Apolipoprotein B-100 Antibody Interaction With Atherosclerotic Plaque Inflammation and Repair Processes

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Background and Purpose—Treatment with IgG against the malondialdehyde (MDA)-modified apolipoprotein B-100 epitope p45 reduces atherosclerosis in experimental models. This study investigated the association between p45 IgG autoantibodies and plaque inflammation in subjects with advanced cardiovascular disease.

Methods—Native and MDA-p45 IgG levels were analyzed by ELISA in 349 carotid endarterectomy patients. In a subcohort of 195 subjects, endarterectomy samples were analyzed by immunohistochemistry and ELISA to determine plaque constituents and inflammation. Peripheral blood mononuclear cells were isolated from healthy donors.

Results—Patients with preoperative events of neurological ischemia had lower levels of native p45 IgG. Low levels of MDA-p45 IgG were associated with increased risk of postoperative cardiovascular death during a mean follow-up of 54 months. High plasma levels of native p45 IgG were associated with increased plaque content of collagen and smooth muscle cell growth factors, as well as lower levels of proinflammatory cytokines. Exposure of peripheral blood mononuclear cells from healthy donors to recombinant MDA-p45 IgG in presence of oxidized low-density lipoprotein reduced the expression of tumor necrosis factor-α and stimulated release of smooth muscle cell growth factors.

Conclusions—This study confirms previous experimental findings of anti-inflammatory properties of apolipoprotein B-100 p45 antibodies and provides the first clinical evidence of associations between p45 IgG autoantibody levels and atherosclerotic plaque inflammation, plaque repair as well as prevalent and incident cardiovascular events in carotid endarterectomy patients. These findings suggest the possibility that treatment with anti-p45 antibodies may have beneficial effects in advanced cardiovascular disease. (Stroke. 2016;47:1140-1143. DOI: 10.1161/STROKEAHA.116.012677.)

Key Words: antibodies ■ atherosclerosis ■ collagen ■ inflammation ■ malondialdehyde

Oxidation of low-density lipoprotein (LDL) in the arterial intima has been identified as an important cause of atherosclerotic plaque development.1 Autoantibodies recognizing epitopes in oxidized LDL (oxLDL) are common in the circulation of both healthy subjects and patients with cardiovascular disease (CVD).2 The function of these autoantibodies remains to be fully understood, but they have been proposed to act as markers of disease severity, to contribute to oxLDL clearance and to modulate inflammatory activity.3,4 IgG autoantibodies against both native and malondialdehyde (MDA)-modified apolipoprotein B-100 (apo B-100) peptides are present in the circulation of most individuals and have generally been associated with less severe atherosclerosis.3,5 Immunization of apo E deficient mice with the MDA-modified apo B-100 peptide MDA-p45 increases MDA-p45 IgG and reduce atherosclerotic plaque formation suggesting that antibodies recognizing modified peptide epitopes in apo B-100 are atheroprotective.6 This concept gained further support from studies demonstrating that anti-p45 IgG inhibited plaque development inflammation in experimental atherosclerosis.7 The mechanism through which anti-p45 IgG inhibits atherosclerosis involves inhibition of inflammation by binding of immune complexes to the inhibitory FcγRII.8 In accordance, administration of this antibody (MLDL1278a) to diet-induced obese non-human primates resulted in a significant decrease in circulating proinflammatory cytokines.9 However, treatment with MLDL1278a failed to decrease carotid plaque inflammation, as assessed by positron emission tomography with 18-F-fluorodeoxyglucose, in the randomized, double-blind Goal of Oxidized LDL and Activated Macrophage Inhibition by Exposure to a Recombinant Antibody (GLACIER) study in subjects with stable CVD.9 The reasons for this remain to be fully understood but may reflect an incomplete understanding of the role of antibodies targeting MDA-modified apo B-100 in clinically

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manifest CVD. With this study, we aimed to reach a better understanding of how antibodies against epitopes in oxLDL influence inflammation and repair processes in advanced human atherosclerotic plaques.

**Materials and Methods**

The study cohort included 349 carotid endarterectomy patients with follow-up through national registers for 54.1±21 months. A total of 93 cardiovascular (CV) events were registered during the postoperative follow-up period. Out of these 27 were fatal (21 acute myocardial infarction and 6 strokes) and 66 nonfatal (29 acute myocardial infarction, 18 strokes, 15 transient ischemic attacks, and 4 Amaurosis fugax). Antibodies against p45 and MDA-p45 were determined by ELISA. Sections and homogenates from 192 carotid plaques were analyzed by Oil Red O staining for lipids, α-actin, and CD 68 immunohistochemistry for smooth muscle cells and macrophages and ELISA for determination of different cytokines and growth factors. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors and exposed to oxLDL and anti-MDA-p45 IgG in cell culture for 48 hours. Tumor necrosis factor (TNF)-α, interleukin (IL)-10, and platelet-derived growth factor (PDGF) were analyzed with multiplex technology (Meck Millipore). Detailed Materials and Methods including statistical methods are available in the online-only Data Supplement.

