Induction of Early Atherosclerosis in LDL-Receptor–Deficient Mice Immunized With β2-Glycoprotein I

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Background—Immunization with β2-glycoprotein I (β2GPI), the probable target of autoimmune anticardiolipin antibodies, results in experimental antiphospholipid syndrome in different mouse strains. The present study was undertaken to evaluate the effect of β2GPI immunization on the progression of atherosclerosis.

Methods and Results—In the first experiment, 3 groups of LDL receptor–deficient (LDL-RD) mice (n=15 per group) were immunized with either β2GPI or ovalbumin or were not immunized and were fed a chow diet for 12 weeks. In a second experiment, 3 groups of LDL-RD mice (n=10 per group) were immunized similarly and fed an atherogenic diet for 6 weeks. All β2GPI-immunized mice developed high titers of anti-β2GPI antibodies as well as a specific lymph node proliferation to β2GPI. The average cholesterol levels did not differ between the mice fed similar diets, regardless of the immunization protocol. Atherosclerosis was enhanced in the β2GPI-immunized mice (mean aortic lesion, 26 000±5700 μm²) in comparison with their ovalbumin-immunized (mean, 3000±1099 μm²; P<0.01) and nonimmunized (mean, 2250±700 μm²; P<0.01) littermates. The average lesion size in the β2GPI-immunized mice fed an atherogenic diet (mean, 98 000±8305 μm²) was larger than the ovalbumin-immunized mice (mean, 81 250±12 933 μm²; P=NS) or the nonimmunized controls (mean, 75 625±7281 μm²; P=NS). The atherosclerotic plaques in the β2GPI-immunized mice appeared to be more mature, and denser infiltration of CD4 lymphocytes was present in the subendothelium of the aortic sinuses from this group of mice.

Conclusions—The results of the present study provide the first direct evidence for the proatherogenic effect of β2GPI immunization and establish a new model for immune-mediated atherosclerosis. (Circulation. 1998;98:1108-1115.)

Key Words: atherosclerosis ■ glycoproteins ■ antibodies ■ lipoproteins

The antiphospholipid syndrome (APS) is characterized by a prothrombotic predisposition manifested by thromboembolic events, thrombocytopenia, recurrent fetal loss, and additional systemic phenomena. The syndrome is associated with high titers of antiphospholipid antibodies (aCLs), which have traditionally been suggested to target negatively charged phospholipids. Circumstantial data imply that patients with systemic lupus erythematosus (SLE) (a high proportion of whom have high titers of aCLs) are prone to develop premature atherosclerosis. However, the mechanisms underlying the proatherogenic predisposition are still unresolved.

The issue of target recognition by aCLs has been the subject of intensive research in recent years. Some authors suggested that aCLs bind native phospholipids, whereas others proposed that certain plasma cofactors, on binding to phospholipids, could allow for aCL attachment. β2-Glycoprotein I (β2GPI) is a 50-kD plasma protein that possesses several in vitro anticoagulant effects (eg, inhibition of prothrombinase activity, ADP-induced platelet aggregation, and factor Xa generation). This glycoprotein has recently been considered to be the target of autoimmune aCLs, which are sometimes referred to as anti-β2GPI antibodies. Several authors have shown that immunization of mice and rabbits with β2GPI results in the production of antibodies that bear similarities to human autoimmune aCLs. Moreover, it has been shown by us and others that immunization with β2GPI leads to manifestations resembling human APS in mice or exacerbates the respective syndrome in susceptible MRL/+ mice.

Macrophages and smooth muscle and endothelial cells play a major role in the development of atherosclerotic plaque. Foam cells (the early, reversible lesions composed of lipid-containing macrophages) gradually develop into complicated plaques during the progression of atherosclerosis. These sequential events are thought to be accelerated by oxidation of plasma lipids. Oxidized LDL (oxLDL) has attracted major interest in view of its various effects on different cellular
components, attesting to its immunogenicity and probable causal effect on atherosclerosis progression.\(^20\),\(^21\)

Several mechanisms could associate aCLs with enhanced atherogenesis, taking into consideration the effects on the cellular components within a mature atherosclerotic plaque. aCL, and anti-oxLDL antibodies were shown to be cross-reactive,\(^23\),\(^24\) and some suggest that aCLs actually target oxidized rather than native phospholipids.\(^25\) Very recently, Hasunuma et al.\(^26\) showed that anti-β2GPI antibodies enhance the in vitro uptake of oxLDL to macrophages (the candidate progenitors of foam cells), postulating that it could contribute to enhanced atherogenesis.

