Published in final edited form as:

J Mol Med (Berl). 2013 October ; 91(10): 1177–1184. doi:10.1007/s00109-013-1066-5.

Atorvastatin counteracts aberrant soft tissue mineralization in a mouse model of pseudoxanthoma elasticum (*Abcc6*^{-/-})

Haitao Guo^{1,2}, Qiaoli Li¹, David W. Chou¹, and Jouni Uitto¹

¹Departments of Dermatology and Cutaneous Biology, and Biochemistry and Molecular Biology, Jefferson Medical College, and Jefferson Institute of Molecular Medicine, Thomas Jefferson University, Philadelphia, PA 19107

²Department of Physiology, Fourth Military Medical University, Xi'an, Shaanxi, PR China, 710032

Abstract

Pseudoxanthoma elasticum (PXE), a multisystem heritable disorder with aberrant mineralization of arterial blood vessels, is caused by mutations in the *ABCC6* gene. Previous studies have suggested that carriers of the *ABCC6* mutations, particularly of p.R1141X, are at increased risk for coronary artery disease. In this study, we used *Abcc6*^{tm1Jfk} knock-out mice to determine the serum lipid profiles and examine the effects of atorvastatin on the aberrant mineralization in this model of PXE. First, serum lipid profiles at 12 weeks of age, after overnight fasting, revealed a statistically significant increase in total cholesterol and triglyceride levels in *Abcc6*^{tm1Jfk} mice compared to their wild-type littermates. Placing these mice at 4 weeks of age for 20 weeks on atorvastatin, either 0.01% or 0.04% of the diet (low statin and high statin groups, respectively), reduced the total triglyceride and cholesterol levels, which was accompanied with significantly reduced mineralization of the dermal sheath of vibrissae, a biomarker of the aberrant mineralization process in these mice. However, if the mice were placed on atorvastatin for 12 weeks at 12 weeks of age, at which time point significant mineralization had already taken place, no difference in the amount of mineralization was noted. These observations suggest that statins, particularly atorvastatin, can prevent, but not reverse, aberrant mineralization in this mouse model of PXE. For a clinical perspective, a survey of 1,747 patients with PXE was conducted regarding their present or past use of statins. The results indicated that about one third of all PXE patients are currently or have previously been on cholesterol-lowering drugs. Thus, a sizable number of patients with PXE could be subject to modulation of their mineralization processes by concomitant statin treatment.

Keywords

Pseudoxanthoma elasticum; Ectopic mineralization; Statin therapy

Introduction

Aberrant deposition of calcium phosphate complexes in connective tissues is linked to a number of pathological conditions in the skin and vascular connective tissues [1, 2]. For example, the risk of death associated with coronary artery calcification in a cohort of over

Author for Correspondence: Jouni Uitto, MD, Ph.D., Department of Dermatology and Cutaneous Biology, Jefferson Medical College, 233 South 10th Street, Suite 450 BLSB, Philadelphia, PA 19107, U.S.A., Tel: 215-503-5785, Fax: 215-503-5788, Jouni.Uitto@jefferson.edu.

Disclosure Statement

The authors have no conflict of interest to report.

25,000 patients was found to be increased by up to 12.5 fold [3]. Multiple factors, both genetic and environmental, can contribute to the pathomechanisms of both metastatic and dystrophic calcification [4, 5]. Significant insight into such factors has been gained by examination of Mendelian genetic disorders with aberrant mineralization of soft connective tissues, with accompanying animal models [6]. The prototype of heritable multisystem ectopic mineralization disorders is pseudoxanthoma elasticum (PXE), with calcium deposition in multiple tissues leading to protean clinical manifestations in the skin, the arterial blood vessels and the eyes [7, 8]. PXE is, in most cases, caused by mutations in the *ABCC6* gene which encodes a putative transmembrane transporter protein, ABCC6. Our understanding of the clinical features and pathomechanistic details of this disorder has been advanced by the study of *Abcc6*^{-/-} knock-out mice which recapitulate the features of PXE, including extensive mineralization in the arterial blood vessels, skin and the Bruch's membrane in the eye [9, 10]. PXE has been suggested to be a metabolic disorder in which the nonfunctional ABCC6 transporter results in deficiency of anti-mineralization factor(s) in circulation, thus allowing calcium and phosphate to precipitate under physiologic conditions resulting in hydroxyapatite formation. However, the details of the chemical nature of such anti-mineralization factor(s) remain unknown [11].

