Intensive Lipid-lowering Therapy Ameliorates Novel Calcification Markers and GSM Score in Patients with Carotid Stenosis

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Objectives/Design. Carotid plaque echogenicity quantified by the Gray-Scale Median (GSM) score has been associated with plaque vulnerability. The aim of this study was to assess whether intensive lipid-lowering treatment with atorvastatin in patients with carotid artery stenosis ameliorates novel vascular calcification inhibitors, such as osteopontin (OPN) and osteoprotegerin (OPG), and improves GSM score.

Methods. Ninety-seven patients with carotid stenosis (>40%), but without indication for intervention, were treated for 6 months with atorvastatin (10 mg–80 mg) to target LDL < 100 mg/dl. Fifty-two age-and sex-matched healthy individuals served as the control group. Blood samples and GSM were obtained at the beginning and after 6 months.

Results. Systolic blood pressure, hsCRP, fibrinogen, OPN and OPG levels differed significantly between patients with carotid stenosis and healthy controls at baseline (p < 0.05). Atorvastatin treatment improved lipid profile and significantly reduced hsCRP (p = 0.002), WBC count (p = 0.041), OPN (p < 0.001) and OPG levels (p < 0.001). GSM score increased considerably after atorvastatin therapy (from 58.33 ± 24.38 to 79.33 ± 22.3; p < 0.001) and that effect appeared related to OPN (p = 0.001), OPG (p = 0.013) and LDL (p = 0.01) reduction.

Conclusions. In patients with carotid stenosis, intensive lipid-lowering therapy with statins attenuates serum OPN and OPG levels and enhances carotid plaque echogenicity, outlining their beneficial effects on plaque stability.

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Introduction

Carotid atherosclerosis and stroke remain one of the leading causes of morbidity and mortality in most industrialized countries. 1 The key factor affecting the risk of cerebrovascular ischemic events in patients with carotid atherosclerosis seems to be the composition of the plaque rather than the degree of luminal encroachment. 2 Plaque prone to rupture, known as vulnerable plaque, characteristically contains large areas of lipids and necrosis, abundant macrophages, less calcified deposits and thin fibrous cap. 2

Accumulating interest has focused on understanding how carotid plaque calcification enhances plaque stability and decreases the likelihood of clinical events. 3 Osteopontin (OPN), an acidic phosphoprotein, and osteoprotegerin (OPG), a member of the tumor necrosis factor-a receptor superfamily, have been recently demonstrated to modulate vascular calcification. 4,5 These bone-matrix proteins inhibit mineral deposition as well as osteoclastogenesis and are expressed constitutively by most vascular cells. 4 Recent studies have shown an association of serum OPN and OPG levels with cardiovascular diseases. 6,7 The immunodetection of OPN and OPG in human carotid plaques indicate their contribution to atherosclerotic process and plaque composition. 5,8,9 Nevertheless, their precise role in atherosclerosis development and their response to pharmaceutical interventions remain obscure.

Among new imaging methods, ultrasound modalities provide a non-invasive, cheap and easily repeatable
approach to carotid atherosclerosis. Most recently, analysis of plaque echogenicity and especially its quantitative index, Gray Scale Median (GSM) score, has emerged as an assessment methodology of carotid plaque vulnerability. Echolucent plaques with low GSM score have been recognized as lipid-rich and less calcified. These unstable plaques correlate with higher rates of cerebrovascular events. Thus increases of plaque echogenicity could be a valuable marker of the efficacy of therapeutic strategies on carotid plaque stability.

The potential usefulness of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), as a part of the conservative treatment of patients with carotid disease has been cited by the National Cholesterol Education Project. Numerous trials show that statin agents induce a resounding and consistent improvement in cardiovascular end-points, which is mainly attributed to plaque stabilization rather than to plaque regression. The beneficial effects of statins are not fully explained by the baseline or treated LDL cholesterol levels and may involve non-lipid mechanisms. These so-called “pleiotropic” properties include anti-inflammatory, immunomodulatory, anti-oxidative and direct anti-atherosclerotic effects resulting in plaque stability. Despite the above molecular evidence, practical examinations to determine the modifying effects of statins on carotid plaque vulnerability are relatively limited.