**Results**

**Association Between p45 Autoantibodies and Preoperative Clinical Events**

The clinical characteristics of the cohort are shown in Table I in the online-only Data Supplement. Subjects with preoperative neurological symptoms (n=237) had lower levels of native p45 IgG than subjects with asymptomatic plaques [median and interquartile range; 0.54 (0.36–0.83) versus 0.67 (0.41–0.93) normalized absorbance units, \( P < 0.05 \)], whereas there was no difference in MDA-p45 IgG levels.

**Association Between p45 Autoantibodies and Clinical Events During Follow-Up**

Kaplan–Meier plots of event-free survival showed increased CV mortality in patients with low levels of MDA-p45 IgG (Figure 1). The association between low levels of MDA-p45 IgG and fatal postoperative CV events remained significant when adjusting for age, sex, total cholesterol, high-density lipoprotein cholesterol, smoking habits, and hypertension in a Cox Proportional Hazard model [MDA-p45 IgG first quartile hazard ratio, 2.9 (95% confidence interval: 1.3–6.6; \( P = 0.01 \))]. There was no association between p45 autoantibody levels and development of combined nonfatal and fatal CV events (data not shown).

**Association Between p45 Autoantibodies and Markers of Plaque Inflammation and Repair**

We next determined how IgG autoantibodies against native and MDA-p45 were associated with expression of markers of inflammation and tissue repair in plaques obtained from 195 endarterectomy patients. The average degree of preoperative stenosis was 84.9%. Examples of staining for lipids (Oil Red O), macrophages (CD68), and smooth muscle cells (α-actin) are shown in Figure I in the online-only Data Supplement. The median and interquartile range of percent plaque area staining for Oil Red O, CD68, and α-actin was 25.9 (15.4–37.8), 24.3 (16.5–34.5), and 30.0 (14.8–34.2), respectively. When comparing p45 autoantibodies in plasma with expression of inflammatory and repair markers in homogenates from carotid atherosclerotic plaques we found that plasma levels of native p45 IgG demonstrated negative associations with the plaque content of IL-6 and TNF-α, but positive associations with collagen and the smooth muscle cell mitogens PDGF and epidermal growth factor \((r=0.22)\), whereas no significant associations were observed for MDA-p45 IgG (Table). There were no significant associations between p45 autoantibodies in plasma and the expression of the macrophage marker CD68 or the smooth muscle cell marker α-actin in immune-histochemical stains of plaque sections (data not shown).

**Effect of MDA-p45 IgG on Human PBMCs**

To determine how antibodies against apo B-100 influence the expression of factors involved in inflammation and repair, we...
exposed PBMCs to different concentrations of oxLDL with or without addition of anti-MDA-p45 IgG. Incubation of PBMCs with up to 100 μg/mL of oxLDL did not affect the release of TNF-α, whereas addition of oxLDL together with anti-MDA-p45 IgG markedly reduced the secretion of TNF-α (Figure 2A). Exposure of PBMCs to oxLDL resulted in a dose-dependent inhibition of IL-10 release that was reversed by addition of anti-MDA-p45 IgG (Figure 2B). OxLDL did not influence the release of PDGF, but addition of anti-MDA-p45 IgG enhanced PDGF secretion at lower oxLDL concentrations (Figure 2C).

**Discussion**

Our observations provide clinical support for the notion that antibodies against the apo B-100 p45 epitope have atheroprotective properties by affecting plaque inflammation and repair. Exposure of human PBMCs to anti-MDA-p45 IgG reduced expression of TNF-α and enhanced the secretion of the anti-inflammatory cytokine IL-10. These observations are in line with previous studies by Li et al., demonstrating that anti-MDA-p45 IgG inhibits the release of proinflammatory cytokines. The in vivo relevance of this finding was supported by the observation that treatment of non-human primates with anti-MDA-p45 IgG reduced circulating levels of TNF-α and IL-1β. In line with this, we found that subjects with high levels of anti-native p45 IgG had a lower carotid plaque expression of IL-6 and TNF-α.

