Effects of impaired vitamin K-dependent protein carboxylation

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effects of impaired vitamin

K-dependent protein carboxylation

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effects of impaired vitamin
K-dependent protein carboxylation

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus,
Prof. Dr. A.C. Nieuwenhuijzen Kruseman
volgens het besluit van het college van Decanen,
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*Tact is the knack of making a point
without making an enemy.*

Isaac Newton

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chapter 1.1

general introduction

Introduction

Matrix Gla protein (MGP) plays a key role in arterial calcification. Gla stands for γ -carboxy glutamic acid (Figure 1), an unusual amino acid formed during posttranslational modification by the action of the vitamin K-dependent enzyme γ -glutamyl carboxylase in the rough endoplasmic reticulum [144, 218]. In this introduction, the general properties of MGP and its role in the vessel wall are discussed with reference to (1.) other vitamin K-dependent proteins and (2.) the roles of MGP in the calcification of bone. Finally, MGP is evaluated as a potential target for prevention and pharmacological intervention of arterial calcification.

Figure 1
Structures of glutamic acid (Glu) and γ -carboxy glutamic acid (Gla).

γ -Glutamyl Carboxylase

The enzyme γ -glutamyl carboxylase is an integral membrane protein and is located at the luminal side of the rough endoplasmic reticulum (RER) and the Golgi complex. The absence of undercarboxylated proprothrombin in the Golgi complex suggests that the majority of carboxylation occurs in the RER. Although the genomic sequences are known for bovine [182, 264], sheep [132], rat [187], mouse [271], whale, toadfish [6], and drosophila carboxylase [120, 243], this introduction will focus on the human enzyme. The enzyme occurs in a large variety of tissues such as liver, lung, kidney, testis [90], spleen, pancreas, thyroid, arterial vessel wall, bone [25, 241], tumor cells [42], and skin [43]. It is generally accepted that in humans the carboxylase from various tissues is the product of the same gene, located on chromosome 2 at position p12 [112]. The gene is 13 kb long, has one transcriptional start site and the 15 exons encode for a mRNA with an open reading frame of 2277 nucleotides [260]. The protein consists of 758 amino acids with a molecular weight of 94 kDa. A hydrophilic region (1 - 51) is followed by a hydrophobic region (51 - 314) and a carboxy-terminal hydrophilic region (315 - 758). There are three to seven putative transmembrane regions. It was shown that the enzyme spans the ER

membrane at least 5 times, with its N-terminus in the cytoplasm and its C-terminus in the lumen of the RER (Figure 2) [229]. The protein contains nine potential N-linked glycosylation sites of which eight are located in the carboxy-terminal hydrophilic region [260].

Figure 2
Hypothetical transmembrane structure of human γ -glutamyl carboxylase. Adapted from Tie *et al.* [229]

At least two regions of γ -glutamyl carboxylase are important for the γ -glutamyl carboxylation reaction: I. the active and vitamin K binding site, and II. the substrate (propeptide) binding site. Two reactions are catalysed by the γ -glutamyl carboxylase enzyme at its active site [137, 247]. The first reaction is the addition of carbon dioxide to a glutamate residue resulting in a Gla-residue and the second one is the oxygenation of vitamin K hydroquinone (KH_2) to vitamin K 2,3-epoxide (KO). Both reactions are catalysed by the carboxylase with a 1:1 stoichiometry [115, 259]. From studies with purified bovine liver enzyme it has been demonstrated that the epoxidase activity is part of the carboxylase enzyme [137]. The model of base strength amplification is a generally accepted hypothetical molecular mechanism [47]. An active site thiol (weak base: Cys-S-) in the carboxylase initiates the chain of reactions by deprotonating vitamin KH_2 , leading to vitamin K alkoxide, which in its own light is a strong base in a hydrophobic environment. The strong base, in turn, deprotonates a Glu residue which in turn will be carboxylated. Oxidation of KH_2 into KO by molecular oxygen provides the energy required for the CO_2 -fixation at the γ -position of a glutamate residue. Carboxylation may proceed by the removal of the γ -proton of the Glu-residue followed by a CO_2 -attack at the same position.

The exact amino acids involved in formation of the active site within carboxylase are not known yet. However, site-directed mutagenesis analyses showed major roles for residues 218, 359, and 394 in the catalytic activity of the enzyme [140, 221]. Furthermore, a vitamin K-dependent protein site of interaction comprising amino acids 343-355 was identified through an immobilized peptide showing that residues 343 and 345 within this peptide

are important in formation of the active site [177]. For identification of the propeptide binding site different methods have been used. These suggest several potential locations (Table 1).

Table 1
Proposed propeptide and active sites in γ -glutamyl carboxylase

Propeptide binding site:		
Residues	Method	Reference
50 - 225	Photoinactivation	Yamada <i>et al.</i> , 1995
234, 406, 513	Site-directed mutagenesis	Sugiura <i>et al.</i> , 1996
438-507 (in lumen ER)	Trypsin-cleavage	Wu <i>et al.</i> , 1997
300 - 758	Affinity probe	Maillet <i>et al.</i> , 1997
495 - 513	Fluorescence Anisotropy	Lin <i>et al.</i> , 2002
Active Site / epoxidation Site:		
Residues	Method	Reference
217/218, 359	Site-directed mutagenesis	Sugiura <i>et al.</i> , 1996
50 - 314	Inactivation with peptide substrate	Kutiopulos <i>et al.</i> , 1994
394	Site-directed mutagenesis	Mutucumarana <i>et al.</i> , 2000
99, 450	Amino acid analysis of 14 C-NEM modified carboxylase	Pudota <i>et al.</i> , 2000
343, 345	Site-directed mutagenesis	Pudota <i>et al.</i> , 2001

Vitamin K

Vitamin K is a generic name for a group of fat-soluble compounds called vitamin K1 (or phyloquinone) and vitamin K2 (menaquinones, Figure 3.1), which both can act as cofactor for γ -glutamyl carboxylase. All K-vitamer have a 2-methyl-1,4-naphthoquinone ring structure in common but differ in the length and degree of saturation of an aliphatic side chain at the 3-position. Vitamin K2 is a group name for structurally related compounds, containing a side chain built of repeating unsaturated 5-carbon (prenyl) units; these isomers are designated menaquinone-*n* (MK-*n*) according to the number (*n*) of prenyl units. For example: menaquinone-4 (MK-4) consists of a naphthoquinone ring structure with a side chain of 4 prenyl units at the 3-position (Figure 3.1). The hydrophobicity of menaquinones increases proportionally with increasing side chain length. A third form of vitamin K, menadione (K3), is a synthetic product. It is the 2-methyl-1,4-naphthoquinone ring structure without a side chain.

Phylloquinone is mainly found in green leafy vegetables like spinach, kale, sprouts and broccoli [11, 13]. These green leafy vegetables contain approximately 1000-8000 μ g phylloquinone per kg. Other minor sources of phylloquinone are fruit, dairy produce and grains. Menaquinones are synthesised by a selected number of bacteria and mostly occur in fermented foods and explains why the daily requirement for vitamin K is low and why vitamin K

Figure 3.1

Structures of vitamin K. A: vitamin K1 or phylloquinone. B: menaquinone-4 (MK-4). C: vitamin K3 or menadione.

Figure 3.2

The vitamin K-cycle. Vitamin K-quinone (K) is first reduced to vitamin K-hydroquinone (KH_2), and then oxidized to vitamin K epoxide (KO). During the oxidation step glutamic acid (Glu) is reduced to γ -carboxy glutamic acid (Gla). Finally KO is reduced to K. 1. NAD(P)H-dependent K-reductase (DT-diaphorase), 2. dithiol-dependent KO-reductase, 3. dithiol-dependent K-reductase.

deficiency is rarely seen in healthy subjects [150, 226]. Besides in the dithiol-dependent pathway, vitamin K (and not KO) may also be reduced by DT-diaphorase, an NAD(P)H-dependent dehydrogenase. Although the DT-diaphorase is highly expressed in the liver, the dithiol-dependent pathway is the most active one for KH_2 generation [249]. like cheeses and other dairy produces [198]. A large pool of menaquinones is synthesised by the intestinal microflora: MK-10 and 11 by bacteroides, MK-8 by enterobacteria, MK-7 by veillonella species, and MK-6 by Eubacterium lentum. Very high concentrations of MK-7 (ranging from 9 to 12 mg/kg) are found in fermented soy beans (natto) [198]. MK-4 is not produced by the intestinal microflora, but relatively high concentrations of it are found in egg yolk, dairy and meats [198]. Besides these dairy sources, MK-4 is also synthesised by the conversion of menadione into MK-4 by a number of tissues and by the conversion of vitamin K1 into MK-4 [188].

The degree to which vitamin K is absorbed in the intestine depends on the side chain, the composition of the food matrix and on the efficiency of liberation from the food matrix. Compared to MK-4 through MK-7, the menaquinones with longer side chains (i.e. MK-8 to MK-13) are not efficiently absorbed [2, 199]. Fat-solubilised vitamin K in for example a meal of spinach with butter has a higher absorption rate than vitamin K from spinach alone [69, 235].

Vitamin K is taken up in the digestive tract by the absorptive enterocytes of the small-intestine and packed with cholesterol, lipids, and lipoproteins into chylomicrons. After exocytosis these chylomicrons enter the blood circulation via the lymphatic system and are rapidly degraded into chylomicron remnants. In this way, most of the absorbed vitamin K1 is delivered to the liver by chylomicron remnants which are cleared from the circulation via apolipoprotein E (ApoE) receptor mediated uptake [126, 205]. The clearance rate is dependent on the ApoE genotype. Three regularly encountered ApoE genotypes are E2, E3, and E4, which promote chylomicron remnant clearance in the order $E2 < E3 < E4$ [255]. As a result, subjects with an ApoE2/2 or ApoE2/3 genotype tend to have higher plasma vitamin K1 concentrations compared to those with the genotypes ApoE3/3, ApoE3/4, or ApoE4/4 [107]. Very little is known about the vitamin K transport to and uptake by extra-hepatic tissues like the arterial vessel wall and bone. It has been shown that vitamin K1 is predominantly carried by the triacylglycerol-rich lipoprotein fraction (TGRLP) [114] and that cultured osteoblasts can internalise vitamin K1 from LDL, chylomicron remnants, VLDL, and HDL by an ApoE-dependent mechanism [146]. In contrast, besides the TGRLP fraction MK-4 is also carried by the LDL and HDL fraction [200]. Another source for extra-hepatic MK-4 is provided by the conversion of vitamin K1 into MK-4 [227].

Not the form in which it occurs in food (vitamin K-quinone), but the reduced form vitamin K hydroquinone (KH_2) is the active cofactor used by the enzyme γ -glutamyl carboxylase. KH_2 is converted into vitamin K epoxide (KO), and KO is subsequently reduced into vitamin K and vitamin KH_2 in two reactions by the action of one or more dithiol dependent vitamin K epoxide reductases (VKOR, Figure 3.2) [223]. This recycling mechanism is called the vitamin K-cycle and explains why the daily requirement for vitamin K is low and why vitamin K deficiency is rarely seen in healthy subjects [150, 226]. Besides in the dithiol-dependent pathway, vitamin K (and not KO) may also be reduced by DT-diaphorase, an NAD(P)H-dependent dehydrogenase. Although the DT-diaphorase is highly expressed in the liver, the dithiol-dependent pathway is the most active one for KH_2 generation [249].

Cofactor activities of K1 and MK-4 in *in vitro* carboxylase studies are comparable as indicated by almost equal concentrations for half-maximal reaction velocity in specific assays for carboxylase and VKOR [26]. For the *in vivo* biological activity vitamin K-deficient rats were used to test the potency of various forms of vitamin K to counteract hypoprothrombinaemia. Two studies suggested respectively a 2-5 [73] and a 8 fold higher activity of K1 [131]. In other words, vitamin K1 has a higher biological γ -glutamyl carboxylase cofactor activity in the liver than has MK-4. Since both vitamins have comparable *in vitro* cofactor activity, differences in biological activity may be explained by different tissue distribution. Supportive for this theory is the observed 10-fold higher hepatic accumulation of K1 than of MK-4 whereas MK-4 was preferentially taken up by a number of extra-hepatic tissues [189].

Vitamin K antagonists

The VKOR is extremely sensitive to the action of 4-hydroxycoumarin derivatives such as warfarin [59]. Oral anticoagulant is another name for the 4-hydroxycoumarin derivatives since these drugs are frequently used for the treatment and prophylaxis of thrombo-embolic diseases and as rodenticides. Inhibition of VKOR by oral anticoagulants results in blockade of KO recycling and thus in inhibition of γ -glutamyl carboxylation of the vitamin K-dependent proteins. DT-diaphorase is relatively insensitive to warfarin and is able to bypass the inhibited dithiol-dependent enzyme during warfarin anticoagulation [248]. During an overdose of anticoagulant the NADH-dependent pathway is therefore the only route by which KH_2 can be made to act as a cofactor in the γ -glutamyl carboxylation [244].

Substrates for γ -glutamyl carboxylase

Since vitamin K is used as a cofactor in the γ -glutamyl carboxylation the products of carboxylase action are called vitamin K-dependent proteins.

Vitamin K-dependent proteins are involved in several physiological processes such as haemostasis, bone and soft tissue mineralization, and cellular proliferation. The coagulation factors II (prothrombin), VII, IX, and X, have pro-coagulant activity, whereas proteins C and S inhibit blood coagulation (see review: [39]). Protein Z is involved in fixation of thrombin via binding to a phospholipid surface [86]. MGP and osteocalcin (or bone Gla protein) are regulators of tissue mineralization, whereas Gas6 is involved in the regulation of cell growth (reviews: [135, 234]). The mRNA sequences of four new putative vitamin K-dependent proteins were recently reported. Two of the proteins are rich in prolines and are therefore called proline-rich Gla proteins (PRGP1 and PRGP2) [109]. Together with the two transmembrane Gla proteins TMG3 and TMG4 [110], these putative proteins form a new class of vitamin K-dependent proteins the functions of which are not known yet. Also, the enzyme γ -glutamyl carboxylase itself was reported to contain three Gla residues per molecule enzyme [9]. In all these proteins the presence of Gla residues is a prerequisite for the Ca^{2+} -binding and/or Ca^{2+} -dependent interaction with negatively charged surfaces.

For correct γ -glutamyl carboxylation all substrates for the γ -glutamyl carboxylase enzyme, are synthesised in a precursor form. This precursor peptide contains a pre-sequence for translocation across the RER membrane, and a propeptide-sequence for recognition by and binding to the γ -glutamyl carboxylase enzyme [96]. As a result of the pre-sequence removal, the immature protein is released into the lumen of the RER and binds to γ -glutamyl carboxylase via its propeptide sequence. Binding of the propeptide to carboxylase is thought to provide the majority of the binding energy required for anchoring a substrate to the enzyme [214]. Except for MGP, all known vitamin K-dependent proteins contain a N-terminal propeptide sequence consisting of residues -18 to -1. The protein sequence of MGP revealed that it contains a sequence segment (residues 15 through 30) that is homologous to the propeptide of other vitamin K-dependent proteins and probably has a comparable role in γ -glutamyl carboxylase recognition [164]. In Figure 4 the sequence alignments of the propeptides from all known human vitamin K-dependent proteins are listed.

Amino acid residues at positions -16, -10, and -6 are highly conserved among the human vitamin K-dependent proteins. From the sequence alignment a X-Z-F-Z-X-X-X-X-A-X-X-Z-Z motif from residues -18 to -6 can be deduced, with Z being an aliphatic hydrophobic residue (Ile, Val, Leu), F phenylalanine, A alanine, and X stands for any amino acid. Except for human Gas6, all human vitamin K-dependent proteins possess Phe at position -16. The amino acids between position -15 and -8 have either polar or charged side chains. Point mutations of the highly conserved hydrophobic residues at -16, -10, and -6 within the propeptide resulted in a dramatic reduction of carboxylation of

Figure 4

Amino acid sequence alignment in one letter code of the propeptides of human vitamin K-dependent proteins. The highly conserved amino acid residues are boxed in grey. #: Predicted peptide sequence from mRNA. *: Residues implicated in the recognition by the γ -glutamyl carboxylase. \$ Inhibition constants toward FIXproGla carboxylation [213].

the attached substrate. The less conserved residues at -18, -17, and -15 contribute to a lesser extend to the affinity towards the carboxylase [80, 92]. Further evidence for the propeptide recognition by carboxylase was provided by carboxylation of substrates in which covalently attached propeptides directed carboxylation of the attached Glu-rich regions [65, 90, 214]. The affinities of propeptides towards carboxylase vary by more than 100,000-fold, despite the homology between propeptides [213]. The propeptide of factor X has the highest affinity for carboxylase, followed by MGP, factor VII, Protein S, PRGP1, Factor IX, Protein C, and factor II. By far the lowest affinity has the propeptide of osteocalcin. Changing only two residues (-6 and -10) of the osteocalcin propeptide to those of the conserved sequence improved its affinity to the level of the other propeptides [214].

Except for MGP, all precursor proteins contain an arginine at -1 and an arginine-rich region at positions -2 to -5. The importance of paired basic residues at position -1 and -2 for proper cleavage of the propeptide was shown by point mutations in blood coagulation factor IX [23]. The cleavage of the propeptide takes place in the trans Golgi compartment [24] and a role for PACE/Furin has been suggested [252]. Although MGP contains an arginine at position +15, the propeptide is not removed due to lack of the arginine-rich region.

Besides the sequence similarity in the propeptide of all mammalian Gla-proteins, there is another similarity in the mature proteins. Price *et al.* found the sequence Gla-X-X-X-Gla-X-Cys at positions +17 to +23 in all mammalian Gla-proteins [164]. *In vitro* carboxylation experiments revealed a possible role for this sequence in substrate recognition by carboxylase. Decarboxylated osteocalcin and decarboxyprothrombin fragment 13-19, both without the

Glu-X-X-X-Glu-X-Cys sequence, are carboxylated with K_m values in the μ molar range [44, 236]. In contrast, Glu-containing peptide substrates lacking the Glu-X-X-X-Glu-X-Cys motive had K_m values in the millimolar range, indicating a possible role for the internal sequence in substrate recognition by the carboxylase. On the other hand, a 59 amino acid peptide consisting of factor IX's propeptide and Gla domain was found to be a better substrate compared to a peptide of the free Gla domain alone, which turned out to be a relatively poor substrate [214].

Matrix Gla Protein: Molecular Background

The mature Matrix Gla protein (MGP) is an 84 amino acid protein which contains five Gla-residues at positions 2, 37, 41, 48, and 52 [167]. Besides the posttranslational γ -glutamyl carboxylation the protein can be phosphorylated at serine residues 3, 6, and 9 [165]. A truncated 77-residue of MGP lacking the COOH-terminal residues Arg-Lys-Arg-Arg-Gly-Thr-Lys is present in human bone [78].

MGP was first isolated from bovine bone [167] and since then the corresponding human, rat [164], mouse [93], avian [256], soupfin shark (*Galeorhinus galeus*) [183], and *Xenopus laevis* [29] proteins or mRNAs were identified. Comparisons of the MGP and osteocalcin gene and protein sequences of several vertebrate and invertebrate species suggest a common ancestor for these two genes [29, 31, 159, 183]. MGP expression (mRNA or protein) has been detected in various tissues and cell cultures like bone, kidney, spleen, lung, cartilage [61], the arterial vessel wall [201], and arterial smooth muscle cells [203]. The human MGP gene (Figure 5), chromosome locus 12p13.1-p12.3, spans 3.9 kb and consists of 4 exons and 3 large introns, which cover almost 80 % of the gene [27]. Besides the presence of a consensus TATA sequence and two CAT boxes in the genomic sequence flanking the 5' end of the gene, putative binding sites for transcription factors AP1 and AP2, cAMP-dependent transcription factors, metal responsive elements, and retinoic acid and vitamin D were identified in the promoter region. Indeed, MGP gene expression has been shown to be strongly induced by retinoic acid and 1,25-dihydroxy-vitamin D₃ in a number of human cell lines including osteoblasts, articular cartilage chondrocytes, and fibroblasts [30] as well as in rat chondrocytes, osteoblasts, and osteosarcoma cells [4, 60, 62]. On the other hand, down regulation of MGP gene expression by retinoic acid was observed in several human breast carcinoma cells and in a rat kidney cell line [104, 206]. This repression of MGP gene expression in human breast carcinoma cells correlated with high estrogen receptor levels, whereas high MGP expression levels were associated with low estrogen receptor expression [206]. Kirfel *et al.* showed binding of a retinoic acid receptor / retinoid X receptor heterodimer to a negative response element between -138 and -102 [57] within the MGP

promoter leading to decreased MGP expression [104]. Another pathway for increasing the MGP expression is via high levels of extracellular calcium. Using cell cultures of human vascular smooth muscle cells (VSMCs), Farzaneh-Far *et al.* showed that this effect is mediated by a yet unknown G protein mediated cation-sensing mechanism [56]. The effect of transforming growth factor β (TGF β) is also contradictory. MGP mRNA expression increases in cultured embryonic lung cells after treatment with TGF β [270], whereas transient transfection of MGP promoter-luciferase constructs into rat VSMCs suggested down regulation by TGF β [57]. Several polymorphisms of the human MGP gene are known (Figure 5). A dinucleotide-repeat (CA) polymorphism ranging from 13 to 18 repeats has been identified at the MGP locus [253]. Six polymorphisms are known to occur in the promoter of the human MGP gene: -7 (G or A), -138 (T or C), -514 (C or T), -2447 (G or A) [55], -814 (A or G), and -2682 (C or T), and two in the coding region: Lys34Glu and Thr83Ala [85]. Two of these promoter polymorphisms (-7 (G or A), -138 (T or C)) have an important impact on *in vitro* promoter activity: the -7A and -138C variants had higher promoter activity compared to the corresponding -7 G and -138T variants. Also, patients with the CC variant at -138 had significantly higher serum MGP levels compared to subjects with the TT variant probably due to decreased binding of the AP1 complex to the promoter [55].

Matrix Gla Protein: Function

Although the molecular mechanism of MGP action is not known, data from various studies demonstrate that it plays a major role in the inhibition of soft-tissue calcification. Most importantly, MGP-deficient mice showed two phenotypes: impaired bone growth and extensive medial calcification of the aorta, leading to death within eight weeks after birth due to ruptures of the thoracic or abdominal aorta [123]. During normal endochondral bone formation MGP is expressed in the epiphyseal growth plate by resting, proliferating, and late hypertrophic chondrocytes in the calcifying zone, but not in the early hypertrophic chondrocytes [124]. In the MGP-deficient mice, however, hypertrophic chondrocytes are absent and the proliferative zone is calcified, which resulted in a disorganization of chondrocyte columns [123]. In line with this finding is the reported induction of apoptosis of proliferative and hypertrophic chondrocytes after expression of antisense MGP cDNA in a cultured mouse chondrogenic cell line [145]. Overexpression of MGP in the maturing chondrocytes of the same cell line also induced apoptosis, whereas higher MGP levels during the hypertrophic phase had an inhibitory effect on calcification and no effect on cell viability [145]. Constitutive overexpression of MGP in chicken embryo limb resulted in *in vivo* inhibition of cartilage mineralization, delayed chondrocyte maturation and termination of endochondral and intra-membranous ossification [266]. All together these data suggest a role for MGP in survival of proliferative and hypertrophic chondrocytes.

Figure 5
Human matrix Gla protein gene structure.

The association between MGP and vascular calcification was initially suggested by the detection of high levels of the corresponding mRNA in proliferating rat VSMCs [203], and the presence of both the mRNA and protein in the arterial vessel wall where it is synthesised by medial and intimal VSMCs and macrophages [201]. Expression of MGP by intimal smooth muscle cells (SMCs) starts already in the intimal xanthoma lesions, increases in the fibrous cap atheroma where it is also present in the foam cells of the lipid core, and extends further in the fibrocalcific plaque [45]. In the fibrocalcific plaques expression of MGP is localised to CD68-positive macrophages surrounding the necrotic core, intimal smooth muscle cells, and around calcium deposits. In all these atherosclerotic plaque stages, MGP is also expressed by the underlying medial VSMCs. Compared to normal arteries, total MGP mRNA expression is lower in peripheral arteries with calcification of the media (Mönckeberg's sclerosis) [202]. However, the expression is increased in a small subset of VSMCs adjacent to the medial calcification. A possible explanation for the increase in MGP mRNA expression around both medial and intimal calcifications may be the proposed G protein mediated cation-sensing mechanism [56].

Despite the observed medial calcification in the MGP deficient mice model and the increased expression of MGP around the site of mineralization in both intimal and medial calcification only little is known about the molecular mechanism of MGP function. As mentioned before, Gla-residues are involved in the Ca^{2+} -binding and/or Ca^{2+} -dependent interaction with negatively charged surfaces. Therefore, it is likely that the Gla-residues within MGP are involved in the inhibition of calcification. Indeed, this was demonstrated in a number of experiments in rats by inhibition of γ -glutamyl carboxylation with warfarin [161, 162]. Rats were treated with both vitamin K and warfarin in such a way that γ -glutamyl carboxylation could proceed in the liver, but was inhibited in extra-hepatic tissues such as the aortic vessel wall resulting in undercarboxylation of MGP. Using this protocol, arterial calcification around the elastic lamellae of the media was found within 3 to 4 weeks of treatment [161]. Treatment of hypertrophic chondrocyte cultures with the vitamin K-antagonist warfarin triggered *in vitro* calcification, whereas this calcification was counteracted by MGP overexpression in the presence of vitamin K [266], confirming the importance of the Gla-residues.

It is hard to envisage that MGP would prevent the precipitation of calcium phosphate simply by binding all calcium in the extracellular space. The remarkable observation of cartilage-producing chondrocytes in the tunica media of the MGP-deficient mice [123] and the above suggested role for MGP in chondrocyte differentiation are supportive for a more complex process of calcification inhibition by MGP. In recent years it has been generally accepted that arterial calcification and inhibition thereof are actively regulated

processes that may be similar to bone formation and in which besides MGP several other proteins are involved. For example, the bone-associated proteins bone morphogenetic protein 2 and 4 [15, 45], bone sialoprotein [45], collagen type I [98], osteocalcin [119], osteonectin [179], and osteopontin [68] are present in calcified human atherosclerotic plaques. Several of these proteins are also found in association with medial calcification. In addition, the calcium crystal deposits are hydroxyapatite, which is the type of crystal normally found in bone [195].

In medial calcification the bone-associated proteins are exclusively expressed in VSMCs, whereas in case of intimal calcification these proteins are synthesised by macrophages, multipotent mesenchymal cells, pericytes, or VSMCs. Cell cultures from isolated human aortic medial VSMC [176], bovine and human pericyte-like aortic cells (CVC) [15], bovine aortic SMCs (BASMCS) [207], and human placenta pericytes [176] are frequently used to study the molecular mechanisms involved in arterial calcification. All these cells can differentiate in culture to form osteoblast-like cells and form multicellular nodules preceding calcification [15, 22, 176, 207, 254]. It is not known whether the calcification is the result of differentiation into osteoblast-like cells or the other way around. The cultured cells synthesise proteins characteristic for osteoblasts, including alkaline phosphatase, collagen type I, osteocalcin, and osteonectin. Furthermore, increased MGP mRNA expression in VSMC nodules is associated with *in vitro* calcification of these nodules [176]. Together with the transition to an osteoblastic phenotype and calcification, the expression of smooth muscle markers SM22 α and SM α -actin is reduced [176, 217], a phenomenon also found in atherosclerotic intimal VSMCs [201] and in the calcified arteries of MGP-deficient mice [217]. Differentiation of CVCs into an osteoblast-like cell can be induced by 25-hydroxycholesterol and TGF- β 1 [254], as well as by an increase in intracellular cAMP levels [230]. Macrophages/monocytes activated by lipopolysaccharide *in vitro* produce tumor necrosis factor- α (TNF- α) and treatment of CVC cultures with this conditioned medium or with TNF- α stimulated osteoblastic differentiation and calcification of CVC through an increase in cAMP [231, 232]. Also, *in vitro* calcification of CVCs is enhanced upon treatment with oxidized-LDL stimulated macrophages/monocytes [232]. However, this mechanism probably involves cell-cell interaction between the vascular cells and activated macrophages/monocytes.

