Differential Expression of Bone Matrix Regulatory Proteins in Human Atherosclerotic Plaques


Abstract—In the present study, we examined the expression of regulators of bone formation and osteoclastogenesis in human atherosclerosis because accumulating evidence suggests that atherosclerotic calcification shares features with bone calcification. The most striking finding of this study was the constitutive immunoreactivity of matrix Gla protein, osteocalcin, and bone sialoprotein in nondiseased aortas and the absence of bone morphogenetic protein (BMP)-2, BMP-4, osteopontin, and osteonectin in nondiseased aortas and early atherosclerotic lesions. When atherosclerotic plaques demonstrated calcification or bone formation, BMP-2, BMP-4, osteopontin, and osteonectin were upregulated. Interestingly, this upregulation was associated with a sustained immunoreactivity of matrix Gla protein, osteocalcin, and bone sialoprotein. The 2 modulators of osteoclastogenesis (osteoprotegerin [OPG] and its ligand, OPGL) were present in the nondiseased vessel wall and in early atherosclerotic lesions. In advanced calcified lesions, OPG was present in bone structures, whereas OPGL was only present in the extracellular matrix surrounding calcium deposits. The observed expression patterns suggest a tight regulation of the expression of bone matrix regulatory proteins during human atherogenesis. The expression pattern of both OPG and OPGL during atherogenesis might suggest a regulatory role of these proteins not only in osteoclastogenesis but also in atherosclerotic calcification. (Arterioscler Thromb Vasc Biol. 2001;21:1998-2003.)

Key Words: bone matrix proteins ■ atherosclerosis ■ vascular calcification ■ osteogenesis ■ osteoclastogenesis

Calcification of the vessel wall is one of the features of atherosclerosis and is by itself considered to be a risk factor for plaque rupture.1-8 Although recent studies indicate that atherosclerotic calcification is an organized process, exact regulatory mechanisms remain unclear.1,2,7 Increasing evidence suggests that atherosclerotic bone formation and calcification share features with skeletal bone formation and calcification,1,8-11 such as chondrocyte and osteoblast differentiation, mineralization, bone matrix deposition, and bone resorption.

Several proteins are involved in the regulation of skeletal bone formation, such as matrix Gla protein (MGP), osteocalcin (OC; also called bone Gla protein), bone sialoprotein (BSP),12 bone morphogenetic protein-2 and -4 (BMP-2, BMP-4), osteopontin (OPN), and osteonectin (ON). Although the immunolocalization of some of these proteins in the human vessel wall has been described, the available data are rather incomplete and restricted to advanced stages of atherosclerosis. Immunoreactivity of 2 proteins in osteoclastogenesis, osteoprotegerin (OPG) and its ligand, OPGL, also named RANKL (receptor activated nuclear factor-kappa B ligand), has not been described before in the human atherosclerotic vessel wall.

In the present study, we examined the protein expression pattern of 7 regulators of bone formation and 2 modulators of osteoclastogenesis in all stages of human atherosclerotic lesions to provide an inventory of the expression of regulators of bone turnover in human atherogenesis.

Methods

Patient Characteristics and Tissue Preparation

Atherosclerotic plaques from abdominal aortas (n=42) were collected from autopsy (Department of Pathology, Academic Hospital Maastricht; n=29) and from patients undergoing vascular surgery (Department of General Surgery, Academic Hospital Maastricht; n=13). Autopsy specimens were obtained from adult men and women aged 55 to 59 years. The cause of death was diverse (eg, cardiovascular disease and cancer). Surgical specimens were obtained from patients (4 women and 7 men aged 51 to 78 years) undergoing vascular surgery, all of whom had symptoms of vascular occlusion. Specimens were processed routinely and embedded in paraffin. Plaque subtypes (5 to 8 per subgroup) were determined according to the classification proposed by Virmani et al.13 Lesion morphology was evaluated on hematoxylin-and-eosin–stained sections.

