ABCC6: A NOVEL PROTEIN FACTOR IN SOFT TISSUE CALCIFICATION

Theses of PhD dissertation

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2013

Introduction

Calcification processes in a human body can be physiological - e.g. bone formation -, or pathologycal. Pathologycal soft tissue calcification, also known as ectopic calcification, is due to inappropriate biomineralization, and usually calcium phosphate salts are involved in the abnormal mineral deposition. Little is known about the mechanisms regulating the calcification processes, several metabolic and environmental factors are contributing to the aberrant mineralization, however, some rare monogenic disorders gave insight into the pathophysiological mechanisms contributing to arterial calcification.

In 2000 it was found that mutations in the *ABCC6* gene are responsible for the development of pseudoxanthoma elasticum (PXE), and recently mutations in the *ABCC6* gene were found to be the genetic background in some cases of Generalized Arterial Calcification of Infancy (GACI). Both diseases are characterized by soft tissue calcification symptoms. Furthermore, a missing allele of *ABCC6* is a genetic risk factor in coronary arterial disease, CAD. Based on these data ABCC6 seems to be a newly identified player in soft tissue calcification processes.

Since the first PXE-causing mutations were discovered in 2000, the number of identified disease-causing variants of ABCC6 has exceeded 350, and most of them are missense mutations caused by amino acid substitutions.

Amino acid substitutions in large plasma membrane proteins, such as ABCC6, generally result in decreased enzymatic activity, major folding and stability deficiency, poor plasma membrane targeting or a combination of these. Therefore, studying the consequence(s) of disease-causing missense mutations can provide important insights into the relationship between protein structure and pathological function.

To understand the biochemical and cellular effects caused by *ABCC6* mutations that lead to arterial and other soft tissue calcification in humans can help to find the appropriate therapy for PXE and GACI patients and may also help to better understand the mechanism of arterial calcification.

In 2007 a disease was described with similar symptoms to PXE, and therefore it was named PXE-like disorder with multiple coagulation factor deficiency. This recently characterized

disease is caused by mutations in the gene of gamma-glutamyl carboxylase (GGCX) enzyme, and the symptoms include soft tissue calcification, just like in the case of PXE. The carboxylase enzyme is a key component of a cycle, in which vitamin K is a cofactor (vitamin K cycle). These data raised a hypothesis that the abnormal calcification symptoms in the above-mentioned diseases may be related to disrupted vitamin K cycle, and that ABCC6 protein may play a role in the maintenance of calcium homeostasis by transporting vitamin K.

Aims

In my PhD research program I focused on the subcellular localization of the ABCC6 protein, on the investigation of the molecular background of disease-causing ABCC6 mutations, and on how disease-causing mutations, resulting in improper localization, could be corrected. We also wanted to test the potential role of ABCC6 in vitamin K transport.

Our specific aims were:

1. To reinvestigate the plasma membrane localization of the wild type ABCC6 transporter and to determine the subcellular localization of disease-causing ABCC6 mutants. We planned to perform several immunocytochemical and immunohistochemical experiments on MDCKII cell line, on primary hepatocytes and also on mouse and human liver tissue.
2. Our aim was to determine the molecular consequences of disease-causing missense mutations and to identify mutants with preserved transport activity but improper subcellular localization (probably due to protein folding problems). These mutants are candidates for pharmacological treatment in order to rescue the mislocalized protein. We planned to perform pharmacological correction experiments of mislocalized ABCC6 mutants *in vitro* and *in vivo*.
3. To test whether correcting the localization of the mutants results in a functional transporter, we aimed to develop an *in vivo* model system, based on determination of soft tissue calcification, to functionally investigate the ABCC6 protein.
4. As a role of ABCC6 in Vitamin K metabolism was suggested, we aimed to prove or disprove this hypothesis investigating the potential role of ABCC6 in the transport of different forms of Vitamin K.

Methods

Cell culturing

Sf9 (Spodoptera frugiperda) cells were cultured on 27°C, in TNM-FH insect medium supplied with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin.

