Atherogenic Diet Accelerates Ectopic Mineralization in a Mouse Model of Pseudoxanthoma Elasticum

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Abstract
Objective: Pseudoxanthoma elasticum (PXE) is a multisystem heritable disorder caused by mutations in the Abcc6 gene. The disease is characterized by ectopic mineralization of the skin, eyes, and arterial blood vessels. Previous studies have suggested that cardiovascular complications in patients with PXE are caused in part by premature atherosclerosis. The aim of this study is to determine the effect of an atherogenic diet on ectopic mineralization.

Methods: We used Abcc6tm1JfK mice (Abcc6−/− mice) as an established preclinical model of PXE. The offspring at age of 4 weeks were divided into two groups and fed the standard control laboratory diet (control group) and the atherogenic diet. Serum lipid profiles and bile acids were measured, and steatosis and tissue mineralization were evaluated by histopathologic analysis and chemical calcium quantification assay, respectively.

Results: After 50–58 weeks of feeding an atherogenic diet, the concentrations of total cholesterol, low-density lipoprotein/very-low-density lipoprotein cholesterol, and bile acids were significantly higher in the Abcc6−/− mice on the atherogenic diet (180.9 ± 14.8 g/L, 145.9 ± 12.9 g/L, and 9.7 ± 1.4 μmol/L, respectively) than in Abcc6+/− mice on a control diet (85.2 ± 4.8 g/L, 25.1 ± 5.5 g/L, and 3.3 ± 0.5 μmol/L, respectively) (P < 0.001). Hypercholesterolemia was accompanied by extensive lipid accumulation in the liver and aorta, a characteristic feature of steatosis. The direct calcium assay demonstrated significantly increased mineralization of the muzzle skin containing the dermal sheath of vibrissae (57.2 ± 4.4 μmol Ca/gram tissue on the atherogenic diet and 43.9 ± 2.2 μmol Ca/gram tissue on control diet; P < 0.01), a reproducible biomarker of the ectopic mineralization process in these mice. An increased frequency of mineralization was also observed in the kidneys and eyes of mice on the atherogenic diet (P < 0.01).

Conclusion: These observations suggest that the atherogenic diet caused hypercholesterolemia and accelerated ectopic mineralization in the Abcc6−/− mice. Our findings have clinical implications for patients with PXE, a currently intractable disorder with considerable morbidity and occasional mortality.

Keywords: pseudoxanthoma elasticum, ectopic mineralization, atherogenesis, mouse model

Introduction
Pathological calcification of connective tissues, also termed ectopic mineralization, is a complicated process leading to deposition of calcium phosphate complexes in the extracellular matrix. This process particularly affects the arterial blood vessels and is common in patients with age-associated disorders.1 In the clinical setting, ectopic mineralization has been encountered in both acquired diseases and heritable Mendelian single-gene disorders with phenotypic similarities. Diseases that cause ectopic mineralization, among which pseudoxanthoma elasticum (PXE) is the paradigm heritable disorder, are prime targets of the efforts to elucidate the precise pathomechanistic pathways.

PXE (OMIM# 264800) is an autosomal recessive connective tissue disorder in which fragmentation and mineralization of elastic fibers result in cutaneous, ocular, and cardiovascular manifestations.2,3 The first organ system affected is often the skin, which develops small yellowish papules primarily in flexural areas of the body.
In this study, we used the Abcc6-knockout mouse as a model of PXE to examine the consequences of an atherogenic diet on serum lipid profiles and the degree of ectopic mineralization. The results demonstrated that the atherogenic diet induced hypercholesterolemia and steatosis accompanied by increased mineralization in the soft connective tissues. These findings have clinical implications for patients with PXE.

Materials and methods

Mice had free access to water and were maintained in the climate-controlled Animal Facility of Thomas Jefferson University. Euthanization was performed by carbon dioxide asphyxiation and opening of the chest 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 protocols were followed according to the animal welfare policies of the U. S. Public Health Service.

Experimental design and diets

The mice were placed on a standard control laboratory diet (Laboratory Autoclavable Meal Rodent Diet 5010; PMI Nutrition, Brentwood, MO) during maintenance and breeding. Offspring were genotyped for the Abcc6 status by previously described polymerase chain reaction protocols and primers.4 At the age of 4 weeks, the Abcc6+/− offspring were divided into two groups and fed specific diets for another 50–58 weeks. Six male and nine female Abcc6+/− mice in the first group continued the standard control laboratory diet. Seven male and three female Abcc6+/− mice in the second group were placed on the atherogenic diet at 4 weeks of age (Teklad Diet TD.02028; Envigo, Madison, WI). This atherogenic diet has higher cholesterol, sucrose, cholesterol, and cholic acid content than the standard control rodent diet. The specific contents of the control diet are provided at http://www.labdiet.com/cs/groups/lolweb/@labdiet/documents/web_content/mdrl/∼edisp/ducu04_028443.pdf, and those of the atherogenic diet are provided at http://www.envigo.com/resources/data-sheets/02028.pdf. These two diets are compared in Table 1. All mice were fasted overnight prior to euthanasia for blood and tissue analysis.

