Eur J Vasc Endovasc Surg (2016) 51, 259–267

Bone Like Arterial Calcification in Femoral Atherosclerotic Lesions: Prevalence and Role of Osteoprotegerin and Pericytes

J.-M. Davaine [a,b,c,h], T. Quillard [a,h], M. Chatelais [a,d], F. Guilbaud [a,d,e], R. Brion [a,d,e], B. Guyomarch [e,f], M.Á. Brennan [a], D. Heymann [a,d,e], M.-F. Heymann [a,g,], Y. Gouëffic [e,f,*]**

[a] INSERM, UMR 957, Nantes F-44035, France
[b] Service de Chirurgie Vasculaire, Centre Hospitalier René-Dubos, Pontoise, France
[c] Service de Chirurgie Vasculaire, CHU Pitié-Salpêtrière, Paris, France
[d] Université de Nantes, Nantes Atlantique Universités, Nantes F-44035, France
[e] Centre Hospitalier Universitaire, Nantes, France
[f] Institut du Thorax, Nantes, France
[g] Department of Medical Oncology, University of Sheffield, Sheffield, UK

WHAT THIS PAPER ADDS

Arterial calcification is very common in arterial lesions and has a major clinical impact. Though recognized as being a highly regulated process, most knowledge is based on studies at the coronary or carotid levels. This work provides detailed analysis of the presence of bone like arterial calcification—known as osteoid metaplasia (OM)—at the femoral level and suggest a high prevalence of OM in femoral atherosclerotic lesions. It further suggests that vascular pericytes and the osteoprotegerin/receptor activator for the nuclear factor kappa B ligand (RANKL)/RANK axis are implicated in the regulation of arterial calcification.

Objective/Background: Arterial calcification, a process that mimics bone formation, is an independent risk factor of cardiovascular morbidity and mortality, and has a significant impact on surgical and endovascular procedures and outcomes. Research efforts have focused mainly on the coronary arteries, while data regarding the femoral territory remain scarce.

Methods: Femoral endarterectomy specimens, clinical data, and plasma from a cohort of patients were collected prospectively. Histological analysis was performed to characterize the cellular populations present in the atherosclerotic lesions, and that were potentially involved in the formation of bone like arterial calcification known as osteoid metaplasia (OM). Enzyme linked immunosorbent assays and cell culture assays were conducted in order to understand the cellular and molecular mechanisms underlying the formation of OM in the lesions.

Results: Twenty-eight of the 43 femoral plaques (65%) displayed OM. OM included osteoblast and osteoclast like cells, but very few of the latter exhibited the functional ability to resorb mineral tissue. As in bone, osteoprotegerin (OPG) was significantly associated with the presence of OM ($p = .04$). Likewise, a high plasma OPG/receptor activator for the nuclear factor kappa B ligand (RANKL) ratio was significantly associated with the presence of OM ($p = .03$). At the cellular level, there was a greater presence of pericytes in OM+ compared with OM− lesions (5.59 ± 1.09 vs. 2.42 ± 0.58, percentage of area staining [region of interest]; $p = .04$); *in vitro,* pericytes were able to inhibit the osteoblastic differentiation of human mesenchymal stem cells, suggesting that they are involved in regulating arterial calcification.

Conclusion: These results suggest that bone like arterial calcification (OM) is highly prevalent at femoral level. Pericyte cells and the OPG/RANK/RANKL triad seem to be critical to the formation of this ectopic osteoid tissue and represent interesting potential therapeutic targets to reduce the clinical impact of arterial calcification.

Article history: Received 12 May 2015, Accepted 5 October 2015, Available online 30 November 2015

Keywords: Atherosclerosis, Femoral artery, Osteoprotegerin, Peripheral arterial disease, Vascular calcification, Vascular pericytes

INTRODUCTION

The prevalence of arterial calcification in atherosclerotic lesions has been reported to be as high as 90% and is identified as an independent risk factor for cardiovascular mortality.[1,2] Arterial calcification also has an impact on

[h] J.-M.D. and T.Q. contributed equally to this work.
* Corresponding author. Centre Hospitalier Universitaire, Nantes, France.
** Corresponding author. INSERM, UMR 957, Nantes F-44035, France.
E-mail addresses: mariefrancoise.heymann@sheffield.ac.uk (M.-F. Heymann); yann.goueffic@chu-nantes.fr (Y. Gouëffic).

http://dx.doi.org/10.1016/j.ejvs.2015.10.004

plaque stability.[3] Microcalcifications in the fibrous cap influence plaque stability and increase the risk of rupture.[4] Conversely, lesions with a high calcic burden are more stable.[5,6] During open surgery, arterial calcification results in difficulty in clamping vessels and in performing arterial anastomoses. Also, arterial calcification influences the technical and clinical success of endovascular repair.[7,8] Identifying the mechanisms underlying this process is thus a priority.

