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Matrix Gla protein is involved in elastic fiber calcification in the dermis of pseudoxanthoma elasticum patients

Dealba Gheduzzi[1,*], Federica Boraldi[1,*], Giulia Annovi[1], Chiara Paolinelli DeVincenzi[1], Leon J Schurgers[2], Cees Vermeer[2], Daniela Quaglino[1] and Ivonne Pasquali Ronchetti[1]

Mature MGP (Matrix γ-carboxyglutamic acid protein) is known to inhibit soft connective tissues calcification. We investigated its possible involvement in pseudoxanthoma elasticum (PXE), a genetic disorder whose clinical manifestations are due to mineralization of elastic fibers. PXE patients have lower serum concentration of total MGP compared to controls ($P<0.001$). Antibodies specific for the noncarboxylated (Glu-MGP) and for the γ-carboxylated (Gla-MGP) forms of MGP were assayed on ultrathin sections of dermis from controls and PXE patients. Normal elastic fibers in controls and patients were slightly positive for both forms of MGP, whereas Gla-MGP was more abundant within control's than within patient's elastic fibers ($P<0.001$). In patients' calcified elastic fibers, Glu-MGP intensively colocalized with mineral precipitates, whereas Gla-MGP precisely localized at the mineralization front. Data suggest that MGP is present within elastic fibers and is associated with calcification of dermal elastic fibers in PXE. To investigate whether local cells produce MGP, dermal fibroblasts were cultured *in vitro* and MGP was assayed at mRNA and protein levels. In spite of very similar MGP mRNA expression, cells from PXE patients produced 30% less of Gla-MGP compared to controls. Data were confirmed by immunocytochemistry on ultrathin sections. Normal fibroblasts *in vitro* were positive for both forms of MGP. PXE fibroblasts were positive for Glu-MGP and only barely positive for Gla-MGP ($P<0.001$). In conclusion, MGP is involved in elastic fiber calcification in PXE. The lower ratio of Gla-MGP over Glu-MGP in pathological fibroblasts compared to controls suggests these cells may play an important role in the ectopic calcification in PXE.
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The high concentration of calcium and phosphate in the extracellular space would lead to tissue calcification unless efficiently inhibited by a series of proteins and glycoproteins that have been found to operate in soft connective tissues to prevent calcium precipitation.[1]

Great attention has been recently paid to fetuin, which accumulates in the mineralized matrix of bone where it modulates apatite formation.[2–4] It also circulates in serum where it probably inhibits mineral precipitation due to the acidic amino-acid residues of its cystatin-like domain.[4,5] Recently, a significant decrease in fetuin was measured in the serum of patients with pseudoxanthoma elasticum (PXE),[6] a genetic disorder characterized by mineralization of elastic fibers,[7–9] and it has been suggested that fetuin could have been removed from serum and 'bound to the mineral deposits'.[5,10]

Another protein involved in inhibition of calcium precipitation in soft connective tissues is matrix Gla protein (MGP), a 10-kDa secreted protein containing 5-glutamic acid residues that must be γ-carboxylated by a vitamin K-dependent γ-carboxylase in order to acquire calcium-binding properties.[11,12] MGP-deficient mice manifest extensive calcification of the aorta and articular cartilage,[12] similar to what has been observed in Keutel syndrome, which is due to mutations in the human MGP gene.[13,14]

Several *in vitro* and *in vivo* studies have shown that MGP is expressed by many peripheral cell types, including fibroblasts and smooth muscle cells.[15–18] In an *in vitro* model of vascular calcification, the expression of MGP mRNA by smooth muscle cells was inversely correlated with mineral formation.[19] Overexpression of MGP was shown to inhibit normal chondrocyte and cartilage mineralization,[20] whereas inhibi-

[1]Department of Biomedical Sciences, University of Modena and Reggio Emilia, Modena, Italy and [2]Department of Biochemistry, University of Maastricht, Maastricht, The Netherlands
Correspondence: Professor IP Ronchetti, PhD, Department of Biomedical Sciences, Via Campi, 287, Modena 41100, Italy. E-mail: ronchetti.ivonne@unimore.it
*These authors contributed equally to this work.

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tion of MGP maturation by warfarin treatment induced calcification of cartilage and aorta, both in animals[21–23] and humans.[24,25] Moreover, in aortas, mineralization induced by warfarin was observed limited to elastic fibers, in the absence of necrotic material, and therefore attributable to an active process.[22,26] Therefore, defects in MGP carboxylation seems to be associated with elastin mineralization. In a recent paper, dermal elastic fiber calcification has been extensively described in patients affected by coagulation disorders due to a genetic defect in vitamin K recycling with consequent deficient protein γ-carboxylation.[27]

In the present study, we investigated on the possible involvement of MGP on elastic fiber calcification in PXE, by measuring MGP serum level and its distribution in the dermis of controls and PXE patients. Moreover, since fibroblasts are responsible for the homeostatic control of connective tissues, we investigated whether dermal fibroblasts isolated from patients had normal efficiency in the expression and maturation of MGP. The following parameters were investigated: (i) the serum MGP level in PXE patients compared to controls. (ii) the relative amount and immunolocalization of both γ-carboxylated and non-carboxylated forms of MGP in the dermis of controls and PXE patients, mainly focusing on elastic fibers; (iii) the expression and immunolocalization of both Gla-MGP and Glu-MGP in human dermal fibroblasts cultured *in vitro* and isolated from normal subjects and PXE patients. Data indicate a deficiency in the homeostatic control of MGP maturation in PXE dermal fibroblasts.

MATERIALS AND METHODS

Blood, Serum, and Plasma Collection

The protocol for the present study was approved by the Medical Ethical Committee of the University of Modena and Reggio Emilia. Healthy subjects were recruited from volunteers; PXE subjects were from the collection present in our reference Center for the Diagnosis of PXE. Patients and controls were of comparable age (ranging from 18 to 65 years). Blood was taken by venipuncture from 30 controls (from 21 to 65 years, mean age 38 ± 14 years; 4 men and 36 women) and from 30 PXE patients (from 13 to 64 years; mean age 41 ± 14 years; 4 men and 36 women), collected in serum tubes, stored for 1 h at room temperature and centrifuged at 3000 *g* for 10 min. Aliquots of serum were frozen and stored at -80°C until used. For plasma, blood from 15 women affected by PXE was collected into Na citrate-containing tubes, centrifuged at 3500 *g* for 10 min at 4°C. Aliquots of plasma-citrate were stored at -85°C until used. As controls, normal pooled laboratory plasma was used.

