Bone 88 (2016) 31–38

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

Direct comparison of regulators of calcification between bone and vessels in humans

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ARTICLE INFO

Article history:
Received 25 August 2015
Revised 30 November 2015
Accepted 16 April 2016
Available online 20 April 2016

Keywords:
Vascular calcification
Atherosclerosis
Regulators of calcification
Gene expression

ABSTRACT

Calcification is not only physiologically present in bone but is a main pathophysiological process in vasculature, favouring cardiovascular diseases. Our aim was to investigate changes in the expression of calcification regulators during vascular calcification in bone and vasculature. Levels of gene expression of osteoprotegerin (OPG), receptor activator of NF-κB ligand (RANKL), osteopontin (OPN), matrix gla protein (MGP), bone sialoprotein (BSP), SMAD6, and runt-related transcription factor 2 (RUNX2) were determined in bone, aorta, and external iliac artery tissue samples of transplant donors. Histological stages of atherosclerosis (AS) in vessels are defined as "no changes", "intima thickening", or "intima calcification". Patients' bone samples were subgrouped accordingly.
We demonstrate that in vessels BSP and OPN expression significantly increased during intima thickening and decreased during intima calcification, whereas the expression of regulators of calcification did not significantly change in bone during intima thickening and intima calcification. At the stage of intima thickening, MGP, OPG, and SMAD6 expression and at stage of intima calcification only MGP expression was lower in bone than in vessel. The expression of BSP and RANKL was regulated in opposite ways in bone and vessels, whereas the expression of MGP, OC, RUNX2, and OPN was regulated in a tissue-specific manner.
Our study is the first direct comparison of gene expression changes during AS progression in bone and vessels. Our results indicate that changes in the expression of regulators of calcification in the vessel wall as well as in bone occur early in the calcification process, even prior to deposition of calcium/phosphate precipitation.

1. Introduction

Vascular calcification is a process where mineral complexes are ectopically deposited in the blood vessel wall [1]. Currently, the term vascular calcification includes atherosclerosis, Mönckeberg's or media sclerosis, and arteriolosclerosis [2]. Common features for all three forms are arterial wall stiffening and thickening [2]. Today, atherosclerosis is seen as the leading cause of cardiovascular mortality in industrialized countries and is recognized as a general cardiovascular risk factor [3]. Atherosclerosis is an active, cell-mediated process, mostly affecting elastic and large muscular arteries like the aorta or the coronary, iliofemoral, and carotid arteries [1,2]. A characteristic feature of atherosclerosis is the presence of lesions enlarging the vessel intima, caused by the deposition of connective tissue, inflammatory proteins, lipids and extracellular components like matrix proteins and calcium [2,4]. Since a variety of studies demonstrated the association of atherosclerosis with bone pathologies, especially osteoporosis [5–7], a crosstalk between the vascular and the skeletal system is most likely. Furthermore, bone mineralization and vascular calcification feature many parallels [8] e. g. vascular cells gain a bone-like phenotype and express bone-related proteins known to regulate bone calcification [1,9]. According to these parallels, it has been proposed that the mechanism of arterial calcification is similar to the one seen in developmental osteogenesis [10].

The expression of regulators of bone calcification in the vasculature is associated with either an increase in calcification as shown for osteocalcin (OC), runt-related transcription factor 2 (RUNX2 or also known as core binding factor alpha 1 (Cbfa-1)), osteonectin, bone morphogenetic protein type 2a, and alkaline phosphatase or with a decrease in vascular calcification as demonstrated for osteopontin (OPN), matrix Gla protein (MGP), and osteoprotegerin (OPG) [1].

In bone, the acidic, noncollagenous RGD-containing (Arg–Gly–Asp sequence) matrix protein [11] bone sialoprotein (BSP) increases bone formation by promoting osteoblast differentiation [12] Knockout of BSP in mice leads to impaired bone growth, mineralization, and bone

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http://dx.doi.org/10.1016/j.bone.2016.04.016
8756-3282/

formation [13]. In contrast to bone, uncalcified human vessels only minimally express BSP [14]. Vascular smooth muscle cells (VSMCs) are able to express BSP, which is induced by factors also promoting their osteoblastic differentiation [15]. During vascular calcification, BSP mediates the migration and attachment of endothelial cells (ECs) [16].

Another extracellular bone matrix protein is MGP, which binds hydroxyapatite with high affinity due to five vitamin K-dependent calcium-binding amino acid gamma-carboxy-glutamic (Gla) residues. It thus maintains calcium in a soluble form and prevents its deposition [14,17]. In bone, MGP inhibits bone formation, whereas in vasculature, it inhibits calcification by preventing hydroxyapatite crystal growth [18,19]. VSMCs and ECs are able to express MGP [9,20,21]. Together with fetuin A it forms the fetuin A mineral complex in plasma, also keeping hydroxyapatite insoluble [22]. The absence or loss of function of MGP, as seen in the human Keutel syndrome, is characterized by abnormal calcification of cartilage and extensive medial vascular calcification [23]. In atherosclerosis, elevated levels of MGP can be found in human calcified plaques [24].

Osteocalcin is the most abundant non-collagenous extracellular matrix protein and contains 3 Gla residues [25]. It is expressed not only by osteoblasts in bone but also by calcifying vascular cells developing an osteoblast-like phenotype [26]. Osteocalcin null mice have increased bone density but otherwise normal arteries [26]. Until now osteocalcin is mostly used as a marker of osteoblastic differentiation of vascular cells but is also under investigation as possible regulator of calcification [27,28].

