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Osteocalcin positive mononuclear cells are associated with the severity of aortic calcification

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Abstract

Objective—To assess the association of circulating bone marrow-derived osteo-progenitors with vascular calcification in mouse models and patients with peripheral artery disease.

Methods—We estimated the percentage of circulating mononuclear cells expressing osteocalcin in 2 mouse models of aortic calcification developed in osteoprotegerin-deficient mice (OPG^{-/-}) using flow cytometry. Aortic calcification was assessed in mice principally by a bioassay of harvested aortas. In patients with peripheral artery disease osteocalcin- positive cells (estimated by flow cytometry) were related to aortic calcification volume assessed from computed tomography.

Results—The amount of extractable aortic calcium was increased in both mouse models used in comparison to controls. The percentage of circulating mononuclear cells expressing osteocalcin was correlated to the amount of extractable aortic calcium in male ($r=0.525$, $p=0.02$) and female OPG^{-/-} ($r=0.564$, $p=0.02$) mice and also in animals in which calcification was accelerated using calcitriol ($r=0.64$, $p=0.01$). Patients with more severe aortic calcification had a greater percentage of circulating OCN⁺ MNCs (median 4.07 %, IQR 3.76–4.39, $n=12$) than those with less severe aortic calcification (median 3.10 %, IQR 2.32–3.60, $n=11$, $p=0.05$).

Conclusions—This study demonstrates that aortic calcification can be robustly quantified in 2 mouse models. In these models and patients with peripheral artery disease circulating osteocalcin positive mononuclear cells are associated with the severity of aortic calcification.

Keywords

Vascular calcification; aorta; osteo-progenitors

1. Introduction

There is controversy regarding the mechanisms of vascular calcification with current theories including passive models and active models based on transformation of vascular smooth muscle cells (VSMCs) to cells capable of mineralisation [1,2]. Recently a further mechanism of vascular calcification has been suggested based on circulating bone marrow-derived progenitors [2,3]. Mouse model and human studies have suggested that bone marrow-derived

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progenitor cells may contribute to a variety of vascular pathologies including calcification, atherosclerosis and intimal hyperplasia [4].

Bone marrow contains both osteoblast and osteoclast precursors which are often termed osteo-progenitors and are associated with bone remodelling [5]. The presence of these osteo-progenitors within the circulation has recently been shown using bone markers identified by flow cytometry [6]. Mononuclear cells (MNCs) expressing osteocalcin (OCN) have been shown to form calcified nodules when plated *in vitro* with osteoblast differentiating medium [6]. Furthermore, bone formation was observed in mouse studies in which purified OCN⁺ MNCs were transplanted into mice [6]. These studies provided evidence for the osteogenic potential of OCN⁺ MNCs. Based on these studies we hypothesized that circulating OCN⁺ MNCs contributed to vascular calcification.

The aim of this study was to examine if there was an association between the numbers of circulating bone marker-positive MNCs and aortic calcification in mouse models and patients. We utilised osteoprotegerin-deficient mice (OPG^{-/-}) [7] which have previously been shown to develop a moderate degree of medial aortic calcification [8]. In some experiments accelerated vascular calcification was induced injecting calcitriol, an active form of vitamin D [9]. We also examined patients with peripheral artery disease in whom aortic calcification was estimated from computed tomographic angiogram (CTA).

2. Methods

2.1. Study design

We hypothesised that bone marker-positive MNCs are released from the bone marrow into peripheral circulation. These cells may home to the diseased artery, differentiate into an osteogenic lineage, proliferate in the vascular lesions and thus contribute to vessel mineralisation. To further assess this hypothesis we had the following aims: a) to assess if OPG^{-/-} mice have more marked aortic calcification than wild type OPG^{+/+} controls. b) To assess the association between OCN⁺ MNCs and aortic calcification in mouse models of vascular calcification and patients with peripheral artery disease.

