Molecular regulation of vascular calcification

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Molecular regulation of vascular calcification

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Molecular regulation of vascular calcification

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List of abbreviations

Ac-LDL	acetylated low density lipoprotein
ALP	alkaline phosphatase
ApoE	apolipoprotein E
ASMA	α -smooth muscle actin
BAP	bone alkaline phosphatase
BCIP	5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt
Bcl-2	B-cell leukemia 2
BMP	bone morphogenetic protein
BSP	bone sialoprotein
Cbfa 1	core binding factor alpha subunit 1
CFDP-1	craniofacial developmental protein 1
Cktsf1-b1	cysteine knot superfamily 1
CRTL-1	cartilage linking protein 1
DIG	digoxigenin
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EVG	Elastica von Giesson
Gla	γ -carboxyglutamate
HE	hematoxylin and eosin
HDL	high density lipoprotein
kDa	kilo dalton
LDL	low density lipoprotein
MGP	matrix Gla protein
MM-LDL	minimally modified low density lipoprotein
mRNA	messenger ribonucleic acid
MS	Mönckeberg's sclerosis
NBT	nitro-blue tetrazolium chloride
OC	osteocalcin
OPN	osteopontin
ON	osteonectin
OPG	osteoprotegerin
OPGL	osteoprotegerin ligand
OxLDL	oxidized LDL
PBS	phosphate buffered saline
pfu	plaque forming unit
PTHrP	parathyroid hormone related peptide
RANK	receptor activator of nuclear factor- κ B
RANKL	receptor activator of nuclear factor- κ B ligand
RGD	Arg-Gly-Asp
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	standard error of the mean
SMC	smooth muscle cell
SPARC	secreted protein, acidic and rich in cysteine
TRAP	tartrate resistant acid phosphatase
TBS	tris buffered saline
TGF β	transforming growth factor β
TNF	tumor necrosis factor
VSMC	vascular smooth muscle cell

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Chapter 1

General introduction

1 Atherosclerosis

1.1 Pathogenesis of atherosclerosis

Atherosclerosis is a disease of the large arteries, and is the primary cause of myocardial infarction, stroke and ischemia of the extremities. In the USA, Europe and Japan, it is the underlying cause of about 50% of all deaths¹. Risk factors for atherosclerosis can be grouped into factors with a genetic compound, and those that are largely environmental². Factors with a genetic compound are elevated levels of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL)³, reduced levels of high-density lipoprotein (HDL)⁴, elevated lipoprotein (a)⁵, elevated blood pressure (hypertension)^{3,6}, elevated homocysteine⁷, family history⁸, diabetes mellitus and obesity³, elevated haemostatic factors (e.g. fibrinogen)³, depression⁹, behavioral traits⁹, gender (male)¹⁰, systemic inflammation¹¹ and metabolic syndrome¹². Environmental risk factors are high-fat diet³, smoking³, low anti-oxidant levels¹³, lack of exercise³ and infectious agents¹⁴. Among these genetic and environmental risk factors, elevated levels of serum cholesterol are probably unique in being sufficient to drive the development of atherosclerosis in humans and experimental animals, even in the absence of other known risk factors¹⁵.

Atherosclerosis starts silently at the first decade of life, progresses with age and mainly affects large arteries. Large arteries consist of three layers (*fig. 1.1*), the tunica intima, tunica media and tunica adventitia and atherosclerosis manifests in the inner layer (intima) of these large arteries. It is a progressive disease characterized by several stages. Atherosclerosis begins with activation of the endothelium of the normal vessel wall, including an alteration in its permeability. Low-density lipoproteins become entrapped

Figure 1.1 morphological characteristics of the vessel wall

within the vessel wall and undergo oxidative modification. Meanwhile, activated endothelial cells produce molecules to attract inflammatory cells.

Monocytes are recruited from the bloodstream and become macrophages. These macrophages are responsible for the uptake of oxidized lipids to form foam cells¹, a characteristic feature of the early lesion, the intimal xanthoma or fatty streak. A subpopulation of smooth muscle cells (SMCs) with a synthetic phenotype migrates towards the intima, where they increase in number, produce extracellular matrix components and form a fibrous cap. Highly oxidized LDL has been shown to be toxic to macrophages¹⁶. With the death of some macrophages, lipid droplets are released and phagocytized by smooth muscle cells, producing smooth muscle foam cells¹⁷. Both macrophage and smooth muscle cell derived lipid filled foam cells have the ability of undergoing necrosis and apoptosis. These necrotic or apoptotic cells can form a nidus for calcification. When the fibrous cap becomes thinner, the plaque is more vulnerable for rupture and thrombosis which may cause acute and near-total occlusion leading to acute clinical complications of myocardial infarction and stroke^{15,18,19}.

Whether the presence of calcification in atherosclerotic plaques increases the appearance of plaque rupture is until now not clear. Recently, two major discussions have dominated the field of vascular calcification. The first focuses on the effect of calcification on plaque stability, the second on the question whether vascular calcification is a passive or an active regulated process. In this thesis we will concentrate on the molecular regulation of vascular calcification and thus on the latter theme.

1.2 Atherosclerotic classification (Virmani)

In this thesis we use the plaque classification proposed by Virmani *et al.*²⁰. This is a rather simple classification that is consistent with the classification proposed by the American Heart Association (AHA)²¹, containing categories of plaque types that are easy to use, and deal with a wide array of morphological variations. Typical examples of these lesions are given in *figure 1.2* (adapted from Virmani *et al.*)²⁰.

Intimal thickening (fig. 1.2a)

This lesion type may occur already soon after birth. The intima becomes thicker mainly due to smooth muscle cells in a proteoglycan rich matrix. Intimal thickening is a pre-atherosclerotic lesion that is able to regress.

Intimal xanthoma or fatty streak (fig. 1.2b)

Like intimal thickening, intimal xanthoma (also known as fatty streak) may occur soon after birth and is known to regress. "Xanthoma" is a general pathological term that describes the accumulations of fat-laden macrophages. Intimal xanthoma primarily

consist of accumulations of macrophage-derived foam cells, T-lymphocytes, and varying degrees of smooth muscle cells in the intima of a large artery.

Pathological intimal thickening (fig. 1.2c)

Pathological intimal thickening is a poorly defined entity sometimes referred to as an "intermediate lesion". True necrosis is not apparent, and there is no evidence of cellular debris. Some lipid may be present deep in the lesion, but dispersed. The fibrous cap overlying areas of lipid is rich in smooth muscle cells in a proteoglycan-rich matrix. Some scattered macrophages and lymphocytes may also be present in the fibrous cap.

Fibrous cap atheroma (fig. 1.2d)

The core of this lesion is true necrotic, containing cholesterol esters, free cholesterol, phospholipids, and triglycerides. The fibrous cap consists of smooth muscle cells in a proteoglycan-collagen matrix, with a variable number of macrophages and lymphocytes. The media underneath the plaque is often thin.

Thin fibrous cap atheroma (fig. 1.2e)

This lesion is a look-a-like of the fibrous cap atheroma, but contains a thin fibrous cap infiltrated by macrophages and lymphocytes with rare SMCs.

Plaque rupture (fig. 1.2f)

Ruptured plaques are thin fibrous cap atheromas with luminal thrombi. These lesions contain an extensive necrotic core with large numbers of cholesterol crystals and a thin fibrous cap infiltrated by foamy macrophages and some T-lymphocytes. The fibrous cap is thinnest at sites of rupture and consists of a few collagen bundles and some smooth muscle cells. The luminal thrombus is in communication with the lipid-rich necrotic core.

Calcified nodule (fig. 1.2g)

Calcified nodules are plaques with luminal thrombi showing calcific nodules protruding into the lumen through a disrupted thin fibrous cap. There is absence of endothelium at the sites of the thrombus, and inflammatory cells (macrophages, T-lymphocytes) are absent.

Fibrocalcific plaque (fig. 1.2h)

Fibrocalcific plaques are referred to as "stable" lesions in the literature. These lesions are predominantly composed of dense collagen containing a few scattered smooth muscle cells and inflammatory cells. When present, the necrotic core is small. These lesions are often heavily calcified and previous hemorrhage and/or thrombosis is difficult to determine.

adapted from Virmani et al.

Figure 1.2 Classification of atherosclerotic plaques. Full color figures see page 141.

1.3 Atherosclerotic calcification

Vascular calcification

Vascular calcification refers to the deposition of calcium phosphate mineral, most often hydroxyapatite, in the vessels of many different tissues. Vascular calcification often occurs in patients with chronic renal failure (kidney), Alzheimer disease and Down syndrome (brain), chronic hemodialysis patients (kidney), and in cardiac patients (heart valves). Vascular calcification in large arteries occurs at two anatomic sites, in the intima or in the medial layer of these vessels. Calcification of the media is referred to as Mönckeberg's sclerosis (MS) and occurs commonly in aged and diabetic individuals²² and in patients with chronic kidney disease²³ (*table 1*). Mönckeberg's sclerosis appears to be a strong predictor of cardiovascular mortality in diabetic patients²⁴, and is independent of atherosclerosis²⁵. However, vascular calcification in the intima is invariably associated with atherosclerosis. Disturbances in lipid metabolism, male sex, and hypertension aggravate atherosclerotic calcification (*table 1*). Atherosclerotic calcification is not restricted to large arteries, and can also occur in the leaflets of aortic valves.

Atherosclerotic calcification

Calcification is a prominent feature of advanced atherosclerosis and is located in the intima of the large arteries. Calcification in atherosclerotic plaques decreases vessel elasticity, augments plaque brittleness, and leads to increased plaque rupture during angioplasty procedures²⁶⁻²⁹. Although atherosclerotic calcification has been associated with increased risk of stroke, myocardial infarction and death^{26,30}, other reports described the opposite^{31,32}.

The main controversy is whether calcification stabilizes or destabilizes the plaque. Demer *et al.* argued that the presence of a soft plaque, with a point of weakness induced by inflammation adjacent to an area of calcification, predisposes the plaque to rupture.

Table 1. Risk factors of vascular calcification.

Risk factor	Atherosclerotic calcification (intimal calcification)	Mönckeberg's calcification (medial calcification)
Age	Yes	Yes
Hypertension	Yes	Reciprocal (medial lesions worsen blood pressure)
Hypercholesterolemia	Yes	No
Male	Yes	No
Diabetes mellitus	Yes	Yes
Chronic kidney disease	No	Yes

Plaque rupture occurs mainly at the interfaces of rigid and compliant structures such as between calcium mineral deposits and the normal arterial wall or the fibrous plaque³³. However, Virmani *et al.* described that calcified area is greatest in healed ruptures, followed by fibro-atheroma, thin cap atheroma, plaque hemorrhage, fibrous plaque, plaque rupture and total occlusion. Although calcification is associated with a large lipid core size, calcification is more often seen in areas overlying collagenous plaques³⁴.

Although its association with atherosclerosis has been noted for centuries^{35,36}, the regulation of vascular calcification has been largely ignored, because of the prevailing view that it was merely a degenerative, passive process of calcium phosphate precipitation that only occurs in end-stage atherosclerosis and simply signifies dying cells.

Recently, by the discovery that bone-associated genes can be expressed in the vessel wall, it has become clear that vascular calcification may not be a passive process, but may be actively regulated³⁷⁻³⁹. Early lesions of young adults have been found to already contain small aggregates of crystalline calcium within the lipid core^{21,40}. The regulation of atherosclerotic plaque calcification has been suggested to share similarity to skeletogenesis. This hypothesis has partly been corroborated by the presence of osteogenic features in plaques^{32,41}.

Aortic valve calcification

Calcification of the tricuspid aortic valve is the most common cause of aortic valve disease in older patients, and is located in the fibrotic layer of aortic valve leaflets. The cause of aortic valve calcification is largely unknown, but typical morphological characteristics associated with calcification in aortic valves are associated with atherosclerotic calcification⁴². Vascular calcification is considered to be a risk factor for valvular calcification⁴³ and recently published data indicate that aortic valve calcification is indeed associated with increased risk of cardiovascular events including stroke and cardiac death⁴³⁻⁴⁵. Moreover, valvular and vascular calcifications are both considered to share similarities with skeletal bone formation⁴⁶.

The composition of a calcified plaque

According to the Virmani classification²⁰, atherosclerotic plaques containing calcium mineral deposits or other osteogenic structures are classified as calcified nodule or as fibrocalcific plaque. The osteogenic elements in calcified plaques can be bone cells, cartilage, calcium mineral, lamellar bone, nucleation sites (matrix vesicles) and bone matrix regulatory proteins. Also, collagen fibrils, mitochondria, and lipids are present in the vessel wall^{21,47,48}.

Calcium mineral within atherosclerotic plaques is known to be hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$, which is the same mineral as is found in bone^{49,50}. Also in the plaque

osteogenic cells like osteoblasts, osteocytes, chondrocytes and osteoclasts are necessary for the formation and degradation of calcium mineral, cartilage and lamellar bone. The origin of these cells in the plaque is not clear. One of the theories is that they are derived from differentiation of vascular cells into chondrogenic and/or osteogenic cells⁵³. One of the most widely used bone differentiation markers is the bone specific isoenzyme alkaline phosphatase (BAP)^{51,52}, and the expression of this marker is enhanced in calcified atherosclerotic plaques⁵³. BAP in osteoblastic differentiation is believed to catalyze the hydrolysis of monophosphate ester at sites of mineralization. The cleaved ionic phosphate groups are incorporated into calcium phosphate in the form of hydroxyapatite mineral⁵⁴. During and after osteoblastic differentiation these cells produce several bone matrix regulatory proteins to induce or inhibit mineralization, cartilage and bone formation. It is well established that lipids are associated with mineralization in bone and other calcified tissues⁵⁵⁻⁵⁷. It has been postulated that lipids are involved in the nucleation of hydroxyapatite mineral. This nucleation is accomplished by specialized structures termed matrix vesicles. Matrix vesicles are membrane bound vesicles that are produced by budding from chondrocytes, osteoblasts, and odontoblasts and contain the necessary calcium-binding proteins and phosphatases for nucleation of hydroxyapatite. Like skeletal bone, human calcified atherosclerotic plaques contain matrix vesicles, which are key elements in organized calcium phosphate crystal formation⁴⁸. Matrix vesicles are thought to initiate calcification by forming bone and mineralizing cartilage⁵⁸. Initial hydroxyapatite crystal deposition occurs along the inner surface of these matrix vesicles⁵⁹. Matrix vesicles-like structures have also been found in calcified arteries and heart valves^{47,60-62}. Kockx *et al.*⁶³ showed that in advanced carotid atherosclerotic plaques, matrix vesicles are derived from vascular smooth muscle cells. They contain BAX, a pro-apoptotic protein of the Bcl-2 family, suggesting that they may be remnants of apoptotic cells. It is thought that cell death may lead to matrix vesicle generation^{58,64}, and Hashimoto⁶⁵ recently demonstrated that also chondrocyte apoptotic bodies have similarities with matrix vesicles. The cytotoxicity of oxidized lipids, which causes cell membrane "blebbing" might induce formation of matrix vesicles⁶⁶.

2 Osteogenesis and vascular calcification

Mineralization of bones and teeth is an exquisitely regulated, cell-mediated process in which the tissue extracellular matrix is embedded in crystalline calcium phosphate deposits. This process gives rise to hard tissues endowed with the mechanical properties required for their normal physiological functions. In contrast, mineralization of soft tissues occurs under pathological conditions.

Fetal bone has a woven structure, which is replaced by a highly ordered structure called lamellar bone. Two mechanisms of bone formation are intramembranous and endochondral ossification. Intramembranous ossification is seen in the craniofacial skeleton, where mesenchymal cells directly become bone matrix producing osteoblasts that form trabecular bone. In contrast, endochondral ossification is a multiple step process. Mesenchymal cells become chondroblasts. These chondroblasts form a hyaline cartilage precursor in the shape of the finally formed bone. Other mesenchymal cells differentiate and make a perichondrium. Central cartilage cells become hypertrophic and no longer divide. The surrounding matrix becomes basophilic and then calcifies. This mineralization may be triggered by the seeding action of calcium-rich matrix vesicles released from chondrocytes. Perichondral cells become bone-matrix producing osteoblasts. Finally, cartilage is replaced by lamellar bone surrounded by a layer of osteoid (non-mineralized matrix). Both mechanisms of ossification, intramembranous and endochondral are able to induce lamellar bone formation within the atherosclerotic plaque. Even within the same plaque, both processes can occur.

The major extracellular matrix protein of bone is type I collagen. The main mineral is hydroxyapatite. The mineral phase comprises 60% of the total bone weight. Glycosaminoglycans, collagen, lipids, cells and water are the remaining constituents of bone.

Mesenchymal stromal cells have the ability to acquire characteristics of several more differentiated cells, like osteoblasts⁶⁷, and are also able to transdifferentiate to myofibroblasts in vitro^{68,69}. In the intima of mice with arterial atherosclerosis SMC-like cells, originating from hemopoietic stem cells were identified^{70,71} which suggests that osteogenic cells are derived from stem cells.

Active calcification can only occur in the presence of bone matrix regulatory proteins. A series of reports provided evidence that several osteogenic bone matrix regulatory factors are expressed in atherosclerotic lesions^{37,72,73} and that the process resembles embryonic bone development^{46,47}.

The first osteo-inductive protein found in atherosclerotic plaques was BMP-2⁷². Since then, several groups described bone matrix regulatory proteins in atherosclerosis, and there is evidence that different cell types present in the plaque are able to produce these proteins^{37,73}. These cell types are vascular smooth muscle cells, macrophages, mast cells or pericytes. They are able to produce matrix Gla protein (MGP)⁷⁴, osteopontin (OPN)^{73,75-78}, bone sialoprotein (BSP)⁷⁸, osteonectin (ON)^{77,78}, alkaline phosphatase (ALP), tumor necrosis factor- α (TNF α)⁷⁹, bone morphogenetic protein 2 (BMP-2)⁷², core binding factor 1 (Cbfa1)⁸⁰, osteocalcin (OC)^{77,78,81}, osteoprotegerin (OPG)⁸²⁻⁸⁵, receptor activator of NF- κ B ligand (RANKL)⁸⁶, type I collagen^{77,87,88}, and fibronectin^{89,90}.

3 Atherosclerotic calcification & osteoporosis

Osteoporosis is characterized by a reduced bone mass and disturbed microarchitecture, which increases the susceptibility to fractures⁹¹. Osteoporosis mainly occurs in postmenopausal women and in elderly women and men. Bone loss is caused by a relative excess of bone resorption over bone formation.

Epidemiologically osteoporosis is associated with both atherosclerosis and vascular calcification⁹²⁻⁹⁸. In osteoporotic patients, the skeletal bone loss occurs at the same time as the formation of bone in the atherosclerotic arterial wall⁹⁹. In rodents, vascular calcification and osteoporosis co-exist under at least three conditions: deficiency of osteoprotegerin¹⁰⁰, deficiency of dietary essential fatty acids⁴³ and hyperlipidemia⁹⁹. The factors that are responsible for this apparent paradox of promotion of vascular calcification and bone formation and osteolysis of bone are still unclear.

Another similarity between atherosclerosis and osteoporosis is found in the working mechanism of the drugs used in the treatment of osteoporosis (bisphosphonates) and atherosclerosis (statins). Both drugs act on the mevalonate pathway (*fig. 1.3*), by inhibiting different steps in the pathways that branch from the synthesis of cholesterol from acetyl CoA. Statins act early in the mevalonate pathway by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, which blocks the conversion of HMG CoA to mevalonate. Mundy and colleagues¹⁰¹ described the bone forming potential of statins, by showing that statin treatment increases the synthesis of BMP-2, causing osteoblast proliferation, maturation and bone formation. Treatment with statins reduces the progression of coronary artery calcification¹⁰² and in atherosclerotic patients, statins reduce cardiovascular events and increase plaque stability¹⁰³. Previous observations in bone tissue¹⁰¹ and in murine embryonic stem cells¹⁰⁴ have shown that statins can induce BMP-2 expression, while Emmanuele *et al.* showed that statins induce BMP-2 expression in human VSMC¹⁰⁵. Although statins induce BMP-2 expression, treatment with statins has been shown to reduce vascular calcification in humans and ApoE3*Leiden mice¹⁰⁶⁻¹⁰⁸, effects that are associated with reduced serum lipid levels. Bea *et al.* showed a significant reduction in the frequency of calcification in innominate arteries of ApoE-/- mice independently of lipid lowering¹⁰⁹. Aortic valve calcification can also be reduced by statin treatment as described by Shavelle *et al.*¹¹⁰. Four mechanisms of action of statins can be used to explain the paradox of the induction of BMP-2 and the reduction of vascular calcification. Lipophilic statins can directly increase the production of BMP-2 and thus induction of osteoblast differentiation. These effects of statins are not equal on bones and plaques because of their different sensitivity for BMP-2. This is also true for the second mechanism of action where statins inhibit G-protein prenylation in the mevalonate pathway resulting in a reduction of both osteoclast activity and osteoblast apoptosis. The third and fourth mechanism

Figure 1.3. Mevalonate pathway. Adapted from Cummings et al.

of action both has effect on the progression of atherogenesis, e.g. reduced LDL cholesterol and markers of inflammation in the circulation. The reduced progression of atherogenesis in a experimental set up with controls that develop advanced atherosclerotic plaques, results in a reduction of vascular calcification.

Further, the currently approved statins for lowering cholesterol are not the most effective drugs for increasing bone mass. High doses might stimulate bone formation more effectively¹¹¹.

Nitrogen containing bisphosphonates interfere with protein prenylation (attachment of a lipid prenyl groups to a protein) by inhibiting the synthesis of farnesyl pyrophosphate (geranylgeranyl-PP). Prenylation is necessary for the proper function of small glutamyl transpeptidases (GTPases) in the osteoclast. These signaling proteins, when anchored in the osteoclast membrane by a lipid prenyl group, regulate osteoclast cell processes, including cytoskeleton organization, vesicle transport, membrane ruffling, and apoptosis.

Although statins and bisphosphonates act in the same general pathway, they may have different effects on bone. By inhibiting osteoclast function, bisphosphonates reduce bone resorption and do not stimulate bone formation¹¹². Conversely, statins seem to increase bone formation, although the effect of statins on bone resorption is not known.

