Serum Factors from Pseudoxanthoma Elasticum Patients Alter Elastic Fiber Formation *In Vitro*

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Pseudoxanthoma elasticum (PXE) is a heritable disorder mainly characterized by calcified elastic fibers in cutaneous, ocular, and vascular tissues. PXE is caused by mutations in *ABCC6*, a gene encoding an ABC transporter predominantly expressed in liver and kidneys. The functional relationship between *ABCC6* and elastic fiber calcification is unknown. We speculated that ABCC6 deficiency in PXE patients induces a persistent imbalance in circulating metabolite(s), which may impair the synthetic abilities of normal elastoblasts or specifically alter elastic fiber assembly. Therefore, we compared the deposition of elastic fiber proteins in cultures of fibroblasts derived from PXE and unaffected individuals. PXE fibroblasts cultured with normal human serum expressed and deposited increased amounts of proteins, but structurally normal elastic fibers. Interestingly, normal and PXE fibroblasts as well as normal smooth muscle cells deposited abnormal aggregates of elastic fibers when maintained in the presence of serum from PXE patients. The expression of tropoelastin and other elastic fiber-associated genes was not significantly modulated by the presence of PXE serum. These results indicated that certain metabolites present in PXE sera interfered with the normal assembly of elastic fibers *in vitro* and suggested that PXE is a primary metabolic disorder with secondary connective tissue manifestations.

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INTRODUCTION

Pseudoxanthoma elasticum (PXE) is a complex disorder with multiorgan involvement and a progressive and uneven severity (Neldner, 1988; Chassaing *et al.*, 2005). This disease is characterized by connective tissue alterations, including calcification of elastic fibers. PXE patients accumulate fragmented calcified elastic fibers, altered collagen fibrils, and proteoglycans in elastic tissues, resulting in lesions predominantly in the skin, retina, and arterial walls (Uitto and Shamban, 1987). Typical skin lesions appear as yellowish papules in flexural areas and are associated with loss of elasticity (Uitto and Shamban, 1987; Neldner, 1988; Uitto *et al.*, 1998). Calcification of elastic fibers of the Bruch's membrane of the eye results in angioid streaks that often lead to subretinal neovascularization and hemorrhages, resulting in the loss of central vision (Weenink *et al.*, 1996). The mineralization of elastic fibers of arteries often causes cardiovascular manifestations such as premature peripheral vascular occlusive disease, intermittent claudication, and/or gastrointestinal bleeding (Mendelsohn *et al.*, 1978; Nishida *et al.*, 1990; Lebwohl *et al.*, 1993). Surprisingly, it was established that the PXE phenotype derives from mutations in an ABC transporter gene called *ABCC6* (Bergen *et al.*, 2000; Le Saux *et al.*, 2000; Ringpfeil *et al.*, 2000; Struk *et al.*, 2000). While mutations in similar genes such as *ABCC2*, *-7*, and *-8* cause a variety of phenotypes (Riordan *et al.*, 1989; Thomas *et al.*, 1995; Toh *et al.*, 1999) consistent with the known function(s) of these transporters, the functional relationship between ABCC6 and elastic fibers defects is obscure. The *ABCC6* cDNA and transport activity of the encoded protein have recently been characterized (Kool *et al.*, 1999; Ilias *et al.*, 2002), but the true nature of the endogenous substrate(s), the overall function of ABCC6, and its influence on connective tissues are largely unknown. The elastic fiber abnormalities associated with the PXE phenotype initially suggested that PXE was a strict connective tissue disorder. In support of this original assumption, skin fibroblasts isolated from PXE patients displayed abnormal characteristics with decreased cell–cell and cell matrix adhesion properties, higher proliferation, and altered synthesis of connective tissue components, including elastin, collagen, and proteoglycans (Lebwohl *et al.*, 1993; Baccarani-Contri *et al.*, 1996; Quaglino *et al.*, 2000). However, abundant *ABCC6* mRNA in human and rodents is almost exclusively found in liver and kidney (Kool *et al.*, 1999; Madon *et al.*, 2000; Scheffer *et al.*,

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Abbreviations: FBS, fetal bovine serum; LOX, lysyl oxidase; LOXL, lysyl oxidase-like; MAGP-1, microfibril-associated glycoprotein-1 PXE, Pseudoxanthoma elasticum; SMC, smooth muscle cell

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2001). Since PXE patients display seemingly normal hepatic and renal function (Uitto *et al.*, 2001), we speculated that the deficiency of ABCC6 activity induces a persisting imbalance in certain circulating metabolite(s) that may indirectly impair the synthetic ability of matrix-producing cells or specifically alter the initial assembly of elastic fibers, thereby compromising their durability and function.

In this study, we compared the deposition of major elastic fiber components and associated gene expression in primary cultures of skin fibroblasts isolated from unaffected individuals and PXE patients maintained in the presence of fetal bovine serum (FBS), serum from normal individuals, or from PXE patients. Our results indicated that although PXE fibroblasts demonstrated elevated expression of genes encoding elastic fiber-associated proteins, these cells deposited structurally normal elastic fibers, when cultured in the presence of FBS or normal human sera. However, we found that the addition of sera from a PXE patient to the culture medium promoted the deposition of abnormal elastic fibers, not only in PXE fibroblasts but also in normal fibroblasts and normal human aortic smooth muscle cells. Our data clearly suggested that unknown circulating metabolites (or the lack thereof) modulated by ABCC6 contribute to the development of the PXE phenotype.