2.2 Mice

Approval for the animal studies 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, and crossed to obtain colonies of OPG^{-/-} and OPG^{+/+} mice [10]. Age and sex-matched OPG^{+/+} were used as controls for all the animal experiments. We designed three studies in mouse models to investigate the relationship between OPG, aortic calcification and circulating osteo-progenitor cells. In study 1 we quantified aortic calcification in old OPG^{-/-} and OPG^{+/+} mice using two different methods, alizarin red-staining and total extractable calcium assays. In study 2 we estimated the number of circulating OCN⁺ MNCs in aged OPG^{-/-} and OPG^{+/+} mice using flow cytometry in order to assess the relationship between these cells and the severity of aortic calcification. In study 3 we investigated the percentage of OCN⁺ cells present in the aortas of OPG^{-/-} mice by enzymatic extraction of the aortic cells and compared them with OPG^{+/+} controls. In study 4 we induced arterial calcification in 14 week old OPG^{-/-} and OPG^{+/+} mice by calcitriol injection and assessed the relationship between aortic calcification and circulating OCN⁺ MNCs. The protocol for these studies is summarised in table 1. All mice were fed a normal chow diet throughout.

2.3 Alizarin red-staining (study 1)

For alizarin red-staining of aortas we compared harvested aortas from 12 month old OPG^{-/-} (n=7) and age matched OPG^{+/+} male mice (n=7) (study 1, table 1). Please see supplementary methods for further details (available on line at www.elsevier.com).

2.4. Calcium bioassay (studies 1, 2 and 4)

Aortic calcification was estimated using a bioassay (QuantichromTM) based on the reaction of phenolsulphonaphthalein with free calcium to produce a stable blue coloured complex. Please see supplementary methods for further details (available on line at www.elsevier.com).

2.5. Flow cytometry of mouse peripheral blood (studies 2 and 4)

For study 2 early morning blood samples (Lithium Heparin, Microvette, USA) were obtained from 40 mice (10 males and females of both OPG^{-/-} and OPG^{+/+} mice) via tail bleeds at 50, 51, 52 and 53 weeks of age (table 1). At 54 weeks of age all 40 mice were euthanized, aortas harvested and stored at -20°C for extraction and quantification of aortic calcium. Please see supplementary methods for further details (available on line at www.elsevier.com).

2.6. Enzymatic digestion of murine aortas and isolation of OCN⁺ cells (study 3)

For this investigation, harvested aortas from 12 month old male experimental OPG^{-/-} (n=16) and age matched control OPG^{+/+} (n=16) were enzymatic digested to obtain aortic immune cells. Please see supplementary methods for further details (available on line at www.elsevier.com). The labelled cells were resuspended in flow cytometer buffer and analysed on the BD FACSCalibur (Becton Dickinson, USA), similar to the analysis used in study 2.

2.7. Calcitriol treatment (study 4)

98 day old male OPG^{-/-} and OPG^{+/+} mice were given daily subcutaneous injections of 5 µg/kg body weight calcitriol (Tocris biosciences, USA) for 3 consecutive days (n=12 per group), as previously described. Peripheral blood was collected via tail bleeds 28 days prior (i.e. 70 days of age) and 1, 2 and 7 days after commencement of the first calcitriol injection, i.e. 99, 100 and 105 days of age for assessment of OCN⁺ MNCs (figure 1) as described above. Nine days after the first calcitriol injection (107 days of age) mice were euthanized and aortas harvested for calcium quantification.

2.8. Patients

Approval for the human studies was obtained from The Townsville hospital ethics committee. Recruited patients had symptoms of intermittent claudication, clinical evidence of lower limb ischemia and according to the treating physician required a 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 a CTA was felt to be contra-indicated, as previously described [11]. Thus no patients with abnormal serum creatinine were included. For selected patients, CTA images were analysed, as previously described, to quantify infrarenal aortic calcification volumes [11,12]. The reproducibility of the technique of measuring calcification volumes has been previously assessed, and found to have an intra and inter-observer coefficient of variation of approximately 1%. Patients' age, gender, risk factors (diabetes, hypertension, dyslipidaemia, and smoking), co-morbidities (coronary heart disease, stroke) and medications were recorded at entry using previously described definitions [11].

