N-Terminal Rather Than Full-Length Osteopontin or Its C-Terminal Fragment Is Associated With Carotid-Plaque Inflammation in Hypertensive Patients

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BACKGROUND
Hypertensive patients develop carotid atherosclerotic plaques with enhanced inflammation. Full-length osteopontin (OPN-FL), a multifunctional protein whose levels are elevated in association with atherosclerosis, is cleaved by thrombin and matrix metalloproteinases to form a C-terminal and a putatively biologically active N-terminal fragment (OPN-C, OPN-N, respectively). We conducted a study to examine whether plaque inflammation in hypertensive patients corresponds to the expression of OPN or of its cleaved forms or both.

METHODS
We collected 42 carotid plaques from 41 consecutive hypertensive patients during carotid endarterectomy. Plaque tissue was used to measure matrix metalloproteinase-12 (MMP-12) and OPN proteins, and for the classification of plaques as showing low- or high-degree inflammation through histological and immunohistochemical evaluation.

RESULTS
Fifteen highly inflamed plaques and 27 plaques with characteristics of low-grade inflammation were collected. Moderate to heavy staining for OPN characterized 87% of the plaques with high-degree inflammation but only 44% of those with low-degree inflammation, corresponding to the percentages of plaques that were heavily stained for the macrophage marker CD68 (93% versus 26%, respectively, $P < 0.01$). Western blot analysis showed that the abundance of OPN-FL and OPN-C was comparable in the two groups. However, the abundance of OPN-N was significantly greater in the highly inflamed plaques (median, 3.8 (range, 0.8–7.3) vs. median, 0.9 (range, 0.2–1.5); $P = 0.017$, respectively). The abundance of MMP-12 was significantly greater in the high- than in the low-degree plaque inflammation group (4.8 (range 1.9–8.8) vs. 1.1 (range 0.3–1.4), respectively; $P = 0.03$).

CONCLUSIONS
The N-terminal fragment of osteopontin, rather than OPN-FL or OPN-C, is associated with carotid plaque inflammation in hypertensive patients. Future studies should assess whether targeting OPN cleavage could present a new approach to preventing high-risk carotid plaques.

Keywords: osteopontin; OPN-N; carotid plaque; inflammation; hypertension; blood pressure.

doi:10.1093/ajh/hps043

Carotid atherosclerosis is a major form of target-organ damage (TOD) in hypertensive patients, and may in turn result in cerebrovascular events. However, and intriguingly, cerebrovascular events are more strongly related to the stability of carotid plaques than to the degree of arterial stenosis, and hypertension is well known to increase the instability of carotid plaques. Osteopontin (OPN) has been implicated as a mediator of atherosclerosis, hypertension, and inflammation. Osteopontin is a glycoprotein with an arginine–glycine–aspartic acid (RGD) motif, which binds to the integrin receptor family. As a matrix protein and a soluble cytokine, OPN is strongly expressed in injured tissues, and was proposed to enhance wound healing by modulating inflammation and fibrosis. Post-translationally, OPN is cleaved by matrix metalloproteinases (MMPs) and thrombin. Thrombin cleaves OPN into an N-terminal fragment (OPN-N) and a C-terminal fragment (OPN-C), two cleavage products that may retain biological activity. Indeed, OPN-N possesses adhesion motifs that may render it more pro-inflammatory than the full-length protein (OPN-FL), whereas the properties of the C-terminal fragment of OPN are less...
well characterized. Moreover, the abundance of OPN and its fragments in atherosclerotic plaque, and how they relate to plaque characteristics, are poorly defined. The purpose of the current study was to evaluate the abundance of OPN-FL, OPN-N, and OPN-C in atherosclerotic carotid-artery plaques from hypertensive patients, and to delineate whether any or all of these OPNs are similarly associated with plaque characteristics, and particularly with the degree of plaque inflammation.

METHODS

Study population

The population for the study included all hypertensive patients who underwent carotid endarterectomy between February 2009 and February 2010 at Soroka Medical Center in Beer-Sheva, Israel, a tertiary medical center. The study was approved by the Human Subjects Committee of Soroka Medical Center and Ben-Gurion University; each participant signed a written statement of informed consent to participate in the study.