Skin Biopsies and Cell Culture

After informed consent, skin biopsies were taken under local anesthesia from the neck or from the axilla of six PXE patients and six normal subjects who underwent surgery and did not show any clinical sign of connective tissue alterations. All PXE patients had severe clinical manifestations, such as coalescent skin papules on the posterior and lateral neck, in the axillae and groin, skin laxity in flexural areas. They were also diagnosed by ultrastructural analysis of skin biopsies and by identification of ABCC6 mutations on both alleles.[28] Fibroblast cultures were established and maintained according to Quaglino *et al*.[29] Briefly, skin biopsies were fragmented and cells were grown as monolayer in 75 cm flasks (Nunc, Roskilde, Denmark) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and used between the third and the tenth passage *in vitro*. Control and PXE cells were used at the same passage within each experiment.

Protein Preparation

Fibroblasts were plated in 75 cm^2 flasks at a density of 8×10^5 cells in 15 ml DMEM with 10% FBS. After 7 days from seeding, cells were removed from the culture flasks with 0.25% trypsin-EDTA in phosphate-buffered saline (PBS) for 10 min at 37°C. Trypsin was blocked by addition of DMEM plus 10% FBS. Cells were centrifuged for 5 min at 1000 r.p.m. at 4°C, washed with cold PBS, centrifuged again and then suspended in isotonic buffer containing 25 mM imidazole, 250 mM sucrose, 10 μg/ml Sigma protease inhibitor mixture and 1 mM NaF, pH 7.5. Cells were sonicated on ice in a Sonifier B-12 (Branson Sonic Power Company, Danbury, Connecticut, USA) with 20 pulses of 2 s each. The sonicate was centrifuged at 10 000 *g* for 10 min to remove cell debris. The membrane-enriched fraction was obtained by centrifuging the supernatant at 229 000 *g* for 60 min in a Type 50-Ti rotor (Beckman Instruments, Fullerton, CA, USA) and stored frozen at -85°C until used. Prior to 2D-PAGE, membrane proteins were dissolved in 250 mM phosphate, 0.5 M KCl, 20% glycerol, and 0.75% CHAPS, pH 7.85, containing 10 μl/ml Sigma protease inhibitor mixture and subsequently precipitated with the ProteoExtract Protein Precipitation Kit (Calbiochem, Darmstadt, Germany) according to the instruction. Proteins were harvested frozen at -20°C.

Electrophoresis

Proteins extracted from a pool of fibroblasts derived from six patients and six controls were used for each assay. Proteins were dissolved in 8 M urea, 2% CHAPS, 65 mM dithioerythritol, 2% pH 3–10 ampholyte (GE HealthCare, UK), and trace of bromphenol blue and were then applied onto Immobiline pH 3–10 nonlinear DryStrips (7 cm long, GE HealthCare). Isoelectric focusing was performed on an IPGphor system (GE HealthCare) at 16°C according to the instruction manual. Immobilized pH gradient strips were reduced (2% dithioerythritol), alkylated (2.5% iodoacetamide) in equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 6.8, 30% glycerol, 2% SDS) and then loaded onto vertical SDS-PAGE gel (15%). The second dimension was run using an electrophoresis Unit (Bio-Rad). Gels were stained with ammoniacal silver nitrate.

Western Blotting

It was carried out after transfer of proteins to nitrocellulose membranes, applying a 200 mA constant current. Membranes were washed with TTBS (50 mM Tris, 150 mM NaCl, pH 7.5, plus 0.1% Tween20) and blocked with 3% BSA in TTBS. Nitrocellulose films were incubated with primary antibodies (mouse anti-γ-carboxylated-MGP or mouse anti-non-carboxylated-MGP) diluted 1:10 000 in TTBS plus 1% BSA. After washes with TTBS, the incubation with the secondary antibody was performed by using anti-mouse IgG conjugated with horseradish peroxidase (GE HealthCare) diluted 1:20 000 in TTBS and 1% BSA and the immunoreaction was revealed by using the SuperSignal West Pico Chemiluminescent Substrate (Pierce) and normalized to β-actin.

Quantitative Real-Time RT-PCR

Total RNA was isolated from cells using the RNeasy Protect cells Mini kit (Qiagen, Valencia, CA, USA). Quality and quantity of RNA were checked by spectrophotometer and agarose gel. Then 3 μg of total RNA was reverse transcribed using Superscript III (Invitrogen) and Oligo dT_{18} primers (Invitrogen) according to the manufacturer's instruction and conversion was confirmed by a PCR with *CLK2* primers. A negative control was carried out to ensure the absence of DNA contamination. A 10 $\times$ diluted cDNA sample was further amplified on an iCycler (BioRad) using SYBR® GreenER™ qPCR SuperMix (Invitrogen), according to the manufacturer's instruction. Primers for conducting real-time RT-PCR are shown in Table 1 and were designed using Universal ProbeLibrary version 2.35 for human (www.roche-applied-science.com) and were synthesized by Invitrogen. All RT-PCR analyses were carried out in triplicate. The thermal cycling parameters were set to 50°C for 2 min, 95°C for 3 min, 45 cycles of 95°C for 30 s, an annealing temperature of 60°C for 30 s and 72°C for 30 s, followed by melting curve analysis with a temperature ranging from 95 to 55°C. Gene expression in each sample was normalized to the expression of an housekeeping gene (CLK2) and compared with control samples, using the $2^{-\Delta\Delta Ct}$ method.[30]

Table 1 Primers used for real-time RT-PCR

	Length	Position	Tm	% GC	Sequence
CLK2					
Sense	20	667–686	59	50	GGACATTTAGCCGCTCATCT
Antisense	21	706–726	59	50	CGTCGTCCTCTACACTCTTGG
MGP					
Sense	21	135–155	59	52	CCGCCTTAGCGGTAGTAACTT
Antisense	20	227–246	59	52	TGCTGAGGGGATATGAAGGT

Immunocytochemistry

Skin biopsies and cultured fibroblasts were processed for electron microscopy. Briefly, small fragments of skin biopsies or fibroblasts scraped from the substrate and centrifuged to form a pellet were immediately fixed in 2.5% paraformaldehyde in Tyrode's saline, pH 7.2, for 16–20 h at 4°C, followed by fixation in 0.5% osmium tetroxide (Fluka AG Chem) in the same buffer for 60 min at room temperature, dehydration in ethanol and propylene oxide and embedding in Spurr resin (Polysciences Inc., Warrington, PA, USA). Ultrathin sections were collected on nickel grids and processed for immunocytochemistry as already described.[31] Unspecific epitopes were neutralized by incubating sections on 0.5% bovine serum albumin in buffer. Monoclonal antibodies towards the noncarboxylated species of MGP and towards the γ-carboxylated form of MGP[32,33] were used in parallel in all experiments where normal and PXE samples had to be compared. The immunoreactions were revealed by secondary antibodies conjugated with 10 nm gold particles (EY Laboratories, San Mateo, CA, USA). Controls for the immunoreactions were performed by omitting the primary antibody or by incubating the sections with nonimmune sera instead of the primary antibody. Sections were then stained with uranyl acetate and lead citrate and observed by transmission electron microscopy (Jeol, EM1200, Tokyo, Japan).

