Oxidative stress in fibroblasts from patients with pseudoxanthoma elasticum: possible role in the pathogenesis of clinical manifestations††

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Abstract

Pseudoxanthoma elasticum (PXE) is a genetic disease characterized by calcification and fragmentation of elastic fibres of the skin, cardiovascular system and eye, caused by mutations of the \(ABCC6\) gene, which encodes the membrane transporter MRP6. The pathogenesis of the lesions is unknown. Based on studies of similar clinical and histopathological damage present in haemolytic disorders, our working hypothesis is that PXE lesions may result from chronic oxidative stress occurring in PXE cells as a consequence of MRP6 deficiency. Our results show that PXE fibroblasts suffer from mild chronic oxidative stress due to the imbalance between production and degradation of oxidant species. The findings also show that this imbalance results, at least in part, from the loss of mitochondrial membrane potential (\(\Delta\psi_m\)) with overproduction of \(H_2O_2\). Whether mitochondrial dysfunction is the main factor responsible for the oxidative stress in PXE cells remains to be elucidated. However, mild chronic generalized oxidative stress could explain the great majority of structural and biochemical alterations already reported in PXE.

Keywords: fibroblast; pseudoxanthoma elasticum (PXE); MRP6; oxidative stress; pathogenesis; elastin mineralization; connective tissue; extracellular matrix

Introduction

Pseudoxanthoma elasticum (PXE) is a genetic disorder characterized by cutaneous, retinal, and cardiovascular lesions, due to progressive calcification and fragmentation of elastic fibres [1,2]. The gene involved is the ATP-binding cassette subfamily C member 6 (\(ABCC6\)), which belongs to the MRP family of membrane transporters [3,4]. More than 80 mutations have been identified and the great majority of these are located in the intra-cytoplasmic C-terminal of the protein MRP6 close to the second Walker motif implicated in the ATP hydrolysis necessary for the activity of this transporter [5,6].

The physiological function of MRP6 is unknown and the pathogenesis of the clinical manifestations in PXE still remains unsolved. From studies on transfected cells [7] and in vitro cultured normal human fibroblasts [8], MRP6 appears to be involved in the extrusion of chemicals from the cell, similarly to other members of the MRP family, although with a different sensitivity to inhibitors and/or competitors compared to other membrane transporters [8].

Interestingly, some individuals affected by inherited haemolytic disorders, such as \(\beta\)-thalassaemia, manifest lesions very similar to those found in PXE [9–11] without any alteration of the \(ABCC6\) gene [12]. The great majority of alterations in these haemolytic disorders have been attributed to the effects of oxidative stress [13,14].

The possibility thus exists that the similarity of the clinical and histopathological lesions in \(\beta\)-thalassaemia and PXE patients may result from oxidative stress occurring in PXE cells as a consequence of MRP6 deficiency. The aim of this research was to explore this hypothesis.

Methods

Cell culture

Dermal biopsies were obtained, after informed consent and approval by the Ethics Committee of the local Faculty of Medicine, from the neck or axilla of 10 control women (age 41 ± 9 years) who underwent surgical treatment for breast cancer and 10 women
affected by PXE (age 44 ± 10 years). PXE patients were diagnosed by skin and eye clinical manifestations and the diagnosis was confirmed by the identification of mutations in the ABCC6 gene [5]. Fibroblasts were used between the third and the eighth passage [15].

**Metabolic assays**

Confluent fibroblasts were trypsinized and suspended in 220 mM mannitol, 70 mM sucrose, 5 mM MOPS, 2 mM EGTA, pH 7.4, at 37°C to a final concentration of 10–15 × 10⁶ cells/ml.

**Cell respiration**

Cells were suspended at 37°C in 139 mM NaCl, 5.5 mM KCl, 5.5 mM KH₂PO₄, 2.8 mM MgSO₄, 6.7 mM HEPES, pH 7.4, to a final concentration of 10–15 × 10⁶ cell/tube and the oxygen uptake was measured using a Clark oxygen electrode at 37°C. In a number of experiments, cells were permeabilized with digitonin (Sigma, St. Louis, MO, USA) (40 µg/1 × 10⁶ cells) for 10 min at 37°C.

