Circulating Concentrations of Stem-Cell-Mobilizing Cytokines Are Associated With Levels of Osteoprogenitor Cells and Aortic Calcification Severity

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Background: To investigate the association between aortic calcification, concentrations of stem-cell-mobilizing cytokines and osteocalcin-positive mononuclear cells in a mouse model and patients with peripheral artery disease.

Methods and Results: We estimated the concentration of the stem-cell-mobilizing cytokines stromal cell-derived factor α (SDF-1α), granulocyte colony stimulating factor and stem cell factor in a mouse model of aortic calcification developed in osteoprotegerin-deficient (OPG−/−) mice, as well as in patient plasma samples. Calcification was estimated by a colorimetric assay of extracts of harvested mice aortas and by computed tomographic angiogram images in patients. The cytokine concentrations were assessed for association with the severity of calcification and the percentage of osteocalcin-positive mononuclear cells (OCN+ MNC) using non-parametric analysis. The serum concentration of stromal SDF-1α and granulocyte-colony stimulating factor (G-CSF) were significantly greater in OPG−/− compared to control mice. The percentage of circulating OCN+ MNC was correlated to the concentration of SDF-1α in OPG−/− mice. These cytokines also correlated with the severity of calcification in OPG−/− mice. Patients with more severe calcification had a higher plasma concentration of the cytokines than those with less marked aortic calcification. The concentrations also correlated with circulating OCN+ MNC and aortic calcification volumes.

Conclusions: The association between stem cell cytokines, OCN+ MNC and calcification suggests a possible role of bone-derived osteoprogenitor cells in the pathogenesis of vascular calcification. (Circ J 2011; 75: 1227–1234)

Key Words: Cytokines; Peripheral artery disease; Stem cells; Vascular calcification

Vascular calcification has been demonstrated to be predictive of cardiovascular events in a range of populations. There is ongoing controversy regarding the mechanisms of vascular calcification, with current theories including passive and active models based on transformation of vascular smooth muscle cells to cells capable of mineralization.

In a previous investigation we reported an association between circulating osteocalcin-positive mononuclear cells (OCN+ MNC) and aortic calcification in 2 mouse models and a patient group with peripheral artery disease. The results fitted with the theory that bone marker-positive MNC are released from the bone marrow into the peripheral circulation, home to the diseased arteries, differentiate into an osteogenic lineage, proliferate and contribute to vessel mineralization.

OCN+ MNC have been previously reported to be a small subset of the bone marrow that possess bone forming abilities in vitro and hence are termed osteoprogenitors. We postulated that these naïve cells were released from the bone marrow environment under the influence of stem-cell-mobilizing cytokines: stromal cell-derived factor α (SDF-1α), granulocyte-colony stimulating factor (G-CSF) and stem cell factor (SCF). Further, it has been suggested that stem-cell-mobilizing cytokines like SDF-1α may facilitate the homing of immature circulating cells to vascular lesions, contributing towards disease progression. On the basis of these previous reports, in the present study we determined the concentrations of stem-cell-mobilizing cytokines in both a mouse model and a patient group. The circulating cytokine concentrations were assessed for association with the percentage of circulating OCN+ MNC and the severity of aortic calcification. For animal experiments we utilized osteoprotegerin-deficient (OPG−/−) mice, which have been previously...
shown to develop a moderate degree of medial aortic calcification.\textsuperscript{9,21,22} We also examined plasma samples obtained from patients with peripheral artery disease in whom aortic calcification was estimated from computed tomographic angiography (CTA).\textsuperscript{9,21,24} Our aim in this study, however, was limited to assessing whether there was a correlation between the circulating concentrations of stem-cell-mobilizing cytokines and aortic calcification.

**Methods**

**Study Design**

We hypothesized a role for the stem-cell-mobilizing cytokines SDF-1\(\alpha\), G-CSF and SCF in the release of naïve osteoprogenitor cells from the bone marrow environment. Further, we also postulated that these circulating osteoprogenitors homed to diseased arteries under the influence of SDF-1\(\alpha\), which then contributes towards aortic calcification. To assess this, we investigated the circulating concentrations of SDF-1\(\alpha\) and G-CSF from serum samples obtained from tail bleeds of OPG\(^{-/-}\) mice. We also quantified SDF-1\(\alpha\), G-CSF and SCF in plasma samples obtained from patients with peripheral artery diseases.