Immunoassay for Serum MGP

Serum MGP was assayed by using the test kit from Biomedica (Vienna, Austria). It is a competitive enzyme-linked immunosorbent assay (ELISA) in which microwell plates are coated with mouse monoclonal antibodies raised against human MGP. The protocol described by Schurgers *et al*[32,33] has been applied.

Coagulation Factors

The procoagulant activity of prothrombin (FIIc) and factor VIIc were measured in a coagulometer (ACL 300 Research; Instrumentation Laboratory, Milan, Italy) using Thromborel S and human coagulation factor II- and VII-deficient plasma (Behringwerke AG, Marburg, Germany). All values were expressed as a percentage of values obtained for pooled normal plasma.

Statistical Analysis

The intensity of immunostaining was evaluated by counting the number of gold particles present in 20 random fields on ultrathin sections of dermal elastic fibers or of cytoplasm of cultured dermal fibroblasts by applying a 1 μm^2 mask on images obtained at the same magnification ($\times$ 20 000). Data were compared by Student's *t*-test with significance at $P<0.05$. The immunoreactivity of the two antibodies was different, therefore only intra-antibody comparison was carried out.

RESULTS

MGP Content in Serum of PXE Patients

Previous studies showed that, in humans, there are no significant differences in the MGP levels between genders and no significant variations with age.[32,33] In the present study, circulating MGP level was measured in the serum of 30 controls and of 30 PXE patients (see Materials and Methods). Figure 1 illustrates that the amount of MGP was statistically lower in patients (5.16 ± 1.07 nM/ml) compared to controls (7.26 ± 1.24 nM/ml) ($P < 0.001$). Unfortunately, the method could not discriminate between the γ-carboxylated and the noncarboxylated forms of MGP.

Immunolocalization of MGP in the Human Dermis

In order to investigate whether MGP is involved in elastic fiber calcification in PXE, ultrathin sections of skin biopsies from normal subjects and from patients affected by PXE were immunostained in parallel by using antibodies specific for the noncarboxylated (Glu-MGP) and for the γ-carboxylated (Gla-MGP) forms of MGP.[32,33]

In normal skin, fibroblasts were almost negative for Glu-MGP, whereas they were slightly positive for Gla-MGP (not shown). In the dermis of PXE patients, fibroblasts were almost negative for both forms of MGP (not shown).

All components of the extracellular matrix, apart from elastic fibers, were always negative for both types of antibodies in all samples of both controls and patients. In contrast, elastic fibers were always positive for MGP. In particular, Glu-MGP was equally present within control's elastic fibers (Figure 2a) and within noncalcified elastic fibers in the dermis of PXE patients (Figure 2b). By contrast, Gla-MGP was always present within control's elastic fibers (Figure 3a), whereas it was almost absent from noncalcified PXE elastic fibers (Figure 3b). The number of gold particles per unit surface area of sectioned elastic fibers and the significance of data are reported in Figures 2 and 3. As already pointed out in the Materials and Method section, the two antibodies had different reactivity and their immunoreactions could not be compared. Therefore, comparison could only be made between control and pathological samples treated with the same antibody under identical experimental conditions. Data clearly show that Gla-MGP was almost absent from elastic fibers in patients.

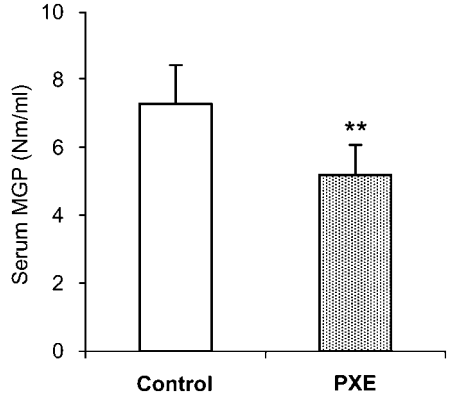


Figure 1 Total MGP measured in the serum of 30 controls and of 30 PXE patients of comparable age. The level of MGP was significantly higher in the serum of normal subjects compared to patients ($P < 0.001$).

As already described in several reports, calcification of elastic fibers in the dermis of PXE can assume different structural forms, which are often present in distinct regions of the same fiber.[8] The two main forms are represented in Figure 4. One of them consists of electron-opaque polymorphic masses of calcified material deforming and fracturing the elastic fibers (Figure 4a, arrows). The second form is characterized by dispersed small-calcified spots occupying the center of fibers (Figure 4a and b, asterisk) and separated from the surrounding apparently normal elastin by an electron opaque ring made of needle-shaped crystals (Figure 4b, inset).

The two antibodies tested reacted in a completely different manner on these two different regions. The antibody that recognizes Glu-MGP strongly reacted with the polymorphous calcified areas and with the finely dispersed calcified core of elastic fibers (Figure 4c, asterisks). By contrast, the antibody towards Gla-MGP was absent from these regions and was only and precisely localized at the calcification front separating the central mineralized core from the surrounding apparently normal elastin (Figure 4d, arrows).

MGP by *In Vitro* Dermal Fibroblasts

Since peripheral cells, namely fibroblasts and smooth muscle cells, are the major producers of MGP *in vivo*,[15,17,34] we wanted to analyse whether dermal fibroblasts might be involved in MGP expression and maturation. Dermal fibroblasts were isolated from skin biopsies of both controls and PXE patients and were analysed for the expression of MGP. The expression of MGP mRNA was similar in controls and in PXE fibroblasts, differences being statistically not significant (Figure 5).