Modifications of respiratory state were induced according to Chance and Williams [16]. Endogenous oxygen uptake was measured in the absence of added substrates; respiratory state 4 was reached by addition of 3 mM sodium succinate or 5 mM pyruvate/malate; respiratory state 3 was induced by addition of 1–3 mM ADP for 3 min. Oligomycin (Sigma; 5 µg/ml), valinomycin (Sigma; 2.7 µM) and FCCP (carbonylcyanide-p-trifluoromethoxyphenylhydrazone) (Sigma; 0.7 µM) were used in some experiments. Mitochondrial oxygen uptake was inhibited by 1 mM KCN.

Each experiment was performed in triplicate by using pools of cells derived from five to seven controls and PXE patients.

**Adenine nucleotide determination**

Cell suspensions were treated with equal volumes of 0.6 N perchloric acid at 0°C. The precipitate was sedimented by centrifugation at 15 000 × g for 15 min and the supernatant was neutralized with a solution containing 2 M K₂CO₃ and 0.5 M triethanolamine.

and the supernatant was neutralized with a solution containing 2 M K₂CO₃ and 0.5 M triethanolamine. The adenylate energy charge was calculated according to Atkinson [18].

**Mitochondrial membrane potential**

Mitochondrial membrane potential (ΔΨ) was estimated from the red : green ratios (FL2/FL1) of the cationic probe JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide; Sigma) [19,20]. Cell suspensions, diluted with the medium used for the respiration assays to a final concentration of 1 × 10⁶ cells/ml, were treated with digitonin (10 µg/1 × 10⁶ cells) and incubated with 3.8 µM JC-1 for 10 min at room temperature in the dark. Modifications of the mitochondrial membrane potential were induced by varying the respiratory state [16]. The fluorescence variations were recorded with an EPICS-XL flow cytometer (Beckman-Coulter, CA, USA) equipped with a single 488 nm argon laser (15 mW).

Green fluorescence was detected through the standard band-pass filter centred at 525 ± 20 nm (FL1); red fluorescence was detected through the long pass filter at 575 ± 15 nm (FL2). A minimum of 1 × 10⁴ cells/sample was acquired in list mode and analysed using System II v. 3.0 software (Beckman-Coulter).

**Estimation of intracellular ROS levels**

The intracellular levels of reactive oxygen species (ROS) were estimated by flow cytometry using a dihydroethidium probe (DH2; Molecular Probes, Eugene, OR) [21]. Confluent fibroblasts were treated with 1 µM DH2 for 60 min at 37°C, washed and analysed for red fluorescence using a 610 nm long pass filter. The effect of vitamin E was assayed by incubating confluent cells for 20 h at 37°C with either 125 µM or 500 µM vitamin E before treatment with DH2.

**Antioxidant enzyme activity**

Cells were suspended in 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM PMSF, kept at ~0°C for 20 min and homogenized in the presence of 0.01% digitonin. Enzyme activities were determined on the supernatant after removal of cell debris and nuclei by centrifugation at 15 000 × g for 30 s at 4°C [22]. Spectrophotometric measurements were performed using a Cobas Mira (ABX Diagnostics, Montpellier, France).

Superoxide dismutase (SOD; E.C.1.15.1.1) activity was determined at 37°C using the commercial kit ‘Ransod’ (Randox, UK). Cu–Zn-SOD activity was differentiated from Mn-SOD on the basis of its sensitivity to 3 mM sodium cyanide.

Catalase (E.C.1.11.1.6) activity was determined by incubating samples for 1 min at 37°C in 66 mM phosphate buffer solution, pH 7.4, and 65 µM H₂O₂ [23]. The reaction was stopped with 32.4 mM ammonium molybdate and the molybdate–H₂O₂ complex was measured at 405 nm. One unit of catalase activity represented the decomposition of 1 µmol H₂O₂ in 1 min at 37°C.

**Oxidative stress in PXE**
Glutathione reductase (E.C.1.6.4.2) activity was measured using the commercial ‘Glutathione Reductase’ kit (Randox, UK). One activity unit was defined as the oxidation of 1 µmol NADPH to NADP/min at 37°C.

Glutathione peroxidase (E.C.1.11.1.9) activity was determined at 37°C using the commercial kit ‘Ransel’ (Randox, UK). One activity unit was defined as the oxidation of 1 µmol NADPH to NADP/min at 37°C.