This study was designed to investigate whether intensive lipid-lowering therapy with atorvastatin attenuates serum OPN and OPG levels and increases GSM value in patients with carotid stenosis and indications for conservative treatment. We also tested the hypothesis that the modification of lipid levels and vascular calcification inhibitors mediates the atorvastatin-induced effects on the GSM score. The present study constitutes a part of an ongoing, prospective project which aims to identify patients with undiagnosed carotid stenosis and to apply optimal therapies. The preliminary baseline data of this project, which derive from most of our participants, have already been published.

Materials and Methods

Subjects

This was an open-label, prospective study. Eligibility for entering this study required recently diagnosed (within 14 days) stenosis of at least one internal carotid artery (ICA). The degree of stenosis was >40%, but there was no indication for revascularization. A group of age- and sex-matched healthy volunteers without any chronic disease were also enrolled. Autoimmune diseases, hypothyroidism, osteoporosis, liver impairment (ALT > 2.5 times higher than the upper normal limit), renal insufficiency (creatinine levels > 2.0 mg/dl), coronary artery disease, atrial fibrillation, malignancy, ongoing or previous use of bisphosphonates, glucocorticoids, lipid-lowering medications and contraindications to the use of statins were exclusion criteria. A total of 155 consecutive Caucasians subjects, aged 50–75, were initially included. Among them, 103 patients had newly-diagnosed ICA stenosis with indications for conservative treatment, while 52 individuals served as healthy controls.

After providing written informed consent, eligible subjects were assigned to the following groups: A) Symptomatic patients with 40–60% stenosis ICA. B) Asymptomatic patients with 40–70% stenosis. C) Healthy subjects. Symptomatic disease was documented when patients had experienced stroke or TIA attributed to ipsilateral carotid stenosis. The diagnosis of ischemic attack was based on the combined evaluation of medical history, neurological examination, and brain computed tomography (CT) or magnetic resonance imaging (MRI) examination, when CT findings were questionable. On the other hand, asymptomatic patients appeared without focal neurological symptoms and negative CT and/or MRI scan.

Methods

Body weight, height, hip and waist circumference were measured and body-mass index (BMI) and waist-to-hip ratio (WHR) calculated. Blood pressure (BP) was measured twice, after keeping participants in a sitting position for 15 minutes. The minute intervals was used for study purposes. All comorbidities, medications, smoking habits, and parental medical history were recorded using structured questionnaires.

At baseline, all participants underwent an ultrasound examination of both carotids. Afterwards, patients with carotid stenosis (groups A and B) were treated for 6 months with atorvastatin. The dose was gradually titrated, at 6 weeks intervals, from 10 mg to 80 mg to target LDL < 100 mg/dl. The number of statin pills, already taken by each patient, was calculated at the end of the study. Compliance was considered effective if at least 90% of estimated study medication had been received. Anti-platelet agents were prescribed to all patients with carotid stenosis (acetylsalicylic acid 100 mg/day or clopidogrel...
75 mg/day). Concomitant pharmaceutical therapy was modified only when deemed medically appropriate during the research period. Ultrasound examination was repeated 6 months later only in the interventional group. The study protocol was approved by the local ethical committee and conducted in compliance with the ethical guidelines of the Declaration of Helsinki.

