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Vascular calcification: expression patterns of the osteoblast-specific gene core binding factor α-1 and the protective factor matrix gla protein in human atherogenesis

Marten A. Engelse[a], Jolanda M. Neele[a], Antonius L.J.J. Bronckers[b], Hans Pannekoek[a], Carlie J.M. de Vries[a,*]

[a]*Department of Biochemistry, Academic Medical Center K1-163, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands*
[b]*ACTA, Department of Oral Cell Biology, Vrije Universiteit, Amsterdam, The Netherlands*

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Abstract

Objective: Increasing evidence suggests that vascular calcification is a regulated process. We studied the vascular expression pattern of a key factor in mineralization and a counteracting, protective factor. Based on the phenotype of null mice, Core binding factor α-1 (Cbfa-1) plays a pivotal role in bone formation, whereas Matrix Gla Protein (MGP) is a potent inhibitor of vascular calcification. **Methods:** We investigated the expression of MGP and Cbfa-1 in cultured, human monocytic cells, endothelial cells and smooth muscle cells (SMC), as well as in normal and atherosclerotic vessel specimens. **Results:** In cultured cells MGP is expressed in endothelial cells and SMC, whereas Cbfa-1 mRNA is predominantly present in macrophages and to a lesser extent in SMC. In the normal vessel wall MGP expression is high at the luminal side and declines toward the center of the media, whereas Cbfa-1 is absent. Moderate, diffuse calcification of the aorta media was observed only in those regions where MGP is low or absent. In atherosclerotic lesions MGP is expressed in endothelial cells and SMC that form fibrous caps, but is never present in macrophages. Cbfa-1 is synthesized in regions without MGP, it is associated with calcified areas and Cbfa-1 may be considered a marker for osteoprogenitor-like cells in the vessel wall. **Conclusions:** Our observations on MGP expression confirm and extend published data and are consistent with a protective function of MGP. Cbfa-1 expression is absent in normal medial SMC and co-localizes with neointimal macrophages and focal calcifications.

Keywords: Atherosclerosis; Gene expression; Macrophages; Smooth muscle; Extracellular matrix

This article is referred to in the Editorial by N.P.J. Brindle (pages 178–180) in this issue.

1. Introduction

Vascular calcification occurs during atherosclerosis and diffuse calcification may already be present in early lesions. With increasing plaque size and complexity vascular calcification progresses, which induces rigidity of the vessel wall (reviewed in Ref. [1]). Mineralization of the arterial vessel wall is regarded as a regulated process, analogous to skeletal bone formation with the same factors involved [1,2]. Normal bone formation requires a delicately balanced expression of mineralization-inducing and inhibiting factors. One of the inhibiting factors is Matrix Gla Protein (MGP), which is a secreted, matrix-associated Gla (gamma-carboxy-glutamate) containing protein that depends on Vitamin K for proper function [3–5]. The profound protective effects of MGP in arterial calcification were demonstrated in studies with warfarin-treated rats and MGP knockout mice [6,7]. In both studies, the ablation of MGP-function caused an excessive mineralization of the arterial tree. Strikingly, the knockout mice die within 2

*Corresponding author. Tel.: +31-20-566-5152; fax: +31-20-691-5519.
E-mail address: c.j.devries@amc.uva.nl (C.J.M. de Vries).

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months due to vessel rupture and subsequent acute thrombosis as a result of this extreme vascular calcification [7]. Furthermore, MGP mRNA expression has been demonstrated in human lesions [8], and it was shown to be preferentially expressed by proliferating rat SMC [9], as well as in calcifying, cultured human SMC [10].

A factor that plays a pivotal role in the induction of bone formation is the runt-domain transcription factor Core binding factor α-1 (Cbfa-1). Cbfa-1 dictates the expression of a set of genes, comprising alpha1(I) collagen, bone sialo protein and osteocalcin, which are expressed predominantly in osteoblasts [11]. Each of these genes has been implicated in bone formation, although their exact functions in this process have not been revealed. The importance of Cbfa-1 in bone formation is demonstrated by the phenotype of Cbfa-1 knockout mice that completely lack mineralized bone structures [11–14]. The expression of MGP is normal in Cbfa-1 null mice, which may indicate that MGP expression is not directly regulated by this transcription factor [12]. Recently, Cbfa-1 has been associated with enhanced calcification in cultured SMC [15,16]. These results imply that Cbfa-1 may be involved in vascular calcification in atherosclerosis.

In this study, we report on the expression of the calcification-modulating factor MGP and the bone inducing factor Cbfa-1 in different cultured vascular cells and in human vascular tissue specimens in relation to calcification and atherogenesis. With respect to MGP we extend published data with a more detailed description of cell-specific expression and its expression in the normal vessel wall. The presence of Cbfa-1 in the human vessel wall is shown for the first time.

