Role of the OPG/RANK/RANKL triad in calcifications of the atheromatous plaques: comparison between carotid and femoral beds

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**Summary**

Recent works demonstrated the difference of calcification genesis between carotid and femoral plaques, femoral plaques being more calcified. It has been clearly demonstrated that the molecular triad Osteoprotegerin (OPG)/Receptor Activator of NFκB (RANK)/RANK Ligand (RANKL) exerts its activities in the osteoimmunology and vascular system. The aim of this study was to determine their expression and their potential role in calcifications of the atheromatous plaques located in two different peripheral arterial beds, carotid and femoral. The expression of OPG, RANK and RANKL was analyzed by immunochemistry in 40 carotid and femoral samples. Blood OPG and RANKL were quantified using specific ELISA assays. OPG staining was more frequently observed in carotid than in femoral plaques, especially in lipid core. Its expression correlated with macrophage infiltration more abundantly observed in carotid specimens. Surprisingly, serum OPG concentration was significantly lower in carotid population compared to femoral population while RANK and RANKL were equally expressed in both arterial beds. Carotid plaques that are less rich in calcium than femoral specimens, express more frequently OPG, this expression being correlated with the abundance of macrophages in the lesions. These data strengthen the key role played by OPG in the differential calcification in carotid and femoral plaques.
Introduction

Vascular calcium deposition can be usefully classified into four histo-anatomical variants, each type being associated with a characteristic spectrum of vascular disease processes: atherosclerotic/fibrotic calcification, cardiac valve calcification, medial artery calcification and vascular calciphylaxis. These four variants arise in response to metabolic, mechanical, infectious and inflammatory injuries [1]. Vascular calcifications, especially of the arteries, often occur with advancing age, atherosclerosis, metabolic disorders (e.g., end-stage renal disease, diabetes mellitus) or with rare genetic diseases [2]. In the past 15 years, the prevailing perspective on vascular calcification has evolved.

Human and animal studies have established that atherosclerosis is driven by a chronic inflammatory process within the arterial wall initiated mainly in response to endogenously modified structures, particularly oxidized lipoproteins that stimulate both innate and adaptive immune responses [3]. The innate response is instigated by the activation of both vascular cells (endothelial cells and smooth muscle cells) and monocytes/macrophages. Subsequently, an adaptive immune response develops against an array of potential antigens presented to T lymphocytes by antigens-presenting cells. Endothelial and smooth muscle cells mediate leukocyte recruitment and vascular remodelling such as fibrosis and calcifications [4] through the production of numerous soluble cytokines by inflammatory and vascular cells [5].

Despite many studies, regulatory factors and molecular pathways of vascular calcifications seem still incompletely understood. These pathobiological mechanisms broadly fall into 2 categories: induction of osteogenesis and loss of inhibitors of mineralization [6]. One emerging pathway in vascular biology involves the osteoprotegerin (OPG) / Receptor Activator of NF-κB (RANK) / RANK Ligand (RANKL) system, cytokines and receptor related to the TNF-TNF receptor families. RANK, RANKL and OPG play essential roles in the regulation of bone metabolism. RANKL binds to RANK which is expressed by
osteoclasts whereas OPG acts as its decoy receptor blocking the RANK-RANKL interaction [7]. OPG is a soluble glycoprotein widely expressed in most human tissues including bone (osteoblasts, mesenchymal stem cells), immune cells (T and B cells) and vessels (endothelial and vascular smooth muscle cells) [2, 8]. RANKL is expressed on osteoblastic, stromal and T cells while RANK is located on the surface of myeloid cell lineages (osteoclasts, monocytic and dendritic cells) [9]. Their expression explain in part their functional involvement in the osteoarticular, immune and vascular systems and their specific implication in the crossroads of immunity and bone metabolism named “osteoimmunology“[7]. Thus, OPG/RANKL couple controls simultaneously the angiogenesis and bone remodeling mechanisms as revealed by OPG deficient mice. Indeed, mice lacking OPG exhibit severe osteoporosis and arterial calcification providing that OPG might be a key molecule linking these vascular and skeletal phenotypes [7, 10]. More recently, Benslimane et al demonstrated that OPG activates endothelial colony-forming cells and consequently induces the formation of microvessels in vivo [11].