**Ultrasound**

An ultrasound scanner (General Electric Logiq700, Riverside, USA) equipped with a 7.5 MHz probe was used. With the patient in the supine position, the carotid artery was investigated bilaterally in a suitable longitudinal and transverse view (the angle of insonation was less than or equal to 60°). The diagnosis of carotid stenosis was based on the recommendations of the Society of Radiologists in Ultrasound. All images from both carotids were directly recorded on a personal computer and GSM score was calculated in longitudinal images using Adobe Photoshop 7.0 software. Image normalization included the definition of the GSM score of the two reference points (blood and adventitia) in the original B-mode image (input values). We then performed the “curves” option of the software so that in the resultant image, the GSM of blood equals 0 to 5 and that of the adventitia equals 185 to 195 (output values). In this way, the gray scale values were adjusted according to the input and the output values of the two reference points. A single operator who was unaware of the patient’s history performed all ultrasound examinations and measurements of plaques.

In the symptomatic group, the GSM value of the culprit lesion, associated with the ipsilateral brain infarct, was used for study purposes. In case of more than one unilateral atherosclerotic plaque, the GSM value of each single plaque was estimated and the average value was considered for statistical analysis. In asymptomatic patients with more than one carotid plaque, we averaged the GSM value of all plaques in both carotids.

**Blood analyses**

Blood samples were obtained between 8.00 and 9.00 a.m after fasting at the beginning and at the end of the study. In the symptomatic group, blood samples were collected at least 14 days after admission to the hospital for stroke or TIA or at least 7 days after discharge from the hospital. Fasting plasma glucose (FPG) and lipid parameters were determined using standard enzymatic methods (Olympus AU560, Hamburg, Germany). Measurements of HbA1c were done by high-performance liquid chromatography (Menarini Diagnostics, Florence, Italy). Plasma OPN and OPG were assayed using quantikine immunoassay EIA kits (R&D Systems Inc., Minneapolis, USA and Metra, San Diego, USA). The intra- and inter-assay coefficients of variance were 2.6% and 5.7% for OPN and 7% and 6.8% for OPG, respectively. High-sensitivity C-Reactive Protein (hsCRP) and fibrinogen were measured by nephelometric assay (Dade Behrin, BNII, Marburg, Germany) and the Clauss method, respectively. Samples were frozen and stored (~80 °C) until analysis in the same assay.

**Statistical analysis**

Data are expressed as mean and ± SD. Normality of distribution was assessed by Kolmogorov–Smirnov test. Comparisons of parametric parameters within and between groups were analyzed by paired-samples and student’s t-test, respectively. One-way ANOVA analysis was applied to assess differences between groups at baseline. Chi-square test was used for categorical data. The univariate associations of OPN, OPG, GSM values at baseline and their changes after treatment were quantified by Pearson correlation. Changes of GSM, as dependent variable, and changes of the rest of significant-related parameters, as independent variables, entered a multiple regression analysis model. All analyses were carried out by software SPSS-13.0 (SPSS Inc, Chicago, USA). Two-tailed p value of <0.05 was considered as statistically significant.

**Results**

Among 155 participants, full measurements were obtained in 149 cases. Six patients with carotid stenosis were excluded, because they did not have a second ultrasound measurement (1 died of pneumonia, 5 patients did not return for the second measurement). Of the remaining 97 individuals with carotid stenosis, all images were of good quality and therefore, were included in statistical analysis. Atorvastatin was well-tolerated and noteworthy adverse effects were not reported. No patient experienced any cardiovascular event during the study period.

**Baseline characteristics**

Baseline characteristics of participants are listed in Tables 1 and 2. Patients with carotid stenosis had
greater waist circumference, WHR, systolic BP, FPG, triglycerides, WBC count, hsCRP, fibrinogen, OPN and OPG levels compared with healthy individuals at baseline ($p < 0.05$). During the study, 6 patients with carotid stenosis (3 symptomatic, 3 asymptomatic) had their anti-hypertensive regimen modified (dose titration or new agent addition), while 6 patients (2 symptomatic, 4 asymptomatic patients) started taking anti-hypertensive agents to target systolic/diastolic BP lower than 130/85 mmHg. Treatment modulation resulted in the downregulation of OPN ($p < 0.001$) and OPG ($p < 0.041$), OPN ($p = 0.004$), OPG ($p < 0.001$) (Table 2). We must emphasize that no patient with carotid stenosis had LDL levels $<100$ mg/dl at baseline, while 84.76% achieved the target at the end of the study. Most importantly, atorvastatin treatment increased GSM considerably (from 58.33 ± 27.35 vs 72 ± 13.08; $p < 0.001$). Compared to healthy individuals, OPG levels were significantly elevated in symptomatic and asymptomatic groups ($p < 0.05$), while OPN was considerably increased only in symptomatic patients ($p < 0.001$) (Fig. 1).