**GSH and GSSG determination**

GSH and GSSG content was measured in a deproteinized cell extract using a Hewlett-Packard 1090 HPLC equipped with a diode-array detector. Aliquots of 2 × 10⁶ cells were centrifuged at 150 × g for 10 min and resuspended with 250 µl 1% picric acid containing 0.5 mM Desferal® (Novartis, Basel). After centrifugation at 2000 × g for 10 min, 100 µl acidic protein-free supernatants were derivatized for HPLC measurements. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured at 357 nm against a standard curve in the range 0.5–5.0 and 0.25–2.5 nmol GSH and GSSG (Sigma), respectively.

**MDA determination**

Malondialdehyde (MDA) was determined by HPLC on cell extracts [24].

**Cellular total antioxidant state (TAS)**

The total antioxidant capacity of cells was estimated using the commercial kit ‘Total Antioxidant Status’ (Randox, UK), measuring at 600 nm the formation of the radical cation ABTS⁺ using the Reagent ABTS® in the presence of H₂O₂ and peroxidase [25]. The method was calibrated using the TROLOX standard included with the kit.

**Protein determination**

Protein content was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, USA) with bovine serum albumin as a standard.

**Structural studies and image analysis**

The number of mitochondria/cell was estimated on fibroblasts incubated with 200 nM MitoTracker Green FM (Molecular Probes, Eugene, OR), and observed using a Leica Laser TCS 4D confocal microscope (Wetzlar, Germany) coupled with an image analyser. Mitochondrial ultrastructure was analysed using a Jeol EM1200 transmission electron microscope.

**Statistical analysis**

Experiments were performed independently at least three times using all cell strains, unless otherwise specified. Statistical significance, taken at p < 0.05, was determined by Student’s t-test and analysis of variance.

**Results**

The intracellular content of reactive oxygen species (ROS) was significantly higher in PXE than in control fibroblasts (Figure 1). The addition of vitamin E to the incubation media drastically reduced the intracellular levels of ROS in both controls and PXE fibroblasts, decreasing the concentration of ROS in PXE cells to the same value as in controls. In agreement with this observation, the total antioxidant status was significantly lower in PXE compared to controls in both cells and culture medium (Figure 2).

The occurrence of persistent oxidative stress in PXE fibroblasts is confirmed by the intracellular levels of MDA, the end-product and reliable marker of lipid peroxidation [26]. The intracellular concentration of MDA was significantly higher in PXE than in control cells (Figure 3).
Therefore, the higher production of H₂O₂ by Mn-SOD were of the same order in both cell types (Table 1). The concentration of malondialdehyde (MDA) is expressed as pmol/mg protein present in cell extracts. In each experiment, a pool of cells from five healthy individuals and five PXE patients was used. Data are expressed as mean ± SD. *p < 0.05 PXE vs. controls.

The equilibrium between reduced and oxidized glutathione in PXE cells was consistently shifted towards the oxidized form when compared to controls. The GSSG:GSH ratio changed from 0.09 in controls to 0.12 in PXE fibroblasts, the difference being statistically significant (p < 0.05).

Assessment of the activity of enzymes involved in maintaining the intracellular redox equilibrium revealed that mitochondrial Mn-SOD was significantly increased, whereas the activities of catalase and of glutathione peroxidase were significantly reduced in PXE cells compared to controls; by contrast, the activities of cytoplasmic Cu–Zn-SOD and of glutathione reductase were of the same order in both cell types (Table 1). Therefore, the higher production of H₂O₂ by Mn-SOD in PXE cells compared to controls, associated with the decreased activity of glutathione peroxidase and catalase, causes an imbalance between over-production and degradation of H₂O₂ in PXE cells (Table 2).

The mitochondrial membrane potential (ΔΨₘ) was evaluated to investigate further the higher level of Mn-SOD activity in PXE cells. Under the same respiratory conditions, the value of ΔΨₘ was significantly higher in PXE compared to control cells (Table 3). Upon addition of succinate or pyruvate, the ΔΨₘ in PXE fibroblasts tended to exceed 140 mV, the value at which the production of ROS takes place. As expected, addition of ADP caused a decrease in ΔΨₘ in both cell types. The effects of valinomycin and of oligomycin validate the technique employed, showing that mitochondrial functions are preserved under the experimental conditions used (Table 3).