PXE is inherited in an autosomal recessive pattern and the heterozygous carriers of the mutations in the *ABCC6* gene do not demonstrate overt clinical findings [12]. However, it has been suggested that heterozygous carrier status of loss-of-function mutations in the *ABCC6* gene, particularly that of p.R1141X, the most common mutation in the Caucasian populations, is associated with a strong increase in the prevalence of coronary artery disease [13, 14]. Thus, these mutations serve as a genetic risk factor in these individuals who do not have the PXE phenotype [12].

In this study, we have used the *Abcc6*^{tm1Jfk} knock-out mouse [10] as a platform to examine the lipid profile in these mice and to test the hypothesis that statins, and specifically atorvastatin, a common cholesterol-lowering drug in clinical use, counteract tissue mineralization in this mouse model of PXE.

Materials and Methods

Mice

Abcc6^{tm1Jfk} mice (referred to hereon as *Abcc6*^{-/-} mice) were developed by targeted ablation of the *Abcc6* gene to serve as a mouse model of PXE [10]. *Abcc6*^{-/-} mice were made congenic by backcrossing heterozygous *Abcc6*^{+/-} mice on 129S1/SvImJ background for five generations. Mice had free access to water and were maintained in the climate-controlled Animal Facility of Thomas Jefferson University on a 12-hour light/dark cycle. Euthanization was done by CO₂ asphyxiation as approved by the American Veterinary Medical Association. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Thomas Jefferson University. Proper handling and care were followed according to animal welfare policies of the U.S. Public Health Service.

Experimental design and diets

Mice were placed on normal laboratory diet (Laboratory Autoclavable Meal Rodent Diet 5010; PMI Nutrition, Brentwood, MO) at 4 weeks of age. A group of *Abcc6*^{+/+} and *Abcc6*^{-/-} mice on 129S1/SvImJ background (n=10 in each group) were sacrificed at 12 weeks of age to evaluate their serum lipid levels.

To study the efficacy of atorvastatin on tissue mineralization, *Abcc6*^{-/-} mice were assigned into Prevention Study and Reversal Study groups with appropriate controls (Table 1). Mice in the Prevention Study group were placed on normal diet with or without atorvastatin at 4

weeks of age and kept on the same diet for an additional 20 weeks. The Prevention Study group consisted of three subgroups with 9 mice each: *Abcc6*^{-/-} mice on normal diet group (Group B), low dose atorvastatin group, which received 0.01 g atorvastatin (Avachem Scientific, San Antonio, TX) per 100 gram of diet (Group C), and high dose atorvastatin group, which received 0.04 g atorvastatin per 100 gram of diet (Group D). Mice in the Reversal Study group were placed on low (Group E) or high (Group F) dose atorvastatin as above at 12 weeks of age and kept on the same diet for an additional 12 weeks, 6–9 mice per group (Table 1). Wild-type littermates (*Abcc6*^{+/+}) on the same genetic background kept on normal diet served as controls for the Prevention and Reversal Study groups (Group A). Retro-orbital bleed was performed every 4 weeks from the mice on normal diet or diet supplemented with atorvastatin to evaluate the effect of atorvastatin on the blood lipid levels. All mice were euthanized at 24 weeks of age and necropsied for tissue analysis.

Measurement of lipids

Total cholesterol and triglyceride levels in serum samples were determined by colorimetric assays. A modified Trinder method was performed to measure total cholesterol content (Cholesterol LiquiColor® Test (Enzymatic); Stanbio Laboratory, Boerne, TX). A glycerylphosphate oxidase method was performed to measure triglyceride content (Triglyceride LiquiColor® Test (Mono); Stanbio Laboratory). To measure total cholesterol and triglycerides, a Bio-Rad Model 680 microplate reader (Hercules, CA) was used to obtain absorbance values of samples at wavelength of 490 nm.