MDCKII cells were cultured in a humidified 37°C, 5% CO_2 incubator, in DMEM culture medium with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. In the localization-correction experiments cells were cultured in the presence of 1 mM 4-PBA.

Animals

All mice were kept under routine laboratory conditions with 12 hours light-dark cycle with access ad libitum to water and standard chow. Mice were euthanized by cervical dislocation. This study was approved by the Directorate of food safety and animal health control, Government Office for Pest County (permission number: XIV-I-001/707/4/2012). C57BL/6J, C3H/HeNHsd and *Abcc6-/-* mouse strains were used.

DNA constructs

The construction of DNA plasmids was based on the standard molecular biological methods. The ten ivestigated disease-causing *ABCC6* mutantions were inserted into the human *ABCC6* cDNA using site-directed mutagenesis by inverse PCR. The cDNA of wt and mutant *ABCC6* was cut from pBluescript SK vector and was inserted into pAcUW21 baculoviral transfer vector. For retroviral transfection, *ABCC6* cDNA was cut from pAcUW21-L vector and was ligated into SpSldS retroviral vector. For sleeping beauty transposon-based transfection, *ABCC6* cDNA was first cut from pAcUW21-L vector and was ligated into a SB-CMV-ΔSma vector. Then in the second step *ABCC6* was cut from this vector and inserted into SB vector containing a puromycin-resistance gene. For the tail vein injection ABCC6 cDNA constructs were sub-cloned into the pLIVE vector.

Expression of wt and mutant forms of ABCC6 in SF9 cells

For transfection of Sf9 cells we used BaculoGold kit (BD Biosciences Pharmingen). We isolated individual virus clones from the supernatant containing the recombinant viruses using the end-point dilution method.

Expression of wt and mutant forms of ABCC6 in MDCKII cells

a) Retroviral transfection: Phoenix-Ampho packaging cells were transfected with the recombinant retrovirus vectors containing wt or mutant ABCC6. The transfection was performed using calcium phosphate transfection kit, according to the manufacturer's protocol. Cell clones overexpressing ABCC6 were selected by end-point dilution method.

b) Sleeping Beauty (SB) transposon-based transfection: For the transfection the SB plasmid containing a puromycin-resistance gene and the wt or mutant forms of *ABCC6*, and a transposase plasmid were used. The transfection solution contained 2 μg SB-ABCC6 plasmid, 0,2 μg 100x transposase and 6 μl FuGene HD reagent in 100μl serum-free DMEM medium. 48 hours after transfection cells were trypsinized and further cultured in cell culture flasks. 96 hours after transfection 3 μg/ml puromycin was added to the culture medium to select for the transfected cells.

Expression of wt and mutant forms of ABCC6 in mouse liver

PLIVE plasmid DNA constructs containing the wt or mutant *ABCC6* were delivered into the mice by hydrodynamic tail vein injection. The tail vein injections were performed with a 27-gauge needle with a volume of 1.8 ml of DNA in physiological salt solution. Mice were injected with 70 μg of plasmid.

For the localization-correction studies mice received 3 intraperitoneal injections of 4-PBA (100 mg/kg/day) prior to performing hydrodynamic tail vein injections.

Agarose gel electrophoresis and immunoblot

Membrane vesicles or whole cell samples were taken up in loading buffer then were sonicated. Proteins were separated in a 7.5% SDS-poliacrylamid gel, then proteins were transferred from the gel to a nitrocellulose membrane using an electroblotting apparatus. After incubation with the appropriate antibodies, blots were revealed by enhanced chemiluminescence.

Immunocytochemistry

Cells were first washed, fixed with 4% paraformaldehide and precooled methanol, then were incubated with blocking buffer for 1 hour at RT. After removal of the blocking buffer, samples were incubated with the appropriate primary antibodies for 2 hours at RT. After washing, cells were incubated with the secondary antibodies for 1 hour. Nuclei were stained with DAPI for 5 minutes. Samples were kept at 4°C until microscope analysis.