When measured at protein level, as illustrated in Figure 6, the total amount of MGP in PXE cells was about 25% less than that found in control fibroblasts ($P < 0.05$). As specified in the Materials and Method section, analyses were performed on a pool of cell membranes from fibroblasts of six patients and six controls in each experiment, and experiments were carried out in triplicate. Therefore, the subfractionation method employed should circumvent the evaluation of MGP eventually taken up by cells from the medium and accumulating within the cytoplasm.

Data on the relative amounts of Glu-MGP (a) and Gla-MGP (b), identified by specific antibodies and normalized to the β-actin content, are shown in Figure 6. The amount of Glu-MGP was very similar in both control (34%) and PXE (32%) fibroblasts. By contrast, the relative amount of Gla-MGP was 66% in controls, whereas it was around 43% in PXE cells, and the ratio between Gla-MGP to Glu-MGP

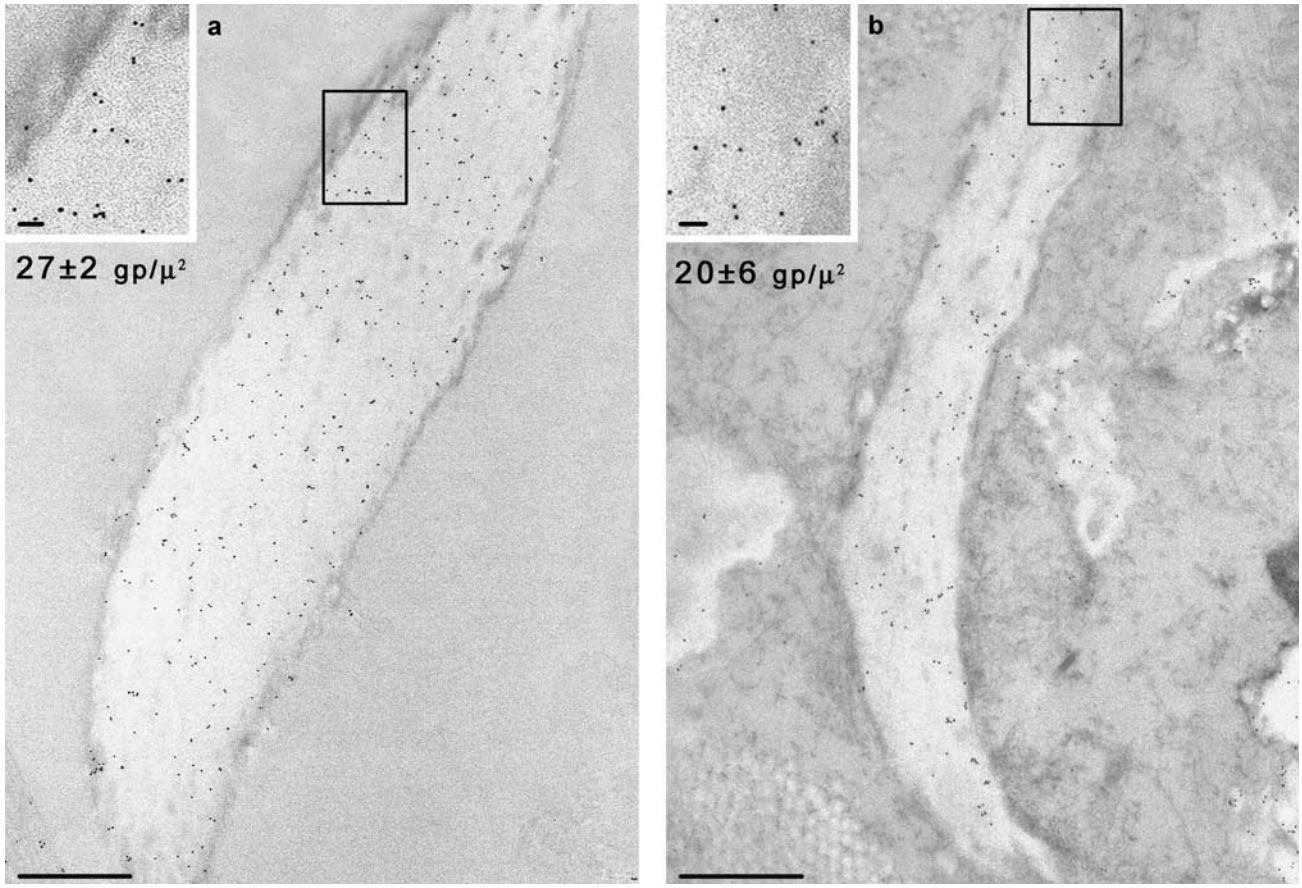


Figure 2 Immunolocalization of noncarboxylated MGP (Glu-MGP) on elastic fibers of the normal human dermis (**a**) and on the nonmineralized dermal elastic fibers of PXE patients (**b**). The number of gold particles (gp) per unit area of elastic fibers was almost the same in the two conditions. Bar: (**a**) and (**b**) 1 μm; insets 0.1 μm.

was more than 30% lower in PXE (1.34) compared to controls (1.94) ($P<0.01$) (Figure 6c).

Immunolocalization of MGP on *In Vitro* Dermal Fibroblasts

In vitro dermal fibroblasts were positive for MGP, as shown by the immunoelectron-microscopy approach applied to thin sections of cultured fibroblasts. The amount and distribution of Glu-MGP were scarce and almost identical in control (a) and PXE (b) fibroblasts (Figure 7). By contrast, Figure 8 illustrates that the immunoreaction towards Gla-MGP was much stronger in control (a) compared to PXE (b) fibroblasts. The number of gold particles per square unit of cytoplasm measured on random fields was $30\pm11/\mu^2$ in controls and $12\pm4/\mu^2$ in PXE cells ($P<0.001$). Moreover, as expected from data of the literature,[32] the immunoreaction for Gla-MGP was precisely and almost exclusively localized on membranes of the endoplasmic reticulum in both control and PXE fibroblasts.

Coagulation Factor

Measurements of coagulation factors II and VII in the plasma of PXE patients did not reveal any significant difference between patients and controls. Values for coagulation factors II and VII were only 3 and 7% higher in PXE patients compared to controls (data not shown).

DISCUSSION

Relevant findings of this work are that human dermal fibroblasts express and produce MGP, and that MGP is present within normal elastic fibers in the human dermis.

