Human Carotid Plaques With High Levels of Interleukin-16 Are Associated With Reduced Risk for Cardiovascular Events

Caitríona Grönberg, MS; Eva Bengtsson, PhD; Gunilla Nordin Fredrikson, PhD; Mihaela Nitulescu, BcS; Giuseppe Asciutto, MD, PhD; Ana Persson, BcS; Linda Andersson, BcS; Jan Nilsson, MD, PhD; Isabel Gonçalves, MD, PhD; Harry Björkbacka, PhD

Background and Purpose—Interleukin-16 (IL-16) functions as a regulator of T-cell growth and acts as an inducer of cell migration. The aim of this study was to determine whether IL-16 measured in human carotid plaques was associated with symptoms (eg, stroke, transient ischemic attack, or amaurosis fugax), markers of plaque stability, and postoperative cardiovascular events.

Methods—Plaques obtained from patients who had ≥1 cerebrovascular ischemic events within 1 month before endarterectomy (n=111) were compared with plaques from patients without symptoms (n=95). Neutral lipids, smooth muscle cell, and macrophage contents were evaluated histologically, and collagen, elastin, and caspase-3 activity were measured biochemically. IL-16, matrix metalloproteinases, and tissue inhibitors of metalloproteinases were measured in plaque homogenates using a multiplex immunoassay. IL-16, CD3, CD4, and FoxP3 mRNA expressions in carotid plaques were analyzed with quantitative real-time polymerase chain reaction.

Results—Carotid plaques from asymptomatic patients had higher levels of IL-16 mRNA. High plaque IL-16 protein levels (above median) were associated with reduced incidence of postoperative cardiovascular events during a mean follow-up of 21 months (hazard ratio, 0.47; 95% confidence interval, 0.22–0.99; P=0.047). IL-16 levels correlated with the plaque-stabilizing components: elastin, collagen, matrix metalloproteinase-2, tissue inhibitors of metalloproteinase-1, tissue inhibitors of metalloproteinase-2 and FoxP3 mRNA.

Conclusions—This study shows that high levels of IL-16 are associated with asymptomatic carotid plaques, expression of factors contributing to plaque stability, and decreased risk of new cardiovascular events during a 2-year period after surgery, suggesting that IL-16 might have a protective role in human atherosclerotic disease. (Stroke. 2015;46:2748-2754. DOI: 10.1161/STROKEAHA.115.009910.)

Key Words: atherosclerosis • carotid stenosis • interleukin-16 • inflammation • polymerase chain reaction

Atherosclerosis is an inflammatory disease driven by the entrapment of low-density lipoprotein particles in the intima of the arterial vessel wall.¹ The entrapment and modification of low-density lipoprotein particles trigger inflammatory events in the vessel wall, which occur early in the atherosclerotic plaque formation.² Advanced rupture-prone (vulnerable) atherosclerotic plaques are characterized by large extracellular lipid deposits, necrosis, and reduced content of connective tissue proteins and infiltration of immune cells, such as macrophages and T cells.³

There is increasing evidence that T-cell subsets modulate the atherosclerotic disease process in different ways. Studies on Th1 cells (T-bet+) have been shown to promote the plaque inflammation, whereas studies on Th2 (GATA3+) cells are associated with protection.⁴ Regulatory T cells (FoxP3+) are clearly involved in dampening the inflammation and have been shown to be protective in atherosclerotic plaque formation.⁵⁻⁷

Interleukin 16 (IL-16; previously known as lymphocyte chemoattractant factor) is a chemokine expressed by a variety of different cells such as CD8+ T cells, macrophages, and endothelial cells.⁸⁻⁹ The IL-16 protein is produced in a pro–IL-16 form, which is later cleaved by caspase-3.¹⁰⁻¹¹ CD4+ T cells have been found in large numbers in the intima, at the site of inflammation and plaque formation.¹² It is known that IL-16 acts as a chemoattractant on CD4+ T cells and is thought to inhibit the T-cell receptor, making the cells incapable of proliferation after antigen stimulation.¹³ IL-16 is a pleiotropic cytokine with proinflammatory, Th1-promoting
properties in diseases such as asthma, allergy, and rheumatoid arthritis. On the contrary, IL-16 has been described as an anti-inflammatory cytokine by induction of FoxP3 expression in human T cells.

