Osteoprotegerin and Osteopontin Are Expressed at High Concentrations Within Symptomatic Carotid Atherosclerosis

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Background and Purpose—The aim of this study was to compare the concentration of osteoprotegerin (OPG), receptor activator of nuclear factor κB ligand (RANKL), and osteopontin (OPN) in stable (asymptomatic) and unstable (symptomatic) carotid atherosclerosis. In addition, we were interested in the effect of angiotensin II blockade on the secretion of these proteins by unstable atherosclerosis.

Methods—Endarterectomy samples removed from patients with recent (within 6 weeks) or no previous focal neurological symptoms were assessed by immunohistochemistry, Western analysis, and explant culture. Concentrations of OPG, RANKL, and OPN were measured by mean optical density (MOD), densitometry of protein bands, and enzyme-linked immunosorbent assay of supernatants from explant culture, and compared between symptomatic and asymptomatic patients.

Results—The concentration of OPG and OPN within the proximal internal carotid (PIC) part of the endarterectomy specimen removed from symptomatic patients was elevated 2- and 4-fold, respectively. Although the concentration of RANKL did not differ according to patients’ symptoms, the quantity of OPG secreted by explants of the PIC was greater in explants from symptomatic patients and could be significantly reduced within 48 hours of incubation with the angiotensin II blocker irbesartan.

Conclusion—OPG and OPN are upregulated in symptomatic human carotid atherosclerosis with possible implications for plaque stability. Angiotensin II blockade is able to downregulate OPG secretion in vitro. (Stroke. 2004;35:1636-1641.)

Key Words: carotid arteries | atherosclerosis | carotid artery plaque

The development of atherosclerosis is a complex process involving the collection of lipids and smooth muscle and inflammatory cells within the intima of arteries of predilection.1 The severity of atherosclerosis at a given site can be graded histologically, with more advanced lesions commonly demonstrating intimal calcification.2 The significance of this intimal calcification is controversial.3–11 Some but not all studies have suggested calcification to predict a more stable plaque less likely to be associated with symptoms. For example, Hunt et al examined carotid endarterectomy specimens from 142 patients and reported a relationship between the absence of calcification and symptoms.9 Calcification was present in 48% of plaques removed from asymptomatic patients compared with 34% in specimens from symptomatic patients (P=0.04).9 Support for a relationship between instability and low levels of plaque calcification comes from studies in the coronary circulation.10,11 For example, Shemesh et al demonstrated much lower total coronary calcium scores (TCS) in patients with an acute myocardial event (median TCS 63) compared with patients with chronic angina (median TCS 906; P<0.01).10

Various mechanisms have been suggested by which calcification might alter atherosclerotic plaque stability. Calcification would be expected to alter the stress distribution within the atherosclerotic plaque, and some12,13 but not all14 studies have suggested calcium to increase the mechanical stability of the plaque. The process of vascular calcification itself might be an important factor linking plaque stability and calcium. In vitro and animal studies simulating arterial calcification suggest that vascular calcification involves a group of noncollagenous matrix proteins originally identified as important in bone mineralization, including osteopontin (OPN), osteoprotegerin (OPG), and receptor activator of nuclear factor κB ligand (RANKL).15–17 These proteins have been demonstrated to have pleiotropic effects that influence matrix turnover, cell migration, and inflammation—all processes believed to be fundamental in atherosclerotic plaque stability.

OPN, a glycoprotein secreted by macrophages, vascular smooth muscle cells (VSMCs), and endothelial cells has been demonstrated to promote macrophage and endothelial chemotaxis.15 In vitro studies of VSMC calcification suggests...
that OPN acts to promote decalcification. The OPG/RANKL/receptor activator of nuclear factor κB (RANK) group of proteins have also been implicated in decalcification. Within bone, OPN binds with RANKL, thereby preventing it from interacting with RANK expressed on osteoclasts. Like OPN, OPG and RANKL have been demonstrated to have pleiotropic effects, such as modulating secretion of proteolytic enzymes by osteoclasts and interactions between inflammatory cells. Differential expression of noncollagenous matrix proteins within atherosclerotic plaque could not only alter the incidence of calcification but also alter the remodeling of the plaque and thus alter rupture risk. Animal and cell culture studies suggest that the expression of OPN and OPG is upregulated in the presence of angiotensin II. The aim of this study was to assess the relative expression of OPG, RANKL, and OPN in clinically stable and unstable atherosclerosis and to measure the effect of angiotensin II blockers on the secretion of these proteins.

