The Level of Hepatic ABCC6 Expression Determines the Severity of Calcification after Cardiac Injury

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Because vascular or cardiac mineralization is inversely correlated with morbidity and long-term survival, we investigated the role of ABCC6 in the calcification response to cardiac injury in mice. By using two models of infarction, nonischemic cryoinjury and the pathologically relevant coronary artery ligation, we confirmed a large propensity to acute cardiac mineralization in Abcc6−/− mice. Furthermore, when the expression of ABCC6 was reduced to approximately 38% of wild-type levels in Abcc6+/− mice, no calcium deposits in injured cardiac tissue were observed. In addition, we used a gene therapy approach to deliver a functional human ABCC6 via hydrodynamic tail vein injection to approximately 13% of mouse hepatocytes, significantly reducing the calcification response to cardiac cryoinjury. We observed that the level and distribution of known regulators of mineralization, such as osteopontin and matrix Gla protein, but not osteocalcin, were concomitant to the level of hepatic expression of human and mouse ABCC6. We notably found that undercarboxylated matrix Gla protein precisely colocalized within areas of mineralization, whereas osteopontin was more diffusely distributed in the area of injury, suggesting a prominent association for matrix Gla protein and osteopontin in ABCC6-related dystrophic cardiac calcification. This study showed that the expression of ABCC6 in liver is an important determinant of calcification in cardiovascular tissues in response to injuries and is associated with changes in the expression patterns of regulators of mineralization. (Am J Pathol 2014, 184: 159–170; http://dx.doi.org/10.1016/j.ajpath.2013.09.015)

In the absence of any systemic mineral imbalance, the calcification of soft tissues is defined as ectopic or dystrophic calcification. Dystrophic mineralization specifically occurs in injured, damaged, and/or necrotic tissues. The dystrophic calcification of cardiovascular tissues presents a significant medical concern because it is associated with common pathological conditions, such as hypercholesterolemia, diabetes, and chronic renal failure, as well as certain infrequent genetic conditions.1,2 Myocardial dystrophic calcification, although less frequent than vascular calcification, may occur...
in long-term survivors after substantial myocardial infarctions as a dramatic manifestation of ectopic calcium deposition. In recent years, we and others have demonstrated the role of ABCC6 mutations in several inheritable pathological conditions, such as pseudoxanthoma elasticum (PXE; Mendelian Inheritance of Man 264800), and in a subset of patients with generalized arterial calcification of infancy (GACI), a disorder otherwise associated with ENPP1 mutations. ABC6 is also associated with acquired forms of pathological calcification that occur in some patients with β-thalassemia. PXE is an autosomal recessive disease characterized by progressive (chronic) dystrophic calcification primarily affecting elastic fibers in dermal, ocular, and vascular tissues. ABC6 is an efflux pump and a member of the superfamily of ATP-binding cassette (ABC) genes. The encoded transmembrane protein is primarily synthesized in the liver and kidneys and localizes to the plasma membrane. Two independent lines of Abcc6 knockout mice replicate the human PXE phenotype. More recently, ABC6 deficiency was linked to an acute cardiac calcification phenotype referred to as dystrophic cardiac calcification (DCC) in several inbred strains of mice, including C3H/HeJ and DBA/2. DCC is an autosomal recessive trait that was described several decades ago in mice and has yet to be described in humans. It corresponds to a spontaneous condition affecting cardiovascular tissues that can also be triggered into an acute phenotype by a specific dietary regimen or direct injury. The Dyscalc1 locus affecting DCC was mapped to chromosome 7 by one of the authors (Z.A.); a single Abcc6 gene mutation leads to a constitutive decrease in protein levels in the liver. Additional loci affecting the penetrance and expression of the DCC phenotype were mapped to chromosomes 4, 12, and 14.

Although the physiological function of ABC6 toward calcification is likely exerted via the systemic circulation of its substrate(s), the exact mechanism by which this protein affects the susceptibility to mineralization in distal tissues has yet to be defined. However, one must distinguish the central differences between the DCC phenotype in mice and the mineralization phenotype of PXE in humans and mice. PXE is characterized by long-term chronic and passive development of extracellular matrix calcification affecting notably elastic fibers. The induced DCC is acute, develops over a short period of time, and affects only muscular tissues. Furthermore, the DCC calcification occurs intracellularly, seemingly initiated within mitochondria. Therefore, the chronic and acute molecular pathways leading to calcification probably share a mechanism of initiation (ABC6 deficiency), but their progressions are most likely distinct. Since the original identification of ABC6 as the causative gene for PXE, the nature of its substrate(s) has thus far been elusive and the characterization of an ABC transporter endogenous substrate(s) is not an easy task. Our present knowledge is limited to experimental data showing the ability of ABC6 to use ATP to extrude conjugated metabolites in vitro and the sporadic and disappointing testing of two potential candidate molecules. To palliate this obstacle, the investigation of the downstream effects of variable ABC6 levels on calcification and associated regulators is useful to garner meaningful clues relating to the physiological function of ABC6.

To this end, we used two complementary models of injury to induce heart mineralization, the cardiac cryoinjury previously developed by one of the authors (Z.A.), which elicits a quick calcification response, and the left coronary artery ligation, representative of ischemic heart injuries. Furthermore, we focused on the mineralization inhibitor, matrix Gla protein (MGP), and its undercarboxylated form (ucMGP), because these are associated with chronic sites of calcification in PXE; we also focused on osteopontin (OPN) and the osteogenic protein, osteocalcin (OC), for their known role in vascular calcification and ABC6-related mineralization.