Endothelial cells are also essential to the development of atherosclerosis, possibly by a prior activation resulting in expression of adhesion molecules.\(^26\) aCLs as well as rabbit anti-β2GPI antibodies have been demonstrated to bind and activate cultured human endothelial cells\(^27,28\) and could thus contribute to a proatherogenic state.

In the present study, we examined the effects β2GPI immunization on the development of atherosclerosis. We have shown that LDL-receptor-deficient (LDL-DRD) mice fed a chow diet and immunized with β2GPI developed accelerated atherosclerosis.

**Methods**

**Animals**

Six-week-old female LDL-DRD mice (hybrids of the C57BL/6J and 129Sv strains) were created by homologous recombination as described by Ishibashi et al.\(^29\) The mice were obtained from the Jackson Laboratory (Bar Harbor, Me.). We used LDL-DRD mice for the experiments because this mouse maintains high cholesterol levels on a chow diet (levels similar to human values) and develops significant atherosclerosis only when fed a high-fat diet. Thus, the problem of the relative resistance of mice to atherosclerosis is overcome. BALB/c mice (8 weeks old) were purchased from the animal house of the Sakler School of Medicine, Tel Aviv University, Israel.

The LDL-DRD and the BALB/c mice were either fed normal chow diet containing 4.5% fat by weight (0.02% cholesterol) or an atherogenic diet containing 1.25% cholesterol, 7.5% casein, and 0.5% (wt/wt) sodium cholate. The mice were maintained on 12-hour dark/12-hour light cycles and were allowed access to food and water ad libitum.

**Antigens and Antibodies**

Human β2GPI was purified from the serum of a healthy adult as previously described by Gharavi et al.\(^13\) Mouse β2GPI was purified from the serum of LDL-DRD mice by a similar method.

For preparation of LDL and copper-oxidized LDL, blood for lipoprotein isolation was collected in EDTA (1 mg/mL) from mice after 12 hours of fasting. LDL (density, 1.019 to 1.063 g/mL) was isolated from the plasma after density adjustment with KBr by preparative ultracentrifugation at 50,000 rpm for 22 hours with a type 50 rotor as previously described.\(^30\) LDL preparations were washed by ultracentrifugation, dialyzed against 0.15 mol/L EDTA (pH 7.4), passed through an Acrodisc filter (0.22-µm pore size) to remove aggregates, and stored under nitrogen in the dark. Copper oxidation of LDL was performed by incubation of postiodized LDL (1 mg of protein/mL in EDTA-free PBS) with copper sulfate (10 µmol/L) for 24 hours at 37°C. Lipoprotein oxidation was confirmed by analysis of thiobarbituric acid-reactive substances (TBARS).\(^30\)

Recombinant heat shock protein (HSP)-65 was kindly provided by Dr M. Singh, Braunschweig, Germany.

Mouse monoclonal anti-β2GPI antibodies Cof-21 and Cof-18 (both of which bind human and mouse β2GPI)\(^31\) was a generous gift of Professor Takao Koike, Hokkaido University, Japan.

Rat monoclonal antibodies H129.19 (L3T4) anti-mouse CD4+ and S3-6.7 (Ly-2) anti-mouse CD8α were from PharMingen; MCA 497 (F4/80) anti-mouse macrophages were from Serotec.

**Immunization Protocol**

In the first experiment, LDL-DRD mice (n=15) were immunized subcutaneously with a single dose of human β2GPI (10 µg per mouse) emulsified in complete Freund’s adjuvant (CFA). The 2 additional groups (15 mice each) either were immunized with ovalbumin (10 µg in CFA) or were not immunized. Fifteen BALB/c mice were immunized similarly with either β2GPI or ovalbumin. All mice in the first experiment were given a normal chow diet and were killed 3 months after the immunization.