4 Atherosclerotic calcification & lipids

Lipids are associated with mineralization in bone and other tissues^{55,57}. Lipid accumulation is clearly associated with atherosclerotic calcification¹¹³. Calcification colocalizes with cholesterol crystals in human atherosclerosis¹¹⁴, is present in the intima of cholesterol-fed rabbits¹¹⁵ or in ApoE-/- mice¹¹⁶, and vascular calcification co-exists with hyperlipidemia.

Lipids have been postulated to be involved in the nucleation of hydroxyapatite mineral, accomplished by matrix vesicles (phospholipid-enriched membrane bound organelles found in the extracellular matrix of calcified tissues)⁵⁹. Cholesterol is present in calcified granules in atherosclerotic plaques, suggesting a potential role for cholesterol and other associated lipids in the nucleation of apatite within these lesions¹¹⁴. Also high tissue lipid content facilitates mineralization¹¹⁷. Watson *et al.* demonstrated that hydroxycholesterol stimulates vascular cell-mediated calcification in vitro¹¹⁸. Hsu showed that cholesterol directly enhanced the ability of vesicles to calcify, providing additional mechanisms of cholesterol-induced vascular calcification¹¹⁹. However, the exact role of lipids in the mineralization process is unknown¹²⁰.

Coronary calcification and aortic valve calcification progress more rapidly in subjects with levels of LDL > 130 mg/dL⁴³, and successful lowering of cholesterol levels with lipid-lowering agents significantly reduces the progression of coronary calcification¹⁰⁶. Oxidized LDL (OxLDL) and acetylated LDL (AcLDL) induce calcification of vascular cells in vitro, by inhibiting their phagocytosis potency¹²¹. HDL is capable of reducing mineralization even when this mineralization is induced by minimally modified LDL (MM-LDL) or cytokines. However, when minimally modified, HDL (MM-HDL) can induce mineralization of vascular cells¹²².

5 Regulation of calcification

Bone morphogenetic proteins

The idea that the regenerative capacity of bone may reside within the bone itself came from the work of Urist¹²³. He demonstrated that isolated adult bone extracts are able to induce ectopic endochondral bone formation in rats. Urist called this extract bone morphogenetic protein, although it was not clear which proteins were responsible for the osteoinductive activity.

It is now evident that bone morphogenetic proteins (BMPs) belong to the large multigene transforming growth factor β (TGF β) super family. Members of the BMP subfamily consist of seven conserved cysteine residues in the mature carboxy-terminal portion of the protein, where the BMPs have their active site. Mature BMPs are

synthesized as large intracellular precursor molecules that are processed to 30 kDa molecular weight dimers before secretion. Although the osteoinductive capacity of many BMPs has been proven, BMP-2, -4, -7, and -9 have been shown to be bone inductive molecules. Interestingly heterodimers of BMP-2/7 and 4/7 were found to be more potent at inducing bone and cartilage formation than the corresponding homodimers^{124,125}. Bone morphogenetic proteins are known to be potent bone inducers that act through specific BMP receptors located on the cell surface of responding mesenchymal cells and promote proliferation and differentiation of these cells into the osteoblastic lineage¹²⁶⁻¹²⁸, and initiate endochondral bone formation.

BMP-2 strongly stimulates adipogenic differentiation of murine mesenchymal precursor cells, and is found in breast tumor cells. Recombinant human BMP-2 has the potential to be used to stimulate bone gap healing in the craniofacial complex in dogs, and induces cell-cycle arrest in the G1 phase and apoptotic cell death of mouse hybridoma cells.

BMP-4 plays an important role in the onset of endochondral bone formation in humans, and a reduction in expression of BMP-4 has been associated with a variety of bone diseases such as fibrodysplasia ossificans. BMP-4 can be stimulated by anti-estrogens, but not by estrogens or other steroid hormones. BMP-4 is expressed in human fetal osteoblast cells. The mRNA of BMP-4 is increased during differentiation of ameloblasts and odontoblasts in tooth. It is also present in bone tissue.

BMP receptors

BMP receptors are members of the serine/threonine receptor kinases. These include the receptors for TGF β , activin and inhibin¹²⁹. BMP receptors consist of two types, type I and II, and both receptor types are required for signal transduction¹³⁰. The type I receptors are characterized by the presence of a highly conserved cysteine rich extracellular domain and a unique GS box (characteristic repetition of serines and glycines) of 30 amino acids, preceding the intracellular serine/threonine kinase domain. Type II receptors have a different extracellular cysteine domain, and lack the intracellular GS box. Ligand studies have shown that type II serine/threonine receptors for activin are able to bind BMP-2 and -7, however the binding affinities for BMPs are lower than for activins.

BMP signaltransduction

Signal transduction for BMPs requires both type I and type II receptors. Outside the cell BMP binds to the type II receptor. This type II receptor becomes phosphorylated, binds to and activates the type I BMP receptor (*fig. 1.4*). The receptor complex is internalized and inside the cell this receptor-complex transduces the signal via a group of proteins called Smads. These Smads (1, 5 and 8) are phosphorylated by type I receptor kinase, bind to Smad 4 and translocated into the nucleus, where they regulate the

Figure 1.4 BMP signaltransduction

transcription of target genes. In addition, a number of negative regulators of BMP action exist within the nucleus such as noggin, chordin and follistatin. It is evident that the positive and negative regulation of bone formation cause a tightly controlled and self-limiting mechanism.

Other bone matrix proteins

BMPs are not the only bone matrix regulatory proteins involved in calcification. Other bone matrix proteins include the gamma carboxy glutamic acid (Gla) containing proteins (matrix Gla protein & osteocalcin), RGD containing glycoproteins (bone sialoprotein & osteopontin), the glycoprotein osteonectin, the tumor necrosis factor receptor family member osteoprotegerin and its ligand receptor activator of nuclear factor- κ B ligand.

Matrix Gla protein (MGP) and osteocalcin (OC) contain 5 and 3 Gla residues respectively. MGP is an inhibitor of cartilage mineralization and has a role in regulating chondrocyte maturation and ossification. MGP^{-/-} mice develop severe aortic calcification, and die due to vessel wall rupture. MGP is found in bone, in serum, the normal and atherosclerotic vessel wall, and in patients with diabetes.

Serum OC is used as an early marker in bone turnover. OC is cleaved by plasmin, and found primarily in bone, but also in patients with chronic arthritis and prostate cancer. OC deficient mice have increased bone formation¹³¹.

The OPN gene encodes a protein of 55-65 kDa with a preserved integrin binding RGD (Arg-Gly-Asp) tripeptide¹³². RGD-mediated cell adhesion and proliferation via OPN and BSP is proposed to act through the integrin $\alpha_v\beta_3$ ¹³³. Inhibition of calcification by OPN requires phosphorylation of the protein. OPN can act as both a cytokine and an extracellular matrix protein. It is expressed by chondrocytes, and is found in several tissues such as brain and heart, in calcified and non-calcified arterial lesions, synovial fibroblasts, mitral valves and in psammoma bodies. Also, OPN is able to attenuate atherosclerosis in female ApoE-/- mice¹³⁴. Numerous factors are known to stimulate osteopontin expression at the RNA level in osteoblasts. These factors include ascorbic acid, TGF β , retinoic acid, endothelin, BMP, and vitamin D. Several of these factors are also known to induce alkaline phosphatase and presumably, elevate phosphate levels.

Bone sialoprotein (BSP) is expressed in highly proliferating bone marrow stromal cells. It is present during angiogenesis and might be implicated in the preferential seeding and growth of metastatic cells in bone. BSP is also present in calcified lesions developed at visceral sites including liver, thyroid, lung and adrenal glands, in tooth and in proliferating neoplastic epithelium in buccal pouches of hamsters. Cementoblasts express BSP¹³⁵ and elevated serum BSP is found in patients with ankylosing spondylitis¹³⁶.

Osteonectin (ON), Sparc and BM-40 are different names for the same protein. ON is expressed at high levels in bone tissue, is widely distributed in other tissues and cell types¹³⁷, and is generally associated with tissue remodeling e.g., tissues undergoing morphogenesis, mineralization, angiogenesis, and pathological responses to injury and tumorigenesis. ON deficient mice develop osteopenia, decreased bone formation¹³⁸ and severe cataract formation¹³⁹. After cleavage of the signal sequence, ON is a 32 kDa protein¹⁴⁰. The secreted form is 43 kDa, in part due to the addition of carbohydrates¹⁴¹. The human protein is divided into three distinct domains. The first domain has been implicated in the mineralization of cartilage and bone. This domain can act as an amine acceptor. ON is a predominant glutaminyl substrate (amine acceptor) in the chondrocyte matrix and is co-expressed with transglutaminase in maturing cartilage¹⁴². ON is also found in the basement membrane¹⁴³ where it presumably binds to type IV collagen. This is followed by a second domain, rich in cysteine, that shows sequence homology with cysteine-rich domains. The third domain is the extracellular calcium-binding domain (residues 138-286). It is largely α -helical and contains a two EF-hand motif high affinity calcium binding site. A EF-hand motif is a helix-loop-helix structural motif which is capable of binding calcium ions in the loop region. This third domain also has a binding epitope of moderate affinity for types I and IV collagen¹⁴⁴. Binding to cytokines is a major characteristic of ON. TGF β , which is associated with the rapid remodeling of connective tissues and has been shown to regulate the expression of extracellular

matrix proteins^{145,146}, was demonstrated to augment ON mRNA levels via a posttranscriptional mechanism in human fibroblasts¹⁴⁷. Specific interactions between ON and collagens could result in the remodeling of extracellular matrix. Cells from ON null mice produce significantly diminished levels of type I collagen in comparison to cells from wild-type mice¹⁴⁸.

Osteoprotegerin (OPG) is a member of the tumor necrosis factor receptor superfamily (TNFR). OPG was isolated independently by two laboratories, and synonyms such as osteoclastogenesis inhibitory factor (OCIF), TNF receptor-related molecule-1 (TR1), or follicular dendritic cell-derived receptor (FDCR-1) have been coined. According to the American Society for Bone and Mineral Research Committee, the term OPG is now recommended. The human OPG gene is a single-copy gene that spans 29 kb and is located on chromosome 8. The secreted glycoprotein exists in both a 60 kDa monomeric form and a disulfide-linked homodimeric form of 120 kDa. Both OPG forms were indistinguishable in their specific activity to inhibit osteoclastogenesis. Osteoclast formation is completely inhibited by OPG. 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, osteoblasts and lining cells in bone, in endothelial cells and occasionally in osteocytes and bone marrow. OPG expression is regulated by the osteoblast-specific transcription factor core binding factor 1 (Cbfa1)¹⁴⁹, and decreased by parathyroid hormone related peptide (PTHrP).

Osteoprotegrin ligand (OPGL), now called receptor activator of nuclear factor- κ B ligand (RANKL), is a membrane-bound ligand expressed by bone marrow stromal cells and is a stimulator of osteoclastogenesis. In bone, osteoblasts and bone marrow stromal cells regulate osteoclast formation by the production of cytokines like OPG and RANKL. Estrogens suppress RANKL induced osteoclast differentiation, while prostaglandin E2 induces expression of RANKL. RANKL binds to receptor activator of NF- κ B (RANK), a transmembrane receptor on hemopoietic osteoclast precursor cells. RANKL is present in bone marrow stromal cells, osteosarcoma cells, odontoblasts, ameloblasts, pulp cells, in tumors associated with bone lysis and in patients with rheumatoid arthritis and Paget's disease.

Fetuin-A (α_2 -Heremans-Schmid glycoprotein (Ahsg)) is a high molecular weight, negatively charged member of the cystatin superfamily of cysteine protease inhibitors. This 62 kDa glycoprotein is synthesized in the liver (hepatocytes) and is ubiquitously present in the extracellular space. Ahsg is a systemic inhibitor of ectopic calcification, acts in all extracellular fluids, is not locally restricted and has a high affinity for

hydroxyapatite. The underlying mechanism involves a direct interaction with the mineral phase and the prevention of large crystal formation, since Ahsg is the major protein of a high molecular weight complex including calcium, phosphate and MGP in serum¹⁵⁰. Ahsg has been shown to antagonize TGFs and BMPs¹⁵¹, it can act as an opsonin¹⁵² and quenches the oxidative burst associated with the uptake of apatite crystals by neutrophils. It forms antibody complexes involved in the removal of apoptotic neutrophils¹⁵³. Strikingly, Ahsg deficient mice have no obvious abnormalities although some female Ahsg deficient mice revealed the existence of soft tissue calcification (unpublished observation)¹⁵⁴. Vascular calcification is the most prominent underlying pathological finding in patients with uraemia, and is a predictor of mortality in this population. In serum of patients on haemodialysis, Ahsg concentrations are significantly lower¹⁵⁵. Ahsg reduction is associated with inflammation and links vascular calcification to mortality in patients on dialysis.

6 Hypothesis

As described in the previous paragraphs atherosclerosis is a disease characterized by several stages. One of these stages is the calcified plaque. In this thesis we focus on the question whether vascular calcification is a passive or an active regulated process and how vascular calcification is regulated. Our main hypothesis is that vascular calcification is a tightly regulated process within the vessel wall.

Due to the presence of osteogenic cells, osteogenic structures (like matrix vesicles, cartilage and full-fledged bone) and the discovery that two bone matrix regulatory proteins, OPN and BMP-2 were present in human atherosclerotic plaques, it is known that atherosclerotic calcification has high similarity with bone calcification (e.g. mineralization). Since bone calcification is regulated by more bone matrix proteins than only the two proteins mentioned above, we hypothesized that several other bone matrix proteins are present in atherosclerotic calcification. Furthermore, it has been suggested that aortic valve stenosis is a disease characterized by the presence of atherosclerotic plaques in its leaflets, including plaques in the calcified stage, and that both atherosclerosis and aortic valve sclerosis are different manifestations of the same disease. Based on these data we hypothesized that several different bone matrix regulatory proteins are present in atherosclerotic calcification in both the aortic wall and aortic valve leaflets.

Since BMP-2 is a highly osteo-inductive protein and one of the first bone matrix proteins described in atherosclerotic plaques our third hypothesis was that this protein is an active regulator of calcification in atherosclerotic plaques.

Based upon the observed correlation of osteoporosis and calcified atherosclerotic plaques and the observation that bisphosphonates inhibit bone resorption and are used in the treatment of osteoporosis, we hypothesized that bisphosphonates will reduce atherosclerotic calcification.

7 This thesis

In *chapter 2 and 3*, we describe that several bone matrix regulatory proteins are present in human atherosclerosis in the intima of large arteries and in the fibrotic layer of aortic valve leaflets, where they contribute to the active regulation of calcification. *Chapter 4* describes fast advanced atherosclerotic calcification in a murine vein graft model induced with BMP-2 adenoviral delivery and shows that atherosclerotic calcification is induced by BMP-2. In *chapter 5* we use a nitrogen containing bisphosphonate for intervention in the atherosclerotic calcification process, since osteoporosis and atherosclerotic calcification are correlated. We found that bisphosphonates have no influence on vascular calcification when given in a dose that enhances bone density. In *chapter 6* we summarize and discuss the data presented in the experimental chapters and provide some novel thoughts on the regulation of vascular calcification and on possibilities to intervene in this process.

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Chapter 2

Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques

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Abstract

In this chapter, 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 MGP, OC and BSP in non-diseased aortas and the absence of BMP-2, BMP-4, OPN and ON in non-diseased aortas and early atherosclerotic lesions. When atherosclerotic plaques demonstrated calcification or bone formation, BMP-2, BMP-4, OPN, and ON were upregulated. Interestingly, this upregulation was associated with a sustained immunoreactivity of MGP, OC and BSP. The two modulators of osteoclastogenesis (OPG and RANKL) were present in the non-diseased vessel wall and in early atherosclerotic lesions. In advanced calcified lesions, OPG was present in bone structures, whereas RANKL 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 RANKL during atherogenesis might suggest a regulatory role of these proteins not only in osteoclastogenesis, but also in atherosclerotic calcification.

Introduction

Calcification of the vessel wall is one of the features of atherosclerosis and is by itself considered to be a risk factor for plaque rupture¹⁻⁶. Although recent studies indicate that atherosclerotic calcification is an organised process, exact regulatory mechanisms remain unclear^{1,2,7}. Increasing evidence suggests that atherosclerotic bone formation and calcification shares features with skeletal bone formation and calcification^{1,8-11}, such as chondrocyte and osteoblast differentiation, mineralisation, 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 immunolocalisation of some of these proteins in the human vessel wall has been described before, the available data are rather incomplete and restricted to advanced stages of atherosclerosis. Immunoreactivity of two proteins involved 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

Patients' 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 (e.g. cardiovascular disease and cancer). Surgical specimens were obtained from patients (4 women, 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, CA, USA). A 15% SDS-page gel with 50 µg whole cell extract protein per sample was electrophoresed. Proteins were transferred to a nitrocellulose membrane at 30 Volts. After 1 hour of blocking with 3% bovine serum albumine (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 10 mM Tris-buffered saline pH 7.5 (TBS), 100 mM NaCl and 0.1% Tween, blots were incubated with antibodies directed to either MGP (1:200)¹⁴, OC (1:800, Anawa Trading SA, Wangen, Zürich, Switzerland), BSP (1:500)¹⁵, BMP-2 (1:350, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), BMP-4 (1:350, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), OPN (1:500)¹⁵, ON (1:1000, Zymed laboratories, Inc., San Francisco, CA, USA)¹⁶, OPG (1:1000), or RANKL (1:1000). Anti-mouse, horseradish peroxidase (HRP) (1:1000, Dako, Denmark) labelled, anti-rabbit HRP (1:2000, Cell Signaling Technology, a New England Biolabs company, UK) and anti-goat HRP (1:2500, Dako, Denmark) were used as the secondary antibodies. Specific antibody binding was visualised with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands).

Immunohistochemical staining

Paraffin sections (4 µm) were deparaffinized and washed three times in Tris-buffered saline (TBS) (5 mM Tris-HCl pH 7.5, 140 mM NaCl). Parallel sections were stained with mouse monoclonal antibodies against MGP (1:25), BMP-2 (1:20, Genetics Institute, Inc., Cambridge, MA, USA), goat polyclonal antibodies against BMP-4 (1:25), OPN (1:125), OPG (1:100), RANKL/OPGL (1:75), rabbit polyclonal antibodies against OC (1:50), ON (1:400) and BSP (1:25). For the mouse monoclonal antibodies, biotinylated sheep anti-mouse IgG (1:250, Amersham, Life Science, Little Chalfont, Buckinghamshire, UK) was used as the second antibody, while biotinylated rabbit anti-goat IgG (1:200, Dako, Glostrup, Denmark) or sheep anti-rabbit IgG (1:1000, Dako) were used as secondary antibody for the polyclonal antibodies. After incubation with an alkaline phosphatase-coupled avidin-biotin complex (ABC complex, Dako), antibodies were visualised with an alkaline substrate kit I (Vector SK-5100, Vector Laboratories, Inc., Burlingame, CA, USA). Sections were counterstained with hematoxylin and mounted with coverslips. In negative controls incubation with primary antibody was omitted.

Since immunohistochemical results of specimens derived from autopsy and surgery did not differ, the data are presented as one group.

Immunophenotyping

Double immunohistochemistry was performed using CD68 (Dako, mouse monoclonal antibody) to identify macrophages, and α -smooth muscle actin (ASMA, Dako, mouse monoclonal antibody) to identify vascular smooth muscle cells.

First, MGP, OC, BSP, BMP-2, BMP-4, OPN, or ON were localised with the above described protocol for immunohistochemical staining. Subsequently, the sections were treated with 0.1% pepsin (Boehringer Mannheim GmbH, Mannheim, Germany) and incubated for 45 min, with ASMA or CD68, both diluted 1:500. Biotin-labelled sheep anti-mouse IgG (1:250, Amersham, Life Science) was used as secondary antibody. The avidin-biotin alkaline phosphatase (1:200, Dako) complex in combination with fast blue substrate (0.2% w/v fast blue-BB-salt (Sigma Chemical CO, St. Louis, MO, USA) was used as chromogen. No counterstaining was performed and sections were mounted. Negative controls were treated accordingly without both primary antibody incubation steps.

In situ hybridisation

Sense and antisense digoxigenin (DIG)-labeled RNA probes for MGP, BMP-2, BMP-4, OPN, and ON mRNAs were transcribed from the T7 promoter of pGEM3Z. Hybridization of 4 μ m paraffin embedded sections, and visualization with alkaline phosphatase-coupled anti-DIG antibodies and indolylphosphate-nitroblue tetrazolium (BCIP/NBT) substrate were performed as described earlier¹⁷. Parallel sections were hybridised with anti-sense and sense probes. In the negative controls the probe was not added to the hybridisation mixture.

Results

Validation of antibodies

Western blotting (*fig. 2.1*) showed that BSP protein (35 kDa) was present in early atherosclerotic plaque 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 RANKL (35 kDa) was only detected in the early lesion. Western blotting of both Gla containing proteins (MGP and OC) was not satisfactory because of the highly 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 due to the insolubility of the protein.

Figure 2.1. Western blotting analysis with antibodies directed to bone matrix proteins. Lane 1: intimal xanthoma, lane 2: fibrous cap atheroma and lane 3: fibrocalcific plaque. Fig. 2.1a: blot stained with α -BSP. Fig. 2.1b: blot stained with α -BMP-2. Note: lane 4= fibrocalcific plaque. Fig. 2.1c: blot stained with α -BMP-4. Note: lane 4= fibrocalcific plaque. Fig. 2.1d: blot stained with α -OPN. Fig. 2.1e: blot stained with α -ON. Fig. 2.1f: blot stained with α -OPG. Fig. 2.1g: blot stained with α -RANKL. Fig. 2.1h: blot stained with MGP antibody. Note: lane 4= synthetic MGP.

Human heart, liver and lung tissue was used as negative controls for immunohistochemistry of antibodies directed to MGP, OC and BSP (fig. 2.2). MGP was absent in the hepatocytes, but the vessels in the liver did contain MGP. OC and BSP were absent in human heart.

Pre-treatment of calcified control sections with 3% citric acid resulted in an equal pattern of protein localisation as non pre-treated sections indicating the lack of a-specific binding of the antibodies to calcium deposits.

Non-diseased aorta

Five bone matrix proteins, MGP, OC, BSP, BMP-4, and OPG were present in the non-diseased aorta (fig. 2.3). MGP (fig. 2.4a) and BSP were present throughout the intima and media, including endothelial cells, SMC and elastic fibres. BMP-4, ON, and OPG proteins were highly expressed, but only in the medial SMC. RANKL immunoreactivity was very weak, and confined to the medial SMC, a pattern that was comparable to OPG. OC, which like MGP, is a Gla containing protein, was only expressed in the endothelial cells lining the lumen. Two bone matrix proteins, BMP-2 and OPN (fig. 2.4b) were absent in the non-diseased aorta.