Calcification

Von Kossa staining was used to assess calcification in specimens from all stages of plaque development. This staining was performed by the standard procedure.
Western Blotting
Validation of all antibodies occurred by Western blotting. Proteins
were isolated by the urea method (Ready Prep Sequential Extraction
Kit, Instruction Manual, Bio-Rad Laboratories). A 15% SDS-PAGE
gel with 50 μg of whole cell extract per sample was electrophoresed.
Proteins were transferred to a nitrocellulose membrane at 3 V. After 1 hour of blocking with 3% bovine serum albumin (Sigma-Aldrich)
in 10 mmol/L Tris-buffered saline, pH 7.5, 100 mmol/L NaCl, and 0.1% Tween, blots were incubated with antibodies directed to either MGP (1:200),13 OC (1:800, Anawa Trading SA, Wangen), BMP-2 (1:500),13 BMP-2 (1:350, Santa Cruz Biotechnology, Inc.), BMP-4 (1:350, Santa Cruz Biotechnology, Inc.), OPN (1:500),13 ON (1:1000, Zymed Laboratories, Inc.),16 OPG (1:100), or OPGL (1:1000). Anti-mouse horseradish peroxidase (HRP; 1:1000, Dako), anti-rabbit HRP (1:2000, Cell Signaling Technology, a New England Biolabs company), and anti-goat HRP (1:2500, Dako) were used as the secondary antibodies. Specific antibody binding was visualized with enhanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech Beneux).

Immunohistochemical Staining
Paraffin sections (4 μm) were deparaffinized and washed 3 times in
Tris-buffered saline (5 mmol/L Tris-HCl, pH 7.5, 140 mmol/L NaCl). Parallel sections were stained with mouse monoclonal anti-
odies against MGP (1:25) and BMP-2 (1:20, Genetics Institute, Inc.); goat polyclonal antibodies against BMP-4 (1:25), OPN (1:125), OPG (1:100), and RANKL/OPGL (1:75); and rabbit polyclonal antibodies against OC (1:50), ON (1:400), and BSEP (1:25). For the mouse monoclonal antibodies, biotinylated sheep anti-mouse IgG (1:250, Amersham Life Science) was used as the secondary antibody. Incubation with an alkaline phosphatase–coupled avidin-biotin complex (ABC complex, Dako), antibodies were visualized with an alkaline substrate kit (Vector SK-5100, Vector Laboratories, Inc.). Sections were counterstained with hematoxylin and mounted with coverslips. In negative controls, antibody binding was visualized with an alkaline substrate kit (Vector SK-5100, Vector Laboratories, Inc.). Specific antibodies against MGP (1:200),14 OC (1:800, Anawa Trading SA, Wangen), BSEP (1:500),13 BMP-2, BMP-4 (Figure 1E), ON, and OPG were expressed in the fibrous cap atheroma and fibrocalcific plaque, whereas OPG (46 kDa) was expressed in the fibrous cap atheroma and fibrocalcific plaque, its ligand (OPGL, 35 kDa) and were slightly upregulated in the fibrous cap lesions include SMCs in the shoulder region of the plaque, covers the large lipid core (Figure III). Cells present in these lesions include SMCs in the shoulder region of the plaque, foam cells, T cells, and endothelial cells.

Medial SMCs underlying the fibrous cap atheroma showed shown that BSP protein (35 kDa) was present in early atherosclerotic plaques but was not detectable in calcified atherosclerotic lesions. BMP-2 and BMP-4 both form dimers (30 kDa) and were slightly upregulated in the fibrous cap atheroma and fibrocalcific plaques. OPN Western blotting resulted in a 67-kDa band in early atherosclerotic plaque. The 43-kDa ON was upregulated in the fibrocalcific atherosclerotic lesion. Whereas OPG (46 kDa) was upregulated in the fibrous cap atheroma and fibrocalcific plaque, its ligand (OPCL, 35 kDa) was only detected in the early lesion. Western blotting of both Glc-containing proteins (MGP and OC) was not satisfactory because of the high insolubility of these proteins. MGP (10 kDa) was detected in a sample of synthetic MGP and was faintly visible in the protein lysates of the atherosclerotic plaques. OC could not be detected with this protein isolation method, probably owing to the insolubility of the protein.

Human heart, liver, and lung tissue were used as negative controls for immunohistochemistry of antibodies directed to MGP, OC, and BSP (Figure II, please see www.atvb.ahajournals.org). MGP was absent in the hepatocytes, but the vessels in the liver did contain MGP, OC and BSP were absent in human heart. Pretreatment of calcified control sections with 3% citric acid resulted in an equal pattern of protein localization as nonpretreated sections, which indicates the lack of aspecific binding of the antibodies to calcium deposits.

