Humoral Immune Response Against Defined Oxidized Low-Density Lipoprotein Antigens Reflects Structure and Disease Activity of Carotid Plaques

Isabel Gonçalves, Marie-Louise M. Gronholdt, Ingrid Söderberg, Mikko P.S. Ares, Borge G. Nordestgaard, Jacob F. Bentzon, Gunilla Nordin Fredrikson, Jan Nilsson

Background—Immune responses against oxidized low-density lipoprotein (LDL) play an important role in atherosclerosis. The aim of this study was to investigate if humoral immune response against specific oxidized LDL antigens, such as aldehyde-modified peptide sequences of apolipoprotein B-100, reflects disease activity and structure of atherosclerotic plaques.

Methods and Results—Plaques were obtained from 114 symptomatic subjects referred to carotid endarterectomy and characterized immunohistochemically and histologically. Plasma levels of IgG and IgM against aldehyde-modified apolipoprotein B-100 amino acid sequences 661 to 680, 3136 to 3155 (peptide 210), and 3661 to 3680 (peptide 240) were determined by enzyme-linked immunosorbent assay. High levels of IgG against peptide 210 were associated with increased plaque content of lipids ($r=0.24, P<0.05$) and hemorrhage ($r=0.27, P=0.005$), with decreased content of fibrous tissue ($r=-0.25, P=0.01$), but also with lower total plaque volume ($r=-0.21, P<0.05$). In contrast, high levels of IgM against peptide 240 were associated with plaques with more fibrous tissue ($r=0.35, P<0.001$), less lipids ($r=-0.34, P<0.001$), and less macrophages ($r=-0.24, P<0.05$). IgM against peptide 210 were found to be associated with plaque fibrous tissue ($r=0.20, P<0.05$), less lipids ($r=-0.21, P<0.05$), and less macrophages ($r=-0.27, P=0.01$).

Conclusion—These findings support the notion that immune responses against oxidized LDL epitopes are involved in atherosclerosis and that the level of circulating antibodies against these structures may reflect disease activity in the arterial wall. (Arterioscler Thromb Vasc Biol. 2005;25:1250-1255.)

Key Words: antibodies ■ atherosclerosis ■ carotid plaque ■ carotid stenosis ■ modified low-density lipoprotein
by coronary angiography\textsuperscript{16,17} and carotid intima-media thickness,\textsuperscript{18–20} We have recently identified aldehyde-modified peptide sequences in apoB-100 that are targeted by autoantibodies present in human plasma.\textsuperscript{19} In this study, we have investigated the association between the plasma levels of 3 of these autoantibodies specific for apoB-100 amino acids 661 to 680 (peptide 45), 3136 to 3155 (peptide 210), and 3661 to 3680 (peptide 240), respectively, by determining binding to the corresponding aldehyde-modified synthetic polypeptides in enzyme-linked immunosorbent assay (ELISA), and atherosclerotic carotid plaque structure as assessed by ultrasonography (grayscale median values), histology, and immunohistochemistry. These peptide sequences were selected because their effect when used in active immunization of experimental animals is well-characterized and because autoantibodies against these sequences are common in humans.

**Materials and Methods**

**Patients**

This study included 114 patients (77 males, 37 females), aged 60.9 ± 7.8 (mean ± SD) years, referred to carotid endarterectomy at Rigshospitalet, University of Copenhagen, Denmark. These patients had previously experienced ipsilateral hemispheric neurological symptoms (20 transient ischemic attack, 49 amaurosis fugax, and 45 stroke) in the last 93 ± 59 days. Plaques were removed from patients with internal carotid artery stenosis > 50%. The severity of carotid stenosis was assessed by duplex imaging by the same observer using internationally established criteria.\textsuperscript{21} Cardiovascular risk factors such as hypertension (systolic blood pressure > 140 mm Hg), diabetes, clinical history of coronary artery disease, claudication, tobacco use (in the past or current), and lipid-lowering medication were recorded. Laboratory analyses including total cholesterol, high-density lipoprotein cholesterol, LDL cholesterol, and triglycerides were performed in fasting blood samples. The study was approved by the local research ethics committee. Patients with excessive alcohol intake, liver disease, cancer, infectious diseases, systemic inflammatory disease, or cerebral hemorrhage were excluded. One or more computer tomography scans were performed to exclude cerebral hemorrhage as the cause for the neurological symptoms. Blood sampling for determination of oxidized LDL autoantibodies and fasting lipoproteins was performed the day before surgery.

