saline containing 0.05% Tween 20.

Urinary 11-dehydro-thromboxane B2 was determined using 11-dhTxB₂ Test Kit (Corgenix, Broomfield, CO, USA).

The tenfold diluted urine samples, 11-dhTxB₂ conjugated to alkaline phosphatase, and purified mouse antibody directed to 11-dhTxB₂ were incubated together in wells coated with a polyclonal anti-mouse antibody. Incubation allowed the endogenous 11-dhTxB₂ present in the samples to compete with the purified alkaline phosphatase-conjugated 11-dhTxB₂ to the bound anti-11-dhTxB₂ antibody. After washing, paranitrophenylphosphate was added to develop color. The concentration of 11-dhTxB₂ was normalized with urinary creatinine. Urinary 8-hydroxy-deoxyguanosine levels were also determined by ELISA according to the manufacturer's instructions (Japan Institute for the Control of Aging, Fukuroi, Japan).

The ELISAs to measure mouse IgM antibodies were performed as follows: native LDL, oxLDL, or oxLDL/β2GPI complexes (0.25 μg/well) were adsorbed on the wells of 96-microwell plates (Immulon 1B, Dynex Technologies, Chantilly, VA, USA) by overnight incubation. The wells were blocked with BSA for 2 hours, and mouse plasma (100-fold diluted) was added and incubated for 1 hour. The wells were subsequently incubated with HRP-labeled goat anti-mouse IgM for 1 hour.

STATISTICAL ANALYSIS

Data are presented as means ± SD. All statistical analyses were performed using the Kaleida Graph software (Synergy Software, Reading, PA, USA). The differences between different groups were analyzed by the Wilcoxon-Mann-Whitney test.

RESULTS

GENOTYPING AND ONSET OF HYPERCHOLESTEROLEMIA

The genotypes of all mice were confirmed by PCR: Both 245 base pair DNA fragment (apoE^{-/-}) and 167 bp DNA fragment (LDLR^{+/-}) for apoE^{-/-} mice, and both 350 bp DNA fragment (LDLR^{-/-}) and 155 bp DNA fragment (apoE^{+/-}) for LDLR^{-/-} mice were used. When atherosclerosis-prone and heterozygous mice were fed with a normal diet their plasma cholesterol levels did not change. In contrast, cholesterol levels were significantly increased with the high-fat diet particularly in atherosclerosis-prone mice [Figure 1].

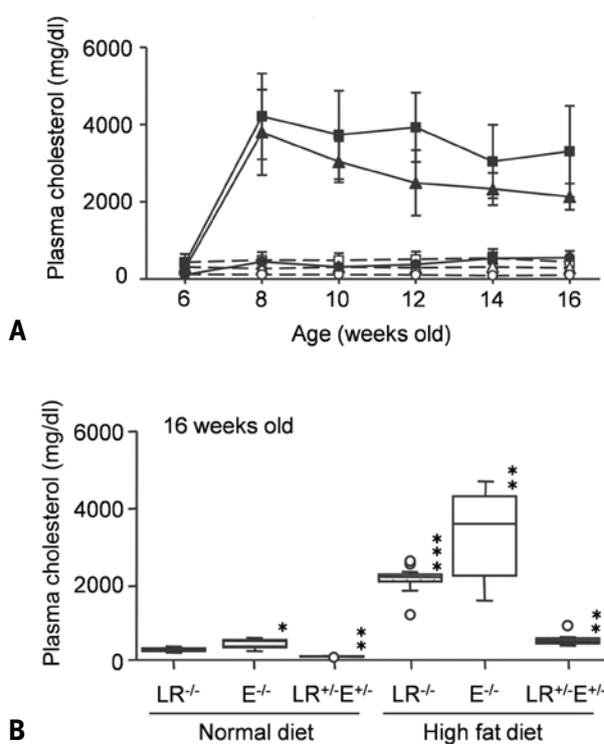
PROGRESSION OF AORTIC ATHEROSCLEROSIS

Significantly larger plaque sizes were observed in both atherosclerosis-prone mice at the age of 16 weeks but there was no significant plaque progression in LDLR^{+/-} apoE^{+/-} mice as compared to LDLR^{-/-} at 16 weeks with normal diet. Independent plaque formation in apoE^{-/-} mice on the high-fat diet was observed [Figure 2].