RESULTS

PXE-derived fibroblasts produce normal elastic fibers when cultured with FBS

Immunostaining of 10-day-old cultures with antibodies specific to elastin and fibrillin 1 revealed that PXE fibroblasts maintained in the presence of 10% FBS produced an abundant network of elastic fibers (Figure 1b and d). The distribution pattern of these elastic fibers did not appreciably differ from those observed in cultures of normal cells (Figure 1a and c). The distribution patterns of immunodetectable fibronectin and chondroitin sulfate-containing moieties produced by PXE cells (Figure 1f and h) and normal fibroblasts (Figure 1e and g) were also similar. Additional immunostainings with antibodies specific to collagen type I, elastin-binding protein, biglycan, and versican did not demonstrate any significant differences in the distribution pattern of these ECM components between cultures of normal and PXE fibroblasts (data not shown).

Morphometric analysis (Figure 2) and statistical evaluation of immunodetected ECM components revealed that amounts of elastin, fibrillin-1, microfibril-associated glycoprotein-1 (MAGP-1), and collagen type I deposited by PXE fibroblasts cultured in the presence of FBS significantly exceeded those present in normal fibroblast cultures. Indeed, PXE cells demonstrated more elastin (+45%), fibrillin-1 (+30%), MAGP-1 (+25%), and collagen type I (+15%) than normal fibroblasts. In contrast, PXE fibroblasts seemed to produce less fibronectin (−15%) and matrix components containing chondroitin sulfate (−25%).

To determine whether the immunodetectable elastin associated with the extracellular fibrillar network represented mature crosslinked elastin, metabolic labeling of cultures with [^{3}H]valine followed by an assay of NaOH-insoluble elastin was used. Our results demonstrated that amounts of deposited insoluble elastin were on average increased by 41% ($P<0.01$) in PXE fibroblast cultures as compared to normal cells (Figure 3). These results were consistent with the immunofluorescence morphometric analysis.

RT-PCR experiments were performed with TaqMan probes specific to *tropoelastin*, *fibrillin-1*, *MAGP-1*, *lysyl oxidase (LOX)*, *LOX-like* (*LOXL*), and *collagen type I* to establish whether the changes resulted directly from increased gene expression. All the tested genes were found to be significantly upregulated ($P<0.01$; Figure 4), except for *LOXL*, which presented an expression level similar to normal fibroblasts. The expression of *tropoelastin* showed the greatest increase (ninefold), while that of *fibrillin-1*, *LOX*, and *collagen type I* displayed moderate upregulation (2–3.5-fold). The level of

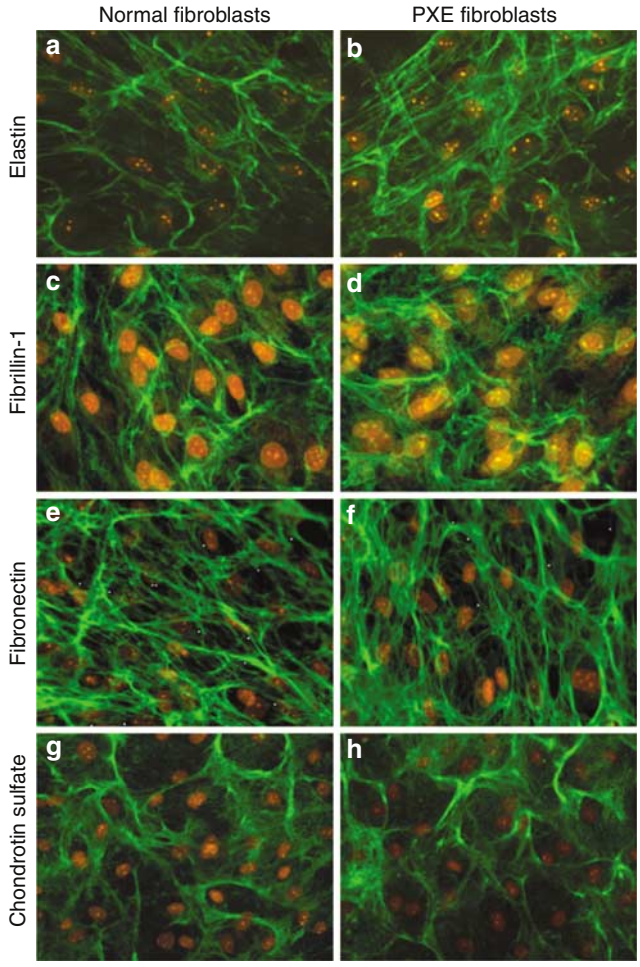

Figure 1. Extracellular matrix components deposited by control and PXE fibroblasts. Fibroblasts were derived from normal and PXE patients and were cultured in medium supplemented with 10% FBS. Fibroblasts were grown for 10 days and the deposited matrix network was revealed by immunofluorescence using antibodies specific to (**a**, **b**) elastin, (**c**, **d**) fibrillin-1, (**e**, **f**) fibronectin, and (**g**, **h**) chondroitin sulfate-containing glycoaminoglycan. Representative photomicrographs are presented. (**b**) No structural difference in the matrix network was visible between normal and PXE cells, except for the overabundance of elastin. Nuclei were counterstained with propidium iodide to reveal cell density.