2. Material and methods

2.1. Cell culture

Cell culture was performed at 37°C in a humidified 5% CO_2 chamber. Monocytic cell line MonoMac6 (MM6) was cultured in 90% (vol/vol) RPMI1640, 10% (vol/vol) fetal bovine serum, supplemented with penicillin, streptomycin and fungizone (GIBCO BRL, Gaithersburg, MD). Human primary smooth muscle cells (SMC) were derived from vessel explants, originating from human umbilical cord arteries. Cultured SMC were characterized by immunofluorescence with the antibody directed against SM α-actin (1A4 from DAKO, Glostrup, Denmark), which was detected with a Cy3-conjugated goat anti-mouse antibody (Jackson Laboratories, Westgrove, PA). With this method, the cells show uniform fibrillar staining. The cells were used at passage five to seven. Human umbilical vein endothelial cells were obtained by trypsinization from fresh umbilical cords, and used after one to two passages. SMC and endothelial cells were cultured in 1% (wt/vol) gelatin (Sigma) coated culture flasks, in 40% (vol/vol) Medium-199 with L-glutamine/L-amino acids, 40% (vol/vol) RPMI1640 with HEPES buffer/L-glutamine, 20% (vol/vol) human serum, supplemented with penicillin, streptomycin and fungizone.

2.2. Atherogenic stimulation of different cell types

MM6 monocytic cells were cultured in standard medium, and subsequently differentiated over 2 days with 100 ng/ml phorbol myristate acetate (PMA). Endothelial cells were grown until confluency, and maintained for an additional 3 days. Tumor necrosis factor-α (100 ng/ml) was added in serum-free standard medium (containing 10 mg/l bovine insulin, 5.5 mg/l human transferrin and 6.7 μg/l sodium selenite (GIBCO BRL)) to the endothelial cells, and the cells were harvested after 0, 6, 24 h, and subjected to RNA isolation using Trizol reagent (GIBCO BRL). SMC were cultured until confluency, and subsequently maintained for 3 days in serum-free medium. Conditioned medium of human peripheral macrophages, stimulated with oxidized-low density lipoprotein (LDL) (see paragraph on preparation of macrophage conditioned medium), was added to the cells in a 10-fold dilution for 0, 8, and 24 h.

2.2.1. Preparation of macrophage conditioned medium

Elutriated, human peripheral monocytes (kindly provided by Dr E. Meul, CLB, Amsterdam, The Netherlands) were cultured in 50% (vol/vol) RPMI1640 and 45% (vol/vol) Medium 199, supplemented with 5% (vol/vol) human serum and antibiotics. Subsequently, the macrophages were incubated for 16 h in culture medium with 30 μg/ml oxidized-LDL (copper-chloride oxidation; 40–50 nmoles/mg thiobarbituric acid-reactive substances). After collection the conditioned medium was stored at −20°C.

2.3. Northern blotting

Northern blots were made as described using Hybond N nylon membranes (Amersham) [17]. MGP or GAPDH probes (Table 1) were radiolabeled with [^{32}P]-αATP (Amersham) using a random oligo-labeling kit (GIBCO BRL), and purified on a oligonucleotide-removal column (Qiagen, Hilden, Germany). Probe hybridization in formamide was performed as described [17]. The blots were stringently washed at 65°C with 0.1×SSC, 0.1% (wt/vol) sodium dodecyl sulfate (SDS). Specific bands were quantitated using PhosphoImager, and Image Quant software.

2.4. Tissue specimens

Human tissue samples were obtained from organ donors or from patients undergoing vascular surgery with informed consent of relatives or the patients, according to protocols approved by the Medical Ethical Committee of the Academic Medical Center. The specimens were fixed in 3.8% (vol/vol) formaldehyde in phosphate-buffered saline (PBS) and paraffin embedded or snap-frozen in

Table 1
Details of probes used in in situ hybridization, Nothern blot analysis and RNase protection analysis

Name	Genbank#	bp	Application
MGP	X53331	102–358	Northern blot analysis In situ hybridization
Cbfa-1	AH005498	642–884 451–2170	RNase protection analysis In situ hybridization
GAPDH	M33197	480–545	Northern blot analysis RNase protection analysis
PCR	Primer sets		
Cbfa-1	Fwd: 5′ CGACAGCCCCAACTTCCTGTG 3′ Rev: 5′ TGCCTTCTGGGTTCCCGAG 3′	451–1007	Semi quantitative RT–PCR
GAPDH	Fwd: 5′ TAGAATTCAGGTCATCCATGACAACTTTGG 3′ Rev: 5′ TAGTCGACATCCACAGTCTTCTGAGTGGCA 3′	545–629	Semi quantitative RT–PCR

The primer sets used for semi quantitative RT–PCR are mentioned. The MGP construct was a kind gift from Dr C. Vermeer (Department of Biochemistry, Maastricht University, The Netherlands).

liquid nitrogen within 5 min after resection. After sectioning (5 μm) and mounting on 3-aminopropyl-triethoxysilane-coated glass slides, the specimens were subjected either to in situ hybridization, immunohistochemistry or von Kossa staining. From snap-frozen tissue samples, mRNA was isolated and subjected to RT–PCR analysis. In this study, lesions were classified according to the guidelines of the American Heart Foundation [18]. Twenty-seven specimens were obtained from 22 individuals ranging in age from 12 to 76 years, comprising apparently normal vascular tissue as well as lesions ranging in complexity from I toVI.

2.5. In situ hybridizations and RNase protection analysis

In vitro transcription of linearized plasmid DNA was performed to obtain radiolabeled anti-sense or sense riboprobes ([^{35}S]-UTP for in situ hybridization and [^{32}P]-UTP for RNase protection [Amersham]); see Table 1 for details on probes. The in situ hybridization assays and RNase protection assays were performed as described previously [19]. As a control for the specificity of the anti-sense riboprobes, matching sense riboprobes were assayed for each gene; the sense probes gave neither background nor an aspecific signal.

