Expression and function of matrix Gla protein in human peritoneal mesothelial cells

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Abstract

Background. Chronic peritoneal dialysis (PD) is associated with peritoneal calcification. Studies in vascular tissue suggest that ectopic calcification is not merely a passive but a regulated process resembling bone mineralization. We investigated whether peritoneal calcification is controlled by matrix Gla protein (MGP) secreted by peritoneal mesothelial cells.

Methods. Human primary mesothelial cells (HPMC) were exposed to constituents of PD fluids and to cytokines relevant to peritoneal integrity. Messenger RNA was quantitated by real-time reverse transcription polymerase chain reaction (RT-PCR), protein abundance by Western blot and in vivo protein expression immunohistochemically. To demonstrate functional relevance, MGP was silenced in HPMC by siRNA transfection and calcium phosphate matrix deposition measured by o-cresolphthalein complexone method and von Kossa staining.

Results. MGP was consistently detected in the mesothelial cell layer of peritoneal tissue specimens from uraemic and non-uraemic patients, in HPMC and in culture medium. MGP mRNA and protein abundance was increased by glucose and IGF1 and decreased by TGFß1. Suppression of MGP increased matrix calcium and phosphorus deposition by 90 ± 6% and 100 ± 4% at 1 mM ambient Ca2+ and phosphorus concentration. Deposition was not increased any further by higher medium Ca2+/phosphorus concentrations nor reduced by inhibition of the phosphate cotransporter Pit1.

Conclusion. MGP is expressed by HPMC and regulated by glucose, IGF1 and TGFß1. It is a potent inhibitor of calcification in vitro and may thus play a role in the regulation of peritoneal calcium homeostasis.

Keywords: glucose; IGF-1; MGP; peritoneal calcification; peritoneal dialysis

Introduction

Limited technique survival remains the major drawback of chronic peritoneal dialysis (PD). While the incidence of severe bacterial inflammation is steadily decreasing due to continued technical improvements, loss of ultrafiltration due to peritoneal sclerosis and neoangiogenesis is becoming the predominant cause of PD failure. In addition, peritoneal calcifications, appearing as diffuse or patchy depositions in the submesothelial interstitial compartment, are commonly observed in patients on long-term PD and are an obligatory feature of peritoneal sclerosis [1–4]. The cellular and physicochemical mechanisms underlying these peritoneal calcifications are only beginning to be understood. Factors such as hypercalcaemia, hyperphosphataemia, severe hyperparathyroidism, supraphysiological dialysate calcium concentration and recurrent or persistent peritoneal inflammation are involved but cannot explain the large individual variation of the process [1–7].

Recent studies have revealed that ectopic, i.e. extraosseous calcification is not merely a passive but an active, regulated biological process [8,9]. Local tissue mineralization is controlled by the release of certain matrix proteins from resident cells. Abnormal expression or bioactivity of some of these proteins, either due to genetic constitution or in response to pathogenic conditions such as local or systemic inflammation, may determine the individual rate of tissue calcification [9,10]. Matrix Gla protein (MGP) plays a crucial role in tissue calcium homeostasis. The 84-amino-acid protein is expressed in cartilage and vascular smooth muscle cells (VSMC) and binds calcium ions or calcium crystals to vitamin-K-dependent carboxylated Gla residues [8]. In addition, MGP binds BMP-2 and thus prevents an osteogenic gene expression profile in VSMC [11]. MGP knockout results in severe cartilage and arterial calcifications and premature death from arterial rupture [12].
Here we demonstrate expression and secretion of MGP in human peritoneal mesothelial cells (HPMC), which is modulated by cytokines, growth factors and supraphysiological glucose concentrations and controls mineral deposition in the perimesothelial matrix of cultured HPMC.