In the second experiment, 3 groups of LDL-DRD mice (n=10 per group, 6 weeks old) were immunized with a regimen resembling the first experiment and fed an atherogenic diet for 6 weeks before they were killed.

**Cholesterol Level Determinations**

At the end of the experiment, 1 to 1.5 mL of blood was obtained by cardiac puncture; 1000 U/mL heparin was added to each sample. Total plasma cholesterol levels were determined by an automated enzymatic technique (Boehringer Mannheim).

**Detection of Anti-β2GPI Antibodies and Inhibition Studies**

Anti-β2GPI antibodies were detected by ELISA using either human or mouse β2GPI (10 µg/mL) for coating and performed as previously described.\(^32\) Inhibition assays were performed to confirm the specificity of the mouse anti-β2GPI antibodies and to check for their possible cross-reactivity with oxLDL. The concentration of serum from LDL-DRD mice giving half of the maximal binding to β2GPI was determined, and different inhibitors (at concentrations of 0 to 200 µg/mL) were used for inhibition (ie, oxLDL, LDL, human β2GPI, BSA) with the anti-β2GPI ELISA.

**Detection of Mouse β2GPI in Immune Complexes**

For detection of mouse β2GPI in immune complexes in sera from mice immunized with human β2GPI, a dot blot assay was performed as previously described.\(^33\) Briefly, 10 µL of protein G-Sepharose beads were added to 20 µL of serum. After 30 minutes of incubation at room temperature, the beads were washed with PBS and resuspended in 10 µL of PBS. Five microliters of the suspension was applied to a BAS 85 nitrocellulose membrane (Schleicher and Schuell), and 5 minutes later, 2 µL of 0.1 mol/L glycine buffer (pH 2.5) was added to the beads. After blocking with 3% BSA and washings, the membranes were incubated for 1 hour with anti-β2GPI biotinylated mouse monoclonal antibody (MAb). After additional washings (3 times, 15 minutes each), the membranes were transferred into a solution of streptavidin–horseradish peroxidase conjugate in PBS (1 : 25 000), and after extensive washing with PBS, a mixture of enhanced chemiluminescence detection reagents (model 300A, Molecular Dynamics). All sera were run in duplicate, and results of experiments were expressed as densitometry units.

**Evaluation of Mouse β2GPI Levels**

For detection of mouse β2GPI levels, we used capture ELISA. Ninety-six ELISA wells were coated with 10 µg of cof-18 (mouse MAb that binds human and mouse β2GPI) in bicarbonate buffer overnight at 4°C and blocked with BSA. After washings, mouse sera from all experimental groups were added for 2 hours. After additional washings, 2 µg/mL of biotinylated cof-21 (mouse MAb that binds human and mouse β2GPI) was added for 1 hour. Subsequently, the wells were incubated with alkaline phosphatase conjugated to avidin, and the reaction was developed with p-nitrophenylphosphate. For quantification of mouse β2GPI levels, the OD values were evaluated by construction of a standard curve with...
purified mouse β2GPI. To confirm specificity, mouse β2GPI was added to some wells containing mouse sera.

Detection of Anti-HSP-65 Antibodies
Antibodies to HSP-65 were detected with ELISA as previously described. This assay was used to exclude the possibility that the immune response to HSP-65, which has been shown to be proatherogenic, influenced atherosclerosis progression

Detection of Anti-oxLDL Antibodies
ELISA plates (Nunc) were coated with either copper-oxLDL, native LDL (at a concentration of 5 μg/mL in PBS), or PBS overnight at 4°C. Serum fractions were diluted to 1:50, and ELISA was performed as previously described. The assay was performed to rule out the possibility of cross-reactivity between anti-β2GPI and anti-oxLDL antibodies, which may have affected lesion formation in the mice.

Proliferation Assays of Draining Lymph Node Lymphocytes From Mice Immunized With β2GPI or Ovalbumin
Draining inguinal lymph nodes were collected from 4 β2GPI- or ovalbumin-immunized mice killed 8 days after the primary immunization. The assays were performed as previously described, with minor modifications. Briefly, 1×10^6 cells/mL were incubated in triplicate for 72 hours in 0.2 mL of culture medium in microtiter wells in the presence or absence of various antigens: concanavalin A, human β2GPI, mouse β2GPI, HSP-65, oxLDL, and BSA in 4 different concentrations (20, 10, 5, and 2.5 μg/mL). Proliferation was measured by the incorporation of [3H]thymidine into DNA during the final 12 hours of incubation. The results were computed as stimulation index: the ratio of the mean cpm of the antigen to the mean background cpm obtained in the absence of the antigen. SDs were always <10% of the mean cpm.