2.9. Isolation of MNCs from patient peripheral blood

At recruitment, an early morning blood sample (20 mL into heparin coated tubes, BD vacutainer, Becton Dickinson, USA) was obtained following an overnight fast. MNCs were

isolated from patients' blood using ficoll-hypaque density gradients (Sigma, USA). Please see supplementary methods for further details (available on line at www.elsevier.com).

2.10. Flow cytometry analysis

Please see supplementary methods for further details (available on line at www.elsevier.com).

2.11. Statistical analysis

Data was analysed using SPSS statistics software (15.0, SPSS Inc USA). Data was assessed for normality using Q-Q plots and was not normally distributed. Non-parametric statistical tests were therefore used to assess our hypotheses, including Mann-Whitney U test, paired Wilcoxon test and Spearman correlations. Assay reproducibility was assessed using coefficient of variation. All the data is represented in medians and inter-quartile ranges (IQR).

3. Results

3.1. Aortic calcification is increased in aged OPG^{-/-} mice

Alizarin red-staining area was greater in the aortic arch, thoracic and suprarenal aorta of OPG^{-/-} compared to OPG^{+/+} mice. Infrarenal alizarin red-staining area was similar in OPG^{-/-} and OPG^{+/+} mice (table 2 and supplementary figure 1). The amount of calcium extractable from the aortas of OPG^{-/-} was significantly greater than that from control mice (table 2).

3.2. OPG^{-/-} mice have increased circulating numbers of OCN⁺ MNC

The percentage of circulating MNCs expressing OCN was found to be greater in 12 month old male and female OPG^{-/-} mice compared to age and sex-matched OPG^{+/+} mice (table 3 and figure 2). In OPG^{-/-} mice the percentage of circulating OCN⁺ MNCs correlated with the amount of calcium extractable from the aorta at necropsy ($r=0.525$, $p=0.02$, and 0.564 , $p=0.02$, for male and female mice, respectively).

3.3. Circulating OCN⁺ MNCs and calcification in an accelerated model of aortic calcification

Calcitriol has been previously demonstrated to accelerate aortic calcification in OPG^{-/-} mice [9]. We investigated the effect of calcitriol injection on circulating OCN⁺ MNCs and aortic calcification in 14 week old OPG^{-/-} male mice. We chose young mice and a short study period because calcitriol induces early mortality, as previously described⁹. Two of the 12 OPG^{-/-} mice (17%) died seven days after the first calcitriol injection. Four of the 12 OPG^{-/-} mice (33%) developed spinal deformities. One of the 12 control OPG^{+/+} mice (12%) died seven days after the first calcitriol injection. No spinal deformities were noted in the control mice. There was no significant difference in the percentage of circulating OCN⁺ MNCs in OPG^{-/-} and OPG^{+/+} mice prior to calcitriol injection. It should be noted that these mice were much younger than those included in study 2 (i.e. 70 compared to ~360 days of age). The percentage of circulating OCN⁺ MNCs increased significantly in response to calcitriol in OPG^{-/-} but not OPG^{+/+} mice (figure 3). The median percentage of circulating MNCs expressing OCN in OPG^{-/-} mice increased significantly from 0.75 % (IQR 0.51–0.85) before receiving the calcitriol injections (70 days of age) to 1.3% (IQR 0.86–1.4, $p=0.01$) seven days after the first calcitriol injection (i.e. at 105 days of age). This represents an increase in the circulating numbers of OCN⁺ MNCs from 3.1×10^3 (IQR $1.6\text{--}3.3 \times 10^3$) to 8×10^3 (IQR $6\text{--}8.3 \times 10^3$) cells/ml after calcitriol injection, $p=0.01$. No significant changes were demonstrated in circulating OCN⁺ MNCs numbers in OPG^{+/+} mice following calcitriol injections (figure 3).