Materials and methods

Materials

Medium 199 was purchased from Biochrom AG (Berlin, Germany), Opti-MEM I reduced serum medium and oligofectamine from Invitrogen (Karlsruhe, Germany). Reagents used for quantitative reverse transcription polymerase chain reaction (RT-PCR) were obtained from Applied Biosystems (Darmstadt, Germany), the monoclonal antibody against phosphorylated and carboxylated MGP from Vita K (Maastricht, Netherlands), the β-actin monoclonal antibody from Abcam (Cambridge, UK) and those against cytokeratin and vimentin monoclonal antibodies from DAKO (Denmark). Basic fibroblast growth factor (bFGF, Roche, Penzberg, Germany), insulin-like growth factor-1 (IGF-1, Calbiochem, Darmstadt, Germany), transforming growth factor-β1 (TGF-β1, R&D Systems, Wiesbaden, Germany), insulin-like growth factor-1 (IGF-1, Calbiochem, Darmstadt, Germany), basic fibroblast growth factor (VEGF, Cell sciences, Canton, MA, USA) were used for analysis of cytokine dependent effects. Unless otherwise stated, all other substances were purchased from Sigma (Taufkirchen, Germany). AGE albumin was generated by incubation of bovine serum albumin with 100 mM phosphate, 200 mM glucose-6-phosphate and 0.5 mM sodium azide at pH 7.4 and 37°C for 6 weeks. Then the AGE albumin preparation was dialysed against 5 L of 100 mM phosphate and 10 mM of EDTA for 24 h and against 0.9 NaCl 0.9% for 12 h. The AGE preparation was subsequently lyased against 5 L of 100 mM phosphate and 10 mM of EDTA for 24 h and stored at pH 7.4 for up to 5 days.

Cytotoxicity analysis (lactate dehydrogenase assay)

For exposure of HPMC cultures to the indicated test media, supernatants were collected and lactate dehydrogenase (LDH) levels measured by a Dimension RxL clinical chemistry analyzer (Dade-Behring, Schwalbach, Germany).

Real-time RT-PCR

RNA was isolated using TRI Reagent (Sigma, Germany) according to the manufacturer's directions, checked for integrity on an agarose gel and quantified photometrically. Total RNA (1 µg) was reverse-transcribed using oligo-dT/random hexamer primers (10:1) at 42°C for 1 h. Total real-time RT-PCR was performed using the ABI Prism 7000 Real-Time PCR system (Applied Biosystems, Darmstadt, Germany) by mixing cDNA with 2× SYBR green PCR Master Mix, Primers and Milliq H2O to a reaction volume of 25 µL and reaction in 40 cycles of 95°C for 15 s and 60°C for 1 min. Genes were amplified by the following primers: MGP (F: AAGACGAAAACCATGAAAGAC, R: CGTTTCTCGGATCCTCTCTTG); 18S (F: AGTTGGTGGAGCGATTTGTC, R: CCGACATCTAAGGGCATT-CAC).

Western immunoblotting

After incubation of HPMC cultures with the substances indicated, cells were lysed in ice-cold lysis buffer and cell extracts treated as described previously [14]. The protein content was measured with Bio-Rad protein assay (Munich, Germany). Total membrane fractions were prepared as described previously [15]. Culture supernatants were concentrated by incubating with desoxycholate acid for 10 min at room temperature (RT), followed by vortexing and centrifugation for 15 min at 5000 rpm after addition of trichloroaetic acid. Pellets were resuspended with 5% sodium dodecyl sulfate (SDS)/0.1 M NaOH and used immediately. Total cell lysate (25 µg protein), membrane preparations or concentrated supernatants were separated on 15% SDS-polyacrylamide gels along with precast molecular standards at 200 V and blotted to polyvinylidene difluoride (PVDF) membrane (Millipore) at 100 V for 90 min, then exposed sequentially to the primary serMGP antibody and horseradish peroxidase-conjugated secondary antibody as described previously [14]. The blots were reexposed to β-actin antibody as a loading control.

Immunocytochemistry, immunohistochemistry

After fixing with cool methanol, cells were permeabilized and blocked for 1 h with fish blocking solution [2% fetal bovine serum (FBS), 2% BSA, 0.2% fish gelatine in PBS], then incubated sequentially with different primary anti-MGP antibodies (1:200) overnight at 4°C and FITC-coupled secondary antibodies (Alexa 488 goat anti-mouse IgG) (1:1000) at RT for 1 h. Thereafter, nuclei were counterstained with bisbenzimide for 2 min and were mounted with Mowiol mounting medium (Calbiochem, Schwalbach, Germany). The localization of proteins was reconfirmed and photographed with a fluorescent microscope (Leica, Germany).