Assessment of Atherosclerosis
Atherosclerotic fatty-streak lesions were quantified by calculating the lesion size in the aortic sinus as previously described, with a few modifications. Briefly, the heart and upper section of the aorta were removed from the animals, and the peripheral fat was carefully cleaned. The upper section was embedded in OCT medium and frozen. Every other section (10 μm thick) throughout the aortic sinus (400 μm) was taken for analysis. The distal portion of the aortic sinus is recognized by the 3 valve cusps, which are the junctions of the aorta to the heart.

The extent of atherosclerosis was evaluated at the level of the aortic sinus. Processing and staining of the tissue with oil red O was carried out according to Paigen et al. Lesion area was quantified by the method of Rubin et al.

Immunohistochemistry of Atherosclerotic Lesions
Immunohistochemical staining for CD4, CD8, and macrophages was done on 5-μm-thick frozen sections of the aortic sinus. The sections were fixed for 4 minutes in methanol at −20°C followed by 10 minutes of incubation with ethanol at −20°C. The sections were then blocked with nonimmune goat serum for 15 minutes at room temperature followed by incubation with CAS blocking reagent for 30 minutes at room temperature. Subsequently, the rat anti-mouse CD4/CD8 MAb was added for 1 hour at room temperature. After washings, affinity-purified biotinylated rabbit anti-rat IgG antibodies (Jackson) were added for 30 minutes at room temperature. After washings, the slides were incubated with 0.3% H₂O₂, followed by additional rinses and incubation with streptavidin-peroxidase conjugate for 30 minutes at room temperature. After washings, the slides were developed with 3-amino-9-ethylcarbazole (AEC) substrate (Dako) for 15 minutes. Sections were counterstained with hematoxylin. Spleen sections were used as a positive control. Staining in the absence of first or second antibody was used as a negative control.

Antibodies to β2GPI, oxLDL, and HSP-65
All mice immunized with a single dose of human β2GPI were found to develop high titers of antibodies against human and mouse β2GPI, starting from 10 days after the immunization (Figure 1A and 1B, respectively). The high titers persisted until the mice were killed, 3 months after the immunization (Figure 2).

Statistical Analysis
The ANOVA test was used to compare values from the 3 experimental groups in each study. *P*<0.05 was accepted as statistically significant.

**Results**

**Body Weight**
In the first experiment, initial body weights for the LDL-RD were 23±2 g, and final body weights were 25±2 g. BALB/c mice weighed 19±2 g at the beginning and 21±2 g at the end of the experiment. In the second experiment, initial weights of the LDL-RD mice were 23±2 g at the beginning and 24±2 g at the end. No differences in the average weight were detected between the study groups given similar diets. One mouse from the β2GPI-immunized group and 2 mice immunized with ovalbumin died of infection during the experiment. Otherwise, none of the mice showed abnormalities in their general health.

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No antibodies were found against oxLDL or HSP-65 (data not shown).
Specificity of Anti-β2GPI Antibodies
The sera of the LDL-RD mice were inhibited in a dose-dependent manner by fluid-phase β2GPI (inhibition of 91% of the binding to β2GPI by a serum dilution of 1:200) but not with native LDL, oxLDL, or ovalbumin (Figure 3).

Circulating Immune Complexes and β2GPI Levels
Circulating immune complexes (CICs) containing mouse β2GPI were determined from the sera of mice taken at the end point of the experiment. The levels of β2GPI CICs were significantly higher in the chow diet–fed LDL-RD mice immunized with β2GPI (mean, 2264±123 densitometry units [DU]) compared with ovalbumin-immunized (1401±156 DU, P<0.05) and with nonimmunized (1168±105 DU, P<0.05) mice (Figure 4).