Bone morphogenetic protein 2 (BMP-2), a potent inducer of bone and cartilage formation [185], has also been suggested as a factor capable of inducing osteoblastic differentiation. Using ligand-blotting, Wallin *et al.* demonstrated binding between MGP and BMP-2 [245]. Osteogenic and chondrogenic differentiation is inhibited in pluripotent mouse mesenchymal cells overexpressing human MGP and treated with BMP-2 [114]. Furthermore,

recombinant human BMP-2 induced osteogenic and chondrogenic differentiation of cultured VSMCs isolated from MGP-deficient mice aortas [14]. Based on these results Böstrom *et al.* hypothesized that the presence of chondrocyte-like cells in MGP-deficient mice aortas are the result of free (not bound to MGP) BMP-2, synthesised by endothelial cells, which induces a chondrogenic differentiation of cells intended for smooth-muscle differentiation. Whether MGP must be carboxylated in order to bind BMP-2 is not known yet.

Both intimal and medial calcification involves the deposition of calcium containing crystals and it is thought that matrix vesicles initiate bone mineralization by serving as nucleation sites [3]. Matrix vesicles are small extracellular remnants produced by budding from the outer cell membranes of chondrocytes, osteoblasts, and odontoblasts. Calcification of the matrix vesicles starts with formation of the first crystals within the vesicles followed by subsequent crystal penetration of the matrix vesicle membrane. Structures similar to matrix vesicles have been identified in calcified arteries [101]. It has been suggested that matrix vesicles are formed as a result of apoptosis [3], and chondrocyte-derived apoptotic bodies have similarities with matrix vesicles [83]. Matrix vesicle-like structures derived from SMCs within human fatty streaks contain BAX protein, a proapoptotic member of the bcl-2 family [106]. The link between matrix vesicle-like structures and apoptotic bodies is supported by the fact that apoptosis precedes *in vitro* VSMC nodule calcification and that these VSMC derived apoptotic bodies can act as nucleation sites for hydroxyapatite formation [175]. Compared to confluent VSMC cultures the VSMC derived apoptotic bodies contain relative high levels of MGP [171]. Together with the before mentioned observation that overexpression of MGP in maturing chondrocytes induced apoptosis [145], this suggest a role for MGP in apoptosis and subsequent vascular medial calcification.

Clinical significance of vascular calcification

Vascular calcification occurs in two distinct forms: intimal calcification, which always occurs in the context of atherosclerosis and medial calcification, which can occur in its absence. Intimal calcification begins as early as the second decade of life, soon after fatty streak formation [215] and increases with age and lesion progression [46, 215]. Intimal calcification only occurs within atherosclerotic plaques and moreover, coronary artery calcification occurs exclusively in atherosclerotic arteries [208]. Plaque rupture is believed to be responsible for many acute coronary disease events, including myocardial infarction [40, 53, 66]. Whether intimal calcification stabilizes atherosclerotic plaques or promotes plaque rupture is still a matter of debate. Calcified lesions and fibrotic hypocellular lesions are much stiffer than cellular lesions [116]. Furthermore, biomechanical data suggests that

calcification reduces the “stresses” in a plaque and does not cause ruptures [34, 91]. It has been suggested that when the entire fibrous cap covering a lesion is calcified, the underlying plaque is encapsulated and protected from rupture [46]. Plaques with a heavily calcified cap are about five times stiffer than cellular lesions or normal vessel wall and very resistant to rupture [117, 184]. On the other hand, coronary artery calcification has been linked with atherosclerotic plaque rupture [54], and coronary artery and aortic calcification are closely associated with increased risk for cardiovascular events [257, 258]. Calcium crystals have been shown to aggravate inflammation [38], if this is the case in an atherosclerotic plaque, enhanced inflammation would be expected to indirectly cause plaque instability [173]. These contradictory data have led to the hypothesis that only when extensive calcification has occurred, the atherosclerotic plaque is resistant to rupture, and early or intermediate stages of calcification may actually enhance plaque vulnerability to rupture [46, 173].

Tunica media calcification is an age dependent process, which occurs independently of atherosclerosis. Medial calcification is associated with uraemia [153], type II diabetes mellitus [48, 51] and diabetic neuropathy [48]. It appears to be a strong independent predictor of cardiovascular and coronary heart disease mortality [48, 51, 118]. Calcification of the arterial media leads to stiffening of the arterial tone [10]. Stiffening of the arterial tone may lead to an increase in cardiac work and systolic blood pressure, which is a highly significant predictor of myocardial infarction and vascular death [125].

Vitamin K, MGP and vascular calcification

Given the proposed role of MGP and its Gla-residues in the prevention of calcium deposition, two factors influencing arterial calcification can be deduced: (1) the level of MGP expression and (2) the vitamin K-status. Low MGP expression might be a risk factor for vascular calcification. In this light it is of interest to investigate whether patients with the TT variant of the -138 (T or C) promotor polymorphism are more sensitive to vascular calcification compared to patients with the CC variant [55]. For both vitamin K1 and K2, inverse correlations between dietary intake and arterial calcification have been shown [67, 94]. The inverse correlation found was strongest for the menaquinones. In hypercholesterolemic rabbits, high menaquinone intake suppressed the progression of atherosclerotic plaques, intimal thickening and pulmonary atherosclerosis [99]. Local vitamin K-deficiency in the arterial vessel wall therefore might lead to undercarboxylated MGP and thus to increased calcification. Present RDA values for vitamin K (1.5 µg/day/kg body weight) are solely based on the hepatic requirement for clotting factor synthesis. It is known, however, that the ratio in which K1 and K2 accumulate is tissue dependent, with relatively high K2 accumulation in the pancreas,

testis and arterial vessel wall [189]. Furthermore, only in extreme cases low dietary vitamin K intake leads to the hepatic synthesis of undercarboxylated clotting factors [226]. For the arterial vessel wall new RDA values need to be defined for vitamin K1 and K2 separately. Patients and subjects at risk for cardiovascular diseases may not optimally be protected against arterial calcification because of a dietary inadequacy of vitamin K. Supplements containing K1 and K2 may contribute to optimal vascular health. Oral anticoagulants such as warfarin have more pronounced effects than vitamin K-deficiency, most notably at young age. This was discovered in women who were anticoagulated during the first trimester of pregnancy, and who gave birth to babies with serious bone defects in 30% of the cases (the "foetal warfarin syndrome" [157]). A regimen of vitamin K + warfarin caused similar bone defects in young animals [170] and calcification of the arterial vessel wall in adult animals [161]. At later age, the effects of oral anticoagulants on arterial calcification are less severe [162]. No data have been published about the effects of oral anticoagulants on the human arterial vessel wall. It may be expected, however, that oral anticoagulant treatment is a risk factor for arterial calcification, especially in combination with the TT variant of the MGP -138 (T or C) promotor polymorphism.

chapter 1.2

introduction to this thesis

Introduction to this thesis

In the first part of this thesis we investigate a novel mutation in the human γ -glutamyl carboxylase gene. The identification of this mutation is described in Chapter 2, followed by the characterisation of the purified mutated human γ -glutamyl carboxylase enzyme (Chapter 3). For this purpose, the mutation was introduced into the human gene, expressed in insect cells, and purified for further enzyme analysis. Next, we developed and characterised antibodies against human MGP (Chapter 4) and synthesised full-length MGP (Chapter 5). These tools were used in the last part of this thesis, which focuses on induced impairment of γ -glutamyl carboxylation and the role of MGP in vascular calcification. In Chapter 6 we describe the inhibition of γ -glutamyl carboxylation in the arterial vessel wall. The results from this study form the basis for our hypothesis that MK-4 serves not only as a cofactor for γ -glutamyl carboxylase, but has a second yet unknown function. This hypothesis is supported by the results from Chapter 7. In this Chapter the effects of MGP and induced impairment of γ -glutamyl carboxylation on VSMC calcification are described. The cellular distribution of MGP in cell cultures is described in Chapter 8. Finally, the findings of these studies and future directions are discussed in Chapter 9.

chapter 2.1

a novel mutation in the γ -glutamyl
carboxylase gene resulting in congenital
combined deficiency of all vitamin
k-dependent blood coagulation factors

Abstract

A mutation in the γ -glutamyl carboxylase gene leading to a combined congenital deficiency of all vitamin K-dependent coagulation factors was identified in a Lebanese boy. He is the first offspring of consanguineous parents and was homozygous for a unique point mutation in exon 11 resulting in the conversion of a tryptophan codon (TGG) to a serine codon (TCG) at amino acid residue 501. The reported mutation affects γ -glutamyl carboxylation of both hepatic and extra-hepatic Gla-proteins, suggesting the presence of only one type of enzyme in all tissues. Oral vitamin K1 administration resulted in resolution of the clinical symptoms. Screening of several family members on this mutation with a RFLP technique based on the presence of 3 BstNI sites in the unaffected allele and 2 BstNI sites in the affected allele revealed 10 asymptomatic members who were heterozygous for the mutation, confirming the autosomal recessive pattern of inheritance of this disease. In 50 non-related normal individuals the mutation was not found. This is the second time that a missense mutation in the γ -glutamyl carboxylase gene is described which has serious impact on normal haemostasis.

Introduction

In mammals vitamin K functions as a cofactor for the endoplasmic enzyme γ -glutamyl carboxylase, which catalyses the posttranslational modification of glutamate residues (Glu) into γ -carboxy glutamate residues (Gla) [225]. The only known function of these Gla-residues is to bind calcium which is essential for their biological activity [142]. The blood coagulation factors II (prothrombin), VII, IX and X were the first vitamin K-dependent proteins in which Gla-residues were discovered [223]. Other Gla-containing proteins are: the anticoagulant proteins C and S [50, 219], protein Z [156], and the extra hepatic Gla-proteins osteocalcin (OC) [74], matrix Gla protein (MGP) [168] and the product of the growth-arrest-specific gene 6 (Gas6) [130].

The enzyme γ -glutamyl carboxylase is an integral membrane protein located at the luminal site of the rough endoplasmic reticulum in a wide variety of tissues, including liver, kidney, bone and aorta. Recently Stafford *et al.* succeeded in cloning the human γ -glutamyl carboxylase gene and in the purification of the corresponding recombinant protein [260]. The enzyme consists of 758 amino acids and has a molecular weight of 94 kDa. The cDNA has an open reading frame of 2277 nucleotides and is encoded by the 15 exons of the carboxylase gene [112]. For the conversion of Glu into Gla residues the enzyme is strictly dependent on the reduced form of vitamin K (KH_2) which is the cofactor in this reaction. The energy for the Glu-Gla conversion is delivered by the simultaneous oxidation of KH_2 into K epoxide (KO). Recycling of KO by the enzymes KO- and K-reductase ensures that vitamin K is efficiently used [240]. Vitamin K deficiency may lead to undercarboxylation of all Gla-containing proteins. Several other reasons may underlie the occurrence of undercarboxylated Gla-proteins, however. First, the recycling of vitamin K may be impaired by a blockade of KO-reductase as a result of coumarin ingestion, which may lead to an exhaustion of the available vitamin K stores. Second, a malfunction of the precursor protein caused by mutation(s) in the propeptide region may result in either poor substrate recognition by carboxylase [79] or in impairment of cleavage of the propeptide from the mature Gla-protein [251]. A third reason stems from a genetic disorder in the γ -glutamyl carboxylase gene.

We have previously reported on a defective γ -glutamyl carboxylase in Devon Rex cats leading to a deficiency of all vitamin K-dependent coagulation factors and shown that the low procoagulant activity in these cats was likely to be caused by a mutation in the γ -glutamyl carboxylase gene resulting in a decreased affinity for KH_2 [209]. Hereditary combined deficiency of all vitamin K-dependent procoagulants and anticoagulants is a rare bleeding disorder reported only by a few authors [12, 18, 37, 95, 154]. Until now, in only one report the hereditary deficiency of all vitamin K-dependent proteins was linked to a

defective γ -glutamyl carboxylase [19]. These authors showed a missense mutation in exon 9 of the γ -glutamyl carboxylase gene leading to the conversion of an arginine into a leucine at residue 394 in the γ -glutamyl carboxylase enzyme (L394R).

In the present communication, we report a missense mutation in the γ -glutamyl carboxylase gene from a G to C that results in the substitution of a tryptophan for a serine leading to a deficiency of all vitamin K-dependent proteins. The mutation is held responsible for the associated bleeding symptoms.

Materials and Methods

Case report

Patient no. 4 (Figure 1) is a full-term breast-fed boy from Baalbeck, Lebanon who presented in 1996 to the emergency room at age 7 days with severe bleeding from the umbilicus. There was no history of circumcision. He was the first offspring of consanguineous asymptomatic parents (patients no. 30 and 2) from the Bekaa valley in Lebanon. There was no family history of bleeding disorders and no history of drug intake during pregnancy. On arrival at the emergency room, he was pale with poor peripheral perfusion, actively bleeding from the umbilicus. He required sutures, blood transfusion and close observation in the intensive care unit. Laboratory evaluation showed markedly prolonged PT and PTT values (both > 100 sec). Renal and liver functions were normal. There was no evidence of fat malabsorption or skeletal abnormalities. He received transfusions of fresh frozen plasma as well as parenteral vitamin K1 which controlled his bleeding and he was discharged from the hospital. Data from further laboratory investigations are summarized in Table 1. Since then, he has had spontaneous bruising, several episodes of oral mucosal bleeding and three episodes of rectal bleeding requiring hospitalisation and infusion of vitamin K1 and fresh frozen plasma. He has no evidence of skeletal abnormalities by X-ray imaging. After diagnosis of deficiency of all vitamin-K dependent factors, he was given 5 mg/day of vitamin K1 orally (phytomenadione, Roche, Basel, Switzerland) resulting in marked improvement of his bleeding tendency. Because the numerous bleeding episodes had resulted in iron deficiency anaemia, he also received iron in therapeutically doses. His vitamin K1 dose was decreased to 2.5 mg/day, and he remained stable with no evidence of bleeding.

Blood collection

After informed consent from his parents, EDTA blood samples were obtained on the patient, his parents and several family members for DNA analysis and citrated samples for coagulation assays.

Figure 1

Pedigree of W501S mutation. Pedigree of the Lebanese family showing the segregation of the W501S mutation in the γ -glutamyl carboxylase gene.

Patient:	30	2	4		
			Age:	7 Days	18 Months 20 Months*
PT	12"	12"	> 100"	> 100"	20.2"
PTT	30"	32"	> 100"	> 100"	36"
Fibrinogen	3.9 g/L	2.8 g/L	2.8 g/L	2.8 g/L	2.8 g/L
Bleeding Time	2' 00"	2' 30"	1' 30"	-	-
Platelets	296,000	273,000	154,000	448,000	301,000
Factor II**	100	116	-	4.8	36
Factor V	85	88	76.5	100	-
Factor VII	62	90	< 1	< 1	30
Factor VIII	100	80	100	100	100
Factor IX	100	60	9	6.4	40
Factor X	100	100	26	< 1	66
Protein C	90	110	-	-	40
Protein S	84	107	-	-	35
ucOC***	100	100	-	-	>200
Vitamin K1	-	-	-	-	> 1 ng/mL
Vitamin K1-O	-	-	-	-	0.38 ng/mL

Table 1

Summary of patient's laboratory data of blood coagulation and vitamin K-dependent proteins plasma levels. (*): After 2 months oral vitamin K1 therapy (5 mg/day). (**): All vitamin K-dependent proteins plasma levels are in percentage of normal. (***) : ucOC stands for undercarboxylated osteocalcin.

Undercarboxylated osteocalcin

The serum concentration of undercarboxylated osteocalcin was measured with the commercially available Takara Kit (Takara Shuzo, Tokyo, Japan) according to the manufacturer's instructions.

Coagulation assays

Prothrombin, factor V, VII, IX and X were determined by one stage coagulation assays using immuno-depleted plasma according to the manufacturer's protocol (Stago, Asnieres-sur-Seine, France). Protein C was determined with a clotting assay after activation with *Agkistrodon c. contortrix* venom and protein S by clotting assay based on the cofactor activity of protein S which enhances the anticoagulant action of activated protein C (Staclot, Asnieres-sur-Seine, France).

Genomic DNA samples

Genomic DNA was extracted from leukocytes by means of the QIAamp Blood Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Polymerase chain reaction

The 15 exons of the γ -glutamyl carboxylase gene were screened for mutations by PCR amplification and subsequent sequencing of both strands. A typical PCR reaction contained: 50 mM KCl, 10mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$, 100 μ M dNTP's (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), 500 ng forward primer and 500 ng reverse primer (Table 2), 1.5 U Taq DNA Polymerase (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), 10 % (v/v) dimethyl sulfoxide (DMSO) and 300 ng human genomic DNA in a total volume of 50 μ L. All PCR amplifications were performed in a DNA Thermal Cycler 480 (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). All amplifications first underwent an initial denaturation of 5 minutes at 95 °C followed by 33 cycles of denaturation for 1 minute at 95 °C, primer annealing for 1 minute at temperatures as indicated in Table 2 and primer extension at 72 °C for 2 minutes. A final 5 minute extension at 72 °C was performed before cooling the reactions to 4 °C. After PCR amplification, products were purified using the commercial "High Pure PCR product purification" kit according to the manufactures instructions (Roche, Basel, Switzerland). Sequence analysis of all purified PCR amplification products were performed by means of the BigDye™ Terminator Cycle Sequencing Kit according to the manufactures instructions (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and analysed on the ABI Prism 310 Genetic Analyzer (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Analysis of exon 11 mutation (W501S)

For simple analysis of the W501S mutation, a 246 bp fragment including exon 11 was amplified using primers Carb-E-XI-5 and Carb-E-XI-3. PCR amplification conditions were the same as described above. After purification, 15 μ L PCR product was digested with 10 U BstNI (New England BioLabs, Beverly, MA) at 37 °C for 3 hours. Restriction enzyme fragments were analysed by gel

electrophoresis on a 4.0 %(w/v) MetaPhor Agarose gel (FMC BioProducts, Rockland, ME).

Exon No.	Elongation Temperature (°C)	Primer	Sequence (5' > 3')
1	47	Carb-E-I-5 Carb-E-I-3	CTAGGGAAGCAAATTCCTCTG ACCGGGAGACACTGGGCGTC
2	60	Carb-E-II-5 Carb-E-II-3	GAGCTGTTGGTGCAGTGATTTT AGAGATTGTCATTCTCCACTCT
3	57	Carb-E-III-5 Carb-E-III-3	GTA CTGTGCAGAGTAGGCACTC TTCACCAGCATGCTTCTATTTT
4	57	Carb-E-IV-5 Carb-E-IV-3	CTGGGCCTGTGGTGATCTCTTG TGATAACCATCCTGACCCAGCCA
5	57	Carb-E-V-5 Carb-E-V-3	GCTCCCCCTCCAATGTTTACCT GTGGGATGGCCATGCTGACCAC
6	60	Carb-E-VI-5 Carb-E-VI-3	TGTA ACTCAGGAGCATGGATT CATTACTGAGAGAGATGAGTCAC
7	57	Carb-E-VII-5 Carb-E-VII-3	TTCGTGCTGTGAATGTGCTTTGA GCTAGTCCCTTCCTGCAAAACTG
8 & 9	57	Carb-E-VIII-5 Carb-E-IX-3	GAGGCCCAGCCAAACTCCT CAAAGCACAAAGGGGGCTCTG
10	57	Carb-E-X-5 Carb-E-X-3	GGCAGTGCATGACATGTCTTGG AAGCAAGGGCTGTTTCTCTTGG
11	57	Carb-E-XI-5 Carb-E-XI-3	GGTGGCTGTGATGTCCTTAGAA AAGACAGAAAAGCCTCTCCTCA
12	57	Carb-E-XII-5 Carb-E-XII-3	GCCATGGGGTGGGATGATGAAC TGCACTCAGTTCTTCTGCTGTTG
13	57	Carb-E-XIII-5 Carb-E-XIII-3	GGAGGCCATAAGCTGGCTAGAG GGCTAGAACATCATTGATAACC
14	57	Carb-E-XIV-5 Carb-E-XIV-3	CTAGCTGGCAGAAGAGGAGTTC TATGATGGCAATGACAAATATTG
15	57	Carb-E-XV-5 Carb-E-XV-3	GGAGGAGTGAGGACCAGGTGC TGTCCATTGCATAGAATGGGTC

Table 2
PCR elongation temperatures and direct sequencing of γ -glutamyl carboxylase gene.

Results

Repeated tests showed that all blood coagulation factors were normal except for the vitamin K-dependent factors II (4.8%), VII (<1%), IX (6.4%) and X (<1%) (Table1). It was established that these low concentrations were not caused by vitamin K-deficiency due to malabsorption, liver disease or accidental anticoagulant ingestion. A missense mutation in exon 9 as reported by Brenner *et al.* [19] was initially thought to be the reason for the coagulation problems found in the patient. However, screening with a specific PCR approach based on the introduction of an Alu I site in the amplified PCR product of exon 9, as described by these authors, failed to show the L394R mutation in the carboxylase gene of the patient. Screening of all exons including intron/exon borders was necessary to identify a potential new mutation in the γ -glutamyl carboxylase gene. Primers that were used for the PCR amplification are listed in Table 2, and the amplified products were subsequently cycle sequenced using either the corresponding forward or reversed primer.

Figure 2
Identification of W501S mutation. A: Schematic representation of the human γ -glutamyl carboxylase gene structure. The 15 exons are indicated in black and in detail the G to C transition in exon 11 at codon 501. This nucleotide substitution results in a Tryptophan to Serine replacement in the protein. B: Sequence analysis of genomic DNA from a normal control person (W), a heterozygous patient (He), and the homozygous patient (Ho). C: RFLP analysis of the W501S mutation, which results in loss of a BstNI site in the amplified exon 11 DNA. Lane 1: patient's DNA homozygous for the mutation; Lane 2: Heterozygous pattern; Lane 3: normal, control.

Sequence analysis of the 15 exons in the carboxylase gene revealed that the patient was homozygous for a unique point mutation in exon 11 resulting in the conversion of a tryptophan codon (TGG) to a serine codon (TCG) at residue 501 (W501S, Figure 2A). Other mutations were found neither in the exons nor in the intron/exon regions. The asymptomatic parents had normal coagulation patterns and were both heterozygous for the W501S mutation (Figure 2A and Figure 1 patients no. 30 and 2). We designed a simple RFLP technique for the detection of the mutation based on the presence of 3 BstNI sites in the unaffected allele and 2 BstNI sites in the affected allele (Figure 2C). Using this technique we analysed 50 normal Caucasian individuals and most members of the pedigree. In none of the Caucasian individuals the mutation was found (data not shown), whereas in the related individuals eight heterozygous members were demonstrated (Figure 1). In none of the heterozygous members procoagulant activities were affected, confirming the autosomal recessive pattern of inheritance of this disease.

Discussion

Here we report a combined congenital deficiency of all vitamin K-dependent blood coagulation factors that is associated with a mutation in the γ -glutamyl carboxylase gene resulting in the conversion of tryptophan into serine at amino acid residue 501 (W501S). Although combined deficiencies of vitamin K-dependent procoagulants have been reported in the literature on several occasions, the L394R mutation in the γ -glutamyl carboxylase gene as described by Brenner *et al.* [19] is the first case in which a naturally occurring mutation is responsible for such a deficiency. The impact of this mutation on the procoagulant activity is explained by these authors as a decreased affinity of substrates for the carboxylase propeptide binding site leading to impaired carboxylation. The fact that this defect could partly be corrected by the administration of a high dose of vitamin K indicated that the L394R mutation does not result in a complete inactivation of γ -glutamyl carboxylase. However, the mechanistic properties resulting in this combined deficiency have not yet been characterized.

In this paper we describe a second case in which a missense mutation in the γ -glutamyl carboxylase gene is responsible for low procoagulant activity. It was confirmed that the L394R mutation was not the reason for the defect. Vitamin K deficiency due to malabsorption, liver disease or anticoagulant ingestion was excluded and because the patient had undetectable vitamin K epoxide serum levels, abnormality of vitamin K epoxide reductase levels could also be ruled out. Interestingly, orally administered vitamin K1 resulted in the resolution of the clinical symptoms, just as was the case in the patient described in the Brenner report. Theoretically, the mutation might affect either the vitamin K binding site or the propeptide binding site. In both cases

the efficacy of orally given vitamin K1 could be explained by the fact that under physiological conditions the concentration of the cofactor is near or below its K_m . The effect of vitamin K administration, the only parameter which can be varied, may be explained by an increase of the rate of carboxylation, resulting in a partial correction of the deficiency. In preliminary expression studies W501A (tryptophan changed into alanine) mutated recombinant γ -glutamyl carboxylase was expressed in High five insect cells. With this mutant carboxylase it was demonstrated in *in vitro* experiments that a serious decrease in γ -glutamyl carboxylase activity had been induced (D. Stafford, personal communication). This suggests that the Trp501 is critical for carboxylase activity, and it is to be expected that the W501S γ -glutamyl carboxylase mutation as found in the present report has the same impact on the carboxylase activity. This would explain the observed low activity of all vitamin K-dependent coagulation factors. Sugiura *et al.* already described that point mutations at the charged residues 513/515 resulted in a γ -glutamyl carboxylase enzyme with reduced affinity for the propeptide [222]. Earlier, we reported a defective γ -glutamyl carboxylase in Devon Rex cats resulting in a bleeding tendency. We have identified the nature of this abnormality as a mutation in the γ -glutamyl carboxylase substrate binding site leading to an increased dissociation constant for reduced vitamin K1 and consequently to an increased requirement for vitamin K1. The clinical phenotype in the Devon Rex cats is similar to the phenotype found in the γ -glutamyl carboxylase W501S mutant of the patient, which suggests that both mutations are located in a corresponding sequence of the γ -glutamyl carboxylase gene. In the patient's plasma, descarboxy-osteocalcin was also demonstrated in high concentrations, which indicates that not only hepatic proteins but also extra-hepatic proteins are affected by the mutation in the γ -glutamyl carboxylase. This is consistent with the present view that only one gene encodes the γ -glutamyl carboxylase in all tissues where it is found. Since Gla-proteins are not only involved in blood coagulation but also in preventing (soft) tissue calcification, the reported mutation may potentially lead to bone malformations and/or increased risk for arterial calcification.

In summary, this is the second time that a naturally occurring mutation is found in human γ -glutamyl carboxylase, which has serious haemorrhagic consequences. The rapid diagnosis of the W501S mutation makes it possible to identify the defect in the γ -glutamyl carboxylase gene in these and further families and to predict associated bleeding disorders in the future. However, to examine the impact of the W501S mutation on the enzymatic aspects of γ -glutamyl carboxylase it is necessary to obtain the recombinant W501S mutant form.

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chapter 2.2

congenital combined deficiencies of all
vitamin K-dependent coagulation factors

Congenital combined deficiencies of all vitamin K-dependent coagulation factors are a rare bleeding disorder. Recently, we reported a missense mutation of the γ -glutamyl carboxylase gene, resulting in a serine codon (TCG) instead of a tryptophan codon (TGG) at amino acid residue 501 of the γ -glutamyl carboxylase enzyme [210]. The carboxylase gene is located on chromosome 2 and has 15 exons which encode a mRNA with an open reading frame of 2277 nt. The γ -glutamyl carboxylase enzyme consists of 758 amino acids and has a molecular weight of 94 kDa [260]. Vitamin K is a necessary cofactor in the γ -glutamyl carboxylation reaction in which specific glutamic acid residues (Glu) in a number of proteins are modified into γ -carboxy glutamic acid residues (Gla), including the procoagulant factors II, VII, IX, and X, the anticoagulant proteins C and S, and the extra-hepatic proteins osteocalcin and matrix Gla protein. These Gla residues serve as binding sites for calcium and are a prerequisite for normal haemostasis [63].

Here we describe a second patient with a congenital deficiency of all vitamin K-dependent coagulation factors which is caused by a defective γ -glutamyl carboxylase consistent with the conversion of a tryptophan codon (TGG) to a serine codon (TCG) at amino acid residue 501. The older sister, parents, grandparents and great grandmother were all asymptomatic and heterozygous for this mutation, indicative for the autosomal recessive pattern of inheritance. The patient was a full term, breast fed female infant admitted at her 3rd day of life for multiple bruises and gastrointestinal haemorrhage. Symptoms resolved after administration of vitamin K (5 mg/day for 3 days) and she was discharged from the hospital. At 3 months she suffered from a spontaneous intracerebral haemorrhage with a 2 cm haematoma in the right frontal lobe and a subarachnoidal haemorrhage. A 3 year old sister was in good health. Parents were first degree consanguineous. There was no other known family history of abnormal bleeding. Physical examination showed a well-developed and nourished infant. There was no evidence of malabsorption, liver disease or medicine intake. Blood urea nitrogen (BUN), creatinin, electrolytes, liver enzymes, and lipoprotein electrophoresis were all normal. On serial testing the patient showed prolonged prothrombin time at 21% (INR: 3.8) and prolonged partial thromboplastin time (aPTT) at 41" (control 28"), that both improved after vitamin K administration (5 mg/day twice) but returned to baseline levels 72 hours after. Fibrinogen and bleeding time were normal. Dosage of coagulation factors showed: factor II:C 9%, factor VII:C 35%, factor X:C 30%, factor IX:C 13%, and factors V:C and XIII:C 100%. Activity of vitamin K-dependent factors slightly improved after vitamin K administration (Table 1). Serum levels of vitamin K estimated 15 days after administration were still elevated (4134 ng/mL). Vitamin K epoxide levels were undetectable. Descarboxy factor II was elevated at 2100 ng/mL (normal < 2). The patient received intramuscularly 5 mg vitamin K twice weekly for two weeks and then 5 mg once a week (for four weeks) and then 5 mg once a month with

significant clinical improvement. Vitamin K administration was stopped after 16 months and a follow-up examination at three and a half years showed no abnormalities. Since then the prothrombin time spontaneously returned to 31% (INR: 2.5).