Peritoneal biopsy specimens were obtained from 10 non-uraemic patients undergoing elective abdominal surgery and eight ureamic patients on peritoneal dialysis undergoing tenkoff catheter revision and renal transplantation, respectively. Immunostaining of human omentum was performed by the paraformaldehyde-saponin procedure (PSP) as previously described by Autschbach et al. [16] using two different anti-MGP antibodies (ser MGP 1:40, cMGP 1:100).

siRNA transfection

For transient transfection studies, cells were divided into six-well tissue culture plates and cultured overnight in normal growth medium. When the cells reached 70–80% confluence, transfections were performed using
siRNA against MGP (sense: GGA UCC GAG AAC GCU CUA A99, antisense: UUA GAG CGU UCU CGG AUC C99, designed by Eurogentech, Cologne, Germany) by a liposome technique using Oligofectamine. According to the manufacturer’s instructions, cells were incubated in Opti-MEM I reduced serum medium containing lipid-siRNA complexes and supplemented with 20% FCS 4 h after transfection. The transfection

Fig. 2. (a) Expression of MGP mRNA in HPMC transfected with MGP siRNA, scrambled siRNA and Oligofectamine alone. Transfection was performed during 3 days, mRNA expression monitored for a total of 8 days (b). Corresponding MGP protein abundance as assessed by Western blotting. A representative example and the densitometric quantification of three independent experiments are given. *P < 0.05.
efficiency was investigated by measuring MGP mRNA and protein abundance and compared to untreated cells.

Calcium and phosphate deposition assay and von Kossa staining
Calcification was assessed by a modification of the o-cresolphthalein complexone method as described previously [17]. Briefly, cells were decalcified with 0.6 N HCl for 24 h. The calcium and phosphate concentrations in the HCl supernatant were determined colorimetrically with a clinical chemistry analyzer. After decalcification, the cells were washed with phosphate-buffered saline and solubilized with 0.1 N NaOH/0.1% SDS. The protein concentration in dissolved cells was measured by Bio-Rad protein assay. The calcium and phosphate content of the cell layer was normalized by protein content.

Mineral deposition was assessed by von Kossa staining. Cells were fixed with cool pure methanol and then treated with 5% silver nitrate for 30 min at room temperature in darkness to detect the calcium deposition.

Fig. 3. Calcium phosphate deposition in HPMC culture with MGP knock-down. After transfection of MGP siRNA and random siRNA, HPMC were incubated with different calcium and phosphate concentrations for a total of 5 days. Calcium deposition in HPMC cultures was visualized by von Kossa staining and phase-contrast micrography (a). Calcium phosphate precipitates were quantified by the o-cresolphthalein complexone method and standardized to the culture protein content. C, control; T, HPMC transfected with MGP siRNA. *P < 0.05.

Statistical analysis
All data represent means of duplicate samples from at least three separate experiments. Data are presented as mean ± SEM. Differences between control and MGP siRNA groups were compared using the two-tailed Student's t-test. A P-value of < 0.05 was considered statistically significant.
Results

MGP expression in cultured HPMC and human peritoneal biopsy specimens

MGP mRNA was strongly expressed by HPMC cells in culture. To investigate the localization of MGP protein in the peritoneum, we performed immunohistochemistry of human peritoneal tissue sections, immunocytochemistry of HPMC monolayers and immunoblotting of subcellular HPMC protein fractions and cell supernatant. MGP protein was abundant in the mesothelial cell layer. Moreover, MGP was detected in vessels in the submesothelial interstitium (Figure 1a). MGP could to a similar extent be detected in the peritoneal mesothelial cells and in the vessels of uraemic patients (Figure 1b). MGP was localized both in the cytoplasm and in membrane fractions of HPMC and could also be recovered from concentrated culture supernatants, suggesting active secretion of MGP by HPMC (Figure 1c and d).