**Mice**

We selected 52-week-old experimental OPG\(^{-/-}\) and control OPG\(^{+/+}\) male mice for the studies. Approval for the animal investigation was provided by the Ethics Committee of James Cook University (#A1099). OPG\(^{-/-}\) breeding pairs (on a C57BL/6 background) were originally obtained from Clea Laboratories, Japan. Age- and sex-matched OPG\(^{+/+}\) were used as controls for all the animal experiments. All mice were fed a normal chow diet throughout.

**Patients**

Approval for the human studies was obtained from the Townsville Hospital Ethics Committee (#H2196). The recruitment criteria and characteristics of the included patients are detailed in a previous publication.\textsuperscript{9} The recruited patients had symptoms of intermittent claudication, clinical evidence...
of lower limb ischemia and according to the treating physician required CTA to further assess their peripheral arteries. Patients were excluded if they had received previous open surgical or endovascular treatment of their infrarenal aorta or if CTA was considered to be contraindicated, as previously described. 24 Patients’ age, sex, risk factors (diabetes, hypertension, dyslipidemia, and smoking), comorbidities (coronary heart disease, stroke) and medications were recorded at entry using previously described definitions. 24 A total of 23 patients were recruited, of whom 17 (74%) were men. The median age of the patients was 75 years (interquartile range (IQR) 65–79), 19 (83%) were current smokers, 11 (48%) had diabetes, 17 (74%) had hypertension, 18 (78%) had dyslipidemia and 18 (78%) had coronary heart disease. Patients were categorized into 2 groups (high and low) based on whether their infrarenal aortic calcification volume was ≥ or < median (805 cm$^3$, IQR 242.5–1384.5) of the entire group. 9

**Mice Serum Samples**

Tail bleeds were collected from 52-week-old male mice into lithium heparin tubes (Microvette, USA); 200 μl of blood was collected per bleed. Blood was allowed to clot for 2h before centrifuging for 20 min at approximately 2,000 g. Serum samples were separated after centrifugation and aliquoted into 1.5-ml Eppendorf tubes and stored at −20°C until required. Repeated thawing and freezing of samples was avoided.

**Patient Plasma Samples**

Whole blood samples were collected in heparin-coated tubes (BD vacutainer, Becton Dickinson, USA), as previous described. 9 The samples were layered onto Ficoll histopaque solution and centrifuged at 400 g for 30 min. The resultant plasma layer was aliquoted and stored at −20°C for cytokine analysis. Repeated thawing and freezing of samples was avoided. For human studies we chose plasma samples since these were recommended by the manufacturer for the planned cytokine enzyme-linked immunosorbent assay (ELISA; R&D, Quantikine, USA).

**ELISA to Estimate Circulating Cytokine Concentrations**

Assays were carried out according to the manufacturer’s instructions (R&D). Standard and samples were assayed in duplicates. The mean coefficient of variance for intra- and inter-assay repeats for SDF-1α, G-CSF and SCF in both

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![Figure 2. Correlation between circulating stem cell cytokines and aortic calcification in OPG−/− male mice groups.](image-url)

Figure 2. Correlation between circulating stem cell cytokines and aortic calcification in OPG−/− male mice groups. (A) Scatter plot illustrating the correlation between circulating serum concentration of SDF-1α and extractable aortic calcium in OPG−/− mice [r=0.68, P=0.01]. (B) Scatter plot illustrating correlation between circulating serum concentration of G-CSF and extractable aortic calcium in OPG−/− mice [r=0.70, P=0.01]. G-CSF, granulocyte-colony stimulating factor; SDF-1α, stromal cell-derived factor α; OPG−/−, osteoprotegerin deficient.
human and mice samples were between 1% and 5%.