Histopathological analysis

Necropsy tissue samples from muzzle skin containing vibrissae and internal organs were fixed in 10% phosphate-buffered formalin, processed routinely, and embedded in paraffin. The tissues were sectioned (6 µm) and stained with hematoxylin and eosin (HE) or Alizarin Red (AR) using standard procedures. Slides were examined for mineralization under light microscope.

Quantitation of tissue mineralization by computerized morphometric analysis

Computerized morphometric analysis of mineralization was performed on HE stained sections with a Nikon Te2000 microscope furnished with an Auto Quant Imaging system (Watervliet, New York, NY). The total area of mineralization in vibrissae was expressed as a percentage of the total area of vibrissae per mouse, and the average percentage of mineralization was determined for each group [15]. All images were analyzed with Image-Pro software (Media Cybernetics, Rockville, MD).

Chemical quantification of calcium and phosphate

To quantify mineral deposition, muzzle skin was harvested and decalcified with 0.15 N HCl for 48 hours at room temperature, followed by assay of calcium in the supernatant. Calcium and phosphate levels in serum samples were also determined. Colorimetric analysis by the *o*-cresolphthalein complexone method (Calcium (CPC) Liquicolor; Stanbio Laboratory, Boerne, TX) was performed to measure the calcium content. Phosphate content was determined with the Malachite Green Phosphate Assay kit (BioAssay Systems, Hayward, CA). Values for muzzle skin were normalized to tissue weight.

Statistical analysis

The comparisons between different groups of mice were completed using the two-sided Kruskal–Wallis nonparametric test, comparable to one-way analysis of variance, but without the parametric assumptions. Fisher's exact test was used to determine the difference between

proportions of mineralization in organs of mice fed with different diets. All statistical computations were completed using SPSS version 15.0 software (SPSS Inc., Chicago, IL).

Clinical survey

Patients in the PXE International Patient Registry were contacted by e-mail and queried about their present or past treatment with cholesterol-lowering drugs, particularly statins. This study was approved by the Genetic Alliance Institutional Review Board.

Results

Abcc6^{-/-} mouse model for PXE

Targeted ablation of the *Abcc6*^{-/-} mice results in aberrant mineralization of soft connective tissues [9, 10]. The first site of mineralization noted at ~5–6 weeks of age of mice kept on normal laboratory diet is the dermal sheath of vibrissae in the muzzle skin (Fig. 1). The mineralization of the vibrissae serves as an early biomarker of the mineralization process, and its quantitation either by histopathologic stains coupled with computerized morphometric analysis or by direct calcium assay of the muzzle skin containing the vibrissae allows monitoring of the overall progression of the mineralization in these PXE mice without sacrificing the animals [15].

Serum lipid profile in *Abcc6*^{-/-} mice

Serum lipid measurements at 12 weeks of age, after overnight fasting, demonstrated a statistically significant increase in serum concentrations of total cholesterol and triglycerides in *Abcc6*^{-/-} mice in comparison to wild-type littermates serving as controls (Fig. 2).

The efficacy of atorvastatin, an HMG-CoA reductase inhibitor, to lower the cholesterol and triglyceride levels and possibly alter the degree of mineralization was tested in subgroups of *Abcc6*^{-/-} mice in two experimental designs. First, the Prevention Study consisted of *Abcc6*^{-/-} mice on normal diet (control, Group B) and mice for whom the diet was supplemented with 0.01% (low statin, Group C) or 0.04% (high statin, Group D) of atorvastatin. These mice were followed for 20 weeks, measuring serum lipids every 4 weeks and then necropsied at 24 weeks to determine the degree of tissue mineralization. Feeding the 4 week old mice with atorvastatin in the high statin group resulted in rapid and significant (47.8%) decrease of triglycerides after 4 weeks on this diet, and the triglyceride levels remained low throughout the study (Fig. 3b). The total serum cholesterol level was not statistically reduced until at 16 weeks on this diet, the reduction being 26.6% compared to control mice on statin-free diet (Group B) (Fig. 3a).