2.6. (Immuno)histochemistry

Antibody 1A4 (DAKO) recognizes SM α-actin and was used to detect SMC, while antibody HAM56 (DAKO) was applied to recognize macrophages in the sections. Cbfa-1-specific antibodies were kindly donated by Dr P. Ducy (Baylor College of Medicine, Houston, TX). The rabbit antibodies were raised against an amino-terminal peptide sequence of Cbfa-1 (amino-acids 69–95) [11] and were applied at a 1:500 dilution. Immunohistochemistry was performed on 5 μm paraffin sections. As a pretreatment, the sections were dewaxed, rehydrated, incubated with 0.3% (vol/vol) hydrogen peroxide to inhibit endogenous peroxidase activity, and blocked with 10% (vol/vol) preimmune goat serum (DAKO) in Tris-buffered saline (TBS; 10 mmol/l Tris, pH 8.0, 150 mmol/l NaCl). The sections were then incubated with the specific antibodies, followed by biotinylated secondary antibodies, which were subsequently detected with streptavidin–horseradish peroxidase conjugates (DAKO). Endothelial cells were specifically recognized with *Ulex europaeus* lectin that was detected with an anti-Ulex lectin–horseradish peroxidase conjugate (DAKO). Peroxidase activity was visualized with aminoethylcarbazole and hydrogen peroxide, which gives rise to a brick-red precipitate. After counterstaining with haematoxilin, the sections were embedded in glycergel (Sigma, St. Louis, MO). Von Kossa staining was performed after standard pretreatment of the sections. The specimens were then incubated for 60 min in 5% (wt/vol) $AgNO_3$ while being exposed to bright light, rinsed thoroughly with water and then incubated in 2.5% (wt/vol) $Na_2S_2O_3$. The sections were subsequently dehydrated and mounted in PERTEX mounting medium (Histolab, Göteborg, Sweden).

2.7. Semi quantitative RT–PCR analysis

Total RNA was reverse-transcribed with Superscript II, and oligo dT primers (GIBCO BRL, Gaithersburg). Cbfa-1 and GAPDH sequences were amplified using specific primersets, indicated in Table 1, and Amplitaq Gold Polymerase (Roche Molecular Systems, Branchburg) after 10 min at 95°C followed by a standard PCR program with 60°C as the annealing temperature. After 24 cycles (GAPDH) and 30 cycles (Cbfa-1), the PCR was stopped and analyzed. In the same experiment, separate control samples were taken at 22, 24 and 26, or 28, 30 and 32 cycles to assure that experiments were performed at the number of PCR cycles, at which the amplification was linear. These samples were analyzed on the same gel as the samples from the vessel specimens, and were calculated to contain relative amounts of PCR product at ratio 1:4:16.

This approach facilitates semi quantitative analysis of Cbfa-1 mRNA expression in vascular specimens.

3. Results

3.1. Expression patterns of Cbfa-1 and MGP in cultured cells

We initiated our study with an inventory on the expression profiles of the calcification inducing factor Cbfa-1 and the protective factor MGP in cultured cells. Macrophages, endothelial cells and SMC, which together are the most important cellular components involved in the initiation and progression of atherosclerosis, were incorporated in our experiments. Moreover, the cells were activated with atherogenic stimuli to mimic the in vivo processes involved in atherogenesis. In MM6 cells no MGP mRNA was detected, while Cbfa-1 mRNA is present and increases 2-fold upon differentiation of the cells after PMA stimulation (Fig. 1; lanes 1–2). In endothelial cells, MGP mRNA expression decreases dramatically after 24 h of stimulation, while Cbfa-1 mRNA expression was below the detection limit of the RNase protection analysis (Fig. 1; lanes 3–5). Additional RT–PCR analysis on the same RNA samples demonstrated Cbfa-1 mRNA to be present at low levels in cultured endothelial cells, but no changes in expression were observed after stimulation (data not shown). In SMC a relatively high expression level of MGP is observed and activation of SMC results in a moderate reduction of MGP expression (1.3±0.02-fold), whereas Cbfa-1 expression is relatively low and increases to 1.7±0.16-fold upon stimulation of the cells (Fig. 1; lanes 6–8).

3.2. MGP expression in the vessel wall

So far, we have demonstrated MGP and Cbfa-1 expression in cultured primary SMC, endothelial cells and in the monocytic/macrophage cell line MM6. Next, we investigated the expression of MGP in normal and atherosclerotic tissue specimens. Vascular expression of MGP has been described to some extent before. Here we demonstrate several significant, novel aspects on vascular MGP expression. As typical examples of MGP expression in the normal medial layer of the vessel wall we show cross sections of a human aorta (Fig. 2a–e) and an iliac artery (Fig. 2f–j) that contain very small neointimas (Ni). The immuno-histochemical data show that the medias (M) are composed solely of SMC (Fig. 2a,f) and contain no macrophages (Fig. 2b,g). An intact layer of endothelial cells covers both vessels (Fig. 2c,h). Von Kossa staining (Fig. 2d,i) revealed a diffuse calcium-rich deposit in the center of the entire media of the aorta specimen, whereas in the iliac artery no such calcified regions were identified. Radioactive in situ hybridizations demonstrated a remarkable gradient of MGP mRNA expression in the media of both aorta and iliac artery, with high MGP expression levels at the internal elastic lamina (IEL) and distinctly lower levels toward the center of the media (Fig. 2e,j). It should be noted that all aorta specimens assayed showed such diffuse medial calcification and were obtained from individuals ranging in age from 31 to 52 years. Moreover, the iliac artery specimens that were incorporated in our study were derived from organ donors aged 34 to 65 years and consistently showed no diffuse calcification in the media.