Owing to the well-recognized role of MGP in preventing mineralization of soft connective tissues[11,12,15,19,20] and the propensity of elastin *per sé* to accumulate calcium,[35] the presence of MGP within normal elastic fibers would suggest that this protein may have a role in preventing elastic fiber calcification. Strategic location of γ-carboxylated MGP within elastic fibers may protect those unique structures against mineralization. Elastic fibers of PXE patients that contain only the noncarboxylated immature MGP are not protected from sequestration of calcium from the serum. Moreover, MGP is involved in PXE calcification, being immature noncarboxylated MGP (Glu-MGP) associated with mineral precipitates and γ-carboxylated MGP (Gla-MGP) associated with the calcification front. These data suggest once more the different and opposite

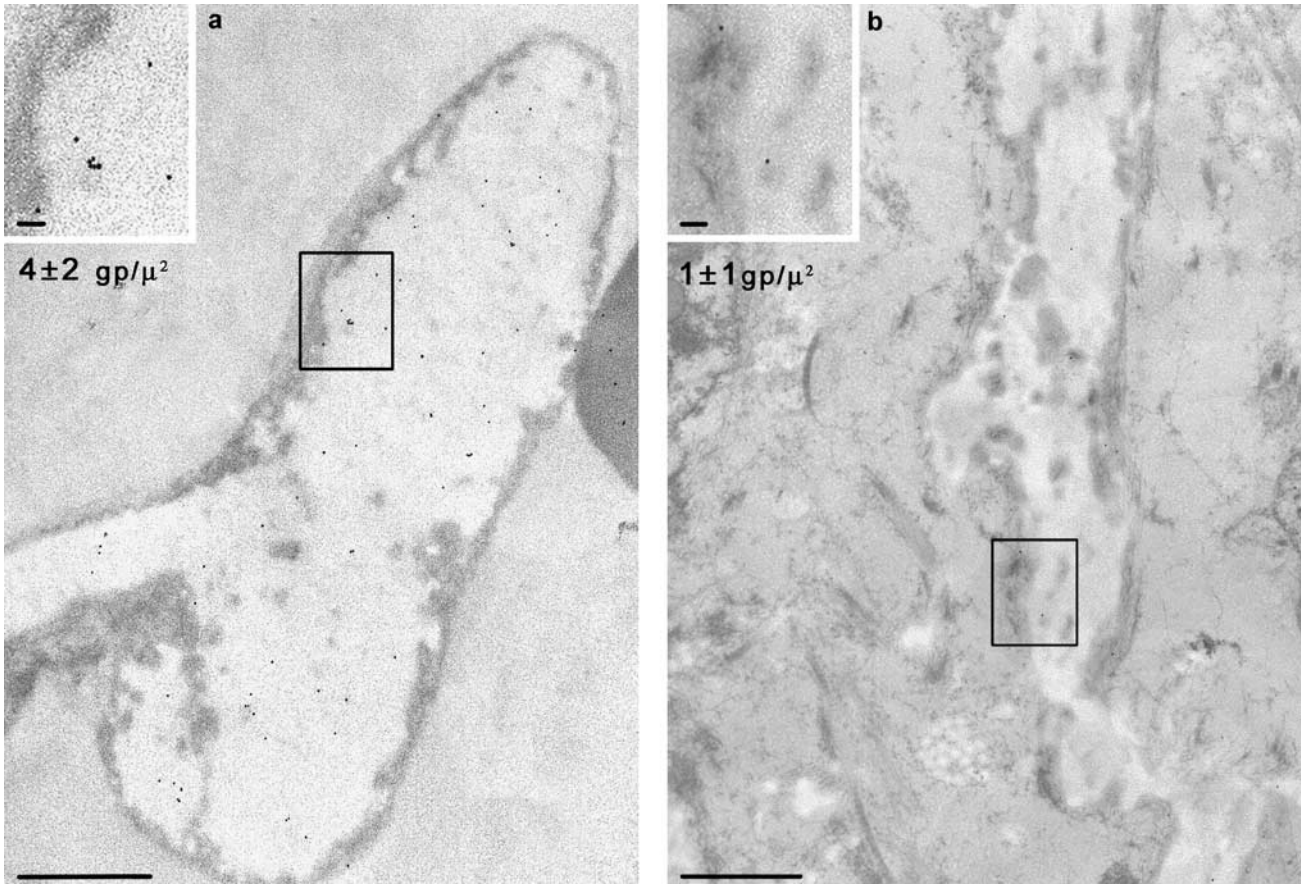


Figure 3 Immunolocalization of γ-carboxylated MGP (Gla-MGP) on elastic fibers of the normal human dermis (**a**) and the nonmineralized dermal elastic fibers of PXE patients (**b**). The number of gold particles (gp) per unit area of elastic fiber was significantly higher in control compared with PXE ($P<0.001$). Bar: (**a**) and (**b**) 1 μm; insets 0.1 μm.