We have recently shown that femoral and carotid plaques exhibit different morphology in comparable groups of patients [12]. Indeed, fibrous cap atheroma was more frequently observed in carotid arteries (75%) while femoral arteries were more calcified (93%). Supported by the literature, we hypothesized that the molecular triad OPG/RANK/RANKL could play a role in the differential histological patterns of these plaques and in the mechanisms of calcification. The aim of the present study was to compare the expression of OPG/RANK/RANKL in a cohort of carotid and femoral endarterectomy specimens.
Materials and Methods

Patients

Atherosclerosis plaques were harvested from patients undergoing carotid or femoral endarterectomy at the department of vascular surgery of Nantes University Hospital (Table 1). There was no significant difference between the two groups in terms of ages, gender, cardiovascular risk factors, coronaropathy, renal failure, statins, anti-platelet therapies and vitamin K antagonists [12]. Endarteriectomies were performed on a consecutive series of patients using conventional surgical techniques. The present study included 40 patients (8 females aged 66-89 years, 32 males aged 53-87 years); 50% of them underwent carotid endarterectomy and 50% femoral endarterectomy. The plaque was removed at the bifurcation from within the lumen as a single specimen. Prior to surgery, serum specimens were collected and stored at -80°C until biological assays. Sample collection and handling was performed in accordance with the guidelines of the Medical and Ethical Committee in Nantes, France, and a written informed consent for each patient was requested.

Histology processing

Atherosclerotic plaques were immediately fixed in 10% formalin overnight, decalcified in Sakura TDE 30 fluid during 24 h and embedded in paraffin. They were embedded in paraffin. Sections (4µm thickness) were stained with hematoxylin eosin (HE) added with safran. Three exclusive sub-types of calcifications can be observed: (i) sheet-like calcification defined as a calcification front within fibrosis, surrounded by numerous calcified micronodules, (ii) nodular calcification characterized by numerous stratified deposits of calcification with multinodular edges, consistent with an aggregation phenomenon, and the presence of very few cells and (iii) osteoid metaplasia consisting of mature bone with typical lamellar structure and often bone marrow.
**Immunohistochemistry**

Immunohistochemistry was carried out on adjacent deparaffinized sections (4 µm thick) taken from the central sections of the plaque (4 sections for each monoclonal antibody). Briefly, tissue sections were first treated with 3% hydrogen peroxide (H2O2) for 5 min to block endogenous peroxydase and with bovine serum albumin 4% to block the non specific bindings. Primary antibodies against RANK, RANKL, OPG and CD68 (Supplementary data 1) were then incubated over night at 4°C in a humidified atmosphere. Biotinylated polyclonal anti-mouse/goat secondary antibody and peroxydase-conjugated streptavidin (Sigma Aldrich, Saint-Quentin Fallavier, France) were applied for 1 hour each with the use of the extravidine peroxydase for 30 min and then revealed with an AEC staining kit (Sigma Aldrich). Preparations were counterstained with hematoxylin eosin. Negative control was analyzed using a similar procedure excluding the primary antibody. Each case was scored by two independent operators using semi-quantitative method, evaluating the heterogeneous positive distribution and the differing intensity of the staining simultaneously. Immunostaining signals were scored according 4 categories by two independent operators including pathologist: (-) absence of signal and positive signal [(+) weak signal, (++) moderate signal and (+++) strong signal].

**OPG, RANKL ELISA**

OPG, RANKL concentrations were analyzed in serum aliquots stored at -80°C. The concentration of total OPG was analysed using an ELISA kit (R&D Systems, Abingdon UK). The concentration of RANKL was measured using an ELISA kit (Biomedica, Vienna, Austria). The OPG and RANKL assays were performed according to the manufacturer’s instructions. All measures were performed in duplicate and the mean values were used in this study.
Statistical analysis

Statistical analysis was performed using a SPSS 10.0 programme. Comparison of the data was assessed by either Chi Square test or two-sample Student’s t-test. For OPG, RANKL analyses, non-parametric Mann and Whitney test was used. P< 0.05 was considered significant.
Results

Carotid and femoral plaques exhibit differential histological morphologies

As expected [12], carotid and femoral atherosclerotic plaques showed different morphology. Indeed, femoral plaques exhibited significantly more sheet-like calcification (86% vs 62%, p<0.05) (Fig. 1A) and nodular calcification (84% vs 58 %, p<0.01) (Fig. 1B); a lot of them presented osteoid metaplasia (63% vs 20%, p<0.001) (Fig.1C). Carotid plaques contained more lipidic deposits (Fig. 2).

OPG staining is more frequently observed in carotid plaques (being less calcified) than in femoral plaques

The expression of OPG, RANK, RANKL and CD68 has been analyzed by immunohistochemistry in 20 carotid atherosclerotic plaques and 20 femoral specimens. (Table1). In carotid specimens, OPG was mainly expressed within the lipid core (64.3%) (Fig. 3A), calcifications (35.7%) and by endothelial cells, macrophages and smooth muscle cells (14.3%) (Fig 3C). In femoral plaques, OPG was more frequently detected associated with calcifications and endothelial cells (90%) (Table 1, Fig. 3 B, D). In 14 cases of the 20 carotid specimens (70% of cases) (Fig. 3G), we observed immunostaining for OPG, while only 10 cases of femoral plaques expressed OPG (50 % of cases). Interestingly, OPG immunopositivity is related to CD68⁺ macrophages population (Table 1, Fig. 3 E, F). Indeed, OPG was more frequently observed in carotid plaques compared with femoral specimens, this expression being correlated with the presence of more numerous macrophages.