**Ultrasound Evaluation**

Carotid high-resolution ultrasonography (Apogee Interspec 400 scanner; ATL Ultrasound Bothell, Wash; 5- to 10-MHz linear array probe) of the plaques was blindly performed preoperatively by one observer. Ultrasonographic data of 9 plaques were accidentally lost. The best longitudinal B-mode image of the carotid plaque and the corresponding color Doppler image showing the outlines of the plaque were recorded on S-VHS, later digitized, and processed by the software program Image-Pro Plus 1.2.01 for Windows (Media Cybernetics, Silver Spring, Md). The carotid plaque was outlined carefully, excluding acoustic shadowing when present. The median of the grayscale of the outlined pixels (grayscale median) was determined according to previously described and validated methodology.\textsuperscript{22–27} The degree of stenoses was determined by Doppler criteria, separating into 0% to 15%, 16% to 49%, 50% to 79%, and 80% to 99% stenosis and occlusion.\textsuperscript{21}

**Peptide ELISA**

Malondialdehyde (MDA)-modified peptides were prepared as described.\textsuperscript{28} MDA-modified peptide 45 (amino acids 661 to 680), 210 (amino acids 3136 to 3155), and 240 (amino acids 3661 to 3680)\textsuperscript{19} were used in the ELISAs. Peptide 45 contained 2 lysine residues and the MDA content of the modified peptide was 0.047 mol/mol peptide; peptide 210 contained 3 lysine and 1 histidine residues, and the MDA content of the modified peptide was 0.080 mol/mol peptide and peptide 240 contained 2 lysine residues, and the MDA content of the modified peptide was 0.048 mol/mol peptide. The peptides were diluted in phosphate-buffered saline, pH 7.4 (20 μg/mL), and used for coating of microtiter plates (Nunc MaxiSorp; Nunc, Roskilde, Denmark) in an overnight incubation (4°C). The coated plates were washed with phosphate-buffered saline containing Tween-20, blocked with Superblock in Tris-buffered saline (Pierce, Rockford, Ill) for 10 minutes at room temperature, incubated with human serum diluted 1:100 in TBS 0.1% Tween for 2 hours at room temperature, and thereafter overnight at 4°C. The deposition of antibodies was detected by adding biotinylated rabbit anti-human IgM or IgG antibodies (Jackson ImmunoResearch, Westgrove, Pa) for 2 hours at room temperature. After washing, bound antibodies were detected by alkaline phosphatase-conjugated streptavidin (DakoCytomation, Glostrup, Denmark) as described.\textsuperscript{28} The results are presented as absorbance (abs) units with the background value (binding to nonpeptide-coated plastic wells) subtracted. Specificity testing of the ELISAs was performed using plasma pooled from healthy controls (n = 33, age 42.7 ± 14.0 years). In this reference, plasma IgM levels for peptides 45, 210, 240, and MDA–LDL were 1.05 abs units, 1.47 abs units, 1.08 abs units, and 1.67 abs units, respectively. The interassay coefficients of variation for the IgM ELISAs were < 5%.

Pre-incubation of control plasma with 500 μg of peptide 210 for 1 hour at room temperature and overnight at 4°C competed 31% of binding to peptide 45, 57% of binding to peptide 210, 53% of binding to peptide 240, and 21% of binding to MDA–LDL. IgG levels for peptides 45, 210, 240, and MDA–LDL were 0.44 abs units, 0.22 abs units, 0.05 abs units, and 0.26 abs units, respectively. The interassay coefficients of variation for the IgG ELISAs were < 15%.

Pre-incubation of control plasma with 500 μg of peptide 210 competed 33% of binding to peptide 45, 70% of binding to peptide 210, 100% of binding to peptide 240, and 0% of binding to MDA–LDL.

**Histological Procedure**

Carotid plaques were removed en bloc by carotid endarterectomy. The specimen was then cut transversally in 3-mm-thick blocks (4 to 14 per patient), paraffin-embedded, and 4-μm sections were cut from the blocks. Sections were then stained with hematoxylin and eosin, Van Gieson stain, and Verhoeff stain for fibrous tissue. Plaque constituents (lipid-rich core, hemorrhage, and fibrous tissue) in all sections were measured morphometrically using the semi-automatic image analyzing software Leitz Texture Analyzing System (TAS, Cambridge, UK). The microscopic image of the plaque section was transferred through a video camera and digitized to a computer screen. Plaque constituents were then outlined manually using a light pen by a pathologist to determine the relative plaque area in each section. The total plaque volume is calculated as the sum of the plaque area in all sections times the plaque length of each section.\textsuperscript{29} The largest variation on reproducibility testing based on 10 randomly chosen plaques for measurements of plaque constituents was found for hemorrhage (0.5% ± 0.3%; mean ± SD). All analyses were performed blindly.