Measurement of serum lipids and bile acids

The cholesterol and triglyceride levels in fasted serum samples were determined by colorimetric assays. The
concentrations of total cholesterol, HDL cholesterol, and low-density lipoprotein/very-low-density lipoprotein (LDL/VLDL) cholesterol were measured with an EnzyChrom™ HDL and LDL/VLDL Assay Kit (BioAssay Systems, Hayward, CA, USA). The triglyceride content was measured with an EnzyChrom™ Triglyceride Assay Kit (BioAssay Systems). The absorbance values of the samples were obtained with an Epoch Model microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). The serum level of total bile acids was determined by an assay kit from Cell Biolabs, Inc. (San Diego, CA, USA). All measurements were performed in duplicate.

**Quantification of serum phosphorus and magnesium**

The serum phosphorus and magnesium contents were determined with a Malachite Green Phosphate Assay Kit and a QuantiChrom™ Magnesium Assay Kit (BioAssay Systems, USA), respectively.

**Histopathological analysis**

Necropsy tissue samples from muzzle skin and internal organs were fixed in 10% phosphate-buffered formalin for 2 days, transferred to 30% sucrose in phosphate-buffered saline overnight, and then embedded in Shandon Cryomatrix (Thermo Fisher Scientific, Waltham, MA, USA). The tissues were sectioned (6 μm) and stained with hematoxylin and eosin using standard procedures. The slides were examined for ectopic mineralization under a light microscope. Oil Red O staining was performed on adjacent sections to detect lipid accumulation (Hitobiotec Corp., Kingsport, TN, USA).

**Chemical quantification of calcium**

To quantify mineral deposition, muzzle skin specimens were harvested and the calcium was solubilized with 0.15 N hydrochloric acid for 48 hours at room temperature, followed by an assay of the solubilized calcium in the supernatant. Colorimetric analysis by the o-cresolphthalein complexone method [Calcium (CPC) Liquicolor; Stanbio Laboratory, Boerne, TX, USA] was performed to measure the calcium content. The values of calcium in muzzle skin were normalized to tissue weight. The calcium in the serum samples was analyzed using the same quantitative assay.

**Statistical analysis**

The data were presented as mean ± standard error (SE). The results in different groups of mice receiving different diets were analyzed using non-parametric Mann-Whitney U test using Prism 8 (GraphPad, San Diego, CA, USA). Fisher’s exact test was used to determine the differences between the proportions of mineralization in the organs of mice fed with different diets. Statistical significance was reached at \( P < 0.05 \).

**Results**

**Serum lipid profiles and bile acids in Abcc6−/− mice fed control or atherogenic diet**

The mice fed the atherogenic diet showed significantly higher serum levels of total cholesterol (52.9% increase) and LDL/VLDL cholesterol (82.8% increase) than the mice fed the control diet; however, the serum HDL cholesterol and triglyceride levels were not significantly different between the two groups (Fig. 1). In addition, a > 1.9-fold increase in the serum bile acid level was noted in mice on the atherogenic diet (Table 2). No sex-related differences in the serum lipid panels or total bile acid levels were observed between the two groups of mice.

To examine the metabolic consequences of the atherogenic diet, the serum concentrations of calcium, phosphorus, and magnesium were determined in all mice at the end of the experimental diet. No significant differences were noted in the serum concentrations of these components (Table 2).

**Steatosis in Abcc6−/− mice fed the atherogenic diet**

Oil Red O staining of the liver and aorta showed significantly higher amounts of lipids in the Abcc6−/− mice on the atherogenic diet than in Abcc6−/− mice on the control diet (Fig. 2A). Thus, the elevations in the serum concentrations of total cholesterol and LDL/VLDL cholesterol were accompanied by steatosis. Although ectopic mineralization affects several tissues in patients with PXE, the clinical manifestations are primarily evident in the skin, eyes, and cardiovascular system. Therefore, the muzzle skin, eyes, and arterial blood vessels (characteristic sites of ectopic mineralization in PXE) were examined for mineralization by hematoxylin and eosin staining, and the lipid distribution was examined by Oil Red O staining.

![Graph: Serum cholesterol and triglyceride concentrations in Abcc6−/− mice maintained on either the control diet or atherogenic diet. The mice were placed on specific diets at 4 weeks of age, and blood samples were obtained at 54–62 weeks of age for analysis of lipid concentrations. The values are expressed as mean ± SE. Control diet, \( n = 15 \); atherogenic diet, \( n = 10 \). *\( P < 0.001 \) compared with mice on the control diet.]
Mineralization was noted in the dermal sheath of vibrissae in the muzzle skin, the retinas, and the arterial blood vessels in the kidneys (Fig. 2B). However, mineralization in these tissues was not associated with deposition of lipids (Fig. 2B).