MGP regulates extracellular calcium deposition in HPMC

To assess the effects of mesothelial MGP on local calcium homeostasis, MGP expression was selectively silenced by transfection of siRNA. MGP mRNA expression was almost completely abolished (to 2% of baseline) within 3 days of transfection of baseline values. After removal of the transfection solution, MGP gene expression slowly recovered but was still reduced by 32% 5 days after the transfection process was stopped. MGP protein levels were reduced by 73 ± 6% and 80 ± 11% by Days 2 and 3 of transfection, respectively (Figure 2a and b). Treatment with scrambled siRNA and Oligofectamine alone did not result in significant modification of MGP mRNA and protein abundance.

Following these validation experiments, cells were transfected with MGP siRNA for 2 days followed by replacement and supplementation of medium with CaCl₂ and H₃PO₄ to achieve 1–1.5 mM ionized calcium and 1.0–2.0 mM phosphate concentrations. After another 3 days of incubation, the degree of calcification was visualized by von Kossa staining and quantitated by measuring precipitated calcium and phosphate using the o-cresolphthalein complexone method. As illustrated in Figure 3a, marked extracellular calcifications developed in knock-down HPMC cultures already at calcium and phosphate concentrations of 1.0 mmol/L. The calcium content normalized to protein content was increased on average by 90 ± 6% and the phosphate content by 100 ± 4% compared to non-transfected HPMC cultures and did not further increase with increasing medium calcium and phosphate concentrations (Figure 3b). Addition of phosphonoformic acid, an inhibitor of the Na⁺-dependent phosphate cotransporter (Pit1), did not reduce tissue calcium deposition in MGP-silenced HPMC cultures at low and high calcium and phosphate concentrations.

Regulation of MGP mRNA and protein expression in HPMC by glucose

The continuous peritoneal exposure to high glucose concentrations is a major factor contributing to the degenerative changes in the peritoneal membrane observed during
chronic PD. The time-dependent effects of glucose on MGP expression were assessed by exposure of HPMC to physiological (0.1%) and typical dialysate concentrations of glucose (2.5 and 4.2%). Within 36 h MGP, mRNA increased to 189 ± 19% and 236 ± 21% and MGP protein to 153 ± 11% and 171 ± 26% with 2.5 and 4.2% glucose as compared to respective controls (P < 0.05, Figure 4). The exposure to high-dose glucose did not exert any detectable cytotoxic effects as judged from the phenotypic appearance of the HPMC and from supernatant LDH concentrations. Also, incubation with isoosmolar D-mannitol, with albumin-derived AGE (500–2000nM for 6 h to 5 days) and with lactate had no effect on MGP expression.

**Effect of growth factors on MGP expression**

HPMC cultured in serum-free medium were exposed to IGF-1, bFGF, VEGF (each 100 ng/mL) and TGF-ß1 (5 ng/mL) in serum-free medium for 24 h. As shown in Figure 5, IGF-1 increased MGP mRNA and protein levels to 161 ± 10% and 132 ± 14% (P < 0.01/0.05), whereas TGF-ß1 decreased MGP mRNA levels to 42 ± 9% and protein levels to 72 ± 8% (both P < 0.01). VEGF and bFGF had no consistent effect. In the presence of high glucose concentrations, TGF-ß was upregulated (148 ± 23% and 193 ± 4% with 2.5 and 4.2% of glucose, P < 0.05). The effect of glucose on MGP, however, was nearly 2-fold
stronger (284 ± 52% and 356 ± 50% with 2.5 and 4.2% of glucose, P < 0.05 compared to TGF-β effect).

Discussion

Peritoneal calcifications are a regular feature of chronic PD, induced by exposure to high calcium and phosphate concentrations and by recurrent or persistent local inflammation. In severe cases, peritoneal calcium deposition results in encapsulating peritoneal sclerosis, a severe and life-threatening disorder. In the present study, we demonstrate that MGP is expressed in human peritoneal mesothelial cells and acts as a functionally important regulator of perimesothelial calcium phosphate precipitation.

Extracellular calcium and phosphate concentrations in healthy humans are sufficiently high to seed crystal, yet widespread tissue calcification does not occur due to complexing of calcium to specific inhibitors such as MGP. We were able to demonstrate MGP expression by human mesothelial cells in vitro and in biopsy specimens from uraemic patients and healthy controls. MGP was abundant in the mesothelial cytoplasm and membrane fractions, similar to vascular smooth muscle cells [18] and chondrocytes [19]. Moreover, MGP was present in the supernatant of cultured cells, indicating active secretion.