**Effects of atorvastatin treatment**

Lipid-lowering therapy elicited marked improvement in most lipid parameters, hsCRP ($p = 0.002$), WBC ($p = 0.041$), OPN ($p < 0.001$) and OPG ($p < 0.001$) (Table 2). We must emphasize that no patient with carotid stenosis had LDL levels $<100$ mg/dl at baseline, while 84.76% achieved the target at the end of the study. Most importantly, atorvastatin treatment increased GSM considerably (from 58.33 ± 24.38 to 79.33 ± 22.3; $p < 0.001$), while the degree of carotid lumen encroachment remained unaffected ($p = 0.823$). The exclusion of patients with modulated anti-hypertensive medications from statistical analysis did not alter all the aforementioned results (data not shown).

Upon examining symptomatic and asymptomatic patients, atorvastatin therapy significantly reduced
most lipid parameters, hsCRP, OPN and OPG (p < 0.05) and increased GSM in both groups (p < 0.01) compared with baseline values. We also noted a significant reduction of fibrinogen only in asymptomatic patients. The amount of changes of all variables did not differ between the symptomatic and asymptomatic groups (p > 0.05) (Table 4, Fig. 1).

**Correlations**

In agreement with our previous study, univariate analysis of carotid atherosclerosis group at baseline confirmed the inverse association of GSM with OPN, OPG and waist circumference (p < 0.01). We next examined the changes of GSM, OPN and OPG in relation to the changes of cardiovascular risk factors after atorvastatin administration. The atorvastatin-induced augmentation of GSM was significantly correlated with the changes of OPN (r = −0.414, p = 0.001), OPG (r = −0.584, p = 0.013) and LDL (r = −0.472, p = 0.01). Besides this, changes of OPN related to OPG alterations (r = 0.538, p = 0.028).

Standard multiple regression analysis revealed that the atorvastatin-related changes of OPN, OPG, and LDL constituted independent predictors of GSM changes (p = 0.008) and seemed to explain 52.3% of its variation.

**Discussion**

The present study demonstrated serum OPN and OPG levels to be increased in patients with carotid stenosis and independently associated with carotid plaque echogenicity and symptomatology. These bone-matrix proteins are now the focus of increasing research in cardiovascular diseases, but their modulation by statins has not yet been explored. In our study, intensive atorvastatin therapy downregulated the levels of both biochemical markers and considerably increased GSM score without regression of plaque size in patients with carotid stenosis. That effect was related to the reduction in OPN, OPG and LDL levels implicating a novel angio-protective mechanism by which statins may enhance carotid plaque stability.

There has been increasing awareness surrounding the association of OPN and OPG with carotid disease. We and other investigators have previously demonstrated increased serum OPN and OPG levels in patients with carotid atherosclerosis. Our results confirmed these studies, while most elevated levels of serum OPN and OPG levels were found in stroke and TIA survivors group indicating the association of bone-remodeling proteins with symptomatology. This notion is further supported by the independent association of serum OPN and OPG with GSM values.