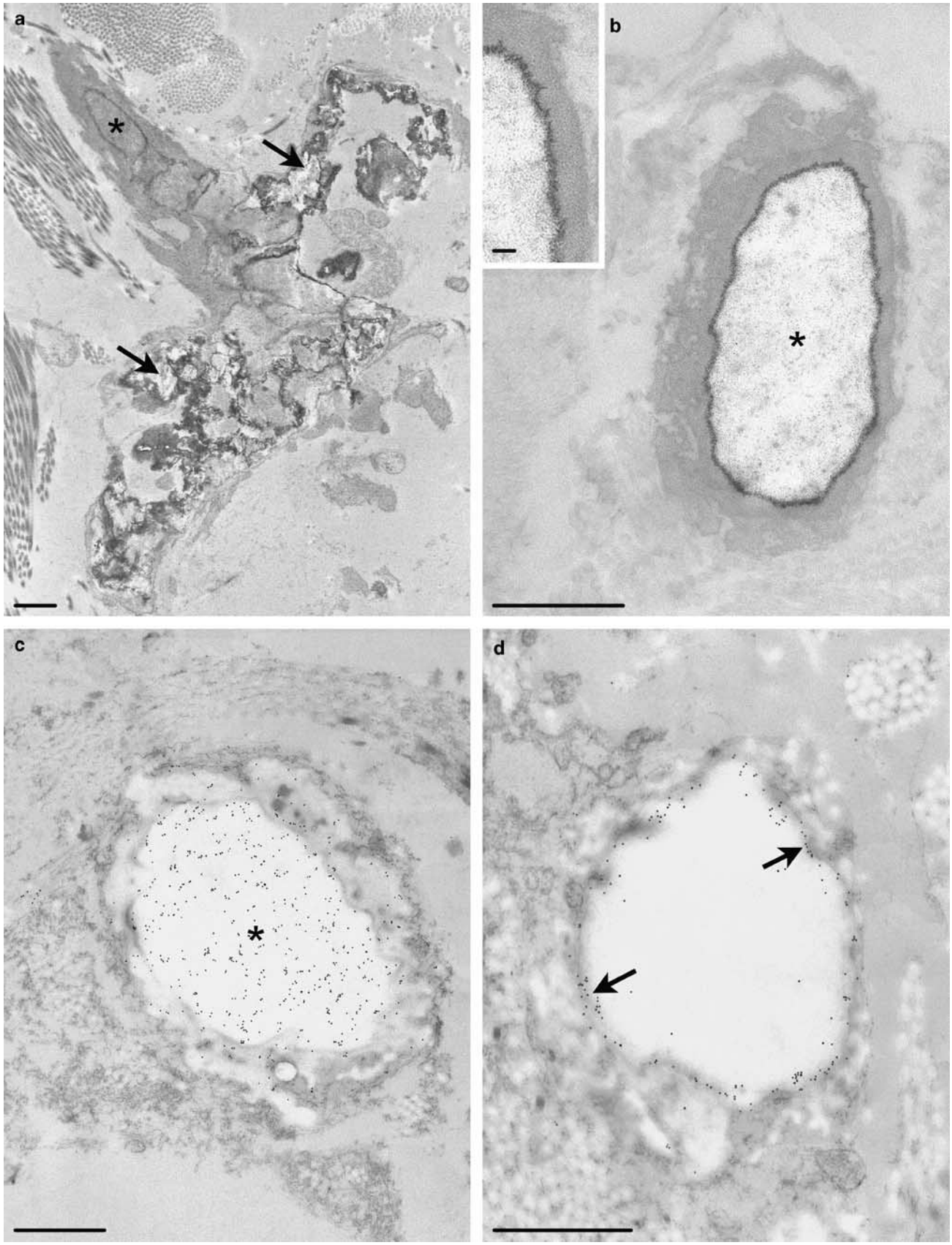
role of the two forms of MGP in favouring/preventing calcium precipitation, respectively. Finally, MGP content, and in particular the ratio between Gla-MGP to Glu-MGP, could be taken as marker for elastic fiber calcification in PXE patients.

In the normal dermis, elastic fibers were immunologically positive for both Glu-MGP and Gla-MGP. By contrast, the noncalcified elastic fibers in PXE patients were almost negative for Gla-MGP. Therefore, elastic fibers of PXE patients would seem less protected from calcium precipitation due to their low content of the active γ-carboxylated form of the protein.

These data may explain findings by Gordon *et al*[36] who found that fragments of skin from PXE patients were able to accumulate more calcium compared to normal skin when incubated in a calcium-containing medium.

The different role of Glu- and Gla-MGP on the mineralization process of elastic fibers was shown by the different localization of the antibodies specific for the two forms of MGP even within the same elastic fiber. Glu-MGP was always and abundantly present in the areas of either bulky or spotty mineralization. By contrast, Gla-MGP was only and precisely localized at the thin frontline separating the central mineralized core from the surrounding apparently normal elastin. These data are in agreement with those obtained by light microscopy on human aortas in the early phase of atherosclerosis and in Monckeberg's sclerosis where the Glu-MGP has been observed to colocalize with calcification, whereas Gla-MGP has been found around elastic fibers.[32] In that report, the low resolution of the technique did not allow the precise localization of the γ-carboxylated form of MGP.[32] By considering the inhibitory role on calcification of mature γ-carboxylated MGP and that calcification seems to progress from the center of elastic fibers towards their periphery, the present data suggest that mature calcium-binding MGP (Gla-MGP) would arrest calcification at these peculiar sites. By contrast, immature Glu-MGP was always associated with bulky mineral precipitates where it might favor calcification. Similar to what is suggested for fetuin,[5,10] it cannot be excluded that immature MGP is captured during calcification.

It has been already demonstrated that MGP is synthesized by numerous cell types, including smooth muscle cells,[17,19,34,37] pneumocytes[15,18] and chondrocytes.[20,38]


a
b
c
d

We wanted to verify whether dermal fibroblasts express MGP and whether they may be responsible for the low amount of the γ-carboxylated form of MGP within elastic fibers in the dermis of patients. Therefore, *in vitro* dermal fibroblasts from normal subjects and from patients affected by PXE, bearing identified ABCC6 mutations,[28] were assayed and compared for MGP expression and maturation. The amount of the MGP protein was measured on cell membrane extracts in order to look for proteins actually belonging to the cells and not taken up from the medium. Data show that dermal fibroblasts express MGP in either the γ-carboxylated and the noncarboxylated forms. However, although the mRNA levels were very similar in control and PXE cells, the amount of protein, and in particular of the mature γ-carboxylated form of MGP, was significantly lower in PXE cells compared to controls ($P<0.01$). Therefore, the data might support the hypothesis of a deficient post-transcriptional maturation of MGP by PXE fibroblasts.

Data obtained by immunoblotting were in perfect agreement with those by immunoelectron microscopy on thin sections of fibroblasts, where control cells were positive for Gla-MGP, whereas PXE fibroblasts were almost negative for the same antibodies.

The lower expression of γ-carboxylated MGP by PXE fibroblasts suggests that these cells may play an important role in calcification of dermal elastic fibers in PXE.

Total serum MGP content was lower in PXE patients compared to controls ($P<0.001$). Unfortunately, the method did not allow to distinguish between the noncarboxylated and the γ-carboxylated forms of MGP. Recently, a reduced amount of fetuin, another serum protein involved in the inhibition of ectopic calcification, has been measured in the serum of PXE patients,[6] and the authors suggested that fetuin might have disappeared from the circulation by remaining entrapped within the calcium/phosphate mineral precipitates typical of PXE.[6] Without excluding a similar possibility, data of the present study seem to suggest that the lower amount of circulating MGP in PXE patients may be related to reduced expression of MGP, and in particular of the active γ-carboxylated form of MGP by peripheral cells, rather than to its capture by mineralized elastic fibers.