The amount of calcium extractable from the aortas of OPG^{-/-} mice (median 0.63 mg, IQR 0.49–0.71) was significantly greater than that from OPG^{+/+} mice (median 0.28 mg, IQR 0.19–0.35, $p=0.01$) 9 days after the first calcitriol injection i.e. 106 days of age. The amount of extractable calcium correlated with the percentage of circulating OCN⁺ MNCs measured 7 days after the first calcitriol injection (105 days of age, $r=0.64$, Figure 4b) but not that measured prior to calcitriol injection (70 days of age, $r=0.31$, Figure 4a) in OPG^{-/-} mice.

The total amount of calcium extractable from the aortas of 15 week old OPG^{-/-} male mice which had received calcitriol (study 4) was significantly greater (median 0.63 mg, IQR 0.49–0.71) than we had identified in our earlier assessment of 12 month old OPG^{-/-} mice which had not received calcitriol (study 2, table 3).

3.4. There are increased numbers of OCN⁺ cells in the aortas of calcification prone OPG^{-/-} mice

The percentage of OCN⁺ viable cells identified in extracts from the aortas of OPG^{-/-} mice was significantly greater than in extracts from the aortas of control mice, $p=0.0001$ (figure 5).

3.5. Association of OCN⁺ MNCs with aortic calcification in patients with peripheral artery disease

A total of 23 patients were recruited, of which 17 (74%) were men. The median age of the patients was 75 years (IQR 65–79), 19 (83%) were current smokers, 11 (48%) had diabetes, 17 (74%) hypertension, 18 (78%) dyslipidemia and 18 (78%) coronary heart disease. Patients with more severe aortic calcification (calcification volume $\geq$ median i.e. 805cm³, IQR 242.5–1384.5) had a greater percentage of circulating OCN⁺ MNCs (median 4.07 %, IQR 3.76–4.39, $n=12$) than those with less severe aortic calcification (median 3.10 %, IQR 2.32–3.60, $n=11$, $p=0.05$, figure 6). Overall, aortic calcification volumes were moderately correlated with circulating OCN⁺ MNCs percentage ($r=0.475$, $p=0.22$).

4. Discussion

This study indicates, for the first time, an association between circulating MNCs expressing OCN and aortic calcification in two mouse models and patients with peripheral artery disease. We believe this is supportive evidence that bone marrow derived cells play a role in vascular calcification. It has previously been demonstrated that human OCN⁺ MNCs are capable of carrying out mineralisation both *in vitro* and when injected into mice [6,13].

Osteoclast and osteoblast-like cells have also been demonstrated within areas of vascular calcification in both human and mice aortas [14]. We have similarly demonstrated the presence of OCN⁺ cells within the aorta of mice with vascular calcification in our study. Currently there is controversy regarding the contribution of bone marrow to vascular disease. While a number of studies appear to support a role of bone marrow derived progenitors in the development of intimal hyperplasia, the role of similar cells in atherosclerosis is less clear [15,16]. Recent studies in ApoE^{-/-} mice for example suggests that local vessel wall progenitors are more important in giving rise to VSMCs in intimal atherosclerotic lesions than those derived from the bone marrow [17,18]. The process involved in vascular calcification may be distinct to those involved in atherosclerosis. The close correlation between bone pathology and vascular calcification supports data presented in this study linking bone marrow-derived cells in this specific arterial problem [5,6,19–22].

In a recent study Gossel and colleagues identified a higher percentage of OCN positive MNCs in patients with coronary atherosclerosis compared to control subjects [23]. These findings are

in line with our association between calcification and circulating concentrations of this cell population.

Despite our consistent data that OCN⁺ MNCs were associated with aortic calcification in two mouse models and patients there are a number of limitations in concluding the definite role of these cells in vascular calcification. Firstly, both mouse models employed were based on OPG deficiency. Thus it is possible that the increased OCN⁺ MNCs was related to this rather than the aortic calcification in these animals. This seems unlikely given the degree of correlation found and the fact that circulating numbers of cells were related to the instigating factors in our calcitriol experiment. Thus circulating OCN⁺ MNCs numbers measured only after calcitriol injections were associated with calcification severity. Furthermore our mice data is supported by human evidence of any association between the circulating number of OCN⁺ MNCs and aortic calcification. We chose mice models without atherosclerosis in order to directly assess the association between vascular calcification and osteo-progenitor cells. Further studies in pro-atherosclerotic mice models would be interesting but were beyond the scope of the current investigation.