OPN is a phosphorylated acidic non-collagenous bone matrix glycoprotein, promoting bone resorption by enhancing osteoclast attachment. Furthermore it enhances cell mediated releases of calcium from vascular deposits and inhibits hydroxyapatite crystal growth via adherence to apatite crystals [29,30]. OPN knock-out mice are susceptible to vascular calcification [31]. In human vessels, OPN protein is highly expressed in ECs and VSMCs with upregulated protein levels at sites of calcification [9,32].

Receptor activator of nuclear factor kappa B ligand (RANKL), a member of the tumour necrosis factor superfamily [33], acts in bone as stimulator of resorption and induces osteoclast differentiation and activation [34]. The circulating glycoprotein OPG acts as decoy receptor of RANKL, thus neutralizing its actions [35]. In the vasculature, ECs are able to express RANKL, receptor activator of nuclear factor kappa B (RANK), and OPG [8]. In human VSMCs, RANKL stimulates osteogenic differentiation and calcification [36]. RANK, RANKL, and OPG are present in calcified regions [8], but not in normal uncalcified vasculature [37]. OPG knock-out leads to severe osteoporosis and vascular calcification in the majority of mice strains [38]. In human vessels, OPG is produced by ECs as well as VSMCs [9,34,39,40]. In clinical studies, high serum levels of OPG were associated with atherosclerosis [5]. RANKL knock-out mice display increased bone mineral density and decreased length of long bones and osteoclast cell number, but show no vascular changes [41].

RUNX2, a pivotal transcription factor, regulates the expression of Mothers against decapentaplegic homolog 6 (SMAD6), OC, OPN, BSP, and other factors [42–44]. It regulates osteogenesis and osteoblastic differentiation of osteoblasts but also of VSMCs [45,46]. RUNX2 knock-out mice die shortly after birth and show no bone formation [45].

SMAD6 (encoded by the MADH6 gene) belongs to the inhibitory smads, which inhibit bone morphogenetic protein (BMP) signaling [47,48] and thus control osteoblast differentiation and promote osteogenesis [30]. Knock-out of SMAD6 in mice, leads to cartilaginous medial metaplasia with ossification and the presence of bone marrow in the aortic wall [49].

The aim of the study was to detect gene expression changes and thereby changes in the local production, of key regulators of calcification in paired samples of vasculature and bone at different atherosclerosis stages. We hereby intended to show similarities and differences between the two tissues during the process of calcification.

2. Material and methods

2.1. Human tissue specimens

The experimental protocol for this study was approved by the Ethics Committee of the Medical University of Graz. Aorta, external iliac artery, and bone (sternum) tissue were obtained from 26 donors of kidney transplants (age = 58 ± 14 years, 13 males, age 55 ± 13 years and 13 females, age 59 ± 15 years).

Human vascular and sternum bone tissue was collected during kidney explantation. At least 1 cm^2 of vascular tissue was collected. Samples from the abdominal aorta were taken below the renal artery orifice and from the iliac artery about 2 cm below the bifurcation of the common iliac artery. A splint of bone was taken from the manubrium sterni (about 0.5 cm × 3 cm × 1 cm). All tissues were roughly freed of unwanted other tissues and were immediately placed in RNAlater. Samples were kept at room temperature until surgery completion, were stored for a maximum of 48 h at 4 °C and were then kept at −20 °C until further processing.

2.2. Real-time PCR

Disintegration of tissue material was performed with the MagNA Lyser (Roche Diagnostics GmbH, Mannheim, Germany) by use of MagNA Lyser Green Beads (Roche Diagnostics GmbH, Mannheim, Germany) for vessels and Precellys-Steel Kit for bone (PEQLAB Biotechnologie GMBH, Erlangen, Germany). RNA was isolated with the RiboPure Kit (Life Tech Austria, Vienna, Austria) according to the manufacturer's instructions. 1 μg RNA was used for reverse transcription with the High Capacity cDNA Reverse Transcription Kit (Life Tech Austria, Vienna, Austria) as indicated by the manufacturer. 2,5 μl/well of cDNA were used for real-time PCR (LC480, Roche Diagnostics GmbH, Mannheim, Germany) with predesigned TaqMan gene expression assays for beta actin, RANKL, OPG, BSP, MGP, OC, OPN, SMAD6, and RUNX2 (Life Tech Austria, Vienna, Austria). Relative Cp-values were generated by division of Cp-value of the gene of interest by the Cp-value of beta actin.

2.3. Determination of calcification type and stage

Thin sections (3 μm) were used for haematoxylin–eosin staining, Elastica-van-Gieson staining and von Kossa staining according to the standard procedures. The type of calcification was determined microscopically by two independent experts at the Institute of Pathology, Medical University of Graz. Atherosclerosis was defined as presence of thickening and/or calcification of the vessel's intima. Seven stages of atherosclerosis were defined for aorta and external iliac artery separately: 0: neither thickening nor calcification of the vessel's intima; 1: thickening of the intima doubling its size; 2: thickening of the intima tripling its size; 3: thickening of the intima more than tripling its size; 4: presence of a single calcification spot; 5: presence of two calcification spots; 6: presence of three or more calcification spots.

For gene expression analysis, stages 1 to 3 were merged to form the stage “intima thickening” and stages 4 to 6 were merged to form the stage “intima calcification”.

For analysis of regulators of calcification in bone, we assigned a stage of atherosclerosis to each patient based on the calcification stages of the respective aorta and external iliac artery. In case of different stages in both vessels, the higher stage was assigned.

2.4. Statistical analysis

Data are presented as mean ± standard deviation (SD) unless otherwise stated. Statistical analysis was performed using SPSS version 22.0 (IBM, Chicago, IL). In the case of non-normally distributed data, differences in continuous parameters were assessed by Mann–Whitney *U* test or Kruskal–Wallis test. A p value < 0.05 was considered significant.