Ig = immunoglobulin
 PBS = phosphate-buffered saline
 ELISA = enzyme-linked immunosorbent assay
 11-dhTxB₂ = 11-dehydro-thromboxane B₂

BSA = bovine serum albumin
 HRP = horseradish peroxidase
 PCR = polymerase chain reaction
 bp = base pair

Figure 1. Diet-dependent elevation of plasma cholesterol levels in mice. **[A]** Time course of plasma cholesterol levels in LDLR^{-/-} (LR^{-/-}: triangle), apoE^{-/-} (E^{-/-}: square), or LDLR^{+/-} apoE^{+/-} (LR^{+/-} E^{+/-}: circle) mice fed with normal chow diet (open symbols) or high-fat diet (closed symbols). **[B]** Comparison of total plasma cholesterol levels in different strains at 16 weeks old. **P* < 0.05, ***P* < 0.001, ****P* < 0.0001 were statistically significant, as compared to the group of LDLR^{-/-} at 16 weeks fed exclusively with a normal chow diet. Each experimental group contains 3–6 mice.



URINARY MARKERS OF PLATELET ACTIVATION AND OXIDATIVE STRESS
 Urinary levels of 11-dhTxB₂ increased gradually in atherosclerosis-prone mice fed a high-fat diet. However, heterozygous LDLR^{+/-} apoE^{+/-} mice showed the highest level of 11-dhTxB₂ at 16 weeks even though atherosclerosis did not develop [Figure 3 A and B]. There were significant differences in urinary 11-dhTxB₂ levels among high-fat diet mice compared to normal-diet mice in all strains at 16 weeks. Urinary 8-OHdG, a marker of oxidative stress, is generated by oxidation with reactive oxygen species (•OH). There were no significant changes in urinary 8-OHdG levels of any strains with or without high-fat diet treatments [Figure 3 C and D].

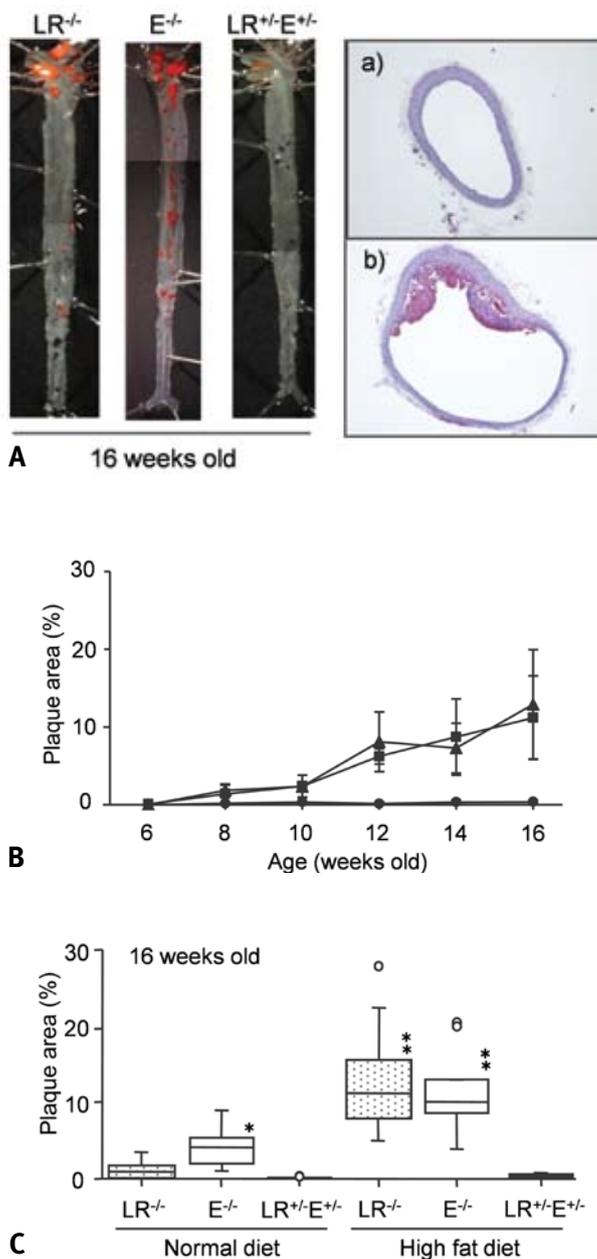
OXIDATION-ASSOCIATED IMMUNOLOGICAL MARKERS

