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The local calcification inhibitor matrix Gla protein in pseudoxanthoma elasticum

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Abstract

Objectives: Recent studies have revealed the involvement of calcification inhibitory proteins in the pathogenesis of pseudoxanthoma elasticum (PXE).

Design and methods: We analyzed serum concentrations of the calcification inhibitor matrix Gla protein (*MGP*) in a large cohort of patients suffering from PXE ($n=101$), 34 first-degree relatives and 67 healthy controls. Moreover, we determined the distribution of the two *MGP* promoter polymorphisms c.-7G>A and c.-138T>C in the three cohorts.

Results: We found significantly lower total MGP concentrations in the sera of PXE patients compared to healthy controls ($p=0.0002$). Furthermore, higher serum MGP concentrations could be correlated with a later PXE onset. Analysis of *MGP* promoter polymorphism frequencies revealed one *MGP* haplotype to be a potential protective co-factor in PXE.

Conclusions: Our findings point to a role of the local calcification inhibitor MGP in PXE manifestation.

Keywords: Matrix Gla protein; Pseudoxanthoma elasticum; PXE; ABCC6; Calcification

Introduction

Progressive calcification and fragmentation of elastic fibers are characteristic hallmarks of pseudoxanthoma elasticum (PXE), caused by mutations in *ABCC6* encoding the multidrug resistance-associated protein 6 (MRP6) [1,2]. The physiological function of MRP6 and its implication in the mineralization typical for PXE are still unknown. Biological calcification was previously reported to be a passive process, but recent evidence suggests a complex regulation involving multiple steps and mediators. Key negative regulators of calcification are fetuin-A, osteopontin and matrix Gla protein (MGP). Recent studies have reported altered expression of systemic and local calcification inhibitory proteins as a result of MRP6 deficiency in PXE patients and *ABCC6* knock-out mice [3,4]. A study published very recently by Gheduzzi et al. presents data on the involvement of MGP in elastic fiber calcification in PXE [5]. MGP is a calcification inhibitor acting locally and mainly synthesized by vascular smooth muscle cells and chondrocytes. MGP was also found in the circulatory system, where it is part of the so-called calciprotein particles, together with fetuin-A and hydroxyapatite [6]. The importance of MGP in preventing pathological calcification is supported by MGP-deficient mice, which develop severe arterial calcification [7]. Moreover, humans with mutations in the *MGP* gene suffer from Keutel syndrome, characterized by cartilaginous but not arterial calcification [8]. Functional activity of MGP is maintained by vitamin K-dependent γ-carboxylation of its five glutamic acid residues. The γ-carboxylated (Gla) form is bound to fetuin-A in the circulation, can moreover interact with bone morphogenetic protein 2 and is therefore essential for inhibiting pathological mineralization [6,9]. The non-γ-carboxylated (Glu) form, as well

Abbreviations: MGP, Matrix Gla protein; MRP, Multidrug resistance-associated protein; PCR, Polymerase chain reaction; PXE, Pseudoxanthoma elasticum; RFLP, Restriction fragment length polymorphism.

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as lowered levels of MGP in the circulatory system, were associated with vascular calcification [10,11]. A genetic association between *MGP* polymorphisms and the risk of atherosclerosis or myocardial infarction has recently been reported [12].

Here, we investigated the role of the local calcification inhibitor MGP in PXE pathogenesis in a large cohort of PXE patients, their first-degree relatives and healthy controls. We found significantly lower total MGP in the PXE patients compared to the healthy controls. No differences in serum MGP levels were found between PXE patients and their healthy first-degree relatives. Moreover, we identified a haplotype of two *MGP* promoter polymorphisms as a protective genetic co-factor in PXE pathogenesis.

