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Research Article

Role of Bone Metabolic Marker Proteins in Calcified Lesions of Carotid Arteriosclerosis

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Abstract

Vascular calcification is an important characteristic of atherosclerosis. According to histological analyses, bone formation tends to occur in heavily calcified carotid lesions devoid of ulceration and hemorrhage. Vascular calcification is not a simple degenerative and necrotic process associated with atherosclerosis but an active process similar to bone formation. Proteins involved in regulating skeletal bone formation are present in human atherosclerotic lesions. These proteins include osteoprotegerin and its ligand, bone sialoprotein, bone morphogenetic protein (BMP)-2 and BMP-4, osteocalcin, osteonectin, matrix Gla protein, and osteopontin. These molecules play important roles as active promotors for calcification or decalcification. These molecules are also important in inflammatory mechanisms in atherosclerosis and calcification.

Keywords: Vascular Calcification; Atherosclerosis; Bone Metabolic Marker Protein

Introduction

Vascular calcification, an important characteristic of systemic atherosclerosis, is frequently observed in the cervical carotid, coronary, and peripheral arteries. Systemic atherosclerosis predisposes one to ischemic events in the body, such as cerebral infarction and cardiac infarction, which lead to high morbidity and mortality. Vascular calcification and atherosclerosis can be visualized by radiological and physiological examinations, such as computed tomography, magnetic resonance imaging, echography, etc. In an illustrative case, the stenosis and calcification of the right cervical carotid artery are revealed by cerebral angiography and three-dimensional computed tomography, whereas no calcification is detected in the left cervical carotid artery (Figure 1). The factors determining the difference in calcification in both carotid arteries is unclear; thus, the mechanism of vascular calcification should be elucidated.

Histopathological analyses have shown that bone formation tends to develop in heavily calcified lesions without ulceration and hemorrhage in carotid arteries [1]. Many proteins are involved in regulating skeletal bone metabolism in human atherosclerotic lesions. These proteins include osteoprotegerin (OPG) and its ligand, bone sialoprotein, bone morphogenetic protein (BMP)-2 and BMP-4, osteocalcin, osteonectin, matrix Gla protein (MGP), osteopontin (OPN), and so on [2-6]. Therefore, vascular calcification is not only a degenerative and necrotic process associated with atherosclerosis but also a bioactive sequela similar to bone formation.

Chronic inflammation is closely involved in the pathogenesis and development of atherosclerosis; thus, inflammatory cytokines derived from macrophages, including interleukin-1β, interleukin-6, tumor necrosis factor (TNF)-α, and oncostatin M, have

Figure 1: The stenosis and calcification of the right cervical carotid artery are revealed by cerebral angiography (left) and three-dimensional computed tomography (right), whereas no calcification is detected in the left cervical carotid artery.

been identified as factors promoting the differentiation of vascular smooth muscle cells (SMCs) into osteoblasts and calcification of the extracellular matrix [7-9]. Molecular imaging analyses have shown that inflammation proceeds to bone formation in atherosclerotic plaques [10,11].

OPG, a member of the TNF receptor superfamily, promotes bone formation through the inhibition of recruitment, proliferation, and activation of osteoclasts by the impaired combination of receptor activator of nuclear factor-κB (RANK) to its ligands, such as RANK ligand (RANKL). OPN is an acidic, phosphorylated glycoprotein first discovered in bone and thought to be involved in the regulation of biomineralization by promoting osteoclast function through $\alpha_v\beta_3$ integrin and by inhibiting apatite crystal growth [12,13].

In this article, we examined the immunohistochemical expression of OPN, OPG, and RANKL in regulating skeletal bone formation in the calcification of carotid artery atherosclerotic lesions, with a literature review.

Methods

Eighteen paraffin sections of atherosclerotic lesions from the carotid artery of 18 patients (17 males and 1 female, aged 58-79 years) were utilized in this study. The calcification of the lesions was verified by hematoxylin-eosin staining and von Kossa staining, capable of visualizing calcium deposits.

For the immunohistochemical staining of OPN, OPG, and RANKL, paraffin sections were deparaffinized in xylene and rehydrated with graded ethanol to water. Following blockage of the endogenous peroxidase activity with 0.3% H_2O_2 in methanol for 30 min, the slides were immunostained with an anti-OPN monoclonal antibody (mouse; Manufacturer, Santa Cruz, CA, USA; diluted 1:1000), at room temperature for 1h. The biotinylated antibody against mouse immunoglobulin (IgG) (goat; Dako, Carpinteria, CA, USA) was applied as a secondary antibody for 30 min.

The slides were immunostained with anti-OPG and anti-RANKL polyclonal antibodies (rabbit; Imgenex, San Diego, CA, USA; diluted

1:1000), at room temperature for 1 h. The biotinylated antibody against rabbit IgG (goat; Dako) was applied as a secondary antibody for 30 min.

Immunoreactions were followed by using the Vectastain streptavidin and biotin complex (ABC) kit, labeled with horseradish peroxidase (Vector Laboratories Inc., Burlingame, CA, USA) for 30 min and developed with freshly prepared 3,3'-diaminobenzidine tetrahydrochloride dissolved in 0.05 M Tris-HCl, pH 7.6, and 0.017% H_2O_2 for 7 min. Negative control studies included substituting normal serum for the primary antibody. Nuclear staining was carried out with hematoxylin for 5 s.