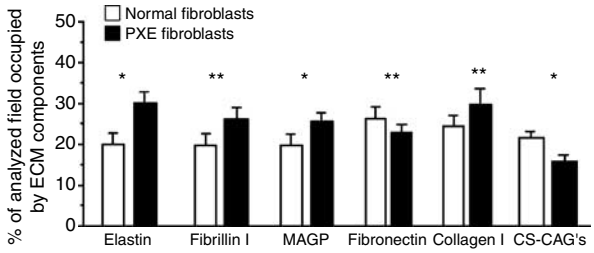

Figure 2. Extracellular matrix components detected by immunohistochemistry. 10-day-old cultures of normal and PXE fibroblasts supplemented with 10% FBS were used. The morphometric analysis of extracellular matrix components was performed using Image-Pro Plus software from Media Cybernetics. MAGP: microfibril-associated glycoprotein-1, CS-GAG: chondroitin sulfate-containing glycoaminoglycan. Each ECM group was analyzed from three separate cultures of three normal and three PXE fibroblast cell lines, and the area occupied by the particular immunodetectable component was quantified. The abundance of each component was then expressed as a percentage of the entire analyzed field (mean ± SD (*$P<0.002$; **$P<0.05$).

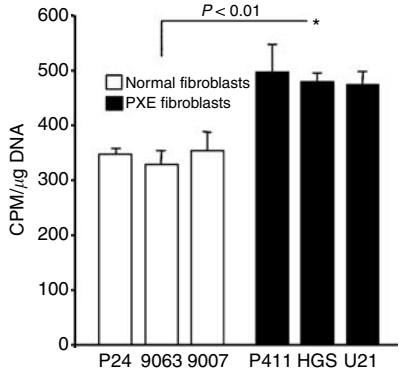

Figure 3. Quantitative analysis of insoluble elastin. The total insoluble elastin labeled with [^{3}H]valine and produced by three normal skin fibroblasts (□) and three PXE skin fibroblasts (■) after 10 days of cultures was quantified. PXE cells deposited on average 41% more insoluble elastin than their unaffected counterparts in the presence of 10% FBS in the culture medium. Values of mean ± SD from four different experiments were collected for statistical evaluation (*$P<0.01$).

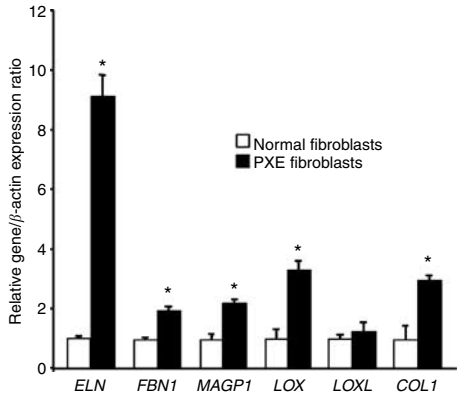

Figure 4. Extracellular matrix gene expression in normal and PXE fibroblasts. The fibroblasts were cultured in medium supplemented with 10% FBS. Total RNA was extracted after 10 days of culture and was reversed transcribed. Real-time PCRs were performed using TaqMan probes (Applied Biosystems) specific for tropoelastin (ELN), fibrillin-1 (FBN-1), microfibril-associated glycoprotein-1 (MAGP-1), lysyl oxidase (LOX), lysyl oxidase-like (LOXL), collagen type I (COL1), and the β-actin cDNAs. Units are ratio of the relative gene expression to β-actin and are shown normalized to expression in normal fibroblasts. Standard errors are indicated ($n=3$, *$P<0.01$).

expression clearly corroborated the increase in deposition of these proteins as detected by immunofluorescence and metabolic labeling.

Since the age of donors from whom normal and PXE fibroblasts were derived could only be matched approximately (average 39 ± 18 years), the level of tropoelastin gene expression and the total insoluble elastin output was verified and no significant variation was observed between our samples (Figure 3).

PXE serum induces abnormal elastogenesis in cultures of normal and PXE fibroblasts

To test the hypothesis that deficiency in ABCC6 transporter activity causes an imbalance in certain circulating factors that modulate the production or durability of elastic fibers, we cultured normal and PXE fibroblasts in media supplemented with human serum derived either from unaffected individuals or from PXE patients. The expression of the relevant genes and the deposition of elastic fiber-related proteins were evaluated with the same methods as those used for cultures in the presence of FBS. Immunohistochemical assessment of 10-day-old cultures demonstrated that dermal fibroblasts derived either from normal individuals or from PXE patients deposited normal elastic fiber network when maintained in the presence of 10% normal human serum (Figure 5a and b). However, the deposition of elastic fibers by normal and PXE fibroblasts was dramatically altered when the media were supplemented with 10% serum from PXE patients (Figure 5c–h). Fibroblasts exposed to PXE serum produced fewer and thinner elastic fibers than those maintained with FBS or normal human serum. Interestingly, the reduction in quantity and thickness of normal elastic fibers in PXE serum-treated cultures seemed to be correlated, to some extent, with the appearance of amorphous elastin aggregates protruding from the fibers, as illustrated in Figure 5.