Methods

Patients and controls

EDTA-anti-coagulated whole blood samples were obtained from 101 German PXE patients and from 34 unaffected first-degree relatives (Table 1). The diagnosis of PXE in all patients was consistent with the reported consensus criteria [13]. The status of the PXE patients was determined by the presence of dermal lesions and ocular findings. The dermal lesions were histologically confirmed by observation of mineralized elastic fibers in biopsy samples following von Kossa staining. All members of the study were thoroughly questioned by medical specialists about personal diseases, organ involvements and family history. Serum samples of 67 Westphalian blood donors were used for analysis of MGP and fetuin-A serum concentrations. DNA samples from 101 age and sex-matched blood donors served as a control cohort for analysis of the *MGP* polymorphisms c.-7G>A and c.-138T>C. Through extensive clinical examination we could exclude impaired organ functions and skin lesions in the controls. All healthy control subjects had blood pressure values >100/55 mmHg and <140/90 mmHg, and negated antihypertensive medication, excluding a vascular disease. Further, all tested subjects were negative for hepatitis B and C virus and had normal serum alanine aminotransferase, serum creatinine and total protein levels. The study was approved by the institutional review board and all patients gave their informed consent.

Table 1
Clinical characteristics of PXE patients, first-degree relatives and healthy controls

	PXE patients (n=101)	Relatives (n=34)	Healthy controls (n=67)
Males (females)	29 (72)	13 (21)	29 (38)
Age [years][1]	48.5 (±14.5)	51.0 (±17.0)	42.3 (±10.4)
Age at disease onset [years][1]	31.2 (±16.8)	n.a.	n.a.
Total MGP [nmol/L][1]	3.85 (±1.53)	3.9 (±1.31)	4.72 (±1.31)
Fetuin-A [g/L][1]	0.55 (±0.11)	0.71 (±0.21)	0.80 (±0.24)
Calcium [mg/L][1]	96.95 (±15.89)	91.75 (±13.86)	64.0 – 140.0
Phosphate [mg/L][1]	52.88 (±28.09)	51.33 (±22.89)	20.0 – 80.0
Organ involvement [mean number][1]	3.1 (±1.49)	n.a.	n.a.

[1]Data are presented as means with the corresponding standard deviation.

Collection of blood and serum samples

For the determination of serum MGP, fetuin-A, calcium and phosphorus concentrations, venous blood samples were collected in serum monovettes (Sarstedt, Nürnberg, Germany). After clotting and centrifugation at 4.000 ×*g* for 15 min sera were stored at −70 °C until assayed as described below.

DNA extraction

Genomic DNA was extracted from 200 µL EDTA-anti-coagulated blood using the QIAamp blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

ABCC6 genotyping

Genotyping of the c.3421C>T mutation and mutational analysis of *ABCC6* was performed as previously described [2,14].

Genotyping of MGP promoter polymorphisms c.-7G>A and c.-138T>C

Mutation numbering refers to the *MGP* cDNA sequence with the A of the ATG translation initiation start site as nucleotide +1 (GenBank Accession No. NM_000900). The restriction length polymorphism analysis of the *MGP* polymorphisms c.-7G>A and c.-138T>C was performed using oligonucleotides described by Farzaneh-Far et al. [15]. Polymerase chain reaction (PCR) was performed in a 50 µL reaction volume containing approximately 65 ng genomic DNA, 25 pmol of each primer (Biomers, Ulm, Germany), 1.5 U HotStar Taq DNA polymerase (Qiagen, Hilden, Germany) in 1× Reaction Buffer and 3.5 mM $MgCl_2$, supplied with the enzyme, and 0.25 mM of each dNTP (Promega, Madison, USA). The PCR conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 15 min. The obtained PCR fragments were digested with 2.5 U NcoI and BsrSI (New England Biolabs, Beverly, USA) overnight at 37 °C and separated on a 1.5% agarose gel.

Measurement of serum MGP, fetuin-A, calcium and phosphate

Measurement of total MGP and fetuin-A concentrations in serum was performed using commercially available ELISA kits (Epitope Diagnostics, San Diego, USA; Biomedica, Vienna, Austria), according to the manufacturer's instructions. Serum concentrations of calcium and phosphate were measured using the Architect ci8200 (Abbott Diagnostics, Wiesbaden, Germany).

Statistical analysis

All values are given as mean±standard deviation. Normality testing for Gaussian distribution of values was performed using the Kolmogorov–Smirnov test. Statistical analysis was performed using the Student's *t*-test. Allele and haplotype frequencies were calculated and compared between cases and