The section from the most stenotic area and the 2 adjacent ones (upstream and downstream) were used for macrophage staining. After rehydration and proteolytic pretreatment with trypsin, the 3 sections were immunostained by sequential incubation with blocking rabbit serum (DakoCytomation), primary monoclonal antibody against human macrophages (CD 68, 1:200; DakoCytomation), secondary biotinylated rabbit antibody (1:300; DakoCytomation), ABComplex-alkaline phosphatase (DakoCytomation), chromogen substrate (Vector Laboratories, Burlingame, Calif), and counterstained with hematoxylin. The microscopic images of the immunostained sections were captured with a Sony 3-chip color video camera (Tokyo, Japan) and analyzed blindly with an automated image analysis equipment. The brightness and color tone were adjusted and a 24-color palette containing 3 red colors specific for the macrophage staining was applied in PaintShop Pro 5 (Jasc Software, Minnetonka, Minn). The percentage of plaque having the macrophage-specific colors was quantified in Sigma Scan Pro 3.0.
TABLE 1. Clinical Characteristics of the Patients

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=114)</th>
</tr>
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<tbody>
<tr>
<td>Age, y</td>
<td>60.9±7.8</td>
</tr>
<tr>
<td>Sex</td>
<td>37 F/77 M</td>
</tr>
<tr>
<td>Degree of stenosis, %</td>
<td>80.4±12.1</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>54 (47)</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>12 (11)</td>
</tr>
<tr>
<td>Coronary artery disease, %</td>
<td>24 (21)</td>
</tr>
<tr>
<td>Smoking (in the past or currently), %</td>
<td>67 (59)</td>
</tr>
<tr>
<td>Fasting lipoproteins</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>6.3±1.6</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.4±0.5</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.5±1.1</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.9±1.1</td>
</tr>
<tr>
<td>Statin use, %</td>
<td>19 (17)</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation. F indicates female; HDL, high-density lipoprotein; LDL, low-density lipoprotein; M, male.

(Jandel Corporation, San Rafael, Calif). An average value of the macrophage density in the 3 sections was used in statistical analysis.

Statistical Analysis

Values are presented as mean±SD. χ² analyses or Fisher exact test analysis was performed to investigate associations with dichotomous variables. Two-group comparisons were performed using the unpaired Student t or Mann–Whitney test, according to the distribution of the samples. Spearman correlation and partial correlations controlling for age and gender were used. Linear regression models considering the histological parameters as the dependent variables were used. When the regression was multivariate, the backward elimination was performed. Values of P<0.05 were considered to indicate statistically significant findings. Statistical analysis was performed using SPSS 12.0.1.

Results

Basic Characteristics and Autoantibodies

The study group consisted of 77 men and 37 women with symptomatic carotid disease and internal carotid artery stenosis >50% referred to carotid endarterectomy. The clinical characteristics of the study group are described in Table 1. The most avidly expressed autoantibodies were IgM against peptide 210 (Figure 1). Significant correlations were found between the different IgM autoantibodies as well as between the different IgG. In contrast, there were no correlations between the respective IgM and IgG autoantibodies for each peptide (Table 2). IgG against peptide 210 was lower in men versus women (0.32±0.46 versus 0.49±0.40 abs units; P<0.02) and in patients with diabetes versus those without (0.10±0.10 versus 0.41±0.49 abs units; P<0.05). Otherwise, there were no statistically significant differences in the antibody levels for patients with or without hypertension, history of coronary artery disease, use of tobacco, or statin medication. With the exception of a positive association between IgG against peptide 45 and LDL cholesterol levels (r=0.21, P<0.05), there were no associations between antibodies and plasma lipoproteins.

Histology and Autoantibodies

Controlling for age and gender, significant correlations were found between high plasma levels of IgM against peptide 210 and an increased plaque content of fibrous tissue (r=0.20, P<0.05; Figure 2A). In contrast, high levels of IgG against the same peptide sequence were associated with a decreased content of fibrous tissue (r=−0.25, P=0.01; Figure 2A). Moreover, high levels of IgM against peptide 210 were associated with a decreased plaque content of lipids (r=−0.21, P<0.05; Figure 2C) and macrophages (r=−0.27, P=0.01; Figure 2E), whereas high IgG levels correlated with an increased plaque content of lipids (r=0.24, P<0.05; Figure 3B). There were also significant correlations between IgG against peptide 210 and the severity of plaque hemorrhage (r=0.27, P=0.005; Figure 3C), as well as with a lower total plaque volume (r=−0.21, P<0.05; Figure 3D).