Inclusion and exclusion criteria

The systolic and diastolic blood pressure (BP) data used in the study were the mean values of three measurements made at different intervals during the hospitalization of each patient by an experienced nurse trained in BP measurement. Hypertension was defined either by an average of three measurements of systolic BP ≥ 140 mm Hg or of diastolic BP ≥ 90 mm Hg made before the patient's surgical procedure, or as documented measurements of systolic BP < 140 mm Hg and diastolic BP < 90 mm Hg made before the surgical procedure in patients receiving antihypertensive treatment. Patients were excluded if they had secondary types of hypertension (except for that caused by atherosclerotic disease of the renal arteries); were pregnant; had an active infection, untreated malignant neoplasm, or chronic autoimmune condition; or were being treated chronically with a corticosteroid medication.

Clinical parameters

The clinical and laboratory parameters recorded from the electronic medical file of each patient were body mass index (BMI) as measured during the patient’s current hospitalization, presence of diabetes mellitus, smoking habit (current, former, never), and most recent medical record (compiled during the patient’s current hospitalization) of total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and triglycerides. Evidence of TOD, including a cerebrovascular event, transient ischemic attack, ischemic heart disease, congestive heart failure, left ventricular hypertrophy, or peripheral vascular disease, was obtained from the patient’s computerized medical file. The regular drug regimen of each patient was also recorded.

Plaque collection and processing

Standard carotid endarterectomy technique was used to remove the intima of the artery of each of the study subjects, together with all components of plaque at the site at which the intima was removed. Immediately after being removed from the carotid artery, plaques were divided along their longitudinal axes into two fragments, to obtain two similarly representative portions of the entire excised plaque. Upon collection, the portion processed for histological studies was immediately fixed for 24 hours in 10% neutral-buffered solution containing 4% formaldehyde, and was subsequently embedded in paraffin. The second portion was immediately snap frozen and stored at −80 °C for biochemical and molecular evaluation.

Immunohistological studies

Each plaque was stained with hematoxylin and eosin (H&E) and evaluated blindly by a certified pathologist for cholesterol clefts, neovascularization, and calcification, each of which was scored as being absent or present. For immunohistochemistry, consecutive histological sections (5-μm thick) were prepared with a Leica RM2165 microtome (Leica Microsystems, Wetzlar, Germany). Neovascularization in the plaque was identified as microvessels having a lumen and containing red blood cells. Cholesterol clefts were identified as tissue surrounding spaces from which cholesterol was dissolved during fixation and paraffin embedding. After deparaffinization, the slides containing plaque specimens were placed in a solution of 3% hydrogen peroxide (1:1) for 10 minutes to block the activity of endogenous peroxidase, and were then heated in a microwave oven for 5 minutes at 100 °C. After the slides has been cooled for 30 minutes, nonspecific binding was blocked with normal serum for 10 minutes, with each slide then being labeled with the following antibodies at the specified dilutions: monoclonal mouse anti-human OPN (1:200) (R&D Systems Minneapolis, MN), anti-α-smooth-muscle actin (1:10,000) (Sigma-Aldrich, St. Louis, MO), and anti-CD68 (1:100) (PGM1, Dako, Glostrup, Denmark), and anti-alpha-smooth-muscle actin (1:10,000) (Sigma-Aldrich, St. Louis, MO). Staining for each antibody was graded according to the following four categories: 0, no staining; 1+, weak staining; 2+, moderate staining; and 3+, heavy staining.

Grading of plaque inflammation

For each H&E-stained plaque, the degree of inflammatory infiltrate was independently evaluated and graded as falling into one of four categories by two of the study investigators, one of them a certified pathologist, who were blinded to the patients’ characteristics. The first two categories of plaque inflammation were: 0 = no inflammatory cells recognized on the slide; and 1 = very few inflammatory cells (<25 cells in no more than two areas of the plaque). Plaques whose inflammation fell into one these two categories were designated as showing low-degree inflammation (Fig. 2A). Plaques having a moderate or heavy inflammatory infiltrate throughout the plaque area were categorized as being of grades 2 or 3, respectively, and were collectively designated as showing high-degree inflammation (Fig. 2B).