Plasma levels of oxLDL/β₂GPI complexes were significantly increased only in LDLR^{-/-} beginning at age 8 weeks (2 weeks after the start of the high-fat diet). ApoE^{-/-} or heterozygous

8-OHdG = 8-hydroxy-deoxyguanosine

Figure 2. Atherosclerotic plaques in aortic trees of mice.

[A] Representative atherosclerotic plaques in mice. Sections of arterial vessel of control (C57BL6) a) or apoE^{-/-} b) mice at age 16 weeks were stained with Sudan IV. Plaque size in mice fed a high-fat diet at different ages **[B]** (LR^{-/-}: triangle, E^{-/-}: square, LR^{+/-} E^{+/-}: circle) and at 16 weeks old **[C]** are indicated. **P* < 0.05, ***P* < 0.001 were statistically significant, as compared to the group of LDLR^{-/-} at 16 weeks fed exclusively with a normal chow diet. Each experimental group contains 3–6 mice.



mice did not produce oxLDL/β₂GPI complexes even at age 16 weeks [Figure 4 A and B]. IgM natural/autoantibody titers against native LDL, oxLDL and oxLDL/β₂GPI complex are depicted in Figure 4C. IgM antibodies against oxLDL,

Figure 3. Urinary markers of platelet activation and oxidative stress in atherosclerosis-prone mice. Levels of urinary 11-dhTxB₂ [A and B] and 8-OHdG [C and D] in atherosclerosis-prone mice are indicated. **P* < 0.05 was statistically significant, as compared to the group of

LDLR^{-/-} at 16 weeks fed exclusively with a normal chow diet. Urinary 11-dhTxB₂ and 8-OHdG in LR^{-/-} (triangle), E^{-/-} (square), or LR^{+/-} E^{+/-} (circle) mice fed with a normal chow diet (open symbols) or high-fat diet (closed symbols). Each experimental group contains 3–6 mice.