These alterations of elastic fibers were consistently observed in cultures treated with all PXE serum samples tested, but were not mineralized, as determined by von Kossa staining (data not shown).

Electron microscopy analysis revealed that PXE sera seemed to affect the early steps of elastic fiber formation probably by interfering with the formation of the microfibrillar scaffold. Indeed, 5-day-old cultures of normal skin fibroblasts cultured with normal human serum produced long and parallel microfibrils (Figure 6a), while fibroblasts maintained with PXE serum deposited irregular and fragmented microfibrils (Figure 6b). Electron micrographs of 10-day-old cultures maintained in the same conditions further demonstrated that cultures treated with PXE sera contained abnormally fragmented fibers in which elastin appeared to aggregate on the periphery of an irregularly assembled microfibrillar scaffold (Figure 6c and d).

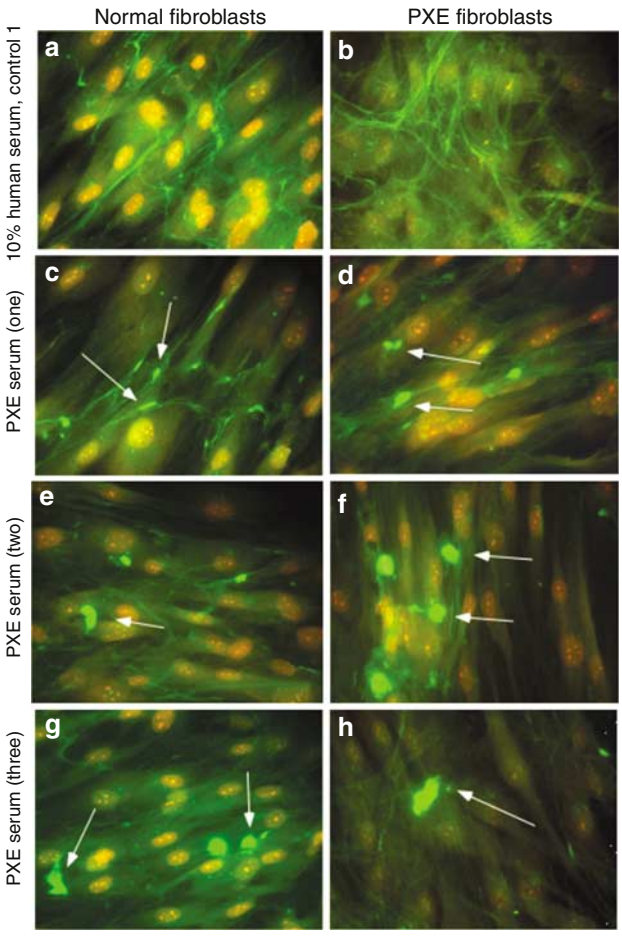

Figure 5. Immunofluorescent labeling of elastin deposited by normal and PXE fibroblasts. The dermal fibroblasts derived from normal and PXE patients were cultured in medium supplemented with 10% serum samples from normal individuals or PXE patients. Fibroblasts were grown for 10 days and the network was revealed by immunofluorescence using an anti-elastin antibody. Representative photomicrographs of normal and PXE fibroblasts grown in normal serum are shown in panels a and b, respectively, while panels c–h represent three different PXE serum samples. Arrows point to large abnormal deposits of immunodetectable elastin. Nuclei were counterstained with propidium iodide to reveal cell density.

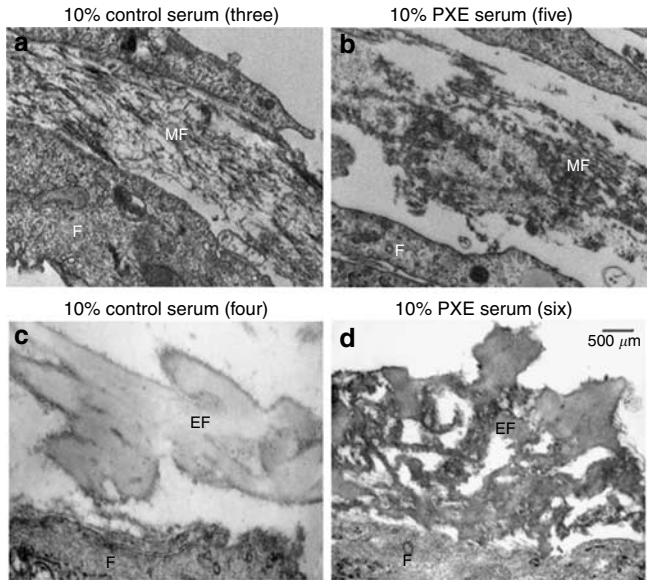

Figure 6. Representative electron micrographs of cultures of skin fibroblasts maintained in 10% human sera. Upper panel: 5-day-old cultures of normal skin fibroblasts grown with (**a**) normal sera and (**b**) from a PXE patient. While cells maintained in the presence of normal human serum produce normal microfibrills, fibroblasts treated with PXE-patient serum deposited irregular and fragmented microfibrils. Lower panel: electron micrographs showing 10-day-old cultures of normal skin fibroblasts maintained in sera from (**c**) unaffected individuals and (**d**) from a PXE subject. Cells maintained with normal serum deposit well-assembled elastic fibers, but cultures treated with PXE serum contain abnormal and fragmented fibers on an irregularly assembled microfibrillar scaffold. MF: microfibrils, EF: elastic fiber, F: fibroblasts. Image scale is indicated on panel d.