Immunohistochemistry

8 μm-thick frozen sections from the appropriate tissues were prepared using a cryotome. Slices were fixed in methanol, then washed in DPBS. After incubation in blocking buffer for 1 hour, the appropriate primary antibodies were added for 90 minutes, followed by the incubation with secondary antibodies for 1 hour. Nuclei were stained with DAPI for 5 minutes. Samples were kept at 4°C until microscope analysis.

Isolation, culturing and immunocytochemistry of mouse primary hepatocytes

Liver cells were isolated following the method published by Bayliss et al, 1996. Cells were plated in collagen-precoated dishes. After attachment of cells, medium was changed and renewed every 24h in the absence of serum. Immunocytochemistry was carried out after 72 hours of cell culturing.

Confocal microscope imaging

Microscope images of fixed samples were acquired using a Zeiss LSM 710 confocal laser scanning microscope. Zeiss Zen software was used to evaluate data.

Cryoinjury of mouse heart and determination of calcium deposition

Cryoinjury was performed on 3-month-old mice. After anaesthesis, aseptic surgery was performed by entering the abdomen through a midline incision. A pre-cooled metal rod was applied with slight pressure to the heart muscle through the diaphragm for 10 seconds. After completion of the procedure, a two-layer abdominal closure was performed. Mice were euthanized 4 days after cryoinjury by cervical dislocation. Heart was taken out, washed in PBS solution, and the necrotic area of the heart was dissected. The heart sections were minced and left in 250 μl 0.15N HCl for 48 hours. The calcium content of the heart sections was determined by Calcium (CPC) LiquiColor Test.

Membrane preparation and measuring protein content

Sf9 cells were harvested, washed, centrifuged, then were homogenized using Potter-Elvehjem tissue grinders. Cell debris was centrifuged and supernatant was ultracentrifuged (32000 rpm, 1 hour, 4°C). The membrane pellet was suspended, homogenized and aliquots of the membrane vesicles were kept at -80°C until use. Protein concentration of the membrane vesicles was determined by Lowry protein assay.

Transport activity assay using membrane vesicles

Sf9 membrane vesicles overexpressing wt or mutant ABCC6 were incubated with [^{3}H]LTC_4 (130 Ci/mmol, PerkinElmer) in the presence or absence of 4 mM MgATP on 37°C in a

transport buffer. The transport reaction was terminated by ice-cold stop solution and the vesicles were transferred onto a nitrocellulose filter. Radioactivity was determined by liquid scintillation counter.

Ex vivo liver perfusion

Mice were anaesthetized, and after laparatomy, the portal vein and the inferior vena cava were cannulated. The blood in the liver was flushed out through the portal vein (in) and inferior vena cava (out), and the perfusate consisting of 0.5 or 50 μM vitamin K3 in 0.3% BSA/PBS was infused into the liver at a constant flow rate of 3 ml/min. Three ml of perfusate was held in the liver after the inferior vena cava and the portal veins were ligated. Five minutes later, the efflux was collected via the inferior vena cava.

Extraction of vitamin K forms

For vitamin K3 detection samples were deproteinated by 5% trichloroacetic acid. After vigorous vortexing samples were centrifuged and the supernatants were transferred into dark glass vials and kept at −20 °C for HPLC-MS/MS analysis.

For vitamin K1 and K2 extraction first ethanol was added for deproteination, then K1 and K2 were extracted with n-hexane. After one minute vortexing samples were centrifuged, the hexane-phase was separated, and dried under a stream of nitrogen. The extracted material was dissolved in isopropanol, and kept at −20°C until HPLC-MS/MS analysis in sealed glass vials.

Measure vitamin K concentration by HPLC-MS/MS

Mass spectrometric measurements were run on an AB Sciex 3200 QTrap tandem mass spectrometer. The components were ionized in positive electrospray ionization (ESI) conditions. Samples were separated prior to mass spectrometric analysis using a Perkin Elmer HPLC system.