Identification of full-length osteopontin, N-terminal osteopontin, and C-terminal osteopontin

Accurate identification of OPN and its fragments required the generation of standards that were used in every Western
blot performed in this part of the study. For the generation of these standards, 1 μg of recombinant full-length human OPN (R&D Systems) was incubated in vitro with either H2O or thrombin, as detailed in Methods. Proteins of recombinant human OPN-FL (lane 1), OPN-FL + thrombin (lane 2), and OPN-FL + H2O (lane 3) were resolved through 10% polyacrylamide gel electrophoresis (PAGE) and blotted onto nitrocellulose membranes, on which they were exposed to an anti-OPN-FL antibody (A), anti-OPN-N antibody (B), or anti-OPN-C antibody (C).
in their clinical characteristics. The mean age of the high-degree plaque inflammation group was 69.3 ± 10.6 years, vs. 67.0 ± 10.6 years for the low-degree plaque inflammation group (P = 0.5), with the two groups containing 73% (11) and 59% (16) men, respectively (P = 0.4). The percentages of patients with diabetes mellitus in the two groups were 57.1% and 38.5%, respectively (P = 0.3), but there was a trend (P = 0.07) toward a higher mean serum concentration of hemoglobin A1C (HbA1C) in the high-degree plaque inflammation group (8.5 ± 2.1% vs. 6.8 ± 1.2%, respectively). The BPs of the high- and low-degree plaque inflammation groups were 154.2 ± 20.9/82.7 ± 11.0 mm Hg vs.
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157.9 ± 20.1/81.1 ± 11.0 mm Hg, respectively (P = 0.9 for systolic BP and P = 0.7 for diastolic BP, respectively). The groups did not differ in their rate of symptomatic cerebrovascular disease (12 (80.0%) vs. 16 (66.7%), respectively, P = 0.4).

There was a trend toward a higher rate of ischemic heart disease in the high-degree plaque inflammation group (n = 5 (33.3%) vs. n = 3 (11.1%); P = 0.08). Lastly, patients in the high-degree plaque inflammation group tended to be less likely to be treated with at least one antihypertensive medication (n = 6 (46.2%) vs. n = 20 (76.9%), respectively; P = 0.08).

Histological features of plaques

Immunohistochemical analyses of the patients’ plaques showed that plaques with high-degree inflammation were more likely to stain moderately or heavily for OPN and macrophages (stained with CD68) than were those with low-degree inflammation, at 87% vs. 44% in the case of OPN and 93% vs. 26% in the case of CD68 staining, respectively (P < 0.01) (Figure 3a1,a2,b1,b2). However, the two groups showed no significant difference in the staining for alpha-smooth-muscle actin (53% vs. 59% for the high- vs. low-degree inflammation groups, respectively (P = 0.7) (Figure 3c1,c2). Cholesterol clefts and neovascularization were significantly more abundant among the plaques with high- than among those with low-degree inflammation (n = 12 (80.0%) vs. n = 11 (40.1%), P = 0.01; and n = 13 (86.7%) and n = 10 (37.0%), P = 0.002, respectively) (Table 2). On the other hand, the two groups showed no difference in the presence of plaque calcifications (data not shown).

Quantitative analysis of osteopontins in plaques with low- and high-degree inflammation

Immunohistochemical analyses of plaques demonstrated a greater abundance of total OPN in atherosclerotic plaques...
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When plaques were sorted according to treatment of their source patients with angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs) or both, there was a trend toward a lower abundance of OPN-FL (0.7 (range, 0.2–1.6) vs. 1.2 (range, 0.5–1.8), P = 0.057) and OPN-N (0.9 (range, 0.1–1.7) vs. 1.4 (range, 0.8–4.1), respectively, P = 0.065) in treated than in nontreated patients. There was a nearly statistically significantly positive correlation between the OPN-N/OPN-C ratio and the concentration of