Also, IgM against peptide 240 were found to be associated with plaques containing more fibrous tissue (r=0.35, P<0.001; Figure 2B), less lipids (r=−0.34, P<0.001; Figure 2D), and less macrophages (r=−0.24, P<0.05; Figure 2F). There were no significant relations between IgM against peptide 45 and plaque structure. Moreover, there were no

TABLE 2. Spearman Correlation Matrix for the Antibodies Against MDA-Modified apoB-100 Peptide Sequences (P45, P210, and P240)

<table>
<thead>
<tr>
<th></th>
<th>P45 IgG</th>
<th>P45 IgM</th>
<th>P210 IgG</th>
<th>P210 IgM</th>
<th>P240 IgG</th>
<th>P240 IgM</th>
</tr>
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<tbody>
<tr>
<td>P45 IgG</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P45 IgM</td>
<td>0.10</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P210 IgG</td>
<td>0.21*</td>
<td>−0.05</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P210 IgM</td>
<td>0.02</td>
<td>0.37†</td>
<td>−0.05</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P240 IgG</td>
<td>0.26†</td>
<td>−0.06</td>
<td>0.19*</td>
<td>0.03</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>P240 IgM</td>
<td>0.11</td>
<td>0.42†</td>
<td>−0.21*</td>
<td>0.59†</td>
<td>−0.02</td>
<td>1</td>
</tr>
</tbody>
</table>

Results are expressed as r values.

*P<0.05; †P<0.001.
significant correlations between IgG against peptides 45 and 240 and plaque structure. However, a significant association was observed between IgG against peptide 240 and the plaque grayscale median value at the pre-operative ultrasound investigation ($r = 0.23$, $P = 0.05$).

A linear regression analysis was performed, including IgG and IgM autoantibodies, age, gender, plasma lipoproteins, smoking, hypertension, diabetes, claudication, and family history of coronary heart disease. Significant independent associations with total plaque volume were found for gender ($P < 0.001$), total cholesterol ($P = 0.001$), high-density lipoprotein ($P = 0.005$), triglycerides ($P < 0.05$), and family history for coronary heart disease ($P < 0.05$), explaining 40% of the variation, whereas IgG against peptide 210 did not remain significantly associated after adjustment for the factors mentioned. Plasma levels of IgM against peptide 240, IgG against peptide 210, and family history for coronary heart disease together explained 17% of the variation in plaque fibrous tissue content ($P = 0.001$), but only IgM against peptide 240 remained independently associated after adjustment for all other factors ($P < 0.02$). IgG against 210 and IgM against 240 together explained 13% of the variation of plaque lipids ($P < 0.005$), but only IgM against peptide 240 showed independent significant association ($P = 0.01$). Both LDL ($P < 0.01$) and IgM against peptide 210 ($P < 0.02$) showed independent association with plaque macrophage content, together explaining 14% of the variation ($P < 0.005$). Finally, independent associations with plaque hemorrhage were found for IgG against peptide 210 ($P < 0.005$), age ($P < 0.05$), smoking ($P < 0.05$), claudication ($P < 0.005$), family history for coronary heart disease ($P < 0.05$), and degree of stenosis ($P < 0.05$) together explaining 27% of the variation ($P < 0.001$).

**Discussion**

The present findings suggest the interesting possibility that monitoring humoral immune responses against oxidized LDL-specific antigens could be used to determine the structure and disease activity of atherosclerotic lesions. High levels of IgG against the aldehyde-modified apoB-100 peptide 210 was associated with small, lipid-rich, fibrous-poor plaques frequently containing signs of hemorrhage. These features are generally considered characteristic for vulnerable, rupture-prone plaques. In contrast, high levels of IgM against aldehyde-modified peptides 210 and 240 were associated with fibrous, lipid-poor plaques containing less macrophages. These characteristics are considered to be typical for stable plaques with little risk for development of clinical events. Because evidence is accumulating of an important role for immune responses against oxidized LDL in the disease process, it seems reasonable that the activity of human immune responses against oxidized LDL could reflect the disease process within atherosclerotic plaques.