Materials and Methods

Specimens Approval for this study was provided by the ethics committees of the Townsville and Mater Hospitals and James Cook University. Informed consent was obtained from participating patients. As part of their preoperative assessment, patients were assessed by consultation with an experienced physician, duplex imaging, magnetic resonance angiography, and head computerized tomography. Patients included in the study either presented with focal neurological symptoms related to their anterior cerebral circulation (transient ischemic attack, amaurosis fugax, and stroke) within 6 weeks of surgery (defined as symptomatic) or presented with no history of neurological symptoms (defined as asymptomatic). Patients with nonfocal, atypical, or distant neurological symptoms were excluded. Patients receiving angiotensin-converting enzyme inhibitors or angiotensin II blockers were excluded. All patients included in the study had a carotid artery stenosis of 70% or greater defined on duplex imaging and magnetic resonance imaging, and head computerized tomography. Patients receiving angiotensin-converting enzyme inhibitors or angiotensin II blockers were included. All patients included in the study had a carotid artery stenosis of 70% or greater defined on duplex imaging and magnetic resonance angiography. A conventional endarterectomy was performed, developing a deep medial plane and removing the specimen en bloc. The common carotid site was tagged for orientation. Specimens were immediately placed in cold culture medium (serum-free Dulbecco modified eagle medium supplemented with antibiotics) and brought to the laboratory for cold culture medium (serum-free Dulbecco modified eagle medium supplemented with antibiotics) and brought to the laboratory for culture. Each specimen was used for immunohistochemistry, Western blotting, or explant culture. Each specimen was used for Western blotting, or explant culture alone to ensure that the diseased site within the specimen was obtained for the assessment in question.

Serum and Plasma Measurements White blood cell count, serum creatinine, fasting cholesterol, triglyceride, low-density lipoprotein, high-density lipoprotein, homocysteine, and C-reactive protein were measured as previously described. Serum OPG and OPN were measured by commercial enzyme-linked immunosorbent assay (ELISA) (R&D Systems) in which the intrasay coefficient of variation was <5%.

Immunohistochemistry Circumferential specimens removed from the diseased internal carotid and common carotid parts of the endarterectomy specimen were formalin-fixed, mounted on end, and wax-embedded. Sections (5 μm) were rehydrated and stained by the immunoperoxidase technique using antibodies to OPN (LF-166, kindly supplied by Dr Larry Fisher, 1 in 1000), OPG (Imgenex, 1 in 1000), RANKL (Imgenex, 1 in 1000), CD68 (Dako, 1 in 100), smooth muscle cell actin (Dako, 1 in 100), and CD3 (Dako, 1 in 100). Samples used to assess staining intensity were processed at the same time to minimize differences in staining conditions. Staining was quantified using optical density. Briefly, computerized images of each section were captured using a digital camera mounted on a Nikon Eclipse E800 microscope and Fujifilm software. Three areas of maximal staining were identified and the mean optical density at these sites measured using Scion software. This optical density was average and background measurements from serial sections in which the primary antibody was omitted were taken away from the resultant value. The reproducibility of the optical density measurement was assessed in 13 specimens examined on 2 separate occasions 5 days apart by the same observer. The concordance correlation coefficient was excellent at 0.97 (95% confidence interval: 0.95 to 0.98).

Western Analysis The proximal internal carotid (PIC) and common carotid (CC) parts of the endarterectomy samples to be used for Western analysis were isolated and stored frozen at −80°C for latter analysis. Frozen samples were ground under liquid nitrogen and total protein extracted and measured by the Bradford technique. Serum OPG and OPN were measured by commercial ELISA (Imgenex, 1 in 500), OPG (LF-166, 1 in 500), and RANKL (Chemicon, 1 in 200) antibodies. Blots were developed with enhanced chemiluminescence (Amer sham, digitally scanned (Bio-Rad, Chemidoc XRS), and analyzed (Bio-Rad, Quantity One). Protein concentrations were assessed by densitometry and results presented as mean:± standard error for ratios of the protein density of specimens removed from symptomatic compared with asymptomatic patients.