Table 2. Histopathological features of plaques with high- and low-degree inflammation

<table>
<thead>
<tr>
<th>Feature</th>
<th>High-degree inflammation</th>
<th>Low-degree inflammation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol clefts, n (%)</td>
<td>12 (80.0)</td>
<td>11 (40.7)</td>
<td>0.01</td>
</tr>
<tr>
<td>Calcifications, n (%)</td>
<td>8 (53.3)</td>
<td>14 (51.9)</td>
<td>0.92</td>
</tr>
<tr>
<td>Neovascularization, n (%)</td>
<td>13 (86.7)</td>
<td>10 (37.0)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Figure 4. Greater protein expression of N-osteopontin (OPN-N) and matrix metalloproteinase-12 (MMP-12) in plaques with high- than in plaques with low-degree inflammation. Lysates prepared from plaques with high- or low-degree inflammation were resolved through sodium dodecylsulfate–polyacrylamide gel electrophoresis and analyzed for full-length osteopontin (OPN-FL), the N-terminal fragment of osteopontin (OPN-N), and the C-terminal fragment of osteopontin (OPN-C) through the use of specific antibodies and with standards prepared as in Figure 1. (A) Representative blots of OPN-FL, OPN-N, and OPN-C in plaques with high- and low-degree inflammation. (B) Scatter plots representing OPN-FL, OPN-C, and OPN-N protein in plaques with high-degree inflammation (n = 12) and in plaques with low-degree inflammation (n = 24). (E) Representative blots of MMP-12 in plaques with high- and low-degree inflammation. (F) Box-and-whiskers plot of MMP-12 protein in plaques with high-degree (n = 6) and low-degree inflammation (n = 6). The Mann–Whitney U test was used to assess the significance of differences in protein levels in the two groups. The horizontal lines in parts B, C, and D of the figure represent the median interquartile range.
HbA1c \( (r = 0.4, P = 0.06) \) and systolic BP \( (r = 0.2, P = 0.07) \), as well as between the plaque content of OPN-N and the concentration of HbA1c \( (r = 0.4, P = 0.08) \). Neither OPN-FL nor OPN-C was correlated significantly with these clinical parameters (Table 3).

**DISCUSSION**

Osteopontin, a protein implicated in inflammation and atherosclerosis, is processed by proteolytic cleavage, yielding peptides that have been suggested as retaining biological activity. The present study demonstrates that OPN-N, rather than OPN-FL, is associated with the degree of inflammation of carotid plaques in patients with hypertension, and tends to be less abundant in patients treated with ACE-I/ARBs than in those not treated.

The past decade has seen a continuous search for markers of the vulnerability of atherosclerotic plaques to rupture. One of the main factors promoting such vulnerability is inflammation. Osteopontin is a mediator related to atherosclerosis and inflammation and has been proposed to play a causative role in the evolution of unstable plaques.5-8 Mice deficient in OPN have shown an attenuated formation of atherosclerotic plaque, of plaque inflammation, and of matrix metalloproteinase activity.9 In hypertensive patients, plasma levels of OPN were shown to be an independent determinant of carotid intimal–medial thickness, which is a marker for carotid atherosclerosis.10 Furthermore, a recent study showed that aggressive treatment with atorvastatin decreased plasma levels of OPN and enhanced the echogenicity of carotid plaques.11 The recognition of OPN as a putative regulator of carotid plaque stability stems from a study of atherosclerotic carotid plaques harvested from patients who underwent carotid endarterectomy. This study showed that plaque levels of OPN in single lesions were predictive of cardiovascular events in other vascular territories.12