Nondiseased Aorta
Five bone matrix proteins (MGP, OC, BSP, BMP-4, and OPG) were present in the nondiseased aorta (Figure III, please see www.atvb.ahajournals.org). MGP (Figure 1A) and ON, and OPG proteins were highly expressed, but only in medial SMCs. OPGL immunoreactivity was very weak and was confined to medial SMCs, a pattern that was comparable to OPG. OC, which, like MGP, is a Glc-containing protein, was only expressed in the endothelial cells lining the lumen. Two bone matrix proteins, BMP-2 and ONP (Figure 1B), were absent in the nondiseased aorta.

The adventitia showed immunoreactivity for 6 bone matrix proteins: MGP, OC, BSP, BMP-2, BMP-4, and ON, MGP, ON, and ON were present in adventitial vessels, OC throughout the entire adventitia except for the vessels, and BMP-2 and BMP-4 only in polymorphonuclear cells.

Intimal Xanthoma or Fatty Streak
Characteristic of this type of early lesion is the accumulation of macrophage-derived lipid-filled foam cells in the intima (Figure III). Intimal xanthoma lesions, MGP, BSP, BMP-4, and ON expression was localized in medial SMCs. The intima showed immunoreactivity of 6 bone matrix regulatory proteins. MGP and BSP were present in intimal SMCs. Macrophage-derived lipid-filled foam cells expressed MGP, BSP (Figure 1C), and ON protein, whereas BMP-2, BMP-4 (Figure 1D), ONP, ON, OPG, and OPGL were absent. Endothelial cells lining the lumen expressed OC, BMP-4, OPG, and OPGL.

Fibrous Cap Atheroma
The fibrous cap atheroma is a type of lesion in which a collagenous-proteoglycan matrix–containing fibrous cap covers the large lipid core (Figure III). Cells present in these lesions include SMCs in the shoulder region of the plaque, foam cells, T cells, and endothelial cells.

Medial SMCs underlying the fibrous cap atheroma showed expression of MGP, BSP, and ON. All 9 proteins investigated showed immunoreactivity in the intima of these lesions. However, localization of the proteins was diverse. BSP, BMP-2, BMP-4 (Figure 1E), ON, and OPG were expressed by the intimal SMCs present in the shoulder regions. Expression of MGP, BSP, OC, BMP-4, ONP, and ON was prominent in foam cells in the lipid core. Some specimens contained cells that can best be described as chondrocyte-like.
cells that reside in small spaces in the intercellular substance. These cells were mainly localized at the borders of the lipid core and showed high expression of MGP and ON protein (Figure 1F) and moderate BSP and BMP-4 immunoreactivity.

**Fibrocalcific Plaque**

This type of advanced atherosclerotic plaque is collagen rich, with large areas of calcification and a necrotic core. Together with areas of calcification, we observed cortical bone structures and cells involved in bone turnover, namely, chondrocyte-like cells, osteoblasts, osteocytes, and osteoclasts (Figure III). All bone matrix proteins examined in the present study were highly expressed in these fibrocalcific lesions. As in the fibrous cap atheroma, medial SMCs underlying the fibrocalcific plaque only showed expression of MGP, ON, and OC, whereas intimal SMCs expressed MGP, BSP, BMP-2, BMP-4, ON, and OPG. CD68-positive macrophages surrounding the necrotic core showed immunoreactivity of MGP, OC, BSP, BMP-4, ON, and OPG. Two types of calcified structures were present in these lesions: calcium mineral deposits and lamellar bone. Around calcium mineral deposits, MGP (Figure 2A) and ON (Figure 2B) were highly expressed, whereas in the deposits, BSP, OC, BMP-2 (Figure 2C), BMP-4, ON, and OPG showed immunoreactivity. Lamellar bone structures, recognized by the presence of cement lines and osteocytes within bone and osteoblasts lining the bone, showed immunoreactivity of ON (Figure 2D) and OC. BMP, BMP-2, BMP-4, ON, and OPG (Figure 2E) lined the bone structures. OC was the only protein present throughout the bone matrix (Figure 2F). OPG could only be demonstrated in association with the extracellular matrix.
surrounding calcium deposits (Figure 2G). High expression of BSP, ON, and OPG was seen in the few inflammatory cells present in these fibrocalcific plaques. ON was also highly expressed in the matrix vesicles present in these lesions (Figure 2H).