Levels of β2GPI were similar in the 3 experimental groups fed chow diet and were in the range of 150 to 220 µg/mL (similar to the levels in humans).

Cholesterol Levels in the Studied Mice
No statistically significant differences were found with respect to the levels of cholesterol in the LDL-RD mice (β2GPI-immunized, 219±25 mg/dL; ovalbumin-immunized, 228±25 mg/dL; and nonimmunized, 211±19 mg/dL). Similarly, no significant differences were noted between the atherogenic diet–fed mice: the mean cholesterol levels in the LDL-RD mice were 2348±56.6 mg/dL in the β2GPI-immunized mice, 2434±44.4 mg/dL in the ovalbumin-immunized mice, and 2549±90 mg/dL in the nonimmunized mice. The cholesterol levels in the β2GPI-immunized BALB/c mice were 78±12 mg/dL and in the ovalbumin-immunized mice, 81±11 mg/dL.

Proliferation of Lymph Node Lymphocytes on Exposure to β2GPI, oxLDL, and HSP-65
Statistically significant stimulation indexes were observed only in the lymphocytes from the LDL-RD mice immunized with β2GPI incubated with human β2GPI (stimulation indexes in β2GPI concentrations of 20, 10, 5, and 2.25 µg/mL were 2.49±0.156, 3.73±0.55, 3.51±0.55, and 3.83±0.625, respectively) (Figure 5). No reactivity of the lymphocytes was observed after in vitro exposure to mouse β2GPI, oxLDL, HSP-65, or BSA in the LDL-RD mice immunized with β2GPI.

Extent of Aortic Sinus Atherosclerosis
Lesion size in the aortic sinus was most prominent in the LDL-RD mice immunized with β2GPI (mean, 26 000±5700 µm²) compared with the LDL-RD mice immunized with ovalbumin (mean, 3000±1099 µm²; P<0.01) and with the nonimmunized mice (mean, 2250±700 µm²; P<0.01) (Figures 6 and 7). No significant differences were noted between the groups of atherogenic diet–fed mice: β2GPI-immunized, 98 000±38305 µm²; ovalbumin-immunized, 81 250±12 933 µm²; and nonimmunized, 75 625±72810 µm². No atherosclerotic lesions were observed in the aortic sinuses of the BALB/c mice immunized with β2GPI or ovalbumin.

Immunohistochemical Analysis of Atherosclerotic Lesions
The lesions of the chow-fed LDL-RD mice immunized with β2GPI appeared to be more mature and contained significantly larger numbers of macrophages. Pronounced infiltration of the aortic sinus valvular and subendothelial intimal...
regions spanning the aortic sinus with CD4+ and CD8+ lymphocytes was noted in the LDL-RD mice immunized with β2GPI (Figure 8). The lymphocytes found in the β2GPI-immunized LDL-RD mice did not appear to reside only in the sites adjacent to the fatty streaks. Only a small number of CD4+ lymphocytes were evident in the aortic sinus of the LDL-RD mice immunized with ovalbumin or the control nonimmunized LDL-RD mice.

Discussion

Atherosclerosis entails the accumulation of lipids in the vessel walls, leading to their subsequent narrowing. In recent years, it has become apparent that the immune system plays a dominant role in mediating atherogenesis, although the exact mechanisms have not been characterized in detail. Several authors postulated that the atherosclerotic process could be triggered or enhanced by immunogens such as the HSP-60/65 or oxLDL. These candidate antigens were shown to promote humoral and cellular immune responses, which were suggested to account for their proatherogenic effects.

The rationale of the present study stems from several in vitro studies suggesting that aCLs could accelerate atherogenesis. Furthermore, clinical studies have shown that patients with SLE are at increased risk of developing premature atherosclerosis, although the exact relationship to antici-

Figure 5. Proliferation of lymphocytes from LDL-RD mice immunized with β2GPI or ovalbumin. Draining inguinal lymph node lymphocytes were collected 8 days after primary immunization from 4 β2GPI-immunized or ovalbumin-immunized LDL-RD mice. Lymphocytes were incubated with different antigens (human β2GPI, mouse β2GPI, HSP-65, oxLDL, and BSA) at different concentrations, and proliferative response was expressed as stimulation index. Hβ2GPI indicates human β2GPI; Mβ2GPI, mouse β2GPI. Values are means of 5 mice per group.