Given the evidence for IL-16 having a role in T-cell biology, we hypothesized that increased levels of IL-16 in the plaque would impact the number of T cells and plaque phenotype. Here, we measured mRNA and protein levels of IL-16 in human carotid plaques, removed by endartectomy, plasma levels of IL-16 from patients with or without symptoms, and associations with plaque markers of stability and postoperative cardiovascular events during a mean follow-up time of 21 months.

Materials and Methods

Patients

Two hundred and six patients who underwent carotid endarterectomy (plaques were removed en bloc) were included in this study after investigation with ultrasound to determine stenosis degree. The clinical characteristics of the patients are summarized in Table 1. Patients were classified as diabetic (fasting glucose >7 mmol/L, or 2-hour glucose >12 mmol/L), hypertensive (systolic blood pressure >140/90 mmHg) and obese (body mass index >30). Patients who qualified for surgery had plaques associated with symptoms (amaurosis fugax, transient ischemic attack, or stroke, n=111) and stenosis degree >70% (n=95). Those who underwent coronary endarterectomy were included in this study after informed consent, and the study was approved by the local ethical board. The day before surgery blood samples were collected. Patients gave informed consent, and the study was approved by the local ethical board.

Postoperative Events

Patients who underwent coronary endarterectomy were included in the study. The Swedish Cause of Death and National inpatient Health Registers were used to identify postoperative cardiovascular events occurring ≤2 years after surgery. International Classification of Diseases-10th Revision (G45, G46, I20 to I25) were considered. Additional details can be found in the online-only Data Supplement.

Carotid Plaque Histology and Homogenate Analysis

The carotid plaques were snap-frozen in liquid nitrogen immediately after surgical removal from the patient. One-millimeter-thick sections of the most stenotic part of the plaque were used for histological analysis. A description of the carotid plaque immunohistochemistry and homogenate analysis has been described before and is given in the online-only Data Supplement.

IL-16 Measurements

Carotid plaque IL-16 mRNA was measured by quantitative polymerase chain reaction and normalized to GAPDH and Cyclophilin A. Because IL-16 is a chemotactic factor for T lymphocytes, we hypothesized that IL-16 mRNA levels would correlate to mRNA levels of the T-cell markers CD3 and CD4. To further confirm the previous connection between IL-16 and increased FoxP3 (Treg marker) levels, we also set out to analyze the levels of FoxP3 mRNA in the carotid plaques. IL-16 protein was measured in carotid plaque homogenate supernatants using Bio-Plex Pro Human Chemokine Assay (BioRad, Hercules, CA), using magnetic beads, according to manufacturer’s instructions. IL-16 in plasma was measured by Proseek Multiplex CVD96x96 reagents kit (Olink Bioscience, Uppsala, Sweden). Extensive information is given in the online-only Data Supplement.

Statistics

Variables are presented as mean (SD), median (interquartile range) or as percentages. Comparison between groups was done with either an independent t test (for normally distributed continuous variables) or a Mann–Whitney U test (for nonparametric continuous variables), and differences in categorical data were calculated with χ² tests. Spearman correlations were used to determine univariate association between variables.