	Before Vitamin K	After Vitamin K
PT	21%	40%
INR	3.8	2
aPTT	41"/28"	36"/28"
Factor II	9%	13%
Factor VII	35%	69%
Factor IX	not determined	13%
Factor X	30%	38%
Protein C	not determined	38%
Factor V	100%	-
Factor XIII	100%	-

Table 1

Summary of patient's laboratory data of blood coagulation and vitamin K-dependent proteins plasma levels. (PT, prothrombin time; INR, international normalised ratios; aPTT, activated partial thromboplastin time)

Genomic DNA extraction from leucocytes, PCR amplification and subsequent sequencing of both strands of all 15 exons were performed according to Spronk *et al.* [210] Except for the W501S mutation as described by Spronk *et al.* no abnormalities in other exons were found. The patient was found to be homozygous for the W501S mutation in contrast to both parents and the asymptomatic sister who were found to be heterozygous (Figure 1). Analysis of seven related individuals showed that five of them were heterozygous (Figure 1) and that none of the procoagulant activities were decreased, confirming the autosomal recessive pattern of inheritance of this disease.

There are several reasons by which impaired γ -glutamyl carboxylation, leading to a decreased activity of the vitamin K-dependent proteins, can be induced. Vitamin K-deficiency and ingestion of coumarin derivatives are the two common ones. Bleeding disorders caused by inherited deficiency of vitamin K-dependent procoagulants have only been reported in a few cases. Diagnosis of the W501S mutation as described by Spronk *et al.* explained the clinical phenotype of the patient. Recognition of the vitamin K-dependent

coagulation factors by γ -glutamyl carboxylase is dependent on the propeptide sequence at the N-terminus of the immature protein. Regions around amino acid residues 234, 406, 513 of the γ -glutamyl carboxylase are involved in propeptide binding [222]. The W501S mutation in proximity to one of these propeptide binding sites on γ -glutamyl carboxylase might influence this binding. Assuming that under physiological conditions the cofactor concentration is marginal, the observed increase in active coagulation factor levels after vitamin K administration may be explained by an increased carboxylation rate. The segregation pattern of the W501S mutation confirms the autosomal recessive pattern of inheritance for combined deficiency of the vitamin K-dependent coagulation factors in this family. In all reported cases until now the clinical presentation and evolution is different [18, 19, 37, 71, 95, 133, 154, 155, 242]. Whether this is caused by other genetic factors or by a different availability of vitamin K remains to be investigated.

This is the second patient in which the W501S mutation in the γ -glutamyl carboxylase gene is described. Identification of the mutation on the carboxylase gene allows diagnosis of potential carriers as well as prenatal diagnosis for this hereditary bleeding disorder.

Figure 1

- A. Pedigree of the family showing the segregation of the W501S mutation in the γ -glutamyl carboxylase gene. Only members of the family available for the study are shown. Affected subjects, carrier subjects, and unaffected subjects are indicated by solid symbols, half-solid symbols, and open symbols respectively. The member indicated with $\otimes$ died of bleeding. The member indicated by the $\rightarrow$ is our proband.
- B. RFLP analysis of the W501S mutation, which results in loss of a BstNI site in the amplified exon 11 DNA. F: Heterozygous father, C: homozygous affected child, M: heterozygous mother, WT: wild-type control.

chapter 3

characteristics of recombinant w501s mutated human γ -glutamyl carboxylase

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Abstract

A mutation (W501S) in the vitamin K-dependent γ -glutamyl carboxylase (VKC) that leads to a congenital bleeding disorder was recently discovered in two patients. To characterize the enzyme defect, recombinant VKC-W501S was expressed in and purified from insect cells. The major effect of the mutation appears to be to decrease the affinity of the carboxylase for the propeptide of its substrates. This observation agrees with recent data of Lin *et al.* (121) that places part of the propeptide binding site within residues 495-513 of VKC. Additionally, we demonstrate that the affinity between descarboxy osteocalcin (d-OC) and VKC remains unaffected by the W501S mutation. This confirms earlier data that the high affinity site for d-OC is not located on the propeptide binding domain of VKC. Two properties of the enzyme suggest an explanation for the observation that vitamin K supplementation ameliorates the effects of the mutation: 1, since full carboxylation requires the propeptide to remain bound to the enzyme sufficiently long for full carboxylation, a reduced affinity can cause its premature release before carboxylation is complete; 2, propeptide binding results in a decrease of the K_m for vitamin K hydroquinone in wild type, but not in mutant carboxylase resulting in increased vitamin K requirement of affected subjects.

Introduction

The vitamin K-dependent post-translational modification of glutamate residues (Glu) into γ -carboxyglutamate residues (Gla) is a specific process performed in all mammalian species [223]. Although originally only some blood clotting proteins were thought to require this modification it is now recognized that a number of proteins involved in bone metabolism and calcium homeostasis also require Gla residues for function [64, 161, 193]. Except for matrix Gla protein, all known Gla proteins are synthesized in a precursor form in which a propeptide sequence serves as a high affinity recognition site for VKC [96, 214]. The conserved propeptide sequence is not only essential for directing γ -glutamyl carboxylation of most of these proteins, but also modulates the K_m for the cofactor vitamin K hydroquinone (KH_2) [35, 209]. During the carboxylation reaction, KH_2 is converted into vitamin K 2,3-epoxide (KO). Recycling of KO via the quinone into the hydroquinone form is mediated by vitamin K epoxide reductase (VKOR). Obviously, undercarboxylation of all vitamin K-dependent proteins may result from a malfunction of either the enzymes involved in this process, VKC or VKOR, from malabsorption of vitamin K, or vitamin K deficiency.

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Congenital combined deficiency of all vitamin K-dependent blood coagulation factors is rare and only a few cases are reported in the literature [12, 20, 37, 95, 154]. To date, the deficiency could only be related unequivocally to a molecular defect of VKC in three patients [19, 138, 210], whereas evidence for a mutation in VKOR has been reported in two families [149, 154]. In all these reports the deficiency resulted in a diversity of phenotypes varying from hemorrhagic diathesis (which in some cases could be corrected by oral administration of vitamin K), to skeletal abnormalities suggesting poor vitamin K status of both the liver and extra-hepatic tissues [12]. Brenner *et al.* [19] showed that a L394R mutation in VKC caused a severe bleeding disorder, which Mutucumarana *et al.* [140] found to be mainly the result of a defective glutamate-binding site.

Recently we reported that a bleeding disorder in two patients with combined deficiency of all vitamin K-dependent proteins was the result of a missense mutation in exon 11 (Trp 501 to Ser: W501S) of the human VKC gene [138, 210]. In both patients the deficiency could be corrected by administration of vitamin K. The W501S mutation is located in a region where several authors reported a possible propeptide binding site. Wu *et al.* [262] described a propeptide binding site near residues 438-507, a region reported to reside in the lumen of the endoplasmic reticulum (ER) [229]. A propeptide-like sequence within VKC (residues 495-513) was suggested by Price and Williamson to function as an intra-molecular inhibitory domain preventing the carboxylation of substrates lacking a propeptide [169]. However, recently

Lin *et al.* [121] characterized this region as a part of the enzyme directly involved in pro-peptide binding. Here we report on the expression, purification and characterization of recombinant W501S mutant carboxylase from insect cells.

Materials and Methods

Materials

All chemicals were of analytical grade or better. Vitamin K1 was purchased from Abbott (Abbott Park, IL), $\text{NaH}^{14}\text{CO}_3$ with a specific activity of 56 mCi/mmol was from ICN Pharmaceuticals (Costa Mesa, CA), CHAPS {3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate}, L- α -Phosphatidylcholine (type X-E) and pepstatin were from Sigma (St. Louis, MO). Leupeptin, aprotinin, and phenylmethylsulfonyl fluoride were from Roche Applied Science (Indianapolis, IN). FLEEL was purchased from Bachem (Philadelphia, PA). ECL Western blotting detection reagents were from Amersham Pharmacia Biotech (Piscataway, NJ). The peptide proFIX19, containing the sequence AVFLDHENANKILNRPKRY, was synthesized by Dr. Frank Church (University of North Carolina, Chapel Hill, NC) and FIXproGla, a 59-residue recombinant peptide containing the propeptide and first 41 residues of the Gla domain of human factor IX, was prepared as described previously [263]. Synthetic descarboxy osteocalcin (d-OC) was synthesized by Dr. Paul Proost (The Rega Institute, Department of Molecular Immunology, Catholic University of Leuven, Belgium), according to Wuyts *et al.* [265]. The consensus propeptide, AVFLSREQANQVLQRRRR, was synthesized by manual solid phase peptide synthesis using tBoc chemistry protocols [196]. The pSK vector was from Stratagene (La Jolla, CA) and the insect cell expression vector (pVL1392) was purchased from Pharmingen (San Diego, CA). The baculovirus viral DNA (BacVector 3000) was purchased from Novagen (Madison, WI). The Sf9 cells (*Spodoptera frugiperda*) were obtained from the Lineberger Cancer Center (University of North Carolina, Chapel Hill, NC), and the High Five cells (*Tricholusia ni*) were a gift from Dr. Thomas Kost of Glaxo-Welcome Inc. (Research Triangle Park, NC). The HPC4 antibody affinity resin was provided by Dr. Charles Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK) and the anti-FLAG M2 monoclonal antibody and MET-FLAG bacterial alkaline phosphatase were from Sigma. Peroxidase-conjugated goat anti-mouse immunoglobulin was from Jackson ImmunoResearch (West Grove, PA).

Expression of Recombinant Wild-type and W501S Mutant Carboxylase

The cDNA encoding the human γ -glutamyl carboxylase [260] was cloned into the pSK- vector either as such or after modification by site-specific mutagenesis [111] to give the W501S mutant. Both constructs contained

amino terminal the sequence encoding the FLAG epitope (DYKDDDDK) and C-terminal the HPC4 tag containing the sequence EDQVDPRLIDGK [216]. The engineered DNA constructs were subcloned into the pVL1392 vector and both wild-type and mutant carboxylase were expressed in baculovirus-infected High Five cells as described previously [213]. Preparation of microsomes from High-Five insect cells followed by solubilization and purification of the microsomal pellet were performed as described previously [140].

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Purified wild-type and mutant carboxylase were analyzed by silver-stained SDS-polyacrylamide gel electrophoresis (10% gels, Bio-Rad) and by Western blot analysis as described [140].

Carboxylase assay

Standard reaction mixtures (125 μ l) contained: 40 nM of purified proteins, 25 mM MOPS pH 7.4, 500 mM NaCl, 0.16% CHAPS, 0.16% phosphatidyl choline, 1.4 mM $\text{NaH}^{14}\text{CO}_3$ (10 μ Ci), 222 μ M of vitamin K hydroquinone (KH_2), 6 mM DTT, and various concentrations of FLEEL or FIXproGla. The mixture was incubated at 20 $^\circ\text{C}$ in sealed tubes, during 30 minutes in case of FLEEL and 60 minutes in case of propeptide containing substrates. Kinetic analysis was performed on equal aliquots subtracted from the incubation mixture at $t=0$, $t=15$, and $t=30$ or $t=60$ min if applicable. Inhibition experiments were done under the same reaction conditions and incubation times as above. All reactions were stopped by adding 0.8 mL of 5% trichloroacetic acid to 100 μ L of reaction mixture and unbound $^{14}\text{CO}_2$ was removed by boiling for 3 minutes. Total incorporation was determined using a Wallac 1414 WinSpectral $^\circ$ liquid scintillation counter (EG&G Wallac, Turku, Finland). Data are expressed as pmol $^{14}\text{CO}_2$ incorporated per minute. Kinetic parameters were determined by fitting to the Michaelis-Menten equation by non linear regression [220].

Results

Expression and purification of W501S and wild-type human γ -glutamyl carboxylase

The expression of both W501S mutant and wild-type carboxylase in insect cells was performed as described [213]. The propeptide sequence is present in all vitamin K-dependent proteins and is important for recognition of the substrate by VKC [96]. In previous studies the purification of both bovine and recombinant VKC was performed in the presence of the propeptide of human coagulation factor IX [261]. Purifications performed in the absence of the propeptide always resulted in less active carboxylase preps. Nowadays, purification of carboxylase is facilitated by the presence of a FLAG tag at the amino terminus and a HPC4 tag at the carboxyl terminus of the recombinant protein. Affinity purification to apparent homogeneity on an HPC4 antibody

column was possible with the wild-type carboxylase (Figure 1). However, mutant carboxylase W501S was less stable than wild type and lower molecular weight bands were visible in all preparations. Recently Stanley *et al.* showed that a consensus propeptide sequence had considerably higher affinity towards VKC than any known propeptide [212]. This consensus propeptide was derived by aligning all known propeptides followed by choosing the most prevalent amino acid at each position. Because the propeptide is known to stabilize VKC, we investigated whether including the propeptide during purification would allow the isolation of a larger fraction of active enzyme. Therefore, we added 5 μ M consensus propeptide into the cultured cells before they were disrupted and included it in every purification step. A control was done in the absence of propeptide. Inclusion of the consensus propeptide resulted in only marginally increased stability on gel and in about 5 times more enzymatic activity (data not shown).

In order to properly characterize the enzyme defect of the W501S, it was necessary to purify the enzyme in the absence of the propeptide and therefore such preparations contained some degraded material.

Figure 1
SDS-polyacrylamide gel electrophoresis and Western blot analysis of wild-type and W501S carboxylase. Purified proteins were electrophoresed on a 10% reduced polyacrylamide gel and silver stained (left panel). For Western blot proteins were transferred onto polyvinylidene difluoride membrane and probed with the anti-FLAG M2 monoclonal antibody and detected using ECL reagents (right panel) SDS-PAGE: Lane 1, wild-type carboxylase; lane 2, mutant W501S; lane 3, mutant W501S + 5 μ M consensus propeptide. Western-Blot: Lane 1, wild-type carboxylase; lane 2, mutant W501S; lane 3, mutant W501S + 5 μ M consensus propeptide. Molecular mass standards are on the left lane of the SDS-PAGE panel.

FIXproGla carboxylation

To test the influence of the W501S mutation on the carboxylation of propeptide-containing substrates we used FIXproGla, a recombinant substrate derived from human blood coagulation factor IX. The K_m for FIXproGla found in wild-type and mutant carboxylase was 0.26 and 21.1 μM , respectively (Table 1, Figure 2A). The 80-fold difference in K_m provides evidence that the mutation is located in a region in the VKC molecule which is important for binding of the substrate via its propeptide sequence.

Parameter	Condition	WT K_m (μM)	W501S K_m (μM)
proIX50		0.26 ± 0.05	21.1 ± 3.4
proIX19	+ FLEEL	0.07 ± 0.008	2.93 ± 0.43
KH2	+ proIX19	6.8 ± 0.95	22.1 ± 3.9
FLEEL		$3,934 \pm 485$	$4,372 \pm 602$
	+ consensus	821 ± 54	944 ± 145
	+ proIX19	956 ± 102	3564 ± 485
dOC		3.3 ± 0.58	1.7 ± 0.22
	+ consensus	1.32 ± 0.17	0.83 ± 0.09

Table 1
Michaelis Menten constants for various reaction components using wild-type (WT) and W501S mutant carboxylase.

Values were determined as described under “Materials and Methods”.
a Determined at 10 mM FLEEL
b Determined at 2 and 50 μM FIXproGla in wild-type and mutant enzyme reactions, respectively
c Determined at 10 μM proFIX19

Influence of proFIX19 on FLEEL carboxylation

It is known from literature that the propeptide of vitamin K-dependent proteins, in addition to directing carboxylation of these proteins, also functions as a stimulator of carboxylase activity [35, 105]. To investigate this stimulatory effect, we examined the influence of proFIX19 on the carboxylation of FLEEL. The half-maximal stimulation of FLEEL with proFIX19 was at 0.07 μM in the wild-type carboxylase and at 2.93 μM in the W501S mutant form (Table 1, Figure 2B). The almost 40-fold difference in half-maximal stimulation indicates a decreased affinity for proFIX19 towards VKC caused by the W501S mutation.

Influence of FIXproGla on the K_m for vitamin KH₂

The propeptide sequence is not only important for recognition of the sub-

strate, when covalently linked to the Gla domain it also decreases the K_m for the cofactor KH_2 by 7-20 fold [209]. In contrast, propeptide alone does not decrease the K_m for vitamin KH_2 in a small substrate like FLEEL [35]. As can be seen in Table 1, the K_m for KH_2 in wild-type and W501S mutant carboxylase was found to be 6.8 and 22.1 μM , respectively. This suggests that the mutated propeptide binding region in W501S results in a lower affinity for KH_2 .

FLEEL carboxylation

The kinetics of FLEEL carboxylation were determined for wild-type and W501S mutant carboxylase molecules. In the absence of proFIX19 the observed K_m values of the wild-type carboxylase and the mutant form were comparable (3934 and 4372 μM respectively, Table 1), indicating that the catalytic domain of the enzyme is not affected by the mutation. However, in the presence of 10 μM proFIX19 only the K_m value of the wild-type form decreased whereas the K_m value of the W501S mutant form only slightly changed (Table 1, Figure 2C). This is consistent with a mutation which has a negative impact on the enzyme-propeptide interaction.

Carboxylation of osteocalcin

The propeptide of osteocalcin (OC) is the only propeptide that has essentially unmeasurable affinity for the carboxylase. This lack of affinity is due to two substitutions in the propeptide: an alanine at -10 and valine instead of leucine at -6. In spite of the low affinity of the osteocalcin propeptide for carboxylase, osteocalcin itself is a low K_m substrate [7, 49, 88] for VKC. Recently we showed that there is another high affinity binding site on VKC for d-OC [88, 89]. The K_m values determined for d-OC in wild-type and W501S mutant carboxylase were 3.3 and 1.7 μM (Table 1, Figure 2D), respectively, and were in good agreement with previous reports, suggesting that the W501S mutation does not influence the binding of d-OC.

Discussion

Patients with hereditary combined deficiency of all vitamin K-dependent procoagulants and anticoagulants are rare and only a few cases have been reported. In the only previous case for which the defect is known it was found to be a mutation in VKC [19]. We recently reported a second case from two patients from Lebanon and determined that the W501S mutation in the VKC gene was the reason for the severe hemorrhagic diathesis [138, 210]. In this paper we investigated the molecular mechanism behind this deficiency. To accomplish this goal, recombinant W501S mutant VKC was expressed in insect cells and purified by affinity chromatography. The propeptides of all vitamin K-dependent precursor proteins show substantial sequence similarity; notably the amino acid residues at -16, -10, -6, -4 and -1 are highly conserved. The propeptide of the substrate functions as a recognition- and binding element

Figure 2

- A: Carboxylation of FIXproGla. Carboxylation of FIXproGla was measured by $^{14}\text{CO}_2$ incorporation. FIXproGla concentrations between 0 and 252 μM were used for wild-type carboxylase (filled circles) and for mutant W501S (open circles). The K_m is $0.26 \pm 0.05 \mu\text{M}$ for the wild-type carboxylase and 21.1 ± 3.4 for mutant W501S.
- B: Stimulation of FLEEL carboxylation by proFIX19. Carboxylation of FLEEL was measured by $^{14}\text{CO}_2$ incorporation and reactions were performed in the presence of 10 mM FLEEL. Concentrations of proFIX19 between 0 and 8 μM were used for wild-type carboxylase (filled circles) and between 0 and 24 μM for mutant W501S (open circles). The half-maximal stimulation concentration of proFIX19 was 0.07 ± 0.008 and $2.93 \pm 0.43 \mu\text{M}$ for the wild-type and mutant W501S, respectively.
- C: FLEEL carboxylation: Carboxylation of FLEEL was measured by $^{14}\text{CO}_2$ incorporation and in the presence of 10 μM proFIX19. Concentrations of FLEEL between 0 and 8 mM were used for both the wild-type carboxylase (filled circles) and mutant W501S (open circles). The K_m is $956 \pm 102 \mu\text{M}$ for the wild-type carboxylase and 3564 ± 485 for mutant W501S.
- D: Osteocalcin carboxylation: Carboxylation of osteocalcin (d-OC) was measured by $^{14}\text{CO}_2$ incorporation. Concentrations of osteocalcin between 0 and 40 μM were used for both the wild-type carboxylase (filled circles) and mutant W501S (open circles). The K_m is $3.3 \pm 0.58 \mu\text{M}$ for the wild-type carboxylase and 1.7 ± 0.22 for mutant W501S.

for VKC and is removed from the substrate after completion of the carboxylation process. The propeptide itself is used for purification of VKC in most purification protocols [261]. In the propeptide purification procedure an enzyme-propeptide complex is formed and after an extensive washing step the enzyme is eluted by free propeptide. In the present protocols an HPC4 antibody tag at the carboxyl terminal end of recombinant VKC facilitates purification and also makes it possible to obtain the enzyme in a propeptide-free form. However, it is known that the stability of VKC is very sensitive to temperature and high concentrations of CHAPS, especially in the absence of propeptide [35]. Recently, Presnell *et al.* [160] showed that a carefully prepared carboxylase preparation still contained about 50% inactive VKC. In our experiments we noticed that purification in the absence of the consensus propeptide resulted in W501S mutant VKC preparations that had approximately 20% of the normal activity. The purified W501S mutant VKC obtained in this way always showed minor protein bands on SDS-page smaller than the 96 kD band normally found in the wild-type preparations (Figure 1). These bands are probably the result of proteolysis of the W501S mutant VKC molecule because of a more relaxed structure that makes the enzyme less stable. Although we presume that these degradation products do not have catalytic activity and that they do not have propeptide binding capacity, we can not exclude that these fragments might impact on the data analysis. Therefore, presentation of values on V_{max} should be regarded with great care.

At least three regions in the VKC molecule are important in the carboxylation process: a) the substrate (propeptide) binding site, b) the active (glutamate binding site) and c) the vitamin K-binding site. There is significant evidence that small peptide substrates lacking a propeptide sequence directly diffuse into the active site and therefore can be used for measurement of the catalytic apparatus of the VKC molecule i.e. the activity of the glutamate binding site. From our experiments we conclude that the active site is not affected by the W501S mutation, since the kinetics for carboxylation of the non-physiological substrate FLEEL are almost identical in both wild-type and mutant VKC. Interestingly, docking of the free propeptide to VKC induces a conformational change that renders the carboxylase a better enzyme for the substrate FLEEL in wild-type VKC. Obviously, this characteristic is lost in W501S mutant VKC since the affinity for FLEEL is not increased by proFIX19.

All data obtained in our experiments are consistent with the W501S mutation playing a role in propeptide binding. For instance, the W501S mutation caused a decrease in the kinetics of the carboxylation of FIX proGla. The 80-fold higher K_m value as found in the W501S mutant form is an indication that the mutation is located in a region that plays a role in substrate recognition. Moreover, the concentration of proFIX19 necessary for half-maximal stimulation FLEEL decreased almost 40 times in the mutant form. For

identification of the propeptide binding site several methods have been used leading to different potential locations (Table 2). The reason for these differences may be variations in the used techniques or that non-contiguous regions of the enzyme are involved in the propeptide binding site. The previously reported binding site near residues 438-507 by Wu *et al.* [262] and the region around residue 513 by Sugiura *et al.* [221] are in agreement with the results as found in our experiments with FIXproGla having a lower affinity in the W501S mutant carboxylase than in the wild type enzyme. Recently Lin *et al.* [121] confirmed Wu's data by showing that mutations, made in the 495-513 region of human carboxylase, specifically affect propeptide binding by decreasing the apparent affinity of carboxylase for the propeptide. Although a mutation of residue W501 was not investigated by these authors, it is likely to expect a decreased apparent affinity also in this W501S mutant form. A consequence of this altered propeptide binding might be an increased turnover rate of propeptide-containing substrates leading to incomplete carboxylation. In addition, several authors showed that a propeptide covalently attached to the Gla domain induces a substantial decrease in the K_m for vitamin KH_2 [88, 209]. It is possible that a decreased affinity for the propeptide caused by the W501S mutation, on its turn, also negatively affects the K_m for its cofactor KH_2 , which results in a decreased affinity for KH_2 , as found in the W501S mutant carboxylase. Taking also into account that in newborns vitamin K nutrition is at risk because (1) the placenta transport of fat-soluble vitamins is poor; (2) the neonatal liver is immature with respect to coagulation factors synthesis; (3) breast milk is low in vitamin K, it is understandable that in these patients the clinical symptoms improve on the administration of vitamin K.

Propeptide binding site:		
Residues	Method	Reference
50 - 225	Photoinactivation	Yamada <i>et al.</i> , 1995
234, 406, 513	Site-directed mutagenesis	Sugiura <i>et al.</i> , 1996
438-507 (in lumen ER)	Trypsin-cleavage	Wu <i>et al.</i> , 1997
300 - 758	Affinity probe	Maillet <i>et al.</i> , 1997
495 - 513	Fluorescence Anisotropy	Lin <i>et al.</i> , 2002
Active Site / epoxidation Site:		
Residues	Method	Reference
217/218, 359	Site-directed mutagenesis	Sugiura <i>et al.</i> , 1996
50 - 314	Inactivation with peptide substrate	Kuliopulos <i>et al.</i> , 1994
394	Site-directed mutagenesis	Mutucumarana <i>et al.</i> , 2000
99, 450	Amino acid analysis of ^{14}C -NEM modified carboxylase	Pudota <i>et al.</i> , 2000
343, 345	Site-directed mutagenesis	Pudota <i>et al.</i> , 2001

Table 2
Proposed propeptide- and active sites on γ -glutamyl carboxylase

Whereas the role of the propeptides in almost all vitamin K-dependent proteins is well defined, this role remains uncertain in case of OC. Stanley *et al.* [213] observed that the affinities of the propetides varied over a broad range and that especially the propeptide of OC showed impaired binding to carboxylase. Nevertheless, several authors found that OC, after thermal decarboxylation, could serve as a low K_m substrate for γ -glutamyl carboxylase in the absence of a covalently attached propeptide [49, 236]. Recently we postulated the presence of a second high affinity binding site on carboxylase capable of specifically binding d-OC. Our results in this paper indicate that in spite of an affected propeptide binding site, the affinity of W501S mutant carboxylase for d-OC did slightly improve, which confirms that d-OC does not utilize the propeptide binding site on VKC.

When comparing carboxylase sequences in different species (Table 3) it is obvious that amino acid 501 is a highly conserved residue in the carboxylase molecule and it is very well possible that specific recognition interactions between carboxylase and its polypeptide substrates are mediated by this region of the molecule. Price and Williamson observed a homology over 24 residues (495-518) between the carboxylase molecule and the integral γ -glutamyl carboxylation recognition site of the matrix Gla protein, suggesting that this region might play a regulatory role in the prevention of non specific carboxylation [169]. However, here we demonstrate that amino acid 501 is directly involved in propeptide binding supporting the theory of Lin *et al.* [121] that residues 495-513 in human carboxylase are part of the propeptide binding site.