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

Figure 2.2. Immunohistochemical staining. Negative controls for the antibodies directed to MGP, OC and BSP. **Fig. 2.2a:** human liver stained with α -MGP. **Fig. 2.2b and c:** human heart stained with respectively α -OC and α -BSP. Note: positive staining is red. Scale bars in figure 2 represent 50 μ m. Full color figures see page 142.

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 (*fig. 2.3*). In intimal xanthoma lesions MGP, BSP, BMP-4, and ON expression was localised in the medial SMC. The intima showed immunoreactivity of six bone matrix regulatory proteins. MGP and BSP were present in the intimal SMC. Macrophage derived lipid filled foam cells expressed MGP, BSP (*fig. 2.4c*) and OC protein, whereas BMP-2, BMP-4 (*fig. 2.4d*), OPN, ON, OPG, and RANKL were absent. Endothelial cells, lining the lumen expressed OC, BMP-4, OPG and RANKL.

Fibrous cap atheroma

The fibrous cap atheroma is the lesion type in which a collagenous-proteoglycan matrix containing fibrous cap is covering the large lipid core (*fig. 2.3*). Cells present in these lesions are smooth muscle cells (SMC) in the shoulder region of the plaque, foam cells, T-cells and endothelial cells.

Medial smooth muscle cells, underlying the fibrous cap atheroma, showed expression of MGP, BSP, and ON. All nine proteins investigated, showed immunoreactivity in the intima of these lesions. However, the localization of the proteins was diverse. BSP, BMP-2, BMP-4 (*fig. 2.4e*), ON and OPG were expressed by the intimal SMC present in the shoulder regions. Expression of MGP, BSP, OC, BMP-4, OPN, and ON was prominent in the 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 (*fig. 2.4f*) protein, and moderate BSP and BMP-4 immunoreactivity.

Figure 2.3. Histological and morphological characteristics of the plaque types. Panels a, c, e, and g represent H&E stainings, panels b, d, f, and h represent Von Kossa stainings. Fig 2.3a and b: non-diseased aorta. Fig 2.3c and d: intimal xanthoma. Fig 2.3e and f: fibrous cap atheroma. Fig 2.3g and h: fibrocalcific plaque. (i=intima; m=media and c=calcium mineral). Scale bars in figure 3 represent 50 μ m. Full color figures see page 143.

Figure 2.4. Immunohistochemical staining of a non-diseased aorta (a and b), intimal xanthoma (c and d), and fibrous cap atheroma (e and f). Fig. 2.4a and b: non-diseased aorta stained with respectively MGP and OPN. Fig. 2.4c and d: intimal xanthoma stained with respectively BSP and BMP-4. Fig. 2.4e and f: fibrous cap atheroma stained with respectively BMP-4 and ON. (i=intima; m=media and sh=shoulder region). Scale bars in figure 2.4a and b represent 50 μ m, scale bars in figure 2.4c, e and f represent 25 μ m and scale bar in figure 2.4d represents 10 μ m. Full color figures see page 144.

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 (fig. 2.3). All bone matrix proteins examined in this study were highly expressed in these fibrocalcific lesions. Like in the fibrous cap atheroma, the medial smooth muscle cells underlying the fibrocalcific plaque showed only expression of MGP, BSP, and ON, whereas intimal smooth muscle cells 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, OPN and ON. Two types of calcified structures were present in these lesions: calcium mineral deposits and lamellar bone. Around calcium mineral deposits MGP (fig. 2.5a) and OPN (fig. 2.5b) were highly expressed, whereas in the deposits BSP, OC, BMP-2 (fig. 2.5c), BMP-4, OPN and ON showed immunoreactivity. Lamellar bone structures, recognized by the presence of cement lines and osteocytes within bone and osteoblasts lining the bone, showed immunoreactivity of OPN (fig. 2.5d) and ON. BSP, BMP-2, BMP-4, OPN, ON, and OPG (fig. 2.5e) lined the bone structures. OC was the only protein present throughout the bone matrix (fig. 2.5f). RANKL could only be demonstrated in association with the extracellular matrix (ECM) surrounding calcium deposits (fig. 2.5g). 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 (fig. 2.5h).

Figure 2.5. Immunohistochemical staining of a fibrocalcific plaque. Fig. 2.5a: fibrocalcific plaque containing calcium mineral deposition immuno-stained with monoclonal antibody against MGP (red staining). Note: Strong MGP positive staining at boundary of the mineral deposits. Fig. 2.5b: fibrocalcific plaque, containing calcium mineral deposits (c) and bone tissue (b) stained with α -OPN (red staining). OPN positive staining could be demonstrated at the boundary of the calcium mineral and bone structures (arrows). Fig. 2.5c: fibrocalcific plaque, containing calcium mineral deposits immuno-stained with a mouse anti-BMP-2 (red staining). BMP-2 positive staining (between arrows) could be visualised surrounding the highly calcified central area (*). Fig. 2.5d: fibrocalcific plaque, containing bone structures (b) stained with α -OPN. OPN positive staining was seen surrounding the bone structures (arrow), but also in a striping pattern within the bone (arrowhead). Fig. 2.5e: fibrocalcific plaque with bone formation (b) stained with goat anti-OPG. OPG could be demonstrated in the osteoblasts (arrow) lining the bone structures (b) and some stromal cells (arrowhead) in the bone marrow (bm). Fig. 2.5f: fibrocalcific plaque stained with α -OC. Fig. 2.5g: fibrocalcific plaque, containing calcium mineral deposits (c), immuno stained with goat anti-RANKL. RANKL was observed in the extracellular matrix surrounding the mineral deposits (arrow). Fig. 2.5h: fibrocalcific plaque with matrix vesicles stained with α -ON. Scale bars in fig. 2.5a, c and h represent 25 μ m, scale bars in fig. 2.5b, d and f represent 50 μ m, scale bar in fig. 2.5g represents 10 μ m. Full color figures see page 145.

In situ hybridisation

In situ hybridisation 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 a 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 2.6*.

Discussion

In vitro and gene expression studies^{8,11,18-21} 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 summarised in *figure 2.7*. 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 (matrix Gla protein (MGP), osteocalcin (OC) and bone sialoprotein (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^{22,23}. MGP is found in bone, in the normal and atherosclerotic vessel wall¹⁸, and in serum of patients with diabetes¹⁴. Mice that lack MGP develop to term, but die within two months as a result of arterial calcification, which leads to blood vessel rupture^{22,24}. Serum OC is used

Figure 2.6. In situ hybridization with riboprobes directed to MGP, BMP-4, OPN and ON. Fig. 2.6a: MGP in situ hybridisation of an intimal xanthoma. Note: dark purple staining are MGP mRNA positive medial smooth muscle cells (arrow). Fig. 2.6b: MGP in situ hybridisation of a fibrous cap atheroma. Note: arrow indicates an MGP mRNA positive intimal smooth muscle cell. Fig. 2.6c: BMP-4 in situ hybridisation of a fibrocalcific plaque. Arrow indicates a BMP-4 mRNA positive intimal smooth muscle cell in the surrounding of a calcified area of the plaque. Fig. 2.6d: OPN mRNA expression in intimal smooth muscle cells in the surrounding of calcium deposits of a fibrocalcific plaque. Fig. 2.6e: OPN in situ hybridisation of a fibrocalcific plaque. Note: osteocytes and osteoblasts express OPN mRNA. Fig. 2.6f: ON mRNA expression in intimal smooth muscle cells in the surrounding of calcium deposits. Scale bar in fig. 2.6a represents 50 μ m, scale bars in fig. 2.6b, d and f represent 25 μ m, and scale bars in fig. 2.6c and e represent 10 μ m. Full color figures see page 147.

Antibody	Intimal xanthoma	Fibrous cap atheroma	Fibrocalcific plaque		
			Cartilage	Calcium	Bone
MGP					
OC					
BSP					
BMP-2					
BMP-4					
OPN					
ON					

Figure 2.7. Immunoreactivity pattern of bone matrix proteins in human non-diseased aorta, intimal xanthoma, fibrous cap atheroma and fibrocalcific plaques.

The figure represents the immuno-histochemical pattern of the bone matrix regulatory proteins MGP, OC, BSP, BMP-2, BMP-4, OPN, and ON in human atherogenesis. Fibrocalcific plaques were divided into cartilage tissue, calcium deposits and bone tissue, structures that were present in these lesions. MGP, OC and BSP were present in early as well as advanced lesions, while BMP-2, BMP-4, OPN and ON were only present in advanced plaques.

as an early marker of bone turnover²⁵, and is increased in women with aortic atherosclerosis²⁶. OC-deficient mice exhibit an increased bone formation²³, but have normal blood vessels. MGP and OC protein are present in calcium deposits in advanced human lesions²⁷, 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 cells²⁸ 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 ankylosis spondylitis²⁹. However, the proposed function of BSP as a regulator of bone mineralization has not yet been confirmed in vivo³⁰. The potential of BSP to nucleate hydroxyapatite³¹, suggests that this protein may act as an activator of calcification. However, in the present study continuous immunoreactivity of BSP in 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 (bone morphogenetic protein 2 and 4 (BMP-2, BMP-4), osteopontin (OPN), and osteonectin (ON))

Two members of the transforming growth factor β (TGF β) superfamily, BMP-2 and

BMP-4, are secreted signalling 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 vertebrates³². Administration of recombinant BMP-2 in animals results in an enhancement of fracture repair³³, 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 anti-estrogens, but not by estrogens or other steroid hormones³⁴. BMP-4 is expressed in human fetal osteoblast cells, and its mRNA level is increased during differentiation of ameloblasts and odontoblasts into teeth³⁵. Although BMP-2 mRNA expression was reported to be present in vascular SMCs of advanced human atherosclerotic plaques³⁶, we are the first to show where BMP-2 and BMP-4 proteins are localized during atherogenesis.

OPN is an acidic phosphorylated glycoprotein that binds calcium and contains a RGD motif for interaction with the integrin family of cell adhesion molecules. It can act as both a cytokine and an ECM protein³⁷. It is expressed by chondrocytes, and found in several tissues such as brain and heart; calcified and non-calcified arterial lesions³⁸, and in mitral valves³⁹. In OPN mutant mice, embryogenesis occurred normally, and mice were fertile⁴⁰. The mutant mice however had disorganization of matrix and alteration of collagen fibrillogenesis. This phenomenon could also be active in atherosclerosis, although no vascular phenotype was reported in these mice.

ON, a bone glycoprotein also known as SPARC (secreted protein, acidic and rich in cysteine) binds tightly to hydroxyapatite and collagen. ON is a protein widely expressed in different tissues such as teeth and psammoma bodies⁴¹. It is involved in cell matrix interaction, wound repair, angiogenesis, vascular permeability, cataract formation⁴² and carcinogenesis. ON-deficient mice have decreased bone formation and decreased osteoblast and osteoclast surface and number, which leads to a decrease in bone formation and remodeling, with a negative bone balance that causes profound osteopenia⁴³ and severe cataract formation. There are no data available regarding the vasculature of these mice. Immunoreactivity of OPN and ON in advanced human atherosclerotic plaques was described previously²⁷ and the localization of these proteins agrees with our results. Thus, also OPN and ON may act as positive regulators of vascular calcification.

Modulators of osteoclastogenesis (osteoprogenin (OPG) and receptor activator of nuclear factor- κ B ligand (RANKL))

OPG, a naturally occurring protein related to the tumor necrosis factor (TNF) receptor family, is an inhibitor of osteoclast formation⁴⁴. 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 fractures⁴⁵. These mice also exhibit medial calcification of the aorta and renal arteries²⁴. This corroborates with the results of a recently published study, that showed that progression of atherosclerotic calcification is associated with increased bone loss in women during menopause⁴⁶. 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 behaviour in mice with bone cancer and it may provide an effective treatment against pain in human bone cancer⁴⁷. 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.

RANKL, also known as OPGL, 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 RANKL. Estrogens suppress RANKL induced osteoclast differentiation, whereas prostaglandin E2 induces expression of RANKL⁴⁸. RANKL binds to RANK, a transmembrane receptor on hemopoietic osteoclast precursor cells. RANKL is also present in bone marrow stromal cells, osteosarcoma cells, odontoblasts, ameloblasts, pulp cells, in tumors associated with bone lysis and in patients with rheumatoid arthritis⁴⁹. Mice with a disrupted RANKL gene show severe osteopetrosis and defects in early differentiation of T and B lymphocytes⁵⁰. There are no data available regarding the vasculature of these mice. In the present study, RANKL was only present in the ECM surrounding the calcium mineral deposits of the plaques. This suggests that RANKL is involved in the regulation of early mineralisation in atherosclerotic lesions.

In contrast to Schinke and Karsenty, who postulated that vascular calcification is a passive process that requires active inhibition²⁴, 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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Chapter 3

Increased expression of bone matrix regulatory proteins in leaflets of patients with aortic valve stenosis

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Submitted

Abstract

Increasing evidence suggests that valvular calcification is a regulated process with similarities to developing bone. We therefore hypothesized that expression of bone matrix regulatory proteins is enhanced during calcification of aortic valves.

We performed a series of immunohistochemical stainings using antibodies directed to matrix Gla protein (MGP), osteocalcin (OC), bone sialoprotein (BSP), bone morphogenetic protein 2 (BMP-2), bone morphogenetic protein 4 (BMP-4), osteopontin (OPN), osteonectin (ON), osteoprotegerin (OPG) and receptor activator of nuclear factor- κ B ligand (RANKL). Cells producing mRNAs of these bone matrix proteins were identified by non-radioactive in situ hybridization.

MGP, OC, BSP, BMP-2 and BMP-4 were present in both normal and calcified aortic valves, while the immunoreactivity of OPN and ON was restricted to valves containing calcifications or bone formation. Modulators of osteoclastogenesis (OPG and RANKL) were present in fibroblasts, smooth muscle and endothelial cells of both non-calcified and calcified aortic valves. In calcified valves OPG protein was present in chondrocytes, osteoblasts and osteocytes, while RANKL was matrix associated and highly expressed in osteoclasts and some macrophages.

Proteins involved in bone metabolism, calcification and ossification are also present in human calcified aortic valves, suggesting an active and tight molecular regulation of valvular calcification.

Introduction

Recent evidence indicates that aortic valve calcification is a well-regulated process, that involves inflammation and lipid accumulation similar to those seen in human atherosclerosis¹⁻³. Vascular calcification is considered to be a risk factor for valvular calcification⁴ and recently published data indicate that aortic valve calcification is indeed associated with increased risk of cardiovascular events including stroke and cardiac death⁴. Moreover, valvular and vascular calcifications are both considered to share similarities with skeletal bone formation⁵.

Matrix Gla protein (MGP), osteocalcin (OC), bone sialoprotein (BSP), bone morphogenetic proteins 2 and 4 (BMP-2, BMP-4), osteopontin (OPN) and osteonectin (ON) all are involved in the regulation of skeletal bone formation. Osteoprotegerin (OPG) and its ligand (RANKL), which are involved in osteoclastogenesis, are associated with the regulation of skeletal bone remodeling. The observation that osteopontin is present in human aortic valves⁶ and our recently obtained evidence that calcification in human atherosclerosis involves several bone matrix regulatory proteins⁷, lead to the hypothesis that these bone matrix regulatory proteins are also expressed during valvular calcification. To further substantiate this hypothesis, we made an inventory of the mRNA and protein expression of bone matrix regulatory proteins in (non-)calcified human aortic valve leaflets.

Here we showed that all 9 bone regulatory proteins are present in human aortic valve leaflets. MGP, OC, BSP, OPG and RANKL are present in both non-calcified and calcified valves, whereas the protein expression of OPN and ON are restricted to calcified valves.

BMP-2 and -4 are present in endothelial cells of non-calcified valves and their enhanced expression is associated with mineralized structures in calcified valves. We conclude that human aortic valve calcification is an active process that is regulated by several molecular regulators of bone and vascular wall calcification and modeling.

Methods

Patients' characteristics and tissue preparation

Leaflets of tricuspid aortic valves were obtained from patients undergoing surgical aortic valve replacement (n=9) or from autopsy (n=3). Autopsy specimens were obtained from two adult men and one woman, aged 51, 81 and 61 years respectively. The cause of death was cancer (2x) or rupture of an abdominal aortic aneurysm. Surgical specimens were obtained from five adult males and four adult women with an average age of 55 years (range 27-79).

Aortic valve leaflet morphology was evaluated on hematoxylin and eosin (H&E) and Elastica von Giesson (EVG) stained sections. Von Kossa, tartrate resistant acid phosphatase (TRAP) and Thionin staining were used to visualize the presence of calcification, osteoclasts and cartilage respectively. For the identification of osteoclasts,

cartilage and hemopoietic cells we used antibodies against cathepsin K, type II collagen and CD138 respectively (see immunophenotyping).

The specimens were divided into two groups:

- Non-calcified aortic valves (n=6, including 3 autopsy specimens)
- Calcified aortic valves (n=6, all surgical specimens)

Immunohistochemical staining

Paraffin sections (4 μ m) were stained with mouse monoclonal antibodies against MGP (1:25)⁷, or BMP-2 (1:20, Genetics Institute, Inc., Cambridge, MA, USA), goat polyclonal antibodies against BMP-4 (1:25 Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA)⁷, OPN (1:500 LF123; gift from Dr L. Fisher), OPG (1:100 Santa Cruz Biotechnology, Inc.)⁷, or RANKL 1:75 Santa Cruz Biotechnology, Inc.)⁷, or rabbit polyclonal antibodies against OC (1:50, Anawa Trading SA, Wangen, Zürich, Switzerland)⁷, ON (1:400 Zymed Laboratories, Inc., San Francisco, CA, USA)⁷ or BSP (1:25)⁸. Biotinylated sheep anti mouse IgG (1:250, Amersham, Life Science, Little Chalfont, Buckinghamshire, UK), rabbit anti goat IgG (1:200, Dako, Glostrup, Denmark) or sheep anti rabbit IgG (1:1000, Dako) were used as the secondary antibodies. After incubation with an alkaline phosphatase coupled avidin-biotin complex (ABC complex, Dako), antibodies were visualised with an alkaline substrate kit I (Vector SK-5100, Vector Laboratories, Inc., Burlingame, CA, USA), counterstained with hematoxylin and mounted. Negative controls lacked incubation with the primary antibody (*fig. 3.1*). Calcified atherosclerotic plaques were used as positive control for respectively MGP, BSP, BMP-2, BMP-4, ON, OPN, OPG and RANKL. Pre-treatment of calcified control sections with 3% citric acid did not affect the staining, indicating the lack of a-specific binding of the antibodies to calcium deposits⁷ (*fig. 3.2*).

Immunophenotyping

Serial sections were stained with antibodies directed to CD68 (Dako, mouse monoclonal antibody) to identify macrophages, α -smooth muscle actin (ASMA, Dako, mouse monoclonal antibody) to identify vascular smooth muscle cells, desmin (ICN, mouse monoclonal antibody) to distinguish smooth muscle cells from myofibroblasts⁹, cytokeratin 8 (Neomarkers, mouse monoclonal antibody) to distinguish synthetic from contractile smooth muscle cells¹⁰, CD3 to identify T-lymphocytes (Dako, rabbit polyclonal antibody), cathepsin K (Oncogene, monoclonal antibody) for osteoclast identification (*fig. 3.3d*), type II collagen for cartilage identification (*fig. 3.3b*), CD138 for hemopoietic cell identification (*fig. 3.3f*), or OC for osteoblast identification (*fig. 3.3c*). Positive controls for CD68, ASMA, desmin, cytokeratin 8, CD3, and cathepsin K were respectively colon, aorta, heart, breast carcinoma, tonsil, and bone.

Fig. 3.1. Immunohistochemical staining of bone matrix regulatory proteins on calcified valves. Immunohistochemistry of osteocalcin (A), matrix Gla protein (C) and osteopontin (E) and their negative controls (B, D and F) on human calcified aortic valves. Scale bars represent 50 μ m. Full color figures see page 148.

Fig. 3.2. Von Kossa staining and immunohistochemical staining of osteopontin on serial sections of a calcified aortic valve. Serial sections of a human calcified aortic valve stained with Von Kossa (A and B) and osteopontin (C and D) without (A and C) and with (B and D) citric acid pretreatment. Scale bars represent 50 μ m. Full color figures see page 149.

For double immunohistochemistry OC, MGP, BMP-2, BMP-4, OPN, ON or BSP stained sections were incubated for 30 minutes with ASMA (1:500) or pre-treated with 0.1% pepsin (Boehringer Mannheim GmbH, Mannheim, Germany) and subsequently incubated for 30 min, with CD3 or CD68, diluted 1:200 and 1:500 respectively. Biotin-labeled sheep anti rabbit IgG (1:1000) or sheep anti mouse IgG (1:250) were used as secondary antibodies. The avidin-biotin alkaline phosphatase (1:200) complex in combination with fast blue substrate (0.2% w/v fast blue-BB-salt, (Sigma Chemical CO, St. Louis, MO, USA)) was used as chromogen. No counterstaining was performed.

Counting and statistics

Each single stained valve (ASMA, CD68, CD3, desmin or cytokeratin 8) was randomly divided into eight fields of 0.08 cm², which comprise approximately 75% of total valve

Fig. 3.3 Identification of chondrocytes, osteoclasts, osteoblasts and hemopoietic cells in human calcified aortic valves by respectively thionin staining (A), type II collagen (B), osteocalcin staining (C), cathepsin K staining (D), TRAP staining (E) and CD138 staining (F). Scale bar in A represent 50 μm and in B, C, D, E and F 25 μm . Full color figures see page 150.

Fig. 3.4: Histological and morphological characteristics of the human aortic valve.

Fig. 3.4a: H&E staining of a non-calcified aortic valve. The three layers of the valve are the fibrosa (f), spongiosa (s) and ventricularis (v). Panel b represent a valve containing calcified tissue H&E stained. Note (*)=cartilage, b=bone, and bm=bone marrow cavity. Scale bars represent 100 μm (a), 50 μm (b). Full color figures see page 150.

area. All cells (positively stained and total amount) in each field were counted by one investigator (CD). The intra observer variety was <10%. Statistical analysis was performed using the Mann-Whitney U test. Data are presented as means $\pm$ SEM and values of $p < 0.05$ were considered to be statistically significant.