To identify the vascular cells, which express MGP in

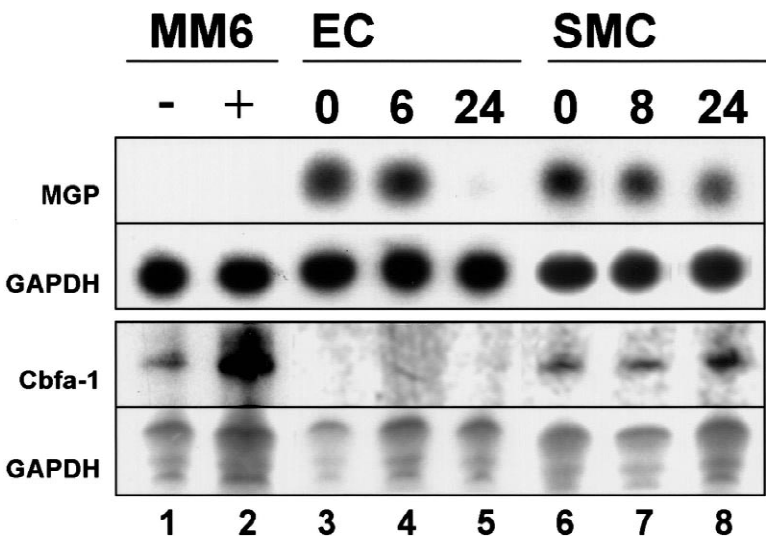

Fig. 1. MGP and Cbfa-1 mRNA expression in (activated) monocytic MM6 cells, endothelial cells and SMC. MGP and Cbfa-1 expression were assayed by Northern blot analysis and RNase protection analysis, respectively. GAPDH expression was tested as a control for equal loading. MM6 monocytic cells were assayed either non-stimulated (lane 1, −) or after stimulated with PMA (lane 2, +). The expression of MGP and Cbfa-1 was also determined in endothelial cells (EC) that were left unstimulated (lane 3) or were stimulated with TNFα for 6 and 24 h (lane 4, 5), in quiescent SMC (lane 6) and in SMC activated with macrophage-conditioned medium for 8 and 24 h (lane 7,8). See Materials and methods for details.

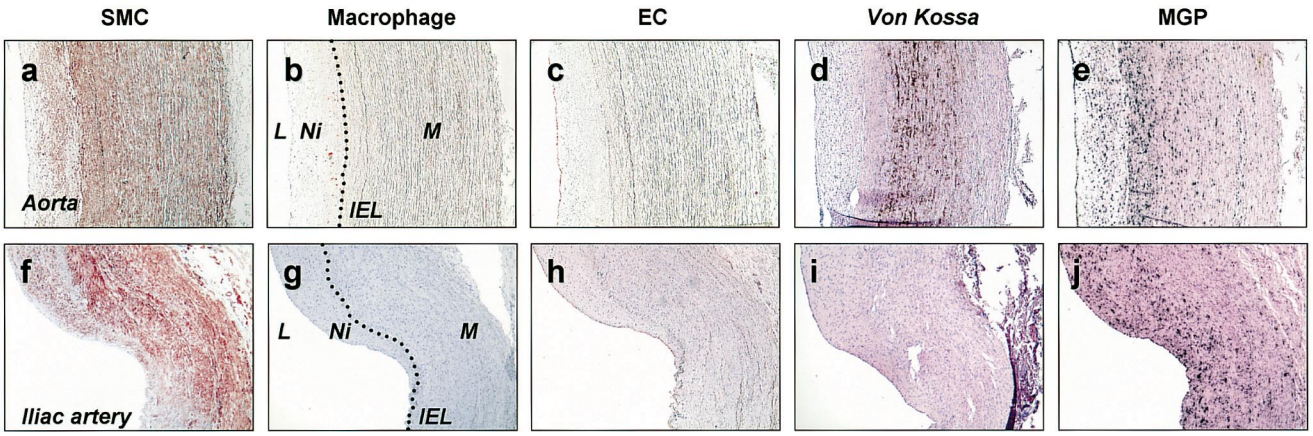

Fig. 2. MGP expression in the vascular media. Early lesions from a human aorta (a–e) and a human iliac artery (f–j) were subjected to immunohistochemistry, von Kossa staining and MGP-specific in situ hybridizations. Consecutive cross sections were assayed to detect SMC (a,f), macrophages (b,g) and endothelial cells (c,h). Additionally, von Kossa staining (d,i) was performed to demonstrate vascular calcification (dark brown-black precipitate). MGP mRNA expression was revealed by radioactive in situ hybridization ((e,j) positive signal results in black dots). The sections were counterstained with haematoxilin to reveal the nuclei (purple). The internal elastic lamina (IEL) is indicated by a dotted line (b,g) and media (M), neointima (Ni) and lumen (L) are indicated accordingly. Photomicrographs of bright field microscopy, original magnification 50×.

vivo, we show additional high-power microscopic examinations of consecutive vascular cross sections of a macrophage-rich lesion (Fig. 3a–d) and a SMC-rich lesion (Fig. 3e–h). Radioactive in situ hybridizations (Fig. 3d,h) revealed that MGP mRNA is not expressed by macrophages but that MGP synthesis is restricted to endothelial cells and SMC, which is consistent with the in vitro data shown in Fig. 1.