	460	470	480	490	500	510	520
Homo sapiens	VTEPQIYFDIWS	INDRFQORI	EDPRVDIVQA	NSPFORTS	WVQPL	LMDLSPWR	KIQEIK
Bos taurus	VTEPQIYFDIWS	INDRFQORI	EDPRVDIVQA	NSPFORTW	WQPL	LMDLSPWR	KIQEIK
Ovis aries	VTEPQIYFDIWS	INDRFQORI	EDPRVDIVQA	NSPFORTW	WQPL	LMDLSPWR	KIQEIK
Rattus norvegicus	VTEPQIYFDIWS	INDRFQRL	EDPRVDIVQ	AVNSPFARTE	WVQPL	LMDLSPWR	KIQDIK
Mus musculus	VTEPQIYFDIWS	INDRFQRL	EDPRVDIVQ	AVNSPFORTW	WVQPL	LMDLSPWR	KIQDIK
Delphinapterus leucas	VTEPQIYFDIWS	INDRFQORI	EDPRVDIVQ	ATNSPFORTW	WQPL	LMDLSPWR	KIQEIK
Opsanus tau	VSDPEIYFDIWS	INERFQORI	ENPQVDIVK	ADNSPFERN	TWL	MPLLVDLSPWR	KIQEIK
Drosophila melanogaster	GRNISIYFDI	CSMNGRFQ	QRSFDPRED	LLRAKWS	PFESTS	WWSLPL	LNELNHMRPKLRTIE
	*****	* * *	*****	* * *	*****	* * *	* * *

Table 3
Alignment sequences of γ -glutamyl carboxylase in different species

“*” indicates positions which have a single, fully conserved residue

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chapter 4

matrix gla protein accumulates at the
border of regions of calcification and
normal tissue in the media of the
arterial vessel wall

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Abstract

Vitamin K-dependent Matrix Gla-Protein (MGP) has been suggested to play a role in the inhibition of soft-tissue calcification. Here we report the expression of recombinant prokaryotic MGP as part of a fusion protein and the preparation of two antibodies that specifically recognize MGP. Monoclonal antibodies were raised against synthetic peptides homologous to the sequences 3-15, and 63-75 of human MGP. Both antibodies recognize recombinant and synthetic human MGP. Immunohistochemical analysis showed that MGP was associated with the extracellular matrix of non-calcified bone and with chondrocytes in cartilage. In the healthy human arterial vessel wall, MGP antigen was demonstrated in association with smooth muscle cells and elastic laminae of the tunica media, and with the extracellular matrix of the adventitia. The co-localization with the elastic laminae was lost at sites of medial calcification: in both human and rat arteries, high amounts of MGP were found in the extracellular matrix at borders of intimal and medial calcification. Our data demonstrate the close association between MGP and calcification. It is suggested that under-carboxylated MGP is biologically inactive and that poor vascular vitamin K status may form a risk factor for vascular calcification.

Introduction

In mammals, vitamin K functions as a cofactor for the endoplasmic reticulum enzyme γ -glutamyl carboxylase, which catalyses the posttranslational modification of glutamate residues (Glu) into γ -carboxy glutamate (Gla) [63, 240]. In all Gla-containing proteins known, the Gla-residues are essential for stabilizing the tertiary structure and for the function of these proteins. Mammalian Gla-proteins are involved in at least three physiological processes: haemostasis, bone metabolism, and vascular biology. Matrix Gla protein (MGP) is a 10 kDa protein containing five Gla residues and is synthesised in bone, cartilage, and soft tissues such as lung, heart and kidney [61, 167]. It has also been shown that MGP is expressed by human medial vascular smooth muscle cells (VSMCs) and that MGP mRNA synthesis is upregulated in calcified atherosclerotic plaques [201]. The molecular mechanism of MGP function is not known, but recent data demonstrate that it plays a major role in the inhibition of soft-tissue calcification. First, MGP-deficient mice showed extensive medial calcification of the aorta, leading to death within eight weeks after birth due to ruptures of the thoracic or abdominal aorta [123]. Second, in the same MGP-deficient mice cartilage-producing chondrocytes were detected in the tunica media of the calcified arteries. Third, a relation between MGP and chondrocyte function was recently suggested by Yagami *et al.*, who showed that in hypertrophic chondrocyte cultures *in vitro* calcification was triggered by treatment with the vitamin K-antagonist warfarin, whereas calcification was counteracted by MGP overexpression in the presence of vitamin K [266]. In the same study constitutive MGP overexpression in chicken limb resulted in *in vivo* inhibition of cartilage mineralization, delayed chondrocyte maturation and termination of endochondral and intramembranous ossification.

The importance of Gla-residues in the prevention of vascular calcification was shown in rats by inhibition of γ -glutamyl carboxylation with warfarin [161]. Rats were treated with both vitamin K and warfarin, in such a way that γ -glutamyl carboxylation could proceed in the liver, but was inhibited in extra-hepatic tissues such as the aortic vessel wall resulting in under-carboxylated MGP. Using this protocol, arterial calcification around the elastic lamellae of the media was found within 3 to 4 weeks of treatment. All data presently available suggest that MGP is a potent inhibitor of tissue calcification, and that vitamin K is necessary for its γ -glutamyl carboxylation and thus for rendering MGP its biological activity. To investigate MGP expression and accumulation in healthy and pathological tissues, we have developed antibodies against two different sequences of human MGP and used these to localize the antigen in bone, trachea and vessel wall.

Materials and Methods

Chemicals and Reagents

All solutions were prepared using reagents of analytical or HPLC grade. Synthetic peptides homologous to the sequences 3-15 and 63-75 of human MGP were synthesised and purified by Pepscan Systems (Lelystad, The Netherlands), peptides homologous to sequences 1-53, and 54-84, and 1-84 (full length) human MGP were synthesised and purified by Dr. T. Hacking as described previously [76]. These peptides will be referred to as MGP³⁻¹⁵, MGP⁶³⁻⁷⁵, MGP¹⁻⁵³, MGP⁵⁴⁻⁸⁴, and MGP¹⁻⁸⁴, respectively.

Construction of recombinant plasmids

Total RNA was extracted from isolated human osteoblasts cultured in RPMI 1640 medium (ICN Pharmaceuticals, CA), supplemented with 1,25(OH)₂-vitamin D₃ (Life Technologies, Breda, The Netherlands). Reverse transcription was performed according to standard procedures with M-MLV reverse transcriptase (Life Technologies). After synthesis of total cDNA, MGP and pro-osteocalcin (proOC) specific DNAs were amplified by PCR. MGP cDNA was equipped with a 5'-SphI and factor Xa digestion site, and with a 3'-HindIII site (primers: 5'-GCA TGC ATT GAA GGT CGT TAT GAA TCA CAT GAA AGC and 5'-TAT AAG CTT GGC CCC TCG GCG CTT CCT), whereas 5'-SphI and 3'-HindIII digestion sites were introduced in the proOC cDNA (primers: 5'-GCA TGC GCA GCC TTT GTG TCC AAG and 5'-AAG CTT GAC CGG GCC GTA GAA GCG CCG). The sticky-end inserts were ligated in SphI and HindIII digested prokaryotic expression vector pQE40 (Qiagen, Hilden, Germany) for in-frame fusion with the 6His-DHFR fusion protein. The recombinant plasmids were transformed into *E. coli* M15[REP4]-strain (NaI^r Str^r rif^r, lac⁻ ara⁻ gal⁻ mtl⁻ F⁺ recA⁺ uvr⁺) and the resulting vectors were confirmed by restriction enzyme digestion and nucleotide sequencing. All molecular biology protocols were according to Sambrook *et al.* [191].

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Large-scale synthesis of recombinant proteins

For the production of recombinant proteins 6His-DHFR-Xa-MGP and 6His-DHFR-proOC, bacteria (1 L cultures) were grown to a density of A₆₀₀ = 0.7 in LB medium. Next, the expression of the recombinant genes was induced by adding isopropyl-b-D-thiogalactopyranoside (IPTG, MBI Fermentas GmbH, St. Leon-Rot, Germany) to a final concentration of 2 mM and growth was continued for another 4 h. Protein samples were analysed by SDS-PAGE and subsequent Coomassie blue staining. The recombinant proteins 6His-DHFR-Xa-MGP and 6His-DHFR-proOC were characterized by immunoblotting using an anti-6His IgG antibody (Qiagen).

Purification of recombinant proteins:

Recombinant proteins were purified by nickel-nitrilotriacetic acid (Ni-NTA,

Qiagen) affinity chelation chromatography. Bacteria from 1 L culture were harvested by centrifugation (30 min at 3,000 x g) and proteins were extracted by dissolving the pellet in buffer A (8 M urea, 300 mM NaCl, 50 mM Tris-HCl, pH 8.0). After centrifugation of the lysate (30 min at 10,000 x g), the supernatant was filtered over a Ni-NTA agarose column in buffer A. After several washes with buffer A, the recombinant 6His-tagged protein was eluted with buffer B (8 M urea, 0.5 M imidazole, 50 mM Tris-HCl, pH 8.0).

Production of monoclonal antibodies

Antibodies were produced against synthetic peptides, according to generally accepted procedures and tested as described by Braam *et al.* [17].

SDS-PAGE and Western blot analysis

Protein samples in SDS sample buffer (40 mM Tris-HCl, pH 6.7, 4 % (w/v) SDS, 10 % (v/v) glycerol, 2 % (v/v) 2-mercaptoethanol, 0.01 % (w/v) bromophenol blue) were boiled for 5 min. Polyacrylamide gels 12.5 % (w/v) in SDS were run under denaturing conditions [113]. After electrophoresis, the gels were either stained with Coomassie blue R-250 or transferred to an Immobilon-P PVDF membrane (Millipore, Bedford, MA) [233]. After washing the membranes in PBS for 15 min, non specific binding sites were blocked with blocking buffer for 1 h at room temperature and incubated at 4 °C overnight with the appropriate antibody in blocking buffer. After washing with PBS-Tween, the membrane was incubated for 1 h at room temperature with biotin-conjugated anti-chicken IgG (Dako) diluted in PBS-Tween. Membranes were washed three times with PBS-Tween, followed by a final wash step of 15 min with PBS and development with ECL Western blotting detection reagents (Amersham Pharmacia). Finally, protein bands were visualized on Hyperfilm ECL (Amersham Pharmacia).

Aortic calcifications in rats

Vascular media calcification was induced in male Lewis rats (8 weeks old) using a modification of the protocol described by Price *et al.*, who injected vitamin K1 and warfarin daily [161]. In our model both K1 and warfarin were given orally and in lower doses (10 mg K1 and 15 mg warfarin per day). After maintaining the animals on this treatment for four weeks, media calcification that had developed in the aortic arch was visualized by Von Kossa staining. The experimental protocols were performed in accordance with local institutional guidelines and approved by the Experimental Animal Ethics Committee of the University of Maastricht.

Histology and immunohistochemistry

Paraffin-embedded human tissues were obtained from the archives of the Department of Pathology of the University Hospital of Maastricht. Rat tissues were from male rats of the Lewis strain. Tissues were collected, fixed by

immersion in 4% formaldehyde in PBS for 12 h at room temperature, and embedded in paraffin. Sections (4 μ m) were stained with haematoxylin and eosin for general histological evaluation and calcification was demonstrated by Von Kossa staining. Immunohistochemistry was performed by using either the monoclonal α 3-15 IgG or α 63-75 IgG as a first antibody and biotinylated anti-mouse IgG (Dako) as the second antibody. Staining was performed with the avidin-biotin immuno alkaline phosphatase kit from Dako. Negative controls were performed by substituting the primary antibody by either PBS, an irrelevant antibody, and by adding competitive amounts of synthetic MGP peptides.

Results

Expression of recombinant MGP and osteocalcin

Initial attempts to express human MGP (10 kDa) and proOC (6 kDa) in a prokaryotic expression system were not successful. This is not unusual with small proteins (< 15 kDa) and has been attributed to improper folding and a high rate of proteolytic degradation. Therefore, both cDNAs were cloned into the commercially available pQE40 QIAexpress system, with DHFR as a fusion protein. To be able to enzymatically split recombinant MGP from the chimeric product, a cleavage site specific for blood coagulation factor Xa was introduced between DHFR and MGP. Extracts from induced bacterial cultures containing either expression vector pQE40 Xa-MGP or pQE40 proOC were analysed by SDS-PAGE and subsequent Western-blot analysis. Depending on the plasmid construct used, recombinant 6His-DHFR-Xa-MGP (pQE40 Xa-MGP), or 6His-DHFR-proOC (pQE40 proOC) were found in the bacterial lysates. On Western-blots, the corresponding protein bands (36 and 32 kDa respectively) could be visualized using antibodies against the 6His epitope (Figure 1). The molecular weights of the observed recombinant proteins are in accordance with the theoretical molecular weights, calculated from their amino acid sequences. For 6His-DHF-proOC a second band at lower molecular weight was observed, which is probably a preliminary termination product generated by rare codon use in the host *Escherichia coli*.

During Ni-NTA affinity chromatography of the 6His-DHFR-Xa-MGP protein from the crude bacterial lysate it became clear that the recombinant fusion protein was only soluble under extreme pH conditions (<2 or >10) or in 8 M urea. Its solubility was not improved by ionic conditions, various detergents, or reducing agents such as dithiothreitol. The theoretical pI of the 6His-DHFR-Xa-MGP protein is 9.8. In an attempt to increase the solubility of the fusion protein, the DHFR moiety was replaced by the much larger maltose binding protein (MBP). Despite its lower theoretical pI of 7.7, however, the solubility of the resulting MBP-MGP construct was not better than that of the DHFR-containing product. Since better yields were obtained with

6His-DHFR-Xa-MGP, the latter protein was used for further experiments. Unfortunately, the non physiological buffer in which the recombinant proteins were dissolved, did not allow its digestion with factor Xa.

Figure 1

Characterization of recombinant products.

A. SDS-PAGE of Ni-NTA purified 6His-DHFR-Xa-MGP (lane 1) and 6His-DHFR-proOC (lane 2). B. Western-blot analysis of crude bacterial lysates from 6His-DHFR-Xa-MGP expression culture (lane 3) and 6His-DHFR-proOC (lane 4), using antibodies against the 6His epitope. C. Recognition of synthetic and recombinant MGP by two antibodies against MGP. Western-blot analysis of prokaryotic recombinant 6His-DHFR-Xa-MGP (lanes 8 and 12), full length synthetic MGP (MGP¹⁻⁸⁴, lanes 7 and 11), synthetic osteocalcin (lanes 6 and 10), and prokaryotic recombinant 6His-DHFR-proOC (lanes 5 and 9). Lanes 5 through 8 are stained with mouse monoclonal antibody α 3-15 MGP, and lanes 9 through 12 are stained with mouse monoclonal antibody α 63-75 MGP.

Screening of antibodies

Clonal hybridoma cell lines producing antibody against MGP were selected with an indirect ELISA by comparing antibody reactivity against 6His-DHFR-Xa-MGP and 6His-DHFR-proOC (negative control). The purified antibodies (α 3-15 MGP mAb and α 63-75 MGP mAb) were found to be of subclass IgG₁, and on Western blots the α 3-15 MGP mAb recognized synthetic MGP¹⁻⁸⁴, the 6His-DHFR-Xa-MGP prokaryotic expression product (Figure 1C), as well as the synthetic MGP¹⁻⁵³ (data not shown). In a similar way, the purified α 63-75 MGP mAb was tested for recognition of the various MGP-related products. The antibody proved to be specific for synthetic MGP¹⁻⁸⁴, and recombinant 6His-DHFR-Xa-MGP, but did not recognize MGP¹⁻⁵³ (data not shown). Neither α 3-15 MGP mAb nor α 63-75 MGP mAb recognized the 6His-DHFR-proOC construct, or synthetic OC¹⁻⁴⁹ (Figure 1C).

Immunohistochemical localization of MGP

Both anti-MGP antibodies were used for immunolocalisation of MGP in human foetal bone, trachea, healthy arterial vessel wall, and blood vessels with medial calcification (Figure 2). In human bone, a weak signal was associated with the extracellular matrix at sites of non-calcified bone (Figure 2A and B), whereas no MGP immunoreactivity was detected at calcified sites (data not shown). In tracheal cartilage, MGP was found mainly intra- and pericellularly in association with a subset of hypertrophic chondrocytes (2C and

D). In the healthy human arterial vessel wall low levels of MGP immuno-reactivity were found in the intima, media, and adventitia (Figure 2E and F). In the adventitia, MGP was mostly associated with the extracellular matrix, in the media with vascular smooth muscle cells and in a striped pattern which co-localized with the elastic laminae. This pattern was lost at sites of medial calcification where protein levels of MGP seem to be strongly increased at sites of calcification. Accumulation of MGP antigen was most pronounced in the extracellular matrix at the borders of intimal (data not shown) and medial calcification (Figure 2G and H).

Both antibodies showed cross reactivity with rat MGP as indicated by the localization of MGP in rat bone, trachea, and vessel wall. The localization of rat MGP antigen was comparable to that in the corresponding human tissues: pericellularly in hypertrophic chondrocytes and vascular smooth muscle cells, and extracellular in the vascular tunica media. To be able to compare MGP accumulation at sites of vascular calcification in human and rat tissues we have subjected rats to the vitamin K + warfarin regimen which is known to result in arterial calcifications after 4 weeks. Like in human calcified arteries, high concentrations of MGP antigen were found at the border of the mineralised areas (figure 2H).

Discussion

Accumulating evidence suggests that MGP plays an important role in the prevention of arterial calcification [123, 161]. Since the availability of fresh human bone is limited and because its poor solubility complicates the purification of native MGP, we have synthesised prokaryotic recombinant MGP. Attempts to express MGP in bacteria as such were unsuccessful, but high yields were obtained if MGP was expressed as part of a fusion protein. Although the insoluble MGP moiety formed only a relatively small part in the total constructs, the chimeric proteins containing either DHFR or MBP protein as water-soluble components were found to be insoluble under physiological conditions. Nevertheless, the recombinant MGP construct was successfully used as an antigen in an indirect ELISA to select positive hybridomas and for screening of antibodies by Western blot analysis. With both monoclonal antibodies (against the MGP N- and C-terminus) we have demonstrated the occurrence of MGP protein at low concentrations in healthy human arterial vessel wall. This is in accordance with a previous study of Shanahan *et al.*, in which MGP mRNA was detected throughout the media and an association of the protein with the elastic lamina was suggested [202]. Substantial accumulation of MGP was found at sites of vascular calcification. Both antibodies demonstrated similar MGP antigen localisation patterns in all tested human and rat tissues.

Figure 2

Immunohistochemistry of human and rat tissues for MGP with mouse monoclonal antibody $\alpha 3-15$ MGP (A, C, E, and G) and mouse monoclonal antibody $\alpha 63-75$ MGP (B, D, F, and H). Positive MGP staining is indicated by a pink/red colour (see arrows). Panel A and B: Human foetal non-calcified bone (b indicates bone). C and D: Human tracheal cartilage; E and F: Healthy human arterial vessel wall, i indicates intima, m indicates media, a stands for adventitia. G: Human coronary artery with medial calcification, m indicates media, c indicates the calcified area; inserts: VK stands for Von Kossa staining with calcified areas in black. H: Medial calcification in the aorta of a warfarin-treated rat (m: media, c: calcified area, VK: insert of Von Kossa staining with calcification in black).

Because of its poor solubility, it is difficult to understand how MGP can perform its function *in vivo* and in which way the protein is transported in blood. Although we were not able to make a soluble fusion protein, one possible explanation for the apparent solubility in plasma is the formation of a complex between MGP and a soluble carrier protein or the association with lipoproteins. Association between MGP and bone morphogenetic proteins has been suggested in this respect [237]. Alternatively, the data available in the literature do not exclude the possibility that reported data on circulating MGP reflect water-soluble degradation products. Our histological findings indicate that extracellular MGP is mainly complexed with or precipitated on the extracellular matrix at areas of calcification.

The proposed function of MGP as inhibitor of calcification is supported by several studies including excessive arterial calcification in MGP-deficient mice [123] and prevention of calcification by MGP over-expression in avian chondrocytes [266]. Available data suggest that inhibition of extra-hepatic γ -glutamyl carboxylation may result in undercarboxylation of vascular MGP and subsequent calcification of the tunica media [161, 162]. It seems plausible, therefore, that carboxylated MGP is the active form which acts as an inhibitor of tissue calcification. Unfortunately, neither of the antibodies described in this paper discriminate between carboxylated and undercarboxylated MGP. When used for immunohistochemical analysis of calcified arteries, MGP was found to be localized at the border between tissue and calcium deposits. This is consistent with *in situ* hybridisation data showing upregulation of MGP mRNA synthesis at sites of calcification [161, 174]. These data may be explained by assuming a feed-back mechanism of increased MGP production, possibly to prevent or minimize further mineral deposition. In case of local vitamin K-deficiency or inhibition of Gla-formation by coumarin anticoagulants, the impaired biological activity of the undercarboxylated MGP produced would give rise to increased calcification and further amplification of undercarboxylated MGP synthesis. Thus, both prolonged vitamin K-deficiency and the use of vitamin K-antagonists (coumarins) may form independent risk factors for vascular calcification. Evidence for this assumption was provided in a population-based study, in which an inverse correlation between dietary vitamin K intake and the occurrence of aortic calcification in elderly subjects was demonstrated [94]. It is still unclear how MGP fulfils its calcification inhibitory activity. An attractive hypothesis was recently postulated by Boström, who proposed that MGP may act as an inhibitor of a cartilage and bone inducing factor [16]. The author further suggests that MGP may occur complexed to BMP. In this concept extracellular transport of the virtually insoluble MGP would take place as a soluble complex with BMP in which BMP function is strongly reduced. Recently, it was shown that *in vitro* MGP can form a complex with BMP-2 [245], and that in cell culture BMP activity is possibly modulated by MGP [14]. Here we like to put forward the hypothesis

that undercarboxylated MGP, synthesised during vitamin K-deficiency or coumarin treatment, has lower affinity for BMP than has carboxylated MGP. This would explain the strong accumulation of MGP-related antigen which was found at all sites of vascular calcification that we have investigated thus far. Obviously, conformation-specific antibodies discriminating between carboxylated and undercarboxylated MGP are required to test this hypothesis.

chapter 5

total chemical synthesis of human matrix
gla protein

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Abstract

Human matrix Gla protein (MGP) is a vitamin K-dependent extracellular matrix protein that binds Ca^{2+} -ions and that is involved in the prevention of vascular calcification. MGP is a 10.6-kD protein (84 amino acids) containing five γ -carboxy glutamic acid (Gla) residues and one disulfide bond. Studies of the mechanism by which MGP prevents calcification of the arterial media are hampered by the low solubility of the protein ($<10 \mu\text{g/mL}$). Because of solubility problems, processing of a recombinant expressed MGP-fusion protein chimera to obtain MGP was unsuccessful. Here we describe the total chemical synthesis of MGP by tBoc solid-phase peptide synthesis (SPPS) and native chemical ligation. Peptide Tyr¹-Ala⁵³ was synthesised on a derivatised resin yielding a C-terminal thioester group. Peptide Cys⁵⁴-Lys⁸⁴ was synthesised on Lys-PAM resin yielding a C-terminal carboxylic acid. Subsequent native chemical ligation of the two peptides resulted in the formation of a native peptide bond between Ala⁵³ and Cys⁵⁴. Folding of the 1-84-polypeptide chain in 3 M guanidine (pH 8) resulted in a decrease of molecular mass from 10,605 to 10,603 (ESI-MS), representing the loss of two protons because of the formation of the Cys⁵⁴-Cys⁶⁰ internal disulfide bond. Like native MGP, synthetic MGP had the same low solubility when brought into aqueous buffer solutions with physiological salt concentrations, confirming its native like structure. However, the solubility of MGP markedly increased in borate buffer at pH 7.4 in the absence of sodium chloride. Ca^{2+} -binding to MGP was confirmed by analytical HPLC, on which the retention time of MGP was reduced in the presence of CaCl_2 .

Circular dichroism studies revealed a sharp increase in α -helicity at 0.2 mM CaCl_2 that may explain the Ca^{2+} -dependent shift in high-pressure liquid chromatography (HPLC)-retention time of MGP. In conclusion, facile and efficient chemical synthesis in combination with native chemical ligation yielded MGP preparations that can aid in unravelling the mechanism by which MGP prevents vascular calcification.

Introduction

Matrix γ -carboxy glutamic acid (Gla) protein (MGP) is an 84-amino-acid protein that was first described in an extract from bovine bone [167]. Following this report, MGP was detected in rats [152], humans [100], mice [93], sharks [183], and chickens [256] either as a protein or as mRNA. MGP is found in high concentrations in the extracellular matrix of bone and cartilage [77, 167] but is also synthesised in a wide variety of other tissues such as lung, heart, kidney, and arterial vessel wall [61]. MGP contains five posttranslationally modified γ -carboxy glutamic acids [167] and, therefore, belongs to the vitamin K-dependent family of proteins (Figure 1).

It was reported that deficiencies in MGP caused impaired bone growth and, more importantly, massive calcification of the arterial vessel wall in mice [123]. To study the molecular mechanisms by which MGP protects against calcification and bone deformation, sufficient amounts of MGP are required. However, the low solubility of MGP limited recombinant production of MGP because an *Escherichia coli*-expressed dihydrofolate reductase-MGP chimera could not be processed further because of insolubility of the fusion protein under physiological conditions [17]. Moreover, conventional purification from bone is also hindered by the insolubility of the protein.

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Figure 1

Amino acid sequence of matrix Gla protein. The grey residues at position 53-54 represent the alanine-cysteine native chemical ligation site. The solid line represents the disulfide-bond. γ denotes γ -carboxy glutamic acid. Sequence from nucleotide analysis [32, 100].

When biologically active proteins cannot be generated by recombinant expression techniques because of insolubility or misfolding, chemical protein synthesis is an excellent alternative that, because of its versatility, can greatly add to structure-function analysis of the protein of interest. Because polypeptide chains of 40-60 amino acids realistically represent the efficiency limit of solid-phase peptide synthesis, native chemical ligation was introduced to join protein segments through native peptide bonds (41). The native chemical ligation technique uses the principle of a chemoselective reaction between two unprotected peptides in aqueous solution in which a carboxyterminal thioester undergoes a thiol-exchange with an amino terminal cysteine sulfhydryl side chain. A subsequent, rapid, intramolecular rearrangement yields a native peptide bond at the site of ligation (Figure 2). Here we report the total chemical synthesis of MGP by solid-phase peptide synthesis and native chemical ligation, yielding highly pure MGP with a full amide backbone and native biochemical features.

Figure 2
 Native chemical ligation strategy for the total chemical synthesis of human matrix Gla protein. The 53-residue N-terminal peptide-thioester (COSR) fragment and the 30-residue C-terminal fragment of MGP were synthesised by stepwise SPPS techniques using Boc-chemistry protocols. The two fragments are initially joined by thioester formation (not observed as a discrete intermediate), and a subsequent spontaneous, rapid rearrangement results in the formation of a native peptide bond at the site of ligation.

Materials and methods

Materials

Most Boc-amino acids and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were from Nova-Biochem. Boc-Lys(Cl-Z)-OH was obtained from Bachem Bioscience; Boc-Arg(p-toluenesulfonyl)-OH and Boc-Asn(xanthyl)-OH were from Midwest Bio-Tech. S-trityl-mercapto-propionic acid was obtained from Peptides International. Boc-Lys-OCH₂Pam-resin and Boc-Leu-OCH₂Pam-resin were obtained from Applied Biosystems. N,N-diisopropylethylamine (DIEA), p-cresol, thiophenol, and benzyl-mercaptan were obtained from Aldrich. N,N-Dimethylformamide (DMF) and HPLC-grade acetonitrile were purchased from Fischer. Trifluoroacetic acid (TFA) was obtained from Halocarbon, and HF was purchased from Matheson Gas.

N^α-Boc-γ, γ-di-cyclohexyl-L-γ-carboxy glutamic acid-OH synthesis

N^α-Boc-γ, γ-di-cyclohexyl-L-γ-carboxy glutamic acid-OH (Boc-Gla[dCHx]-OH) was synthesised as described [148].

Peptide synthesis

The sequence of MGP to be synthesised [32, 100] is shown in Figure 1. Peptides were prepared by manual solid-phase peptide synthesis (SPPS) on a 0.3 mmol scale (MGP 1-53) and 0.35 mmol scale (MGP 54-84) using the in situ neutralization/HBTU activation procedure for Boc chemistry essentially as previously described [196]. Each synthetic cycle consisted of Na-Boc removal by a 1-2-min treatment with neat TFA, a 1 min DMF-flow wash, and a 10 min coupling (Arg, Asn: 20 min) with 1.0 mmol preactivated Boc amino acid in the presence of excess DIEA, followed by a second DMF-flow wash. For 3 min, 1.1 mmol Boc amino acids (except Boc-Gla[dCHx]-OH) were preactivated with 1.0 mmol HBTU (0.5 M in DMF) in the presence of 3 mmol DIEA. For 3 min, 0.44 mmol Na-Boc Gla(dCHx)-OH was preactivated with 0.4 mmol HBTU in the presence of 1.5 mmol DIEA. After each coupling step, yields were determined by measuring residual free amine with the quantitative ninhydrin assay [192]. After coupling of Gln residues, a DCM flow wash was used before and after deprotection using TFA to prevent possible high-temperature (TFA/DMF)-catalysed pyrrolidonecarboxylic acid formation [196]. Side-chain-protected amino acids were Boc-Arg (p-toluenesulfonyl)-OH, Boc-Asn(xanthyl)-OH, Boc-Asp(O-cyclohexyl)-OH, Boc-Cys(4-methylbenzyl)-OH, Boc-Gla(di-O-cyclohexyl)-OH, Boc-Glu(O-cyclohexyl)-OH, Boc-His(dinitrophenyl)-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Ser(benzyl)-OH, Boc-Thr(benzyl)-OH, and Boc-Tyr(2-Br-Z)-OH. Other amino acids were used without side-chain protection. After chain assembly was completed, the peptides were deprotected and cleaved from the resin by treatment with anhydrous HF for 1 h at 0 °C using 4% anisole (MGP 1-53) or p-cresol(MGP 54-84) as a scavenger. The imidazole side chain-

dinitrophenyl (Dnp)-protecting groups remained on His residues on peptide MGP 1-53 because the Dnp-removal procedure is incompatible with C-terminal thioester groups. However, Dnp is gradually removed by thiols during the ligation reaction yielding unprotected His. After cleavage, both peptides were precipitated and washed with ice-cold diethylether. Subsequently, the precipitate was dissolved in aqueous acetonitrile and lyophilised.