**Aortic Calcification Measurement**

**In Mice** Aortas were digested with lysis buffer (1 N hydrochloric acid) and centrifuged at 30,000 g for 30 min to extract calcium. The bioassay experiments were set up in a 96-well plate and carried out according to the manufacturer’s instructions. Calcium concentration was estimated by optical density assessment at 595 nm (Tecan plate reader, Qlab, Brisbane, QLD, Australia). The mean coefficient of variance for the intra- and interassay repeats for the calcium assays in mice samples was between 1% and 3%.

**In Patients** Infrarenal aortic calcification volumes were measured from CTA using workstation protocols we have previously demonstrated to be reproducible.23

**OCN+ MNC Quantification**

**In Mice** Blood samples were treated with ammonium chloride and potassium chloride (0.829 g NH4Cl, 0.1 g KHCO3 and 0.372 mg EDTA/100 ml) lysis buffer to eliminate red blood cells from the sample. After centrifugation the cell pellets were resuspended and incubated in blocking buffer (1:25, purified rat anti-mouse CD16/CD32, BD Biosciences, USA) for 20 min at room temperature (RT) to eliminate non-specific binding. The cells were washed, and incubated with primary OCN antibody (goat anti-mouse, Biomedical Technologies, BT 592, USA) or an isotype control (goat IgG, I-5000, Vector Antibodies, USA) at 10 μg/ml for 30 min at 4°C. The cells were labeled with phycoerythrin (PE)-conjugated secondary antibody (donkey anti-goat IgG, 1:75 dilutions, Jackson’s Immunoresearch, USA) and incubated for 30 min at 4°C. Washed cells were resuspended in flow cytometry buffer and analyzed on the BD FACS Calibur (Becton Dickinson).

**In Patients** An initial hemocytometer cell count was performed using trypan blue staining to determine the viable MNC count. The cell pellet was then incubated with a human blocking reagent (20 μl/107 cells, Miltenyi Biotech, USA) and 10% donkey serum for 20 min at RT to eliminate non-specific antibody binding. The MNC were incubated with
human OCN specific antibody (goat anti-OCN, Santa Cruz Biotechnology, USA, V-19, 1:100 dilution) for 30 min at 4°C followed by washing in phosphate-buffered saline containing 2 mmol/L EDTA (Ajax Fine Chem, Australia) and 0.5% chicken albumin (Sigma, St Louis, MO, USA) and centrifugation at 400 g for 10 min. PE-conjugated donkey anti-goat IgG (1:75 dilution, Jackson Immunoresearch, USA) was used as the secondary antibody. The cell suspension was incubated for 30 min at 4°C in the dark. The cells were further washed and incubated in anti-PE microbeads (Miltenyi Biotech, 20 μl/10^7 cells) for 20 min at 4°C. After further washing, the MNC were added to a magnetic separation column (Miltenyi Biotech, USA). The bound fraction was eluted and washed and centrifuged at 400 g for 10 min. Both positively and negatively selected cell fractions were resuspended in flow cytometry buffer and immediately analyzed on the BD FACSCalibur (Becton Dickinson).

**Statistical Analysis**

Data were analyzed using SPSS statistics software (16.0, SPSS Inc, Chicago, IL, USA). Data were assessed for normality using Q-Q plots and were not normally distributed. Non-parametric statistical tests were therefore used to assess our hypotheses, including the Mann-Whitney U test and Spearman correlations. Assay reproducibility was assessed using coefficient of variation. All the data are represented by medians and IQR.