Figure 6. Determination of atherosclerotic aortic lesion size. Aortic sinuses were cut and stained with oil red O. Assessment of plaque size was performed by an unbiased observer using a grid. Values are means of 15 mice per group.
out the possibility that the HSP-65 present in CFA was responsible for the accelerated atherosclerosis. The inhibition studies and lymphocyte proliferation assays excluded the possibility that immune response to \( \beta_2 \)GPI was cross-reactive with oxLDL; cross-reactivity of aCLs with oxLDL could have affected the progression of atherosclerosis, because the latter antibodies are considered by some authors to be protective.42,43

Several mechanisms could explain the enhanced atherosclerosis observed in the study: anti-\( \beta_2 \)GPI antibodies have recently been shown to activate endothelial cells,27,28 and it is possible that recruitment of immunopotent cell results in accelerated atherosclerosis. Such immune-mediated recruitment could account for the abundance of the CD4+ and CD8+ lymphocytes as well as the accumulation of macrophages within the atherosclerotic plaques.

An additional mechanism that could account for the enhanced atherogenesis in the \( \beta_2 \)GPI-immunized mice is an effect on macrophages. These latter cells were shown to accelerate the in vitro uptake of radiolabeled oxLDL in the presence of anti-\( \beta_2 \)GPI antibodies.26 This finding was explained by the association of anti-\( \beta_2 \)GPI with \( \beta_2 \)GPI, thus preventing the competition of the latter with oxLDL for the scavenger receptor–mediated binding to the macrophage. In this study, we have shown (Figure 1B) that the anti-human \( \beta_2 \)GPI antibodies also bound mouse \( \beta_2 \)GPI and could thus have influenced the uptake of lipids to resident macrophages, as was shown in the in vitro assays.26 Moreover, \( \beta_2 \)GPI possesses anticoagulant properties, and its “neutralization” by antibodies could contribute to a prothrombotic state, which could accelerate the atherosclerotic process.

The issue of binding of anti-human \( \beta_2 \)GPI antibodies to native mouse \( \beta_2 \)GPI in vivo was addressed by evaluation of the presence of immune complexes. Indeed, we have found higher levels of CICs containing mouse \( \beta_2 \)GPI in the sera of the mice immunized with human \( \beta_2 \)GPI, providing a possible explanation for the proatherogenic effect of the immunization. It should be mentioned in this respect that despite higher levels of CICs containing mouse \( \beta_2 \)GPI, the serum levels of this anticoagulant molecule did not differ between the experimental groups, thus excluding the possibility that it may have affected atherosclerosis progression.

Figure 7. Oil red O staining of atherosclerotic lesions. A, Average-size characteristic aortic sinus lesion from a chow diet–fed LDL-RD mouse immunized with \( \beta_2 \)GPI; B, representative lesion from an ovalbumin-immunized LDL-RD mouse. Magnification \( \times 15 \).
The control group in this experiment was immunized with ovalbumin. Use of an irrelevant protein for immunization might have been more appropriate in resolving $\beta_2$GPI specificity by these antibodies. However, this issue was addressed by evaluation of binding of the antibodies to immobilized or to circulating mouse $\beta_2$GPI (Figure 4).

NZWxBXB F1 (W/B F1) male mice develop a spontaneous, SLE-like syndrome with production of several autoantibodies and CICs. One of the features of the disease in these mice is a degenerative coronary vascular disease with myocardial infarction and thrombocytopenia. The production of antibodies to platelets and to $\beta_2$GPI/CL may play a pathogenic role in mediating the coronary vasculopathy, although lesions are not atherosclerotic but rather result from a prothrombotic state.

In conclusion, this study provides the first demonstration of a direct causal association between a cellular and a humoral immune response to $\beta_2$GPI and atherosclerosis progression. This observation is of particular interest because $\beta_2$GPI-binding antibodies formerly thought to invariably exist in APS patients are being increasingly reported in various infections. Because the association of infections with atherosclerosis has been raised by several authors (reviewed in References 47 and 48), the occurrence of anti-$\beta_2$GPI antibodies could provide a possible explanation for this link.

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References


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