We also found that anti-MDA-p45 IgG stimulates the release of PDGF, an important growth factor for arterial smooth muscle cells, suggesting that antibodies against the apo B p45 epitope may promote vascular repair responses. This possibility was further supported by the finding that plaques from carotid endarterectomy patients with high circulating levels of anti-native-p45 IgG had a higher content of PDGF, epidermal growth factor, and collagen, indicative of ongoing repair processes.

An important question is whether these effects of p45 antibodies are of importance for the risk of developing CV events. In support of this notion, we found reduced levels of native p45 IgG in carotid endarterectomy patients with preoperative symptoms and that low levels of MDA-p45 IgG was associated with an increased risk of fatal postoperative CV events. These observations are in contrast to the finding in the GLACIER trial that treatment with recombinant MDA-p45 IgG failed to reduce carotid plaque inflammation. However, it is possible that the level of plaque inflammation in the stable CVD patients participating in the GLACIER trial was too low for the antibody treatment to have an effect. Thus, it cannot be excluded that treatment with MDA-p45 antibodies would be effective in patients with more severe disease such as carotid endarterectomy patients.

In conclusion, this study confirms previous experimental findings of anti-inflammatory properties of apo B-100 p45 antibodies and provides the first clinical evidence of associations between p45 IgG autoantibody levels and atherosclerotic plaque inflammation, plaque repair as well as prevalent and incident CV events in carotid endarterectomy patients. These findings suggest the possibility that treatment with p45 antibodies may have beneficial effects in subjects with advanced CVD.

### Table. Spearman’s Correlations (r) Between p45 IgG Autoantibodies and Inflammation and Apoptosis Markers in Plaque Homogenates and Plasma

<table>
<thead>
<tr>
<th></th>
<th>IgG-p45nat</th>
<th>P Value</th>
<th>IgG-p45MDA</th>
<th>P Value</th>
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<tr>
<td>Inflammatory markers</td>
<td></td>
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<tr>
<td>IL-6</td>
<td>−0.15</td>
<td>0.035</td>
<td>−0.09</td>
<td>NS</td>
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<td>TNF-α</td>
<td>−0.22</td>
<td>0.003</td>
<td>−0.07</td>
<td>NS</td>
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<tr>
<td>MCP-1</td>
<td>−0.14</td>
<td>NS</td>
<td>−0.09</td>
<td>NS</td>
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<tr>
<td>RANTES</td>
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<td>NS</td>
<td>0.01</td>
<td>NS</td>
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<tr>
<td>IL-10</td>
<td>0.09</td>
<td>NS</td>
<td>0.04</td>
<td>NS</td>
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<tr>
<td>Repair markers</td>
<td></td>
<td></td>
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<tr>
<td>Collagen</td>
<td>0.15</td>
<td>0.036</td>
<td>0.10</td>
<td>NS</td>
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<tr>
<td>PDGF</td>
<td>0.24</td>
<td>0.001</td>
<td>0.11</td>
<td>NS</td>
</tr>
<tr>
<td>EGF</td>
<td>0.22</td>
<td>0.003</td>
<td>0.10</td>
<td>NS</td>
</tr>
</tbody>
</table>

EGF indicates epidermal growth factor; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; NS, nonsignificant; PDGF, platelet-derived growth factor; RANTES, Regulated on Activation, Normal T Cell Expressed and Secreted; and TNF-α, tumor necrosis factor α.

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**Figure 2.** Cytokines and platelet-derived growth factor (PDGF) secreted from oxidized low-density lipoprotein (oxLDL) stimulated peripheral blood mononuclear cells (PBMCs) with or without anti-malondialdehyde (MDA)-p45 IgG. PBMCs from healthy donors were stimulated with increasing concentrations of oxLDL with or without anti-MDA-p45 IgG. OxLDL (A), IL-10 (B), and PDGF (C) were determined with multiplex technology in the cell culture media after 48 hours. Values are mean and standard deviation. Note that values with low standard deviation may appear as filled symbols. *P<0.05, **P<0.001, and ***P<0.001 for OxLDL versus oxLDL/anti-MDA-p45 IgG. IL indicates interleukin; and TNF-α, tumor necrosis factor α.
Sources of Funding
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Disclosures
Dr Nilsson is coinventor on patents for immunomodulation of atherosclerosis assigned to Cardiovax, CA.