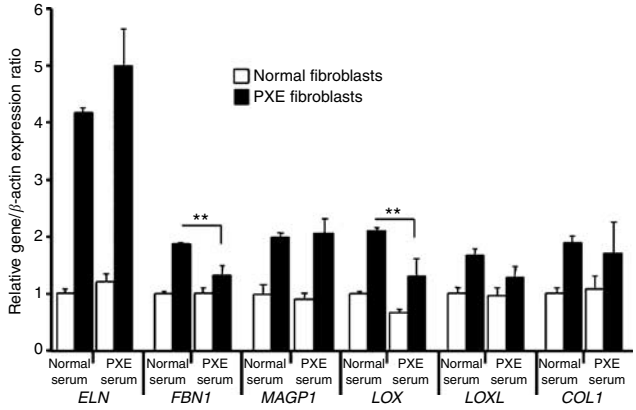

Figure 7. Extracellular matrix gene expressions in normal and PXE fibroblasts. The fibroblasts were cultured in medium supplemented with 10% normal or PXE serum. Total RNA was extracted after 10 days of culture and was reversed transcribed. Real-time PCRs were performed using TaqMan probes (Applied Biosystems) specific for tropoelastin (ELN), fibrillin-1 (FBN-1), microfibril-associated glycoprotein-1 (MAGP-1), lysyl oxidase (LOX), lysyl oxidase-like (LOXL), collagen type I (COL1), and the β-actin cDNAs. Units are ratio of the relative gene expression to β-actin and are shown normalized to expression in normal fibroblasts. Standard errors are indicated ($n=3$, $^{**}P<0.05$).

Results of metabolic labeling with [^{3}H]valine, followed by assay of insoluble elastin, also demonstrated that cultures of PXE fibroblasts deposited more insoluble elastin than normal fibroblasts (+66%, $P<0.01$) when maintained in media supplemented with normal human serum. Addition of serum from PXE patients to culture media produced a similar trend (+91%, $P<0.01$) in both experimental groups (normal *vs* PXE fibroblasts); however, the stimulation of insoluble elastin deposition in the presence of PXE serum was slightly lower than that induced by normal human serum (data not shown).

Results of gene expression studies performed with RNA extracted from fibroblast cultures confirmed that PXE fibroblasts expressed significantly more *tropoelastin* than normal cells when maintained with normal human serum, and that the presence of PXE serum had no significant effect on mRNA synthesis. Similar results were obtained with *MAGP-1* and *collagen type I* expression. However, *fibrillin 1* and *LOX* showed a moderate decrease when PXE fibroblasts were exposed to PXE serum (Figure 7). Interestingly, after transient transfection with a vector carrying the *ABCC6*

cDNA to test whether the observed phenotype could be rescued, we found that overexpressing *ABCC6* in normal and PXE fibroblasts had no influence on the level of expression of *tropoelastin* and the other elastic fiber-associated gene (data not shown) regardless of the type of serum used (FBS, normal or PXE). These results suggested that ABCC6 function in fibroblasts has little or no influence on elastic fiber production.

PXE serum induces abnormal elastogenesis in cultures of normal smooth muscle cells

Since PXE is also characterized by calcification of elastic fibers in vascular tissues, we tested whether the presence of PXE serum would affect production of elastic fibers in cultures of smooth muscle cells (SMC) derived from normal human aorta. The results demonstrated that normal human serum stimulated the production of normal elastic fibers, whereas the presence of PXE serum grossly impaired the deposition of extracellular elastin by these normal SMC (Figure 8). The SMC cultures maintained with PXE serum showed elastin aggregates identical to those observed in cultures of dermal fibroblasts. It is noteworthy that normal SMC express higher amount of *ABCC6* mRNA than skin fibroblasts (Beck *et al.*, 2003) and yet displayed the same response to PXE serum.

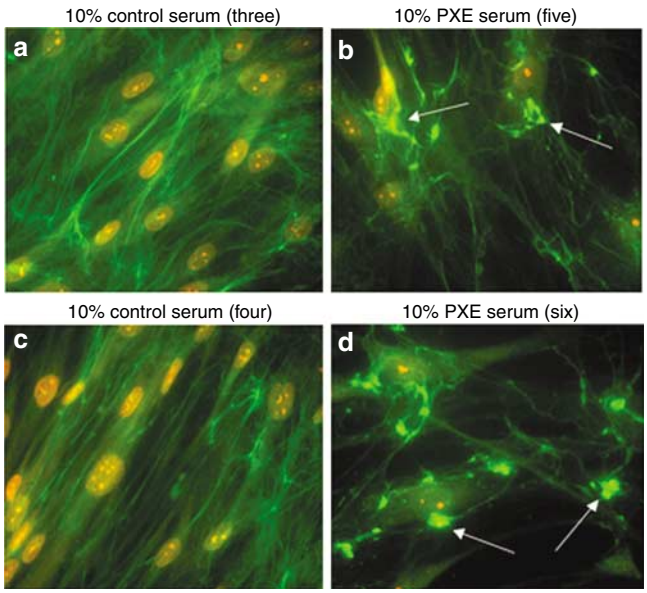

Figure 8. Immunofluorescent labeling of elastin deposited by aortic smooth muscle cells. The smooth muscle cells were derived from a normal 6-year-old individual in culture medium supplemented with 10% serum samples from normal individuals or PXE patients. The cells were grown for 5 days and the network was revealed by immunofluorescence using an anti-elastin antibody. Representative pictures of smooth muscle cells grown in normal serum are shown in panels a and c, respectively, while panels b and d represent cells cultured with PXE serum samples. Arrows point to large abnormal deposits of immunodetectable elastin. Nuclei were counterstained with propidium iodide.