There were significant associations between the different IgM, as well as between the different IgG. To a certain extent, this was explained by cross-reactivity of the same antibodies with different peptides most likely caused by recognition of MDA adducts. Binding competition studies revealed that this was particularly true for antibodies binding to peptides 210 and 240. However, the cross-reactivity of these antibodies with peptide 45 was much less prominent despite a similar degree of MDA modification, suggesting that the antibody...
binding also depended on the peptide sequence. In contrast, there was no association between IgG and IgM against the same peptide. Moreover, immune responses against different sites in apoB-100 were not consistent in their association with plaque structure. The oxidative modification of LDL is a complex process and its in vivo kinetics remains largely unknown. However, it is known that LDL with minor modifications is present in the circulation, whereas more severely oxidized LDL are found inside atherosclerotic plaques.32

High levels of IgG against peptide 210 were not only associated with more lipid-rich lesions but also associated with a smaller plaque size. The latter observation is in agreement with several studies demonstrating an inverse association between oxidized LDL IgG and carotid intima-media thickness.19,20 However, in prospective studies, high titers of IgG against oxidized LDL have also been associated with an increased risk for development of cardiovascular events.33,34 This is in accordance with the present observation that IgG levels may reflect the presence of vulnerable plaques.

The functional role of oxidized LDL autoantibodies remains to be fully understood. The atheroprotective effect of immunization with oxidized LDL antigens has generally been associated with expression of specific IgG.13 Direct evidence for a protective effect of IgG has also been obtained from studies in mice treated with recombinant human IgG specific for the MDA-modified peptide 45 sequence.15 Because these studies favor an atheroprotective role of oxidized LDL IgG, the present observation of an association of these IgG with more unstable plaques appears paradoxical. One possibility is that this reflects a fundamental difference in the immune response to oxidized LDL that is activated endogenously as part of the atherosclerotic disease process and that activated in response to immunization. Apolipoprotein E−/− mice lacking functional CD4+ T cells have less atherosclerosis,35 suggesting that the net effect of adaptive immunity is proatherogenic. Assuming that similar mechanisms are involved also in the human disease process, IgG levels against oxidized LDL antigens may act as markers of this adaptive immune response. Experimental studies evaluating the effect of active immunization have consistently used adjuvants favoring Th2 type immune responses characterized by induction of antiinflammatory cytokines and increased IgG secretion.10–13 Accordingly, IgG may in this situation serve as marker for a shift from a pro-inflammatory Th1 response toward an antiinflammatory Th2 response and reach sufficiently high levels to have protective effects in itself.

IgM against oxidized LDL phospholipids inhibit the scavenger receptor-mediated uptake of oxidized LDL and apoptotic cells in macrophages.3 Immunization of apolipoprotein E−/− mice with Streptococcus pneumoniae has been shown to result in increased expression of oxidized LDL-specific IgM, inhibition of atherosclerosis, and reduced levels of oxidized LDL in plasma.36 The latter observation suggests the possibility that these IgM may help to clear oxidized LDL from the circulation. This notion has also been supported by clinical studies demonstrating inverse associations between IgM against MDA-modified apoB-100 peptides and plasma-oxidized LDL.18 However, recent findings of an unaltered clearance of oxidized LDL in immunodeficient mice argue against this effect of oxidized LDL IgM.37 The possibility that antibody opsonization of oxidized LDL in plaques may influence its removal from the extravascular space by mediating uptake via Fc or complement receptors and that IgG and IgM may differ in this respect should be considered.38 There is also a possibility that formation of oxidized LDL immune complexes in plaques may lead to complement activation and tissue damage. It remains to be clarified if the association between IgG against peptide 210 and plaque hemorrhage reflects activation of such processes.

The present observations need to be interpreted with due caution because they are based on a relatively small number of samples and represent associations present at a single time point. It would be of considerable interest to study the association of these immune responses with plaque structure over an extended time period using ultrasound, both extravascular and intravascular, or magnetic resonance imaging. Moreover, antibody levels were only compared with plaque tissue from a single arterial segment. It is uncertain how representative these plaques are of lesions in other arteries. It should also be kept in mind that the present findings only demonstrate the existence of an association between antibodies against oxidized LDL antigens and plaque structure but do not clarify whether these antibodies have a direct effect on plaques or if they only serve as secondary markers.

In summary, these studies add further support to the notion that immune responses against epitopes in oxidized LDL are involved in atherosclerosis and suggest that the level of circulating antibodies against these structures may reflect disease activity in the artery wall.

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References


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Antibodies Against oxLDL and Plaque Structure

1255


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