Table 1. Baseline Clinical Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Asymptomatic (n=95)</th>
<th>Symptomatic (n=111)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>67.1 (SD, 6.6)</td>
<td>71.3 (SD, 9.3)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Body mass index</td>
<td>26.9 (SD, 4.0)</td>
<td>26.5 (SD, 4.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Sex, men/women</td>
<td>65/30</td>
<td>75/36</td>
<td>NS</td>
</tr>
<tr>
<td>Degree of stenosis, %</td>
<td>90 (IQR, 80–95)</td>
<td>90 (IQR, 75–95)</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>24.2</td>
<td>44</td>
<td>0.003</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>79</td>
<td>71</td>
<td>NS</td>
</tr>
<tr>
<td>Smoking (in the past or current, %)</td>
<td>84.2</td>
<td>78.4</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting lipoproteins, mmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>4.3 (IQR, 3.4–5.1)</td>
<td>4.3 (IQR, 3.6–5.1)</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>2.2 (IQR, 1.8–3.1)</td>
<td>2.5 (IQR, 2–3.3)</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.1 (IQR, 0.9–1.4)</td>
<td>1.1 (IQR, 0.9–1.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides cholesterol</td>
<td>1.3 (IQR, 0.9–1.8)</td>
<td>1.3 (IQR, 1–1.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>142 (SD, 13.0)</td>
<td>139 (SD, 13.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine, mmol/L</td>
<td>84 (IQR, 72–100)</td>
<td>91 (IQR, 77–104)</td>
<td>NS</td>
</tr>
<tr>
<td>High-sensitive CRP</td>
<td>3.8 (IQR, 1.8–6)</td>
<td>4.1 (IQR, 2.0–7.1)</td>
<td>NS</td>
</tr>
<tr>
<td>White blood cell count, 10^9/L</td>
<td>7.8 (IQR, 6.4–8.9)</td>
<td>7.6 (IQR, 6.5–9.2)</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c, mmol/L</td>
<td>6.4 (IQR, 5.4–7.0)</td>
<td>6.8 (IQR, 5.7–7.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Statins, %</td>
<td>91.6</td>
<td>83.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are represented as percentages, mean with SD, or when not normally distributed as median with IQR. CRP indicates c-reactive protein; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; and NS, nonsignificant.
Table 2. Spearman Correlations and Linear Regression Between IL-16 (Plaque Protein, mRNA, and Plasma Levels) and Plaque Components, Matrix Metalloproteinases and Their Inhibitors, and T-Cell–Related mRNAs

<table>
<thead>
<tr>
<th>Plaque components</th>
<th>IL-16 Protein n=48</th>
<th>IL-16 mRNA n=48</th>
<th>IL-16 Plasma n=48</th>
<th>Linear Regression IL-16 Protein n=48</th>
<th>IL-16 mRNA n=48</th>
<th>IL-16 Plasma n=48</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-actin, % of area</td>
<td>NS</td>
<td>NS</td>
<td>n=0.177*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD68, % of area</td>
<td>n=0.153*</td>
<td>NS</td>
<td>NS</td>
<td>n=0.086*</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Oil Red 0, % of area</td>
<td>n=0.340†</td>
<td>NS</td>
<td>NS</td>
<td>n=0.199*</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Collagen, mg/g</td>
<td>n=0.366†</td>
<td>NS</td>
<td>NS</td>
<td>n=0.269†</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Elastin, mg/g</td>
<td>n=0.308†</td>
<td>NS</td>
<td>NS</td>
<td>n=0.240†</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Caspase-3 activity</td>
<td>n=0.234†</td>
<td>NS</td>
<td>NS</td>
<td>n=0.228†</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MMPs</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>MMP-1</td>
<td>n=0.362‡</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MMP-2</td>
<td>n=0.281‡</td>
<td>NS</td>
<td>n=0.192†</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MMP-3</td>
<td>NS</td>
<td>NS</td>
<td>n=0.309‡</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MMP-9</td>
<td>n=0.428‡</td>
<td>NS</td>
<td>n=0.183*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>MMP-10</td>
<td>n=0.343‡</td>
<td>NS</td>
<td>n=0.194†</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>n=0.400‡</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>n=0.149*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>mRNA</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CD3</td>
<td>n=0.209*</td>
<td>n=0.191*</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CD4</td>
<td>NS</td>
<td>n=0.404‡</td>
<td>NS</td>
<td>n=0.246*</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>FOXP3</td>
<td>n=0.200*</td>
<td>n=0.465‡</td>
<td>NS</td>
<td>n=0.520†</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

IL indicates interleukin; MMP, matrix metalloproteinase; NS, nonsignificant; and TIMP, tissue inhibitors of metalloproteinase.

Level of statistical significance is annotated as *P<0.05, †P<0.01, and ‡P<0.001.

IL-16 levels and plaque phenotypic markers. Linear regression was used to determine independent associations between IL-16 levels and plaque phenotypic markers. Non-normally distributed variables were log transformed before linear regression analysis. Kaplan–Meier survival curves were used to analyze event-free survival over time during follow-up. Binary logistic regression and Cox regression were used to determine association between IL-16 and symptoms and cardiovascular events within 2 years after surgery, when controlling for confounders. Potential confounders (age, sex, hypertension, diabetes mellitus, smoking, and dyslipidemia) were included in the corrected model if they had a P value of ≤0.05 when comparing the patient groups and IL-16 levels. If no confounders could be identified, age and sex were used. A multivariate partial least square regression analysis was performed using Simca version 13.0.2 (Umetrics, Umeå, Sweden). Statistical analyses were performed using IBM SPSS Statistics version 20 and in GraphPad Prism version 6.