**Effects of atherogenic diet on tissue mineralization in Abcc6−/− mice**

The degree of mineralization in Abcc6−/− mice on the atherogenic diet, as determined by the content of calcium in the muzzle skin, was significantly higher (30.4% increase) than that in mice on the control diet (Fig. 3). In addition to mineralization of the dermal sheath of vibrissae in the muzzle skin, mineralization was evaluated by histopathological analysis in the kidneys and the eyes by counting the number of mice with mineralization as a percent of the total mice examined. The results demonstrated that 70.0% of mice (7 of 10) on the atherogenic diet had mineralization in the kidneys, a significantly higher proportion than the 13.3% of mice (2 of 15) on the control diet. The incidence of eye mineralization was also higher in mice on the atherogenic diet (26.7%, 4 of 15 mice) than in mice on the control diet (6.7%, 1 of 15 mice).

### Table 2

| Concentration in group | Calcium (g/L) | Phosphorus (g/L) | Magnesium (g/L) | Bile acid (µmol/L) |
|------------------------|---------------|------------------|-----------------|-------------------|
| Control diet           | 8.03 ± 0.21   | 9.29 ± 0.30      | 2.19 ± 0.09     | 3.33 ± 0.47       |
| Atherogenic diet       | 8.40 ± 0.22   | 9.50 ± 0.50      | 2.11 ± 0.14     | 9.73 ± 1.43       |

*P < 0.001.

### Figure 2.

Demonstration of the presence of steatosis in the liver and absence of lipid accumulation at sites of ectopic mineralization in Abcc6−/− mice fed the atherogenic diet. (A) Comparison of lipid accumulation in the liver and aorta of Abcc6−/− mice on the atherogenic versus control diet, as revealed by Oil Red O staining. Scale bar = 200 µm. (B) The Abcc6−/− mice on the atherogenic diet developed ectopic mineralization (the arrows) in muzzle skin containing the dermal sheath of vibrissae, kidney, and eye as demonstrated by hematoxylin and eosin staining. However, Oil Red O staining did not show lipid accumulation in these tissues in the areas of ectopic mineralization. Scale bar = 400 µm.
The first site of mineralization, noted as early as approximately 5–6 weeks of age in Abcc6−/− mice kept on the control diet, was the dermal sheath of vibrissae in the muzzle skin.4 Mineralization of the vibrissae serves as an early biomarker of the overall mineralization process, and its quantitation by a direct calcium assay of the muzzle skin allows determination of the overall extent of mineralization in these mice.4,15 In the present study, when the Abcc6−/− mice were challenged with an atherogenic diet (TD.02028) containing higher fat, sucrose, cholesterol, and cholic acid content than the standard control rodent diet, their serum levels were significantly higher with concurrent increase of mineral deposition in the soft connective tissues of the muzzle skin. Serum concentrations of calcium, phosphorus, and magnesium were not different from those in mice on the control diet, suggesting that mineral homeostasis was not altered by the atherogenic diet. The atherogenic diet also exacerbated ectopic mineralization in the kidneys and eyes despite normal serum mineral homeostasis. Although the detailed pathomechanisms of the apparent increase in ectopic mineralization as a result of atherogenesis is not clear, recent studies have suggested that inflammatory atherosclerosis precedes and drives ectopic mineralization.25 In the present study, the increased tissue mineralization in mice fed the atherogenic diet was associated with elevated serum levels of total cholesterol and LDL/VLDL cholesterol, risk factors for cardiovascular disease. In contrast, cholesterol-lowering drugs such as atorvastatin have been shown to ameliorate the extent of ectopic mineralization in the same mouse model.13 These findings have clinical relevance for the management of PXE in humans. In this context, the prevalence of PXE (approximately 1:30,000) suggests that more than 150,000 individuals are affected by PXE worldwide. Sequence variants in the ABCC6 gene are associated with plasma levels of lipoprotein.10–11 Early detection of hypercholesterolemia and atherosclerotic disease is of paramount importance to institute possible prevention strategies and monitor treatment. Limiting the intake of dietary saturated fat and cholesterol is expected to provide significant benefits to a large number of patients in terms of preventing cardiovascular complications and worsened tissue mineralization.

One limitation of the study is that the degree of vascular mineralization as a result of hypercholesterolemia was not analyzed in depth. This is due to the fact that PXE is a late onset, yet progressive disease. While multisystem mineralization is reproduced in the Abcc6−/− mice, mineralization of the vasculature occurs later in life.4 Further studies should examine the Abcc6−/− mice to preceded death or a moribund state when vascular mineralization becomes fully penetrant.

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