Furthermore, biochemical determinations confirmed that the process of mitochondrial oxidative phosphorylation in PXE cells was essentially normal. PXE cells

**Table 1. Activities of enzymes involved in maintaining the intracellular redox state**

<table>
<thead>
<tr>
<th>Enzymes assayed</th>
<th>Control</th>
<th>PXE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn-superoxide dismutase (CN-insensitive; mitochondrial)</td>
<td>48 ± 4</td>
<td>74 ± 3**</td>
</tr>
<tr>
<td>Cu–Zn-superoxide dismutase (CN-sensitive; cytoplasmic)</td>
<td>313 ± 22</td>
<td>314 ± 34</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>30 ± 1</td>
<td>23 ± 3*</td>
</tr>
<tr>
<td>Catalase</td>
<td>8000 ± 691</td>
<td>6762 ± 561</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>6.1 ± 0.3</td>
<td>5.7 ± 0.4</td>
</tr>
</tbody>
</table>

In each experiment, duplicates of pools of cells from five healthy individuals and five PXE patients were used. The results are expressed as mean ± SD. *p < 0.05; **p < 0.01 PXE vs. controls.

**Table 2. Imbalance between the rate of H₂O₂ production and degradation**

<table>
<thead>
<tr>
<th>Activity ratio</th>
<th>Control</th>
<th>PXE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn-SOD/catalase</td>
<td>6 × 10⁻³</td>
<td>11 × 10⁻³</td>
</tr>
<tr>
<td>Mn-SOD/glutathione peroxidase</td>
<td>1.6</td>
<td>3.22</td>
</tr>
</tbody>
</table>

The ratios between the rate of H₂O₂ production and that of its reduction are estimated from the ratios between the activity of mitochondrial Mn-SOD and those of catalase and glutathione peroxidase, as reported in Table 1.

**Table 3. Mitochondrial membrane potential ΔΨ in control and PXE fibroblasts. The variations of ΔΨ are estimated from the variations of red:green fluorescence intensity ratio. Values in mV are calculated according to the method described by Reers et al [20]**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Variations of red:green fluorescence ratios</th>
<th>ΔΨ (mV) calculated from the fluorescence ratio variations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PXE</td>
</tr>
<tr>
<td>Basal endogenous substrate</td>
<td>0.53 ± 0.15</td>
<td>0.74 ± 0.20**</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.88 ± 0.21</td>
<td>1.15 ± 0.22*</td>
</tr>
<tr>
<td>Pyruvate/malate</td>
<td>0.78 ± 0.16</td>
<td>0.87 ± 0.19*</td>
</tr>
<tr>
<td>Succinate + oligomycin</td>
<td>1.25 ± 0.16</td>
<td>1.40 ± 0.20</td>
</tr>
<tr>
<td>Pyruvate/malate + oligomycin</td>
<td>1.04 ± 0.13</td>
<td>1.01 ± 0.13</td>
</tr>
<tr>
<td>Succinate + valinomycin</td>
<td>0.13 ± 0.09</td>
<td>0.19 ± 0.09</td>
</tr>
<tr>
<td>Pyruvate/malate + valinomycin</td>
<td>0.13 ± 0.09</td>
<td>0.19 ± 0.09</td>
</tr>
<tr>
<td>Succinate + ADP</td>
<td>0.63 ± 0.07</td>
<td>0.61 ± 0.10</td>
</tr>
<tr>
<td>Pyruvate/malate + ADP</td>
<td>0.68 ± 0.06</td>
<td>0.70 ± 0.20</td>
</tr>
</tbody>
</table>

*2 mM ADP; **1 mM ADP. Data represent results of three independent experiments performed on cultured fibroblasts obtained from six different patients and controls. Data are expressed as mean ± SD. *p < 0.05; **p < 0.01 PXE vs. control.
had a lower rate of oxygen uptake that became similar to that of controls upon addition of ADP, indicating that the rate of ATP synthesis was controlled by its utilization [20] (Table 4). Moreover, the amount of metabolically available energy for PXE fibroblasts was comparable with that of controls, as indicated by measurement of the energy charge of the adenylate system (Table 5).

Investigations by confocal and electron microscopy failed to reveal significant differences in the number and structure of mitochondria between control and PXE fibroblasts (data not shown).