Results

Of the 206 carotid plaques in this study, 95 were asymptomatic and 111 were symptomatic, ie, related to a preoperative cerebrovascular. The 2 patient groups were similar in baseline characteristics. Patients treated for a symptomatic stenosis were older (P<0.0001) and had diabetes mellitus more often (P=0.005) than those treated for an asymptomatic stenosis (Table 1).

Expression of IL-16 and T-Cell Markers

IL-16 mRNA levels correlated positively with the mRNA levels of CD3 (r=0.191; P=0.032), CD4 (r=0.404; P=0.0001) and also FoxP3 (r=0.465; P<0.0001), the regulatory T-cell transcription factor, in the carotid plaques (Table 2). IL-16 protein measured in carotid plaque homogenates correlated with CD3 (r=0.204; P=0.014) and FoxP3 mRNA (r=0.200; P=0.018; Table 2). These correlations are consistent with a role of IL-16 in T-cell recruitment to the plaque.

IL-16 and Caspase-3

Caspase-3 activity correlated with the IL-16 protein levels in the carotid plaque (P=0.001; r=0.234; Table 2). Given that caspase-3 cleavage of IL-16 is required for generation of the released form of IL-16, the correlation between IL-16 and caspase-3 activity in plaques may not reflect a correlation between IL-16 and apoptosis.

IL-16 and Preoperative Symptoms and Postoperative Cardiovascular Events

The IL-16 mRNA expression was significantly higher in plaques from patients without symptoms than in plaques from patients with symptoms (P=0.043). The plaques with IL-16 mRNA expression in the highest tertile were associated with fewer symptoms than the plaques with IL-16 mRNA expression in the lowest tertile (odds ratio, 0.38; 95% confidence interval, 0.16–0.92; P=0.031) when adjusting for the potential confounders age, sex, and diabetes (Figure 1). A significant linear association was also found across tertiles of IL-16 mRNA expression and symptoms (P=0.031) when adjusting...
for potential confounders (Table I in the online-only Data Supplement). These findings indicate that increased IL-16 expression in plaques is associated with fewer symptoms independently of other risk factors. No difference was seen for IL-16 protein levels in carotid plaques, or in plasma, when comparing asymptomatic and symptomatic patients (Figure 1). No correlations could be detected between IL-16 levels measured in plaques (mRNA and protein) or in plasma.

To determine whether IL-16 levels are associated with a lower risk for a new event, the patients were followed up 2 years after undergoing carotid endarterectomy. Higher IL-16 protein levels in carotid plaques (above median) were associated with reduced incidence of new cardiovascular events during a mean follow-up of 21 months (Kaplan–Meier log-rank test, \( P = 0.03 \); Figure 2). The association remained significant in a Cox proportional hazard model (hazard ratio, 0.47; 95% confidence interval, 0.22–0.99; \( P = 0.047 \)) when adjusting for age and sex. Of the 31 events, 21 were recorded in the group below median (14 were classified as symptomatic and 7 were asymptomatic, ie, 33% asymptomatic) and 10 in the group above median (7 were symptomatic and 3 were asymptomatic, ie, 30% asymptomatic). In the Cox regression analysis when adding symptoms at inclusion as a confounder (as well as age and sex), the IL-16 group above median has a hazard ratio of 0.436 (confidence interval 0.204–0.931, \( P = 0.032 \)) when compared with IL-16 group below median, indicating that the an altered event rate in patients with symptomatic plaques cannot explain the association between IL-16 and events during follow-up. No associations were found between IL-16 mRNA in the carotid plaques or IL-16 levels in plasma and the occurrence of postoperative cardiovascular events.

**IL-16 and Plaque Phenotype**

To determine whether the levels of IL-16 correlated with markers of plaque composition, the following components were analyzed: smooth muscle cells (\( \alpha \)-actin staining), macrophages (CD68 staining), collagen (biochemical analysis), elastin (biochemical analysis), and neutral lipids (Oil Red O).

No correlation was found between IL-16 mRNA expression levels and these markers of plaque composition.

