Undercarboxylated Osteocalcin Is a Biomarker of Carotid Calcification in Patients with Essential Hypertension

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Abstract
The development of vascular calcification is an active, highly regulated process with similarities to bone formation. Osteocalcin (OC), a vitamin K-dependent protein expressed by osteoblasts, contains 3 γ-carboxyglutamic acid residues derived from the vitamin K-dependent posttranslational modification of glutamic acid residues. Circulating undercarboxylated OC (ucOC) is increased in vitamin K deficiency and serum ucOC is reported to be a clinical marker of vitamin K status. Vitamin K deficiency is associated with vascular calcification as well as osteoporosis. We evaluated the relationship between ucOC and carotid artery calcification in 92 patients with essential hypertension. Ultrasound of the common carotid artery was performed to identify vascular calcification and subjects were divided into 2 groups: a calcification (+) group and a calcification (−) group. Serum creatinine and ucOC levels were higher in the calcification (+) group than in the calcification (−) group and serum ucOC correlated with serum creatinine. To identify the independent determinant factor for carotid artery calcification, we applied both ucOC and estimated glomerular filtration rate as independent factors in logistic regression analysis. Serum ucOC was an independent determinant of carotid calcification, suggesting that circulating ucOC may be an important biomarker of carotid artery calcification.

Introduction
Vascular calcification is a common pathological feature of advanced atherosclerosis, aging, diabetes and hypertension, and it is associated with an increased risk of morbidity and mortality [1]. The deposition of calcium in the vasculature is an active and highly regulated process similar to bone formation [2]. Bone mass is determined by a long-term net balance between bone formation and bone resorption. Osteocalcin (OC) is a bone-specific protein of 49 amino acids that is synthesized by osteoblasts [3]. Since a fraction of newly synthesized OC is released into the circulation, its serum concentration generally reflects mature osteoblastic activity and bone formation [4, 5]. OC contains 3 γ-carboxyglutamic acid residues derived from the vitamin K-dependent posttranslational modification of glutamic acid residues [6, 7]. The serum...
undercarboxylated OC (ucOC) level has been reported to be increased in elderly women [8], particularly those with hip fractures [9], and to be negatively correlated with bone mineral density [10]. ucOC is also reported to be a clinical marker of vitamin K status and intake [11], as deficiency in vitamin K leads to increased circulating ucOC. Vitamin K deficiency is associated with both vascular calcification and osteoporosis [12, 13].

Recently, Gössl et al. [14] reported that patients with coronary atherosclerosis have increased circulating endothelial progenitor cells that display an osteogenic phenotype, including OC, providing a potential mechanism for the link between OC and atherosclerosis or vascular calcification. In the present study, we investigated the relationship between carotid artery calcification and serum ucOC in patients with essential hypertension (EHT).

**Methods**

**Study Subjects**

The ethics committee of the Ehime University Graduate School of Medicine provided approval for the study. Informed consent was obtained from all participating patients. Ninety-two patients with EHT were enrolled in this study. Hypertension was defined as either the use of antihypertensive medications, or as a systolic blood pressure (SBP) ≥ 140 mm Hg or a diastolic blood pressure (DBP) ≥ 90 mm Hg. The SBP and DBP were the average of 3 measurements taken with a brachial sphygmomanometer with the patient in the seated position. Patients with congestive heart failure, previous myocardial infarction, angina pectoris, atrial fibrillation, or a history of stroke, malignant tumor or autoimmune disease were excluded. None of the patients had been treated with warfarin (Coumadin) and/or anti-osteoporosis drugs which influence the ucOC levels.

**Blood Sampling**

Blood samples were drawn from the forearm after 20 min of rest in the supine position. Clinical chemistry tests were done within 30 min after blood sampling; blood samples used for ucOC were centrifuged and stored at −80°C until assayed by an enzyme-linked immunosorbent assay kindly provided by SRL Inc. (Tokyo, Japan). The coefficient of variation for both the intra- and interassays of ucOC was less than 5%. The serum levels of creatinine (serum Cr), low-density lipoprotein (LDL-cholesterol), triglyceride, and high-density lipoprotein (HDL-cholesterol) were measured using an automated analyzer (model TBA-200FR; Toshiba Inc., Tokyo, Japan). Estimated glomerular filtration rate (eGFR) was calculated using a Modification of Diet in Renal Disease (MDRD) study equation with standardized serum Cr (4-variable equation) [15]. Hemoglobin A1c (HbA1c) was analyzed on an ADAMS-A1c HA-8160 (ARKRAY Inc., Kyoto, Japan) based on high-performance liquid chromatography assay.