**Results**

**OPG–/– Mice Have Elevated Circulating Serum Concentration of Stem-Cell-Mobilizing Cytokines**

The median serum SDF-1α concentration (Figure 1A) was significantly greater in OPG–/– (median 3.26 ng/ml, IQR 3.03–3.59, P=0.01, n=10) compared to OPG+/+ mice (median 1.65 ng/ml, IQR 1.44–1.72, n=10). Similarly, the median serum G-CSF concentration (Figure 1B) was significantly greater in OPG–/– (median 412.70 pg/ml, IQR 398.65–430.94, P=0.01, n=10) compared to OPG+/+ mice (median 236.22 pg/ml, IQR 228.20–260.10, n=10).
Association of SDF-1α and G-CSF With Aortic Calcification and Circulating OCN+ MNC in OPG−/− Mice Samples

Serum SDF-1α and G-CSF concentrations correlated with the amount of extractable aortic calcium in OPG−/− (Figure 2, SDF-1α: r=0.68, n=10, P=0.01; G-CSF: r=0.70, n=10, P=0.01) but not OPG+/+ mice (SDF-1α: r=0.45, n=10, P=0.21; G-CSF: r=0.43, n=10, P=0.30, not shown in Figure 2). Serum SDF-1α and G-CSF concentrations also correlated with circulating OCN+ MNC in experimental OPG−/− (SDF-1α: r=0.50, n=10, P=0.01; G-CSF: r=0.61, n=10, P=0.01, Figure S1), but not for control OPG+/+ mice (SDF-1α: r=0.38, n=10, P=0.21; G-CSF: r=0.42, n=10, P=0.15, not shown in Figure 2).

Patients With Severe Infrarenal Aortic Calcification Have Higher Plasma Concentration of Stem-Cell-Mobilizing Cytokines

Patients with more severe aortic calcification (calcification volume ≥ median ie, 805 cm³, median 60.50, IQR 50.63–84.25, n=11). The patients with more severe aortic calcification had higher circulating plasma concentration of G-CSF (Figure 3B, median 77.55 pg/ml, IQR 65.63–92.50, P=0.03, n=12) than those with less severe aortic calcification (median 50.75, IQR 41.25–68.75, n=11). Also, the patients with more severe aortic calcification had higher circulating plasma concentrations of SCF (Figure 3C, median 2,285 pg/ml, IQR 2,180–2,401, n=12, P=0.02) than those with less severe aortic calcification (median 1,813, IQR 1,703–1,944, n=11).

Association of SDF-1α, G-CSF and SCF With Infrarenal Aortic Calcification Volumes and Circulating OCN+ MNC in Patient Samples

Plasma concentrations of SDF-1α, G-CSF and SCF were significantly correlated with circulating OCN+ MNC. For SDF-1α and OCN+ MNC the Spearman correlation coefficient was 0.59 (Figure 4A, n=23, P=0.003) while that for G-CSF and SCF was 0.64 (Figure 4B, n=23, P=0.001) and 0.64 (Figure 4C, n=23, P=0.001), respectively.

Figure 5. Correlation between circulating stem cell cytokines and infrarenal aortic calcification volumes in patients with peripheral artery diseases. (A) Scatter plot illustrating the correlation between circulating plasma concentration of SDF-1α and infrarenal aortic calcification volumes in patients [r=0.48, P=0.01]. (B) Scatter plot illustrating the correlation between circulating plasma concentration of G-CSF and infrarenal aortic calcification volumes in patients [r=0.52, P=0.01]. (C) Scatter plot illustrating the correlation between circulating plasma concentration of SCF and infrarenal aortic calcification volumes in patients [r=0.48, P=0.03]. SDF-1α, stromal cell-derived factor α; G-CSF, granulocyte-colony stimulating factor; SCF, stem cell factor.
The Spearman correlation coefficient between the plasma SDF-1α concentration and infrarenal aortic calcification volume was found to be 0.48 (Figure 5A, n=23, P=0.01). Also, the correlation coefficient between the plasma G-CSF concentration and infrarenal aortic calcification volume was 0.52 (Figure 5B, n=23, P=0.01), while that for plasma SCF was found to be 0.48 (Figure 5C, n=23, P=0.03).

Discussion

The results of this study indicate, for the first time, a 3-way association between stem-cell-mobilizing cytokines, circulating osteoprogenitor percentages (OCN+ MNC) and aortic calcification. This investigation, together with our previous report, provides supportive evidence of an active circulating cell theory in the pathogenesis of vascular calcification.