**In Situ Hybridization**

In situ hybridization revealed that MGP mRNA expression pattern colocalizes with the protein. In intimal xanthoma, medial vascular SMCs adjacent to the adventitia expressed MGP mRNA, whereas in fibrocalcific plaques, MGP mRNA was mainly present in vascular SMCs and osteoblastic cells in calcified and ossified regions in the plaque.

In situ hybridization of BMP-2 and BMP-4 in fibrocalcific plaques showed mRNA expression in intimal vascular SMCs, mainly surrounding the calcified areas, and in osteoblasts, whereas mRNA expression was absent in medial vascular SMCs. This mRNA expression pattern was comparable to immunohistochemical localization of these proteins.

In addition, in situ hybridization of OPN and ON revealed an mRNA expression profile that resembled the protein expression profile. OPN mRNA expression was present in SMCs and in bone structures of the fibrocalcific plaque, in the osteocytes and osteoblasts. ON mRNA was highly expressed in matrix-producing SMCs and in chondrocyte-like cells. For in situ hybridization results, see Figure IV at www.atvb.ahajournals.org.

**Discussion**

In vitro and gene expression studies demonstrate that human vascular calcification is a regulated process with similarities to bone modeling and remodeling. Our observations using a panel of antibodies against several bone matrix regulatory proteins support these findings and are summarized in Figure 3. We observed that all of these bone matrix proteins were expressed in the arterial wall. One set of proteins, known to belong to inhibitors of calcification, was present at all stages of human atherosclerosis. This suggests a continuous inhibition of calcification in the atherosclerotic vessel wall. The expression of a second set of proteins, known to belong to the activators of calcification, was restricted to advanced and calcified lesions. Given these observations, a possible mechanism of atherosclerotic calcification is that vascular calcification is the result of a time- and plaque-stage–restricted activation of proteins such as BMP-2, BMP-4, OPN, and ON that overrules the continuous expression of inhibitory proteins such as MGP, OC, and BSP.

**Inhibitors of Calcification (MGP, OC, and BSP)**

Analysis of knockout mice has shown that both vitamin K-dependent, γ-carboxyglutamic acid (Gla)–containing proteins MGP and OC are inhibitors of calcification. MGP is found in bone, in the normal and atherosclerotic vessel wall, and in serum of patients with diabetes. Mice that lack MGP develop to term but die within 2 months of birth as a result of arterial calcification, which leads to blood vessel rupture. Serum OC is used as an early marker of bone turnover and is increased in women with aortic atherosclerosis. OC-deficient mice exhibit an increased bone formation but have normal blood vessels. MGP and OC protein are present in calcium deposits in advanced human lesions, which corroborates our results.

BSP is a secreted glycoprotein and contains an Arg-Gly-Asp (RGD) sequence. It is expressed in highly proliferating marrow stromal cells and cementoblasts and might be implicated in the preferential seeding and growth of metastatic cells in bone. Elevated serum BSP is found in patients with ankylosing spondylitis. However, the proposed function of BSP as a regulator of bone mineralization has not yet been confirmed in vivo. The potential of BSP to nucleate hydroxyapatite suggests that this protein may act as an activator of calcification. However, in the present study, continuous immunoreactivity of BSP during all stages of human atherosclerosis was observed, and this may suggest that BSP is also involved in the inhibition of arterial calcification.

**Activators of Calcification (BMP-2, BMP-4, OPN, and ON)**

Two members of the transforming growth factor-β superfamily, BMP-2 and BMP-4, are secreted signaling molecules present in bone tissue. Individual BMPs are prominent at many sites during embryonic development and organogenesis. BMP-2 can induce ectopic bone and cartilage formation in adult vertebrates. Administration of recombinant BMP-2 in animals results in an enhancement of fracture repair, and BMP-2 is currently being evaluated in clinical studies.