**Ultrasound Analysis of the Common Carotid Artery**

Trained two-scan readers, who were blinded to the clinical data of the participants, performed the ultrasound using a Sonos 5500 (Philips, Eindhoven, The Netherlands) and a 7.5-MHz probe as previously described [16, 17]. After the subjects had rested for at least 10 min in the supine position with a slight hyperextension of the neck, the bilateral common carotid artery, carotid bulb, and extracranial internal and external carotid arteries were observed with optimal visualization. The intima-media thickness (IMT) of the far wall was measured in the common carotid artery from the anterior, lateral, and posterior approaches. The value of thickest IMT in these observations was defined as maxIMT. To evaluate the distribution of atherosclerosis in the carotid arteries, we used a plaque scoring method, which was the summation of bilateral thickness >1.1 mm, as described previously [17]. Calcification was defined by an acoustic shadow within the atherosclerotic lesion on ultrasound.

**Statistical Analysis**

All values are expressed as means ± standard deviation. Comparisons between the 2 groups were performed by unpaired t tests. Pearson's correlation coefficients were calculated to assess the association between continuous variables. χ² test was used to analyze the distribution of drug use and of patients with or without diabetes between the calcification (+) group and the calcification (–) group. Logistic regression analysis was applied to assess the relationship between carotid calcification and eGFR or ucOC. All analyses were performed using StatView version 5 (SAS Institute, Inc., Cary, N.C., USA). A p value of <0.05 was considered to be statistically significant.

**Results**

**Patient Characteristics**

The clinical characteristics of the study subjects are summarized in table 1. Fifty patients (54%) were treated with anti-hypertensive drugs. Twenty-eight patients (30%), 41 patients (44%), 9 patients (9.7%) and 6 patients (6.5%) were taking an angiotensin II receptor blocker, Ca channel blocker, β-blocker and diuretic, respectively. Fourteen patients (15%) also had diabetes mellitus.

**Carotid Artery Calcification**

The subjects were divided into 2 groups, a calcification (+) group and a calcification (–) group, according to evidence of calcification in the carotid arteries detected by ultrasound. Between the 2 groups, there were no significant differences in age, body mass index, SBP, DBP, HbA1c, LDL-cholesterol or HDL-cholesterol (table 1). Similarly, the frequencies of patients taking angiotensin II receptor blocker, Ca channel blocker, statin and anti-platelet drug were not different between the calcification (+) group and the calcification (–) group (p = 0.272, 0.888, 0.415 and 0.361, respectively). In contrast, serum Cr and
ucOC levels were significantly higher in the calcification (+) group than in the calcification (−) group (table 1). The evaluation of atherosclerosis in the carotid artery by ultrasound demonstrated that both maxIMT and plaque score were higher in the calcification (+) group than in the calcification (−) group (fig. 1).