TAMPAL resin

0.6 mmol S-trityl mercaptopropionic acid was activated with 0.54 mmol HBTU in the presence of 2 mmol DIEA and coupled for 10 min to Boc-Leu-OCH₂Pam-resin (99.61% coupling). The resulting TAMPAL resin was used as a starting resin for polypeptide chain assembly following removal of the trityl protecting group and formation of the thioester bond with any desired amino acid using standard peptide coupling protocols. Treatment of the final peptide with anhydrous HF yielded the C-terminal activated mercaptopropionic acid-leucine (MPAL) thioester peptide that can directly participate in native chemical ligation [75]. Trityldeprotection was achieved by 2 x 1 min treatment with a solution of 1.25% triisopropylsilane and 1.25% H₂O in TFA.

HPLC

Analytical reversed-phase HPLC was performed on a Hewlett Packard HPLC 1050 system using Vydac C-18 columns (5 μ m, 0.46 x 5 cm). Semipreparative reversed-phase HPLC was performed on a Rainin HPLC system using a Vydac C-18 column (10 μ m, 2.5 x 25 cm). Linear gradients of acetonitrile in water/0.1% TFA were used to elute bound peptides. The flow rates used were 1 mL/min (analytical) and 15 mL/min (semipreparative).

Mass spectrometry

ESI-MS was performed on an API-III triple quadrupole mass spectrometer (PE-Sciex). Peptide masses were calculated from the experimental *m/z* ratios from all the observed protonation states of a peptide using MacSpec software (Sciex). Theoretical masses of peptides and proteins were calculated using MacProMass software (Beckman Research Institute).

Native chemical ligation

The ligation of unprotected synthetic peptide segments was performed as described earlier [75]. In short, peptides were dissolved in 0.1 M phosphate buffer containing 6 M guanidine, 1 %(v/v) benzylmercaptan, and 1 %(v/v) thiophenol (pH 7). The ligation reaction was performed at 37 °C under frequent vortexing, and the reaction was monitored by HPLC and ESI-MS until completion.

CD spectroscopy

CD spectra were recorded on an AVIV stopped-flow CD spectrometer Model 202SF at 25 °C. MGP was dissolved in 25 mM boric acid at pH 7.4, and the concentration was determined by quantitative amino acid analysis. CD spectra are presented as the molar ellipticity versus wavelength in 0.5-nm increments. The mean residual weight ellipticity was calculated using Equation (1).

$$[\theta]_{MRW} = (\theta \times 100 \times Mr) / (c \times d \times n) \quad (1)$$

where $[\theta]_{MRW}$ is the mean residue ellipticity ($\text{deg cm}^2 \text{dmol}^{-1}$), θ the observed ellipticity at 222 nm, Mr the molecular weight of MGP, c the concentration in mg/mL, d the path length in centimetres, and n the number of residues [194]. The ellipticity of MGP at 222 nm was recorded as a function of the CaCl_2 concentration. To a volume of 250 μL of MGP (3.4 mM), CaCl_2 was added to a final concentration of 20, 40, 79, 157, 311, 618, 1225, and 2411 μM . MGP concentrations were corrected for dilution of the sample by CaCl_2 (20 μL maximal addition). For the calculation of α -helical content of MGP, the averages of the mean residual weight ellipticities at 223, 222.5, 222, 221.5, and 221 nm were used. The percentage of α -helix in MGP was determined as the ratio of the mean residual weight ellipticity at 222 nm and the maximal theoretical mean residual weight ellipticity at 222 nm ($[\theta]_{\text{max}}$; [33]) using Equation (2).

$$[\theta]_{\text{max}} = -39500(1 - (2.57/n)) \quad (2)$$

SDS-PAGE and Western blot analysis

Protein samples in SDS sample buffer (40 mM Tris-HCl at pH 6.7, 4 % (w/v) SDS, 10 % (v/v) glycerol, 2 % (v/v) 2-mercaptoethanol, 0.01 % (w/v) bromophenol blue) were applied to polyacrylamide gels 20 % (w/v) in SDS under denaturing conditions. After electrophoresis, the gels were transferred to Immobilon-P PVDF membranes (Millipore). After washing the membranes in PBS for 15 min, non-specific binding sites were blocked with blocking buffer (3 % w/v non-fat dry milk in PBS) for 1 h at room temperature and incubated at 4 °C overnight with an α 3-15 MGP antibody in blocking buffer [17]. After washing three times with 0.3 % (v/v) Tween-20 in phosphate-buffered saline (PBS-Tween), the membrane was incubated for 1 h at room temperature with HRP-conjugated rabbit anti-mouse IgG in PBS-Tween. The membrane was washed with PBS-Tween, followed by a final wash step of 15 min with PBS and development with ECL Western blotting detection reagents (Amersham Pharmacia). Finally, protein bands were visualized on Hyperfilm ECL (Amersham Pharmacia).

Results

Synthesis of matrix Gla protein peptides

To establish chemical access to the 84-residue human matrix Gla protein, a two-fragment synthesis was chosen with a suitable ligation site at Ala⁵³-Cys⁵⁴, conducted by the need for an N-terminal cysteine on the C-terminal peptide at the site of ligation (Figure 1). The synthesis of the 84-residue MGP therefore required synthesis of two polypeptides: NH₂-Tyr¹-Ala⁵³-COSR (MGP 1-53) and NH₂-Cys⁵⁴-Lys⁸⁴-COOH (MGP 54-84). MGP 1-53 was synthesised on C-terminal thioester generating resin (TAMPAL) to yield the C-terminal mercaptopropionic acid-Leu (MPAL)-activated thioester. MGP 54-84 was synthesised on Lys-PAM resin to yield an N-terminal free cysteine and a C-terminal carboxylic acid group after cleavage of the peptide from the resin. The synthesis (0.3 mmol) of MGP 1-53 on TAMPAL resin yielded 2.08 g peptide resin (theoretical yield: 2.27 g corrected for resin sampling), with an average coupling efficiency of 99.56% (range 99.33%-99.96%) based on ninhydrin assays after each coupling step. After the N^α-Boc group was removed, 330 mg peptide-resin was cleaved with anhydrous HF, yielding 216 mg dry crude peptide that was further purified on preparative high-pressure liquid chromatography (HPLC; Figure 3A). From preparative HPLC, 29 mg of pure MGP 1-53 was obtained. ESI-MS revealed a molecular mass of 7282.3 ± 0.3 D (Figure 3B), which agreed well with the theoretical average mass of 7282.6 D of MGP 1-53-MPAL, with dinitrophenyl (Dnp) groups attached to His⁴ and His⁴⁷ (Figure 3B).

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The synthesis of MGP 54-84 (0.35 mmol) on Lys-PAM resin yielded 2.06 g peptide-resin (theoretical yield: 2.11 g) with an average coupling efficiency of 99.52% (range 99.23%-99.83%). Yielding 185 mg dry crude peptide, 300 mg peptide resin was cleaved with anhydrous HF. From a preparative HPLC run of 80 mg of crude MGP 54-84, 21 mg of pure MGP 54-84 was obtained (Figure 3A). ESI-MS showed an apparent molecular mass of 3872.8 ± 0.7 D (Figure 3C), which fitted well with the calculated average isotopic mass of 3873.4 D.

Synthesis of matrix Gla protein by native chemical ligation

Native chemical ligation of MGP peptides was performed with 27.8 mg (3.8 μmol) MGP 1-53 and 17.1 mg (4.4 μmol) MGP 54-84. The peptides were dissolved in 2 mL ligation buffer with 2% thiophenol and 2% benzylmercaptan, resulting in a final peptide concentration of 1.9 mM MGP 1-53 and 2.2 mM MGP 54-84 at pH ≈ 7. After 1 h at 37 °C, multiple peaks could be observed on analytical HPLC (Figure 3D). In addition to the starting materials (54-84 and 1-53-MPAL[2 Dnp]), the following fragments were identified: a +26-D derivative of 54-84 (peak 1); 1-53-MPAL without Dnp (peak 2); 1-53-MPAL with 1 His-Dnp (peak 3); MGP 1-53-benzylmercaptan thioester exchange (peak 4); 1-53-MPAL with 1 His-Dnp (peak 5); and the ligated

Synthesis of MGP Fragments

Figure 3

Total synthesis of human matrix Gla protein by native chemical ligation. Synthesis of MGP fragments: (A) HPLC chromatogram (C18: 22.5%-41% acetonitrile, 1.23%/min) of the starting synthetic peptide segments MGP 54-84 and MGP 1-53. (B) ESI-MS spectrum of MGP 1-53 shows the m/z ratios (fourth through ninth ionised states) with an calculated mass of 7282.3 ± 0.3 D (theoretical average mass of 7282.6). (C) ESI-MS spectrum of MGP 54-84 shows the m/z ratios (second through sixth ionised states) with an calculated mass of 3872.8 ± 0.7 D (theoretical average mass: 3873.4 D). Native chemical ligation of MGP fragments: (D) HPLC chromatograms of the ligation reaction, started by the addition of 2 % (v/v) thiophenol and 2% benzyl-mercaptan to the peptide mixture MGP 1-53 and MGP 54-84. At $t = 1$ h, ligated product (1-84) is shown as well as the C-terminal segment starting material. The unreacted N-terminal material can be accounted for as multiple intermediates eluting between 7 and 13 min (see text). At $t = 24$ h, ligation was almost complete. (E) ESI-MS spectrum shows the m/z pattern of the ligated material (fifth through fifteenth ionised states) with a calculated mass of $10,604.9 \pm 0.7$ D (theoretical average mass of the reduced 84-residue MGP: 10,604.8 D). Folding and disulfide formation of MGP: (F) HPLC chromatograms of the purified, reduced MGP polypeptide ($t = 0$), the crude, folded material ($t = 24$ h), and the purified final product (MGP). (G) ESI-MS spectrum shows the m/z pattern (fifth through fourteenth ionised state) with a calculated molecular mass of $10,602.9 \pm 1.0$ D (theoretical average mass of MGP containing one disulfide: 10,602.8 D).

84-residue polypeptide (1-84; Figure 3D). The difference in retention time of peaks 3 and 5 can be explained by Dnp groups that are attached to different His residues in fragment 1-53. ESI-MS revealed a mass for the 1-84 MGP polypeptide of 10,604.9 D, which fitted well with the theoretical average isotopic mass of 10,604.8 D (Figure 3E). After 24 h, ligation was almost complete, and the mixture was applied to preparative HPLC (C18: 22.5%-41% acetonitrile, 0.31%/min) to isolate the 1-84-polypeptide chain, yielding 17 mg (42% yield) dry peptide after lyophilisation.

Folding of matrix Gla protein

The 84-residue polypeptide chain of MGP (16 mg) was folded in 3 M guanidine (pH 8.0) at a final concentration of 0.2 mg/mL polypeptide. Overnight stirring exposed to air at 4 °C resulted in a quantitative shift of the peptide peak on analytical HPLC from 11.36 to 9.55 min (Figure 3F). This shift was likely because of a conformational change caused by the formation of the disulfide loop between Cys⁵⁴ and Cys⁶⁰. ESI-MS showed a decrease in mass of the folded polypeptide from 10,604.9 D to 10,602.9 D, the loss of 2.0 D representing the loss of two hydrogen atoms from the free sulfhydryl groups on formation of the disulfide bond (Figure 3G). After purification on preparative HPLC (C18: 22.5%- 41% acetonitrile, 0.31%/min), 6 mg of MGP containing five Gla residues and an internal disulfide were isolated (35% yield). When MGP was brought into Tris buffer (50 mM Tris, 100 mM NaCl at pH 7.4) at a concentration of 0.2 mg/mL at room temperature, MGP initially dissolved, but it precipitated within minutes after addition of buffer (data not shown). Addition of guanidine to the Tris buffer prevented precipitation at guanidine concentrations >2.5 M. The insolubility of synthetic MGP resembles that of native, purified MGP, which precipitates at concentrations >10 µg/mL [164]. However, when MGP was brought into Boric acid buffer (25 mM boric acid at pH 7.4) at 0.2 mg/mL, no precipitation was observed, and peak area analysis by analytical HPLC showed that >90% of MGP was still in solution after overnight incubation (data not shown).

Ca²⁺ binding to matrix Gla protein

Evidence of Ca²⁺-binding to MGP was obtained by analytical HPLC in the absence or presence of 5 mM CaCl₂. The retention time of MGP decreased from 9.6 min in the absence of CaCl₂ to 8.8 min in the presence of 5 mM CaCl₂ (Figure 4). This shift in retention time was either caused by a charge effect or by a conformational change of MGP on binding of Ca²⁺-ions. The two additional peaks that elute before and after the main peak may represent MGP molecules that have bound a different number of Ca²⁺-ions (Figure 4).

To investigate a possible change in secondary structure contents of MGP on binding of Ca²⁺-ions, folded MGP was subjected to circular dichroism (CD) studies. In the absence of CaCl₂, CD spectroscopy showed a typical protein

Figure 4
 Ca^{2+} -binding to synthetic MGP. HPLC chromatograms (C18: 22.5%-41% acetonitrile, 1.23%/min) of synthetic MGP in the absence or in the presence of 5 mM CaCl_2 .

structure spectrum composed of a mixture of α -helix, β -structure, and random coil (Figure 5). From the mean residual weight rotation at 222 nm, it was estimated that MGP contained 21% α -helix in the absence of CaCl_2 . However, addition of varying amounts of CaCl_2 (20 μM -2.4 mM) to MGP resulted in a sharp increase in mean residual weight rotation at 222 nm at ~ 0.2 mM CaCl_2 (Figure 5B). This effective concentration of CaCl_2 lies in the range of Gla-dependent Ca^{2+} binding to bone Gla protein [84] and to Gla-containing coagulation factors ([143], and references therein), which occurs between 0.1 and 1 mM CaCl_2 . It was calculated that MGP contained 21% α -helix in the absence of CaCl_2 , which increased to 26% at 0.3 mM CaCl_2 , representing an increase in α -helicity of $\approx 25\%$.

To compare synthetic MGP with native MGP, SDS-PAGE was performed under denaturing conditions. After Western blotting, MGP was detected with monoclonal antibodies against a synthetic peptide 3-15 from MGP [17]. Figure 6 shows MGP isolated from human bone (kindly donated by Dr. Reidar Wallin from Wake Forest University) in comparison with synthetic MGP (Mr: 10.6 kDa) and the fragments from which MGP was synthesised. As can be observed, the synthetic MGP in lane 1 comigrates with a purified sample of human MGP in lane 2 (barely visible) and a crude preparation of human MGP in lane 4. The MGP fragment 1-53 with a mass of 7.3 kDa in lane 3 has increased mobility compared to full-length MGP, and because of the single

Figure 5

Circular dichroism spectrum of synthetic matrix Gla protein. (A) The spectrum for matrix Gla protein at 25 °C in boric acid shows 21% α -helical structure as can be estimated from the mean residual weight (MRW) ellipticity at 222 nm. (B) The increase (25%) of mean residual weight ellipticity of MGP at 222 nm as a function of CaCl_2 concentration.

free cysteine in MGP 1-53, a dimer of MGP 1-53 is visible at ≈ 15 kDa. MGP fragment 54-84 could not be detected with the monoclonal antibody. This SDS-PAGE observation provides additional proof for the native-like condition of chemically synthesised MGP.

Discussion

Matrix Gla protein, an essential Ca^{2+} -chelator that prevents vascular calcification, was successfully synthesised using solid-phase synthesis and native chemical ligation. The 84-residue polypeptide was folded to obtain the native-like (low-solubility) conformation containing one disulfide bond. Synthetic MGP comigrated with MGP derived from human bone on SDS-PAGE under denaturing conditions. The mass of the folded matrix Gla protein as measured using electrospray ionisation mass spectrometry was identical to the theoretical average isotopic mass. The ability of MGP to form its internal disulfide shows that the initial thioester bond between Ala⁵³ and

Figure 6

SDS-PAGE of MGP. Synthetic MGP (lane 1), purified MGP from human bone (lane 2), MGP peptide 1-53-COSR (lane 3), and crude MGP from human bone (lane 4) were applied to 12.5% SDS-PAGE, Western blotting, and detection with a monoclonal antibody against peptide 3-15 from human MGP.

Cys⁵⁴ has rearranged into a peptide bond as depicted in Figure 2, resulting in the regeneration of the functional Cys⁵⁴ sulfhydryl side chain. When proteins fold, ESI-MS shows a characteristic shift of maximal intensity from the higher m/z states in the reduced protein to the lower m/z states in the folded protein. This is because the native, folded conformation of proteins typically can carry fewer charges under ESI-MS ionisation conditions than the unfolded, extended polypeptides [36]. The minor shift from the twelfth charged state for the reduced MGP to the eleventh charged state for the folded, oxidized MGP (Figure 3 E,G) is most likely a result of the relatively high amount of positively chargeable residues arginine (14) and asparagine (7) in MGP. This means that these residues stay exposed to the bulk solvent in the folded MGP, and this might play a role in the observed low solubility of MGP.

Using HPLC and CD spectroscopy, it was shown that synthetic MGP could bind Ca²⁺, inducing a shift in the HPLC retention time of MGP and an increase in α -helicity as measured as a change in mean residual weight ellipticity at 222 nm. From the increase in mean residual rotation at 222 nm, it was calculated that the content of α -helix increased by 25% in the presence of CaCl₂. Therefore, the shift in retention time of synthetic MGP on HPLC in the presence of CaCl₂ can be explained by an increase in α -helicity of MGP on binding of Ca²⁺-ions.

Similar behaviour was shown for bone Gla protein (osteocalcin), in which α -helicity was observed to increase from 8%-38% as a result of Ca²⁺-binding

[84]. Although no homology between the proteins is obvious, some similar functional properties during bone metabolism can be anticipated, and it is therefore interesting that in both proteins, ≈ 20 residues are in α -helical conformation in the presence of CaCl_2 . The synthetic, native-like MGP generated in our study will be used for structural studies and as an antigen to raise antibodies required for the development of immunoassays. Determination of MGP in plasmas from patients with cardiovascular disease and healthy individuals will give the necessary insight into a possible role of MGP in the development of atherosclerosis.

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chapter 6

tissue-specific utilization of
menaquinone-4 results in prevention of
arterial calcification in warfarin-treated
rats

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Summary

The effects of vitamin K (phyloquinone: K1 and menaquinone-4: MK-4) on vascular calcification and their utilization in the arterial vessel wall were compared in the warfarin-treated rat model for arterial calcification. Warfarin-treated rats were fed diets containing K1, MK-4, or both. Both K1 and MK-4 are cofactors for the endoplasmic reticulum enzyme γ -glutamyl carboxylase but have a structurally different aliphatic side chain. Despite their similar *in vitro* cofactor activity we show that MK-4 and not K1 inhibits warfarin-induced arterial calcification. The total hepatic K1 accumulation was three fold higher than that of MK-4, whereas aortic MK-4 was three times that of K1. The utilization of K1 and MK-4 in various tissues was estimated by calculating the ratios between accumulated quinone and epoxide species. K1 and MK-4 were both equally utilised in the liver, but the aorta showed a more efficient utilization of MK-4. Therefore, the observed differences between K1 and MK-4 with respect to inhibition of arterial calcification may be explained by both differences in their tissue bioavailability and cofactor utilization in the reductase/carboxylase reaction. An alternative explanation may come from an as yet hypothetical function of the geranylgeranyl side chain of MK-4, which is a structural analogue of geranylgeranyl pyrophosphate and could interfere with a critical step in the mevalonate pathway.

Introduction

Vitamin K is a group name for a number of related compounds, which all have a methylated naphthoquinone ring structure, but differ from each other by the length and degree of saturation of their aliphatic side chain at the 3-position [204]. Two K-vitamins were studied in this paper: phyloquinone (K1) and menaquinone-4 (MK-4). The only known function of vitamin K in mammals is to act as a cofactor for γ -glutamyl carboxylase. This endoplasmic reticulum enzyme catalyses the posttranslational conversion of specific glutamic acid residues into γ -carboxyglutamic acid residues (Gla) in a limited number of proteins [224, 240]. Reduced vitamin K (KH_2) is used as the active cofactor in this reaction. During the carboxylation reaction, KH_2 is converted into vitamin K-epoxide (KO) and one or two dithiol-dependent reductases mediate the efficient recycling of the epoxide formed. The dithiol-dependent pathway is inhibited by coumarin derivatives such as warfarin [58]. Mainly in the liver, a second pathway for KH_2 formation is formed by an NAD(P)H-dependent dehydrogenase (DT-diaphorase), which uses K and not KO as a substrate. Relatively high intakes of vitamin K are required to generate adequate amounts of KH_2 via the latter pathway because the KO formed cannot be re-used. In extrahepatic tissues such as arterial blood vessels and cartilage, the DT-diaphorase is less active than in liver [246], which explains why vitamin K1 effectively counteracts the effect of warfarin in the liver, but not in bone [170] and the arterial vessel wall [161]. Thus, the synthesis of Gla-proteins can be blocked in extrahepatic tissues by a combination of warfarin and vitamin K1, without affecting the (hepatic) synthesis of coagulation factors and thereby avoiding the risk of bleeding [164]. Using this regimen Price *et al.* showed that warfarin induced calcifications of the elastic lamellae in arteries and heart valves of rats within 3-5 weeks through inhibition of the γ -glutamyl carboxylation of matrix Gla protein [161, 162].

Three observations suggest that in the arterial vessel wall MK-4 is preferentially absorbed and used as a cofactor in the γ -glutamyl carboxylation reaction. First, it has been reported that in rats after equal intakes of K1 and MK-4 hepatic accumulation of K1 is higher than that of MK-4 whereas higher MK-4 tissue concentrations were found in the arterial vessel wall [188]. Second, it has been shown (also in rats) that even if K1 is the sole source of vitamin K, MK-4 accumulates in a limited number of tissues including the aorta; this is suggestive for a conversion of K1 into MK-4 in these tissues [188]. Third, in humans a strong and inverse correlation was found between K2 intake and the risk of cardiac events and aortic arteriosclerosis, whereas no protective effect was observed for dietary K1 [67]. The (warfarin + K1)-treated rat provides an excellent model in which the effects of calcification-inhibiting drugs can be compared [163]. Here we report an improvement of the model in such a way that both warfarin and K-vitamins

are administered orally. With this protocol we compared the effects of K1 and MK-4 on vascular calcification. Moreover, the utilization of both vitamers in liver and aorta was estimated from the tissue concentrations of the respective epoxides formed.

Materials and Methods

Chemicals

All reagents were of analytical grade or better and were from commercial suppliers. Phylloquinone was obtained from Sigma (St.Louis, MO) and menaquinone-4 was a kind gift from EISAI (Tokyo, Japan). Tromborel S (a commercial human thromboplastin supplemented with calcium) and human clotting factor II-deficient plasma were from Behringwerke AG (Marburg, Germany).

Animals and diets

All studies were performed in male rats of the WKY strain (University of Maastricht), which were 8 weeks old at the start of the experiment. The animals were housed in normal cages in an environment with a 12 hour light-dark cycle, controlled temperature (20 ± 2 °C) and humidity ($50 \pm 10\%$). Animals had free access to water and the various diets, which were prepared with an irradiated (0.9 Mrad) vitamin K-deficient diet (Hope Farms, Woerden, The Netherlands). Solutions of vitamin K1 and MK-4 were prepared in corn oil (CPC Bestfoods, Heilbronn, Germany), whereas warfarin was dissolved in phosphate-buffered saline (PBS: 150 mmol/L NaCl, 10 mmol/L sodium phosphate, pH 7.4). In all cases the amount of vitamin K and warfarin to be administered per rat per day was mixed in the vitamin K-deficient food in a professional food processor. The following abbreviations for the diets are used: diet K1&MK-4&W containing 1.5 mg vitamin K1, 1.5 mg vitamin MK-4, and 3 mg warfarin per gram diet; diet K1&W containing 1.5 mg vitamin K1 and 3 mg warfarin per gram diet; diet MK-4&W containing 1.5 mg vitamin MK-4 and 3 mg warfarin per gram diet. During all experiments, the food intake was checked to be regular (see below). The Experimental Animal Ethics Committee of the University Maastricht approved all experimental protocols.

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Experimental procedures

Blood was collected either by puncture of the tail vein (150 μ L blood in 15 μ L of 100 mM trisodium citrate) or from the abdominal aorta at the end of the experiment (4.5 mL blood in 500 μ L of 100 mM trisodium citrate). The samples were centrifuged for 15 min at 3000 x g and the supernatant plasma was frozen at -80 °C until use. Rats were killed by exsanguination while they were under Na-nembutal (Ceva Santa Animle, Libourne, France; 100 μ L/g body weight; 60 mg/mL) anesthesia, and organs were perfused with saline (150 mM NaCl, sodium nitropruside) via the portal vein. Liver, abdominal

aorta, testis, kidney and heart were frozen at -80°C until serial vitamin K analysis. Carotid artery, aortic arch, and abdominal aorta (from 1 cm above and just below the femoral bifurcation) were first placed in 4 % (v/v) phosphate buffered formaldehyde within 30 minutes of death and fixed for 24 hours at room temperature. Embedding, sectioning, and histological analysis were carried out at the Department of Pathology, University Maastricht, The Netherlands. Arterial tissues (carotid artery and aorta) were completely sectioned in $4\text{ }\mu\text{m}$ sections and every fifth section was screened for calcification by Von Kossa staining. The presence of calcification was assessed by 2 observers blinded as to treatment.

Plasma prothrombin assay

Prothrombin concentrations were determined in the one-stage assay using a coagulometer (KC-4, Amelung, Germany), a commercial thromboplastin preparation (Thromborel S) and clotting factor II-deficient plasma. Prothrombin concentrations were calculated with the aid of a reference curve from pooled normal rat plasma (same strain, sex and age as rats used in the experiment).

Vitamin K and KO determination

Concentrations of vitamin K1, K1-O, MK-4, and MK-4-O were analysed simultaneously in one HPLC-run. After thawing, tissues were weighed and homogenised in 1 volume ethanol and 2 volumes of water using a blender (Ultra Turrax, Janke and Kunkel, Staufen, Germany). At this stage samples were supplemented with 100 ng vitamin MK6 as internal standard, extracted with 4 volumes of n-hexane, and pre-purified on silica columns as described previously [227]. Quantification of vitamin K was performed by HPLC with fluorescence detection (excitation at 244 nm, emission at 430 nm) after post-column reduction on a $10 \times 0.2\text{ cm}$ column filled with zinc powder (Riedel-DeHaën, Seelze, Germany) at 40°C [82]. The mobile phase consisted of methanol / acetonitril / Reduction Buffer / water at a ratio (v/v) of 360:90:4:3 and was degassed continuously with helium. Reduction buffer contained: 1 M ZnCl_2 , 1 M NaOAc , 1 M AcOH in MeOH .

Data and statistical analysis

Plasma prothrombin values and tissue vitamin K concentrations are presented as means $\pm$ S.D. for 4 rats. Comparisons between groups were performed with a nonparametric test for two-independent-samples using SPSS for Windows Release 10.0.7. Differences were considered to be significant at a level of $p < 0.05$.