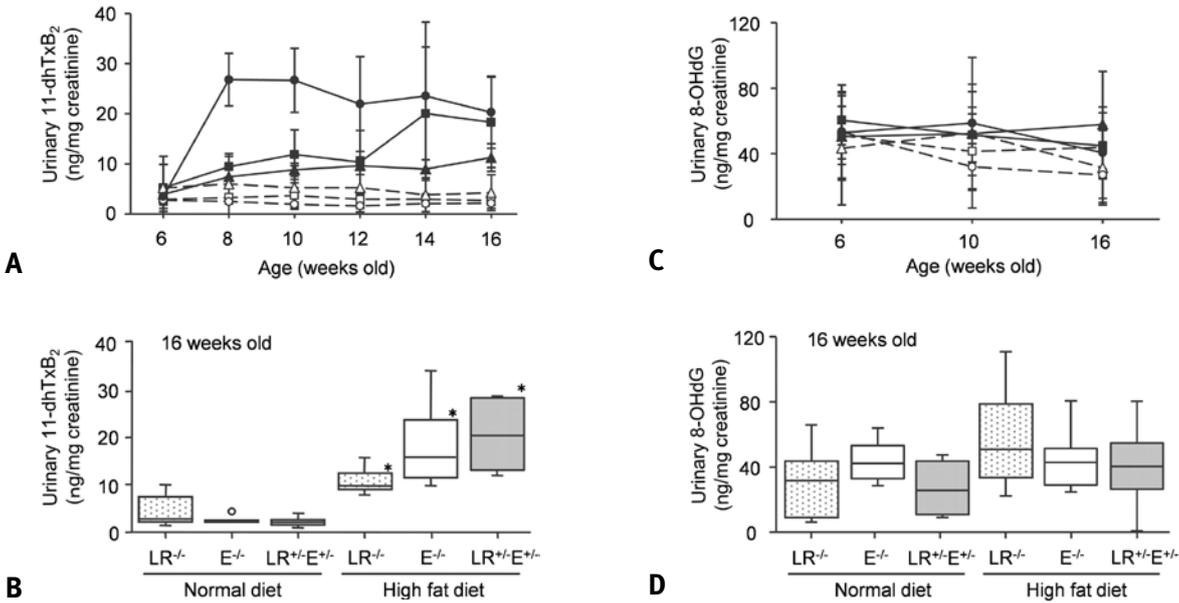
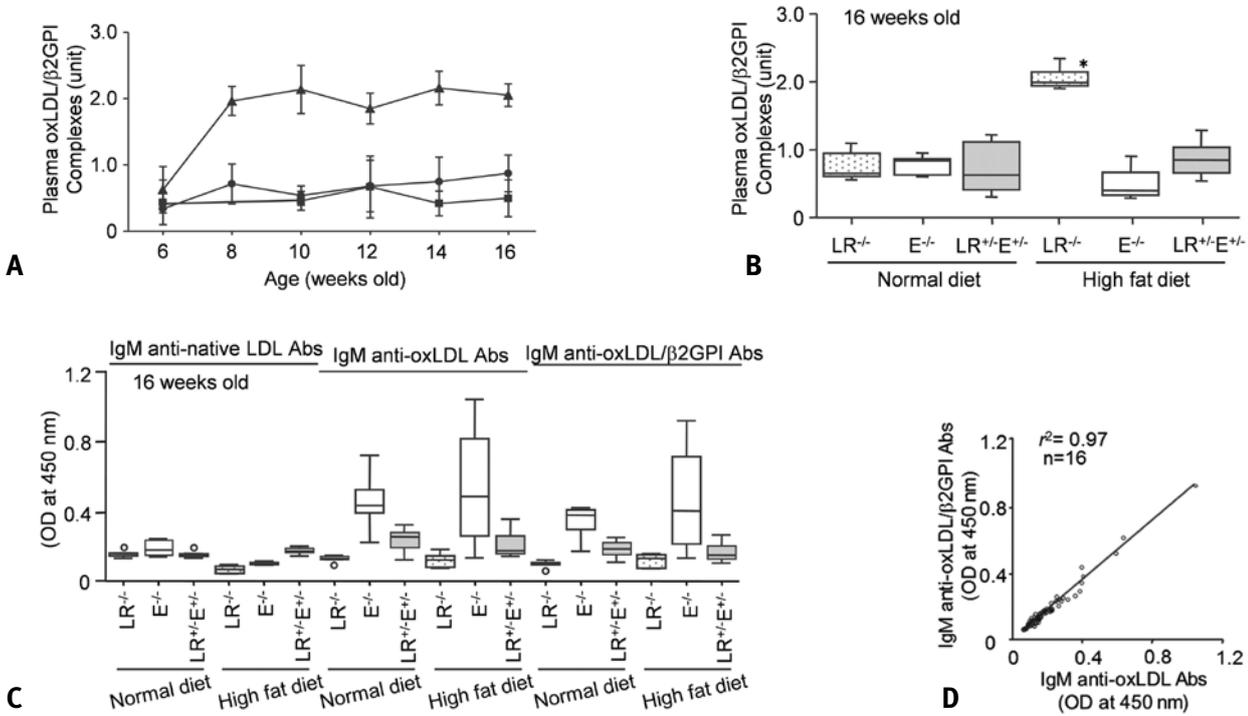


Figure 4. In vivo oxidation-associated immunological markers in mice. [A and B] OxLDL/β2GPI complexes in plasma of atherosclerosis-prone mice (LR^{-/-}: triangle, E^{-/-}: square, LR^{+/-} E^{+/-}: circle). [C] Titers of IgM natural/autoantibodies against native LDL, oxLDL, and oxLDL/β2GPI

complexes. [D] Correlation between IgM against oxLDL and oxLDL/β2GPI complexes. **P* < 0.05 was statistically significant, as compared to the group of LDLR^{-/-} at 16 weeks fed exclusively with a normal chow diet. Each experimental group contains 3–6 mice.



oxLDL/ β 2GPI were significantly high in apoE^{-/-} mice. There was a strong correlation ($r^2 = 0.97$) between IgM antibodies against oxLDL with antibodies to oxLDL/ β 2GPI complexes [Figure 4D]. This indicates that these natural or autoantibodies are directed to epitopes on oxLDL particles but not to β 2GPI moieties.