Up to now, there has been limited evidence concerning the effects of statins on OPN. In vitro studies from cultured rat aortic smooth muscle cells (SMCs) or aorta tissue obtained from hypothyroid mice have shown statins to dose-dependently reduce OPN expression. Preliminary data from Tanaka et al. demonstrated that 6 months treatment with ator- vastatin (20 mg) reduced plasma OPN levels by 19% in a small sample of hypercholesterolemic patients. In our study the hypolipidemic regimen elicited 32% reduction in OPN levels. A plausible explanation for that marked effect derives from the interplay between lipid-lowering and cardiovascular OPN expression.

However our findings do not sustain this theory,
because atorvastatin reduced serum OPN independently of lipid parameters changes. Alternatively we postulated the atorvastatin-induced modulation of OPN expression via non-lipid mechanisms such as mevalonate pathways and isoprenylated molecules. Taking into consideration that OPN constitutes a novel cardiovascular risk factor the above beneficial effects of atorvastatin are quite promising, but remain to be proved by longitudinal studies.

In the light of the “pleiotropic” properties of statins, a recent study showed that atorvastatin treatment of human osteoblasts stimulated dose-dependently the production of OPG. In contrast, treatment with atorvastatin suppressed, in a dose-dependent manner, the production of OPG from endothelial and vascular SMCs. Perhaps the different cell-type may explain the above discrepancy. In our study we documented for first time the suppression of serum OPG levels by intensive statin therapy. Concerning that OPG is strongly related to cardiovascular diseases, our finding is of clinical relevance. Future studies will address the question of whether statin-induced reduction of OPG is another vasculoprotective mechanism or a sensitive bystander of atherosclerotic process.

To our knowledge this is the first study using GSM values to quantify the effects of lipid-lowering therapy on carotid plaque composition. We documented that intensive atorvastatin therapy did not limit carotid narrowing, but significantly increased carotid plaque echogenicity. It is well-established that statin administration curtails carotid plaque progression, reduces cerebrovascular events rate and decreases perioperative morbidity and mortality in patients undergoing carotid surgical or endovascular revascularization. Stabilization of lesions by modification of structure and content, rather than simple improvement in the luminal diameter explains the above mechanistic effects and provides a new therapeutic target in carotid disease, whose mainstay of therapy remains surgical. Moreover the present study underscores that ultrasound image analysis is a practical index to monitor plaque texture changes during the surveillance period.

Although the extent to which statins influence plaque biology in vivo has engendered controversy, several potential mechanisms account for the augmentation of plaque echodensity. Statins predominantly reduce plasma concentration of LDL, a well-established cardiovascular risk factor. A recent meta-analysis of all randomized trials testing statin drugs found a 21% risk reduction for stroke, closely associated with reductions in LDL levels. Next to the lipid-lowering effects, statin treatment has potential “pleiotropic” anti-atherosclerotic effects. In our study atorvastatin administration improved lipid profile and decreased inflammatory burden. Meanwhile the increment of GSM value was significantly correlated with the reduction of OPN, OPG and LDL levels. These results were supported by multivariate regression analysis, after adjustment for potential confounders. Taken all together we postulated that the enhancement of carotid plaque echogenicity was attributed to the “pleiotropic” functions of statins besides to lipid-lowering.

Extensive research suggests that subintimal lipid deposition, lipid oxidation and inflammatory cells

\[ \text{Fig. 1. Serum levels of osteopontin and osteoprotegerin and GSM values at baseline and at the end of the study. Data are mean} \pm SD. ||p<0.05 \text{ difference of variables at baseline between A and B groups; *p<0.05 difference of variables at baseline between A and C groups; **p<0.05 difference of variables at baseline between B and C groups; #p<0.05 changes of variables within groups. A, symptomatic group; B, asymptomatic group; C, healthy control group.} \]
Data are means ± SD. FPG, Fasting plasma glucose; hsCRP, high sensitivity C-Reactive Protein; WBC, White Blood Cells. \( P_1 \), \( P_2 \) values of changes of variables between groups. \( P_3 \), \( P_4 \) values of changes of variables within groups.

Comparison within and between groups for HbA1c was performed only in diabetic patients.

References


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