Figure 1

Arterial calcification induced by (warfarin + vitamin K1)-treatment. Eight week old male WKY rats received a diet containing 3 mg warfarin and 1-6 mg vitamin K1 per gram of food for 4 weeks. Rats were killed and carotid arteries were fixed in 4 % phosphate buffered formaldehyde. Tissues were completely sectioned and every fifth section was screened for calcification by Von Kossa staining (positive staining in black and indicated with arrows). The figure shows examples of typical sections from animals treated with vitamin K1 and warfarin (diet K1&W) containing calcification (A, B, C, D, E, and F), and from animals treated with vitamin MK-4 and warfarin (diet MK-4&W: panels G, H, I, J). A: 1 mg vitamin K1 and 3 mg warfarin per gram of diet, B: 2 mg vitamin K1 and 3 mg warfarin per gram of diet, C: 3 mg vitamin K1 and 3 mg warfarin per gram of diet, D: 4 mg vitamin K1 and 3 mg warfarin per gram of diet, E: 5 mg vitamin K1 and 3 mg warfarin per gram of diet, F: 6 mg vitamin K1 and 3 mg warfarin per gram of diet. G, H, I, J: 1.5 mg menaquinone-4 and 3 mg warfarin per gram of food.

Results

Medial calcification of arteries by dietary (warfarin + K1) regimens

In a dose-finding study, arterial media calcification was induced by feeding 6 groups of four rats a diet containing warfarin and increasing doses of vitamin K1. All animals received 3 mg of warfarin per g food, whereas the concentration of K1 increased from 1 to 6 mg/g of food with 1 mg increments per group. The warfarin dose is based on the warfarin injection protocol used by Price *et al.* [161]. After four weeks, citrated blood was taken from the tail vein and subsequently the animals were killed. Sections of the aortic arch and carotid artery were prepared and stained for calcium deposits. Examples are shown in Figure 1. It turned out that all diets had induced vascular media calcification. Although there were individual variations in the extent of calcification, no trend was observed towards decreased calcification at higher vitamin K intakes and even the highest intake of K1 did not prevent or notably diminish calcification. The plasma prothrombin concentration was measured and compared to normal pooled rat plasma. Even in the lowest K1 intake group the circulating prothrombin concentrations were comparable to those in the reference plasma, showing that on a hepatic level vitamin K had effectively counteracted the warfarin treatment. In a separate experiment (data not shown) we tried to decrease the treatment period, but it turned out that calcification could not be detected in all animals. For further experiments we have chosen to work with calcifying diets containing 3 mg of warfarin and 1.5 mg of K1 per g of food, and with a treatment period of 4 weeks.

Differential effects of K1 and MK-4

Four groups of four rats each were compared. One group served as a control and received standard food. In the three experimental groups, all animals received 3 mg of warfarin per g food. These groups differed in that their diets were supplemented with either 1.5 mg K1, 1.5 mg MK-4, or 1.5 mg K1 + 1.5 mg MK-4 per g of food. After four weeks of treatment the animals were killed and longitudinal sections were made of the aorta and carotid arteries. As is shown in Table 1, only in the K1&W-group the tissues were calcified. Neither the MK-4&W nor the K1&MK-4&W treatment resulted in detectable arterial calcifications (Figure 1). Total food intake was compared in the experimental and control animals, and no significant differences were found between the different groups. Also the weight gain after four weeks of treatment was equal between the four groups.

Treatment	Number of rats	Total Food Intake #	Weight Gain\$	Rats with Calcification	
		g/day	%	Aorta	Carotid Artery
Control	4	19.7 ± 2.16	22.6 ± 3.82	0	0
K1&W	4	19.2 ± 3.67	24.1 ± 1.64	4	4
MK4&W	4	20.6 ± 3.43	19.3 ± 5.61	0	0
K1&MK4&W	4	21.3 ± 4.18	25.9 ± 4.36	0	0

Table 1

Effect of vitamin K1 and vitamin MK-4 on food intake, weight gain, and arterial calcification

Food intake was calculated from four rats per group and 24 hours differences between two food supplies on nine randomly selected days (average ± SD). No significant differences between groups ($p > 0.05$).

\$ Weight gain is expressed as the average ($n=4$; ± SD) percentage increase in body weight after 4 weeks of treatment. No significant differences between groups ($p > 0.05$).

Tissue accumulation of K vitamins and epoxides

To estimate the utilization of K1 and MK-4 in different tissues the ratios between the accumulated quinone and epoxide forms were assessed (Table 2). The observed K1 and MK-4 tissue concentrations varied in a broad range, whereas the calculated quinone and epoxide percentages of the sum of vitamin K metabolites showed less inter-animal variation. Epoxides were abundantly present in the livers of all rats on a warfarin-containing diet. In the K1&MK-4&W group, the total liver K1 concentration (quinone + epoxide) was about 3-fold ($p < 0.05$) that of MK-4, and comparable to that in the K1&W group. Similarly, the total liver MK-4 concentration in the K1&MK-4&W group was comparable to that in the MK-4&W-treated animals, indicating that uptake of one vitamer was not influenced by the other. In all cases, half of the total hepatic K1 or MK-4 occurred in the epoxide form, indicating a difference in hepatic uptake rather than in utilization of both forms of vitamin K. As compared to the liver, the aortas in the K1&MK-4&W group contained substantially less vitamin K (MK-4: 30% of liver, K1: 3% of liver, see Table 2), but - like in the liver - the accumulated amounts of K1 and MK-4 in the single vitamin diets were comparable to those in the K1&MK-4&W diet. In the latter group the total aortic MK-4 was 3 times that of K1 ($p < 0.05$), suggesting a preference for MK-4 uptake by the arterial vessel wall. Up to 96 % of all vascular MK-4 had accumulated in its epoxide form (Table 2), whereas for K1 this was 73% ($p < 0.05$). Although total vitamin K uptake in testis, kidney, or heart was lower than in the aorta, these extra-hepatic tissues showed similar patterns of vitamin K accumulation and utilization.

It is well known that in certain extra-hepatic tissues K1 may be converted into MK-4 [228]. No MK-4, either in its quinone or its epoxide form, was detected in the extra-hepatic tissues of animals treated with warfarin and vitamin K1, thus confirming the reported inhibition of its conversion by coumarins [228].

Treatment	Tissue	K1	K1-O	MK4	MK4-O
		µg/g	µg/g	µg/g	µg/g
K1&W	Liver	75.2 (± 53)	74.8 (± 40)		
	Aorta	0.6 (± 0.2)	0.9 (± 0.4)		
	Testis	0.03 (± 0.01)	0.08 (± 0.03)		
	Kidney	0.3 (± 0.1)	0.5 (± 0.3)		
	Heart	1.4 (± 0.2)	0.7 (± 0.5)		
MK4&W	Liver			30 (± 18)	22.3 (± 7)
	Aorta			0.7 (± 0.3)	10.5 (± 7)
	Testis			0.04 (± 0.04)	0.8 (± 0.8)
	Kidney			0.1 (± 0.04)	1.8 (± 0.9)
	Heart			0.5 (± 0.1)	4.2 (± 2)
K1&MK4&W	Liver	94.3 (± 15)	94.9 (± 30)	18.8 (± 7)	48.3 (± 16)
	Aorta	1.5 (± 1)	4 (± 2)	0.7 (± 0.3)	18.5 (± 9)
	Testis	0.04 (± 0.02)	0.08 (± 0.01)	0.01 (± 0.01)	1.3 (± 1)
	Kidney	0.2 (± 0.01)	0.4 (± 0.01)	0.1 (± 0.02)	2.8 (± 0.7)
	Heart	1.3 (± 0.3)	0.5 (± 0.01)	0.3 (± 0.02)	3 (± 0.1)

Treatment	Tissue	K1	K1-O	MK4	MK4-O
		%	%	%	%
K1&W	Liver	47 (± 8)	53 (± 8)		
	Aorta	42 (± 6)	58 (± 6)		
	Testis	30 (± 6)	70 (± 6)		
	Kidney	32 (± 7)	68 (± 7)		
	Heart	72 (± 17)	28 (± 17)		
MK4&W	Liver			55 (± 9)	45 (± 9)
	Aorta			9 (± 8)	91 (± 8)
	Testis			3 (± 1)	97 (± 1)
	Kidney			6 (± 3)	94 (± 3)
	Heart			11 (± 2)	89 (± 2)
K1&MK4&W	Liver	51 (± 4)	49 (± 4)	28 (± 8)	72 (± 8)
	Aorta	27 (± 3)	73 (± 3)	4 (± 0.4)	96 (± 0.4)
	Testis	32 (± 3)	68 (± 3)	1 (± 2)	99 (± 2)
	Kidney	30 (± 1)	70 (± 1)	2 (± 0.1)	98 (± 0.1)
	Heart	70 (± 7)	30 (± 7)	10 (± 1)	90 (± 1)

Table 2
Tissue concentrations of vitamin K (total of quinone and epoxide) and percentage of quinones and epoxides after four weeks of treatment.
Rats received diets supplemented with 1.5 mg vitamin K1 + 3 mg warfarin (K1&W), 1.5 mg vitamin MK-4 + 3 mg warfarin (MK-4&W), or 1.5 mg vitamin K1 + 1.5 mg vitamin MK-4 + 3 mg warfarin (K1&MK-4&W) per gram of food.

Discussion

K1 and MK-4 have a 2-methyl-naphthoquinone group in common, and differ in the side chain attached to the 3-position. In K1 this side chain consists of 4 isoprenoid residues one of which is unsaturated, whereas MK-4 has a geranyl-geranyl side chain containing 4 isoprenoid residues, which are all unsaturated. Although both K1 and MK-4 function as a cofactor for γ -glutamyl carboxylase [240] the data in this paper demonstrate a marked difference between both vitamers: in contrast to vitamin K1, MK-4 prevents warfarin-induced vascular media calcification. Feeding rats a diet containing warfarin and K1 induced vascular media calcification within 4 weeks. The dose of K1 used exceeded the minimal daily K1 requirement for conventional male rats (0.5 µg/g diet) [103] by 3,000 fold, and even at still higher doses calcification could not be prevented by K1. However, no calcification was observed when MK-4 was added to the diet. Although it is unclear whether data from our animal model may be extrapolated to humans, these results are in line with a recently reported reduced risk for myocardial infarction,

cardiac death, and atherosclerotic calcifications in subjects with a high menaquinone intake [67]. No relation between dietary K1 intake and cardiac events or aortic atherosclerosis was shown in the same study. In our studies we have used MK-4 as a representative of the group of menaquinones, and we demonstrate that the differences between K1 and MK-4 with respect to inhibition of arterial calcification may be explained by both a better bioavailability and a more effective utilization of MK-4 in the vessel wall. Several mechanisms may underlie these observations.

In vivo studies in vitamin K-deficient rats suggested a 8 fold higher activity of K1 in counteracting hypoprothrombinaemia [73]. These data are consistent with previous data from our group, in which we demonstrated a 10-fold higher hepatic accumulation of K1 than of MK-4 whereas MK-4 was preferentially taken up by a number of extra-hepatic tissues [189]. This might be caused by the different distribution over the plasma lipoproteins of these vitamers, and by the resulting different transport to the various tissues [200]. The higher amount of vitamin K1 compared to that of MK-4 which results in equal amounts of the epoxides of both vitamers can be explained by the 4-fold lower KM of MK-4 compared with K1 [26].

An *in vivo* method to estimate the *in vivo* utilization of different K-vitamers was first worked out by Reedstrom and Suttie [181], who blocked the recycling of K-epoxides by treating rats with warfarin and measuring the accumulated epoxides. Based on the assumption that the clearance rate of both epoxides is similar, the ratio between K1 epoxide and MK-4 epoxide reflects the relative utilization of both vitamins. We observed that in liver the utilization of K1 and MK-4 was similar, but that in extra-hepatic tissues such as the aorta MK-4 was preferentially converted into its corresponding epoxide, suggesting a more efficient use by extra-hepatic carboxylase. In addition, these data suggest that in the vessel wall sufficient amounts of MK-4 hydroquinone are generated to be used as cofactor in the posttranslational γ -glutamyl carboxylation reaction. The utilization of MK-4 by γ -glutamyl carboxylase implies synthesis of carboxylated and thus biologically active Gla-proteins, most probably including MGP. Price *et al.* showed already the vital importance of MGP carboxylation in preventing arterial calcification [162]. Recently, a strong link between bone loss and arterial calcification was suggested by Price *et al.* [163], although the underlying mechanism remains to be elucidated.

An alternative explanation for the differences between K1 and MK-4 might be given by an as yet hypothetical function for the geranylgeranyl side chain of MK-4. Various Japanese studies have demonstrated a strong decrease of bone loss and even an increased bone mineral density by a high-dose MK-4 treatment (45 mg/day) [1, 151]. The reported effects are comparable to those

obtained with bisphosphonate treatment. Remarkably, the optimal dose of MK-4 is much higher than can be understood from the regular function of vitamin K. Like bone loss, also vascular media calcification can be completely abolished by both N-containing bisphosphonate treatment [163] and by high-dose MK-4 treatment (this study), but not by treatment with K1. N-containing bisphosphonates act as competitive inhibitors in the mevalonate pathway, where they inhibit the pyrophosphorylation of farnesol and geranylgeraniol. One might speculate that the effect of MK-4 is partly related to its side chain, which might – like bisphosphonates – inhibit the pyrophosphorylation of geranylgeraniol, and thus interfere with the prenylation of signaltransduction proteins. However, this remains to be established.

In conclusion: our study indicates that in a rat model arterial calcification is prevented by a high dose of MK-4. Further studies are needed to unravel the underlying mechanism and to demonstrate the potential role of MK-4 in prevention of vascular calcification in humans.

chapter 7

regulation of human VSMC calcification by matrix Gla protein

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Summary

The effects of γ -glutamyl carboxylation and the vitamin K-dependent matrix Gla protein (MGP) on VSMC calcification were measured using two *in vitro* models. In the first model, confluent human VSMCs spontaneously retract and form multicellular nodules which calcify within 28 days of culture. In the second model calcium ions were added to human VSMC cultures which then calcify rapidly, within 48 hours.

The formation of VSMC nodules was completely blocked by inhibition of γ -glutamyl carboxylation with the vitamin K antagonist warfarin. Vitamin K1 counteracted the effect of warfarin, suggesting a Gla-protein dependent pathway. In contrast to vitamin K1, which had no effect on nodule formation, menaquinone-4 (vitamin K2, MK-4) inhibited nodule formation by 50%. However, if warfarin and vitamin K1 were added after nodule formation had already occurred, calcification was stimulated by warfarin and inhibited by vitamin K1, indicating a role for Gla-proteins in prevention of vascular calcification. In the second model in which calcification was induced rapidly with 3.6mM ionic calcium, both synthetic full length MGP (1-84) and the N-terminal Gla domain (1-53) prevented calcification of VSMCs, whereas the C-terminal domain of MGP (54-84) did not.

These results indicate that (1.) Gla proteins are involved in human VSMC nodule formation and calcification, (2.) MGP inhibits VSMC calcification and Gla-residues are necessary for this activity, and (3.) MK-4 not only acts as a cofactor for γ -glutamyl carboxylation, but has a second yet unknown function.

Introduction

The posttranslational conversion of specific glutamate residues into γ -carboxy glutamic acid (Gla) residues is catalysed by the endoplasmic reticulum enzyme γ -glutamyl carboxylase. Reduced vitamin K (KH_2) is used as the active cofactor in this reaction and is converted into vitamin K epoxide (KO) during the carboxylation reaction. One or two dithiol-dependent reductases mediate the efficient recycling of vitamin KO back into KH_2 . Coumarin anticoagulants such as warfarin inhibit the dithiol-dependent reductases and thus the synthesis of Gla-residues [58]. Matrix Gla protein (MGP) is a vitamin K-dependent protein that is highly expressed by proliferating VSMCs and is thought to play a role in the inhibition of vascular calcification [203]. The evidence for this inhibitory effect stems from its theoretical calcium-binding Gla domain [167] and from MGP deficient mice, which develop excessive vascular calcification as well as increased cartilage calcification [123]. Furthermore, rats treated with both warfarin and vitamin K develop vascular media calcification within 4 weeks, probably as a consequence of undercarboxylated MGP [161, 162]. However, the mechanism underlying the calcification inhibitory activity of carboxylated MGP is not clear since the type of cells present in the vascular media from the MGP null mice resemble chondrocytes, rather than the expected vascular smooth muscle cells (VSMCs). Therefore, it is not known whether MGP can inhibit human VSMC calcification.

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Isolated human aortic VSMCs maintained in confluent culture form a distinct "hill-and-valley" morphology, with cell retraction in some areas and cell accumulation into multicellular foci (nodules) in other areas [174]. These multicellular VSMC nodules start to form within 14 days and start calcifying after 21 days in cell culture. The formation of nodules appears to be a prerequisite for spontaneous calcification [174]. Proudfoot *et al.* previously found that MGP mRNA expression was upregulated in nodular, calcifying, human VSMCs *in vitro* [174]. To elucidate the role of carboxylated MGP in *in vitro* calcification, we modulated MGP activity using warfarin, vitamin K or synthetic MGP [76].

Materials and Methods

Cell Culture

The culture medium used was M199 (GIBCO, Breda, The Netherlands) buffered with 3.7 g/L $NaHCO_3$ and 5% CO_2 , and supplemented with 100 IU/mL penicillin (Sigma), 100 mg/mL streptomycin (Sigma), 250 ng/mL amphotericin B (Sigma), and 4 mmol/L of L-glutamine (Sigma). Heat-inactivated foetal calf serum (FCS) was purchased from Sigma. Human VSMCs were obtained from nonatherosclerotic areas of aortas from organ donors of

various ages (males and females from 3 to 65 years of age). The cells were prepared from explants of aortic tissue and were identified as smooth muscle cells (SMCs) by positive staining with monoclonal antibodies against α -SM actin (A2547, Sigma). Cells were maintained in M199 medium containing 20% FCS and were used between passages 3 and 15. At least three different isolates from individuals of different ages were used in the experiments.

Nodule formation

Day 1 (1 day after passaging) VSMC cultures were incubated in M199 medium containing 20% FCS in the presence of 10 μ M vitamin K1, 10 μ M MK-4, 10 μ M warfarin, or 500 ng/mL full length synthetic MGP (sMGP¹⁻⁸⁴) [76]. The medium was changed every 2 days. Vitamin K1 and warfarin were obtained from Sigma (St.Louis, MO) and vitamin K2, menaquinone-4 (MK-4), was a kind gift from Eisai (Tokyo, Japan).

VSMC calcification

Day 14 VSMC cultures were incubated in M199 medium containing 20% FCS in the presence of 10 μ M vitamin K1, or 10 μ M warfarin. The medium was changed every 2 days. After confluence, VSMCs were cultured in the presence of sMGP¹⁻⁸⁴, sMGP¹⁻⁵³, or sMGP⁵⁴⁻⁸⁴ with or without extra calcium (3.6 mM Ca^{2+}) for 48 hours. The sMGP¹⁻⁸⁴ used in these experiments was synthesised in the Glu form as two synthetic peptides (the Glu domain: sMGP¹⁻⁵³, and the carboxy-terminal part: sMGP⁵⁴⁻⁸⁴), which had been linked by native chemical ligation [76].

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Detection of Calcification

Von Kossa staining: Cells were grown in 12-well plates, washed with PBS three times, and then fixed in 4 % (v/v) formaldehyde in PBS for 45 minutes at 4 °C. The cells were washed with distilled water and exposed to 5 % (w/v) aqueous AgNO_3 and an intensive light source for 60 minutes at RT. The cells were then exposed to 2.5 % (w/v) sodium thiosulfate for 5 minutes (black = positive staining).

Alizarin Red S staining: Cells were prepared and fixed as described above. The cells were washed in distilled water and then exposed to 2 % (w/v) Alizarin red S (Sigma) for 5 minutes (red/orange = positive staining).

Calcium concentration: Cells were washed with Hank's Balanced Salt Solution without CaCl_2 and MgCl_2 (Sigma) and incubated in 100 mM HCl at RT overnight. Samples were collected and centrifuged at 13,000 rpm for 1 minute. 50 μ L of sample was mixed with 30 μ L H_2O , 10 μ L cresolphthalein solution (0.1 % (w/v) Phthalein purple, 28 % (v/v) ammonia buffer), 200 μ L ammonia buffer (0.24 % (w/v) NH_4Cl , 5 % (v/v) NH_4OH , pH 10.5) and the

absorption was measured at 540 nm [70].

Western Blotting

sMGP¹⁻⁶⁴ (500 ng/mL) samples for protein analysis were prepared by dissolving them either in medium or 8 M urea. Solutions were incubated for 15 minutes at RT and then centrifuged at 13,000 rpm for 5 minutes. Supernatants and precipitates were dissolved in sample buffer containing 100 mmol/L β -mercaptoethanol, boiled, and applied to a 14% polyacrylamide gel containing 8 M urea. Gels were transferred to Immobilon-P (Millipore, Bedford, MA) by using an electroblotting system (Bio-Rad, Veenendaal, The Netherlands). Western blot analysis was performed using the monoclonal antibody α 3-15 MGP [211]. This antibody was detected with an anti-mouse horseradish peroxidase - conjugated antibody and stained by chemiluminescence (Amersham Pharmacia, Uppsala, Sweden).

Figure 1

Effects of warfarin and vitamin K1 on *in vitro* nodule formation by human VSMC. Panel A: Dose dependent inhibition of nodule formation by warfarin. The total number of nodules formed in control medium was set to 100%. Panel B: 10 μ M vitamin K1 and no warfarin, 10 μ M vitamin K1 and 10 μ M warfarin, untreated control culture. Results are expressed as mean $\pm$ SE (n=3), and p value determined using a Student's t test. * indicates significant difference from the control (p< 0.05).

Results

Two approaches were used to study the influences of γ -glutamyl carboxylation and MGP on VSMC cultures. In the first setup the effect on nodule formation was measured. In the second approach VSMC calcification was measured.

Nodule Formation

Effects of γ -glutamyl carboxylation on human VSMC nodule formation

To examine the role of γ -glutamyl carboxylation on human VSMC nodule formation, either warfarin or vitamin K1 were added to VSMC cultures 1 day after the start of cell culture (before nodules formed). Total numbers of nodules were counted after 28 days and expressed as percentage of the total number of nodules in an untreated control culture. Nodule formation was almost completely inhibited in the presence of warfarin, whereas vitamin K1 treatment had no effect (Table 1).

To investigate whether the effect of warfarin on nodule formation is brought about via its well-known specific inhibitory activity of the dithiol-dependent vitamin K-reductase, non-nodular human VSMC day 1 cultures were cultured for 7 days in the presence of different warfarin concentrations. As can be seen in Figure 1A, the nodule formation inhibiting activity of warfarin is a dose dependent effect. Vitamin K1 had no effect on nodule formation but completely counteracted the inhibitory effect of warfarin (Figure 1B).

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	Number of Nodules	Percentage
Control	17.3 $\pm$ 1.5	100 $\pm$ 8.8
Warfarin	0.3 $\pm$ 0.6	2 $\pm$ 3.3
Vitamin K1	20.5 $\pm$ 2.1	118 $\pm$ 12.2

Table 1
Modulation of VSMC nodule formation.

Human aortic VSMCs were cultured in the presence of either 10 μ M warfarin or 10 μ M vitamin K1. After 28 days the total numbers of nodules were counted (average of n=3) and expressed as percentage of untreated cultures (control).

Effects of synthetic MGP on human VSMC nodule formation

Day 1 VSMC cultures were grown in the presence of 500 ng/mL full length synthetic MGP (sMGP¹⁸⁴) to examine its role in human VSMC nodule formation. MGP is known to be poorly soluble. At a concentration of 500 ng/mL, however, sMGP¹⁸⁴ retained solubility in the culture medium, as demonstrated by centrifugation of the medium and subsequent Western blot analysis (Figure 2). After 7, 14, 21, and 28 days the numbers of nodules were counted. The number of nodules formed in the presence of 500 ng/mL of MGP is about 50 % of that found in the control medium (Figure 3).

Figure 2

MGP solubility in culture medium

Synthetic MGP (sMGP¹⁸⁴, 500 ng/mL) was dissolved in medium or 8 M urea. The solution was incubated for 15 minutes and then centrifuged at 13,000 rpm for 5 minutes. The supernatant (sn) and precipitate (ppt) were separated on SDS-PAGE, blotted, and probed with α -3-15 MGP mAb. This blot shows that 500 ng/mL MGP remained soluble in the supernatant when added to culture medium.

Figure 3

Effect of synthetic MGP (sMGP¹⁸⁴) on human VSMC nodule formation. Day 1 cultures were grown for up to 28 days in the presence of 500 ng/mL sMGP¹⁸⁴ (hatched bars) and the total number of nodules was compared to control cultures (open bars, set to 100% nodule formation). Results are expressed as mean $\pm$ SE (n=3), and p value determined using a Student's t test. *: p < 0.05.

Different effects of vitamin K1 and MK-4 on human VSMC nodule formation

To compare the effects of vitamin K1 and MK-4 (vitamin K2, menaquinone-4) on VSMC nodule formation, non-nodular human VSMC day 1 cultures were cultured for 7 days in the presence of vitamins with or without warfarin (Figure 4). Vitamin K1 had no effect on nodule formation and completely counteracted the inhibitory effect of warfarin (Figure 1B). On the other hand, MK-4 alone inhibited nodule formation by approximately 50% compared to the control culture. Also in the presence of both warfarin and MK-4 nodule formation was 50% compared to the control culture. The same results were obtained by adding a mixture of 5 μM vitamin K1 and 5 μM MK-4 (Figure 4). These data suggest that inhibition of nodule formation by MK-4 occurs via a yet unknown pathway distinct from the Gla-protein(s) dependent pathway.

Figure 4

Effect of MK-4 on human VSMC *in vitro* nodule formation. The total number of nodules formed in control medium was set to 100%. Results are expressed as mean $\pm$ SE ($n=3$), and p value determined using a Student's t test. *: $p < 0.05$.

VSMC Calcification

Effects of γ -glutamyl carboxylation on human VSMC calcification

To examine the role of γ -glutamyl carboxylation in human VSMC calcification, either warfarin or vitamin K1, were added to nodular VSMC cultures at day 14. After 14 days of treatment, the presence of calcified material was determined by Von Kossa staining and the total amount of acid-extractable calcium was quantitated by the cresolphthalein method. Calcification was stimulated by warfarin ($p = 0.02$) whereas vitamin K1 inhibited calcification ($p = 0.01$) in VSMC nodular cultures (Figure 5). This suggests that vitamin K-dependent proteins are involved in the inhibition of VSMC nodule calcification.

Figure 5

Effects of warfarin and vitamin K1 on human VSMC calcification.

Warfarin (10 μ M) or vitamin K1 (10 μ M), were added to nodular VSMC cultures (day 14). At day 28, cells were stained with Von Kossa (upper panel, positive staining in black). Total acid-extractable calcium was quantitated by the cresolphthalein method (lower panel). Warfarin stimulated calcification (* $p=0.02$) while vitamin K1 inhibited calcification (** $p=0.01$).

Effect of MGP on human VSMC calcification

To find out whether MGP could inhibit calcification, synthetic MGP was added to human VSMC cultures in which calcium apatite formation was induced within 48 hours by incubating with twice the physiological concentration of calcium ions (3.6 mM Ca^{2+}) (this model is demonstrated in Figure 6). Either sMGP¹⁻⁸⁴, the Gla domain sMGP¹⁻⁵³, or the carboxy-terminal part sMGP⁵⁴⁻⁸⁴ were added to human VSMC cultures with or without 3.6 mM Ca^{2+} for 48 hours and calcification was measured by the cresolphthalein method. At 500 ng/mL, sMGP¹⁻⁸⁴ significantly ($p = 0.01$) inhibited VSMC calcification by approximately 60% compared to controls (Figure 7A), whereas no inhibition was observed at 50 and 100 ng/mL sMGP¹⁻⁸⁴. Furthermore, the N-terminal part of MGP (1-53, Gla) inhibited calcification at a concentration of 500 ng/mL, while the C-terminal part (54-84) did not (Figure 7B).

Figure 6

Calcification in VSMC monolayers. Human VSMC monolayers were incubated in either control medium containing 1.8 mM Ca^{2+} or medium containing 3.6 mM Ca^{2+} for 48 hours. Subsequently cultures were stained for calcification using Alizarin red or Von Kossa. Unlike VSMCs exposed to 1.8 mM Ca^{2+} (control), human VSMC cultures exposed to twice the physiological Ca^{2+} -concentration calcified within 48 hours.

Discussion

Human aortic VSMC nodule formation and calcification occur spontaneously *in vitro*, without the addition of exogenous factors [174]. The mechanisms involved in these two processes are not known. In this article, we show that a complete inhibition of γ -glutamyl carboxylation blocks nodule formation but stimulates nodule calcification. Furthermore, exogenously added fully carboxylated synthetic MGP inhibited VSMC calcification.