BMP-4 plays an important role in the onset of human endochondral bone formation, and a reduction in BMP-4 expression is associated with a variety of bone diseases.
Expression of BMP-4 can be stimulated by antiestrogens but not by estrogens or other steroid hormones. BMP-4 is expressed in human fetal osteoblast cells, and its mRNA level is increased during differentiation of ameloblasts and odontoblasts into teeth. Although BMP-2 mRNA expression was reported to be present in vascular SMCs of advanced human atherosclerotic plaques, we are the first to show where BMP-2 and BMP-4 proteins are localized during atherogenesis.

OPG is a naturally occurring protein related to the tumor necrosis factor receptor family, is an inhibitor of osteoclastogenesis. OPG and OPGL, also known as RANKL, is a membrane-bound ligand expressed by bone marrow stromal cells and is a stimulator of osteoclastogenesis. In bone, osteoblasts/stromal cells regulate osteoclast formation by the production of cytokines like OPG and OPGL. Estrogens suppress OPGL-induced osteoclast differentiation, whereas prostaglandin E2 induces expression of OPGL. OPGL binds to RANK, a transmembrane receptor on hemopoietic osteoclast precursor cells. OPGL is also present in bone marrow stromal cells, osteosarcoma cells, odontoblasts, ameloblasts, and pulp cells; in tumors associated with bone lysis; and in patients with rheumatoid arthritis. Mice with a disrupted OPGL gene show severe osteopetrosis and defects in early differentiation of T and B lymphocytes. There are no data available regarding the vasculature of these mice. In the present study, OPGL was only present in the extracellular matrix surrounding the calcium mineral deposits of the plaques. This suggests that OPGL is involved in the regulation of early mineralization in atherosclerotic lesions.

In contrast to Schinke and Karsenty, who postulated that vascular calcification is a passive process that requires active inhibition, the major conclusion derived from our data is that atherosclerotic calcification is an active process regulated by inhibitors and activators of calcification and bone formation. According to our data, inhibitor proteins continuously prevent calcification, whereas the restricted presence of activators provides an imbalance, finally resulting in atherosclerotic calcification. Whether bone structure formation in the arterial wall is also mediated by changes in osteoclast formation remains to be elucidated.

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Modulators of Osteoclastogenesis (OPG and OPGL)
OPG, a naturally occurring protein related to the tumor necrosis factor receptor family, is an inhibitor of osteoclast formation. OPG is present in bone marrow stromal cells, osteoblast-like cells, and osteosarcoma cells; in odontoblasts, ameloblasts, and pulp cells in teeth; in (pre-)osteoblasts and lining cells in bone; in endothelial cells; and occasionally in osteocytes. OPG-deficient mice exhibit a decrease in total bone density with a high incidence of bone fractures. These mice also exhibit medial calcification of the aorta and renal arteries. This corresponds with the results of a recently published study that showed that progression of atherosclerotic calcification is associated with increased bone loss in women during menopause. Recently, the results of the first clinical trial with OPG supported its potential as a therapeutic agent for osteoporosis. These results also suggest that OPG might play a role in the association between osteoporosis and vascular calcification. OPG also blocks pain-related behavior in mice with bone cancer, and it may provide an effective treatment against pain in human bone cancer. The presence of OPG in the borders of bone structures harmonizes with its function as an inhibitor of bone resorption, most likely by inhibition of osteoclastogenesis. No immunoreactivity with OPG could be demonstrated in or around calcified areas of the vessel wall, which might coincide with the arterial calcification in OPG-deficient mice.

OPGL, also known as RANKL, is a membrane-bound ligand expressed by bone marrow stromal cells and is a stimulator of osteoclastogenesis. In bone, osteoblasts/stromal cells regulate osteoclast formation by the production of cytokines like OPG and OPGL. Estrogens suppress OPGL-induced osteoclast differentiation, whereas prostaglandin E2 induces expression of OPGL. OPGL binds to RANK, a transmembrane receptor on hemopoietic osteoclast precursor cells. OPGL is also present in bone marrow stromal cells, osteosarcoma cells, odontoblasts, ameloblasts, and pulp cells; in tumors associated with bone lysis; and in patients with rheumatoid arthritis. Mice with a disrupted OPGL gene show severe osteopetrosis and defects in early differentiation of T and B lymphocytes. There are no data available regarding the vasculature of these mice. In the present study, OPGL was only present in the extracellular matrix surrounding the calcium mineral deposits of the plaques. This suggests that OPGL is involved in the regulation of early mineralization in atherosclerotic lesions.

References


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