**Table 1.** Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>Calcification (+)</th>
<th>Calcification (−)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (% male)</td>
<td>92 (58)</td>
<td>14 (43)</td>
<td>78 (60)</td>
<td>0.225</td>
</tr>
<tr>
<td>Age, years</td>
<td>61 ± 11</td>
<td>61 ± 16</td>
<td>60 ± 12</td>
<td>0.764</td>
</tr>
<tr>
<td>BMI</td>
<td>25.0 ± 3.6</td>
<td>24.0 ± 3.3</td>
<td>25.1 ± 3.6</td>
<td>0.326</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>149 ± 20</td>
<td>143 ± 19</td>
<td>150 ± 19</td>
<td>0.242</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>88 ± 15</td>
<td>77 ± 18</td>
<td>90 ± 13</td>
<td>0.002</td>
</tr>
<tr>
<td>Pulse pressure, mm Hg</td>
<td>61 ± 16</td>
<td>66 ± 13</td>
<td>60 ± 16</td>
<td>0.203</td>
</tr>
<tr>
<td>Cr, mg/dl</td>
<td>1.12 ± 1.01</td>
<td>1.66 ± 1.79</td>
<td>1.03 ± 0.78</td>
<td>0.031</td>
</tr>
<tr>
<td>eGFR</td>
<td>63.0 ± 24.4</td>
<td>56.3 ± 33.2</td>
<td>64.2 ± 22.6</td>
<td>0.270</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.24 ± 1.27</td>
<td>5.47 ± 0.62</td>
<td>5.20 ± 1.35</td>
<td>0.486</td>
</tr>
<tr>
<td>LDL-cho, mg/dl</td>
<td>126 ± 38</td>
<td>108 ± 21</td>
<td>130 ± 40</td>
<td>0.099</td>
</tr>
<tr>
<td>HDL-cho, mg/dl</td>
<td>54 ± 15</td>
<td>50 ± 15</td>
<td>55 ± 14</td>
<td>0.225</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>150 ± 99</td>
<td>162 ± 163</td>
<td>147 ± 83</td>
<td>0.606</td>
</tr>
<tr>
<td>ucOC, ng/ml</td>
<td>5.9 ± 4.2</td>
<td>8.6 ± 5.7</td>
<td>5.4 ± 3.7</td>
<td>0.007</td>
</tr>
<tr>
<td>Diabetes</td>
<td>14 (15)</td>
<td>3 (21)</td>
<td>11 (14)</td>
<td>0.235</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>receptor blocker</td>
<td>28 (30)</td>
<td>6 (42)</td>
<td>22 (28)</td>
<td>0.272</td>
</tr>
<tr>
<td>Ca channel blocker</td>
<td>41 (44)</td>
<td>6 (42)</td>
<td>35 (45)</td>
<td>0.888</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>9 (9.7)</td>
<td>0 (0)</td>
<td>9 (11)</td>
<td>0.180</td>
</tr>
<tr>
<td>Diuretic</td>
<td>6 (6.5)</td>
<td>0 (0)</td>
<td>6 (8)</td>
<td>0.283</td>
</tr>
<tr>
<td>Statin</td>
<td>13 (14)</td>
<td>1 (7)</td>
<td>12 (15)</td>
<td>0.415</td>
</tr>
<tr>
<td>Anti-platelet agent</td>
<td>14 (15)</td>
<td>2 (14)</td>
<td>12 (15)</td>
<td>0.361</td>
</tr>
</tbody>
</table>

Figures in parentheses are percentages. cho = Cholesterol.

**ucOC and Carotid Artery Calcification**

To further investigate the relationship between ucOC and other clinical parameters, Pearson’s correlation coefficients (r) were calculated (table 2). Serum ucOC correlated with eGFR and serum Cr but did not correlate with age, body mass index, blood pressure, HbA1c, LDL-cholesterol or HDL-cholesterol, indicating that ucOC levels are associated with renal function. Since carotid artery calcification was associated with both ucOC and renal function, we determined whether ucOC and eGFR are independent determinant factors for carotid artery calcification using a logistic regression analysis. Serum ucOC was an independent determinant of carotid calcification (table 3).

**Discussion**

Vascular calcification is an important clinical entity in cardiovascular disease thought to be significantly affected by vitamin K levels. In this study, we demonstrate for the first time that ucOC is associated with carotid artery calcification in patients with EHT and that this association is independent of renal function.

The prevalence of calcification increases with age and occurs preferentially in men [18, 19]. Prabhakaran et al. [19] reported that calcified carotid plaque was an independent predictor of vascular events in the Northern Manhattan cohort study (1,118 subjects). In that study, the mean age of the subjects was 68 ± 8 years and the prevalence of carotid plaque with calcification was 20.1% [19]. Our study showed that there was no difference in age and gender between the calcification (+) group and the
Table 2. Correlation (r) between ucOC and clinical parameters

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.16</td>
<td>0.127</td>
</tr>
<tr>
<td>BMI</td>
<td>0.058</td>
<td>0.600</td>
</tr>
<tr>
<td>Pulse pressure</td>
<td>0.168</td>
<td>0.111</td>
</tr>
<tr>
<td>Cr</td>
<td>0.426</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>eGFR</td>
<td>-0.297</td>
<td>0.004</td>
</tr>
<tr>
<td>HbA1c</td>
<td>0.045</td>
<td>0.677</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>0.120</td>
<td>0.394</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>-0.091</td>
<td>0.396</td>
</tr>
</tbody>
</table>

Table 3. Logistic regression analysis of eGFR and ucOC as independent determinants for carotid artery calcification

<table>
<thead>
<tr>
<th></th>
<th>β</th>
<th>Odds ratio</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFR</td>
<td>-0.012</td>
<td>0.999</td>
<td>0.908</td>
</tr>
<tr>
<td>ucOC</td>
<td>2.294</td>
<td>1.178</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Calcification (–) group. The reason for this difference may be due to the relatively younger age of patients in our study (60±13) and the lower prevalence of calcification (15.2%). We also evaluated the aortic arch calcification by chest X-ray. All 14 patients with carotid calcification had aortic calcification, 47 patients without carotid calcification had aortic calcification (60%) and in 31 patients we could detect neither aortic nor carotid calcification. There was a 66% prevalence of aortic calcification in the hypertensive patients in our study. We also analyzed the relationship between aortic arch calcification evaluated by chest X-ray and serum ucOC concentration. However, we do not show the relation between them. In our study, only 15% (14 patients) were positive for carotid calcification and 65% were positive for aortic arch calcification. This suggested that patients with carotid calcification had a more severe calcified state than patients with aortic arch calcification but without carotid calcification.