Effects of atorvastatin on tissue mineralization in *Abcc6*^{-/-} mice

Assay of mineralization in mice in the prevention study by computerized morphometric analysis of the dermal sheath of vibrissae or the content of calcium in the muzzle skin demonstrated a significant 26.9% decrease in mice kept on high statin diet (Group D), while the lower amount of statin did not alter the degree of mineralization in comparison to the normal diet (Fig. 4). Thus, significant reduction in serum total cholesterol and triglyceride levels in these mice was accompanied by a reduced mineralization of the dermal sheath of vibrissae. These results suggested that statins, particularly atorvastatin, in relatively high daily quantities, are able to counteract the progression of mineralization in soft connective tissue in the skin.

This study was extended to an experiment to determine whether the mineralization, once established, could be reversed by feeding the *Abcc6*^{-/-} mice with statins, again in low (Group E) and high (Group F) concentrations, in comparison to mice kept on normal diet

without statin (Group B). These mice were first kept on control diet for 12 weeks, and at this point at which significant mineralization had already taken place, the mice were placed on statin-containing diets for an additional 12 weeks. Assay of total cholesterol at 24 weeks of age revealed 18.4 and 20.4% reduction in Groups E and F, respectively, but this change was not statistically significant ($P>0.05$; $n=6-9$). However, by as early as 4 weeks the triglyceride values were reduced in Group F by 71.8% ($P<0.01$ in Group F vs. Group B or E). Assay of the degree of mineralization either by computerized morphometric analysis of histopathologic sections from the muzzle skin or by direct calcium chemical assay revealed no difference in the degree of mineralization between groups B, E and F, suggesting that once the mineralization has taken place, the mineral content is not reduced by treatment by atorvastatin, but the deposition of new calcium phosphate complexes is inhibited (Fig. 5).

In addition to mineralization of the dermal sheath of vibrissae in the skin, mineralization was examined in the kidneys, the heart and the eyes by histopathological analysis by counting the number of mice that demonstrated mineralization as a percent of the total ($n=6-10$) (Table 2). No statistically significant differences were noted in the relative number of tissues involved in these mice.

Assay of serum calcium and phosphate did not demonstrate significant changes at the time of the sacrifice of the mice (Table 3). Finally, the mean body weight of the *Abcc6*^{-/-} mice was not affected by the inclusion of statin into their diets, the mean body weight (g) being 27.3 ± 1.1 , 26.6 ± 1.1 , 27.5 ± 0.9 , 24.8 ± 1.3 , and 23.6 ± 0.9 in groups B, C, D, E and F, respectively.

Clinical survey

To explore the clinical perspective of the potential importance of statins in patients with PXE, 1,747 patients in the PXE International Patient Registry, which consists of a total of ~4,000 patients, for whom an e-mail address was available, were contacted regarding present or past use of statins. Of the total of 539 respondents, 24.1% reported current and 6.7% reported previous use of cholesterol-lowering drugs. Thus, a sizable number of patients with PXE is potentially subject to modulation of their mineralization processes by concomitant statin treatment for cholesterol-lowering purposes.

Discussion

This study demonstrates that atorvastatin, a commonly prescribed cholesterol-lowering drug, can result in reduced mineral deposition of soft connective tissues in the skin of *Abcc6*^{-/-} mice, an animal model of PXE, a multisystem mineralization disorder. The reduced mineral deposition with atorvastatin, particularly in higher doses, was associated with reduced total cholesterol and triglyceride serum concentrations. Statins have previously been shown to affect mineralization processes, particularly in the context of bone metabolism [16–18]. With respect to these effects, statins have been suggested to augment the expression of bone morphogenic protein-2, a potent stimulator of osteoblast differentiation, and to promote mineralization by activated osteoblasts. This effect has been suggested to result from reduced protein prenylation, particularly of RhoA, a GTP-binding protein. Reduced prenylation of RhoA is postulated to result in increased activity of BMP-2 resulting in increased vascular endothelial growth factor activity and angiogenesis coupled with osteoblast differentiation and bone formation [19]. At the same time, statins have been reported to inhibit bone formation and produce a net reduction in bone density in rats, concluding that statins do not have *in vivo* anabolic effects on bone in rodents [20].