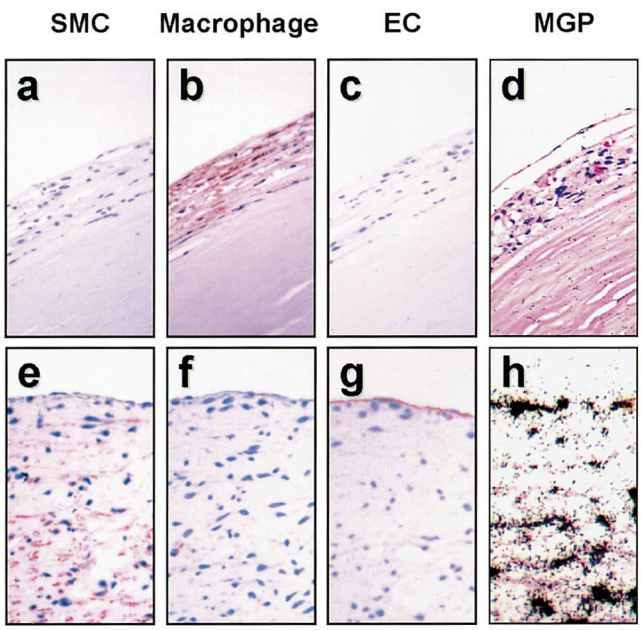

Fig. 3. MGP expression in a macrophage-rich region (a–d) and in a SMC rich-region covered by an intact layer of endothelial cells (e–h). Consecutive sections were stained immunohistochemically for the presence of SMC (a,e), macrophages (b,f) and endothelial cells (c,g). MGP mRNA expression was assayed by radioactive in situ hybridization (d,h). Photomicrographs: bright field microscopy, original magnification 200×.

Subsequently, we determined the pattern of MGP expression at different stages of atherosclerosis. As typical examples, we show MGP expression in an early aortic lesion (Fig. 4a,b), an iliac artery with a fatty streak (Fig. 4c,d), an iliac artery containing advanced fibrous lesions (Fig. 4e,f) and an aorta containing advanced atheromatous lesions (Fig. 4g,h). Again, in the media of these arteries, we observed a gradient of MGP mRNA expression. In the neointima of early SMC-rich lesions, MGP mRNA is strongly expressed (Fig. 4a,b), while in macrophage-rich areas of an early fatty streak MGP expression is reduced (Fig. 4c,d; see *). A similar dichotomy was observed in advanced stages of atherogenesis. In advanced fibrous lesions, MGP mRNA is abundant in the fibrous cap, which consists mainly of SMC (Fig. 4e,f; see **). In contrast, atheromatous areas are devoid of MGP mRNA expression, which is demonstrated in an atheromatous inclusion beneath a fibrous cap (Fig. 4f; see *), and in an advanced atheromatous lesion (Fig. 4g,h; see *).

3.3. Cbfa-1 expression in the vessel wall

We observed expression of Cbfa-1 in cultured macrophage-like cells, in SMC and at a very low level in endothelial cells. So far, the expression of this transcription factor has not been studied in the (calcified) vessel wall. This knowledge prompted us to study the expression of Cbfa-1 in the vessel wall in atherosclerosis. We obtained a limited number of pairs of atherosclerotic and apparently normal arteries from different patients, and examined the expression of Cbfa-1 by semi-quantitative RT–PCR. In apparently normal vessels, we observed low expression of Cbfa-1 mRNA (Fig. 5; lanes 1, 3, 5), whereas in atherosclerotic vessels Cbfa-1 mRNA expression was consistently increased (Fig. 5; lanes 2, 4, 6). In addition, a high level of