IL-16 protein measured in the plaques showed univariate correlations with the plaque stability–associated markers elastin \( (r = 0.308; P < 0.0001) \) and collagen \( (r = 0.366; P < 0.0001) \), as well as with CD68 staining \( (r = 0.153; P = 0.033) \) and neutral lipid staining \( (r = 0.340; P < 0.0001) \), which are considered to promote plaque instability (Table 2). In a linear regression model including all phenotypic markers, however, only collagen \( (r = 0.269; P = 0.006) \), elastin \( (r = 0.240; P = 0.047) \), and caspase-3 activity \( (r = 0.228; P = 0.019) \) displayed significant independent correlations with IL-16 protein (Table 2).

The correlations to classical markers of plaque stability, described in the section above, raised an interest in the relationship between IL-16 and extracellular matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases. IL-16 plaque protein levels showed univariate correlations with the plaque-stabilizing MMP-2 \( (r = 0.281; P < 0.0001) \), tissue inhibitors of metalloproteinase-1 \( (r = 0.400; P < 0.0001) \), and tissue inhibitors of metalloproteinase-2 \( (r = 0.149; P < 0.0001) \), as well as MMPs usually increased in plaques with a more unstable phenotype, such as MMP-1, MMP-9, and MMP-10 (Table 2).21–22 The associations with MMPs indicate a higher extracellular matrix turnover rate in plaques with high IL-16 levels. In a linear regression model, however, none of the MMPs or their inhibitors displayed independent significant correlations with IL-16 protein (Table 2).

To further explore associations between IL-16 and all measured variables, including markers of plaque stability or instability, and to correlate them to the clinical outcome variables (preoperative symptoms and occurrence of postoperative cardiovascular events during follow-up) when all intermarker correlations were accounted for, we performed a multivariate partial least square regression analysis. The score plot from the partial least square regression analysis shows a separation of patients with and without preoperative symptoms along the x axis, whereas postoperative events separates along the y axis (Figure 3A). The loading plot shows the variables responsible for the separation in the score plot (Figure 3B). Plaque IL-16 levels, both mRNA and protein, were associated with markers of a stable plaque phenotype (eg, collagen, elastin, MMP-2, and MMP-10 (Table 2).
tissue inhibitors of metalloproteinase-2, and FoxP3 mRNA) and had reduced preoperative symptoms and postoperative events in the upper right quadrant of the loadings plot, indicating that patients located in the same quadrant in the score plot have less clinical symptoms, a more stable plaque phenotype and higher plaque IL-16 levels. This coclustering of IL-16, plaque stability markers, and less clinical symptoms is in line with the linear regression analysis and indicates that high IL-16 levels in plaques are predominantly associated with reduced plaque vulnerability and a favorable clinical outcome. In addition, patients with plaque IL-16 protein levels above median had fewer ruptured plaques than patients with plaque IL-16 protein levels below median (odds ratio 0.42; 95% confidence interval, 0.17–1.0; \(P=0.050\)), when adjusting for age and sex.

**Discussion**

Our data support the hypothesis that IL-16 is associated with a decreased risk of cardiovascular events possibly by stabilizing the plaques. We have found that high IL-16 mRNA levels in human carotid plaques are associated with reduced clinical symptoms independently of potential confounders. Furthermore, high IL-16 protein levels in carotid plaques were associated with reduced incidence of cardiovascular disease independently of potential confounders. To understand why IL-16 was associated with atheroprotection, we measured markers of plaques stability. We found independent correlations between IL-16 and elastin and collagen, suggesting that high IL-16 levels associate with a more stable plaque phenotype. Taken together, the net effect of high IL-16 levels in plaques seems to be protection as symptoms, and incident cardiovascular events were reduced in patients with higher IL-16 levels.

IL-16 has been described as a proinflammatory chemokine in several different diseases including asthma, allergy, and rheumatoid arthritis.\(^1\)\(^4\)\(^-\)\(^1\) There are only a few studies on IL-16 supporting a possible anti-inflammatory function.\(^1\)\(^9\)\(^,\)\(^2\)\(^4\) The 121 amino acid C-terminal secreted part of pro–IL-16 has been described to both interact with CD4 and inhibit T-cell responses through desensitization of the T-cell receptor.\(^2\)\(^5\) This could give a potential explanation for the beneficial effect of high IL-16 levels in dampening the T-cell response, and thereby the inflammatory drive in the plaque. Many previous studies have shown the beneficial role of Tregs in atherosclerosis,\(^5\)\(^,\)\(^2\)\(^6\) and the correlation between IL-16 levels at the site of the inflamed plaque with FoxP3 expression could hold the key to the fewer atherosclerotic manifestations displayed in the group with higher level of IL-16. It remains to be seen whether and how IL-16–mediated T-cell recruitment directly affects plaque stability.