The femoral territory is unique in terms of both its anatomy and its pathology, and is very challenging for endovascular procedures when highly calcified, but little is known about the calcification process at the femoral level.

Arterial calcification results from a highly regulated process sharing many similarities with bone formation.[9] Bone like tissue, characterized by the presence of osteoid matrix, osteoblast and osteoclast like cells, and the major bone regulatory cytokines, has been observed in atheromatous lesions.[6] Smooth muscle cells (SMC) are the most frequently studied,[10] but macrophages and pericytes recently emerged as key players in this process.[6,11,12] In particular, pericytes are mesenchymal precursors directly involved in the development of arterial calcification.[6,12] The osteoprotegerin (OPG)/receptor activator for the nuclear factor kappa B (NF-κB) ligand (RANKL)/receptor activator for NF-κB (RANK) triad, which regulates bone remodeling, is also involved in the formation of arterial calcification.[13] OPG is associated with calcification of the coronary arteries, cardiovascular mortality, and peripheral arterial disease (PAD).[14] It was recently reported that the presence of osteoid tissue, known as osteoid metaplasia (OM), in carotid lesions was associated with the asymptomatic nature of the plaques. These results also support the role of pericytes and OPG in this process.[6]

The aim of this study was to provide detailed characteristics of osteoid tissue at the femoral level and to further assess the involvement of pericytes and the OPG/RANK/RANKL triad in this process.

METHODS

Patients, biological samples, and imaging data

Atheromatous plaques were harvested from 43 patients undergoing endarterectomy of the common femoral artery and of its bifurcation between February 2008 and June 2009. Clinical presentation was reported according to the Rutherford classification of PAD.[15] All patients participating in the study provided written informed consent. The clinical research protocol was approved by the institutional medical ethics committee of Nantes University Hospital. When a computed tomography (CT) scan was available, the importance of calcification of the lesions was assessed separately by two authors (J.M.D. and Y.G.) and categorized as non-calcified, mildly calcified, moderately calcified or highly calcified. Where there was disagreement, the analysis of a third vascular surgeon was requested. Surgical endarterectomy was performed together with best medical treatment.

OPG and RANKL measurements

For each patient a blood sample was collected on the day prior to surgery, centrifuged (5 minutes at 1,465 *g*), and plasma was then aliquoted and stored at −80 °C for subsequent analysis.

OPG and RANKL levels were measured in plasma by enzyme linked immunosorbent assay (DY805 ELISA kit; R&D Systems, Minneapolis, MN, USA). Osteoblastic pericyte conditioned medium was obtained as follows: the pericytes were cultured for 16—18 days in an osteoblastic inducing medium. The cells were then washed and left in starvation medium for 48 hours before the collection of the cell supernatants. The experiments were carried out in duplicate and repeated three times.

Histological and immunological analyses

The atherosclerotic plaques were fixed in 10% formalin overnight, decalcified in Sakura TDE 30 fluid for 24 hours, and embedded in paraffin. Ten serial sections from the middle of the atherosclerotic lesion were processed. These sections were stained with (i) hematoxylin and eosin, (ii) Masson's trichrome, and (iii) tartrate resistant acid phosphatase (TRAP). Immunohistochemistry (IHC) was carried out to localize and semi-quantify endothelial cells with CD31 antibody (Dako, Glostrup, Denmark); pericytes and endothelium with CD146 antibody (Abcam, Cambridge, UK); pericytes with NG2 antibody (Millipore, Billerica, MA, USA); smooth muscle actin (SMA), a marker for SMC with SMA antibody (R&D Systems); macrophages with CD68 antibody (Immunotech, Marseille, France); and OPG (R&D Systems). TRAP staining was performed by incubating sections in 1 mg/mL naphthol AS—TR phosphate, 60 mM NN dimethylformamide, and 1 mg/mL fast red salt solution (pH 5.2; Sigma—Aldrich, St. Louis, MO, USA) for 1 hour.

For each IHC staining, a negative control was performed using a similar procedure excluding the primary antibody. Plaques were analyzed for the presence of calcification, categorized as sheet like, nodular, and clear center calcifications, and osteoid metaplasia. The prevalence of each type of arterial calcification of this cohort of femoral lesions has been described in detail previously.[16]

Images of the whole sections were obtained with the NanoZoomer digital slide scanner (Hamamatsu Photonics, Hamamatsu, Japan). For each patient, the whole slide was scanned and made available for analysis. As a result, the whole section was quantified using a dedicated Image J (National Institutes of Health, Bethesda, MD, USA) macro applied systematically to all samples (selection parameters were applied to all samples for each staining). Numbers were expressed as a percentage of the stained area over the area of the plaque for each patient, and final results are the mean of the whole cohort.

Cell culture and differentiation assays

Reagents were obtained from Sigma—Aldrich unless otherwise stated. Human pericytes (C12980; PromoCell, Heidelberg, Germany) were placed for 18 days in an