We measured the circulating concentrations of SDF-1α, G-CSF and SCF in a mouse model and a human patient group. These cytokines have been previously reported to promote the release of naive cells from the bone marrow environment into the peripheral circulation. SDF-1α is also believed to facilitate the recruitment of undifferentiated circulating cells to diseased arteries, and has also been previously reported to be associated with circulation endothelial progenitor cells. Our current study demonstrated that the circulating concentrations of these cytokines were markedly higher in OPG−/− mice compared to controls. The higher cytokine concentrations may be important in stimulating osteoprogenitor release from the bone marrow and thus susceptibility to vascular calcification.

Plasma concentrations of SDF-1α and G-CSF were approximately 2–3-fold higher in patients with aortic calcification than those reported in healthy subjects (normal G-CSF concentrations: 27 pg/ml; normal SDF-1α concentrations: 34 ng/ml). Plasma SCF levels were only slightly higher than reported normal values (2,100 pg/ml). A positive association between the concentrations of these cytokines, aortic calcification volumes and OCN+ MNC suggests a role for circulating osteoprogenitors in arterial mineralization in patients. The results obtained from the patient samples were in line with the findings obtained from our OPG−/− mouse model investigation. Our previous studies also noted a direct correlation between the circulating osteoprogenitor population and the severity of aortic calcification in the same mouse model (r=0.52, P=0.02, n=10) and patient group (r=0.47, P=0.02, n=23) investigated in this present study. These cumulative results suggest a role for stem-cell-mobilizing cytokines in stimulating the release of the immature progenitor cells from the bone marrow environment. It is suggested that after the release of OCN+ MNC into the peripheral circulation, chemotaxants such as SDF-1α facilitate cell homing and survival in diseased lesions. Within diseased arteries these osteoprogenitor cells may differentiate into osteoblasts and contribute to vessel wall mineralization. This conclusion is supported by our previous finding of an increased OCN+ population in the calcified aorta. This increase in the aortic deposition of OCN+ cells could be a result of SDF-1α activity within the vasculature.

Despite our consistent data there are a number of limitations in concluding a definite role for these stem cell cytokines. Firstly, both mouse models used were based on OPG deficiency. These OPG−/− mice are reported to undergo osteoporosis together with vascular calcification. Thus it is possible that the increase in OCN+ MNC was related to bone loss occurring in osteoporosis rather than to aortic calcification in these animals. The finding that the OCN+ MNC increase in response to the vascular-calcification-promoting agent calcitriol and their association with aortic calcification in humans do, however, suggest that the osteoprogenitor cells are important in the calcification process. Also, the human group investigated in this study was small. A larger sample would be ideal to confirm the association between circulating osteoprogenitors and aortic calcification. However, this could not be achieved because of the complicated recruitment criteria in which patients were excluded from the study if they had received previous open surgical or endovascular treatment of their infrarenal aorta. The unavailability of a healthy control group for comparative studies was also considered to be a limitation in our studies.

Conclusions

This investigation of the association of stem-cell-mobilizing cytokines, circulating bone marrow-derived osteoprogenitors and vascular calcification, overall, suggests that bone-derived cells may play a role in arterial wall mineralization. The results obtained from these studies, however, are preliminary in relation to the “circulation cell theory” and are only limited to the assessment of correlative association between the cytokines and the severity of vascular calcification. Further studies are required to assess this theory of aortic calcification. Evaluation of labeled bone marrow cells or injected progenitor cells would provide further assessment. Another way of determining the role of progenitor cells would be to carry out experiments designed to block the effect of stem-cell-mobilizing cytokines and study its effect on the severity of vascular calcification.

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References

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**Supplemental Files**

**Figure S1.** Scatter plot illustrating correlation between circulating serum concentration of SDF-1α (A) and G-CSF (B) with circulating OCN+ MNC percentage in OPG–/– mice.

**Figure S2.** Examples of flow cytometry dot plots comparing the percentage of circulating OCN+ MNC in 12-month-old OPG–/– (A) and OPG++ control (B) mice.

Please find supplemental file(s); http://dx.doi.org/10.1253/circj.CJ-10-1056