DISCUSSION

Homozygous LDLR^{+/-} and apoE^{+/-} mice developed hypercholesterolemia, but the severity and manifestations differed markedly. On a normal diet, apoE^{-/-} mice developed a more severe hypercholesterolemia as compared with LDLR^{-/-} mice. Plasma cholesterol levels above 2000 mg/dl were seen in both atherosclerotic strains in response (hyper-responsiveness) to dietary cholesterol [16,17]. In the present study, we observed a similar response regarding plasma cholesterol levels. We recently studied the response of heterozygous LDLR^{+/-} apoE^{+/-} mice to *H. pylori* infection. At age 16 weeks, the heterozygous mice did not develop significant hyperlipidemia or progression of atherosclerosis. Nevertheless, infection with the *H. pylori* SS1 strain led to severe atherosclerosis [15]. In that study, we proposed that chronic infection may induce Th1-oriented immune response specific to heat shock protein-60 that can promote progression of atherosclerosis in mice. In the present study, we reproduced the previous observation of no elevation of plasma cholesterol and no atherosclerosis progression in the heterozygous LDLR^{+/-} apoE^{+/-} mice. However, we encountered an unexpected finding, i.e., that urinary 11-dhTxB₂ levels were greatly increased in the heterozygous mice similar to the hypercholesterolemic and atherosclerosis-prone apoE^{-/-} and LDLR^{-/-} mice. 11-dhTxB₂ is an activation marker of platelets and we assumed that platelet activation would promote a pro-coagulation state, followed by inflammation and/or oxidative stress in atherosclerotic plaques. It seems that this explanation does not apply for the heterozygous mice. Furthermore, we found that severe inflammation in atherosclerotic plaques did not influence urinary levels of 8-OHdG, a well-known oxidative stress marker, in any of the experimental groups.

We were also interested in measuring oxLDL/ β 2GPI complexes, a novel oxidative stress marker associated with the progression of atherosclerosis by ELISA that was recently established in our laboratory. Multicenter clinical trials using the AtherOx[®] ELISA kit to measure oxLDL/ β 2GPI complexes to evaluate their clinical significance are currently underway not only in Japan but also in the United States and some European countries. Our mouse study aimed at determining these complexes is one of the first attempts to understand their role in the progression of atherosclerosis in LDLR^{-/-} mice. As shown in Figure 4A, we were unable to detect oxLDL/ β 2GPI complexes in apoE^{-/-} mice, probably due to lack of specificity

of HRP-labeled rabbit anti-mouse oxLDL antibodies. This is due to the lack of apoB100 in LDL-like particles in the apoE^{-/-} mice. In contrast, rabbit anti-mouse LDL antibodies, which we prepared by immunization with LDL from pooled sera of LDLR^{-/-} mice, worked well. One possible explanation is that the antibodies used in our ELISA may be directed to the C-terminal region of apoB100, which cannot recognize apoB48 in apoE^{-/-} mice. The observation that IgM natural or autoantibodies against oxLDL present in apoE^{-/-} mice during the progression of atherosclerosis is consistent with previous reports [18]. Furthermore, as shown in Figure 4D, the strong correlation between anti-oxLDL and anti-oxLDL/ β 2GPI complexes clearly indicates that IgM antibodies raised in atherosclerosis-prone apoE^{-/-} mice are directed to epitopic moieties on LDL particles not on the β 2GPI molecule.

The current study is an initial quantitative comparison of novel biomarkers involved in oxidative stress, inflammation and immune response that occur during the development of atherosclerotic plaques along with a marker of platelet activation in LDLR^{-/-}, apoE^{-/-}, and heterozygous LDLR^{+/-} apoE^{+/-} mice. More extensive and detailed studies of immunoregulatory mechanisms will undoubtedly provide further insights into the pathophysiology in atherosclerosis.

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