DISCUSSION

ABCC6 as several other ABC transporters of the same subfamily ''C'' are thought to actively export metabolites from the basolateral or apical sides of polarized cells (Madon *et al.*, 2000; Beck *et al.*, 2003). ABCC6 is particularly abundant in renal proximal tubules and hepatocytes (Madon *et al.*, 2000; Beck *et al.*, 2003) and is also present in numerous epithelial cell types (Beck *et al.*, 2005). Remarkably, nonpolarized cells such as arterial SMC, dermal fibroblasts, and other connective tissue cells that are capable of elastic fiber synthesis, present moderate or low levels of ABCC6 expression (Gorgels *et al.*, 2005). However, the basic functions of these cells seem to be significantly affected by *ABCC6* deficiency and could be responsible for the major phenotypic changes observed in PXE. However, the endogenous substrates of ABCC6 are presently not known and the pathomechanism underlying the PXE phenotype is only a matter of speculation. Indeed, it has been proposed that ABCC6 deficiency in affected skin, notably in dermal fibroblasts, could directly promote the deposition of an aberrant extracellular matrix, including abnormal elastic fibers (Beck *et al.*, 2003; Boraldi *et al.*, 2003). An alternate hypothesis stated that ABCC6 could instead be involved in a detoxification process in liver and/or kidney, and that in the absence of a functional transporter certain compounds accumulate or are depleted, resulting in the progressive fragmentation and systemic calcification of elastic fibers (Uitto *et al.*, 2001). If indeed the lack of ABCC6 in kidneys or liver indirectly influences elastic fibers in the dermis, arteries, or ocular tissues, then certain metabolites must be circulating to promote the systemic alteration of extracellular matrix (Gheduzzi *et al.*, 2003) observed in PXE patients. The experiments reported here were carried out to specifically evaluate these hypotheses.

In this study, we investigated the effects of serum samples derived from PXE subjects and from unaffected individuals on the deposition of extracellular matrix components in cultures of normal and PXE-derived dermal fibroblasts. All tested fibroblasts were derived from lesional skin biopsies of different PXE patients and from equivalent skin areas of unaffected donors. Results of immunohistochemistry, metabolic labeling, and gene expression studies showed that PXE fibroblasts cultured in medium supplemented with FBS produced an abundant extracellular matrix characterized by the presence of a structurally normal network of elastic fibers (Figures 1–3). The increased deposition of major elastic fiber components as well as other ECM molecules by PXE fibroblasts was associated with an overall increase in ECM gene expression, with the notable exception of *LOXL* (Figure 4). The high level of expression of these genes *in vitro* suggested a permanent phenotypic shift of PXE fibroblasts that appeared independent of the environment of the cells. Interestingly, the *LOXL* gene that encodes an enzyme essential for elastic fiber homeostasis (Maki *et al.*, 2002; Hornstra *et al.*, 2003; Liu *et al.*, 2004) was not elevated in PXE fibroblasts. This would suggest that these cells produced an under-crosslinked elastin, and thus less durable fibers that could possibly be responsible for the abnormal PXE phenotype, as suggested previously (Baccarani-Contri *et al.*,

1996; Bacchelli *et al.*, 1999; Quaglino *et al.*, 2000). However, our experiments showed that PXE fibroblast produced fully polymerized elastin. This fact would suggest that, despite their apparent abnormal *in vitro* phenotype, PXE fibroblasts might only play a limited role towards the elastic fiber defects observed in PXE patients. In addition, the presence of PXE serum equally resulted in the abnormal production of elastic fibers in cultures of normal and PXE fibroblasts and normal SMC. Thus, it appears that the impaired elastogenesis occurring in these conditions is independent of the overproduction of elastic fiber components observed in PXE fibroblasts and the expression of ABCC6, as shown by the rescue experiments.

The current understanding of elastic fiber formation in the pericellular space indicates that the secreted tropoelastin molecules must be assembled on a preformed microfibrillar scaffold in order to be crosslinked into insoluble and resilient elastin by LOX (Bedell-Hogan *et al.*, 1993). The major component of this scaffold is fibrillin-1, which assembles into head-to-tail periodically beaded structures (Kielty *et al.*, 2002; Rock *et al.*, 2004). MAGP-1 is thought to bind the beaded regions of the fibrillin-containing microfibrils and subsequently promote the accretion of tropoelastin (Clarke and Weiss 2004). The microfibrilar scaffold thus critically influences tropoelastin deposition by providing the foundation and organization of mature elastic fibers (Sherratt *et al.*, 2003). One can then reasonably assume that any defect in the microfibrils assembly is very likely to alter the normal formation of elastic fibers. Indeed, mutations in the fibrillin 1 gene lead to Marfan syndrome (Dietz and Pyeritz, 1995).