Transient knock-down of MGP in human peritoneal cells resulted in rapid extracellular calcium phosphate precipitation even at low physiological ambient calcium and phosphorus concentrations. Hence, our in vitro findings suggest that peritoneal calcification does not simply result from chemical precipitation of minerals but represents a regulated biological process involving a similar molecular mechanism as involved in vascular calcification [20]. To some degree, the pattern of regulation appears to be tissue specific. For instance, we found a slight upregulation of MGP expression by IGF-1, whereas an inhibitory effect of IGF-1 was found in chondrocytes [21], a cell type in which IGF-1-induced hypertrophy is physiologically associated with increasing extracellular calcification. Also, in cultured VSMC, extracellular calcification depends on an increase in intracellular calcium and phosphate and subsequent release of matrix vesicles [17,22]. By contrast, inhibition of the phosphate transporter PIT-1 in our experiments did not affect the marked extracellular calcification that occurred in MGP-silenced mesothelial cells, compatible with a partially different mechanism of extracellular crystal formation.

MGP is involved in the uptake of fetuin [18] and inhibits BMP-2-dependent mesenchymal cell differentiation [11] and chondrocyte maturation and thus calcification [23]. We also detected BMP-2 in human peritoneal mesothelial cells (data not shown), compatible with the existence of a similar cross-talk in the peritoneal tissue, which may also include MGP derived from peritoneal vessels [24]. This, however, needs to be demonstrated.

Our in vitro findings do not necessarily reflect the much more complex in vivo situation. Biopsy data moreover demonstrated progressive loss of the mesothelial cell layer with time on PD; thus, the protective role of MGP may be lost and thus its protective action. This, however, may be different with patients using biocompatible PD solution. Continuous peritoneal dialysis (CPD)-associated peritoneal sclerosis involves several pathogenic mechanisms. The high glucose concentration of peritoneal dialysis solutions is detrimental to the peritoneal membrane by leading to neovascularization, excessive accumulation of extracellular matrix and ultimately peritoneal fibrosis [13,25]. Furthermore, there is evidence that the use of high dialysate glucose concentrations is associated with peritoneal calcification in long-term PD [6]. For this reason, we incubated HPMC cells with different glucose concentrations. In contrast to our expectation, glucose induced a consistent time- and dose-dependent upregulation of MGP expression both on the mRNA and the protein level. In contrast to this direct effect of high-dose glucose, TGF-β1, a crucial mediator of glucose-induced injury to HPMC [26], suppressed MGP expression in accordance with findings in VSMC [27]. As suggested from previous studies, we demonstrate glucose-induced upregulation of TGF-β expression, which has a suppressive effect on MGP. The stimulatory effect of glucose on MGP, however, was 2-fold higher and thus predominates over the TGF-β effect on MGP expression. The observed upregulation of MGP expression by high glucose concentrations might be interpreted as an intrinsic regulatory mechanism to prevent calcification events triggered by other glucose-mediated, pro-precipitative effects. Moreover, it is tempting to speculate that the stimulation of MGP release may mediate calcification-independent adverse effects of high-dose glucose in the physiopathological process of peritoneal membrane degeneration in long-term PD. Of note, MGP has been demonstrated to induce endothelial VEGF and neoangiogenesis [28], a key process leading to ultrafiltration failure in PD. To elucidate the ultimate impact of MGP on peritoneal membranes on patients chronically exposed to PD solutions, respective experimental in vivo models are required.

Other components of PD solutions which have been implicated in peritoneal membrane transformation, such as osmolality per se, lactate and advanced glycated end products had no effect on mesothelial MGP expression.

In summary, MGP expression by the mesothelial cell layer may be an important physiological mechanism countering peritoneal membrane calcification promoted by high calcium, phosphate and parathormone (PTH) concentrations and by recurrent or persistent peritoneal inflammation. Since many patients on chronic PD suffer from progressive peritoneal calcifications, MGP represents an interesting target for intervention studies in animal models of chronic PD and subsequent clinical trials.

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Conflict of interest statement. None declared.

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