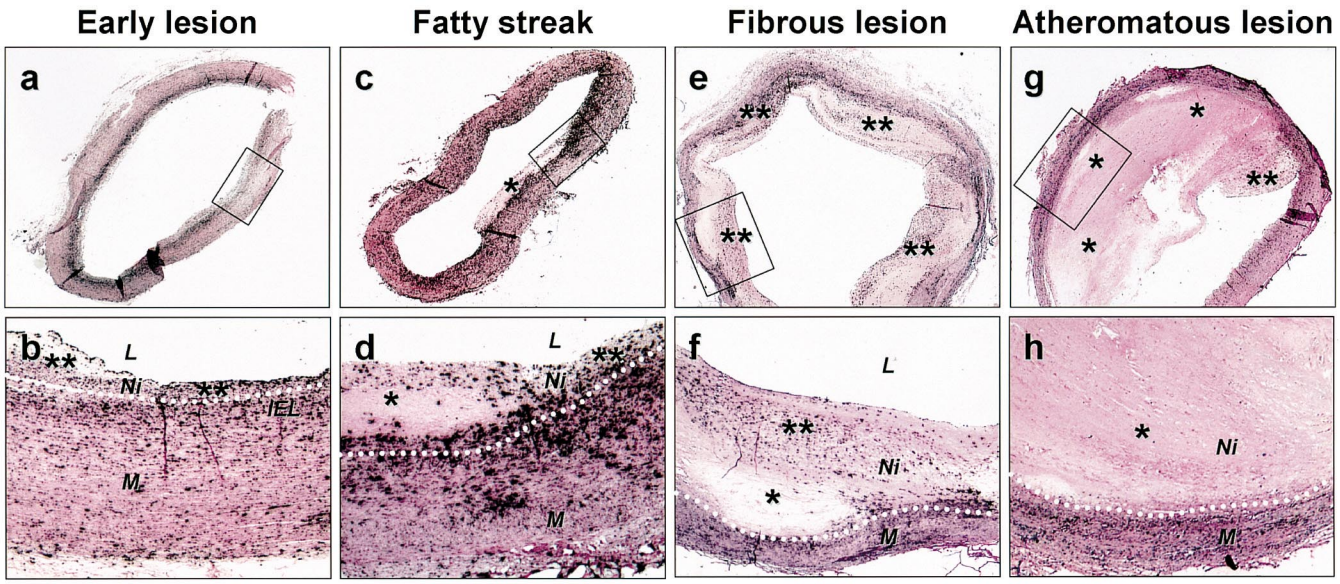

Fig. 4. MGP expression at different stages of atherosclerosis. Radioactive in situ hybridizations, specific for MGP mRNA, were performed on typical examples of an aorta with early lesions (a,b), an iliac artery with a fatty streak (c,d), an iliac artery with advanced fibrous lesions (e,f), and an aorta with an advanced atheromatous lesion (g,h). Panels a, c, e and g show the cross section of the whole vessel, while panels b, d, f and h demonstrate details of the boxed areas, respectively. The internal elastic lamina (IEL) is indicated by a dotted line. Atheromatous areas (*), fibrous areas (**), internal elastic lamina (IEL), lumen (L), media (M) and neointima (Ni) are indicated accordingly. Photomicrographs: bright field microscopy, original magnification 5× (e,g), 10× (a,c), 25× (f,h) and 50× (b,d).

Cbfa-1 mRNA expression was detected in two atherosclerotic artery specimens derived from two additional patients (Fig. 5; lanes 7 and 8).

To assess which cell types in the vasculature contribute to Cbfa-1 expression, we performed radioactive Cbfa-1 mRNA-specific in situ hybridization studies. After in situ hybridization, cell-specific immunostaining was performed to reveal colocalization of Cbfa-1 expression with a specific cell type within the same section. In Fig. 6a,b we show expression of Cbfa-1 in subsets of macrophages in two different lesions by bright-field microscopy and epipolarization, respectively. Previously, we have shown that a remarkable phenotypic variation exists among macrophages within atherosclerotic lesions, which is again exemplified by the non-homogeneous expression of Cbfa-1 in these cells [20]. In another aorta lesion (Fig. 6c), we combined Cbfa-1 specific hybridization with SMC-specific immunostaining and revealed Cbfa-1 expression to a lesser extent in neointimal SMC and in endothelial cells at the luminal side of the lesion. Cbfa-1 is an intracellular protein, which allows the identification of those cells that are recognized with specific antibodies, as the cells that actually synthesize Cbfa-1. The antibodies we applied in this study have been used succesfully in band-shift assays [11]. Based on the amino-acid sequence and its function as transcription factor Cbfa-1 is predicted to be translocated

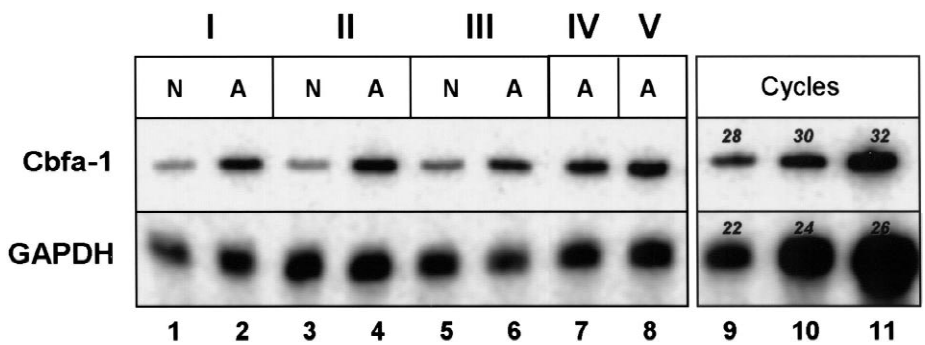

Fig. 5. Cbfa-1 expression in normal and atherosclerotic vessels. Semi quantitative, radioactive RT–PCR specific for Cbfa-1 expression, at 30 PCR cycles, was performed on apparently normal vessels (N) and atherosclerotic (A) vessels that were obtained from different patients (I–V, in lanes 1–8). GAPDH expression, at 24 PCR cycles, was tested as a control for equal loading (lanes 1–8). In lanes 9–11, samples with an increasing number of PCR cycles are shown to demonstrate that the PCR reactions were performed in the linear range of the reaction; Products were obtained after 28, 30 and 32 cycles for Cbfa-1, and after 22, 24, 26 cycles for GAPDH, respectively.