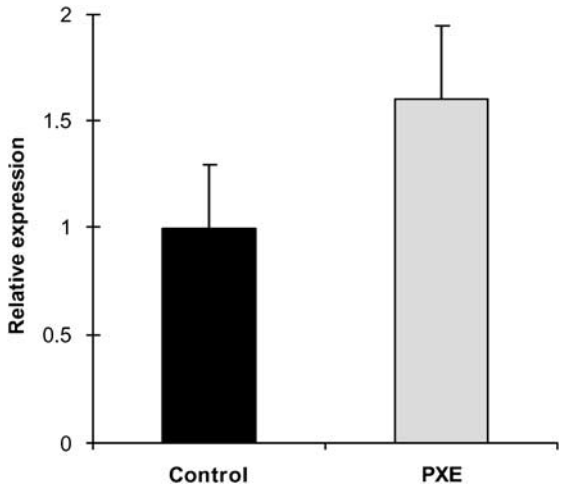


Figure 5 MGP mRNA expression in control and PXE fibroblasts assayed by RT-PCR. Differences between control and PXE cells were not significant.

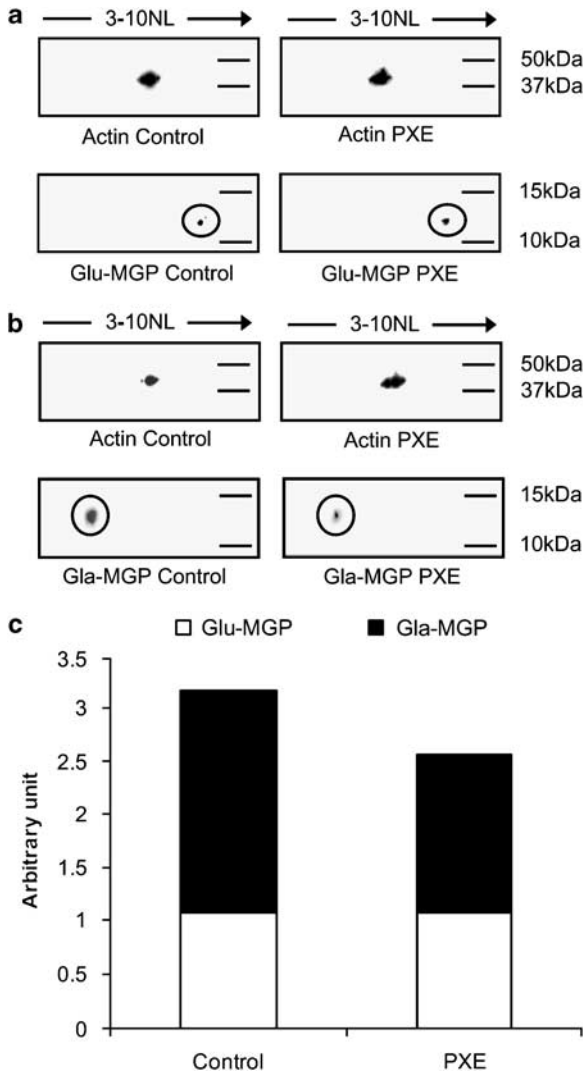


Figure 6 Noncarboxylated-MGP and γ-carboxylated MGP expressed by *in vitro* fibroblasts were assayed at protein level by western blot. Data were normalized to β-actin. PXE fibroblasts expressed 25% less MGP compared to controls (**a**, **b**). In addition, both control and PXE cells produced similar amount of noncarboxylated MGP, whereas γ-carboxylated MGP was more than 30% higher in control compared to PXE fibroblasts (**c**).

Figure 4 Immunolocalization of glu- and Gla-MGP in calcified dermal elastic fibers in PXE patients. The polymorphous precipitates deforming elastic fibers (**a**, arrows) coexisted with areas of fine disperse calcification precipitates (**a** and **b**, asterisk), which were very often separated from the surrounding apparently normal elastin (**b**) by a ring of needle-shaped mineral crystals (**b**, inset). Noncarboxylated-MGP was localized on the polymorphous precipitates and on the mineralized core of elastic fibers (**c**, asterisk); γ-carboxylated MGP was always and only localized at the mineralization front (**d**, arrows). Bar: 1 μm.

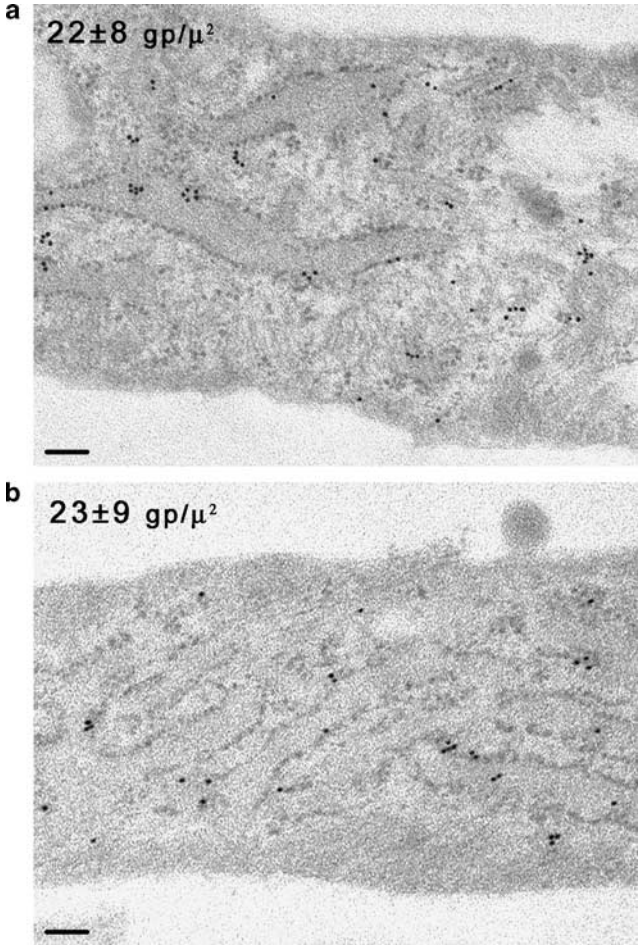


Figure 7 Immunolocalization of noncarboxylated MGP (Glu-MGP) on *in vitro* fibroblasts from control (**a**) and PXE patients (**b**). In both cases, MGP localized on membranes of the endoplasmic reticulum. The amount of noncarboxylated MGP, evaluated as number of gold particles (gp) per unit area, was similar in control (**a**) and PXE fibroblasts (**b**). Bar: 0.1 μm.