The present study showed that serum ucOC was an independent determinant factor for carotid calcification. OC is the most abundant non-collagenous bone matrix protein produced by osteoblasts. A number of studies have shown that OC has a high affinity for hydroxyapatite, with some in vitro cell studies demonstrating that OC has a function in the recruitment and differentiation of osteoclast precursors into mature, bone-resorbing osteoclasts, suggesting a role in bone resorption [20]. In human carotid plaque, protein levels and the mRNA expression of OC were greater in calcified plaques than non-calcified ones [21]. In addition, Adjiang et al. [22] reported that OC is highly expressed in calcified aortic regions of indoxyl sulphate-treated hypertensive rats. These results indicate that OC is closely associated with vascular calcification; however, the precise function of OC in vascular calcification has not yet been elucidated.

OC contains 3 γ-carboxyglutamic acid residues that are derived from vitamin K-dependent posttranslational modification [6, 7]. Vitamin K functions as a cofactor for the endoplasmic reticulum enzyme γ-glutamyl carboxylase, which catalyzes the conversion of glutamate residues into γ-carboxyglutamate (Gla) [6, 23]. Circulating ucOC is a valuable nutritional marker that reflects both the vitamin K and D status in the skeleton [24]. Serum ucOC levels are also reported to be increased in elderly women [8], particularly those with hip fractures [9], and to be negatively correlated with bone mineral density [10].

Our study is the first to indicate that ucOC is associated with carotid calcification in EHT patients, although it remains unknown whether ucOC directly affects vascular calcification. Matrix Gla protein (MGP) is also a vitamin K-dependent protein that has 5 Gla residues and is synthesized in bone, cartilage, and soft tissue such as lung, heart and kidney [25, 26]. MGP is also expressed by human vascular smooth muscle cells, and MGP mRNA synthesis is upregulated in calcified atherosclerotic plaques [2]. Interestingly, MGP-deficient mice showed extensive medial calcification of the aorta, leading to death within 8 weeks after birth because of ruptures of the thoracic or abdominal aorta [27]. This indicates that MGP plays an important role in the inhibition of vascular calcification. The γ-glutamyl carboxylation of MGP is essential for its inhibitory effect on calcification in vitro [28], and in vivo, undercarboxylation of MGP (ucMGP) is a risk factor for vascular calcification [29]. In the Rotterdam trial in 4,800 elderly patients, low vitamin K2 intake was associated with a higher incidence of severe aortic calcification and increased mortality [30]. Thus, circulating levels of ucOC and ucMGP are likely to be closely linked because of their coregulation by vitamin K levels [11] and association with vascular calcification. However, since serum vitamin K or ucMGP were not measured in our study, it remains to be determined whether the ucOC level is directly associated with carotid calcification or is a marker of γ-glutamyl carboxylation or vitamin K deficiency.
Chronic kidney disease is a strong risk factor for cardiovascular mortality [31, 32]. Despite intensive investigation, the precise mechanisms responsible for this strong association remain unknown. One candidate mechanism may be accelerated vascular calcification, which is highly prevalent in chronic kidney disease [33–35]. In the present study, ucOC correlates with renal function, consistent with the association of both of them with calcification. However, ucOC is independently associated with vascular calcification. This suggests that the mechanism(s) or pathways linking ucOC to vascular calcification are independent of renal function.

There are some limitations to our study. The study size was relatively small, especially the number of patients with carotid calcification; moreover, some patients were taking medication that may have influenced vascular calcification and circulating ucOC. In addition, we did not measure the circulating total OC concentration, and some studies have reported that the ucOC/OC ratio is a more reliable biomarker of vitamin K status [36]. The ucOC level reflects osteoblast activity; however, we could not show another parameter which indicates the osteoblast function. Finally, we did not measure MGP and ucMGP which show the status of vitamin K as well as vascular calcification.

In conclusion, we investigated the relationship between carotid artery calcification and serum ucOC in patients with EHT. Patients with carotid artery calcification have higher circulating levels of ucOC than patients without carotid calcification, making circulating ucOC a potential biomarker of carotid artery calcification.

Acknowledgement

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References