Results

We focused on two areas in the paraffin sections, namely, the area with apparent calcification and the area without obvious calcification, with negative von Kossa staining. In all cases, OPN was immunostained in areas without obvious calcification, with negative von Kossa staining (Figure 2). Moreover, OPN was positively immunostained in areas with apparent calcification (Figure 3). However, OPG and RANKL were negatively immunostained either in areas with apparent calcification or in areas without obvious calcification, with negative von Kossa staining.

Figure 2: A: Hematoxylin-eosin (HE) staining; B: von Kossa staining; C: osteopontin (OPN) immunostaining. OPN is positively immunostained in the specimens with obvious calcification, namely, positive von Kossa staining.

Figure 3: A: Hematoxylin-eosin (HE) staining; B: von Kossa staining; C: osteopontin (OPN) immunostaining. OPN is positively immunostained in the specimens without calcification, namely, negative von Kossa staining.

Discussion

Vascular calcification is classified into two types: endothelial calcification associated with atherosclerosis and Mönckeberg's medial sclerosis, mainly observed in small arteries [14]. Several factors regulate vascular calcification.

First, Demer., *et al*. and Giachelli., *et al*. reported that the expressions of BMP-2 and OPN are essential factors in the process of bone formation in atherosclerotic lesions [15,16]. BMP-2, a superfamily of TGF-β, is expressed in vascular SMCs, myofibroblasts, pericytes, endothelial cells, and macrophages. BMP-2 activates muscle segment homeobox homolog-2 in membranous ossification and runt-related transcription factor 2 in endochondral ossification.

Second, the roles of calcium and phosphate are also important in atherosclerotic lesions. Hyperphosphatemia and high levels of the calcium-phosphate product promote vascular calcification. For example, patients with uremia are prone to widespread ectopic extraskeletal calcification resulting from an imbalance of systemic inorganic phosphate. Clinically, it seems that the early control or prevention of hyperphosphatemia may reduce coronary calcification and its associated morbidity and mortality for patients undergoing dialysis [17].

Based on the experiment in *MGP-/-*mice, MGP suppresses BMP-2 and is considered an inhibitory factor of vascular calcification through the inhibition of differentiation of vascular SMCs into osteoblasts [18].

RANK, RANKL, and OPG are regulatory factors of bone immunosystem [19]. RANKL is identified in T cells and is expressed in osteoblasts and osteocytes. RANKL binds to osteoclasts, dendric cells, and their precursors; promotes differentiation into multinuclear osteoclasts; and acts as a regulatory factor of functional maturation survival extension in multinuclear osteoclasts. OPG is expressed in T cells and osteoblasts together with RANKL and inhibits the function of RANK/RANKL by combining with RANKL as a soluble decoy receptor [20]. Severe osteoporosis and vascular calcification are observed in OPG-/- mice [20].

Higgins., *et al*. [21] measured the concentrations of OPN, OPG, RANKL, and alkaline phosphatase in sera and carotid endarterectomy (CEA) specimens to determine the central roles in the calcification or demineralization of atherosclerotic lesions. In CEA tissue segments, the calcification levels were inversely associated with the OPG levels and positively associated with the RANKL levels. In turn, tissue levels of OPG were associated with homologous serum levels of OPG, and tissue levels of RANKL were associated with serum levels of homologous RANKL. Their study suggests that serum levels of OPG and RANKL may be valuable biomarkers for estimating the degree of calcification in atherosclerotic lesions in the carotid artery.

OPN is abundant at sites of ectopic calcification in human atherosclerotic lesions [22-27], diabetic arteries [28], uremic arteriolopathy [29], and native and prosthetic valves [30-33]. In calcified arteries and valves, OPN is highly localized to the surfaces of calcified deposits [34,35]. In addition, OPN potently inhibits calcium deposition in calcifying SMCs [36]. This suggests the role of OPN in regulating vascular calcification. In our study, OPN was present in areas with apparent calcification and in areas without calcification. This reveals the possible role of OPN that prevents calcification in the carotid artery, and advancements in the calcification process exceed the prevention effect of OPN for calcium deposits.

OPN has been studied as a multifunctional protein that is upregulated in various acute and chronic inflammatory conditions, such as wound healing, fibrosis, autoimmune disease, and atherosclerosis. OPN is expressed at sites with atherosclerotic plaques, especially those associated with macrophages and foam cells. In the context of atherosclerosis, OPN is generally regarded as a proinflammatory and proatherogenic molecule. However, the role of OPN in vascular calcification, which is closely related to chronic and active inflammation, is that of a negative regulator because it is an inhibitor of calcification and an inducer of decalcification [37].

Conclusions

Vascular calcification is not a simple degenerative and necrotic process associated with atherosclerosis but an active process similar to bone formation. Various molecules act in this process as active promotors for calcification or decalcification. These molecules play important roles in inflammatory mechanisms in atherosclerosis and calcification.

Conflict of Interests

The authors declare that there is no conflict of interest.

Ethical Approval

This study was approved by the ethical committees of Teikyo University and the International University of Health and Welfare.

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