In situ hybridisation

Sense and anti-sense digoxigenin (DIG) labeled RNA probes for MGP, BMP-2, BMP-4, and ON mRNAs were transcribed from the T7 promoter of pGEM3Z, following a protocol described earlier⁷. Parallel sections were hybridised with anti-sense and sense RNA probes. In the negative controls no probe was added to the hybridisation mixture.

Protein extraction and immunoblotting

Protein extracts were prepared from 3 non-calcified and 3 calcified aortic valves by homogenizing the leaflets in 8M urea. 20 μ g protein of each sample was separated by polyacrylamide gel electrophoresis followed by transfer to a nitrocellulose membrane (Protran, Schleicher & Schuell). The membrane was blocked with 3% bovine serum albumin (BSA) and then incubated with rabbit anti-OPN antibody 1:1000 (LF123; gift from Dr L. Fisher) for 1 hour in blocking buffer. After three washes, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling) diluted 1:2000 for 1 hour in blocking buffer. OPN was visualized using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech).

Results

Aortic valve morphology

Calcified structures were found in the fibrosa, a distinct layer of dense collagenous fibrous tissue subjacent to the endothelium of the aortic surface (*fig. 3.4a*). Calcified aortic valves may contain several different structures associated with bone mineralization, like amorphous calcium phosphate, cartilage and lamellar bone structures (*fig. 3.4b*).

Non-calcified aortic valve leaflets contain three major cell types, smooth muscle cells, myofibroblasts and fibroblasts. Smooth muscle cells are essentially localized in the fibrosa, whereas fibroblasts and myofibroblasts are uniquely segregated in the ventricularis, a distinct layer of elastic tissue subjacent to the endothelium of the ventricular surface. Major differences were found in the cellular composition of non-calcified and calcified aortic valves (*table 3.1*). The ventricularis of non-calcified valves (*fig. 3.5a*) contained less α -smooth muscle actin positive cells than calcified valves (*fig. 3.5d*) ($13 \pm 5\%$ and $33 \pm 5\%$ respectively, $p = 0.03$). The vast majority of the ASMA positive cells in the fibrosa were negative for desmin, and can thus be classified as

Table 3.1. Quantification of ASMA, CD3, CD68, and desmin positive cells in non-calcified and calcified aortic valves.

	Non-calcified (n=6)	Calcified (n=6)	p value
ASMA (%)	13±5	33±5	p=0.03*
Desmin (%)	7±6	3±1	p=0.94
CD3 (%)	2±2	19±8	p=0.02*
CD68 (%)	3±1	17±4	p=0.01*
*p<0.05, significant.			

myofibroblasts. In the fibrosa of calcified aortic valves, myofibroblasts were mainly localised around the calcified structures.

There was no difference in percentage of contractile smooth muscle cells (ASMA and desmin positive, cytokeratin 8 negative) between non-calcified valves (7±6%) and the calcified valves (3±1%, p=0.94). Macrophages and T-cells were almost absent in non-calcified aortic valves (*fig. 3.5b and 3.5c*), whereas both cell types were present in the surroundings of calcified structures (macrophages 3±1% versus 17±4%, p=0.01 and T-cells 2±2% versus 19±8%, p=0.02) of the aortic valves (*fig. 3.5e and f*).

Non-calcified valves and bone matrix regulatory proteins

OPN and ON were absent in non-calcified aortic valves, whereas the BMPs showed very low immunoreactivity and were expressed in the fibroblasts and endothelial cells of the ventricularis. Three bone matrix regulatory proteins (MGP, OC, and BSP) showed clear immunoreactivity. MGP was present in the ventricularis and associated with elastic fibres (*fig. 3.6a*), while OC was mainly present in the mesenchymal spongiosa, a thin layer of loose mesenchymal tissue between the fibrosa and the ventricularis (*fig. 3.4a*). BSP was diffusely present in all three layers of the valves, with a high expression in the myofibroblasts (*fig. 3.6b*) of the fibrosa and in endothelial cells. The modulators of osteoclastogenesis, OPG and RANKL, were also present in non-calcified human aortic valves (*fig. 3.6c and d*). Endothelial, smooth muscle cells and some fibroblasts in the ventricularis showed immunoreactivity with OPG, while RANKL was only present in the endothelial cells.

Calcified valves and bone matrix regulatory proteins

In contrast to non-calcified aortic valves, all bone regulatory proteins showed clear immunoreactivity in calcified aortic valves. MGP (*fig. 3.7a*) and BSP were observed at

the boundaries of calcium deposits. OC was present in lamellar bone (*fig. 3.7b*) and bone-associated cells, such as osteoblasts and bone marrow cells, at the boundaries of amorphous calcium phosphate and in smooth muscle cells (*fig. 3.8*). Immunoreactivity of ON and OPN was prominent in amorphous calcium phosphate depositions, especially at the borders (*fig. 3.7c*) and in lamellae of mature bone. ON showed also immunoreactivity with chondrocyte-like cells, which were positive for ASMA. BMP-2 and BMP-4 showed no prominent expression within amorphous calcium phosphate, while the myofibroblasts surrounding these deposits showed high immunoreactivity. BMP-4 also showed immunoreactivity in the osteoblasts (*fig. 3.7d*).

As in non-calcified valves, both modulators of osteoclastogenesis, OPG and RANKL were present in calcified human aortic valves, especially in endothelial cells and chondrocyte-like cells, while mature chondrocytes showed only immunoreactivity with OPG (*fig. 3.7e*). OPG protein was further present in myofibroblasts, osteoblasts, and in the surroundings of osteoclasts. RANKL showed high immunoreactivity with osteoclasts (*fig. 3.7f*) and with macrophages in the surroundings of amorphous calcium phosphate.

In situ hybridisation vs immunohistochemistry

Fibroblasts present in the ventricularis of non-calcified aortic valves expressed MGP mRNA, whereas in calcified valves, MGP mRNA was mainly present in the surroundings of calcified and ossified regions.

BMP-2 and BMP-4 mRNA were expressed in valves in the endothelial cells (*fig. 3.9a*) and some myofibroblasts of non-calcified, whereas osteoblasts in calcified valves expressed both BMP-2 and BMP-4 mRNA. Immunoreactivity of BMP-4 in non-calcified valves was low (*fig. 3.9b*), however clearly present in endothelial cells (*fig. 3.9b*) and myofibroblasts (*fig. 3.9c*).

ON mRNA (*fig. 3.9d*) and protein (*fig. 3.9e*) were highly expressed in chondrocyte-like cells, which showed immunoreactivity with ASMA (*fig. 3.9f*).

The mRNA expression pattern of MGP, BMP-2, BMP-4, and ON was comparable to the immunohistochemical localisation of these proteins.

Osteopontin immunoblotting

Immunoblotting of 3 non-calcified and 3 calcified aortic valve leaflets using polyclonal antibody generated against the amino terminus of human recombinant OPN, revealed three different forms of OPN protein (*fig. 3.10*). In all non-calcified valves (lane 4-6) two forms were present, the native form (30 kDa) and the glycosylated form (55 kDa). However, in two out of three calcified valves (lane 1-3) three OPN forms were detected. A 30 kDa band of native OPN, a 55 kDa band of glycosylated OPN and a 69 kDa band of phosphorylated OPN, indicating that the post-translational modifications of OPN are not equal in non-calcified and calcified valves, and that only the calcified valves contain phosphorylated OPN.

Fig. 3.5. Immunohistochemistry of ASMA, CD68 and CD3 on human aortic valves. A, B, and C: represent immunohistochemical stainings of non-calcified valves, while D, E and F showed immunohistochemical stainings of calcified valves. Aortic valves stained with antibodies directed to ASMA (A and D), CD68 (B and E) and CD3 (C and F). Note: In the calcified valves ASMA, CD68 and CD3 positive cells (red staining) are located in the surroundings of mineralized tissue. (f=fibrosa; s=spongiosa; v=ventricularis and b=bone). Scale bars represent 150 μ m. Full color figures see page 151.

Fig. 3.6. Immunohistochemical staining of bone matrix regulatory proteins on non-calcified valves. A and B: non-calcified valve stained with antibodies directed to MGP (A) and BSP (B). Both proteins are present in these valves (red staining). Note: MGP is present in the ventricular layer of the valve and BSP is clear expressed in the fibroblasts (arrow and inset) in the fibrosa. C and D: non-calcified valve stained with antibodies directed to OPG (C) and RANKL (D). Scale bars represent 100 μ m (A) and 25 μ m (B, C and D). Full color figures see page 152.

Fig. 3.7. Immunohistochemical staining of bone matrix regulatory proteins on calcified valves. A and B: MGP, OC and BSP are present in calcified aortic valves. MGP (A) showed expression (arrow) at the borders of amorphous calcium phosphate deposits, while OC (B) is present in bone matrix of lamellar bone. C and D: In calcified valves OPN (C) is localised in the cement lines (arrow), and BMP-4 (D) is present in the osteoblasts lining lamellar bone (arrow). E and F: OPG (E) showed immunoreactivity in the chondrocytes (arrow) and its ligand RANKL (F) is present in osteoclasts (arrow). Scale bars represent 50 μ m (A and C) and 25 μ m (B, D, E and F). Full color figures see page 153.

Fig. 3.8. Double immunohistochemical staining of human calcified aortic valve with osteocalcin and ASMA (A). Note: osteocalcin is also expressed by a subpopulation of smooth muscle cells. Figure B represents a higher magnification. Scale bar in A represents 50 μm and B 25 μm . Full color figures see page 154.

Fig. 3.10. Immunoblotting of osteopontin. Lanes 1-3 represent 20 μg of protein extracted from calcified valves, lanes 4-6 represent 20 μg of protein extracted from non-calcified aortic valves. Membrane was immunostained with OPN antibody. 69 kDa band represent phosphorylated OPN, 55 kDa band glycosylated OPN and the 30 kDa band native OPN.

Fig. 3.9. *In situ hybridisation (ISH).* A, B and C: representation of respectively ISH of BMP-4 (A), and immunohistochemistry with antibodies directed to BMP-4 (B) and ASMA (C). D, E and F: ISH of ON (D, left inset is magnification of chondrocyte like cells and right inset is ISH of ON with sense probe), and immunohistochemistry with antibodies directed to ON (E) and ASMA (F). Note: BMP-4 is present in endothelial cells (arrow). ON is present in chondrocyte like cells (arrow and inset). Scale bars represent 50 µm. Full color figures see page 155.

Discussion

The data from the present study show that the expression profile of bone matrix regulatory proteins in human heart valves is comparable to the recently described profile in human atherosclerotic plaques⁷. This suggests that the regulation of human aortic valve and human atherosclerotic calcification share common features, including the expression of bone matrix regulatory proteins.

To gain more insights into the possible function of the examined bone matrix regulatory proteins in valvular calcification, we compared the present data with our human atherogenesis study⁷ and with available data from genetically modified mice (table 3.2). We found that the immunolocalization of various bone matrix regulatory proteins in aortic valves is equal to that found in atherosclerotic plaques⁷. However, data from genetically modified mice models of several bone matrix regulatory proteins do not always show a clear phenotype in the aorta or aortic valve.

MGP and OC, both gamma carboxyglutamic acid (Gla) residues containing proteins, are activated by the vitamin K dependent enzyme γ -carboxylase and subsequently bind to hydroxyapatite¹¹. In atherosclerotic plaques, MGP is expressed in SMC, macrophages, and chondrocytes. OC is highly expressed by osteoblastic cells¹². MGP knockout mice have severe calcifications of their aorta¹¹ and OC-deficient mice exhibit an increased bone formation¹³ (table 3.2). There are no data available on aortic valves in both of these models.

BSP and OPN are RGD (arginine-glycine-aspartic acid) containing glycoproteins. They both have high affinity for binding to $\alpha_v\beta_3$ integrins and might regulate cell adhesion and migration. BSP and OPN are functional in bone formation and degradation¹⁴. In the atherosclerotic plaque BSP and OPN are both present in the vascular smooth muscle cells and foam cells^{15,16}. There are no data published on BSP knockout or transgenic mice, whereas OPN mutant mice had a disorganised matrix and alteration of collagen fibrillogenesis¹⁷ (table 3.2).

Interesting is the shift in post-translational modification of OPN in calcified valves, where it is at least in part phosphorylated. Since phosphorylation is necessary for its inhibitory effect on calcification as shown by Speer *et al.*, it seems likely that OPN in calcified human valves has an inhibitory function on calcification¹⁸.

ON, also known as SPARC (secreted protein acidic and rich in cysteine), binds to collagen and hydroxyapatite. It is involved in cell matrix interaction, wound repair, angiogenesis, vascular permeability, cataract formation¹⁹ and carcinogenesis. ON-deficient mice have decreased bone formation and decreased osteoblast and osteoclast surface and number, causing profound osteopenia²⁰. Therefore ON is involved in activation of skeletal calcification. Our data show that ON immunolocalization is restricted to calcified valves, and in combination with our atherosclerotic plaque data thus also involved in the activation of vascular calcification.

BMPs, members of the TGF β super family, are secreted signalling molecules which

Table 3.2. Bone matrix regulatory proteins in human aortic valves and genetically modified mice models.

	Human aortic valves		Knockout mice phenotype			References
	non-calcified	calcified	valves	aorta	bones	
MGP	+	+	no data	calcification		11
OC	+	+	no data		↑ bone formation	13
BSP	+	+	no knockout mice available			
BMP-2	-	+	no knockout mice available			
BMP-4	+/-	+	no knockout mice available			
OPN	-	+	disorganized matrix , no clear phenotype			17
OPN-/- & MGP-/-				Enhanced calcification		18
ON	-	+			↓ bone formation osteopenia	20
OPG	+/-	+		calcification	osteoporosis	27
RANKL	+	+/-			osteopetrosis	28

have been implicated in embryogenesis, organogenesis and morphogenesis²¹. BMP-2 and BMP-4 have been shown to promote the development of bone and cartilage by inducing the differentiation of undifferentiated mesenchymal cells into the osteoblastic cells or cartilage cells, respectively²². A reduction in expression of BMP-4 has been associated with a variety of bone diseases^{23,24}. BMP-2 and BMP-4 proteins were recently localized in human atherosclerotic plaques^{7,25,26}, and the present data show immunolocalization in calcified aortic valves. Since BMP-2 and -4 are essential developmental genes, genetically modified mice are not viable.

OPG is a secreted member of the tumor necrosis factor receptor gene superfamily, acts as an inhibitor of osteoclast formation and is produced by osteoblasts and osteoclasts. RANKL is an activator of osteoclast formation, but its activation is inhibited by OPG. Mice lacking OPG have severe osteoporosis and vascular calcification²⁷ while RANKL knockout mice develop severe osteopetrosis due to the absence of osteoclasts²⁸.

Severe calcification of the human aortic valve is often followed by aortic valve replacement (AVR) surgery. Although AVR is initially successful, in 10 to 20% of adult recipients, bioprosthetic valves become calcified within 10 years after implantation²⁹. Medication to prevent or reduce valvular calcification before or after AVR is not available yet. One possible target is lipid therapy. High levels of LDL are associated with aortic valve calcification, and lipids have been suggested to serve as a nidus for calcification,

whereas oxidized lipids promote osteoblastic differentiation of progenitor cells^{3-5,30}. Moreover, delipidation of bioprosthetic valves significantly reduces their calcific degeneration, and hyperlipidemic LDL receptor null mice, fed a high fat diabetogenic diet, suffered from active induction of vascular calcification^{31,32}.

Since statins have particular benefit in both arterial calcification and osteoporosis³¹, and aortic valve calcification has similarities with atherosclerotic calcification and bone formation, statins may have the potential in retarding the rate of ectopic valvular calcification. Thus far, two studies described the use of statins in atherosclerotic patients and demonstrated a reduction in calcium volume score³³. Mohler et al. indicated that lipid-lowering therapy is not associated with reduced aortic valve ossification⁵, while Shavelle *et al.*³⁴ recently showed that patients treated with statins had a 63% lower rate of aortic valve calcium and a much smaller percentage of patients progressed to definite aortic valve calcification. Thus, lipid-lowering therapy by statins, may decrease aortic valve calcium accumulation.

Another issue that is not yet resolved, is the origin of the bone-like cells in calcified aortic valves. Circulating osteogenic stem cells have been identified, but there is no evidence that they infiltrate into aortic valves. Other studies suggested a local origin by showing that valvular SMC, pericyte-like cells and valvular cells have the capacity to calcify^{31,35}. Bone formation in the aortic valves could be initiated by the differentiation of bone marrow derived cells³⁶ such as bone marrow stromal cells³⁷ or blood mononuclear cells³⁸. The knuckle epitope of BMP-2 is capable to activate the osteoprogenitor cells and stimulate calcification³⁹, whereas also inflammatory cytokines and lipids can promote calcification⁴⁰. Since our data show the presence of CD138, identifying bone marrow cells, in calcified aortic valves we agree with the initiation of bone formation by the differentiation of bone marrow derived cells.

The major conclusion derived from our data, is that human aortic valve calcification is an active process, associated with the expression of known regulators of calcification and bone modeling. The constitutive immunoreactivity of MGP, OC, BSP, and RANKL in normal aortic valves, and the enhanced immunoreactivity of BMP-2, BMP-4, OPN, ON and OPG in calcified aortic valves, suggests that a shift of the balance between bone formation and bone resorption in favor of bone formation, results in aortic valve calcification. Further dissection of these molecular pathways may provide new targets for interventions in human valvular calcification.

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Chapter 4

BMP-2 induces atherosclerotic calcification and fibrosis in carotid artery vein grafting in hypercholesteremic ApoE3* Leiden mice

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In preparation

Abstract

To study the effect of adenovirus mediated overexpression of bone morphogenetic protein 2 (BMP-2) on vascular calcification, venous interposition grafting was performed in the carotid artery of ApoE3*Leiden mice, fed a high cholesterol-rich diet for four weeks. After surgery, mice were intravenously injected with either 2×10^9 plaque forming units (pfu) of adenovirus carrying the BMP-2 gene (Ad.BMP-2) or control vector. 28 days after surgery, the vein grafts were dissected, processed and analyzed. In both groups the intima showed intimal thickening, foam cell accumulation, necrosis, cartilage formation and calcium mineral deposits. No difference in plaque area, relative smooth muscle cell content and cartilage were found. In the Ad.BMP-2 group the relative macrophage content showed a slight increase (from $41.0 \pm 2.6\%$ to $50.1 \pm 3.5\%$; $p=0.08$) although not significant. The area of bone formation increased substantially (from $0.0 \pm 0.0 \text{ mm}^2$ to $0.016 \pm 0.01 \text{ mm}^2$; $p=0.03$) and the area of calcification (from $0.01 \pm 0.006 \text{ mm}^2$ to $0.04 \pm 0.01 \text{ mm}^2$; $p=0.06$) showed a tendency to increase.

Several bone matrix regulatory proteins were present in both groups, but the localization differed. In the control group these proteins were most prominent in cartilage, while in the Ad.BMP-2 treated group bone matrix regulatory proteins were highly expressed in calcified areas in the grafted vein.

In conclusion, adenoviral BMP-2 overexpression accelerates calcification and ossification in atherosclerotic lesions in vein grafts in the carotid artery in mice, which is associated with an altered bone matrix regulatory protein expression profile.

Introduction

Bone morphogenetic proteins (BMPs) play important roles in the migration of osteoblast progenitor cells, the proliferation of mesenchymal cells and their differentiation into chondrogenic and osteogenic cells^{1,2}. BMPs are able to induce endochondral bone formation at ectopic sites. A prominent feature of atherosclerosis is calcification and in calcified atherosclerotic plaques, organized bone including bone marrow may appear³. Bone morphogenetic protein 2 (BMP-2) is a highly osteoinductive protein and is present in human atherosclerotic plaques⁴. We recently showed that besides BMP-2 other bone matrix regulatory proteins are present in human atherosclerotic plaques⁵. These proteins include BMP-4, matrix Gla protein, osteocalcin, osteopontin, osteonectin, osteoprotegerin and receptor activator of nuclear factor- κ B ligand (RANKL). In addition transforming growth factor β (TGF β), one of the main factors controlling the formation of connective tissue matrix, plays a role in endochondral bone formation, exerts similar effects as BMP-2 and is present in atherosclerotic plaques⁶⁻⁸. Although bone matrix regulatory proteins have a specific expression pattern in atherosclerotic plaques, data on the function of these proteins in atherosclerotic calcification are lacking.

Since BMP-2 is a highly osteoinductive protein, involved in the differentiation into chondrogenic and osteogenic cells and is present in atherosclerotic plaques we hypothesize that BMP-2 gene accelerates atherosclerotic calcification. Since a simple administration of purified, water-soluble BMP protein alone did not induce local bone formation *in vivo*⁹, we used adenovirus-mediated transfer of the BMP-2 gene.

In the present study we examined the effect of adenovirus mediated BMP-2 overexpression, using an adenoviral vector system, in the carotid artery vein graft model in hypercholesterolemic ApoE3*Leiden mice¹⁰. In this model in which the venous interposition is placed in the carotid artery in hypercholesterolemic ApoE3*Leiden mice, a rapid development of accelerated vein graft atherosclerosis including calcification is observed.

Four weeks after adenoviral BMP-2 administration the composition of the intima in the vein graft was studied as well as the expression profile of bone matrix regulatory proteins and TGF β . We demonstrate that BMP-2 overexpression accelerates calcification and ossification in these vein grafts.

Methods

Adenoviral vectors

An adenoviral vector encoding human BMP-2 under control of a CMV promoter was kindly provided by Dr. D. Gazit¹¹. As a control an adenoviral constructs carrying no insert (Ad.control) was used as described¹². For all adenovirus preparations the particle per plaque forming unit (pfu) ratio was between 15 and 20 and the titre 10¹⁰ pfu/ml.

Mice and tissue preparation

All experiments were approved by the committee on animal welfare of TNO. Specific Pathogen-Free Transgenic APOE*3 Leiden mice were crossbred for 18 generations with C57BL/6 mice.

Four weeks before surgery and continued after surgery, male ApoE3*Leiden mice (n=8), aged 8-10 weeks were fed a cholesterol-enriched high-fat diet containing 0.5% cholate (HFC: casein 20%, choline chloride 1%, methionine 0.2%, cocoa butter 15%, cholate 0.5%, cholesterol 1%, sucrose 40.5%, cornstarch 10%, corn oil 1%, cellulose 5.1% and mineral mixture 5.1%) to improve intestinal cholesterol uptake and suppress bile acid synthesis, both leading to increased plasma cholesterol levels. All mice received water and food *ad libitum* during the entire experiment.