References
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SUPPLEMENTAL MATERIAL

Materials and Methods

Patient data

Three hundred-forty-nine patients who underwent carotid endarterectomy between 2005 and 2010 at Scania University Hospital were included in the study. All patients gave written informed consent and the local ethical committee approved the study. Clinical charts were reviewed to gain information about comorbidities and medical history. The Swedish Cause of Death and National in-patient Health Registers were used to identify postoperative CV events that occurred until December 2012 using the G45, G46, I20 to I25 ICD-10 codes.

Analysis of p45 IgG

Antibodies against p45 epitopes were measured using by ELISA as reported elsewhere. Briefly, the 96-holes plates (Nunc, Roskilde, Denmark) were coated with 20 µg/ml of native p45 and malondialdehyde (MDA)-modified p45 (TAG Copenhagen A/S, Copenhagen, Denmark), and incubated overnight at 4 °C. Biotinylated rabbit polyclonal antibody to human IgG (Abcam ab7159) or goat anti-human IgM (ICN 67-321, Biomedicals, Inc., Aurora, OH) were used to detect bound autoantibodies. Values are presented as ratios (normalized absorbance units) to reference plasma with high levels p45 autoantibodies. The reference plasma was included on each ELISA plate to control for inter-plate variation. The intra- and inter-assay coefficients of variation are for the native and MDA-p45 autoantibody ELISAs 9-10% and 23%, respectively.
Carotid plaque homogenate analysis

After surgical removal, the plaques were immediately snap-frozen in liquid nitrogen. A one-mm fragment at the most stenotic region was used for histology and the rest of the plaque was homogenized. For measuring collagen content in plaque homogenate Sircol soluble collagen assay (Biocolor, Carrickfergus, UK) was used as describe. Eight µm sections were fixed in Histochoice (Amresco, Solon, OH). For immunohistochemistry, monoclonal mouse anti-human CD68 diluted 1:100 in 10% rabbit serum and monoclonal mouse anti-human smooth muscle alpha actin diluted 1:50 in 10% rabbit serum (both from DakoCytomation, Glostrup, Denmark) were used to stain for macrophages and smooth muscle cells, respectively. Stained areas were quantified blindly using Biopix iQ 2.1.8 (Gothenburg, Sweden). IL-6, TNF-α, MCP-1 and RANTES in plaque homogenates and plasma, as well as PDGF and EGF in plaque homogenates, were analyzed by ELISA as previously described.

Stimulation of PBMCs

LDL was oxidized by exposure to 5 µM CuCl2 for 18h at 37°C as previously described. PBMCs were purified from healthy donors with Ficoll-Paque (GE Heathcare). The cells were cultured in 2% human serum in the presence of 5-100 µg/ml oxLDL with and without addition of MDA-p45 IgG (kindly provided by Cardiovax, Los Angeles, CA). Dose-response experiments identified 60 µg/ml of oxLDL as an effective concentration of MDA-p45 IgG (data not shown). Cell culture media was analyzed with multiplex technology (Meck Millipore) for detection of TNF-α, IL-10 and PDGF.
Statistical analysis

Continuous variables are presented as mean ± standard deviation (SD) or median and interquartile range depending on distribution. Pearson’s Chi-square test was used for categorical variables. Student’s t-test were used for continuous variables whenever normally distributed, while Mann-Whitney U test and Spearman’s rank correlation were used for non-normally distributed variables. Freedom from postoperative events was calculated by life-tables according to Kaplan-Meier survival analysis and is presented as survival estimate ± standard error. Correction for the above mentioned variables was done through Cox regression analysis. A P-value of < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 22.0 (IBM Corp., Amonk, NY).

References


Supplemental table I. Clinical characteristics of the study cohort

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<th>CV death (27)</th>
<th>no CV death (322)</th>
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<td>71.8 ± 8.49</td>
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<td>75.2 ± 6.89</td>
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<td>66.7 (62)</td>
<td>66 (169)</td>
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<td>66.7 (18)</td>
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<td>74.2 (69)</td>
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<td>.818</td>
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<td>7.19 ± 9.09</td>
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<td>.403</td>
<td>7.37 ± 2.26</td>
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Categorical variables are expressed in percentages (calculated in the group of patients with and without CV deaths, respectively), continuous variables as median ± standard deviation. Systolic pressure > 140 mmHg; § not active smoking for at least 6 months before surgery. hsCRP indicates high sensitive C-reactive protein; WBCC, white blood cells count.
Supplemental figure I. Carotid plaque stained for (A) lipids with Oil Red O, (B) macrophages with CD68 immunohistochemistry and (C) smooth muscle cells with α-actin immunohistochemistry. Bars equal 1.0 mm.