RANK and RANKL are similarly expressed in carotid and femoral plaques

One hundred percent of carotid specimens and 95% of femoral plaques expressed RANK and RANKL respectively and to similar extent (Fig. 4). However, although the
immunostaining intensity appeared similar, their cell localization was different. Indeed, in carotid plaques, RANK immunoreactivity was mainly located in lipid core and in macrophages (Fig. 4A) while its localization is observed in calcifications and specifically around of them, and in endothelial cells in femoral plaques (Fig. 4B). RANKL immunostaining was observed preferentially in macrophages, calcifications and in endothelial cells of carotid plaques (Fig. 4C). In femoral specimens, RANKL was located in calcifications and endothelial cells. (Fig. 4D).

**Serum OPG is significantly lower in the carotid population compared with the femoral group**

Serum concentrations of OPG and RANKL were determined in all patients enrolled (Fig. 5). Although serum concentration of RANKL was higher for carotid population (0.66 ± 0.39 pmol/L versus 0.19 ± 0.06 pmol/L), these data were not significant. In contrast to RANKL, serum concentration of OPG was significantly lower in the carotid population compared to the femoral group. The concentration of OPG of carotid population was 4.03 ± 0.8 ng/mL (mean ± SD) while this concentration was 6.53 ± 0.64 ng/mL in femoral group (p < 0.02) (Fig. 5).
Discussion

Mounting evidence suggests that the OPG/RANK/RANKL triad may participate in multiple aspects of the processes governing vascular calcification. The implication of this triad in vascular physiopathology is supported by the expression of these molecules by the normal tissues of cardiovascular system (heart, arteries and veins). Both endothelial cells and vascular smooth muscle cells constitutively express OPG, and such levels are particularly high in aortic and renal arteries [2]. Furthermore, OPG is physically associated with the von Willebrand factor localized in the Weibel-Palade bodies of endothelial cells, can bind to the complex VIII [13] and is rapidly secreted in response to inflammatory stimuli [14]. In contrast, RANKL and RANK are frequently undetectable in normal vessels and non calcified arteries or valves [8]. OPG/RANK/RANKL exerts its activities simultaneously on endothelial cells and smooth muscle cells. OPG promotes endothelial cell survival through neutralizing pro-apoptotic TRAIL [15]. The triad is also involved in angiogenesis supporting its involvement in revascularization of numerous tissues. Thus, OPG induces endothelial colony-forming cell activation and is a positive regulator of microvessel formation in vivo [11]. Similarly, Cross et al also reported a pro-angiogenic effect of OPG which was expressed by tumour endothelial cells [16]. RANKL has also been implicated in endothelial cell metabolism, and induces angiogenesis in vitro and in vivo by acting as a chemotactic factor for endothelial cells and by inducing their migration [7]. This molecule promotes endothelial proliferation and survival [17], suggesting a potential involvement in the tumour development and increased vascular permeability [18].

It has been recently established that activated macrophages modulate bone mineralization process and consequently all factors modulating macrophage migration can alter this mechanism [19]. OPG is preferentially observed in carotid plaques, arteries that contained more macrophages. Thus this recruitment of macrophages in carotid plaque could
be mediated by OPG and Mosheimer et al demonstrated that OPG affects monocyte migration and protein kinase C and phosphatidylinositol 3-kinase/Akt activation via syndecan-1. OPG-induced activation of monocyte chemotaxis toward different chemokines is due to the interactions of OPG with heparin and chondroitin sulphates [20]. Similarly, RANKL enhances monocyte migration through its binding to its receptor RANK which activates PI3-kinase, phosphodiesterase, and Src kinases [21].

We have demonstrated in a previous study [12], that femoral endarterectomies were more calcified that carotid specimens, with osteoid metaplasia although the phosphocalcic ratio was similar between both types of plaques. OPG immunostainings (Fig.3) are consistent with these results. Indeed, OPG postivity was more frequently observed in carotid plaques compared to femoral plaques (70% versus 50%). OPG expression might reflect a protective mechanism against vascular calcifications. The senescence of vascular smooth muscle cells promotes the development of vascular calcification which is responsible of the progression of atherosclerosis [22]. OPG directly inhibits calcification of vascular smooth muscle cells, potentially by a mechanism whereby OPG is secreted via vesicle release from viable or apoptotic vascular smooth muscle cells and potentially limiting the matrix vesicle-driven mineral nucleation and deposition of hydroxyapatite in the vascular wall [23]. In pathology, vascular calcification and osteoporosis frequently occur together and share many of the same risk factors (eg, aging, inflammatory disease, glucocorticoid use, chronic renal failure, or estrogen deficiency). Osteoporotic patients have a higher incidence of arterial calcification and conversely, epidemiological studies indicate that the incidence of osteoporosis is elevated in people with atherosclerosis, cardiovascular disease, or aortic calcifications [24]. Addition to early onset osteoporosis, two-thirds of OPG knockout mice unexpectedly have late medial calcification of renal and aortic arteries [10]. Clearly, the vascular role of OPG is multifaceted.
and depends of the interplay with its multiples ligands, RANKL and TRAIL, and a bidirectional modulation involving osteogenic, inflammatory and apoptotic responses.