While the mineral deposits in soft tissues of *Abcc6*^{-/-} mice have been shown to consist of calcium hydroxyapatite [21, 22], the pathomechanistic pathways resulting in aberrant

mineral deposits are currently unknown. Thus, it is not clear how statins might elicit reduction of mineral deposition in these mice and whether the effect is related to the inhibition of the cholesterol pathway. It should be noted that the dosages of atorvastatin used in our study, selected on the basis of previous mouse studies reported in the literature with documented cholesterol-lowering effect [23, 24], are high in comparison to clinical use in humans. The lower concentration of atorvastatin, 0.01% of the diet, corresponds to a dietary dose of about 110 mg of atorvastatin for a 70 kg human adult, with the assumption that a mouse weighs about 25 grams and eats about 5 grams of food daily. It should be noted, however, that even the higher dose appeared not to be toxic to the mice, as their weight gain during the 24 week experimental study was not different from the mice on control diet, and the serum calcium and phosphate levels were essentially unchanged.

It was of interest to note that the *Abcc6^{tm1Jfk}* knock-out mice at 12 weeks of age kept on normal laboratory diet showed increased total cholesterol and triglyceride levels. These observations differ from those reported by Gorgels *et al.* [9] on *Abcc6^{tm1Aabb}* knock-out mice, who noted somewhat decreased serum total cholesterol levels in 8 month old mice as compared to the corresponding wild-type mice [9]. However, the total cholesterol and triglyceride values at 2.5 month old mice were not different in their study. The possible reasons for the differences include different mouse diets and different genetic backgrounds, the mice in our study being congenic on 129S1/SvImJ background [10] while the *Abcc6^{tm1Aabb}* mice developed by Gorgels *et al.* [9] were on C57BL/6 background.

In summary, the results of the present study indicate that atorvastatin at a relatively high daily dose is able to prevent, but not to reverse, ectopic mineralization of soft connective tissues in *Abcc6^{-/-}* mice, a model for PXE. A clinical survey conducted by querying a group of patients with PXE in the Registry of the patient advocacy organization, PXE International, revealed that about one third of them were currently or had history of being treated with cholesterol-lowering drugs. Thus, a substantial number of PXE patients are subject to modulation of the mineralization process by statins, with potentially beneficial consequences.

Acknowledgments

The authors thank Dian Wang for animal care. Adele Donahue and Alix E. Grand-Pierre assisted in histopathology. Changxia Shao provided help in statistical analyses. Sharon Terry and Abbie Moore at PXE International assisted in clinical survey. Carol Kelly assisted in manuscript preparation. These studies were supported by NIH/NIAMS grant R01 AR28450.

Abbreviations

PXE	pseudoxanthoma elasticum
HE	hematoxylin-eosin
AR	Alizarin red

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Figure 1.

Demonstration of aberrant mineralization in the dermal sheath of vibrissae in *Abcc6*^{-/-} mice; for genotype and treatment schedule of different groups of mice, see Table 1. The mice were sacrificed at 24 weeks of age, and tissues of muzzle skin containing the vibrissae were processed for histopathology and stained with hematoxylin eosin (HE) or with Alizarin Red (AR). Note the absence of mineralization in the wild-type mice (*Abcc6*^{+/+}, Group A), while there is extensive mineralization in *Abcc6*^{-/-} mice kept on normal diet (Group B). Representative samples of histopathology of mice treated with atorvastatin (Groups C–F). HE stained sections were used for computerized morphometric quantitation of the degree of mineralization, as summarized in Figs. 4a and 5a. The original magnification in all frames is the same, X100.

Figure 2. Serum concentrations of total cholesterol and triglycerides in *Abcc6*^{+/+} control mice and in *Abcc6*^{-/-} knock-out mice of 12 weeks of age. (The values are mean $\pm$ S.E.; n=10; Statistical significance: ** P <0.01 vs. the *Abcc6*^{+/+} mice).

Figure 3.