Explant Culture Freshly excised endarterectomy samples were washed in tissue medium to remove thrombus and 10-mm² specimens were cut from the proximal internal and common carotid arteries under sterile conditions. The samples were then cut into quarters, 2 samples saved for initial plaque assessment and 2 available for culture. Initially, 1 sample each from the common and internal carotid artery were placed in tissue culture wells, intima up, in 1.5 mL of medium and incubated at 37°C in a humidified 5% CO₂ atmosphere. In subsequent experiments, 2 samples were used each from the common and internal carotid arteries. One sample from each site was incubated in the presence of irbesartan (1 mg/mL), whereas the other was incubated without medication as control. The dose of irbesartan was based on published safe serum levels of these medications achieved in patients on treatment. The culture medium was replaced every 48 hours and the harvested medium centrifuged (10 000g for 60 seconds) to remove particulate debris and stored frozen at −80°C until analyzed for OPG and RANKL by ELISA, and total protein was analyzed using the Bradford method. Explants from days 0 and 8 were stored frozen for later assessment. The viability of explant cultures was investigated using histology, immunohistochemistry, and ATP measurement in a preliminary study. In this initial viability study, 24 biopsy specimens (5 mm²) taken from 4 carotid endarterectomies were cultured for up to 8 days. Tissue morphology and cell type were examined in 5-μm sections stained with hematoxylin and eosin and using antibodies to CD68, CD3, and smooth muscle α-actin. Sections were examined by a senior pathologist (A.K.Y.L.) blinded to the culture conditions to assess cell morphology, type, and number on high-power fields (×40). Cell counts were graded as: 0, <5; 1, 5 to 9; II, 10 to 20; III, >20. Intraobserver agreement was 92%. Examination of sections taken from explants cultured up to 8 days demonstrated distinct cell membranes, nuclear staining, and preservation of viable cells. For example, the median macrophage counts were graded as I for both freshly excised and plaques cultured for 8 days. Tissue viability of explants was further assessed by measurement of ATP concentra-
tions using a commercially available kit (Sigma). Viability of specimens treated with irbesartan (n=4) or untreated (n=4) was assessed by comparison of tissue ATP with that in specimens frozen before culture (n=4). No significant difference between samples was detected (preculture, 2.1±0.3; cultured 8 days, 1.9±0.3; cultured 8 days in the presence of irbesartan 2.4±0.3 ng ATP/mg tissue; P>0.5).

Data Analysis
Blood measurements, immunostaining optical densities, Western analysis densitometry, and ELISA concentrations were expressed as mean and standard error and were compared by t test. Nominal data were compared by χ² test with Yates correction.

Results
A total of 44 patients (24 with recent symptoms and 20 without symptoms) were included in the study. Mean age was 70±2 years, 36 (81%) were male, and 10 (23%) patients had diabetes. Gender, mean age, prevalence of diabetes, serum creatinine, cholesterol, triglyceride, low-density lipoprotein, high-density lipoprotein, homocysteine, and OPN were similar in symptomatic and asymptomatic patients. Serum OPG (2.1±0.1 versus 1.7±0.1; P=0.03) and C-reactive protein (5.6±1.1 versus 2.8±0.5; P=0.05) were higher in symptomatic patients.

OPG Is Expressed at Higher Concentrations in Symptomatic Plaques
Immunohistochemistry demonstrated OPG within the intima of atherosclerotic biopsy specimens taken from the proximal internal and common carotid arteries (Figures 1 and 2). The protein is expressed in a sheet-like pattern near areas of vascular calcification (Figure 2A and 2B). On high-power magnification, the protein can also be demonstrated adjacent to VSMCs and inflammatory cells on serial sections (Figure 2C through 2F). The mean optical density of OPG staining was significantly greater in the PIC of symptomatic plaques (Table 1). On Western analysis, the concentration of OPG within the proximal internal carotid artery was 2-fold higher in endarterectomy specimens removed from symptomatic compared with asymptomatic patients (Figure 3). No significant difference could be demonstrated in the concentration of OPG at the common carotid artery in relation to symptoms (Table 1, Figure 3).

OPG Is Secreted in Greater Amounts From Symptomatic Carotid Explants
Explants taken from the common (n=20) and proximal internal carotid (n=20) of endarterectomy specimens removed from 12 symptomatic and 8 asymptomatic patients were cultured for 8 days and the secretion of OPG into medium was monitored (Table 2). The amount of OPG secreted by explants was similar throughout the culture period (days 0 to 2, 210±16 pg/mg compared with days 6 to 8, 204±18 pg/mg). However, the OPG secreted by proximal internal carotid specimens removed from symptomatic patients was significantly greater than those removed from asymptomatic patients (Table 2). Considering all explants together (PIC and CC), the mean OPG secreted per 48-hour period from 24 symptomatic explants was 227±10 pg/mg compared with 159±11 pg/mg from 16 explants removed from asymptomatic patients (P<0.0001).
OPG Secretion From Carotid Atherosclerotic Explants Is Reduced by Irbesartan

To investigate the effect of angiotensin II blockade on OPG secretion, further proximal internal carotid explants were incubated with and without irbesartan. The angiotensin II blocker led to a rapid reduction in the secretion of OPG by explants from symptomatic patients without significantly affecting that secreted by explants from asymptomatic patients (Figure 4). The amount of OPG secreted by explants from symptomatic patients was reduced within 48 hours of incubation with irbesartan (108 ± 16 with and 167 ± 17 without irbesartan, n = 10, P = 0.03).