Significant differences of serum concentrations of OPG are noted between carotid and femoral patients. Serum concentration of OPG was significantly lower in carotid population compared to the femoral population (4.03 ng/mL vs 5.53 ng/mL, P<0.02). These results support the difference of calcification of the 2 arterial beds. The data concerning the serum concentrations of OPG are surprisingly opposite to immunohistochemical results. We could expect to observe serum concentrations of OPG higher for carotid than for femoral patients. This difference between local and systemic environment could be explained by several compensatory mechanisms involving bone and inflammatory regulation. Pro-inflammatory mediators such as TNF-α are able to induce OPG expression in vascular smooth muscle cells and endothelial cells (and particularly in atheromatous plaques) [2, 24] but are also implicated in the development of vascular calcification by inducing ALP [25, 26]. Moreover, the calcification processing stimulates the secretion of pro-inflammatory cytokines capable of simultaneously increasing OPG production and enhancing osteogenic differentiation [27, 28]. On the other hand, elevated serum concentrations of OPG are found in a range of cardiovascular pathologies, suggesting the potential value of OPG as a biomarker of vascular risk and prognosis. Cellular studies have suggested a number of mechanisms by which such elevated concentrations of OPG could promote the progression and instability of atherosclerosis. [27].

Is the prognosis for femoral patients worse than for carotid patients? It is difficult to answer given the short duration of patient follow-up. An explanation could be provided by the fact that the age of development is different between the 2 groups of plaques. Indeed, carotid plaques seem to be observed sooner than femoral plaques with a prevalence of lipid core plaques [29] and increased OPG staining in the carotid then represents a “younger” plaque
lesion with more inflammatory activity revealed by high macrophage infiltration than an “older” femoral plaque lesion with significant calcification [30, 31]. Moreover, patients with carotid plaques exhibit a significant symptomatic or non symptomatic stenosis (>70%) are usually operated earlier than those with femoral plaques who are all symptomatic.

**Conclusions:** Carotid plaques, less calcified than femoral specimens, express more frequently OPG, this expression being correlated with the abundance of macrophages population. The OPG/RANK/RANKL molecular triad may therefore play a role in the differential calcification in carotid and femoral plaques however its intervention remains unclear.

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References


Figure legends

Figure 1: Femoral plaques exhibit three different histologic morphologies. (A) Plaque with sheet-like calcification; (B) Plaque with nodular calcifications; (C) Plaque with osteoid metaplasia. HES staining, original magnification: X10.

Figure 2: Main typical histological feature of carotid plaque (sheet-like calcification). HES staining, original magnification: X10. *: Lipid core with microcalcifications.

Figure 3: OPG staining is more frequently observed in carotid plaques than in femoral plaques. OPG expression was studied by immunohistochemistry in carotid plaques (A, C) and in femoral plaques (B, D). A: OPG positive staining in lipid core with cholesterol crystals; C: OPG is expressed by endothelial cells (arrow) and calcified matrix (*); B: femoral nodular calcification showing OPG immunostaining; D: OPG expressed in endothelial cells (arrow) and macrophages (arrow head). (E, F): CD68 expression in carotid plaques (E) and in femoral plaques (F). G: quantitative evaluation of positive and negative OPG plaque between the two arterial beds. Original magnification: X10 (A, B, E, F); X20 (C-D).

Figure 4: RANK and RANKL are similarly expressed in carotid and femoral plaques. RANK and RANKL expressions were studied by immunohistochemistry in carotid plaques (A, C) and in femoral (B-D). A : RANK positive expression in macrophages (arrow head) and in lipid core (LC); B : RANK positive expression in calcifications (*) and vessels (arrow dead); C : RANKL immunostaining in calcifications (*) and vessels (arrow head), some macrophages are also positively stained; D: RANKL immunolocalization in calcification (*) and vessels (arrow). Original magnification: X10.
Figure 5: Differential concentration in serum OPG between carotid and femoral populations. Serum OPG was measured by ELISA assay. ** p < 0.02.