Vein graft procedure

Venous interposition grafting was performed in the carotid artery of ApoE3*Leiden

mice with the vena cava harvested from a donor mouse and grafted between the two ends of the right carotid artery as described before^{10,13}. Immediately after surgery, mice were intravenously injected with 200 µl of either 10¹⁰ pfu/ml Ad.BMP-2 or control vector. After 28 days, mice were anesthetized with Hypnorm (Bayer, 25 mg/kg) and Dormicum (Roche, 25 mg/kg). The venous graft was dissected and pressure-perfusion (100 mm Hg) was performed with 3.7% formaldehyde in 0.9% NaCl (wt/vol). The venous grafts were taken out, fixed overnight in 3.7% phosphate buffered formaldehyde, dehydrated and embedded in paraffin.

Serial cross sections (5 µm) were cut from the entire length of the graft.

Histological stainings

All samples were routinely stained with hematoxylin-phloxine-saffron (HPS). Weigert's elastin staining was used to visualize elastic laminae, von Kossa staining for calcium deposits, thionin staining for cartilage¹⁴, and Sirius red for collagen.

Immunohistochemical staining

Phenotyping

Smooth muscle cells were visualized by immunohistochemistry with antibodies directed to α -smooth muscle cell actin (ASMA) (1:750 Dako), and monocytes/macrophages with antibodies directed to Mac-3 (1:200 Pharmingen).

Bone modulators

Paraffin sections were stained with goat polyclonal antibodies against BMP-2 (1:50 Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), BMP-4 (1:25 Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), OPN (1:500 LF123; gift from Dr L. Fisher), OPG (1:200 Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), RANKL 1:100 Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), or OC (1:400 Anawa Trading SA, Wangen, Zürich, Switzerland), or rabbit polyclonal antibodies against ON (1:400 Zymed Laboratories, Inc., San Francisco, CA, USA) or BSP (1:25)⁵, or TGF β (1:30 R&D Systems). For goat and rabbit polyclonal antibodies, biotinylated rabbit anti goat IgG (1:200, Dako, Glostrup, Denmark) or sheep anti rabbit IgG (1:1000, Dako) was used as secondary antibody. Sections were incubated with alkaline phosphatase coupled avidin-biotin complex (Dako) and visualised with alkaline substrate kit I (Vector SK-5100, Vector Laboratories, Inc., Burlingame, CA, USA), counterstained with hematoxylin and mounted. Negative controls lacked incubation with primary antibody. BMP-2 specific immunohistochemistry was performed on murine liver sections.

Morphometrical analysis

Vessel wall thickening, plaque, lumen and the calcified area, as well as the cartilage, macrophage and smooth muscle cell content were measured using a computerized morphometry system (Quantimet, Leica, The Netherlands).

Statistics

Ad.BMP-2 treated ApoE3*Leiden mice were compared with control ApoE3*Leiden mice by a nonparametric Mann-Whitney *U* test. Data are presented as means $\pm$ SEM and values of $p < 0.05$ were considered statistically significant.

Results

General

Body weights were monitored at the day of surgery and at sacrifice. Average body weight at the day of surgery was 27.4 g ($\pm$ 1.1) for the control group and 22.4 g ($\pm$ 1.1) for the Ad.BMP-2 group, probably due to difference (2-3 weeks) in age between the groups. At sacrifice the average body weight was 28.2 g ($\pm$ 1.0) for the control group and 25.0 g ($\pm$ 1.8) for the Ad.BMP-2 treated animals. No significant change in body weight in the control group between surgery and sacrifice was registered, $p = 0.38$. The Ad.BMP-2 group showed significant enhancement in body weight between surgery and sacrifice, $p = 0.02$).

To check adequate BMP-2 gene expression after intravenous injection of the adenovirus, we performed a BMP-2 specific staining of the liver in which BMP-2 protein expression is normally absent. 28 days after surgery, liver cells of the Ad.BMP-2 treated mice showed immunoreactivity while the liver cells of the control mice showed no immunoreactivity (*fig. 4.1a and b*), indicating that BMP-2 expression is induced by adenoviral administration.

Plaque morphology

Total vessel area (1.5 ± 0.2 mm² versus 1.7 ± 0.2 mm²; $p = 0.20$), plaque area (1.1 ± 0.2 mm² versus 1.4 ± 0.2 mm²; $p = 0.34$) and lumen area (0.34 ± 0.05 mm² versus 0.26 ± 0.08 mm²; $p = 0.49$) were not different in the Ad.BMP-2 versus the control group (*fig. 4.2a and b*). There was no difference in relative SMC content ($26.5 \pm 7.4\%$ versus $28.3 \pm 2.8\%$; $p = 0.89$) between the Ad.BMP-2 and the control group. However, the area of non-osseous calcification (0.04 ± 0.01 mm² versus 0.01 ± 0.06 mm²; $p = 0.06$) and the area of ectopic bone formation (0.016 ± 0.01 mm² versus 0.0 ± 0.0 mm², $p = 0.03$) (*fig. 4.2c*) was enhanced in the Ad.BMP-2 treated mice versus the controls. The relative amount of macrophages ($50.1 \pm 3.5\%$ versus $41.0 \pm 2.6\%$; $p = 0.08$), was not

Fig. 4.1. Cross-sections of the liver of respectively a Ad.BMP-2 treated mouse (A) and a control mouse (B) stained by immunohistochemistry with a polyclonal antibody directed to BMP-2. A: BMP-2 is visualized in red. Arrows indicate BMP-2 present in liver cells. Cross-section of atherosclerotic plaques of respectively the Ad.BMP-2 treated group (C) and the control group (D) stained for TGFβ (visualized in red). Note: TGFβ was hardly present in the control group (Fig. 4.1D) and clearly enhanced in the Ad.BMP-2 group, with TGFβ located primarily at the adventitial side of the intima (Fig. 4.1C). Scale bars in panel A and B represent 50μm, in panel C and D 100μm. Full color figures see page 156.

Fig. 4.2. Cross-sections of a vein graft of respectively the Ad.BMP-2 treated group (A and C) and the control group (B and D). There was no difference in total vessel area, plaque area and lumen area between the Ad.BMP-2 treated mice (A) and the controls (B). However, the area of non-ossious calcification and ectopic bone formation was enhanced in the Ad.BMP-2 treated mice (C) versus the controls (D). Arrow in panel C indicate ectopic bone formation. Arrow in panel D indicate cartilage. Cross-sections of atherosclerotic plaques in Ad.BMP-2 treated mouse (E) and control mouse (F) containing non-calcified cartilage. Cartilage is visualized in purple by a thionin staining (arrow). Scale bars in panel A and B represent 100 μ m, and in panel C, D, E and F 50 μ m. C and D 100 μ m. Full color figures see page 157.

Fig. 4.3. Cross-sections of atherosclerotic plaques in both the Ad.BMP-2 treated group (left panels) and the control group (right panels). Positive staining was visualized in red.

In the Ad.BMP-2 treated group only osteocalcin was present in calcified cartilage, BMP-4 in young chondrocytes, BMP-2 in the macrophages, BMP-2 and osteoprotegerin in intimal calcification and in adventitial calcification/ossification five proteins showed immunoreactivity (BMP-2, BMP-4, osteocalcin, BSP and osteoprotegerin). In the control group BMP-2, BMP-4, BSP and osteonectin were present in both calcified cartilage and non-calcified cartilage. Osteopontin was present in calcified cartilage, while osteoprotegerin showed immunoreactivity in non-calcified cartilage. In young chondrocytes RANKL and osteonectin were present, in macrophages osteopontin and osteocalcin and in intimal calcification only osteopontin was expressed. Scale bars in panels with 10x magnification represent 50µm, and in panels with 25x magnification 20µm. Full color figures see page 158.

changed in plaques of the Ad.BMP-2 group. There was no difference in the area of cartilage between the Ad.BMP-2 and the control group ($0.49 \pm 0.18 \text{ mm}^2$ versus $0.25 \pm 0.15 \text{ mm}^2$; $p=0.3$) (fig. 4.2d), although non-calcified cartilage was present in only one of the Ad.BMP-2 treated mice versus four of the control mice (fig. 4.2e and f). Non-calcified cartilage, calcified cartilage and amorphous calcification were located in the shoulder regions in the intima and at the adventitial side of the plaque. However ectopic bone formation which was only present in the Ad.BMP-2 group, was restricted to the adventitial side of the plaques.

Collagen content was measured on Sirius Red stained sections. These measurements revealed no significant difference in collagen content between the Ad.BMP-2 group and the controls ($12.4 \pm 6.1\%$ versus $12.9 \pm 2.4\%$; $p=1.0$). However, all plaques of the Ad.BMP-2 treated mice contained enhanced TGFβ protein expression compared to the control mice after histological analysis.

Expression profile of bone matrix regulatory proteins

Immunohistochemistry with bone matrix regulatory proteins revealed a clear shift of protein expression profile in atherosclerotic plaques of Ad.BMP-2 treated mice versus the controls.

In the vein grafts of the controls, the majority of bone matrix regulatory proteins were expressed in chondrocytes and in non-calcified and calcified cartilage in the intima, as is summarized in figure 4.3. In the vein grafts of the Ad.BMP-2 treated group, the expression profile of the majority of bone matrix regulatory proteins had shifted to areas of calcification and bone formation in the intima and the adventitial side of the vein.

Immunohistochemistry with TGFβ didn't show a clear shift in localization of the protein. However, TGFβ was hardly present in the control group (fig. 4.1d) and clearly enhanced in the Ad.BMP-2 group, with TGFβ located primarily at the adventitial side of the intima (fig. 4.1c).

Discussion

Bone morphogenetic proteins are potent bone inducers that act through specific receptors located on the cell surface of responding mesenchymal cells, promote proliferation and differentiation of these cells into the osteoblastic lineage^{15,16}, and initiate endochondral bone formation. It has been well established that BMP-2 can induce ectopic bone formation both in vivo and in vitro. Adenovirus mediated overexpression of the BMP-2 gene and direct injection of recombinant human BMP-2 are able to induce ossification in vitro¹⁷ and in the musculature¹⁷⁻¹⁹ of several animal models²⁰. BMP-2 is also involved in other processes like vascular remodeling²¹. We previously showed that BMP-2 expression is enhanced in calcified human atherosclerotic plaques and calcified aortic valve leaflets⁵.

The present study demonstrates that BMP-2 overexpression accelerates atherosclerotic calcification in the carotid artery vein graft model after intravenous injection of an adenovirus carrying the BMP-2 gene. This model is known for its development of advanced atherosclerotic plaques, including calcification within 28 days¹⁰. The vein grafts of Ad.BMP-2 treated mice showed an enhancement in bone formation and a tendency in enhancement of calcification. Strikingly, the amount of non-calcified cartilage seems to be reduced in the Ad.BMP-2 treated mice. Bone morphogenetic proteins are known to induce both endochondral (through cartilage intermediate) and direct (intramembranous) bone formation, however our data suggest that intramembranous bone formation was enhanced after Ad.BMP-2 treatment.

The expression profile of several bone matrix regulatory proteins (BMP-2, BMP-4, BSP, OC, OPN, ON, OPG and RANKL) was influenced by overexpression of BMP-2. The main observation was a shift from expression in cartilage tissue in controls to intimal and adventitial calcification of the Ad.BMP-2 treated group. This suggests an intermediate role of other bone matrix regulatory proteins in the BMP-2 induced acceleration of vascular calcification.

A number of different growth factors have been identified to influence bone growth, collagen synthesis, and fracture repair both in vitro and in vivo²², including TGF β and BMPs^{23,24}, both members of the TGF β superfamily. Like BMPs, TGF β is known for its role in development, proliferation, differentiation and extracellular matrix biology, and is important in matrix remodelling and collagen synthesis²⁵. Lutgens *et al.*⁶ and Mallat *et al.*^{7,26} described that inhibition of TGF β in murine atherosclerotic plaques reduces plaque stability partly due to the reduction of the amount of fibrosis. In the present study we showed that adenovirus mediated overexpression of BMP-2 induces a more fibrous plaque with an enhanced TGF β protein expression. The induction of collagen was also seen in several cell lines transfected with the BMP-2 gene²⁷. The cells produced more BMP-2, which causes an upregulation of alkaline phosphatase activity, osteocalcin, osteopontin and type I collagen²⁸⁻³⁰. It has also been described³¹ that BMP-2 is able to

induce scar formation and fibrosis in fetal wound healing. These data suggest a key role for BMP-2 in the development of a stable plaque phenotype.

The results from our study may have important clinical implications. Recent pre-clinical experiments have served the idea that BMP-2 will be a useful therapeutic agent, acting to induce new bone formation at sites of bone injury in osteoporotic patients. Mostly BMP-2, a secreted protein, was placed on a matrix or collagen sponge, and implanted at the site of bone fracture healing. Since osteoporosis is associated with atherosclerotic calcification³², systemic treatment with BMP-2 in order to reduce osteoporosis may enhance vascular calcification.

In conclusion, atherosclerotic plaques of Ad.BMP-2 treated mice have accelerated calcification and ossification, a pro-fibrotic phenotype and an altered bone matrix regulatory protein expression profile. This suggests that BMP-2 is an active regulator of atherosclerotic plaque calcification and fibrosis in the murine carotid artery vein graft model.

Table 4.1.

Parameters	Ad.BMP-2	Control
Body weight begin (g)	27.4 ± 1.1	22.4 ± 1.1*
Body weight end (g)	25.0 ± 1.8	28.2 ± 1.0*
Total vessel area (mm ²)	1.5 ± 0.2	1.7 ± 0.2
Plaque area (mm ²)	1.1 ± 0.2	1.4 ± 0.2
Lumen area (mm ²)	0.34 ± 0.05	0.26 ± 0.08
Relative SMC content (%)	26.5 ± 7.4	28.3 ± 2.8
Relative MQ content (%)	50.1 ± 3.5	41.0 ± 2.6
Relative collagen content (%)	12.4 ± 6.1	12.9 ± 2.4
Cartilage (mm ²)	0.49 ± 0.18	0.02 ± 0.01
Non-osseous calcification (mm ²)	0.04 ± 0.01	0.01 ± 0.06
Ectopic bone formation (mm ²)	0.02 ± 0.01	0.00 ± 0.00*

Values are represented as mean ± SEM.
*p<0.05 for statistical significant difference between Ad.BMP-2 mice and Control mice.

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Chapter 5

A third generation bisphosphonate, risedronate, has no effect on the development of atherosclerosis in ApoE^{-/-} mice in doses that affect bone density

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Abstract

Bisphosphonates, pyrophosphate analogs, are potent inhibitors of bone resorption. At the cellular level the target of bisphosphonate-action is the osteoclast, the bone resorbing cell. At the molecular level enzymes in the cholesterol biosynthesis pathway were shown to be inhibited by bisphosphonates. A third generation nitrogen containing bisphosphonate risedronate is used clinically for the treatment of postmenopausal osteoporosis. Since in older women osteoporosis and atherosclerosis are correlated and atherosclerotic calcification shares molecular pathways with bone formation we hypothesized that risedronate, according to its mechanism of action, could reduce vascular calcification at doses effective on bone density and microarchitecture. We therefore used a mouse model of atherosclerosis and subcutaneously injected ApoE^{-/-} mice with different dosages (5 $\mu\text{g.kg}^{-1}$. injection⁻¹ or 25 $\mu\text{g.kg}^{-1}$ .injection⁻¹) of risedronate twice a week. We used two different starting ages (5 and 29 weeks old mice) and two different types of diet (Western type and normal chow). Results revealed that risedronate had no effect on the development of atherosclerosis in ApoE^{-/-} mice at doses that had significant effects on bone density and microarchitecture.

Introduction

Bisphosphonates are nonhydrolyzable analogs of pyrophosphate and, like pyrophosphate have the ability to bind to bone mineral and hydroxyapatite crystals. N-containing bisphosphonates are non-metabolized potent inhibitors of osteoclastic bone resorption and inhibit farnesyl-diphosphate synthase, an enzyme in the mevalonate pathway. This results in inhibition of posttranslational prenylation, which is necessary for appropriate anchoring of small glutamyl transpeptidases such as Rho, Ras, Rac and Rab to the osteoclast membrane. This inhibits the regulation of membrane ruffling and apoptosis in the osteoclast^{1,2}. N-containing bisphosphonates such as the pyridinyl bisphosphonate risedronate have been successfully used in the treatment of postmenopausal osteoporosis^{3,4} and Paget's disease^{5,6}. Since in older women osteoporosis and atherosclerosis are correlated and atherosclerotic calcification shares molecular pathways⁷ with bone formation we hypothesized that bisphosphonates would reduce vascular calcification. We therefore examined the chronic effects of the third generation, N-containing bisphosphonate risedronate on atherosclerotic calcification in a mouse model of atherosclerosis. First risedronate was tested in a prevention set-up in which treatment began at 5 weeks of age and continued for 24 weeks. Because treatment of osteoporosis mainly starts at an older age when atherosclerotic calcification may already be present, we also studied the effect of risedronate in older ApoE^{-/-} mice that had already developed calcified atherosclerotic lesions (regression set-up). In this part of the study a high (25 $\mu\text{g/kg}$) and low (5 $\mu\text{g/kg}$) dose of risedronate was used as well as two different diets, normal chow and Western type diet.

Materials and Methods

Animals

Male ApoE $-/-$ mice on a C57Bl6 background were obtained from Iffa Credo (Lyon, France). All animal work was approved by the institutional committee for the welfare of laboratory animals of the University of Maastricht and was performed in compliance with the Dutch government guidelines. Mice were kept in accordance with standard animal care requirements of the University of Maastricht, housed 5 to 6 per cage with free access to food and water, and maintained on a 12-hour light-dark cycle. Three experiments were performed: prevention Western type diet (pWT) (n=48), regression Western type diet (rWT) (n=45) and regression normal chow diet (rNC) (n=31). Mice in the prevention WT and the regression WT received a WT diet (0.21% cholesterol, Hope Farms), beginning at week 5 of age until the end of the experiment, while mice of the regression NC diet experiment were kept on NC from week 5 after birth.

Body weights were measured at the beginning and at the end of the experimental period.

In vivo treatment of animals with drug

All mice were injected subcutaneously twice a week with 200 μ L of with either risidronate (kindly provided by P&GP) diluted in phosphate buffered saline (PBS) or phosphate buffered saline (PBS), alone as a control. In the prevention WT experiment injections began at 5 weeks of age and continued for 24 weeks. The dose of risidronate was 5 μ g.kg $^{-1}$ .injection $^{-1}$. A high (25 μ g.kg $^{-1}$ .injection $^{-1}$) and a low (5 μ g.kg $^{-1}$ .injection $^{-1}$) dose of risidronate was given in the regression WT experiment, which began at 29 weeks of age and continued for 12 weeks. A control group received PBS injections for 12 weeks. In the last experiment risidronate was given at a dose of 5 μ g.kg $^{-1}$ .injection $^{-1}$ for 12 weeks starting 35 weeks of age (regression NC experiment). All experimental groups are represented in *table 5.1* and *table 5.2*.

Table 5.1

Groups	Subgroups	Dose	Injection volume	Age of mice	Treatment period	Diet
Prevention (n=48)	Risedronate	5 μ g/kg	200 μ l	5-29 weeks	24 weeks	WT
	Controls	PBS				
Regression (n=45)	Risedronate	5 μ g/kg	200 μ l	29-41 weeks	12 weeks	WT
	Risedronate	25 μ g/kg				
	Controls	PBS				
Non-diet (n=31)	Risedronate	5 μ g/kg	200 μ l	35-47 weeks	12 weeks	NC
	Controls	PBS				

Table 5.2

	week 5	week 29	week 35	week 41	week 47
Prevention WT low dose	5µg/kg				
Regression WT low dose		5µg/kg			
Regression WT high dose		5µg/kg			
Regression NC low dose			5µg/kg		

The risedronate dose used is in the same range of two recent published article⁹. Nakamura *et al.* used a dose of 10 µg.kg⁻¹.day⁻¹ s.c. for 30 days and showed effects of risedronate treatment on bone formation and bone resorption in mice. This dose of 70 µg.kg⁻¹.week⁻¹ is comparable to the dose of 50 µg.kg⁻¹.week⁻¹ used in the present study. Shimshi *et al.* used a dose of 6 mg p.o. twice weekly for 8 weeks in ApoE^{-/-} mice, which corresponds with the human-equivalent dose of 0.3 mg.kg⁻¹.day⁻¹ and showed effects of risedronate treatment in plaques.

Tissue preparation

Mice were sacrificed after a 24-hour fasting period. Blood (0.5 to 1.0 mL) was obtained from the right cardiac ventricle, mixed with 50 µL 0.5 M EDTA, centrifuged at 1500 rpm for 10 min and plasma was stored at -80°C until lipoprotein analysis. The arterial tree was perfused through a catheter inserted into the left ventricular apex for 3 minutes with PBS, followed by 3 minutes with 10% phosphate-buffered formalin (pH 7.4), both containing 0.1 mg/mL sodium nitroprusside (Sigma)¹⁰. The aortic arch, including its main branch points (brachiocephalic trunk, left common carotid artery, and left subclavian artery) was embedded longitudinally in paraffin and cut into ~ 40 sections. A series of twenty 4 µm-thick sections, which represented the central area of the arch with an intact morphology of the complete arch and branch points, were used for analysis (see further, histological and morphometric analysis).

Lipid Profile

For the assessment of lipid profiles at the end of the experiments, standard enzymatic techniques, automated on the Cobas Fara centrifugal analyzer (Hoffmann-La Roche), were used. Total plasma cholesterol and HDL were measured by using kit No. 07 3663 5 and kit No. 543004 (Hofmann-La Roche), total glycerol was measured by using kit

No. 337-40 A/337-10B (Sigma Chemical Co) and free glycerol was measured by using kit No. 0148270 (Hoffmann-La Roche). Standardized mouse serum (Precipath) was used as an internal standard. LDL was calculated by the equation: total cholesterol - [(total glycerol - free glycerol)/2.2] = HDL.