Nodule Formation

The complete inhibition of VSMC nodule formation by warfarin and reversal with vitamin K1, suggests the involvement of Gla-protein(s) in this process. Three vitamin K-dependent proteins are known to be expressed by the arterial vessel wall: protein S, MGP, and growth-arrest-specific gene-6 protein (Gas6). Protein S is expressed by VSMCs and endothelial cells and possibly involved in the local inhibition of thrombosis [8, 52]. Gas6 prevents serum starvation-induced death of fibroblasts and VSMCs, and may act as a growth-potentiating factor [72, 141, 239]. Gla-residues were shown to be essential for the functions of Gas6 in cell culture [141]. Also, the calcification inhibitory function of MGP requires the presence of Gla-residues [161, 162]. The paradoxical effects of warfarin and sMGP¹⁻⁸⁴ on nodule formation can be explained by two hypotheses. First, a yet unknown Gla-protein may stimulate VSMC nodule formation in its carboxylated form. Second, the Gla-residues of MGP are not involved in the inhibition of VSMC nodule formation. Whether higher concentrations of sMGP result in an even greater degree of inhibition, could not be tested because of the insolubility properties of MGP.

Different effects of vitamin K1 and MK-4 on human VSMC nodule formation

Although both vitamin K1 and MK-4 serve as a cofactor in γ -glutamyl carboxylation, several data suggest a second function for MK-4. Wang *et al.* reported the growth inhibition of hepatocellular carcinoma cells by MK-4

[250] and several studies showed apoptosis inducing activity for MK-4 [97, 136, 147, 267]. Also, a nuclear vitamin K2 binding protein homologues to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been detected in human osteoblasts [87]. Taken together, these data suggest a new role for vitamin K2 outside the field of γ -glutamyl carboxylation.

Figure 7

Effect of full-length MGP (Panel A) and MGP fragments (panel B) on calcification. sMGP¹⁻⁸⁴ was added to cultures with or without extra calcium ions for 48 hours and calcification was measured by the cresolphthalein method. At 500 ng/mL, MGP and the 1-53 portion of MGP significantly inhibited calcification compared with controls (* $P=0.01$ and ** $P<0.02$ respectively).

VSMC Calcification

Using full length synthetic MGP, we demonstrated that exogenous addition of MGP inhibited calcification of human VSMCs *in vitro* and that this activity required the N-terminal (Gla-containing) moiety of MGP. Based on the phenotype of the MGP null mouse [123] and results from the warfarin-treated rats [161] it seems reasonable to propose a direct role for MGP in the inhibition of VSMC calcification. It is unclear whether descarboxy-sMGP¹⁸⁴ has the same calcification inhibitory activity as sMGP¹⁸⁴, since this peptide is not yet available. Other peptides which are interesting for *in vitro* calcification inhibition studies are for example the full length MGP with or without serine-phosphorylation at positions 3, 6 and 9. Future use of peptides in the VSMC *in vitro* model might contribute to understanding the molecular mechanism of MGP in arterial calcification. The mechanism by which MGP may inhibit calcification remains a matter of speculation. Full-length synthetic MGP binds calcium ions, which induces an increase in α -helicity [76]. Furthermore, MGP binds to hydroxyapatite with a relative high association constant compared to other hydroxyapatite-binding proteins [190]. The binding of MGP to hydroxyapatite, however, is increased in the presence of calcium ions [190]. Similar hydroxyapatite binding behaviour has been reported for osteocalcin [84], suggesting that MGP binds to hydroxyapatite via calcium ions which induce an increase in α -helical structure of the Gla domain. By binding to calcium ions or crystals, MGP is expected to prevent crystal growth.

In conclusion, these *in vitro* studies have shown that Gla-containing proteins play an important role in nodule formation and calcification. The dramatic blockade of nodule formation by warfarin was a surprising observation, given that it is a stimulator of calcification. This finding revealed that although nodule formation is a prerequisite for calcification [174], nodule formation and calcification should be studied separately to elucidate the processes leading to VSMC calcification. The mechanisms regulating nodule formation are not known and it is therefore difficult to speculate on the mechanism of action of Gla-proteins in this process. In addition, these *in vitro* experiments have highlighted that MGP is an inhibitor of human VSMC calcification and that the Gla domain is important for this function.

chapter 8

possible role for apoptotic bodies in the
cellular release of matrix gla protein

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Summary

Matrix Gla protein (MGP) is a vitamin K-dependent protein expressed by various tissues, including the arterial vessel wall, where it is synthesised by vascular smooth muscle cells (VSMCs). MGP is thought to play a role in the inhibition of soft-tissue calcification. Its association with the extracellular matrix, the presence of a predicted signal-peptide, and Gla-residues within its N-terminal domain suggest it to be an extracellular protein.

Here we report the intracellular localisation of MGP in cultured human aortic vascular smooth muscle cells (VSMCs), human breast cancer epithelial cells (T47D), and human osteoblastic osteosarcoma cells (MG-63). Using Western blot, ELISA or immunoprecipitation techniques and a monoclonal antibody raised against amino acids 3-15 of human MGP, MGP was detected in cell lysates but not in the conditioned medium of these three cell types. Stimulating or inhibiting γ -glutamyl carboxylation with vitamin K1 and warfarin respectively, had no effect on the localisation or secretion of MGP. The intracellular localisation of MGP was investigated using confocal analysis and MGP was found diffusely throughout the cytoplasm but was highest at the nuclear periphery. Although MGP was not detected in conditioned media, it was present in VSMC-derived apoptotic bodies and small vesicular membrane structures. The presence of MGP in these membrane structures suggests that this may be the mechanism by which MGP is released by cells. Since apoptotic bodies and vesicles are prone to calcification, MGP may regulate mineralization at these sites.

Introduction

Mammalian Gla-proteins are involved in at least three physiological processes: haemostasis, bone metabolism, and vascular biology. γ -Carboxy glutamate residues (Gla) are the result of a posttranslational modification of glutamate residues (Glu). This reaction is catalysed by the endoplasmic reticulum enzyme γ -glutamyl carboxylase, which uses vitamin K as a cofactor. In all known Gla-containing proteins, the Gla-residues are essential for stabilizing the tertiary structure and for the function of these proteins.

Matrix Gla protein (MGP) was first described by Price *et al.* in 1983 and since then its synthesis has been shown in bone, cartilage, lung, heart, kidney, arterial smooth muscle cells (VSMCs) and in calcified atherosclerotic plaques [61, 167, 201]. Although the precise molecular mechanism of MGP function is not known, recent data demonstrate that it plays a major role in the inhibition of soft-tissue calcification. Targeted deletion of the MGP gene in mice resulted in extensive medial calcification of the aorta, leading to death within eight weeks after birth due to ruptures of the thoracic or abdominal aorta [123]. In these MGP-deficient mice, cartilage-producing chondrocytes were detected in the tunica media of the calcified arteries. A relation between MGP and chondrocyte function was recently suggested by Yagami *et al.*, who showed that *in vitro* calcification in hypertrophic chondrocyte cultures was triggered by treatment with the vitamin K-antagonist warfarin, whereas calcification was counteracted by MGP overexpression in the presence of vitamin K [266]. In the same study, constitutive MGP overexpression in chicken limb resulted in *in vivo* inhibition of cartilage mineralization, delayed chondrocyte maturation and termination of endochondral and intramembranous ossification. The importance of MGP Gla-residues in the prevention of vascular calcification was shown by inhibition of γ -glutamyl carboxylation in rats with warfarin [161, 162]. Within 3 to 4 weeks of treatment, arterial calcification was found around the elastic lamellae of the media. Furthermore, in humans, mutations in MGP that predict a non-functional protein have been shown to cause Keutel syndrome, a rare disorder characterised by abnormal cartilage calcification and pulmonary stenoses [139]. Post mortem examination of a young Keutel patient also revealed extensive vascular calcification [134]. It is currently thought that for MGP to prevent calcification it must be secreted and be associated with the extracellular matrix [161, 266]. Prevention of calcification is likely to involve binding of calcium ions or crystals [166, 190] and/or binding of calcification-inducing proteins [14, 245]. MGP related antigen is also found in plasma and, like osteocalcin, is thought to be released into the circulation via its tissue of synthesis. Whether MGP circulates as a free protein or in a complex is not known, although MGP can occur as a plasma complex with calcium, phosphate and fetuin in rats treated with etidronate [166].

Since secretion of MGP is thought to be pivotal to its calcification-inhibitory activity, we have investigated its release from cultured cells. Surprisingly, we found that in human VSMCs and in cell lines constitutively expressing high levels of MGP, the protein remained intracellularly and was not secreted. The intracellular location of MGP persisted even when the cells were incubated with excess vitamin K1 or MK-4. Further experiments suggested that MGP may only be released by VSMCs in association with apoptotic bodies or vesicles.

Materials and Methods

Chemicals and Reagents

All solutions were prepared using reagents of analytical or HPLC grade. Vitamin K1, warfarin, and Dulbecco's modified Eagle's medium (DMEM) and M199 medium were purchased from Sigma (St.Louis, MO). Vitamin K2 (menaquinone-4) was a kind gift from Eisai (Tokyo, Japan). Eagle's minimum essential medium (EMEM) with Earle's balanced salt solution without L-glutamine was obtained from Bio-Whittaker (Verviers, Belgium).

Selection of cell-types

In previous studies it was shown that MGP mRNA is expressed by various human cell types such as VSMCs [203], epithelial cells [206], and osteosarcoma cells [30]. Therefore, a range of cells was tested for MGP expression and these cells (T47D, MG-63, HEK 293, VSMCs, and SAH) were used in this study.

Cell Cultures

VSMCs were prepared from medial explants of human aortic tissue and cultured in 20% FCS in M199 medium, as described previously [176]. T47D is a human breast cancer epithelial cell line, HEK-293 is a human embryonic kidney cell line, MG-63 is a human osteoblastic osteosarcoma cell line, and SAH is a human hepatocyte cell line and was a kind gift from Dr. R. Kuijter (University Maastricht, The Netherlands). T47D, HEK-293 and MG-63 were obtained from the European Collection of Cell Cultures (ECACC). T47D and HEK-293 were cultured in DMEM supplemented with 10% FCS and L-glutamine. MG-63 and SAH were cultured in EMEM supplemented with 10% FCS and L-glutamine. To examine the effect of γ -glutamyl carboxylation on the MGP localisation, cells were treated with 10 μ M vitamin K1, 10 μ M vitamin K2, or 10 μ M warfarin for seven days. Medium was changed every 2-3 days and conditioned medium was collected without further treatment.

Immunoprecipitation of MGP

A commercially available immunoprecipitation kit (Roche Diagnostics, Almere, The Netherlands) was used for the immunoprecipitation of MGP from cellular extracts and conditioned medium. In brief, cells were washed twice

with ice-cold PBS after removal of the conditioned medium and suspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 % (v/v) Nonident P40, 0.5 % (w/v) sodium deoxycholate). To each cellular extract or conditioned medium sample 5 µg monoclonal α3-15 MGP IgG [17] was added and incubated at 4 °C for 3 hours. Protein G-agarose was added and after overnight incubation at 4 °C the complexes were collected by centrifugation. Complexes were washed twice in lysis buffer, twice in a high salt washing buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1 % (v/v) Nonident P40, 0.05 % (w/v) sodium deoxycholate) and finally once in a low salt washing buffer (50 mM Tris-HCl, pH 7.5, 0.1 % (v/v) Nonident P40, 0.05 % (w/v) sodium deoxycholate). The immunoprecipitated proteins were analysed on SDS-polyacrylamide gel electrophoresis (PAGE) and subsequent Western blot analysis.

SDS-PAGE and Western blot analysis

Protein samples in SDS sample buffer (40 mM Tris-HCl, pH 6.7, 4 % (w/v) SDS, 10 % (v/v) glycerol, 2 % (v/v) 2-mercaptoethanol, 0.01 % (w/v) bromophenol blue) were boiled for 5 min. Polyacrylamide gels 12.5 % (w/v) in SDS were run under denaturing conditions [113] in 8 M urea. After electrophoresis, the gels were transferred to an Immobilon-P PVDF membrane (Millipore, Bedford, MA) [233]. After washing the membranes in PBS for 15 min, non specific binding sites were blocked with blocking buffer (3 % (w/v) non-fat dry milk powder in PBS) for 1 h at RT and incubated at 4 °C overnight with biotin-conjugated α3-15 MGP IgG in blocking buffer. After washing with PBS-Tween (0.3 % (v/v) Tween-20 in PBS), the membrane was incubated for 1 h at RT with streptavidin-HRP (Dako, Glostrup, Danmark) diluted in PBS-Tween. Membranes were washed three times with PBS-Tween, followed by a final wash step of 15 min with PBS and development with ECL Western blotting detection reagents (Amersham Pharmacia, Uppsala, Sweden). Finally, protein bands were visualized on Hyperfilm ECL (Amersham Pharmacia).

Single antibody ELISA

Single antibody ELISA was performed by coating microtiter plates (60 Protein Immobilizer, Sanver TECH, Heerhugowaard, The Netherlands) with monoclonal α3-15 MGP IgG (500 ng/well) in carbonate buffer (0.1 mol/L sodium carbonate, pH 9.6) for 1 h at RT [17]. Plates were stabilised using a 5 % (w/v) sucrose solution. Cells were washed twice with ice-cold PBS after removal of the conditioned medium and suspended in lysis buffer. Samples (cell lysates and conditioned media) were diluted with lysis buffer as indicated and supplemented with a constant amount of tracer (biotinylated MGP³⁻¹⁵ peptide, 1.8 ng per mL of sample). Next, 100 µL of the mixtures were transferred to the microtiter plate wells and incubated for 2 h at RT. After three washing cycles with PBS-Tween, the amount of tracer bound was quantified by incubation with avidin-labelled horse radish peroxidase (Dako,

10 µg/well) and subsequent staining with 3,3',5,5' tetramethylbenzidine reagent (Pierce, Rockford, IL). After 15 minutes the staining process was stopped by adding 2 mol/L H₂SO₄ and plates were read at 450 nm. Reference curves were prepared by serial dilution of MGP³⁻¹⁵ peptide. Experimental samples were tested in duplicate in 2-fold dilutions and read from the reference curve.

Immunofluorescence microscopy

Cells were washed twice with PBS prior to one of the following protocols. Cells were either fixed by incubation in 4 % (v/v) formaldehyde in PBS for 45 minutes at 4 °C and subsequent permeabilised with 0.5 % (v/v) NP-40, 0.1 % (v/v) Triton X-100 in PBS, fixed without permeabilisation, or not fixed and not permeabilised. Primary antibody, monoclonal α3-15 MGP IgG, was added for 1 hour at room temperature. Coverslips were washed three times with PBS, then incubated with FITC-conjugated anti-mouse IgG (DAKO) for a further hour at room temperature. Negative controls were performed by substituting the primary antibody by either PBS, or an irrelevant antibody. After several washes in PBS, coverslips were mounted face down in Mowiol (Calbiochem) containing 1 µg/ml DAPI. Immunostained samples were viewed using a Zeiss axiovert 10 microscope with a plan-APOCHROMAT 63x/1.40-oil immersion lens and equipped with a Digital Pixel Instruments 12-bit CCD camera. Images were captured using IP Lab Scientific Imaging Software (Scanalytics). Additionally, a Zeiss LSM 410 confocal laser scanning microscope was used (63x/1.40 oil immersion lens) for imaging of MGP within cells.

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Immunohistochemistry

Paraffin-embedded human tissues were obtained from the archives of the Department of Pathology of the University Hospital of Maastricht. Tissues were collected, fixed by immersion in 4% formaldehyde in PBS for 12 h at room temperature, and embedded in paraffin. Sections (4 µm) were stained with hematoxylin and eosin for general histological evaluation. Immunohistochemistry was performed by using the monoclonal α3-15 MGP IgG as a first antibody and biotinylated anti-mouse IgG (Dako) as the second antibody. Staining was performed with the avidin-biotin immuno alkaline phosphatase kit from Dako. Negative controls were performed by substituting the primary antibody by either PBS, an irrelevant antibody, and by adding competitive amounts of synthetic MGP peptides.

Inducing apoptosis in VSMCs

Apoptosis was induced in human VSMCs by addition of αFas IgM (100ng/mL, clone CH11, Upstate Biotechnology) and cycloheximide (10 µg/mL) in serum-free M199 for 24 hours. Apoptotic bodies were collected from cultures by centrifuging cell culture supernatants at 2,500 rpm, as described previously

[175].

Cell Type	Condition	Cell Lysate	Conditioned Medium
		ng/mg	ng/mg
T47D	Control	21.2 ± 0.3	b.d.l.
	Vitamin K1	27.7 ± 4.7	b.d.l.
	Menaquinone-4	28.1 ± 1.5	b.d.l.
	Warfarin	25.8 ± 4.5	b.d.l.
VSMC	Control	9 ± 0.4	b.d.l.
	Vitamin K1	9.5 ± 1.5	b.d.l.
	Menaquinone-4	8.6 ± 0.7	b.d.l.
	Warfarin	4.5 ± 1.8	b.d.l.
MG-63	Control	26.4 ± 4.4	b.d.l.
	Vitamin K1	34 ± 2.6	b.d.l.
	Menaquinone-4	32.9 ± 4.3	b.d.l.
	Warfarin	22.5 ± 3.1	b.d.l.
SAH	Control	b.d.l.	b.d.l.
	Vitamin K1	b.d.l.	b.d.l.
	Menaquinone-4	b.d.l.	b.d.l.
	Warfarin	b.d.l.	b.d.l.

Table 1

MGP concentration in cell lysates and conditioned media of T47D, VSMCs, MG-63, and SAH cells. Concentrations are in mean ± 2SD (n=3), b.d.l. stands for below detection limit (2 ng/mL).

Results

Expression of MGP in various Cell Types

To investigate secretion of MGP by VSMCs and other cell lines known to express MGP mRNA, both cell lysates and corresponding conditioned media (collected after 3 days) were subjected to Western blot analysis. A monoclonal antibody against the 3-15 epitope of MGP (α 3-15 MGP IgG) was used for detection [17]. MGP (13.6 kD) was present in the cell lysates of VSMCs, MG-63, HEK-293, and T47D cells but no MGP was detected in the corresponding conditioned media (data not shown). Also, no MGP was observed in the cell lysate and conditioned media of SAH cell cultures. This cell line was therefore used as a negative control in subsequent experiments. Since MGP is thought to be a secreted protein we further investigated cell conditioned media for its presence using both ELISA and immunoprecipitation.

ELISA for MGP

The presence of MGP related antigen in cell lysates of VSMC, MG-63, and T47D

cells was confirmed using a single antibody competition ELISA for human MGP [17] (Table 1). However, no MGP was detected in the corresponding conditioned media. Since correct γ -glutamyl carboxylation is necessary for the biological activity of MGP [162], it may also influence the location and distribution of the protein (i.e. secretion). Therefore, cell cultures were treated with either vitamin K1, menaquinone-4 or, warfarin and MGP was measured in both the cell lysate and corresponding conditioned media. However, neither vitamin K nor warfarin treatment influenced the secretion of MGP by VSMC, T47D, and MG-63 cell cultures: MGP was present in the cell lysate and absent in the corresponding conditioned media (Table 1).

Immunoprecipitation of MGP

An MGP concentration in the conditioned medium below the detection limit of Western analysis or ELISA could cause a false negative result. Therefore, cell lysates and conditioned media were concentrated via immunoprecipitation using the α 3-15 MGP IgG. Subsequently the samples were subjected to Western analysis. Using this method MGP was detected in the immunoprecipitated cell lysates but not in the conditioned media of MG-63, VSMCs, T47D, or HEK-293. Again, treatment of the cells with either warfarin or vitamin K1 had no effect on the distribution of MGP (Figure 1A).

These observations were confirmed using an antibody against the 63-75 epitope of MGP (α 63-75 MGP IgG) (Figure 1B and 1C). Firstly, samples were concentrated via immunoprecipitation using the α 3-15 MGP IgG and analysed by Western blot using the α 63-75 MGP IgG (Figure 1B). In the second approach, the α 63-75 MGP IgG was used for both the immunoprecipitation and Western analysis (Figure 1C). With neither of both approaches, MGP was detected in the conditioned medium, which strongly suggests that MGP is not secreted by these cultured cells.

Figure 1: Immunoprecipitation of MGP. After treatment of T47D cells, MG-63 cells, VSMCs, HEK-293 and SAH cells with either vitamin K1 (K) or warfarin (W) for 72 hours, cell lysates (C) and conditioned medium (M) were incubated with α 3-15 MGP mAb (panels A and B) or α 63-75 MGP mAb (panel C). After precipitation using protein G agarose, samples were separated on SDS-PAGE and blotted to PVDF-membrane. Panel A was stained with biotin conjugated α 3-15 MGP mAb, panel B and C were stained with α 63-75 MGP mAb and rabbit anti-mouse peroxidase-conjugated IgG. Synthetic MGP peptide (sMGP) was used as a positive control. MGP was absent in the SAH cell lysate and conditioned media, confirming the absence of MGP expression in this cell type.

Immunofluorescence microscopy

Given the presence of MGP in cell lysates from various cell types, we determined whether MGP was localised at the extracellular or intracellular side of the cell membrane. Initially, this was investigated by evaluating the effect of fixation and permeabilisation of the cells, followed by subsequent immunofluorescence analysis. When non-fixed and non-permeabilised MG-63 cells were stained with the $\alpha 3$ -15 MGP IgG, staining was weak or absent (Figure 2A). Also, after fixation without permeabilisation no MGP was detected in the extracellular space (Figure 2B). These results indicate that MGP is absent in the extracellular space. On the other hand, fixation and permeabilisation of the cells prior to antibody staining showed the presence of intracellular MGP (Figure 2C). Similar observations were made with human VSMCs, and T47D (Figures 2A,C). No staining was observed in the case of the SAH cells, again indicating absence of MGP (Figure 2). These studies suggest that MGP is located inside the cell, rather than at the cell surface. The specs of staining were not observed in all cases and might reflect antibody precipitation. γ -Glutamyl carboxylation of MGP did not change the intracellular localisation of MGP as was shown by adding vitamin K1, MK-4, or warfarin to the culture medium (Figures 2D, E, F).

Confocal microscopy

The intracellular location of MGP was further investigated using confocal microscopy. In MG-63 cells stained with the $\alpha 3$ -15 MGP IgG, MGP was found diffusely throughout the cytoplasm, some of which was in rounded structures with more dense staining near the edges of the cells (Figure 3). There was an intense perinuclear staining, which suggests localisation of MGP in the RER. Little or no MGP was detected in the nucleus or associated with the cellular membrane. Similar results were obtained for T47D cells and VSMCs (data not shown).

MGP in vivo

MGP protein has been detected in the extracellular matrix of bone and cartilage (Figure 4) as well as in VSMCs and elastic lamellae of the artery wall [167, 174]. MGP expression has been reported for malignant human cultured epithelial cells [32, 206] and we have investigated epithelial cell expression of MGP within human tissues (Figure 4). Immunohistochemical staining of human gland epithelial cells showed high levels of MGP deposition within cubic shaped epithelial cells, whereas MGP staining was lower or absent in more pavement shaped cells (Figure 4). Also in this *in vivo* situation, MGP was localised in the cytoplasm of the cells and no MGP staining was observed in association with the extracellular matrix.

Figure 2:

Immunofluorescence analysis of MG-63 cells, VSMCs, T47D cells, and SAH cells. Cells were stained with $\alpha 3$ -15 MGP MAb and subsequently with FITC-conjugated anti-mouse IgG (positive signal in green). Nucleus stained in blue. Cells were stained directly (A), after fixation (B), after fixation and permeabilisation (C), and after 72 hours treatment with 10 μ M vitamin K1 (D), 10 μ M vitamin K2 (E), or 10 μ M warfarin (F) (all three with fixation and permeabilisation).

Release of MGP via apoptotic bodies

We previously reported that apoptosis is involved in calcification of VSMC multicellular nodules [175]. We also found that isolated VSMC-derived apoptotic bodies could calcify in a similar manner to matrix vesicles. Since MGP is involved in both calcification [123] and apoptosis [145] and is expressed by VSMC calcifying nodular cells [176], we analysed apoptotic bodies for the presence of MGP. By Western blot analysis it was shown that apoptotic bodies released by VSMCs, upon exposure to α Fas IgM and cycloheximide, contained MGP (Figure 5A). In addition, extracellular vesicular structures in human VSMC cultures stained intensely using the $\alpha 3$ -15 MGP IgG (Figure 5B). These bodies were also observed in MG-63 cells (Figure 2C). These

bodies/vesicles, of a size similar to apoptotic bodies, could be the mechanism by which MGP is exported from cells.

Figure 3: Immunofluorescence confocal microscopy of human osteoblastic osteosarcoma cell line (MG-63), using the α 3-15 MGP IgG antibody. Positive MGP staining in black.

Discussion

The immature vitamin K-dependent matrix Gla protein contains a predicted signal peptide of 19 amino acid residues, which is necessary for translocation across the rough endoplasmic reticulum membrane and subsequent post-translational modifications [167]. Several studies indicate that MGP plays a major role in the prevention of soft-tissue calcification [123, 161, 176]. Until recently most studies on MGP were based on mRNA or genomic DNA and only a few studies focussed on the protein itself, probably because of the insolubility of purified or synthesized MGP under physiological conditions [17, 76]. MGP mRNA is expressed by many tissues but the protein only accumulates at specific sites such as the extracellular matrix of bone, cartilage, and the arterial vessel wall [77, 167, 176].

In this study, we provide experimental evidence for an intracellular localisation and secretion via apoptotic bodies of MGP in cultured cells. We primarily analysed human VSMCs and compared these to other human cells that are known to express high or low levels of MGP mRNA. ELISA and Western analysis showed the presence of MGP in cell lysates of human breast cancer epithelial cells (T47D), human osteoblastic osteosarcoma cells (MG-63), human embryonic kidney cells (HEK-293), and human aortic VSMCs. No MGP was detected in the corresponding conditioned media by immunoprecipitation and subsequent Western analysis. Two different

antibodies raised to N- or C-terminal epitopes of MGP were used and both precipitated MGP in cell lysates but not from conditioned medium. Possible extracellular degradation of MGP by a protease can be excluded, since with both the N-terminal and C-terminal MGP antibodies no MGP fragments were detected. Our observations differ from studies of MGP secretion from non-human cells in culture. In rat cells in culture, MGP has been detected in conditioned medium from lung, kidney and osteosarcoma cells [28, 61, 180]. Also in mouse preosteoblastic M2 cells, MGP was detected intracellularly in newly plated cultures, but translocated to the extracellular matrix within 3 days of culture [269].

Figure 4: Immunohistochemical staining of human gland epithelial cells (A) and chondrocytes (B) in the trachea with α 3-15 MGP MAb. Positive staining is indicated with arrows.

The finding of intracellular MGP may simply reflect its storage prior to release in cultured cells but it is also possible that MGP has an intracellular role. Further analysis of MGP within cells revealed that it was abundant and diffusely distributed throughout the cytoplasm, with a higher concentration around the nucleus. The perinuclear staining is likely to represent the RER since γ -glutamyl carboxylation occurs at this site. Native MGP in its Gla-form is predicted to bind calcium ions and could therefore have a role in cellular calcium homeostasis and signalling.

MGP was found not only intracellularly in cultured human cells but also in human tracheal epithelial cells and cartilage *in vivo*. However, as mentioned previously, MGP has been found in the extracellular matrix of bone, cartilage and the vasculature. This raises the possibility that MGP is only secreted by specific cells or that it only accumulates in specific extracellular matrices. MGP is therefore thought to be a secreted protein.

Release of MGP in the form of apoptotic bodies/vesicles

Our experiments suggest that MGP is an intracellular protein. However, the extracellular location of MGP *in vivo* indicates that it must be secreted by cells via a presently unknown mechanism. We found that VSMCs undergoing

Figure 5:
MGP in apoptotic bodies/vesicles.

A. Both VSMCs and apoptotic bodies (AB) were resuspended in 8M urea, diluted 1:1 in 2x SDS-PAGE sample buffer and 10 μ g was electrophoresed on a 12% polyacrylamide gel. The gel was subsequently blotted and probed with the α 3-15 MGP monoclonal antibody as described in the methods section. MGP was detected in both VSMCs and apoptotic bodies. The positive control for MGP in this Western was a recombinant form of MGP (rMGP), linked to DHFR, as described previously.