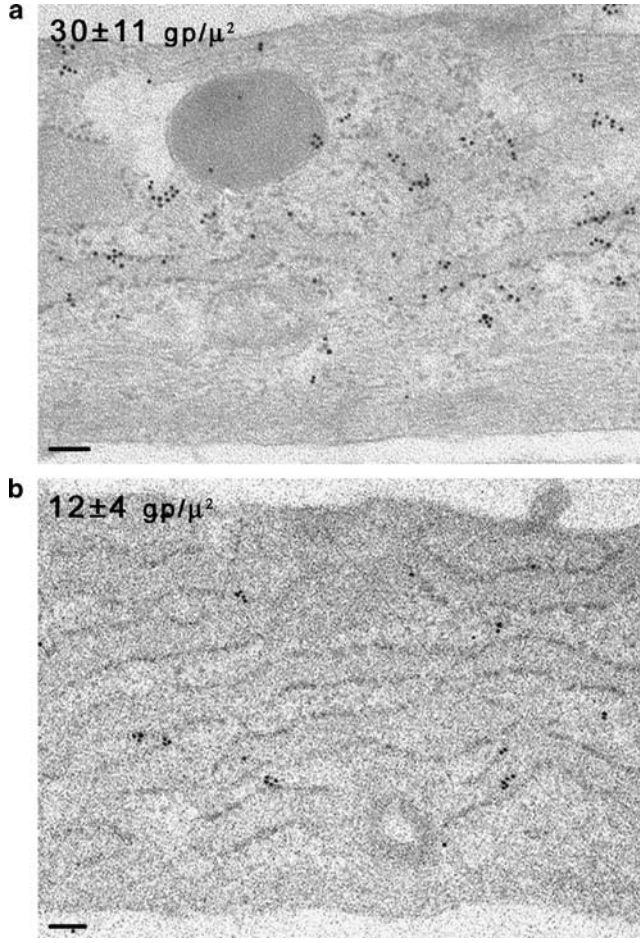


Figure 8 Immunolocalization of γ-carboxylated MGP (Gla-MGP) on *in vitro* fibroblasts from controls (**a**) and PXE patients (**b**). In both cases, MGP localized on membranes of the endoplasmic reticulum. The amount of γ-carboxylated MGP, evaluated as number of gold particles (gp) per unit area, was much higher in control (**a**) than in PXE fibroblasts (**b**) ($P<0.001$). Bar: 0.1 μm.

By immunoelectron microscopy, Glu-MGP was equally present within noncalcified elastic fibers of both controls and patients; by contrast, Gla-MGP was present in controls whereas it was almost absent from nonmineralized elastic fibers of patients. Similar findings were also found for dermal fibroblasts cultured *in vitro* where PXE fibroblasts appeared to be significantly less positive for Gla-MGP compared to controls. Therefore, the ratio of Gla-MGP to Glu-MGP would seem of paramount importance in understanding the occurrence of ectopic mineralization.

From these data it would seem that local cells, such as fibroblasts, play an important role in preventing dermal elastic fiber calcification. It has been repeatedly shown that MGP, although it is a circulating protein, acts as inhibitor of the extracellular matrix calcification in the vicinity of cells expressing the protein.[12,34,37] Therefore, our findings that PXE fibroblasts express lower amount of the γ-carboxylated form of MGP compared to controls may suggest that calcification of elastic fibers, at least in the dermis of PXE patients, might depend on local scarce availability of the active carboxylated form of MGP.[26] This hypothesis is in agreement with data obtained on transgenic animals showing that local, and not systemic, expression of MGP inhibits extracellular matrix calcification,[34] and with findings showing that only living smooth muscle cells producing MGP are able to prevent calcification of aortic rings in organ culture.[37]

How to reconcile ABCC6 mutations in PXE with impaired expression and maturation of MGP is an open question. In the absence of knowledge on the physiological role of the membrane transporter responsible for PXE, we approach the problem of clinical manifestations in PXE by trying to understand the mechanisms of elastic fiber calcification, to identify cells involved and to characterize alterations of connective tissue metabolism relevant for the progression of disease.

It has been repeatedly reported that fibroblasts from patients, despite their very low or absent expression of the

PXE causative gene,[39] exhibit and maintain, at least up to the tenth passage *in vitro*, behavioral,[29] biochemical,[40,41] and degradation[42] properties different from controls. Therefore, PXE fibroblasts seem to bear permanent metabolic alterations that are maintained when cultured *in vitro* and that, *in vivo*, could be responsible for local clinical manifestations.

We have recently reported that PXE fibroblasts *in vitro* suffer from a mild chronic oxidative stress with production of significantly higher amount of malondialdehyde compared to controls.[43] Since malondialdehyde is the final product of phospholipid peroxidation induced by oxidative stress, the organization of cell membranes in PXE would be different from controls (unpublished data). This, in turn, may affect the activity of enzymes localized on membranes of the endoplasmic reticulum, such as the enzyme γ-carboxylase,[44,45] whose activity is necessary for MGP γ-carboxylation. Therefore, disturbance of the γ-carboxylase system, however induced, might lead to deficient expression of active MGP. Very recently, skin and vessel alterations similar to those in PXE have been observed in patients suffering from vitamin K-dependent coagulation factor deficiency associated with mutations in genes coding for enzymes involved in the γ-carboxylation pathway and recycling.[27] Therefore, maturation of MGP by the vitamin K-dependent γ-carboxylase, an endoplasmic reticulum enzyme, has fundamental importance in preventing extracellular matrix calcification and, in particular, calcification of dermal elastic fibers similar to those in PXE.[27] PXE patients do not suffer from coagulation deficiency and those of the present study had normal levels of factors II and VII. Therefore, it is not that MGP deficiency in PXE patients may depend on alterations in the vitamin K-dependent γ-carboxylase system *per se*.

The present report indicates that elastic fiber calcification in PXE is associated with scarce expression and accumulation within elastic fibers of the mature γ-carboxylated form of MGP, an inhibitor of ectopic calcification in soft connective tissues. Moreover, since the reduced expression of the mature γ-carboxylated form of MGP by PXE dermal fibroblasts is associated with significant lower amount of circulating MGP in patients compared to controls, we suggest that fibroblasts may be, at least partially, responsible for the low level of circulating MGP. This is in agreement with recent observations that mature MGP is synthesized by cells in periphery,[34] where it would exert local inhibition of ectopic calcification.[12,34,37]

In conclusion, impaired maturation of MGP would be part of the metabolic alterations of PXE fibroblasts and could play a relevant role in time-dependent stability of elastic fibers in PXE.

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