

SERUM FROM PSEUDOXANTHOMA ELASTICUM PATIENTS ALTER THE STRUCTURE OF ELASTIC FIBERS

Pseudoxanthoma elasticum (PXE) is a hereditary disease that causes calcification of elastic fibers in the skin, arteries, and eyes. PXE is caused by a mutation in the *ABCC6* gene, which encodes an ABC transporter. *ABCC6* is only expressed in high amounts in the liver and kidneys, while the PXE phenotype affects the skin, the arteries, and the eyes. *ABCC6* is a metabolite pump secreting unknown compounds from polarized cells. The relationship between *ABCC6* function and elastic fibers is not known. The PXE phenotype initially suggested that PXE was a connective tissue disorder but since *ABCC6* has shown little or no expression in the skin, the arteries, or the eyes, PXE is now thought to be a metabolic disease. Consequently, it was hypothesized that *ABCC6* lack of expression in the liver/kidneys causes unknown molecule(s) to accumulate in the blood at toxic levels, and ultimately causes elastic fiber calcification. The presence of these unknown molecules in the serum of PXE patients was recently demonstrated.¹ However, the nature of this molecule has yet to be determined. A mouse model of PXE has been recently generated and replicates the human PXE phenotype. Because the mouse model of PXE could serve as a source of serum to isolate and characterize the unknown PXE molecule(s), we performed cell cultures and immunofluorescence stainings to determine if the PXE molecule(s) could also be detected in mouse PXE serum. Results revealed that PXE fibroblasts maintained in 10% mouse PXE serum produced normal networks of elastic fibers.

INTRODUCTION

Pseudoxanthoma elasticum (PXE) is a hereditary disorder distinguished by dermal, ocular, and vascular lesions that result in connective tissue alterations, including the calcification of elastic fibers. The accumulations of calcified fibers, altered collagen fibrils, and proteoglycans result in lesions predominantly in the skin, eyes, and arterial walls.² This progressive disorder is characterized by multi-organ involvement. Skin manifestations are the most common characteristic of PXE; it is normally the first physical sign of this developing disorder. Typical skin lesions appear as yellowish papules and plaques with loss of elasticity. These lesions are primarily seen on the neck, interior elbows, axilla, groin, and periumbilical area.²⁻⁴ Retinal angioid streaks results from fragmentation and calcification of the elastic component of the Bruch's membrane. Angioid streaks are affiliated with hemorrhage and subretinal neovascularization, leading to severe loss of vision.⁵ The calcification of elastic fibers of arteries often causes cardiovascular manifestations such as premature peripheral vascular occlusive disease, and intermittent claudication.⁶ The diagnoses of PXE is confirmed histologically, in biopsies of leisonal skin, and by fragmented calcified elastic fibers shown by the use of von Kossa staining. The PXE phenotype is caused by mutations in the *ABCC6* gene,⁷ which encodes an ABC transporter. *ABCC6* is only expressed in high amounts in the liver and kidneys, while the PXE phenotype affects the skin, arteries, and eyes.

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Student Researcher: Ailea Apana

Mentor: Olivier Le Saux, PhD, University of Hawaii,
John A. Burns School of Medicine, Honolulu, Hawaii

disorder, but since *ABCC6* has shown little or no expression in the skin, arteries, or eyes, PXE is now thought to be a metabolic disease. Several studies have brought support to this notion. Alterations in plasma, lipoproteins, and vitamin D metabolism from PXE patients support that PXE is a metabolic disease. In a PXE mouse model, high density lipoprotein and creatinine levels have been found to be altered.⁸ The mechanism that links liver or kidney *ABCC6* activity to the calcification of elastic fibers in other tissues remains unknown. It was, however, hypothesized that an unknown molecule(s) is no longer detoxified by the liver/kidneys, but rather accumulates in the blood at toxic levels and ultimately causes elastic fiber calcification.⁴

METHODS

Fibroblasts and serum samples

This study was approved by the University of Hawaii Institution Review Board. The fibroblasts that we used were commercial skin fibroblasts, which were purchased from Cambrex Corporation. The human PXE serum used was obtained from a French patient. The mouse PXE serum was obtained from a PXE mouse model, and the fetal bovine serum (FBS), was bought from GIBCO Life Technologies in Burlington, Ontario.