B. Both panels show two different human VSMC isolates stained with the α 3-15 MGP monoclonal antibody. The top panel contains monolayer cells as well as a multicellular nodule which stains intensely for MGP. In both isolates MGP staining is seen throughout the cytoplasm and some cells have intense perinuclear staining. Vesicular structures containing MGP are indicated by arrows.

apoptosis released apoptotic bodies which contained MGP. Therefore, apoptotic body or vesicle release could facilitate MGP export from cells. Since MGP is prone to precipitation, it is possible that apoptotic bodies 'chaperone' MGP and maintain its solubility. Our findings are supported by an electron microscopic study of adult cartilage in which MGP was detected within chondrocytes and matrix vesicles [122]. Matrix vesicles in cartilage and bone are membrane-bound bodies, often generated in association with apoptosis, which bud-off from chondrocytes and osteoblasts [3, 102]. Our previous studies have shown that VSMC apoptotic bodies have similarities with matrix vesicles in that they can accumulate calcium ions and calcify *in vitro* [175], suggesting that they may be key sites for calcium crystal nucleation in vascular calcification. The presence of MGP in both VSMC apoptotic bodies and matrix vesicles indicates that calcification of these vesicles may be regulated by MGP. Although apoptotic cells are normally rapidly phagocytosed, both apoptotic bodies and matrix vesicle-like structures have been detected in blood vessels which indicates that phagocytosis is inhibited in the diseased vessel wall. We have recently shown that modified lipids can inhibit apoptotic body phagocytosis by VSMCs *in vitro* thereby enhancing calcification [172]. It is likely that MGP is present in apoptotic bodies to ensure that calcification does not occur before they are phagocytosed. It is also possible that MGP has a role in phagocytosis since Gla proteins are predicted to bind acidic phospholipids and the Gla protein Gas6 has been shown to promote uptake of apoptotic bodies via binding to phosphatidylserine [141]. Furthermore, it has recently been shown that MGP has a role in preventing apoptosis at specific stages of chondrocyte differentiation [145] and has also been linked with apoptosis in other cell types [5, 21]. It is therefore tempting to speculate that one of the mechanisms by which MGP might inhibit vascular calcification is via inhibition of VSMC apoptosis.

Non-phagocytosed apoptotic bodies are expected to either calcify or undergo secondary necrosis, releasing MGP. This may be particularly important in atherosclerotic plaques since MGP is deposited in calcified lesions [201]. Therefore, the accumulation of MGP in plaques could be due to apoptosis and a lack of phagocytosis. MGP may then be trapped due to the avascular, lipid-rich nature of the plaque. Alternatively, MGP may remain in plaques because it is modified or undercarboxylated [197].

Another potential fate of non-phagocytosed apoptotic bodies is migration to the blood vessel lumen. Vascular cell-derived apoptotic microparticles have been detected in circulating blood from normal individuals and are more abundant in patients with complicated atherosclerosis [128]. MGP levels are also elevated in serum from patients with severe atherosclerosis [17]. From these observations we can speculate that in the blood vessel or tissue where MGP mRNA is expressed, the protein is released from cells in the form of

apoptotic vesicles that travel through the matrix to the lumen. In calcified blood vessels, the vesicles may contain more MGP and/or there is an increase in production of these vesicles, either retained in the diseased tissue or released into the circulation. However, it remains to be investigated whether MGP is associated with apoptotic vesicles *in vivo*. At present, tissue factor is the only other protein known to be released from VSMCs in the form of apoptotic vesicles [129].

In conclusion, we have shown that MGP is an intracellular protein which is released via apoptotic bodies. MGP is a potent inhibitor of calcification *in vivo* but its mechanism of action remains unknown. Our study now highlights that MGP could have an intracellular function as well as an extracellular role associated with apoptotic vesicles.

chapter 9.1

discussion and summary

The posttranslational process of γ -glutamyl carboxylation is found in various species including drosophila [120, 243], suggesting early development of this process during evolution. After translocation of the precursor protein across the rough endoplasmic membrane, specific glutamic acid residues (Glu) are converted into γ -carboxy glutamic acid residues (Gla). In this process are involved: the transmembrane enzyme γ -glutamyl carboxylase which catalyses the reaction, vitamin K as a cofactor, and at least two known reductases for the recycling of vitamin K (Chapter 1, Figure 3.2). The resulting protein is called a Gla-protein or vitamin K-dependent protein.

Gla-proteins may be classified in three groups: I. Gla-proteins involved in blood haemostasis: prothrombin, coagulation factors VII, IX, and X, protein C, protein S, and protein Z. II: Gla-proteins involved in tissue calcification: matrix Gla protein (MGP) and osteocalcin (OC). And III: other Gla-containing proteins such as praline-rich Gla-proteins and Gas6. In all these proteins the presence of Gla-residues is a prerequisite in the Ca^{2+} -binding and/or Ca^{2+} -dependent interaction with negatively charged surfaces.

In 1929 the Danish scientist Henrik Dam described the first disorder due to a malfunction of the γ -glutamyl carboxylation process. Since then, several pathological symptoms resulting from inhibition or malfunction of one of the components involved in the γ -glutamyl carboxylation process have been described. Taking a close look at the carboxylation process (Chapter 1, Figure 3.2) several reasons may underlie the occurrence of non-carboxylated Gla-proteins and the subsequent (pathological) phenotype displayed. First, a blockade of vitamin K-reductase as a result of coumarin ingestion or a mutation may lead to impaired recycling of vitamin K and finally to an exhaustion of the available vitamin K stores. Second, a mutation in the Gla-protein may result in either poor substrate recognition by carboxylase [79] or in impairment of propeptide cleavage from the mature protein [251]. Third, the rare disorder of a mutation in the γ -glutamyl carboxylase gene leading to a defective enzyme. Two Lebanese patients with the same mutation in the γ -glutamyl carboxylase gene are described in Chapters 2.1 and 2.2. Both patients are the first offspring of consanguineous parents and are homozygous for a point mutation in exon 11 of the γ -glutamyl carboxylase gene. The mutation causes the conversion of a tryptophan codon (TGG) to a serine codon (TCG) at amino acid residue 501 of γ -glutamyl carboxylase (W501S). As a result of the mutation both hepatic and extra-hepatic Gla-proteins are affected (non-carboxylated) leading to a hereditary combined deficiency of all vitamin K-dependent procoagulants and anticoagulants. For rapid detection of the mutation a RFLP technique based on the presence of 3 BstNI sites in the unaffected allele and 2 BstNI sites in the affected allele was developed (Chapter 2.1). Using this screening technique the autosomal recessive pattern of inheritance of this disease was shown.

Hereditary combined deficiency of all vitamin K-dependent procoagulants and anticoagulants is a rare bleeding disorder reported only by a few authors [12, 19, 37, 95, 154]. Until now, only in two other cases mutations leading to a defective γ -glutamyl carboxylase enzyme have been reported [19, 209]. This suggests that most mutations in the γ -glutamyl carboxylase enzyme are not compatible with embryonic development and therefore are not detected. In other words, only mutations leading to a rather mild dysfunction of γ -glutamyl carboxylase are not lethal for the embryo. This is supported by the fact that in the two patients which we investigated oral vitamin K1 administration resulted in resolution of the clinical symptoms. Before birth, the vitamin K-dependent blood coagulation factors are synthesised at levels (ca. 20%) far below adult levels [158]. After birth, synthesis is increased and from then on higher vitamin K1 amounts are needed for complete γ -glutamyl carboxylation.

A second explanation for the effect of oral vitamin K1 administration is given by the molecular nature of the W501S mutation (Chapter 3). The impact of the W501S mutation on the activity of γ -glutamyl carboxylase was characterised using recombinant γ -glutamyl carboxylase expressed in insect cells. It was demonstrated that the mutation is most likely localised in the propeptide binding domain of carboxylase. The conversion of tryptophan residue 501 to serine results in a decreased affinity for substrate (propeptide) binding to γ -glutamyl carboxylase. For example, the blood coagulation factor IX derived substrate FIXproGla binds with an 80 fold higher K_m value to W501S-carboxylase than to wild type enzyme. Binding of a propeptide covalently linked to the Gla domain induces a substantial decrease in the K_m for vitamin KH_2 [88, 209]. It is possible that the decreased propeptide affinity of W501S-carboxylase negatively affects the K_m for the cofactor KH_2 . Given the low vitamin K1 levels in newborns, additional vitamin K1 supplementation leads to a KH_2 concentration above the K_m -value and thus to partial improvement of carboxylation.

In order to gain more insight in the role of MGP in vascular calcification, monoclonal antibodies (Chapter 4) and synthetic full length MGP (Chapter 5) were prepared. The two monoclonal antibodies described were raised against synthetic peptides homologous to the sequences 3-15 and 63-75 of human MGP and both antibodies recognize recombinant and synthetic human MGP. The two antibodies were used for immunohistochemical localisation of MGP in human calcified areas. MGP was associated with the extracellular matrix of non-calcified bone and with chondrocytes in cartilage. In the healthy human arterial vessel wall, MGP antigen was demonstrated in association with smooth muscle cells and elastic laminae of the tunica media, and with the extracellular matrix of the adventitia. The co-localization with the elastic laminae was lost at sites of medial calcification: in both human and

rat arteries, high amounts of MGP were found in the extracellular matrix at borders of intimal and medial calcification. Native MGP is difficult to isolate from human bones and due to solubility problems, processing of a recombinant MGP-fusion protein chimera to obtain MGP was unsuccessful. Therefore, in Chapter 5 we describe the total chemical synthesis of MGP by tBoc solid-phase peptide synthesis (SPPS) and native chemical ligation. Two peptides, Tyr¹-Ala⁵³ and Cys⁵⁴-Lys⁸⁴, were separately synthesised and subsequently coupled with native chemical ligation.

As described above, a blockade of vitamin K-reductase by coumarin inhibits γ -glutamyl carboxylation leading to undercarboxylated Gla-proteins. In Chapter 6 we describe an animal model in which the extra-hepatic γ -glutamyl carboxylase was inhibited using warfarin, whereas the hepatic enzyme remained unaffected. Using this model we demonstrated that, in contrast to vitamin K1, menaquinone-4 (MK-4) prevents warfarin-induced arterial calcification. Furthermore, we demonstrated both a better bio-availability and a more effective utilisation of MK-4 in the arterial vessel wall. Although both vitamin K1 and MK-4 have a 2-methyl-naphthoquinone group in common, and both function as a cofactor for γ -glutamyl carboxylase, our observations demonstrate a marked difference between both vitamins. These results are consistent with the strong inverse correlation found between vitamin K2 intake and artery calcification, myocardial infarction and total cardiac death [67]. Kawashima *et al.* showed that high menaquinone intake suppressed the progression of atherosclerotic plaques, intimal thickening and pulmonary atherosclerosis in hypercholesterolemic rabbits [99].

MGP is expressed by vascular smooth muscle cells (VSMCs) in the arterial vessel wall and thought to play a role in the inhibition of soft-tissue calcification. In Chapter 7 we added the synthetic full length MGP to calcifying human aortic VSMCs cultures. We showed that *in vitro* at a concentration of 500 ng/mL synthetic full length MGP inhibited VSMCs calcification. In this Chapter we also demonstrate a difference between vitamin K1 and menaquinone-4 on VSMCs nodule formation. Vitamin K1 had no effect, whereas MK-4 inhibited nodule formation by 50%. This inhibitory activity of MK-4 was not γ -glutamyl carboxylase dependent and therefore suggests a second, yet unknown, function for vitamin K2.

Various Japanese studies have demonstrated a strong decrease of bone loss and even an increase of bone mineral density by a high-dose MK-4 treatment (45 mg/day) [1, 151]. Remarkably, the used dose is much higher than can be understood from the regular function of vitamin K. Also, using cell and tissue culture experiments it was shown that: (a) among all the poly-isoprenoids known as vitamin K side chains, only geranylgeraniol was capable of

inhibiting osteoclast activity [81], and (b) MK-4 specifically induced apoptosis of osteoclasts in experimental model systems [97]. Together with the observed differences between vitamin K1 and K2 described in Chapter 6, this has led to a new hypothetical function for the geranylgeranyl side chain of MK-4. The reported inhibitory effect of MK-4 on bone loss is comparable to those obtained with bisphosphonate treatment. Like bone loss, also vascular media calcification can be completely abolished by both bisphosphonate treatment [163], and by high-dose MK-4 treatment (Chapter 6), but not by treatment with K1. Bisphosphonates are well known inhibitors of osteoclastic bone resorption. Nitrogen-containing bisphosphonates are specific inhibitors of the enzyme farnesyl-pyrophosphate synthase and in bone this leads to reduced production of the compounds farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate [186]. The latter compounds are needed for the prenylation of signal-transducing proteins such as rho, rac, and ras, which stimulate osteoclast activity and bone resorption [238]. In this way it can be understood that bisphosphonates lead to disturbed signal transduction, followed by loss of cellular organization and leading to inactivation of the osteoclasts and their subsequent apoptosis. The function of the geranylgeranyl side chain in MK-4 would thus be that it serves as a competitive inhibitor in the prenylation of signal transduction proteins, in such a way that the naphthoquinone group in MK-4 could block either the phosphorylation of farnesyl and geranylgeranyl or the protein coupling to farnesyl- and geranylgeranyl-pyrophosphate. As a consequence, high doses of MK-4 may bind to and thus saturate the geranylgeranyl-binding sites in the prenylating enzyme system, which makes clear why high doses of MK-4 are required for *in vivo* effects comparable with those obtained with bisphosphonates.

The association of MGP with the extracellular matrix, the presence of a predicted signal-peptide, and Gla-residues within its N-terminal domain suggests it to be an extracellular protein. In Chapter 8 we show an intracellular localization of MGP in cultured human aortic VSMCs, human breast cancer epithelial cells (T47D), and human osteoblastic osteosarcoma cells (MG-63). These data are in contrast with the extracellular location of MGP *in vivo* and indicates that the protein must be secreted via a presently unknown mechanism by cells. We found that VSMCs undergoing apoptosis released apoptotic bodies which contained MGP. In addition, in normal cell cultures which expressed MGP, numerous small MGP containing vesicular structures could be detected. Therefore, apoptotic body or vesicle release could facilitate MGP export from cells.

During the past decade it became clear that vitamin K-dependent proteins are not only involved in the blood coagulation process. An example of such a protein is the matrix Gla protein, which has an important role in the prevention of soft tissue calcification. Together with the proposed second, yet

unknown, function of menaquinone-4 this makes the scientific field of γ -glutamyl carboxylation, vitamin K, and matrix Gla protein of potential relevance in cardiovascular research.

chapter 9.2

discussie en samenvatting

De γ -glutamylcarboxylering van specifieke glutaminezuurresiduen is een posttranslationele modificatie die plaats vindt bij verschillende diersoorten. De aanwezigheid van het enzym γ -glutamyl carboxylase in de fruitvlieg suggereert een vroege ontwikkeling van het proces gedurende de evolutie [120, 243]. Nadat een immatuur eiwit over de membraan van het ruw endoplasmatische reticulum is getransporteerd, worden specifieke glutaminezuurresiduen omgezet in γ -carboxyglutaminezuren (Gla). Bij deze omzetting zijn de volgende componenten betrokken: het transmembraan enzym γ -glutamyl carboxylase welk zorgt voor katalysatie van de reactie, vitamine K als co-factor, ten minste twee reductases voor de recycling van vitamine K, en het te carboxyleren eiwit (Hoofdstuk 1, Figuur 3.2). Dit substraat eiwit wordt een Gla-eiwit of een vitamine K-afhankelijk eiwit genoemd.

Gla-eiwitten kunnen in drie groepen worden onderverdeeld: I. Gla-eiwitten betrokken bij bloedstolling: prothrombine, factoren VII, IX en X, proteïne C, proteïne S en proteïne Z. II: Gla-eiwitten betrokken bij verkalking van weefsels: matrix Gla proteïne (MGP) en osteocalcine (OC). En III: andere Gla-eiwitten zoals de proline-rijke Gla-eiwitten en Gas6. Bij al deze eiwitten is de aanwezigheid van Gla-residuen een vereiste voor de binding van calcium-ionen (Ca^{2+}) en/of de calcium-afhankelijke interactie met negatief geladen oppervlakken.

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De Deense wetenschapper Henrik Dam beschreef in 1929 als eerste een aandoening die werd veroorzaakt door een slecht werkend carboxylase proces. Sindsdien zijn verscheidene pathologische symptomen beschreven welke het gevolg zijn van remming of slecht functioneren van componenten betrokken bij de γ -glutamyl carboxylase reactie. Kijken we naar het carboxylerings proces (Hoofdstuk 1, Figuur 3.2) dan wordt duidelijk dat diverse oorzaken kunnen leiden tot de aanmaak van niet gecarboxyleerde Gla-eiwitten en het daaropvolgende (pathologische) fenotype. Als eerste, remming van het vitamine K-reductase als gevolg van coumarine inname of een mutatie kan resulteren in een slecht hergebruik van vitamine K en uiteindelijk tot uitputting van de beschikbare vitamine K voorraad. Als tweede, een mutatie in het Gla-eiwit kan leiden tot een slechte herkenning van het substraat door het enzym γ -glutamyl carboxylase [79] of tot het niet verwijderen van het propeptide van het uiteindelijke eiwit [251]. Als derde, een mutatie in het γ -glutamyl carboxylase gen kan leiden tot een slecht werkzaam enzym. Dit laatste is echter een zeldzame aandoening. In de hoofdstukken 2.1 en 2.2 worden twee patienten beschreven met dezelfde mutatie in het γ -glutamyl carboxylase gen. Beide patienten zijn de eerste nakomelingen van bloedverwante ouders en zijn homozygoot voor een punt mutatie in exon 11 van het γ -glutamyl carboxylase gen. De mutatie veroorzaakt de omzetting van een tryptofaan codon (TGG) naar een serine codon (TCG) van aminozuur residu 501 (W501S) in γ -glutamyl carboxylase.

Als gevolg van deze mutatie zijn zowel de hepatische als extra-hepatische Gla-eiwitten niet gecarboxyleerd, hetgeen leidt tot een erfelijke gecombineerde deficiëntie van alle vitamine K-afhankelijke eiwitten. Voor een snelle detectie van de mutatie werd een RFLP-techniek opgezet, op basis van drie aanwezige BstNI restrictie enzym herkenningsplaatsen in het onaangedane allel en twee BstNI plaatsen in het aangedane allel (Hoofdstuk 2.1). Met behulp van deze methode werd het autosomale recessieve overervingpatroon van deze ziekte aangetoond.

Erfelijke gecombineerde deficiëntie van alle vitamine K-afhankelijke eiwitten is een zeldzaam voorkomende bloedingaandoening, waarvan maar door enkele auteurs melding wordt gemaakt [12, 19, 37, 95, 154]. Tot nu toe zijn maar twee patiënten bekend waarbij een mutatie resulteert in een defect γ -glutamyl carboxylase enzym [19, 209]. Dit suggereert dat de meeste mutaties in het γ -glutamyl carboxylase enzym reeds in de embryonale fase tot de dood leiden en dien ten gevolge niet gevonden worden. Met andere woorden, alleen mutaties welke leiden tot een milde disfunctie van γ -glutamyl carboxylase zijn niet lethaal voor het embryo. De bevinding dat orale vitamine K toediening in de hier onderzochte patiënten resulteerde in herstel van de klinische symptomen, ondersteunt dit. Voor de geboorte worden de vitamine K-afhankelijke stollingseiwitten aangemaakt op een niveau (ca. 20%) ver beneden het niveau gevonden bij volwassenen [158]. Na de geboorte neemt de synthese toe en is een hogere vitamine K inname nodig voor een volledige γ -glutamyl carboxylering.

Een tweede oorzaak voor het effect van orale vitamine K toediening werd gevonden in de moleculaire achtergrond van de W501S mutatie (Hoofdstuk 3). Recombinant γ -glutamyl carboxylase werd tot expressie gebracht in insect cellen om het effect van de W501S mutatie op de enzymatische functie te onderzoeken. Er werd aangetoond dat de mutatie waarschijnlijk in het propeptide bindende gedeelte van γ -glutamyl carboxylase zit. De conversie van tryptopfaan residu 501 tot een serine leidt tot een afgenomen herkenning en binding van het substraat (propeptide) aan γ -glutamyl carboxylase. Bijvoorbeeld, het van bloedstollingsfactor IX afgeleide substraat FIXproGla bindt 80 maal slechter aan W501S γ -glutamyl carboxylase dan aan het wildtype enzym. Het is aangetoond dat een propetide welk covalent gekoppeld is aan een Gla-domein een substantiële afname in de K_m voor KH_2 induceert [88, 209]. Het is daarom omgekeerd ook mogelijk dat de verlaagde affiniteit voor een propeptide, veroorzaakt door de W501S mutatie, de K_m voor cofactor KH_2 negatief beïnvloed. Om meer inzicht te verkrijgen in de rol van MGP in vasculaire verkalking, werden monoklonale antilichamen (Hoofdstuk 4) en synthetisch MGP (Hoofdstuk 5) gemaakt. De twee monoklonale antilichamen die hier worden beschreven werden opgewekt tegen synthetische peptiden homoloog aan de

sequenties 3-15 en 63-75 van humaan MGP en herkennen beiden zowel recombinant als synthetisch MGP. De twee antilichamen werden gebruikt voor immunohistochemische lokalisering van MGP in humane verkalkte gebieden. MGP is geassocieerd met de extracellulaire matrix van niet verkalkt bot en met chondrocyten in kraakbeen. In de gezonde humane arteriële vaatwand werd MGP antigeen aangetoond in associatie met gladde spier cellen en de elastische lamina van de tunica media en met de extracellulaire matrix van de adventitia. De co-lokalisatie met de elastische lamina ging verloren in gebieden van mediale verkalking. In zowel humane als rat arteriën werden grote hoeveelheden MGP gevonden in de extracellulaire matrix aan de grensvlakken van intima en mediale verkalking. Natuurlijk voorkomend MGP is moeilijk te isoleren uit humaan bot en door oplosbaarheids problemen was de verwerking van recombinant chimeer MGP niet succesvol. Daarom wordt in Hoofdstuk 5 de totale chemische synthese van MGP door middel van tBoc solid-phase peptide synthesis (SPPS) en native chemical ligation beschreven. De twee peptiden, Tyr¹-Ala⁵³ en Cys⁵⁴-Lys⁸⁴, werden afzonderlijk gemaakt en vervolgens gekoppeld door middel van native chemical ligation.

Zoals hierboven beschreven leidt een blokkade van het vitamine K-reductase door coumarine tot remming van de γ -glutamyl carboxylering en zo tot niet gecarboxyleerde Gla-eiwitten. In hoofdstuk 6 wordt een dier model beschreven waarin het extrahepatische γ -glutamyl carboxylase werd geremd door middel van warfarine, terwijl het hepatische enzym niet werd beïnvloed. Met behulp van dit model werd aangetoond dat menaquinone-4 (MK-4), in tegenstelling tot vitamine K1, de warfarine geïnduceerde arteriële verkalking voorkomt. Daarnaast werd een betere biologische beschikbaarheid en een meer efficiënt gebruik van MK-4 door de arteriële vaatwand aangetoond. Hoewel vitamine K1 en MK-4 beiden een 2-methylnaphtoquinone groep hebben en beiden fungeren als co-factor voor γ -glutamyl carboxylase, tonen deze resultaten een opmerkelijk verschil aan tussen beide vitaminen. De hier gepresenteerde resultaten zijn in overeenstemming met de gevonden sterke inverse correlatie tussen vitamine K2 inname en respectievelijk arteriële verkalking, het risico op een hart aanval en totale cardiale dood [67]. Deze bevindingen worden ondersteund door de resultaten van Kawashima *et al.* Zij hebben aangetoond dat een hoge menaquinone inname de ontwikkeling van atherosclerotische plaques onderdrukt in hypercholesterolemische konijnen [99].

MGP wordt tot expressie gebracht door vasculaire gladde spier cellen (VSMCs) in de arteriële vaatwand en speelt een belangrijke rol in de remming van verkalking in zachte weefsels. In hoofdstuk 7 wordt de toevoeging van synthetisch MGP aan verkalkende humane aorta VSMCs culturen beschreven. Er werd aangetoond dat in dit *in vitro* systeem een synthetische

MGP concentratie van 500 ng/ml de verkalking van VSMCs remt. Met dit model werd ook aangetoond dat vitamine K1 en MK-4 een verschillend effect hadden op de vorming van VSMCs nodules. Vitamine K1 had geen effect, terwijl MK-4 de vorming van nodules met 50% remde. Deze remmende activiteit van MK-4 was niet afhankelijk van γ -glutamyl carboxylase en dit suggereert een tweede, tot op heden nog niet bekende, functie voor MK-4.

Verscheidende Japanse studies tonen een sterk verminderd botverlies en zelfs een toename in botdichtheid aan bij inname van een hoge dosis MK-4 (45 mg/dag) [1, 151]. Opmerkelijk was dat de optimale MK-4 dosis veel hoger is dan kan worden verwacht op basis van een rol als co-factor voor γ -glutamyl carboxylase. Door gebruik te maken van cel en weefsel cultuur experimenten werd aangetoond dat: (a) van alle poly-isoprenoiden bekend als vitamine K zijketens alleen geranylgeraniol osteoclast activiteit remt [81], en (b) MK-4 specifiek apoptose induceert in osteoclasten [97]. Samen met de resultaten beschreven in Hoofdstuk 6 heeft dit geleid tot een nieuwe hypothetische functie voor de geranylgeranyl zijketen van MK-4.

Deze gemelde remmende effecten van MK-4 op bot verlies zijn vergelijkbaar met de resultaten verkregen uit studies met bisfosfonaat behandeling. Daarnaast kan mediale verkalking evenals botverlies worden tegengegaan door behandeling met bisfosfonaten [163] of een hoge dosis MK-4 (Hoofdstuk 6), maar niet door behandeling met vitamine K1. Bisfosfonaten zijn bekende remmers van osteoclastische bot resorptie. Stikstof bevattende bisfosfonaten zijn specifieke remmers van het enzym farnesyl-pyrofosfaat synthase en dit leidt in bot tot een gereduceerde productie van de componenten farnesyl-pyrofosfaat en geranylgeranyl-pyrofosfaat [186]. Deze laatste component is nodig voor de prenylering van signaal overbrengende eiwitten zoals rho, rac en ras welke de osteoclast activiteit en bot resorptie stimuleren [238]. Op deze manier zorgen bisfosfonaten voor een verstoorde signaal overdracht, gevolgd door inactiviteit en apoptose van de osteoclasten. In dit systeem zal de geranylgeranyl zijketen van MK-4 fungeren als een competitieve remmer van de prenylering van signaal overbrengende eiwitten. De naphthoquinone groep van MK-4 kan de fosforylering van farnesyl en geranylgeranyl blokkeren of de eiwit koppeling aan farnesyl- en geranylgeranyl-pyrofosfaat. Als een gevolg hiervan kan een hoge dosis MK-4 binden aan de geranylgeranyl-bindende plaatsen in het prenylerings enzym systeem en deze dus verzadigen. Dit maakt ook meteen duidelijk waarom een hoge dosis MK-4 nodig is voor *in vivo* effecten, vergelijkbaar met die verkregen met bisfosfonaten.

De associatie van MGP met de extracellulaire matrix, de aanwezigheid van een voorspeld signaal-peptide en de Gla-residuen in zijn N-terminus, suggereren dat MGP een extracellulair eiwit is. In Hoofdstuk 8 laten we een

intracellulaire lokalisatie zien voor MGP in een humane aorta VSMCs cultuur, een humane borstkanker epitheel cel (T47D) cultuur en een humane osteosarcoma cel (MG-63) cultuur. Deze gegevens staan in contrast tot de extracellulaire lokalisatie van MGP *in vivo* en suggereren dat het eiwit via een tot nu toe onbekend mechanisme door de cellen wordt uitgescheiden. We vonden dat VSMCs in apoptose MGP bevattende apoptotische lichaampjes vrijgeven. Tevens werden in normale cel culturen, welke MGP tot expressie brengen, membraan blaasjes gedetecteerd met daarin MGP. Apoptotische lichaampjes of membraan blaasjes kunnen dus de afgifte van MGP door cellen verzorgen.

Gedurende de laatste decennia wordt het steeds duidelijker dat vitamine K-afhankelijke eiwitten niet alleen betrokken zijn bij de bloedstolling, maar ook een belangrijke rol spelen in andere processen. Een voorbeeld van zo een vitamine K-afhankelijk eiwit is het matrix Gla proteïne, dat betrokken is bij het voorkomen van zacht weefsel verkalking. Samen met de voorgestelde tweede functie van menaquinone-4 maakt dit het wetenschappelijke veld van γ -glutamyl carboxylering, vitamine K en matrix Gla proteïne van potentieel belang in cardiovasculair onderzoek.

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“Sommige mensen wachten hun hele leven op applaus van de wereld en pas vlak voor hun dood komen ze tot de ontdekking dat ze voor een lege zaal hebben staan spelen”

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