5. Conclusion

Our investigation on the role of circulating bone marrow-derived osteo-progenitors in the pathogenesis of vascular calcification, overall, is suggestive and not entirely conclusive. Our study demonstrated a possible association between cells previously identified as osteogenic progenitors and aortic calcification. Further work, however, is required to confirm the circulatory path of these osteo-progenitors and their final lineage within the vessel wall.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Outline of the timing of calcitriol injections and tail bleeds in study 4. (OCN: osteocalcin; OPG^{-/-}: osteoprotegerin-deficient; OPG^{+/+}: osteoprotegerin- present)

Figure 2a

Figure 2b

Figure 2.

Examples of flow cytometry dot plots comparing the percentage of circulating OCN⁺ MNCs in 12 month old OPG^{-/-} (a) and OPG^{+/+} control (b) male mice. (OCN: osteocalcin; MNC: mononuclear cells; OPG^{-/-}: osteoprotegerin-deficient; OPG^{+/+}: osteoprotegerin-present)

Figure 3.

Circulating percentage of OCN⁺ MNC in OPG^{-/-} and control OPG^{+/+} male mice receiving calcitriol injections from 98 days of age.

Shown are box plots illustrating the median and inter-quartile range of OCN⁺ MNCs in blood obtained at 98, 99, 100 and 105 days of age from OPG^{-/-} and OPG^{+/+} mice (n=12 per group) in relation to calcitriol injections. (OCN⁺: osteocalcin positive; MNC: mononuclear cells; OPG^{-/-}: osteoprotegerin-deficient; OPG^{+/+}: osteoprotegerin-present; %: percentage)

Before calcitriol injections

Figure 4a

After 3 calcitriol injections

Figure 4b

Figure 4.

Relationship between extractable aortic calcium and the percentage of circulating OCN⁺ MNCs before and after calcitriol injections in OPG^{-/-} and control OPG^{+/+} mice. ▲ = OPG^{-/-}; □ = OPG^{+/+}

a) In blood samples obtained 28 days prior to the first calcitriol injection (age 70 days) the percentage of OCN⁺ MNCs was unrelated to aortic calcium in either OPG^{-/-} (n=12, ▲, r=0.31, p=0.27) or OPG^{+/+} (n=12, □, r=0.231, p=0.91). b) Seven days after the first calcitriol injection (age 105 days) the percentage of OCN⁺ MNCs was correlated with aortic calcium estimates in OPG^{-/-} (n=12, ▲, r=0.64, p=0.01) but not OPG^{+/+} mice (n=12, □, r= 0.46, p=0.73).

(OCN⁺: osteocalcin positive; MNC: mononuclear cells; OPG^{-/-}: osteoprotegerin-deficient; OPG^{+/+}: osteoprotegerin-present; %: percentage; mg: milligrams)

Figure 5.

Percentages of extractable OCN⁺ viable cells in extracts from aortas of OPG^{-/-} and control male mice. Shown are box plots representing medians and inter-quartile range. The median percentage of OCN⁺ cells were significantly increased in experimental male OPG^{-/-} mice (2.23 %, IQR 1.9–2.52, n=16) when compared to control OPG^{+/+} mice (0.66 %, IQR 0.59–0.66, n=16, p=0.0001) (OCN⁺: osteocalcin positive; OPG^{-/-}: osteoprotegerin-deficient; OPG^{+/+}: osteoprotegerin-present; %: percentage, IQR: interquartile range)

Figure 6.