In a study investigating elevated serum levels of IL-16 and the risk of having an acute coronary event (algorithm based and combined with other biomarkers), there was a 2-fold increased risk for the group with high IL-16 levels.\(^2\)\(^7\) IL-16 polymorphism has also been reported to be associated with an increased risk of an ischemic stroke.\(^2\)\(^8\) Our measurements of plasma IL-16 did not reflect the levels we found in the plaque or the associations to a more stable plaque phenotype and decreased incidence of postoperative cardiovascular events. This highlights the potential interest of measuring IL-16 locally at the site of disease and not systemically. Pro–IL-16 is cleaved by caspase-3 to produce the secreted C-terminal chemotactic part of pro–IL-16 and the N-terminal cell cycle regulatory part, of which the IL-16 mRNA transcript encodes for both.\(^2\)\(^9\)\(^,\)\(^3\)\(^0\) It is possible that the protection from symptoms observed in our study reflects increased IL-16–mediated cell cycle regulation and not increased secretion of IL-16, which has been associated with inflammation.
Caspase-3 is thought to be an active player in apoptosis. Recent studies, however, have shown a role of caspase-3 as an immunologic silencer of the apoptotic event by inhibiting interferon production. Therefore, increased apoptosis, in contrast to necrosis, could promote a more stable plaque phenotype. In our study, the correlation among levels of IL-16, caspase-3 activity, and a stable plaque phenotype could reflect increased caspase-3–mediated apoptosis and silencing of the immune system in addition to caspase-3–mediated increased IL-16 levels.

In general, because of alternative splicing, mRNA decay, and post-translational modifications, only ≈40% of all cellular proteins can be predicted from the corresponding mRNA measurements. In the case of IL-16, we also know that certain cell types (such as CD8+ T cells) constitutively express bioactive IL-16 that can be released on stimuli, whereas other cell types need to first activate the IL-16 gene and produce mRNA, which will lead to protein. In this assay, we measure all forms of IL-16 protein, both the secreted part and the pro–IL-16, in the plaque homogenates. Therefore, we cannot say which form (or both) is associated with decreased risk of atheroma formation. Interestingly, the secreted IL-16 measured in the plasma does not give the same results as the measurements done in the homogenates, which measures both the secreted and the pro–IL-16. The measurements of IL-16 in plaque homogenates have the advantage of providing information from almost the entire plaque when compared with a single section in immunohistochemistry assays, which does not take into account the heterogeneity of the plaque.

There are a few limitations in this present study that should be considered. An underlying assumption made is that the phenotype of the plaque removed can identify patients who may have similar plaques in other locations that will affect future clinical outcome. The assay used to measure IL-16 protein cannot distinguish between the pro form and the secreted form of IL-16, or answers the question whether the IL-16 is functionally active. The associations that we observed between high IL-16 levels and reduced clinical symptoms do not provide proof of causality. Further clinical and experimental studies are needed to confirm the plaque-stabilizing role of IL-16 in atherosclerosis and to determine the molecular mechanisms explaining the potentially protective role of IL-16.

One of the strengths of this study is the extensive histochemical and biochemical characterizations of the plaques and the follow-up of the patients, allowing the assessment of mRNA and protein levels of IL-16 and their relationship to factors contributing to stabilization of the plaques and the future outcome for the patients. Studies like the present may have limited immediate applicability in the clinic, but they can identify novel targets and processes worthy of more detailed analysis. For instance, there are not that many known inflammatory mediators that have been proven to be antiatherosclerotic or shown to associate with increased plaque stability in humans. This makes IL-16 an interesting target to study as a goal for future therapies, which will likely have to not only promote plaque stability but also limit lipid deposition and inflammation.