Consistent with this assumption, our results indicated that the abnormal elastic fibers we observed were unlikely to result from the insufficient supply of one or more of the elastic fiber components or crosslinking activity. More specifically, the ultrastructural examination of the abnormal elastic fibers suggested that a defective microfibrilar scaffold (Figure 6b) was responsible for the observed alterations. Indeed, one can speculate that the secreted tropoelastin molecules would be unable to assemble properly along the disorganized microfibrils and would be forced to aggregate into the amorphous structures we observed. It is interesting to note that the deposited elastin was crosslinked as efficiently as in controls, indicating that the secreted tropoelastin molecules assumed a structure compatible with the progressive crosslinking activity of LOX (Bellingham *et al.*, 2001) even in the absence of orderly assembly on the microfibrils. We propose that such aggregates containing crosslinked but improperly anchored elastin are reminiscent of the structurally invalid elastic fibers observed in PXE patients, even though these structures were not calcified in the experimental conditions we used. The lack of mineralization was not unexpected, since the cell culture media were not favorable to mineralization (Sugitani *et al.*, 2003) and also because samples of serum from PXE patients contained levels of calcium and phosphate within the normal range. Moreover, it is known that mineralization of elastic fibers does not occur on early stages of their assembly (Davis 1993) and that, in PXE patients as well as in mouse models of PXE, the mineralization of elastic fibers occurs progressively in the second decade of their life (Neldner 1988) or at about 3–5 weeks of age (Gorgels *et al.*, 2005; Klement *et al.*, 2005).

The overall clinical manifestations of the PXE phenotype suggest that the lack of ABCC6 function relates to deterioration of elastic fibers. Although the *in vitro* conditions of our experiments do not reflect *in vivo* conditions, results presented in this report showed unambiguously that metabolites in the serum of PXE patients interfered with the initial assembly of elastic fibers produced by normal human skin fibroblasts and normal aortic SMC. It is the first indication that the pathology of PXE derives from the defective modulation of circulating factors by ABCC6 transport activity probably in kidney and/or liver.

We hope that this report will initiate a series of comprehensive analytical studies that may eventually identify these serum factors and precisely determine the level of their action during the complicated process of elastic fiber formation. We also anticipate that the identification of factors interfering with elastic fiber assembly would lead to the development of therapeutic measures alleviating the symptoms of this still puzzling disease.

MATERIALS AND METHODS

Fibroblasts, smooth muscle cells, and serum samples

Primary fibroblasts were derived from biopsies of affected skin from three PXE patients and equivalent biopsies from three unaffected individuals (Table 1). These skin samples were obtained with the patients' consent and were generous gifts of Drs F.M. Pope, Z. Urban, and from PXE International, Inc. All donors were females, except one male PXE patient. The dates of birth of five patients and unaffected subjects were known, whereas the ages of two subjects could not be determined. The smooth muscle cells were derived from a single 6-year-old female without evidence of pathology. The serum samples were collected with informed consents of the donors and were prepared by centrifugation from coagulated blood collected in tubes with no additives. The serum samples were subsequently frozen and stored at −20°C. For the experiments, both individual and pooled serum samples were used in separate cultures. The institutional review boards of the participating Universities or institutions approved the collection and use of these samples and experiments adhered to the Declaration of Helsinki Principles. The *ABCC6* genotype of fibroblasts and serum donors was determined according to published criteria and methods (Le Saux *et al.*, 2001; Chassaing *et al.*, 2004).

Cell cultures

The fibroblasts derived from the skin biopsies were routinely passaged by trypsinization. All experiments were performed with skin fibroblasts at passages 4–6. Cells were maintained in DMEM containing 1% antibiotics/antimycotics and supplemented with either 10% FBS from GIBCO Life Technologies (Burlington, ON) or 10% heat-inactivated serum from unaffected subjects or PXE patients. Transient transfections were performed using the GeneJammer transfection reagent (Stratagene, La Jolla, CA) as described by the manufacturer. Briefly, cells were plated in 24-well plates at 2×10^5 cells per well and incubated at 37°C until 80% confluence. Cells were rinsed once with serum-free medium without antibacterial

Table 1. Fibroblast cells and serum samples

Cells	Diagnosis	Mutations	Age (years)
Fibroblast P24 (F)	Normal	wt/wt	54
Fibroblast 9063 (F)	Normal	wt/wt	25
Fibroblasts 9007 (F)	Normal	wt/wt	?
Aortic SMC (F)	Normal	wt/wt	6
Fibroblast P411 (F)	PXE	IVS8+2delTG/del23-29	44
Fibroblast HGS (F)	PXE	?/?	21
Fibroblast U21 (M)	PXE	R1141X/R1141X	?
Serum samples			
Control #1 (M)	Normal	wt/wt	45
Control #2 (M)	Normal	wt/wt	34
Control #3 (pool)[1]	Normal	wt/wt	Av. 68
Control #4 (pool)[1]	Normal	wt/wt	Av. 18
PXE 1 (F)	PXE	R391G/del23-29	50
PXE 2 (F)	PXE	IVS21+1 G>T/4104delC	51
PXE 3 (F)	PXE	IVS13-29 T>A/R1141X	41
PXE 4 (M)	PXE	?/?	52
PXE 5 (F)	PXE	?/?	69
PXE 6 (M)	PXE	Y768X/Y768X	18

[1]Control #3: pool of 10 samples, age >65, control #4: pool of 15 samples age <30. Av.: average, SMC: smooth muscle cell.

agents before transfection. Cells were then transfected with 1 μg of pCDNA3.1 vector carrying the *ABCC6* cDNA. After 3 hours, the transfection mixture was removed and replaced with complete growth medium. After 72 hours, cells were harvested for RNA extraction.