Comparison of the percentage of OCN⁺ MNCs in peripheral blood of patients with low and high infrarenal aortic calcification volumes. The infra renal aortic calcium volumes were measured from CTA and patients were categorised into those with calcification $\geq$ or $<$ median (805 cm³). The median number of OCN⁺ MNCs in patients with infrarenal aortic calcification volumes $\geq$ or $<$ median were 4.07 % (IQR 3.76–4.39, n=12) and 3.10 % (IQR 2.32–3.60, n=11), respectively, p=0.05. (OCN⁺: osteocalcin positive; MNC: mononuclear cells; CTA: computed tomographic angiogram; IQR: interquartile range; %: percentage)

Summary of mice studies

Table 1

Study	Number of mice per group	Gender	Age of mice at start of study (in weeks)	Study mice	Controls	Age of mice during tail bleeds (in weeks)	Age of mice at aortic harvesting (in weeks)
1: Calcium staining	7	Males	54	OPG ^{-/-}	OPG ^{+/+}	-	54
1: Calcium bioassay	7	Males	54	OPG ^{-/-}	OPG ^{+/+}	-	54
2: Circulating OCN ⁺ MNC quantification	10	Males	50	OPG ^{-/-}	OPG ^{+/+}	50,51,52,53	54
	10	Females	50	OPG ^{-/-}	OPG ^{+/+}	50,51,52,53	54
3: Aortic OCN ⁺ MNC quantification	16	Males	54	OPG ^{-/-}	OPG ^{+/+}	-	54
4: Accelerated model of aortic calcification	12	Males	8	OPG ^{-/-}	OPG ^{+/+}	10,14	15

OPG^{-/-}: osteoprotegerin deficient; OPG^{+/+}: osteoprotegerin present; OCN⁺: osteocalcin positive; MNC: mononuclear cells

Table 2
Comparison of aortic calcification estimates in 12 month old, male OPG^{-/-} and OPG^{+/+} mice.

Assessment	OPG ^{-/-}		OPG ^{+/+}		P value
	Median	IQR	Median	IQR	
Alizarin red staining area (%)					
Whole aorta	0.89	0.81–1.0	0.46	0.41–0.58	0.001
Aortic arch	1.40	1.23–1.54	0.8	0.78–0.88	0.001
Thoracic	0.88	0.81–0.9	0.5	0.4–0.58	0.001
Suprarenal	0.73	0.65–0.8	0.4	0.35–0.48	0.001
Infrarenal	0.5	0.34–0.56	0.4	0.31–0.47	0.165
Extractable calcium (mg)	0.49	0.38–0.6	0.18	0.1–0.26	0.001

Staining area and calcium amounts were compared by Mann Whitney U test.
(OPG^{-/-}: osteoprotegerin deficient; OPG^{+/+}: osteoprotegerin present; IQR: inter quartile range; %: Percentage; Mg: milligram)

Table 3
Comparison of circulating OCN⁺ MNCs and extractable aortic calcium in 12 month OPG^{-/-} and OPG^{+/+} mice.

Gender	Assessment	OPG ^{-/-}		OPG ^{+/+}		P value
		Median	IQR	Median	IQR	
Males	OCN ⁺ MNCs (%)	2.23	2.11–2.37	1.46	1.2–1.51	0.02
	Extractable calcium (mg)	0.41	0.39–0.51	0.18	0.1–0.28	0.02
Female	OCN ⁺ MNCs (%)	2.14	1.35–2.34	0.98	0.57–1.23	0.01
	Extractable calcium (mg)	0.30	0.19–0.4	0.15	0.08–0.23	0.02

The percentage of circulating OCN⁺ MNCs were estimated by calculated the median of results from blood collected at 50, 51, 52 and 53 weeks of age from each group and gender (n=10 each) of mice. Statistical comparison was by Mann Whitney U test. Aortas were harvested at 54 weeks for extractable calcium quantification. (OCN⁺: osteocalcin positive; MNC: mononuclear cells; OPG^{-/-}: osteoprotegerin deficient; OPG^{+/+}: osteoprotegerin present; %: percentage; IQR: interquartile range; Mg: milligram