Evaluation of Systemic Effects

In each experimental group twenty organs (brain, lung, heart, thymus, liver, spleen, kidneys, stomach, intestine, pancreas, bone (skull, femur), adrenal glands, bladder, skin, prostate, seminal vesicle, salivary glands, lymph nodes and tonsil) were excised from 4 randomly selected risedronate treated (prevention WT low dose group) and 4 control mice. Analysis was performed by light microscopy on 4 μ m sections stained with hematoxylin and eosin.

Histological and Morphometric analysis

For histological analysis of atherosclerosis, 4 sections of the complete aortic arch including the main branch points (20 μ m apart) were stained with hematoxylin and eosin (*fig. 5.1a and b*). Atherosclerotic lesions were classified by light microscopy, based on histological criteria, according to the classification by Virmani¹¹. The number of atherosclerotic plaques, the presence or absence of lipid cores, chondroid and calcification were determined. Lawson staining (modified Elastica von Giesson, Verhoef staining), which exclusively stains the elastic laminae, was used to delineate the internal elastic lamina, to permit an easier distinction between media and intima. Four Lawson-stained sections (20 μ m apart) were used for morphometrical analysis. All morphometrical parameters were determined using a microscope coupled to a computerized morphometry system (Quantimet 570, Leica, The Netherlands). Plaque area excluding the underlying media was measured for each individual plaque, and measurements of chondroid and calcified area were performed. To facilitate the detection of calcified areas in the plaques, two sections (28 μ m apart) were stained with Von Kossa. The relative collagen area (the percentage of area that stained positively for Sirius Red)(*fig. 5.1c and d*) was determined for each atherosclerotic plaque using the same morphometric system.

Immunohistochemistry:

Antibodies directed to α -smooth muscle actin (ASMA^{TM} monoclonal antibody, 1:500; Sigma), Mac3 (1:30, Pharmingen) (*fig. 5.1e and f*) and CD3 (A0452 polyclonal antibody, 1:200; Dako) were used to identify smooth muscle cells, macrophages and T-cells respectively. The relative α -smooth muscle actin area was determined by dividing the ASMA-positive area by total plaque area using the morphometric system. To determine the relative amounts of macrophage and T-lymphocyte content in an

Fig. 5.1. Cross-sections of the first branch (right carotid artery) of the aortic arch of respectively a risedronate treated mouse (a, c and e) and a control mouse (b, d and f) stained with H&E (a and b), Sirius Red for the detection of collagen (c and d) and with a monoclonal antibody directed to Mac3 for the detection of macrophages (e and f). a: arrow indicate cholesterol crystals. b: arrow indicate calcification. c and d: collagen is visualized in red. e and f: arrows indicate macrophages, visualized in red. Full color figures see page 160.

Fig. 5.2. Cross-sections of a femur H&E stained (a) and a three-dimensional micro-CT scan of the left leg (femur, knee joint and tibia) (b). Full color figures see page 161.

atherosclerotic plaque, the number of Mac3- or CD3- positive cells in the lesion was divided by the total number of cells in the entire atherosclerotic plaque.

The expression profile of bone morphogenetic protein 2 (BMP-2), osteoprotegerin (OPG) and receptor activator of nuclear factor- κ B (RANKL) were studied with antibodies directed to BMP-2 (1:12.5, Santa Cruz), OPG (1:200, Santa Cruz) and RANKL (1:100, Santa Cruz). Incubation of the primary and secondary antibodies was performed using standard procedures. After incubation with an alkaline phosphatase-coupled avidin-biotin complex (ABC complex, Dako), antibodies were visualised with an alkaline substrate kit I (Vector SK-5100, Vector Laboratories, Inc., Burlingame, CA, USA). Sections were counterstained with hematoxylin and mounted with coverslips. The primary antibody was omitted in negative controls. Each staining was performed on one section per mouse.

Bone structural measurements:

After the mice were sacrificed, bones of the left leg (femur, knee joint and tibia) were extracted, stored in PBS and frozen at -20°C . A micro-CT scanner (μ CT80, Scanco Medical AG, Bassersdorf, Switzerland) was used to produce three-dimensional reconstructions of the distal femurs at an isotropic resolution of 10 microns (fig. 5.2). A volume of interest (VOI) was selected that comprises the trabecular bone in the femoral epiphysis. After thresholding the images, structural parameters were determined for this VOI using the Scanco 3D evaluation software. Parameters included in this study

are bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular spacing (Tb.Sp).

Statistical analysis

All data are expressed as mean $\pm$ SEM. Means between groups were compared by the use of the non-parametric Mann-Whitney U test, and data were considered statistically significant at $p < 0.05$.

Results

General

In the prevention (pWT low dose) experiment 3 out of 24 risedronate and 1 out of 24 PBS treated animals died. In the regression (rWT high and low dose) experiment no risedronate treated animals died while 3 out of 19 PBS treated animals died. In the regression normal chow (rNC low dose) experiment no animals died. In all three experiments, the starting body weights were not different between treated and control groups (*table 5.3*). Risedronate treatment induced a small ($<10\%$) loss of body weight but only in the normal chow (rNC low dose) experiment (risedronate group: body weight decreased from $35.0 \pm 0.6\text{g}$ to $31.7 \pm 0.6\text{g}$; $p=0.001$, controls: body weight loss from $35.7 \pm 0.7\text{g}$ to $33.7 \pm 0.7\text{g}$; $p=0.58$).

The atherogenic Western type diet resulted in a doubling of cholesterol, triglycerides and LDL plasma levels. Treatment with risedronate had no effect on this increase in lipoprotein levels. Similarly in the normal chow (rNC low dose) experiment risedronate did not affect plasma lipoprotein levels.

Toxicology

Histological analysis of all 20 organs did not reveal any differences between PBS and risedronate treated animals in any of the experiments.

Bone

Bone measurements revealed that risedronate treatment in the prevention WT low dose group (pWT low dose) and in the regression WT low dose group (rWT low dose) has effects on bone parameters, however in the regression WT high dose (rWT high dose) there were no differences. In the pWT low dose group, volume fraction, trabecular numbering and thickness were increased, while trabecular spacing was decreased. Risedronate treatment in the regression WT low dose group (rWT low dose) showed an increase in trabecular thickness and a reduction in trabecular spacing. Risedronate treatment in the regression NC low dose group (rNC low dose) had no effect on the various bone parameters (*table 5.4*).

Table 5.3

Physiological parameters	Prevention (n=48)		Regression (n=45)		Non-diet (n=31)	
	Risedronate (n=24)	Control (n=24)	Risedronate 5 (n=12)	Risedronate 25 (n=11)	Risedronate (n=16)	Control (n=15)
Body weight begin (g)	22.1 ± 0.5	22.3 ± 0.3	36.2 ± 1.3	36.8 ± 1.2	35.0 ± 0.6	35.7 ± 0.7
Body weight end (g)	32.1 ± 1.4	33.0 ± 0.9	34.5 ± 1.7	34.3 ± 1.4	31.7 ± 0.6	33.7 ± 0.7
Cholesterol (mM)	19.4 ± 1.7	20.0 ± 0.9	18.4 ± 2.0	19.6 ± 0.8	10.2 ± 0.4	10.5 ± 0.3
Triglycerides (mM)	1.0 ± 0.1	1.0 ± 0.2	1.2 ± 0.3	1.0 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
HDL (mM)	0.19 ± 0.03	0.23 ± 0.04	0.37 ± 0.07	0.38 ± 0.10	0.26 ± 0.04	0.39 ± 0.05
LDL (mM)	18.9 ± 1.8	18.9 ± 1.0	17.5 ± 1.8	18.7 ± 0.6	9.7 ± 0.4	9.9 ± 0.3

Table 5.4

Bone parameters	Prevention (n=48)		Regression (n=45)		Non-diet (n=31)	
	Risedronate	Control	Risedronate 5	Risedronate 25	Risedronate	Control
Bone volume fraction	0.50 ± 0.01*	0.40 ± 0.02	0.41 ± 0.02	0.40 ± 0.03	0.42 ± 0.02	0.41 ± 0.02
Trabecular number (N/mm)	6.76 ± 0.06*	6.07 ± 0.17	6.09 ± 0.06*	5.98 ± 0.08	6.11 ± 0.09	5.93 ± 0.20
Trabecular thickness (mm)	0.080 ± 0.002*	0.067 ± 0.003	0.068 ± 0.003	0.070 ± 0.004	0.070 ± 0.003	0.074 ± 0.002
Trabecular space (mm)	0.137 ± 0.002*	0.162 ± 0.005	0.156 ± 0.003*	0.160 ± 0.003	0.156 ± 0.004	0.163 ± 0.006

Table 5.5

Atherogenic parameters	Prevention (n=48)		Regression (n=45)		Non-diet (n=31)	
	Risedronate	Control	Risedronate 5	Risedronate 25	Risedronate	Control
Total plaque area (mm ²)	1.6 ± 0.2	1.8 ± 0.4	1.7 ± 0.2	2.1 ± 0.2	1.5 ± 0.1	1.5 ± 0.1
Calcified area (mm ²)	3.4 ± 0.9	5.4 ± 2.7	7.0 ± 1.6	2.7 ± 0.8	3.2 ± 0.9	5.0 ± 1.4
Controid area (mm ²)			0.24 ± 0.10	0.13 ± 0.06		
Smooth muscle cells (%)					0.6 ± 0.4	0.4 ± 0.3
Macrophages (%)					28.5 ± 16.1	26.0 ± 15.4
Collagen content (%)					49.5 ± 9.2	46.6 ± 12.3

Fig. 5.3. Cross-sections of the complete aortic arch including the main branch points (a, c, e, g and i) and a higher magnification of the first branch (right carotid artery) (b, d, f, h and j) of various groups and controls. a and b: regression Western type diet group with risedronate dose of 5 $\mu\text{g/kg}$ (rWT low dose). c and d: regression Western type diet group with risedronate dose of 25 $\mu\text{g/kg}$ (rWT high dose). e and f: control group for the regression Western type diet experiment. g and h: regression normal chow group with risedronate dose of 5 μg . i and j: control group for the regression normal chow experiment. Scale bars in fig. 5.3a, c, e, g and i represent 300 μm , and in fig. 5.3b, d, f, h and j the scale bars represent 200 μm . Full color figures see page 162.

Atherosclerosis

All atherosclerotic lesions in the aortic arch of mice in the prevention (pWT low dose), regression (rWT high and low dose) and normal chow (rNC low dose) experiments were analyzed (table 5.5).

Risedronate at a dose of 5 µg/kg had no effect on total plaque area. This was true for the prevention (pWT low dose) and regression (rWT low dose) experiment (Western type diet) and the normal chow (rNC low dose) experiment. Similarly the high dose of 25 µg/kg in the rWT high dose experiment did not affect total plaque area. Detailed phenotypic analysis of the plaque did not show any differences between control and risedronate treated groups (fig. 5.3). The percentage of macrophages, smooth muscle cells and collagen was not different. Chondroid and calcified area in the plaque were also not affected by risedronate treatment in any of the four groups.

Immunohistochemistry

Evaluation of sections, immunohistochemically stained with antibodies directed to BMP-2, OPG and RANKL (fig. 5.4) revealed expression of all three bone related proteins in atherosclerotic plaques of ApoE^{-/-} mice. Expression of BMP-2 was mainly present in and around calcium mineral deposits in the plaques. Especially hypertrophic chondrocytes and calcifying chondrocytes showed BMP-2 expression, although BMP-2 was also present in some SMC in areas surrounding calcification. Like BMP-2, OPG and RANKL were also present in hypertrophic chondrocytes and calcifying chondrocytes. Additional, OPG showed immunoreactivity with endothelial cells in plaques. BMP-2, OPG and RANKL were present in both, risedronate treated animals and controls of all experimental groups. Distribution of these proteins were not effected by risedronate administration, in either the prevention WT experiment, the regression WT experiment or in the regression NC experiment.

Discussion

Risedronate, at doses that affected BMD and protected bone microarchitecture, had no effect on amorphous vascular calcifications. As no ectopic bone formation was detected in this animal model, the effect on ectopic bone formation could not be documented. The main findings of the present study are that chronic treatment with two different doses of the N-containing bisphosphonate risedronate has no effects on atherosclerotic lesion instability, development and regression in ApoE^{-/-} mice. Risedronate had no effects in mice fed a normal chow diet nor on mice fed an atherogenic Western type diet. Detailed phenotypical analysis of the atherosclerotic lesion, including quantification of the area of calcification in the plaque, did not yield

Fig. 5.4. Immunohistochemical staining of three bone matrix regulatory proteins (BMP-2, OPG and RANKL) on murine atherosclerotic plaques of various experimental groups. **a, b and c** represent atherosclerotic plaques of prevention Western type diet mice that received a risedronate dose of 5 $\mu\text{g/kg}$. **d, e and f** are the controls. **g, h and i** represent atherosclerotic plaques of regression Western type diet mice that received a risedronate dose of 5 $\mu\text{g/kg}$. The left panels are immunohistochemically stained with BMP-2, the middle panels with OPG and the right panels with RANKL. Positive staining was visualized in red. Mainly the chondrocytes (arrows) are positively stained for all three bone matrix proteins. Scale bars represent 20 μm . Full color figures see page 163.

Fig. 5.5. TRAP staining for the identification of osteoclasts. A single osteoclast (arrow) present in an atherosclerotic plaque of the regression NC experiment (fig. 5.5a) and in the accessory control group (fig. 5.5b). Positive staining is visualized in brown. Scale bars represent 20 μ m. Full color figures see page 164.

any differences between risedronate and PBS treated animals, in either in the normal chow or Western type diet fed animals. Plasma lipoprotein levels increased with the high fat diet, but were unaffected by bisphosphonate treatment. Furthermore, a five-fold higher dose of risedronate did not result in any significant changes in plaque development, while protecting bone.

These findings are in accordance with several studies in humans which showed absence of effect of treatment with bisphosphonates on atherosclerosis lesion formation and progression using alendronate¹² and ibandronate¹³. However, other studies in humans demonstrated possible negative effect of bisphosphonates on atherosclerosis^{9, 14, 15}. Shimshi *et al.*⁹ demonstrated that ApoE^{-/-} mice treated with alendronate and risedronate had an increased risk of plaque rupture. There are several possibilities to explain these results. First of all the dose of risedronate might have been too low to have any effects on vascular calcifications. However, published data on risedronate administration in mice and rats¹⁶ resulting in a dose response relation⁸ indicate that we have used an appropriate dose regimen. For instance Nakamura *et al.* described that risedronate treatment in OPG^{-/-} mice (10 μ g.kg⁻¹.day⁻¹ for 30 days) showed effects on bone resorption and bone formation⁸. Boyce *et al.* reported that one year of risedronate treatment in a dose of 5 μ g.kg⁻¹.day⁻¹ subcutaneously administered for 1 week followed by 3 weeks without treatment enhanced bone density after ovariectomy¹⁷. Lin *et al.* showed that risedronate increased bone density in rats in a dose regimen of 5 μ g.kg⁻¹.day⁻¹ sc twice a week for 60 days¹⁶. In a bone metastasis model in nude mice

(approximately 20 g) risedronate sc at a dose of $200 \mu\text{g.kg}^{-1}.\text{day}^{-1}$ for 28 days slowed or inhibited the development of bone metastasis¹⁸. A proof of concept study on vascular calcification showed that the bisphosphonates ibandronate and alendronate in an oral dose of $50 \mu\text{g.kg}^{-1}.\text{day}^{-1}$ and $1000 \mu\text{g.kg}^{-1}.\text{day}^{-1}$ for 14 and 28 days completely inhibited vascular calcification in the warfarin induced medial calcification rats¹⁹. However, these doses were higher than used in clinical practice and the warfarin model is different from the currently studied model, in that it results in ectopic bone formation in the blood vessels. Furthermore, evidence that we used the appropriate dose was revealed by the increase in bone mineral density observed after low dose risedronate treatment.

Another possibility is that chronic inhibition of the mevalonate pathway by risedronate induced counter-regulatory pathways that reduce calcification. Possible candidates are the downregulation of BMP-2, OPG and upregulation of RANKL. Immunohistochemical analysis of BMP-2, OPG and RANKL protein expression in the different treatment groups do not support this hypothesis since the expression of all three proteins appeared not to be regulated by chronic risedronate administration.

A third possibility is that the high fat diet may have overruled the possible effects of risedronate. This is unlikely, however since the drug did not have an effect in animals receiving a normal chow diet.

A fourth possibility to explain the negative finding of this study is that the proposed mechanism whereby bisphosphonates enhance bone density and microarchitecture, which is by the inhibition of osteoclasts, does not occur in atherosclerotic lesions in mice. We and others have shown the presence of osteoclasts in human atherosclerotic lesions which colocalize with lamellar bone structures^{7,20,21}, suggesting that these osteoclasts are associated with the transition of calcified cartilage into lamellar bone. Thus far, no published data are available on the presence of osteoclasts in atherosclerotic plaques in mice. However, we found a single osteoclast present in an atherosclerotic plaque of the regression NC experiment and in the accessory control group (*fig. 5.5*). Unlike in human plaques, lamellar bone structures have never shown in advanced atherosclerotic plaques of ApoE^{-/-} mice. The fact that lamellar bone is absent and osteoclasts appear sporadically present in atherosclerotic plaques of ApoE^{-/-} mice suggests that our negative results in mice can not be extrapolated to humans. Risedronate had little effect on the bone structure in the regression groups. Significant changes in bone structural parameters were found in the regression WT low dose group, the changes were within the expected range (10-20% for TB/BV). This can be explained by the fact that the animals in the regression groups were older (29 to 41 weeks) than the animals in the prevention WT low dose group (5 to 29 weeks). These younger animals have a higher bone turnover during their growth. A significant increase in bone density and structural quality (as expressed by increased trabecular thickness and number and

reduced trabecular spacing) was found, however, for the prevention group. A possible explanation for this finding is that the bones in these animals are still in a growth process, which includes osteoclastic activity. Inhibition of osteoclastic activity by risedronate, at this stage might hinder the removal of surplus bone that can exist during growth²². Inhibition of bone removal results in a higher bone mass in these mice.

Suppression of osteoclasts in bone might alter bone matrix regulatory proteins in bone and in atherosclerotic plaques. However, our data revealed no altered protein expression profile of BMP-2, OPG or RANKL.

The association of osteoporosis with vascular calcification has been widely reported²³⁻²⁷. Thus far only two articles were published on the effect of bisphosphonates on arterial calcification in osteoporotic patients. One small pilot study indicated that oral alendronate administration did not accelerate the rate of coronary artery calcification¹². However Tanko *et al.*²⁴ recently described the effect of bisphosphonates on aortic calcification in osteoporotic patients, and concluded that a 3-year treatment with effective doses does not pose any altering in vascular calcification. Our data show that risedronate did not affect vascular calcification in atherosclerotic lesions of ApoE-/- mice.

In conclusion these findings indicate that risedronate treatment has a positive effect on bone mineral density and bone microarchitecture but does not alter atherosclerotic vessel wall plaque progression or calcification.

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Chapter 6

General discussion

In this thesis we investigated aspects of the molecular regulation of vascular calcification. We hypothesized that bone matrix regulatory proteins that are associated with skeletal bone formation are also involved in the initiation and progression of vascular calcification. We further hypothesized that enhanced BMP-2 gene expression accelerates atherosclerotic calcification and ossification and that bisphosphonates reduce vascular calcification.

The main conclusions derived from the studies are that both atherosclerotic and aortic valve calcifications are indeed actively regulated processes involving bone matrix regulatory proteins. This regulation involves both activators and inhibitors of calcification and bone formation. We also conclude that BMP-2 is one of the major active regulators of vascular calcification and that in an atherosclerotic mouse model bisphosphonates have no influence on vascular calcification when given in a dose that enhances bone density.

1 Active stimulation and inhibition of vascular calcification

Many studies revealed that vascular smooth muscle cells and pericytes have the potential to undergo osteogenic differentiation¹⁻⁴. While our studies clearly show active regulation of vascular calcification including activators and inhibitors, Schinke described another model of vascular calcification. He described that the passive deposition of calcium phosphates in the arterial wall could only occur when active inhibitors of calcification were no longer able to prevent precipitation⁵, thus proposing an active inhibition model. A third model is described by Price and colleagues⁶. They reasoned that since vascular calcification is linked to osteoporosis, calcium and phosphate concentrations in the blood are enhanced, which stimulates the formation and growth of crystal nuclei in the arterial wall. Soft tissue calcification is promoted by crystal nuclei generated at sites of bone resorption that travel via the blood and occasionally lodge in soft tissue structures. The observation that reduction of bone resorption by bisphosphonates has no effects on serum levels of free calcium and phosphate, but reduces arterial medial calcification by inhibiting the release of a complex of calcium phosphate mineral and MGP from bone into the blood, does not support a strictly passive model. In fact, these data support the active inhibition model described by Schinke. Our data suggest not only active inhibition but also active stimulation of vascular calcification.

Giachelli⁷ described that the uptake of inorganic phosphate by SMC that were exposed to inorganic phosphate levels similar to those seen in patients with end stage renal disease (>1.4 mmol/L) is facilitated by mediation of the sodium dependent phosphate co-transporter type III, Pit-1. Pit-1 also induces the expression of Cbfa-1 in these aortic SMC, resulting in an enhanced expression of osteogenic markers and calcification of vascular cells⁸. These results indicate that Cbfa-1, a bone matrix regulatory protein, is stimulated by inorganic phosphate levels, which can indirectly induce vascular calcification. They also suggest that the increased risk for vascular calcification in patients with end stage renal disease may be explained by such an active model, rather than the simple precipitation of calcium and phosphate as suggested in the passive model. Vattikuti *et al.* suggested that tissue calcium phosphate deposition may serve as a metabolic stressor that induces BMP-2, thereby enhancing calcification⁹. This points to the importance of bone matrix regulatory proteins, such as BMP-2, besides calcium and phosphate serum levels in the regulation of vascular calcification. Thus also recent data from several other laboratories suggest an active regulation of vascular calcification rather than a passive precipitation of calcium and phosphate in the vessels. This active regulation involves at least active inhibition and most probably also active stimulation.

Vascular calcification is not only regulated by bone matrix regulatory proteins, but also involves mechanisms of skeletal bone formation as demonstrated in *chapters 2 and 3* of this thesis, where we described cartilage, matrix vesicles and lamellar bone

formation in calcified human atherosclerotic plaques and aortic valves. The finding that in human atherosclerotic lesions mature bone tissue is frequently anchored to amorphous mineralized matrix¹⁰, resembling intramembranous ossification, suggests that amorphous mineral matrix serves as a scaffold for mature bone formation even in atherosclerotic plaques. The chondrocytes that form the cartilage in the vessel wall are thought to align to calcified amorphous mineral deposits^{11,12}. Both chondrocytes and calcified amorphous mineral deposits form nuclei for endochondral ossification⁹. One of the candidate regulators of both intramembranous and endochondral vascular ossification is BMP-2.