Cell culture

The skin fibroblasts were passaged by trypsinization. Our experiment was performed with the skin fibroblasts at passages 3-5. The cells were maintained in DMEM containing 1% antibiotics/antimycotics and supplemented with

10% FBS as described previously.¹ The cells were incubated at 37°C until confluence. Every three days the cells were rinsed with phosphate buffered saline (PBS) and their medium was changed. The pre-culture cells were grown for seven days in a T25 flask before being passed by trypsinization into a 24-well plate. After the cells were passed they were grown for another 12 days in three different wells. The first well contained 10% mouse PXE serum (mPXES). This is the serum that we were testing to see if characteristics of PXE could be detected. The second well contained 10% human PXE serum (hPXES). The human serum was a positive control; tests have been previously conducted to show PXE characteristics in this serum.¹ The third and last well contained 10% FBS; this was a negative control. The cells were maintained with these three different types of serum and incubated at 37°C until they reached confluence and began producing elastin fibers. After a 12-day period of growth the cells were prepared for immunofluorescence stainings.

Immunostaining

After 12 days of culture the medium was removed and the cells were washed four times with PBS. The cells were fixed in 100% methanol at -20°C for 30 minutes and incubated with an anti-elastin antibody for one hour at room temperature. The cells were then washed with PBS four times to remove the first antibody. All cultures were then incubated with a secondary antibody (a goat anti-rabbit IgG conjugated with FITC, for 45 minutes at room temperature in the dark. The fibroblasts were washed in PBS four additional times to remove the secondary antibody. Nuclei were stained with propidium iodide. All cultures were examined with an Olympus DSU microscope.

Trainings

Before performing immunofluorescence stainings on cell cultures, both

paraffin and frozen sections of normal mouse lung tissues were trained.

RESULTS

Fibroblasts produced normal elastic fibers when cultured with mPXES; immunofluorescence stainings of skin fibroblasts with antibodies specific to elastin revealed that PXE fibroblasts maintained in 10% mPXES produced normal looking networks of elastic fibers. The patterns of these fibers did not show signs of the PXE phenotype. The elastic fiber network observed with 10% mPXES closely resembled that seen with fibroblasts grown in the presence of 10% FBS (negative control), with long, well-organized strands of elastic fibers.

Fibroblasts were grown for 12 days and elastic fibers were revealed by immunofluorescence stainings using an antibody specific to elastin. Nuclei were counterstained with propidium iodine. Characteristics of the unknown PXE molecules include: PXE molecules characteristics identified thus far; heat sensitivity (not labile at 56°C, 30 min); estimated molecular weight (10 to 30 kDa); other (not an IgG, not linked to serum albumin, active in normal human or fetal bovine sera).

DISCUSSION

The PXE phenotype affects the skin, arteries, and eyes. The ABCC6 gene expression, however, is found in the liver/kidneys. It was hypothesized that the lack of ABCC6 gene expression in the liver/kidneys causes the PXE phenotype and as a consequence one or more molecules is no longer detoxified by the liver/kidneys. Thus, one could speculate that this unknown molecule(s) accumulates in the blood at toxic levels, and ultimately causes elastic fiber calcification.

In this study, we examined the effects of three different serum samples

on human skin fibroblasts. Immunofluorescence stainings specific to elastin were performed on culture of human skin fibroblasts and revealed that the fibroblasts cultured with mPXES produced normal networks of elastic fibers when compared to a negative control (FBS) and a positive control (hPXES). Because the PXE mice reproduce the human PXE phenotype faithfully,^{8,9} it is likely the mice also possess equivalent PXE molecules in their serum. Our experiments, however, failed to detect these molecules in our experimental conditions, that is, we observed no interaction between mouse PXE molecules and human elastic fibers. Previous experiments with hPXES on fetal bovine chondrocytes and rat fetal lung cells also did not reveal any interaction between human PXE molecule(s) and animal elastic fibers. Therefore, one could speculate that there is no cross-species interaction and that the PXE molecule(s) interact only with the parts of elastic fibers that are different between species. This new characteristic of the PXE molecule(s) might be useful in determining where the interaction occurs since elastin and other elastic fiber proteins are fairly well conserved between rodent and human.

This new information completed a list of previously identified characteristics of the PXE molecule(s) that includes: heat resistance; that the molecule is not an immunoglobulin, that it is not related to serum albumin, and; that it has a molecular weight between 10 and 30 kDa. Future experiments will focus on using mouse PXE serum on mouse cells producing elastic fibers. Should this experiment yield positive results, we would then have an unlimited supply of PXE serum with which to isolate and characterize the PXE molecule.

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