Discussion

Fibroblasts isolated from the dermis of PXE patients and cultured in vitro suffer from chronic oxidative stress. This conclusion is supported by the finding that, in PXE fibroblasts compared to control cells: (a) the level of ROS is significantly higher and is reduced to values similar to those of controls by addition of vitamin E; (b) the total antioxidant status, i.e. the fraction of antioxidant pool available for further anti-ROS activity, is significantly lower; (c) the end product of lipid peroxidation (MDA) is significantly higher; and (d) the ratio GSSG:GSH is shifted towards the oxidized form. Moreover, the observed increase of mitochondrial Mn-SOD activity in PXE cells, which is not compensated for by variation in catalase or glutathione peroxidase activities, suggests that mitochondria may be involved in the generation of oxidative stress in PXE cells.

In normal cultured fibroblasts, under basal conditions, the mitochondrial membrane potential ($\Delta \Psi_m$) does not exceed 105 mV [27], close to the optimal values for the synthesis of ATP by ATP synthetase [28]; by contrast, in PXE fibroblasts, the $\Delta \Psi_m$ is significantly higher and tends to exceed 140 mV. This is a very critical value above which an exponential increase in ROS production has been measured [29]. To avoid the harmful effects of ROS, cells have developed mechanisms to keep the $\Delta \Psi_m$ at values well below 140 mV. Regulation of membrane potential requires the concerted action of a number of mechanisms that include the allosteric ATP inhibition of cytochrome c oxidase at high ATP: ADP ratios [30] and the activation of extrinsic and intrinsic uncoupling of oxidative phosphorylation [31].

The present data suggest that some of these mechanisms do not function adequately in PXE fibroblasts, with a consequent increase in $\Delta \Psi_m$ and overproduction of ROS. This conclusion is consistent with the observation that activity of Mn-SOD, the enzyme primarily involved in the removal of $O_2^-$ formed in mitochondria during respiration [32] by converting superoxide radicals into $H_2O_2$ [33], is significantly higher in PXE cells than in controls. However, the overproduction of $H_2O_2$ is not compensated for by an increase in catalase and glutathione peroxidase activities, which should remove the excess $H_2O_2$. This would lead to an imbalance between production and removal of $H_2O_2$ in PXE cells.

Although the present data do not firmly establish the sequence of events leading to impairment of the control of $\Delta \Psi_m$ in PXE mitochondria, they provide strong evidence that the hyperpolarization of mitochondrial membrane in PXE fibroblasts induces overproduction of ROS, which in turn results in continuous oxidative stress. Whether the loss of mitochondrial $\Delta \Psi_m$ control is strictly related to MRP6 deficiency, or is an independent event that contributes eventually to the severity of lesions in PXE patients, is merely speculative without knowledge of the physiological substrate of MRP6.

Interestingly, the occurrence of mild chronic oxidative stress may well explain several of the biochemical, histological and clinical lesions typical of PXE. A number of metabolic and biological processes are sensitive to variations in the intracellular redox equilibrium. Besides causing DNA damage, ROS activate cytoplasmic signal transduction pathways in resident fibroblasts related to growth, differentiation and senescence, as well as connective tissue production and degradation [34]. A direct effect of ROS on the production of TGF$\beta$, with consequent stimulation of extracellular matrix gene expression [35] and the expression of metalloproteases in cardiac fibroblasts,

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**Table 4. Rate of oxygen uptake by control and PXE fibroblasts under different experimental conditions**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Untreated fibroblasts (nAtoms $O_2$/min/1 $\times 10^6$ cells)</th>
<th>Ferroblasts treated with digitonin (nAtoms $O_2$/min/1 $\times 10^6$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control PXE</td>
<td>Control PXE</td>
</tr>
<tr>
<td>None</td>
<td>3.26 ± 0.31 2.54 ± 0.26*</td>
<td>3.55 ± 0.38 2.83 ± 0.30*</td>
</tr>
<tr>
<td>Succinate</td>
<td>3.35 ± 0.36 2.72 ± 0.22</td>
<td>4.82 ± 0.39 3.59 ± 0.34*</td>
</tr>
<tr>
<td>Succinate + ADP</td>
<td>3.43 ± 0.30 2.92 ± 0.27</td>
<td>5.42 ± 0.40 4.77 ± 0.45*</td>
</tr>
<tr>
<td>KCN</td>
<td>~0</td>
<td>~0</td>
</tr>
</tbody>
</table>

Respiratory control index (RCI) is the ratio between state 3 and state 4 respiratory rate. The data represent results of three independent experiments performed on pools of cultured fibroblasts obtained from six different healthy individuals and five PXE patients. Data are expressed as mean ± SD. * $p < 0.05$ PXE vs. control cells.