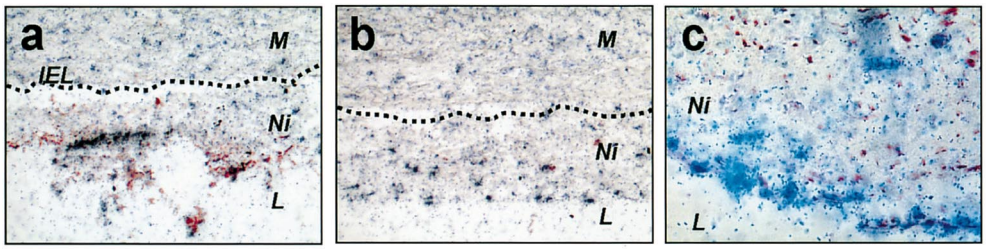

Fig. 6. Cbfa-1 mRNA expression in aorta lesions in cross sections of three distinct specimens. Each specimen was tested by radioactive in situ hybridization for Cbfa-1 mRNA, and subsequently immunohistochemically stained to simultaneously detect macrophages (a, b) or SMC (c) in the same section. In situ hybridization signal is visualized by bright-field microscopy (black grains) (a, b) or by epipolarization (bright-blue) (c). Original magnification of the photomicrographs 200× (a) and 630× (b, c). The lumen is indicated (L).

to the nucleus. Positive Cbfa-1 immunohistochemistry was, however, detected throughout the cell, reflecting the cytoplasmic origin of protein synthesis. We have observed similar staining throughout the cell in murine dental development [21]. We studied Cbfa-1 expression at different stages of atherosclerosis. As typical examples, we show the luminal side of an early lesion (Fig. 7a–e) and the center of an advanced atheromatous lesion (Fig. 7f–j). Immunostaining with specific antibodies on consecutive sections, showed the localization of SMC (Fig. 7a,f), macrophages (Fig. 7b,g), and Cbfa-1 protein (Fig. 7c,h), whereas calcified deposits were visualized with von Kossa staining (Fig. 7d,i). In the early lesion no calcification was observed and Cbfa-1 protein staining colocalizes strongly with macrophages and to a lesser extent with neointimal SMC. In the advanced atheromatous lesion, substantial calcium deposits, as shown by von Kossa staining, are present that colocalize with cells expressing Cbfa-1 protein (Fig. 7h,i). The cells in this area did not react with SMC or macrophages-specific antibodies (Fig. 7f,g). Cbfa-1 was originally isolated from T cells as PEPB2αA [22], which prompted us to perform an additional T-cell specific immunostaining. T cells are present in atherosclerotic lesions, but are not associated with the (calcified) areas in which Cbfa-1 is expressed (Engelse and van der Wal, data not shown). In this figure we also show MGP in situ hybridizations on consecutive sections (Fig. 7e,j). Both in early and advanced lesions Cbfa-1 is expressed in those regions, in which no MGP expression is present. These data show that the expression patterns in atherosclerotic lesions of these two factors, which exhibit opposite functions in bone formation, do not colocalize.

4. Discussion

Vascular calcification may be considered a regulated process involving factors that induce bone formation such as Cbfa-1, as well as modulating components such as MGP. Based on our observations, we postulate that differ-