OPN Expression Is Greater in Symptomatic Than in Asymptomatic Atherosclerosis

Staining for OPN was demonstrated adjacent to areas of intimal calcification. The mean optical density of OPN staining was significantly greater in the PIC of symptomatic plaques (Table 1, Figure 1). On Western analysis, the concentration of OPN within the proximal internal carotid artery was 4-fold higher in endarterectomy specimens removed from symptomatic compared with asymptomatic patients (Figure 3).

RANKL Expression Is Similar in Symptomatic and Asymptomatic Atherosclerosis

Unlike OPG and OPN, the concentration of RANKL within the PIC and CC of carotid atherosclerosis was unrelated to symptoms (Table 1, Figures 1 and 3).

Discussion

In this study, we have demonstrated increased concentrations of OPG and OPN within symptomatic or unstable compared with asymptomatic or stable atherosclerosis. Western analysis suggests a 2- and 4-fold upregulation of OPG and OPN, respectively, in symptomatic atherosclerosis (Figure 3). The increased concentration of these proteins is limited to the main site of atherosclerosis, ie, the proximal internal carotid. We also found a smaller increase in the concentration of serum OPG but not OPN in patients with symptomatic carotid artery disease. An elevated concentration of serum OPG in patients with more severe atherosclerosis has been previously reported in relation to coronary artery disease and presumably relates to release of the cytokine from the diseased arteries.31

The possible sources of OPG and OPN within atherosclerosis include VSMCs, macrophages, and endothelial cells.15–17 In vitro studies have demonstrated the upregulation of OPG and OPN expression in these cell types by proinflammatory cytokines such as IL-1, IL-6, and TNFα.32,33 Immunohistochemistry demonstrated that OPG and OPN were mainly distributed around areas of calcification but also were

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<tr>
<th>Protein</th>
<th>PIC</th>
<th>CC</th>
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<tr>
<td></td>
<td>Symptoms</td>
<td>No Symptoms</td>
</tr>
<tr>
<td>OPG</td>
<td>115±8</td>
<td>90±4</td>
</tr>
<tr>
<td>RANKL</td>
<td>164±15</td>
<td>151±12</td>
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<tr>
<td>OPN</td>
<td>162±8</td>
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Figures presented are mean optical densities minus background staining from negative slides ± standard error.

OPN indicates osteopontin; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor κB ligand; PIC, proximal internal carotid; CC, common carotid.

Figure 3. Western analysis of the concentration of OPG, OPN, and RANKL in carotid atherosclerosis. Results are presented as the ratios of protein densities for atherosclerosis removed from the proximal internal (A) and common carotid (B) arteries comparing symptomatic to asymptomatic patients. The OPG and OPN concentrations are 2- and 4-fold greater in atherosclerosis removed from the proximal internal carotid artery of symptomatic compared with asymptomatic patients (A) (P < 0.01). Protein concentrations do not differ significantly in the common carotid artery (B). An example blot for proximal internal carotid samples is shown (C).
associated with inflammatory cells and VSMCs (Figure 2). Thus, it is likely that these 2 cell types are mainly responsible for release of these proteins.

The high concentrations of OPG and OPN could have important implications for plaque stability, and work is underway to investigate the functional consequences of these changes. The high concentrations of these proteins could have important implications for plaque stability, and work is underway to investigate the functional consequences of these changes.

Angiotensin II has been implicated in atherosclerosis progression, and trials using angiotensin II blockade suggest an improved outcome beyond any blood pressure-lowering effect. For example, in the PROGRESS trial in which patients with symptoms of stroke or transient ischemic attack were treated with a regimen including an angiotensin-converting enzyme inhibitor, there was a relative risk reduction of stroke of 28% over 4 years, despite only a small effect on blood pressure. We suspected that angiotensin II blockade might be able to reduce levels of OPG and OPN secreted by atherosclerosis, because angiotensin II has previously been shown to upregulate these proteins in animal and cell culture studies. We found that in vitro irbesartan reduces the secretion of OPG by unstable atherosclerosis explants as early as 48 hours after treatment (Figure 4). This effect could eliminate the potential plaque destabilizing effects of OPG, such as decalcification and matrix degradation.

In conclusion, this study demonstrates increased concentrations of OPG and OPN within unstable atherosclerosis. The high concentrations of these proteins could have important implications for plaque stability, and work is underway to investigate the functional consequences of these changes.

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