Immunostaining

PXE and normal human fibroblasts were plated in six-well plates (6 cm^2/well) at an initial level of 100,000 cells/dish and subsequently maintained in medium supplemented with appropriate serum for 10-days. Each well contained a 4 cm^2 glass coverslip that was subsequently used for immunostainings. Media were changed every 72 hours (at days −4 and −7). After 10 days of culture, confluent cells were fixed in cold 100% methanol at −20°C for 30 minutes and blocked with 1% normal goat serum for 1 hour at room temperature. Cultures used for immunohistochemical assessment of microfibrillar components (MAGP-1) were fixed in 0.5% paraformaldehyde for 15 minutes, treated with phosphate-buffered saline containing 50 mM of dithiothreitol for an additional 10 minutes, and alkylated with 100 mM iodoacetamide for 15 minutes. Finally, these cultures were blocked in phosphate-buffered saline containing 0.1 M ammonium chloride. The cells were then incubated for 1 hour with different antibodies: 20 μg/ml of specific polyclonal antibodies recognizing tropoelastin (Elastin product, Owensville, MI), fibrillin-1 (Biomeda Corp., Foster City, CA), MAGP-1 (Elastin product, Owensville, MI), collagen type I (generous gift of Dr Larry W. Fischer from The National Institute of Health, Bethesda, MD), and elastin-binding protein (Hinek *et al.*, 1993), as well as with 10 μg/ml of monoclonal antibodies to fibronectin (Chemicon, Temecula, CA), chondroitin sulfate (Sigma, St Louis, MO), and LOX (gift from Dr Csiszar to AH). All cultures were then incubated for an additional hour with the appropriate fluorescein-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Sigma, St Louis, MO). Nuclei were counterstained with propidium iodide. For negative controls the same procedure was followed, but the primary antibody was omitted. All cultures were then mounted in elvanol, and examined with a Nikon Eclipse E1000 microscope. The images were obtained with a cooled CCD camera (QImaging, Retiga EX). The morphometric analysis of extracellular matrix components was then performed using Image-Pro Plus software from Media Cybernetics (Silver Springs, MD) as described previously (Hinek *et al.*, 2000).

RNA expression studies

Fibroblasts were cultured for 10 days in the same conditions as those used for immunostaining, except for transient transfection assays. RNA was extracted using TRI reagent. Reverse transcription of total RNA (1 μg) from fibroblasts was performed using the Superscript first-strand synthesis kit (Invitrogen, Carlsbad, CA) and random hexamer primers. The generated cDNA was subsequently used as template for real-time PCR amplification with a DNA Engine Opticon 2 (MJ Research/Bio-Rad, Waltham, MA). TaqMan probes specific for *beta-actin*, *tropoelastin*, *fibrillin-1*, *MAGP-1*, *LOX*, and *LOXL and collagen type I* were purchased from Applied Biosystems (Foster City, CA). The PCR cycling conditions started with two incubation steps at 50°C for 2 minutes and 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute with plate read.

Insoluble elastin assay

In each experiment, fibroblasts from unaffected individuals and PXE patients were densely plated in 60-mm culture dishes (100,000 cells/dish) to quickly reach confluence. After 7 days in culture, an aliquot of 20 μCi of [^{3}H]valine (Amersham Canada Ltd, Oakville, ON) was added to fresh media. Cultures were then incubated for the next 3 days. Quadruplicate cultures, 10-day-old, belonging to each experimental group were terminated and insoluble elastin was assessed as described previously (Hinek and Wilson, 2000). After the removal of the media, cell layers containing insoluble elastin deposited in ECM were washed in 0.1 M acetic acid, then scraped in 0.1 N NaOH, sedimented by centrifugation, and boiled in 0.5 ml of 0.1 N NaOH for 45 minutes to dissolve all matrix components except elastin. The resulting pellets containing the insoluble elastin were then dissolved by boiling in 200 μl of 5.7 N HCl for 1 hour, and the aliquots were mixed with scintillation fluid and counted. Aliquots taken from each culture were also used for DNA determination using the DNeasy Tissue System from Qiagen (Valencia, CA). Final results reflecting amounts of metabolically labeled insoluble elastin were expressed as CPM/μg DNA.

Electron microscopy

Cultures of human fibroblasts, 5- and 10-day-old, maintained with serum from normal individuals and from PXE patients were prepared for electron microscopic examination. The cells were fixed in the presence of 2% glutaraldehyde dissolved in 0.1 M cacodylate buffer containing 0.25% tannic acid and post-fixed with 1% osmium tetroxide in the same buffer. The samples were then dehydrated in ethanol and embedded in Spurr low-viscosity resin, which assures high contrast of elastin even when thin sections are stained with uranyl acetate and lead citrate (Hinek *et al.*, 1976).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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