In conclusion, IL-16 protein levels in plaques correlates with plaque-stabilizing components of the extracellular matrix, as well as FoxP3 and caspase-3 activity, which all may (in their own way or synergistically) promote a more stable plaque phenotype, therefore displaying less clinical manifestations. Consequently high IL-16 levels in carotid plaques are associated with fewer symptoms and a lower incidence of cardiovascular disease, indicating a role for IL-16 in protection from the clinical complications of atherosclerosis.

Acknowledgments
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Disclosures
None.

References


Human Carotid Plaques With High Levels of Interleukin-16 Are Associated With Reduced Risk for Cardiovascular Events
Caitríona Grönberg, Eva Bengtsson, Gunilla Nordin Fredrikson, Mihaela Nitulescu, Giuseppe Asciutto, Ana Persson, Linda Andersson, Jan Nilsson, Isabel Gonçalves and Harry Björkbacka

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SUPPLEMENTAL MATERIAL

Materials and Methods

Sample preparation

The carotid plaques were snap-frozen in liquid nitrogen immediately after surgical removal from the patient. One-mm-thick sections of the most stenotic part of the plaque were saved for histological analysis. The remaining parts of the plaques were homogenized as has been previously described.¹

Postoperative events

The Swedish national inpatient health register was analyzed in order to identify postoperative cardiovascular events, with corresponding International Classification of Diseases, Tenth Revision (ICD-10) codes G45, G46, I20 to I25, I60 to I69 and I97 from October 2005 to December 2012. This is a nationwide validated register where more than 99 percent of all somatic (including surgery) and psychiatric hospital discharges are registered.² In doubtful cases, information was gained through telephone interviews and medical chart reviews. All causes deaths were verified against the National Population Register.

All events occurring in the postoperative period were analyzed singularly. Events occurring in the first 24 hours postoperatively were considered as procedure-related and excluded from the follow-up analysis. Furthermore, all cerebrovascular events were analyzed in combination. The variables death of cardiovascular origin, non-fatal stroke (non-hemorrhagic), non-fatal acute myocardial infarction (AMI), and any TIA or amaurosis fugax were analyzed singularly and in combination. Any arterial cardiac or vascular intervention that had not already been planned at the time of inclusion such as carotid surgery or stenting, coronary artery bypass grafting (CABG), percutaneous coronary artery intervention (PCI) or a surgical/endovascular intervention for peripheral artery disease or abdominal aortic aneurysm was also registered and analyzed singularly.

Patients suffering of more than one episode of the same event (i.e. patients with for example multiple strokes or AMIs) were classified as suffering of multiple events. In these cases only the first chronological event was taken into account in the survival analysis. A CABG or a PCI performed during the first 2 weeks after an AMI as well as a later surgical or endovascular intervention for a symptomatic contralateral carotid artery stenosis/ipsilateral restenosis were considered as consequence of the correlated cardiac/neurologic ischemia and not recorded as supplementary events.

Patients who underwent coronary endarterectomy were included in the study. The Swedish Cause of Death and National in-patient Health Registers were used identify postoperative cardiovascular events occurring up to two years after surgery. The 10th revision of the International Classification of Diseases (ICD-10): G45, G46, I20 to I25 were considered.

Histology

Eight µm sections were fixed in Histochoice (Amresco, Solon, OH), submerged in 60% isopropanol, transferred to 0.4% Oil Red O, in 60% isopropanol, for duration of 20 minutes to stain for lipids. Masson trichrome using Ponceau-acid fuchsin (Chroma-Gesellschaft, Schmidt GmbH, Germany), Phosphomolybdic Acid and Fast Green (Sigma Aldrich Chemie Gmbh, Germany) was used to assess plaque collagen content. Alpha-actin was used as a marker for vascular smooth muscle cells by using a primary mouse monoclonal anti-human antibody
clone 1A4 (DakoCytomation, Glosterup Denmark), diluted in 10 % rabbit serum 1:50, and a secondary rabbit anti-mouse biotin labeled antibody (DakoCytomation, Glosterup Denmark), diluted 1:200 in 10 % rabbit serum. To stain macrophages a primary monoclonal mouse anti-human antibody, towards CD68 was used (DakoCytomation, Glosterup Denmark), diluted in 10% rabbit serum 1:100, and as a secondary polyclonal rabbit antibody (DakoCytomation, Glosterup Denmark), diluted 1:200, were used. Biopix iQ 2.1.8 (Gothenburg, Sweden) was used to quantify the different stainings in the plaque (% of area) after scanning with ScanScope Consloe Version 8.2 (LRI imaging AB, Vista California, USA) and photographed with Aperio Image Scope v.8.0 (Aperio, Vista California, USA).