2 Molecular regulation of vascular calcification

2.1 BMP-2, an important active regulator of vascular calcification

BMP-2 is an osteoinductive protein, which induces intramembranous bone formation via *Msx-2* upregulation but also endochondral bone formation and neovascularisation via *Cbfa-1* upregulation¹³. Based on the data described in *chapter 4* we conclude that atherosclerotic calcification and ossification are accelerated by BMP-2 and associated with an altered bone matrix regulatory protein expression profile illustrated by an upregulation of BMP-4, OC, OPG, OPN and a downregulation of RANKL. This is supported by data from Bächner *et al.* They demonstrated that multipotent mouse embryonic cells, also known as vascular progenitor cells¹⁴ when stimulated with BMP-2 differentiate along osteogenic and chondrogenic lineages¹⁵. Moreover, BMP-2 can potently induce the expression of *Cbfa-1*, *Sox-9* and *Msx-2*, transcription factors that are upregulated in the calcified vasculature^{4,16,17} (*fig. 6.1*). In diabetes Cheng *et al.* provided evidence that aortic myofibroblasts respond to BMP-2/*Msx-2*, resulting in medial calcification independent of *Cbfa-1*¹⁸. In atherosclerotic calcification however, the *Cbfa-1* dependent endochondral ossification appears to predominate since in plaques *Cbfa-1* gene expression is much higher than *Msx-2* gene expression¹⁹.

Besides these direct effects of BMP-2 on vascular calcification it also has indirect effects by altering the expression of other bone regulatory proteins (*fig. 6.2*). Cellular transfection with BMP-2²⁰ causes an upregulation of alkaline phosphatase activity, osteocalcin, osteopontin and type I collagen²¹⁻²³. Expression of two other bone matrix regulatory proteins BMP-4 and BSP is enhanced after BMP-2 induction in rat calvarial osteoblasts²⁴. OPG mRNA expression, a potent inhibitor of osteoclast mediated bone resorption is produced by osteoblasts and is present in human atherosclerotic plaques. Its expression is stimulated by BMP-2²⁵. BMPs play a critical role in bone remodeling and maintenance of the structural integrity of the skeletal system, due to their ability to induce osteoblastic differentiation²⁶. The inhibitory effect of MGP on BMP-2 is

Fig. 6.1 The role of BMP-2 vascular calcification.

Fig. 6.2 The role of BMP-2 on bone matrix regulatory proteins

concentration dependent²⁷. Since BMP-2 has both direct and indirect effects, we consider BMP-2 as a major activator of vascular calcification.

2.2 Possible novel regulators of vascular calcification

To identify possible novel regulators of vascular calcification we performed a DNA micro-array experiment, using a DNA micro-array with 8000 human genes spotted in duplo (Clontech). We compared the expression profiles of human non-calcified fibrous

cap atheromas and fibrocalcific plaques. After hybridization, about 10% of the spotted genes were differentially expressed. We selected four differentially expressed bone associated genes for further validation, bone morphogenetic protein 3 (BMP-3), cartilage linking protein 1 (CRTL-1), craniofacial developmental protein 1 (CFDP-1), and cysteine knot superfamily 1 (Cktsf1-b1). While the DNA array revealed an upregulation of BMP-3 and CRTL-1 in non-calcified plaques and an upregulation of CFDP-1 and Cktsf1-b1 in calcified plaques, quantitative Real Time PCR on non-calcified fibrous cap atheromas, fibrocalcific plaques and on human non-calcified and calcified aortic valves showed a significant upregulation of only BMP-3 in both human fibrous cap atheromas (*fig. 6.3a*) and non-calcified aortic valves (*fig. 6.3b*). BMP-3 is a member of the transforming growth factor β superfamily, and known as a negative regulator of bone density. Daluiski *et al.* showed that BMP-3 is an antagonist of osteogenic BMPs. BMP-3 inhibits BMP-2 mediated induction of Msx-2 and blocks BMP-2 mediated differentiation of osteoprogenitor cells into osteoblasts²⁸. BMP-3 knockout mice are viable and show increased trabecular bone density, however no vascular or valvular phenotype was reported²⁹. The upregulation of BMP-3 in non-calcified atherosclerotic plaques and aortic valves is in accordance with our earlier findings of the expression of inhibitors of calcification in non-calcified tissues (*chapter 2 and 3*). The enhanced expression of BMP-3 might inhibit the differentiation of vascular cells into osteogenic cells, resulting in the active inhibition of calcification and ossification in atherosclerotic plaques and aortic valves.

3 Vascular calcification: good or bad?

The deposition of solid calcified areas in atherosclerotic lesions occurs predominantly in advanced lesions³⁰, with only speckled deposits in early lesions³¹. This indicates that the presence of solid calcified deposits is an indicator of advanced atherosclerotic disease and represents an increased risk for end stage organ damage³². Yet there has been a large debate in the literature on the effect of calcification on the stability of the plaque. Others stated that plaque calcification would further stabilize the plaque^{10,33}, because soft plaques are more prone to rupture than calcified plaques^{34,35}. Echomorphologic studies indicate that echolucent and ulcerated atherosclerotic plaques are associated with a higher risk for ischemic cerebrovascular events, whereas echo-dense plaques are more prevalent in asymptomatic plaques³⁵⁻³⁷. It has also been proposed that the presence of both hard and soft tissue in the plaque makes it more vulnerable. That the presence of calcification in coronary atherosclerosis has been associated with a less favorable prognosis is supported by several studies. Autopsy studies have shown that calcification increases the risk of myocardial infarction independently of age³². Niskanen

Fig. 6.3

et al. showed that calcification in peripheral vascular lesions increases the likelihood of ischemia. Vascular calcification has also been documented in patients with clinically significant coronary artery disease³⁸ and it is a sensitive and specific predictor for future cardiac events³⁹. Coronary calcification contributes to plaque rupture⁴⁰ by increasing the stress at the interface of calcified and compliant structures in the arterial wall.

Thus the presence of calcification in the plaque increases the heterogeneity of the tissue and thereby the likelihood of rupture. Flanking calcification may function as an anchor on an individual plaque, reducing circumferential stress and the likelihood of subsequent rupture³³. Shear stress increases at the edges of the calcified segment of the adjacent plaque, increasing the propensity for rupture⁴¹.

4 Is regression of calcification in atherosclerotic lesions possible?

Considering that vascular calcification is a risk factor, one has to find targets to prevent or reduce vascular calcification. The increased understanding of the molecular regulation of vascular calcification may provide novel possible targets for regression of vascular calcification. Because of the reciprocal effects of some factors on vascular and bone cells, one might be able to reduce both atherosclerotic calcification and osteoporosis. In the mouse, a bisphosphonate, risedronate inhibits both osteoclast function^{42,43} and cholesterol synthesis⁴⁴, but has no effect on vascular calcification as described in chapter 5, at least in the dose and model tested. Thus far, only limited data on the efficacy of bisphosphonates in the treatment of atherosclerosis are available in the literature⁴⁵. Recently Tanko *et al.*⁴⁶ described the effect of bisphosphonates on aortic calcification in osteoporotic patients, and concluded that a 3-year treatment with

effective doses does not pose any altering in vascular calcification.

As bisphosphonates, statins interfere with the mevalonate cholesterol biosynthesis pathway, albeit at a different point. Statins also have reciprocal effects on vascular and bone cells. Treatment with statins has been shown to reduce vascular calcification in humans^{47,48}. These effects are associated with reduced serum lipid levels. Bea and colleagues demonstrated a significant 56% reduction in vascular calcification, independent of the lipid lowering effect of statins⁴⁹. They also showed that statins have stabilizing effects on advanced atherosclerotic lesions. Whether stabilization of advanced plaques is due to the reduction of vascular calcification is still unclear.

In vitro, statins have bone forming potential by increasing the synthesis of BMP-2 by osteogenic progenitor cells⁵⁰. They also substantially reduce the risk of bone fractures in older women⁵¹. Studies are ongoing to examine the effects of statins in treatment of osteoporosis⁵².

A major protein capable of inhibiting BMP-2, and thus vascular calcification, is MGP. MGP contains Gla residues that are activated by vitamin K⁵³. Jie *et al.* demonstrated that in postmenopausal women, the presence of atherosclerotic calcification in the abdominal aorta is associated with a marginal vitamin K status⁵⁴. Warfarins are vitamin K antagonists and used in anti-coagulation therapy in cardiac patients⁵⁵. Warfarins induce vascular calcification by blocking vitamin K and hence MGP, thereby reducing the inhibition of BMP-2 activity^{27,56}. However, the warfarin induced vascular calcification can also be inhibited by osteoprotegerin, that is also regulated by BMP-2^{57,58}. Thus, in the active regulation of vascular calcification, the BMPs are key molecules.

BMP-3, which we identified in our micro-array, might also have therapeutic potential in inhibiting the progression of vascular calcification. Whether it has reciprocal effects on vascular- and bone cells is not known yet and its capacity to reduce calcification in plaques is still elusive. However, BMP-3 might be beneficial in preventing vascular calcification by inhibiting BMP-2.

5 Limitations of the study

In this thesis we showed that valvular and vascular calcifications are actively regulated by both active inhibitors and active stimulators (*chapter 2 and 3*). Since these studies were designed to be descriptive, we did not perform molecular interventions with these regulators of vascular calcification, except for BMP-2. This key molecule was used to determine the effects on vascular calcification in mice (*chapter 4*). However, interventions with the other regulators of vascular calcification would be elusive and most suited to further dissect the molecular pathways involved.

A second limitation is the choice to use small animal models for in vivo interventions

(chapter 4 and 5). With the use of mouse models, we restricted the possibility to investigate vulnerability of the plaques, since these are rather limited, if not absent in mice. The use of larger animal models, such as the rabbit will enable to study the role of vascular calcification in plaque vulnerability. Another limitation of the use of mice is the apparent lack of lamellar bone structures and the paucity of osteoclasts in advanced lesions, which may reduce the effectiveness of therapeutic interventions such as bisphosphonates that act on osteoclasts.

Randomized clinical studies will be necessary to translate the findings of our animal studies to humans.

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Summary

Atherosclerosis is a chronic inflammatory disease that affects the inner layer (intima) of mainly large arteries. It is a disease characterized by several stages, starting at the first decade of life and progressing with age. As the primary cause of myocardial infarction, stroke and ischemia of the extremities, atherosclerosis is responsible for about 50% of all deaths in the western society. The etiology of atherosclerotic disease is multifactorial, and both hereditary and environmental factors accelerate atherosclerosis. Atherosclerotic plaques can lead to clinical manifestations by blocking the blood flow in the vessel or by thrombus formation. The different stages of atherosclerosis are morphologically well described; plaques responsible for blockade of the blood flow are mainly large and stable, while the thrombus forming plaques are ruptured, eroded or show calcified nodules. Vascular calcification in the intima is invariably associated with, and is a prominent feature of atherosclerosis. Disturbances in lipid metabolism, male sex, and hypertension aggravate atherosclerotic calcification. Calcification in plaques decreases vessel elasticity, and augments plaque brittleness. Atherosclerotic calcification is not restricted to large arteries, and can also occur in leaflets of aortic valves. Aortic valve calcification is associated with increased risk of cardiovascular events including stroke and cardiac death.

In the field of vascular calcification two main questions have been raised. The first question is whether atherosclerotic calcification stabilizes or destabilizes the plaque. The second question is whether vascular calcification is a passive, or active regulated process. Although the association of vascular calcification with atherosclerosis has been noted for centuries, its regulation has been largely ignored, because of the prevailing view that calcification was merely a degenerative, passive process of calcium phosphate precipitation that only occurs in end-stage atherosclerosis and simply signifies dying cells. By the discovery that bone-associated genes can be expressed in the vessel wall, it has become clear that vascular calcification may not be a passive process, but may be actively regulated. In this thesis we focus on the question whether vascular calcification is a passive or active regulated process and how vascular calcification is regulated.

Calcified atherosclerotic plaques can contain osteogenic elements such as bone cells, cartilage, calcium mineral, lamellar bone, nucleation sites (matrix vesicles) and bone regulatory proteins. The presence of these elements and the discovery that two bone matrix regulatory proteins, OPN and BMP-2 were present in human atherosclerotic plaques, demonstrate that atherosclerotic calcification has high similarity with bone calcification. Since bone calcification is regulated by more bone matrix proteins than only OPN and BMP-2, we hypothesized that other bone matrix proteins are also present in human atherosclerotic calcification, both in the vessel wall and aortic valve leaflets. In the second part of this thesis interventions with BMP-2, a highly osteo-inductive protein and one of the first bone matrix proteins described in atherosclerotic plaques, and bisphosphonates, drugs used in the treatment of

osteoporosis were performed in mouse models of atherosclerotic calcification. We hypothesized that enhanced BMP-2 gene expression accelerates atherosclerotic calcification and ossification and that bisphosphonates reduce vascular calcification.

In *chapter 2* we examined the protein expression of 7 bone matrix regulatory proteins and 2 modulators of osteoclastogenesis in all stages of human atherosclerosis. We observed that all these bone matrix proteins are expressed in the arterial wall. One set of proteins, known to belong to inhibitors of calcification, was present in 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. From this study we conclude that vascular calcification is actively regulated by both, inhibitors and activators of calcification.

Recent evidence indicates that aortic valve calcification is a well-regulated process that involves inflammation and lipid accumulation similar to those seen in human atherosclerosis. Vascular calcification is considered to be a risk factor for valvular calcification and recently published data indicate that aortic valve calcification is indeed associated with increased risk of cardiovascular events. Moreover, valvular and vascular calcifications are both considered to share similarities with skeletal bone formation. We therefore examined and described in *chapter 3* the protein expression of 7 bone matrix regulatory proteins and 2 modulators of osteoclastogenesis in both non-calcified and calcified aortic valves. Our data showed that the expression profile of bone matrix proteins in human aortic valves is comparable to the in *chapter 2* described profiles in human atherosclerotic plaques.

According to our data described in *chapter 2 and 3*, inhibitory proteins continuously prevent calcification, whereas the restricted presence of activators provide an imbalance, finally resulting in vascular and/or valvular calcification.

One of the activators of vascular calcification is BMP-2. BMP-2 is a member of the TGF β super family, and is a potent inducer of bone formation and the first osteo-inductive protein found in atherosclerotic plaques. Since BMP-2 is highly osteo inductive, enhances proliferation and differentiation of osteogenic cells, and is present in atherosclerotic plaques we examined the intervention of BMP-2 gene expression on the atherosclerotic calcification process in *chapter 4*. Our data revealed not only that enhanced BMP-2 gene expression accelerated calcification and ossification in atherosclerotic plaques, but that enhanced BMP-2 gene expression also caused an altered bone matrix regulatory protein expression profile.

Another intervention we performed with bisphosphonates, a drug used for the treatment of osteoporosis, and is described in *chapter 5*. Therefore we used the N-containing bisphosphonate, risedronate in an atherosclerotic mouse model. Our data showed that risedronate enhanced bone mineral density of skeletal bones, but had no influence on the initiation and regression of atherosclerotic calcification in mice.

In *chapter 6* we summarize and discuss the data presented in the experimental chapters and provide some novel thoughts on the regulation of vascular calcification and on possibilities to intervene in this process.

The main conclusions derived from the studies described in this thesis are that both atherosclerotic and aortic valve calcifications are indeed actively regulated processes involving bone matrix regulatory proteins. This regulation involves both activators and inhibitors of calcification and bone formation. Furthermore, we conclude that BMP-2 is one of the major active regulators of vascular calcification, and that in an atherosclerotic mouse model bisphosphonates have no influence on vascular calcification when given in a dose that enhanced bone density.

Samenvatting

Atherosclerose (aderverkalking) is een chronische systemische aandoening die de binnenste laag (intima) van voornamelijk de grote slagaders aantast. Kenmerkend voor deze ziekte zijn de verschillende stadia, beginnend in het eerste decennium van ons leven en verergerend naarmate men ouder wordt. Atherosclerose is de belangrijkste oorzaak van een hartinfarct, herseninfarct en beperkte bloedvoorziening in de extremiteiten en is hierdoor verantwoordelijk voor ongeveer 50% van alle sterfgevallen in de Westerse samenleving. De oorzaak van atherosclerose is multi-factorieel. Zowel erfelijke als omgevingsfactoren versnellen de vorming van atherosclerose. Atherosclerotische plaques kunnen leiden tot klinische verschijnselen doordat de bloedstroom in een bloedvat door de plaque geblokkeerd wordt of omdat er een bloedstolsel ontstaat. De verschillende stadia van atherosclerose zijn morfologisch goed beschreven; plaques verantwoordelijk voor de blokkade van de bloedstroom zijn voornamelijk groot en stabiel, terwijl de bloedstolsel vormende plaques geruptureerd (gescheurd) of geërodeerd zijn, of gecalcificeerde (verkalkte) structuren vertonen. Vaatverkalking in de intima is niet alleen geassocieerd met atherosclerose, het is ook een belangrijk kenmerk van atherosclerose. Veranderingen in vetstofwisseling, het mannelijk geslacht en hoge bloeddruk dragen bij aan het ontstaan van atherosclerotische verkalking. Verkalking in plaques vermindert de elasticiteit van het bloedvat, verhoogt de breekbaarheid van de plaque en leidt tot een toegenomen risico voor het ruptureren van de plaques gedurende dotteren. Atherosclerotische verkalking beperkt zich niet tot de grote slagaders, het kan ook voorkomen in de aortakleppen. Aortaklepverkalking is geassocieerd met een toegenomen risico voor hart- en vaatandoeningen inclusief herseninfarcten en hartstilstand.

In het onderzoeksgebied van vaatverkalking zijn twee belangrijke vragen aan de orde. De eerste vraag is of atherosclerotische verkalking de plaque stabiliseert of destabiliseert. De tweede vraag is of vaatverkalking een passief of een actief gereguleerd proces is. Hoewel de associatie tussen vaatverkalking en atherosclerose al eeuwen geleden gemaakt is, heeft men zich nooit verdiept in de regulatie ervan, omdat men dacht dat verkalking een degeneratief, passief proces van calciumfosfaat neerslag was dat alleen voorkwam in het laatste stadium van atherosclerose als gevolg van een ophoping van dode cellen. Door de ontdekking dat bot geassocieerde genen tot expressie kunnen komen in de vaatwand, is het duidelijk geworden dat vaatverkalking niet zomaar een passief proces is, maar dat het mogelijk actief gereguleerd wordt.

Verkalkte atherosclerotische plaques kunnen bot gerelateerde elementen bevatten zoals botcellen, kraakbeen, calciummineraal, volledig uitgegroeid bot, aanhechtingsplaatsen voor botvorming en bot regulerende eiwitten. Door de aanwezigheid van deze elementen en door de ontdekking dat er twee botmatrix regulerende eiwitten, OPN en BMP-2 aanwezig zijn in humane atherosclerotische plaques weet men nu dat atherosclerotische verkalking grote overeenkomsten vertoont

met botverkalking. Doordat botverkalking gereguleerd wordt door meer botmatrix eiwitten dan alleen OPN en BMP-2, hebben wij de hypothese opgesteld dat andere botmatrix eiwitten ook aanwezig zijn in humane atherosclerotische verkalking, zowel de vaatwand als in aortakleppen. In het tweede deel van dit proefschrift worden twee interventie studies beschreven. De ene interventie is gedaan met BMP-2, een krachtig botvorming inducerend eiwit en tevens een van de eerste botmatrix eiwitten beschreven in atherosclerotische plaques. De andere interventie met bisfosfonaten, een medicijn gebruikt voor de behandeling van osteoporose (botontkalking). Beide interventies zijn uitgevoerd in een muismodel dat atherosclerotische verkalking ontwikkelt. Onze hypothese was dat een verhoogde BMP-2 genexpressie zorgt voor een versnelde atherosclerotische verkalking en botvorming en dat bisfosfonaten vaatverkalking kunnen verminderen.

In *hoofdstuk 2* hebben we gekeken naar de eiwit expressie van 7 botmatrix regulerende eiwitten en 2 modulatoren van osteoclastogenese (vorming van botafbraak cellen) in alle stadia van atherosclerose. We hebben geobserveerd dat al deze botmatrix eiwitten tot expressie komen in de vaatwand. Eén set eiwitten, behorend tot de remmers van verkalking, was aanwezig in alle stadia van atherosclerose. Dit suggereert een continue remming van verkalking in de atherosclerotische vaatwand. De expressie van een tweede set van eiwitten, behorend tot de activatoren van verkalking, was beperkt tot de vergevorderde en verkalkte plaques. Uit de resultaten van deze studie concluderen wij dat vaatverkalking actief gereguleerd wordt door zowel remmers als activatoren van verkalking.

Recent bewijs geeft aanwijzingen dat aortaklepverkalking een goed gereguleerd proces is met aanwezigheid van ontsteking en vetophoping in overeenstemming met humane atherosclerose. Vaatverkalking wordt beschouwd als een risicofactor voor klepverkalking en recentelijk gepubliceerde data wijzen in de richting dat aortaklepverkalking weldegelijk geassocieerd is met een verhoogd risico op hart- en vaataandoeningen. Bovendien vertonen zowel klep- als vaatverkalkingen grote overeenkomsten met skeletale botvorming. Daarom hebben we in *hoofdstuk 3* de eiwit expressie van 7 botmatrix regulerende eiwitten en 2 modulatoren van osteoclastogenese onderzocht en beschreven in zowel niet verkalkte als verkalkte aortakleppen. Onze data laten zien dat het eiwit expressiepatroon in de humane aortakleppen vergelijkbaar is met het in *hoofdstuk 2* beschreven eiwitpatroon in humane atherosclerotische plaques.

Volgens onze data beschreven in *hoofdstuk 2 en 3*, zijn het de remmende eiwitten die continu voorkomen dat er verkalking plaats vindt, terwijl de beperkte aanwezigheid van de activatoren zorgen voor een disbalans, die uiteindelijk resulteert in vaat- en/ of klepverkalking.