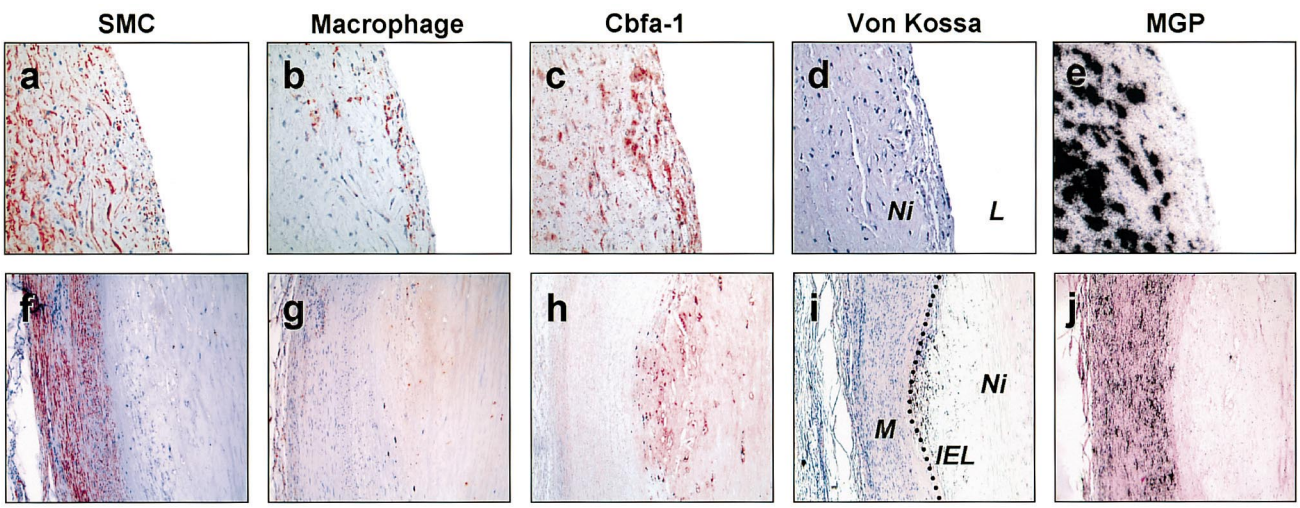

Fig. 7. Cbfa-1 expression in an early aorta lesion (a–e) and in the center of an advanced, atheromatous iliac artery lesion (f–j). Immunohistochemical analyses, specific for SMC (a, f), macrophages (b, g) and Cbfa-1 protein (c, h) were performed. In addition, von Kossa staining revealed calcified areas (d, i) and in situ hybridizations show MGP mRNA expression (e, j). The internal elastic lamina is indicated by a dotted line (i), media (M), neointima (Ni) and lumen (L) are indicated accordingly. Photomicrographs: bright field microscopy, 200× (a–e), 50× (f–j).

ent calcification processes occur in the medial layer of the vessel wall and in the atherosclerotic lesion. It should be emphasized that the diffuse, medial calcification we observed in the aorta specimens, is different from Mönckeberg's sclerosis, which is characterized by dense, focal medial calcifications [23]. We believe that the diffuse calcification of the media may be modulated by MGP, which is abundantly expressed both in the muscular, iliac arteries and in the elastic aorta. A gradient of MGP expression is observed in the media such that MGP is highly expressed in the SMC at the internal elastic lamina, with relatively low levels toward the middle of the media. Areas with high MGP expression are potentially more resistant to calcification than other parts of the vessel. Indeed, von Kossa staining revealed moderate, diffuse mineralization only at the center of the aortic vessel wall. Remarkably, iliac artery specimens did not reveal any medial calcification, while specimens derived from the aorta-iliac artery bifurcation showed an intermediate phenotype of calcification (data not shown). In our study, medial calcification did not colocalize with elevated levels of Cbfa-1 expression. These observations may indicate that moderate levels of MGP expression in the iliac artery-, but not in the aorta specimens, are sufficient to protect against medial calcification. Alternatively, while assuming that MGP expression itself is sufficient, the percentage of MGP that is properly γ-carboxylated, which is essential for calcium binding, may be lower in the aorta. This rationale has previously been discussed with respect to data that show a 70% reduction of γ-carboxylase activity in atherosclerotic vessels [1,24]. In addition, it has been demonstrated that reduced dietary Vitamin K intake, which is essential for the function of MGP, leads to an increased risk for vascular calcification [25]. Finally, the presence of multiple elastin laminas in the aorta, which are absent in the muscular iliac artery, may explain the differences in calcification between these vessel types, since it has been shown that laminas are preferential sites of mineralization [6].

In contrast to data published by Shanahan et al. [9] we show that MGP expression in the atherosclerotic lesion is confined to endothelial cells and SMC, but is absent in macrophages. Advanced, fibrous lesions containing an extensive fibrous cap over a relatively small atheroma express high levels of MGP. As MGP is a secreted protein, a clear correlation between enhanced plasma levels of MGP and advanced stages of atherosclerosis is to be expected and has indeed recently been shown to occur [26]. However, advanced, unstable lesions with a high macrophage and lipid content, and relatively small fibrous caps express low levels of MGP. Therefore, the mere concentration of plasma MGP does not reveal the clinical status of an individual, as unstable lesions express low MGP levels, but are more prone to rupture and for that reason clinically more relevant.

We demonstrated increased Cbfa-1 expression when cultured macrophages and SMC are activated with atherogenic stimuli. Previously, it has been shown that Cbfa-1 is expressed during calcification of human and bovine SMC in vitro [15,16]. In the developing skeleton, Cbfa-1 is essential to the genesis of osteoprogenitor cells, but additional cell-matrix interactions are required to allow differentiation into mature osteoblasts (reviewed in Ref. [27]). Based on this knowledge we propose that even though Cbfa-1 is expressed in vascular cells in early lesions, it may be expected that additional factors such as the vascular microenvironment and extracellular matrix components eventually determine whether such a cell transdifferentiates into an osteoblast-like cell. This is in line with our observation that Cbfa-1 expression in early lesions is not associated with vascular calcification. In advanced lesions we observed in close vicinity of calcified areas, cells that express Cbfa-1, but no longer react with SMC, macrophage or T-cell specific antibodies. We speculate that in calcifying areas Cbfa-1 may induce 'transdifferentiation' of macrophages or SMC into cells with osteblast-like traits, involving their loss of vascular cell-specific markers. Alternatively, these cells are infiltrated and may originate from the adventitia.

In summary, based on our data and those of others we suppose that regions with downregulated MGP synthesis are prone to calcification. Furthermore, we hypothesize that medial calcification of the aorta vessel wall is Cbfa-1-independent. This hypothesis is in line with the cAMP-induced calcification observed in calcifying vascular cells derived from primary aortic SMC, which has also been shown to be independent of Cbfa-1 [28]. Finally, we show the first data that suggest a function for Cbfa-1 in atherosclerotic calcification, analogous to that in normal bone development [11–14].

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