Quantitative real-time PCR on human carotid plaque

Carotid plaques were homogenized and total RNA was extracted as previously described. The total RNA integrity and quantity was analyzed on BioRad Experion and Thermo Scientific NanoDrop instruments. Total RNA from each patient (500 ng) was reverse transcribed with SuperScriptIII RT and random primers (Invitrogen). Expression of Cyclophilin A (4326316E), GAPDH (4326317E), ACTB (beta actin gene) (HS0160665_g1), CD4 (HS00181217_m1), IL-16 (HS00189606_m1), FoxP3 (HS00203958_m1) and CD3 (HS00174158_m1) was determined by using FAM-labeled TaqMan Assays (Applied Biosystems) and a 7900HT real-time PCR System (Applied Biosystems). Ct values were determined in RQ Manager 1.2.1. Relative mRNA expression was calculated from standard curves, constructed by serial dilution of gel-purified PCR products. CD4 and IL-16 were normalized to the housekeeping genes Cyclophilin A and GAPDH. CD3 was normalized to ACTB.

Carotid plaque homogenate analysis

Elastin and collagen content in plaque homogenates (n=206) were measured with the Fastin Elastin assay (Biocolor, Carrickfergus, Northern Irland, UK) and the Sircol soluble collagen assay (Biocolor, Carrickfergus, Northern Irland, UK) as described previously. Active caspase 3, cleaving at the Asp175/Ser176 site, and MMPs (1, 2, 3, 9, 10) were measured in plaque homogenate using Human Caspase-3 ELISA (Invitrogen, Life Technologies, Carlsbad, CA) and Mesoscale human MMP ultra-sensitive kit (Mesoscale, Gaithersburg, MD, USA), respectively, according to the manufacturer’s instructions. TIMPs (1 and 2) were analyzed in carotid plaque homogenate supernatants using MILLIPLEX MAP Human TIMP Magnetic Bead Panel (Milliplex, MA, USA), according to the manufacturer’s instructions. IL-16 was measured in carotid plaque homogenate supernatants using Bio-Plex Pro Human Chemokine Assay (BioRad, CA, USA), using magnetic beads, according to manufacturer’s instructions. All results were normalized to plaque wet weight.

Analysis of IL-16 in plasma

IL-16 was analyzed in plasma from the 206 patients included in the cohort. IL-16 was analyzed by the Proximity Extension Assay (PEA) technique using Proseek Multiplex CVD96x96 reagents kit (Olink Bioscience, Uppsala, Sweden) at the Clinical Biomarker Facility, Science for Life Laboratory, Uppsala. Oligonucleotide-labeled antibody probe pairs were allowed to bind to IL-16 in the plasma and addition of a DNA polymerase led to an extension and joining of the two oligonucleotides and formation of a PCR template. Universal primers were used to pre-amplify the DNA templates parallel. Finally, the individual DNA sequences were detected and quantified using specific primers by microfluidic real-time
quantitative PCR chip (96.96, Dynamic Array IFC, Fluidigm Biomark). The chip was run with a Biomark HD instrument. The mean coefficient of variance for intra-assay variation and inter-assay variation were 5% and 11%, respectively. Data analysis was performed by a preprocessing normalization procedure using Olink Wizard for GenEx (multid Analyses, Sweden). Data is presented as arbitrary units. General calibration curves to calculate the approximate concentrations are available on the Olink homepage (http://www.olink.com).

Supplemental Table I. Odds ratios and 95% confidence intervals for symptom by IL-16 mRNA tertiles.

<table>
<thead>
<tr>
<th></th>
<th>1st tertile</th>
<th>2nd tertile</th>
<th>3rd tertile</th>
<th>( P ) for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>1.0</td>
<td>0.87 (0.38-1.98)</td>
<td>0.44 (0.19-1.01)</td>
<td>0.051</td>
</tr>
<tr>
<td>Adjusted</td>
<td>1.0</td>
<td>0.92 (0.38-2.23)</td>
<td>0.38 (0.16-0.92)</td>
<td>0.031</td>
</tr>
</tbody>
</table>

References