Most of the studies that have evaluated the potential role of OPN in atherosclerosis have measured the full-length protein. However, it is also well established that after translation, OPN undergoes proteolytic cleavage by thrombin into two fragments. This cleavage reveals a cryptic motif (SVVYGLR) located in the N-terminal fragment of OPN that is thought to potentiate certain bioactivities of OPN. This concept was supported by Lai et al.,13 who showed that the production by vascular smooth-muscle cells of free radicals related to oxidative stress was greater in response to OPN-N than in response to OPN-FL. The adhesive ability of the N-terminal fragment of OPN is also enhanced in comparison to that of full-length OPN.14 In the current study we demonstrated that the abundance of OPN was greater in atherosclerotic plaques that were preclassified as showing high-grade inflammation than in those showing low-grade inflammation. However, when we examined different fragments of OPN, we demonstrated that OPN-FL is not the protein most strongly associated with inflammation and atherosclerosis, but that this protein is instead the N-terminal fragment. From this observation it might be suggested that the post-translational cleavage of OPN-FL is a key step in creating the inflammatory properties of OPN in atherosclerotic plaque.

The abundance of OPN-N over OPN-C in the plaques with high-degree inflammation harvested in our study can be explained by the greater MMP activity of the former as than of the latter fragment: In addition to its thrombin cleavage site, OPN-FL has several MMP cleavage sites. Most of these sites are located in the OPN-C fragment.15 Because it is likely that plaque inflammation correlates with plaque MMP activity, it is plausible that in plaques with high-degree inflammation, the OPN-C fragment would undergo greater degradation than would the OPN-N fragment. Indeed, in the study described here we demonstrated greater expression of MMP-12 in plaques with high- than in those with low-degree inflammation.

The present study has several noteworthy limitations. First a comparison of the blood vessels examined in the study with blood vessels from healthy controls was clearly not possible. Additionally, we could not define a specific cellular source, within the plaques that we examined, of the OPN fragments investigated in the study because of the reactivity of anti-OPN antibodies with several nonspecific bands produced by electrophoresis. Additionally, the study was cross-sectional and observational, without patient follow-up or outcome data. Therefore, rather than clearly defining whether cleavage products of OPN could be clinically useful beyond being markers of plaque inflammation, the present study can be useful in prompting further studies that, through the investigation of specific pathophysiologic and biochemical mechanisms, would reveal whether OPN-N can be used as an interventional target for plaque stabilization.

In conclusion, the cleavage product OPN-N is the form of OPN that most strongly associates with the inflammation and instability of carotid atherosclerotic plaques in patients with hypertension. We therefore propose that with regard to

**Table 3. Spearman correlations of full-length osteopontin, N-terminal osteopontin, and C-terminal osteopontin associated with carotid atherosclerotic plaque, and of the ratio of N-terminal to C-terminal osteopontin, with clinical parameters in 42 atherosclerotic plaques**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HbA1c ( (r;P) )</th>
<th>Systolic blood pressure ( (r;P) )</th>
<th>Diastolic blood pressure ( (r;P) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full length OPN</td>
<td>–0.05;0.49</td>
<td>0.1;0.23</td>
<td>–0.1;0.24</td>
</tr>
<tr>
<td>N-terminal OPN</td>
<td>0.4;0.08*</td>
<td>0.1;0.29</td>
<td>0.1;0.29</td>
</tr>
<tr>
<td>C-terminal OPN</td>
<td>0.2;0.21</td>
<td>–0.04;0.39</td>
<td>–0.07;0.33</td>
</tr>
<tr>
<td>N-terminal OPN/C-terminal OPN</td>
<td>0.4;0.06*</td>
<td>0.2;0.07*</td>
<td>0.1;0.21</td>
</tr>
</tbody>
</table>

* Suggested correlation \((0.05 < P < 0.1)\). 
Abbreviations: HbA1c, hemoglobin A1c; OPN, osteopontin.
interference in the secretion of MMP, inhibiting the MMP-mediated cleavage of OPN could be a complementary and perhaps even more potent approach to increasing the stability and limiting the level of inflammation of atherosclerotic plaque.

ACKNOWLEDGMENTS

This study was supported in part by grant 28181565311 from the Israel Ministry of Health to T.W., and by an internal grant by Soroka University Medical Center to T.W. We thank Y. Haim for her technical assistance.

DISCLOSURE

None of the authors of this paper has any conflict of interest regarding any of the products named in the paper.

REFERENCES