Eén van de activatoren van vaatverkalking is BMP-2. BMP-2 behoort tot de TGF β superfamilie, en is een krachtige aandrijver van botvorming en het eerst botinducerende eiwit dat gevonden is in atherosclerotische plaques. Omdat BMP-2 zo sterk osteo-inductief is, zorgt voor versnelde deling en differentiatie van osteogene (bot) cellen, en omdat BMP-2 aanwezig is in atherosclerotische plaques hebben we in *hoofdstuk 4* de effecten van een toegenomen BMP-2 genexpressie op het atherosclerotische verkalkingproces beschreven. Onze data tonen aan dat een verhoogde BMP-2 genexpressie niet alleen maar leidt tot versnelde verkalking en botvorming in atherosclerotische plaques, maar ook een veranderd botmatrix regulerend eiwit expressiepatroon veroorzaakt.

De effecten van de interventie met bisfosfonaten, een medicijn dat gebruikt wordt bij de behandeling van osteoporose (botontkalking) wordt beschreven in *hoofdstuk 5*. We hebben hiervoor het N-bevattende bisfosfonaat risedronate gebruikt in een atherosclerotisch muismodel. Uit de resultaten van deze studie blijkt dat risedronate wel zorgt voor een verhoogde botdichtheid van skeletbeenderen, maar geen invloed heeft op zowel het ontstaan van als het terugdringen van verkalkte atherosclerotische plaques in muizen.

In *hoofdstuk 6* worden de bevindingen die beschreven worden in de experimentele hoofdstukken samengevat en bediscussieerd. Dit laatste hoofdstuk verschaft ook een aantal nieuwe gedachten over de regulatie van vaatverkalking en over de mogelijkheden om in dit proces in te grijpen.

De hoofdconclusies van de studies die in dit proefschrift worden beschreven zijn dat zowel atherosclerotische verkalking als ook aortaklepverkalking wel degelijk actief gereguleerde processen zijn waarbij botmatrix regulerende eiwitten betrokken zijn. Bij deze regulatie zijn zowel de activatoren als ook de remmers van verkalking en botvorming betrokken. Bovendien concluderen we dat BMP-2 één van de belangrijkste actieve regulatoren is van vaatverkalking, en dat behandeling met bisfosfonaten in een atherosclerotisch muismodel géén invloed heeft op vaatverkalking, hoewel de gegeven dosis wel zorgt voor een verhoging van de botdichtheid van de skeletbeenderen.

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Kitty, wij zijn ongeveer tegelijk begonnen bij Pathologie, maar hebben niet veel samengewerkt. Maar als ik jouw advies nodig had, was je beschikbaar met jou schat aan kennis en ervaring. Kitty, heel veel succes met alles! Manon, niet altijd alleen maar werken hè!

De vele uren die ik op het lab het doorgebracht waren onvergetelijk. Ik zal het echt gaan missen. Ik wil bij voorbaat alle mensen van het lab bedanken voor al het werk en vooral jullie aanwezigheid als ik weer eens iets nodig had, of met een vraag kwam, ook de naastgelegen labs. Guido altijd weer een rare situatie om over te schakelen naar het Nederlands zodra we door de poort van pathologie liepen. Degenen van ons eigen lab bedank ik natuurlijk ook. Ron, het was jammer dat je al snel na mijn komst wegging, maar Intervet is een uitermate geschikte vervanging. Loes, Monique en Wendy, inmiddels ook niet meer werkzaam bij de cardio's, bedankt voor de leuke tijd. Wendy, ik hoop dat je het nu wel naar je zin hebt. Anique, ik kan nu eindelijk een groot deel van mijn

spullen opruimen. Ine en Coby, al zag ik jullie niet zo vaak omdat jullie altijd enorm druk met coupes bezig waren op het ander lab. Ruben en Barry, eindelijk jongens op het lab. Jullie waren een perfect duo. Ruben, de puppy kalender hangt nog steeds. De data kloppen niet meer maar de puppies zijn nog steeds een genot om naar te kijken. Barry, jou E-mail adres en telefoonnummer zal bewaren zodat ik je altijd kan bereiken als er weer eens iets met mijn computer is. Meestal weet jij een heel eenvoudige oplossing. Anouk, na het vertrek van vele collega's ben jij dé vaste kracht op het lab. Anouk, bedankt voor het "quantimetten" en succes met alle experimenten! Petra, jij hebt erg veel labwerk voor mij gedaan. Het was zeer aangenaam om i.p.v. twee handen, over vier handen te beschikken. Ondanks dat het genotyperen van de transgene muizen jou niet altijd vrolijk maakte, deed jij het elke keer weer zonder te mopperen (ik voelde mij wel schuldig hoor!). Ook de in situ hybridisaties roepen in elk geval bij mij, geen vrolijke gedachten op, maar jij kwam telkens weer met coupes die mij positief stemde. Dit heb ik erg weten te waarderen. Petra, hartelijk bedankt voor de enorm leuke samenwerking. En....ik ben blij dat jij mijn paranimf wil zijn.

Mensen die ik zeker niet mag vergeten zijn de pathologen (Robert-Jan, Adriaan, Ton e alle anderen die ik vergeten ben), de "farmacologen" (Agnieszka ik zal jou op concours nog wel tegen komen, Nicole, Jack, Peter), de "mannen" van biochemie (Henri, Leon, Barry en Cees), en de "cardiologen" (Bianca, Alexandra, Denny en Henny)

De samenwerking met Leiden en P&G hebben beide een mooi hoofdstuk in het proefschrift opgeleverd. Margreet, bedankt voor alle experiment en Paul bedankt voor het kritisch nalezen van het manuscript. Prof Piet Geusens, bedankt voor alle informatie over osteoporose en vasculitis en natuurlijk het fantastische congres in Madrid.

In de periode dat ik elke dag naar Maastricht kwam met de trein heb ik vele uren in dit vervoersmiddel doorgebracht. Eerst vanuit Boxmeer en later vanuit Tegelen (opstapplaats Reuver). In de trein heb ik vele mensen leren kennen, maar Anita, jij bent mijn echte treingenoetje. Met z'n tweetjes zijn we heel vaak op en neer naar Maastricht gegaan. Later zijn ook Bart en Bianca met grote regelmaat met ons meegegaan. 's Morgens hadden we elkaar meestal niet zo heel veel te vertellen, 's middags daarentegen was altijd voldoende stof om over te kletsen. Ook was het zo nu en dan nodig om eventjes met z'n tweetjes de stad in te gaan om met volle tassen huiswaarts te keren. Bart, jammer dat we elkaar niet meer zo vaak spreken, maar de mail is toch een prefect medium om over vanalles op de hoogte te blijven.

Bianca, jij ging bijna altijd met Anita en mij naar Maastricht, behalve dinsdag en woensdag. Dinsdag ging jij altijd met de auto omdat je dan heel vroeg werkbespreking had. Omdat jij in Belfeld woont, konden we ook vaker carpoolen. De leuke gesprekken die wij hadden zal ik niet vergeten. Ook onze discussies, niet alleen over het werk, maar natuurlijk ook over paarden.

Voor ik in Maastricht ben begonnen heb ik bij Intervet in Boxmeer een enorme leuke tijd gehad. Ik heb daar hele mooie herinneringen aan. Zelfs als ik nu nog wel eens daar binnenloop is iedereen nog even aardig. Er zijn een paar mensen die ik extra wil bedanken. Paul, om met jou te beginnen, bijna alles wat ik de op het lab heb gedaan (qua experimenten) heeft zijn oorsprong bij jou. Toen ik na mijn stage bij jou ben gaan werken heb je mij zo vrij gelaten dat ik heel veel zelf heb kunnen ontdekken. Als ik er echt niet meer uitkwam en geen ideeën/mogelijkheden meer had, dan belde ik jou of liep even langs. Jij wist mij elke keer weer te verbazen met het scala aan ideeën/mogelijkheden die jij nog voorhanden had. Daarna ging ik dan snel weer verder om "op tijd" naar huis te gaan. Paul, door jou oplettendheid ben ik geen analist gebleven, en toch AIO geworden. Nog bedankt voor de vacature toentertijd!

Lisette, eerst even koffie drinken en kletsen om daarna snel aan de slag te gaan. Zo begon elke morgen voor ons. Eén van de overeenkomsten tussen ons is het Baculo-virus expressie systeem. Jij werkt nu nog steeds met dit zeer veilige systeem, terwijl ik er na mijn stage nooit meer iets mee gedaan heb, behalve als jij op vakantie was. In de jaren dat we samen het kantoortje deelden zijn we behalve collega's ook vriendinnen geworden. Helaas zien we elkaar niet meer zo heel vaak, maar de kapper (Luci) is dan weer een perfecte gelegenheid om af te spreken. De planning om het mountainbiken weer op te pakken hebben we erin weten te houden, alleen laat de uitvoering nog te wensen over. Lisette en Pieter, we moeten nu toch echt afspreken om te gaan fietsen, en daarna heerlijk te eten met een goed glas wijn erbij.

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Karin, de lay-out en de buitenkant van het boekje zijn van jou hand. Enorm veel werk heb je ermee gehad, onder andere omdat ik toch wel een pietje precies ben. Gelukkig heb ik (als de photoshop-koningin) jou kunnen helpen met de figuren. Het proefschrift ziet er schitterend uit. Karin heel erg bedankt voor alle uren die je erin gestoken hebt, en nu is het niet meer nodig om op een oude krant met een fles whisky op kantoor te overnachten!

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Curriculum Vitae

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Full color figures

Figure 1.2 Classification of atherosclerotic plaques

Figure 2.2. Immunohistochemical staining. Negative controls for the antibodies directed to MGP, OC and BSP. Fig. 2.2a: human liver stained with α -MGP. Fig. 2.2b and c: human heart stained with respectively α -OC and α -BSP. Note: positive staining is red. Scale bars in figure 2 represent 50 μ m.

Figure 2.3. Histological and morphological characteristics of the plaque types. Panels a, c, e, and g represent H&E stainings, panels b, d, f, and h represent Von Kossa stainings. Fig 2.3a and b: non-diseased aorta. Fig 2.3c and d: intimal xanthoma. Fig 2.3e and f: fibrous cap atheroma. Fig 2.3g and h: fibrocalcific plaque. (i=intima; m=media and c=calcium mineral). Scale bars in figure 3 represent 50 μ m.

Figure 2.4. Immunohistochemical staining of a non-diseased aorta (a and b), intimal xanthoma (c and d), and fibrous cap atheroma (e and f). Fig. 2.4a and b: non-diseased aorta stained with respectively MGP and OPN. Fig. 2.4c and d: intimal xanthoma stained with respectively BSP and BMP-4. Fig. 2.4e and f: fibrous cap atheroma stained with respectively BMP-4 and ON. (i=intima; m=media and sh=shoulder region). Scale bars in figure 2.4a and b represent 50 μ m, scale bars in figure 2.4c, e and f represent 25 μ m and scale bar in figure 2.4d represents 10 μ m.

Figure 2.5. Immunohistochemical staining of a fibrocalcific plaque. Fig. 2.5a: fibrocalcific plaque containing calcium mineral deposition immuno-stained with monoclonal antibody against MGP (red staining). Note: Strong MGP positive staining at boundary of the mineral deposits. Fig. 2.5b: fibrocalcific plaque, containing calcium mineral deposits (c) and bone tissue (b) stained with α -OPN (red staining). OPN positive staining could be demonstrated at the boundary of the calcium mineral and bone structures (arrows). Fig. 2.5c: fibrocalcific plaque, containing calcium mineral deposits immuno-stained with a mouse anti-BMP-2 (red staining). BMP-2 positive staining (between arrows) could be visualised surrounding the highly calcified central area (*). Fig. 2.5d: fibrocalcific plaque, containing bone structures (b) stained with α -OPN. OPN positive staining was seen surrounding the bone structures (arrow), but also in a striping pattern within the bone (arrowhead). Fig. 2.5e: fibrocalcific plaque with bone formation (b) stained with goat anti-OPG. OPG could be demonstrated in the osteoblasts (arrow) lining the bone structures (b) and some stromal cells (arrowhead) in the bone marrow (bm). Fig. 2.5f: fibrocalcific plaque stained with α -OC. Fig. 2.5g: fibrocalcific plaque, containing calcium mineral deposits (c), immuno stained with goat anti-RANKL. RANKL was observed in the extracellular matrix surrounding the mineral deposits (arrow). Fig. 2.5h: fibrocalcific plaque with matrix vesicles stained with α -ON. Scale bars in fig. 2.5a, c and h represent 25 μ m, scale bars in fig. 2.5b, d and f represent 50 μ m, scale bar in fig. 2.5g represents 10 μ m.

Figure 2.6. *In situ* hybridization with riboprobes directed to MGP, BMP-4, OPN and ON. **Fig. 2.6a:** MGP *in situ* hybridisation of an intimal xanthoma. Note: dark purple staining are MGP mRNA positive medial smooth muscle cells (arrow). **Fig. 2.6b:** MGP *in situ* hybridisation of a fibrous cap atheroma. Note: arrow indicates an MGP mRNA positive intimal smooth muscle cell. **Fig. 2.6c:** BMP-4 *in situ* hybridisation of a fibrocalcific plaque. Arrow indicates a BMP-4 mRNA positive intimal smooth muscle cell in the surrounding of a calcified area of the plaque. **Fig. 2.6d:** OPN mRNA expression in intimal smooth muscle cells in the surrounding of calcium deposits of a fibrocalcific plaque. **Fig. 2.6e:** OPN *in situ* hybridisation of a fibrocalcific plaque. Note: osteocytes and osteoblasts express OPN mRNA. **Fig. 2.6f:** ON mRNA expression in intimal smooth muscle cells in the surrounding of calcium deposits. Scale bar in **fig. 2.6a** represents 50 μ m, scale bars in **fig. 2.6b, d and f** represent 25 μ m, and scale bars in **fig. 2.6c and e** represent 10 μ m.

Fig. 3.1. Immunohistochemical staining of bone matrix regulatory proteins on calcified valves. Immunohistochemistry of osteocalcin (A), matrix Gla protein (C) and osteopontin (E) and their negative controls (B, D and F) on human calcified aortic valves. Scale bars represent 50 μ m.

Fig. 3.2. Von Kossa staining and immunohistochemical staining of osteopontin on serial sections of a calcified aortic valve. Serial sections of a human calcified aortic valve stained with Von Kossa (A and B) and osteopontin (C and D) without (A and C) and with (B and D) citric acid pretreatment. Scale bars represent 50 μ m.

Fig. 3.3 Identification of chondrocytes, osteoclasts, osteoblasts and hemopoietic cells in human calcified aortic valves by respectively thionin staining (A), type II collagen (B), osteocalcin staining (C), cathepsin K staining (D), TRAP staining (E) and CD138 staining (F). Scale bar in A represent 50 μ m and in B, C, D, E and F 25 μ m.

Fig. 3.4: Histological and morphological characteristics of the human aortic valve.

Fig. 3.4a: H&E staining of a non-calcified aortic valve. The three layers of the valve are the fibrosa (f), spongiosa (s) and ventricularis (v). Panel b represent a valve containing calcified tissue H&E stained. Note (*)=cartilage, b=bone, and bm=bone marrow cavity. Scale bars represent 100 μ m (a), 50 μ m (b).

Fig. 3.5. Immunohistochemistry of ASMA, CD68 and CD3 on human aortic valves. A, B, and C: represent immunohistochemical stainings of non-calcified valves, while D, E and F showed immunohistochemical stainings of calcified valves. Aortic valves stained with antibodies directed to ASMA (A and D), CD68 (B and E) and CD3 (C and F). Note: In the calcified valves ASMA, CD68 and CD3 positive cells (red staining) are located in the surroundings of mineralized tissue. (f=fibrosa; s=spongiosa; v=ventricularis and b=bone). Scale bars represent 150 μ m.

Fig. 3.6. Immunohistochemical staining of bone matrix regulatory proteins on non-calcified valves. A and B: non-calcified valve stained with antibodies directed to MGP (A) and BSP (B). Both proteins are present in these valves (red staining). Note: MGP is present in the ventricular layer of the valve and BSP is clearly expressed in the fibroblasts (arrow and inset) in the fibrosa. C and D: non-calcified valve stained with antibodies directed to OPG (C) and RANKL (D). Scale bars represent 100 μm (A) and 25 μm (B, C and D).

Fig. 3.7. Immunohistochemical staining of bone matrix regulatory proteins on calcified valves. A and B: MGP, OC and BSP are present in calcified aortic valves. MGP (A) showed expression (arrow) at the borders of amorphous calcium phosphate deposits, while OC (B) is present in bone matrix of lamellar bone. C and D: In calcified valves OPN (C) is localised in the cement lines (arrow), and BMP-4 (D) is present in the osteoblasts lining lamellar bone (arrow). E and F: OPG (E) showed immunoreactivity in the chondrocytes (arrow) and its ligand RANKL (F) is present in osteoclasts (arrow). Scale bars represent 50 μ m (A and C) and 25 μ m (B, D, E and F).

Fig. 3.8. Double immunohistochemical staining of human calcified aortic valve with osteocalcin and ASMA (A). Note: osteocalcin is also expressed by a subpopulation of smooth muscle cells. Figure B represents a higher magnification. Scale bar in A represents 50 μm and B 25 μm .

Fig. 3.9. *In situ* hybridisation (ISH). A, B and C: representation of respectively ISH of BMP-4 (A), and immunohistochemistry with antibodies directed to BMP-4 (B) and ASMA (C). D, E and F: ISH of ON (D, left inset is magnification of chondrocyte like cells and right inset is ISH of ON with sense probe), and immunohistochemistry with antibodies directed to ON (E) and ASMA (F). Note: BMP-4 is present in endothelial cells (arrow). ON is present in chondrocyte like cells (arrow and inset). Scale bars represent 50 μ m.

Fig. 4.1. Cross-sections of the liver of respectively a Ad.BMP-2 treated mouse (A) and a control mouse (B) stained by immunohistochemistry with a polyclonal antibody directed to BMP-2. A: BMP-2 is visualized in red. Arrows indicate BMP-2 present in liver cells. Cross-section of atherosclerotic plaques of respectively the Ad.BMP-2 treated group (C) and the control group (D) stained for TGFβ (visualized in red). Note: TGFβ was hardly present in the control group (Fig. 4.1D) and clearly enhanced in the Ad.BMP-2 group, with TGFβ located primarily at the adventitial side of the intima (Fig. 4.1C). Scale bars in panel A and B represent 50μm, in panel C and D 100μm.

Fig. 4.2. Cross-sections of a vein graft of respectively the Ad.BMP-2 treated group (A and C) and the control group (B and D). There was no difference in total vessel area, plaque area and lumen area between the Ad.BMP-2 treated mice (A) and the controls (B). However, the area of non-ossious calcification and ectopic bone formation was enhanced in the Ad.BMP-2 treated mice (C) versus the controls (D). Arrow in panel C indicate ectopic bone formation. Arrow in panel D indicate cartilage.

Cross-sections of atherosclerotic plaques in Ad.BMP-2 treated mouse (E) and control mouse (F) containing non-calcified cartilage. Cartilage is visualized in purple by a thionin staining (arrow).

Scale bars in panel A and B represent 100 μ m, and in panel C, D, E and F 50 μ m. C and D 100 μ m.

Fig. 4.3. Cross-sections of atherosclerotic plaques in both the Ad.BMP-2 treated group (left panels) and the control group (right panels). Positive staining was visualized in red.

In the Ad.BMP-2 treated group only osteocalcin was present in calcified cartilage, BMP-4 in young chondrocytes, BMP-2 in the macrophages, BMP-2 and osteoprotegerin in intimal calcification and in adventitial calcification/ossification five proteins showed immunoreactivity (BMP-2, BMP-4, osteocalcin, BSP and osteoprotegerin). In the control group BMP-2, BMP-4, BSP and osteonectin were present in both calcified cartilage and non-calcified cartilage. Osteopontin was present in calcified cartilage, while osteoprotegerin showed immunoreactivity in non-calcified cartilage. In young chondrocytes RANKL and osteonectin were present, in macrophages osteopontin and osteocalcin and in intimal calcification only osteopontin was expressed. Scale bars in panels with 10x magnification represent 50 μ m, and in panels with 25x magnification 20 μ m.

Fig. 5.1. Cross-sections of the first branch (right carotid artery) of the aortic arch of respectively a risedronate treated mouse (a, c and e) and a control mouse (b, d and f) stained with H&E (a and b), Sirius Red for the detection of collagen (c and d) and with a monoclonal antibody directed to Mac3 for the detection of macrophages (e and f). a: arrow indicate cholesterol crystals. b: arrow indicate calcification. c and d: collagen is visualized in red. e and f: arrows indicate macrophages, visualized in red.

Fig. 5.2. Cross-sections of a femur H&E stained (a) and a three-dimensional micro-CT scan of the left leg (femur, knee joint and tibia) (b).

Fig. 5.3. Cross-sections of the complete aortic arch including the main branch points (a, c, e, g and i) and a higher magnification of the first branch (right carotid artery) (b, d, f, h and j) of various groups and controls. a and b: regression Western type diet group with risedronate dose of 5 µg/kg (rWT low dose). c and d: regression Western type diet group with risedronate dose of 25 µg/kg (rWT high dose). e and f: control group for the regression Western type diet experiment. g and h: regression normal chow group with risedronate dose of 5 µg. i and j: control group for the regression normal chow experiment. Scale bars in fig. 5.3a, c, e, g and i represent 300 µm, and in fig. 5.3 b, d, f, h and j the scale bars represent 200 µm.

Fig. 5.4. Immunohistochemical staining of three bone matrix regulatory proteins (BMP-2, OPG and RANKL) on murine atherosclerotic plaques of various experimental groups. **a, b and c** represent atherosclerotic plaques of prevention Western type diet mice that received a risedronate dose of 5 $\mu\text{g/kg}$. **d, e and f** are the controls. **g, h and i** represent atherosclerotic plaques of regression Western type diet mice that received a risedronate dose of 5 $\mu\text{g/kg}$. The left panels are immunohistochemically stained with BMP-2, the middle panels with OPG and the right panels with RANKL. Positive staining was visualized in red. Mainly the chondrocytes (arrows) are positively stained for all three bone matrix proteins. Scale bars represent 20 μm .

Fig. 5.5. TRAP staining for the identification of osteoclasts. A single osteoclast (arrow) present in an atherosclerotic plaque of the regression NC experiment (fig. 5.5a) and in the accessory control group (fig. 5.5b). Positive staining is visualized in brown. Scale bars represent 20 μ m.