Results

- We proved the basolateral plasma membrane localization of both the human ABCC6 and the mouse Abcc6 transporter in primary hepatocytes and in intact liver tissue.
- We have established an *in vitro* model system to investigate the subcellular localization of ABCC6 variants, generating stable MDCKII cell lines overexpressing the wt or mutant forms of ABCC6 by sleeping beauty transposon-based transduction system.
- We have set up an *in vivo* model system suitable to investigate the expression and subcellular localization of human ABCC6 protein in mouse liver.
- We have determined the transport activity and subcellular localization of ten disease-causing ABCC6 mutants *in vitro* and *in vivo*.
- We could determine the molecular background of the disease phenotype in case of the ten investigated disease-causing ABCC6 mutants.
- 4-phenyl-butyrate (4-PBA) treatment was applied as a potential pharmacological rescue method. In MDCKII cell lines six mislocalized mutants were found to reach correct plasma membrane localization after the treatment, and the localization of four of the mutants was also corrected in mouse liver *in vivo*. These results serve as basis for future human therapeutic treatments.
- A functional *in vivo* method - based on determination of soft tissue calcification - was set up, which is suitable to investigate the functional activity of human wt and mutant ABCC6 variants expressed in mouse liver.
- Effective methods were established to extract vitamin K1, K2, K3 and K3-GS forms, and a sensitive detection method was developed for all these vitamin K forms.
- Utilizing the above techniques the potential role of ABCC6 in vitamin K metabolism was investigated, and we proved that ABCC6 does not play a role in the transport of vitamin K3-gluthation conjugate.
- We have also proved that dietary supplementation of vitamin K1 or K2 could not prevent PXE-related calcification, however, ABCC6 might be involved in vitamin K absorption, distribution or metabolism.

Discussion

Our results, demonstrating basolateral plasma membrane localization of ABCC6 both in mouse and human hepatocytes, gives a solid perspective for understanding the physiological role of ABCC6.

The *in vitro* and *in vivo* model systems we developed were suitable to investigate the subcellular localization of wt and mutant variants of ABCC6 transporter. We also investigated the effect of 4-PBA, an FDA-approved drug, on the subcellular localization of ABCC6 mutants.

Using the *in vitro* transport activity assay and investigating the subcellular localization of the ABCC6 variants we could determine the molecular background leading to the development of the disease phenotype.

An interesting observation of this study was that non-polarized cells, polarized cells and mouse hepatocytes did not give identical results when investigating the subcellular localization of the mutants. The *in vivo* system, when overexpression of ABCC6 variants were executed in intact mouse liver, is surely closer to the physiological conditions of the human ABCC6 hepatic transporter compared to cell lines.

Our results have valuable information about the subcellular localization of disease-causing ABCC6 mutants and about the potential correction of intracellularly localized mutants by 4-PBA treatment. Some of the – otherwise intracellularly localized - mutants were found with correct plasma membrane localization upon 4PBA treatment. This is an important finding, as those intracellularly expressed mutants which could be rescued by 4-PBA leading to correct plasma membrane localization are ideal candidates for further studies and may serve as basis of potential human therapies for those PXE or GACI patients who carry such disease-causing *ABCC6* mutations.

We have also developed an *in vivo* method using *Abcc6* KO mice to monitor the function of ABCC6. This *in vivo* system is based on the determination of calcium deposition, and is suitable to study the functional activity of the disease-causing ABCC6 mutants and to test whether those mutants which were previously found to be “rescued” to the plasma membrane after 4-PBA treatment are also functionally active. Results obtained from this combined *in vivo* model will be important steps toward future human therapies.

In addition, our finding that expression of ABCC6 in the liver alone was sufficient to reduce calcification, indicates that most probably the liver is the target organ of a future gene therapy in case of ABCC6-dependent genetic diseases.

Based on clinical observations, a potential role of ABCC6 in the transport of vitamin K was suggested. We tested this hypothesis, investigating K3-GS transport in an *ex vivo* system, and studying the effect of dietary K1 and K2 on the calcification in wt and *Abcc6-/-* mice.

Our results demonstrated that Abcc6 is not involved in the transport of K3-GS and that neither K1, nor K2 could prevent calcification in *Abcc6-/-* mice, therefore vitamin K1 and K2 at the periphery is not the limiting factor in the pathological calcification processes in PXE patients.

Publications related to this thesis

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