Serum total cholesterol (a) and triglyceride (b) concentrations in *Abcc6*^{-/-} mice in the Prevention Study. The mice were placed on atorvastatin at 4 weeks of age, and blood samples were obtained at 4 week intervals as shown. Mice in Group B were maintained on normal diet, while mice in Groups C and D were fed normal diet supplemented with low (Group C) or high (Group D) content of atorvastatin. (The values are mean $\pm$ S.E.; n=9; Statistical significance: * P <0.05; ** P <0.01 vs. Group B; # P <0.01 vs. Group C).

Figure 4.

Quantitation of aberrant mineralization of the dermal sheath of vibrissae in the Prevention Study by computerized morphometric analysis (a) and by direct assay of calcium in the tissue (b). The mice in Groups C and D were maintained for 20 weeks on low and high atorvastatin containing diet, respectively, and the degree of mineralization was compared with that in *Abcc6*^{+/-} control mice (Group A) and in *Abcc6*^{-/-} mice (Group B) maintained on normal diet. (The values are mean $\pm$ S.E.; n=9; Statistical significance: * P <0.05 vs. Group B).

Figure 5.

Quantitation of aberrant mineralization of the dermal sheath of vibrissae in the Reversal Study by computerized morphometric analysis (a) and by direct assay of calcium in the tissue (b). *Abcc6*^{-/-} mice were placed on low and high statin diet at 12 weeks of age and sacrificed at the age of 24 weeks. The mineralization in the treated mice was compared with *Abcc6*^{-/-} mice (Group B) maintained on normal diet. (The values are mean ± S.E.; n=6–9).

Table 1Experimental groups of *Abcc6*^{+/+} and *Abcc6*^{-/-} mice by genotype and diet *

Group	Genotype	<i>n</i>	Diet
Controls			
A	<i>Abcc6</i> ^{+/+}	10	Normal diet
B	<i>Abcc6</i> ^{-/-}	9	Normal diet
Prevention (4 w + 20 w)			
C	<i>Abcc6</i> ^{-/-}	9	Normal diet + 0.01% Atorvastatin
D	<i>Abcc6</i> ^{-/-}	9	Normal diet + 0.04% Atorvastatin
Reversal (12 w + 12 w)			
E	<i>Abcc6</i> ^{-/-}	9	Normal diet + 0.01% Atorvastatin
F	<i>Abcc6</i> ^{-/-}	6	Normal diet + 0.04% Atorvastatin

* The mice were placed on atorvastatin-containing diets at 4 weeks of age and followed for another 20 weeks (Prevention) or at 12 weeks of age followed by another 12 weeks (Reversal). The mice were sacrificed at the age of 24 weeks and compared with control mice, either *Abcc6*^{+/+} or *Abcc6*^{-/-}, maintained on normal diet.

Table 2Soft tissue mineralization in *Abcc6*^{-/-} mice fed on normal diet or diet supplemented with atorvastatin

Group*	Mineralization in soft tissues (%)			
	vibrissae	kidneys	heart	eyes
Controls				
A	0	0	0	0
B	100	63	11	11
Prevention				
C	100	56	22	44
D	100	100	0	67
Reversal				
E	100	45	0	44
F	100	100	33	67

* For the genotypes of mice and the schedule of treatment with atorvastatin, see Table 1.

Table 3

Calcium and phosphate concentrations in the serum of *Abcc6*^{-/-} mice fed on normal diet or diet supplemented with atorvastatin

Group *	Serum concentration (mean ± SEM)		
	Calcium (mg/dL)	Phosphate (mg/dL)	Ca/P ratio
A	10.8 ± 0.5	11.8 ± 0.7	0.94 ± 0.08
B	10.1 ± 0.3	10.0 ± 0.6	1.02 ± 0.03
C	9.9 ± 0.4	9.9 ± 0.7	1.04 ± 0.01
D	9.4 ± 0.4	12.0 ± 1.1	0.83 ± 0.08 ⁺
E	10.0 ± 0.2	10.7 ± 0.6	0.96 ± 0.06
F	10.4 ± 0.5	11.7 ± 0.7	0.91 ± 0.08

* For the genotypes of mice and the schedule of treatment with atorvastatin, see Table 1.

⁺ $P < 0.05$ vs. Group B.