**Table 5. Adenine nucleotides in cultured fibroblasts**

<table>
<thead>
<tr>
<th>Concentration (nMol/1 $\times 10^6$ cells)</th>
<th>Control</th>
<th>PXE</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>5.39 ± 1.24</td>
<td>4.99 ± 1.22</td>
</tr>
<tr>
<td>ADP</td>
<td>4.30 ± 0.92</td>
<td>3.81 ± 0.62</td>
</tr>
<tr>
<td>AMP</td>
<td>1.52 ± 0.42</td>
<td>1.31 ± 0.31</td>
</tr>
<tr>
<td>E(Ad)</td>
<td>0.67</td>
<td>0.68</td>
</tr>
</tbody>
</table>

E(Ad) = energy charge of the adenylate system, calculated as described by Atkinson [18]. The data represent results of three independent experiments performed on cultured fibroblasts obtained from six different patients and controls. Data are expressed as mean ± SD.
has recently been shown [36]. In agreement with these data, we have recently observed that metalloprotease activities are significantly higher in PXE fibroblasts compared to controls [37].

In cultured human dermal fibroblasts, ultraviolet (UV)-generated ROS increase the level of elastin mRNA expression in a dose-related manner, suggesting that ROS may also contribute to the elastin deposition observed in photo-aged skin [38]. Interestingly, an increase in elastin has been measured by biochemical [39] and morphometric analyses [40] in the skin of PXE patients.

In vitro, treatment of elastin with agents capable of generating ROS, such as UV, copper sulphate/ascorbic acid and xanthine/xanthine oxidase, cause elastolytic changes and/or production of elastin fragments [41]. Both in vivo and in vitro studies have recently shown that elastic fibre degradation, regardless of how it is induced, favours elastic fibre-associated mineralization [42].

Reactive oxygen species and proteolytic degradation of matrix components may also play a crucial role in angiogenesis [43,44], suggesting that oxidative stress and unbalanced metalloprotease activity may be involved in the pathogenesis of retinal neovascularization, which is one of the most severe clinical manifestations in PXE, as it causes recurrent haemorrhage [1]. Consistent with this hypothesis are the results of studies on age-related macular degeneration [45]. In this disease, neovascularization of the retina has been correlated with oxidative stress, even if produced through different mechanisms [46–48].

Experimental evidence shows that changes in the redox equilibrium of the cell and oxidative stress may hinder cytokinesis by affecting spindle formation, chromosome migration and tubulin stability [49,50]. These data may explain the alterations in cell duplication observed in PXE cells [15] as well as the presence of polynucleated fibroblasts often observed in the skin of PXE patients (personal observation).

Moreover, it has been recently found that high levels of intracellular ROS delay cell attachment to the substrate and attenuate the anchorage dependence of growth [51]. This phenomenon has been described in cultured PXE fibroblasts [15].

The content of highly sulphated glycosaminoglycans (GAGs), and in particular of heparan sulphate and chondroitin sulphate, has been found to be significantly increased in the plasma and urine of PXE patients [52] and in the culture medium of PXE fibroblasts [53,54]. There is no evidence that the synthesis of these compounds is influenced by ROS; however, since GAGs may function as scavengers of free radicals during oxidative stress [55], one hypothesis could be that the increased concentration of GAGs may represent a compensatory response to oxidative stress. An alternative hypothesis is that the high levels of sulphated GAGs may derive from augmented proteolytic cleavage of proteoglycans, as suggested by the observation that PXE fibroblasts have higher proteolytic activities compared to controls [37]. Whether the abnormally high amount of GAGs interferes with the formation, stability and, eventually, the calcification of elastic fibres is under investigation [56]. The present data seem to suggest that overproduction of ROS could be the unifying pathogenetic mechanism for the most relevant lesions of PXE, i.e. alterations in cellular and extracellular matrix components [2], degradation [57] and mineralization of elastic fibres and neovascularization of the retina [1]. This interpretation is supported by the observation that identical clinical and histopathological alterations have been observed in a number of haemolytic disorders [9,10,13] in which oxidative stress has been well documented [58,59].

Therefore, MRP6 deficiency seems to be responsible for chronic oxidative stress in PXE cells and substantial progress toward understanding the effects of ROS on fibroblasts and extracellular matrix metabolism may lead to therapeutic strategies aimed at preventing at least some of the damage that occurs in PXE.

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