Materials and Methods

Animals

C57BL/6J mice, designated herein as wild-type (WT) controls, were derived from mice purchased from Jackson Laboratories (Bar Harbor, Maine). Abcc6−/− mice were obtained from the laboratory of one of the authors (A.A.B.B.) at the Netherlands Institute for Neuroscience (Amsterdam) via a materials transfer agreement. Abcc6−/− mice were generated on a 129/Ola background and backcrossed >10 times into a C57BL/6J background. Age-matched Abcc6−/− and Abcc6+/+ littersmates used in this study were all derived from the original breeding pairs from one of the authors (A.A.B.B.) and were kept separate from the WT line of control mice. All animals were housed and cared for in an approved animal care facility in the bioscience building of the University of Hawaii School of Medicine (Honolulu, HI). The mice were kept under routine laboratory conditions with a 12-hour light-dark cycle with access ad libitum to water and standard chow. The Institutional Animal Care and Use Committee of the University of Hawaii approved this study. Experiments have been conducted according to national guidelines.

Liver-Specific Expression of ABC6 cDNA in Mice

Inducing the transient expression of a specific cDNA by hydrodynamic tail vein injection (HTVI) has been described previously. Briefly, a cDNA encoding human ABC6 or β-galactosidase was subcloned into the pLIVE vector carrying the mouse albumin promoter and a fetoprotein enhancer that ensured a liver-specific expression (Mirus Bio, Madison, WI). Plasmid DNA constructs were delivered to 3-month-old Abcc6−/− mice by HTVI. The injections were performed with a 27-gauge needle with a...
volume of 1 to 2 mL of a DNA-containing TransIT EE solution, according to the manufacturer’s instruction (Mirus Bio). Mice were injected with 40 to 60 μg of plasmid. At least three mice were injected with ABCC6 or empty pLIVE vector equivalent and subjected to cryoinjury. Mice were euthanized by standard CO2 procedures 7 days after HTVI.

Myocardial Cryoinjury

At 72 hours after tail vein injection, cardiac injury was instilled through trans-diaphragm cryoinjury, as previously described by one of the authors (Z.A.).28,29 Mice were sacrificed by CO2 asphyxiation 7 days after injury to ensure that the cardiac calcification phenotype was fully developed. Hearts were quickly removed, rinsed in PBS, imaged, and either frozen or appropriately embedded for later immunological/histological analyses.

Myocardial Ischemia through Ligation of the Left Coronary Artery

Mouse models of ischemic myocardial infarction have been described extensively in the literature.44 We used permanent occlusion of the left anterior descending coronary artery. In brief, mice were anesthetized with 240 mg/kg of tri-bromoethanol (Avertin; Sigma, St. Louis, MO) by i.p. injection, endotracheally intubated, and ventilated on a mouse ventilator (687 series; Harvard Apparatus, Holliston, MA). Subsequently, an oblique 8-mm incision was made 2 mm away from the left sternal border toward the left arm pit and the muscles were separated. Next, the chest cavity was opened and a chest retractor was inserted and opened to spread the wound 8 to 10 mm in width. The pericardium was picked up with forceps, pulled apart, and placed behind the arms of the retractor. The left coronary artery was ligated 1 to 2 mm below the tip of the left auricle in its normal position, which is expected to induce approximately 40% to 50% ischemia of the left ventricle. A 7-0 silk ligature was passed underneath the left coronary artery and tied. Occlusion was confirmed by observing discoloration of the anterior wall of the left ventricle, followed by modulation of the electrocardiogram (data not shown). The chest cavity was closed, with pressure applied to the chest wall to reduce the volume of free air. The muscles and skin were closed layer by layer with 6-0 absorbable and nylon sutures, respectively. Mice were sacrificed by CO2 asphyxiation 28 days after surgery, and hearts were quickly removed, rinsed in PBS, imaged, and processed for immunological/histological analyses.

Immunohistochemistry and Histological Analysis

For immunofluorescent and histochemical staining, hearts were quickly harvested after euthanasia, placed in optimal cutting temperature (OCT) compound, and stored at −80°C. Direct histological visualization of calcium deposition after Alizarin Red S staining on frozen sections was performed on the site of cardiac injury. Briefly, frozen slides (5 μm thick) were thawed, fixed in 100% acetone for 15 minutes, and incubated in Alizarin Red S solution (Sigma, St. Louis, MO) for 5 minutes. Selected sections from each treatment group were examined for calcium deposition, and images were collected using an Axioscope 2 fluorescent microscope (Zeiss, Thornwood, NY).

Immunofluorescent staining of cardiac injury and ABCC6 expression in the liver was performed using frozen OCT sections (5 μm thick). On heart sections, a mouse monoclonal anti-ucMGP antibody (VitaK BV, Maastricht, The Netherlands) was used to specifically detect the uncarboxylated MGP. OC and OPN were visualized with rabbit polyclonal antibodies (Millipore, Temecula, CA). ABCC6 expression was detected on liver sections using antibodies against human ABCC6 (clone M6II-31) and mouse ABCC6 (S-20) (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibody used was Alexafluor 488 (Invitrogen, Carlsbad, CA). Stained sections were mounted in mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). The distribution of OC, OPN, and MGP in the heart and human and mouse ABCC6 in the liver was determined by imaging using an Axioscope 2 fluorescent microscope. Individual images were collected and processed with Photoshop CS3 (Adobe, San Jose, CA).

Quantification of Regulators of Calcification

OC, OPN, and total MGP levels were quantified using whole tissue protein extract and commercially available enzyme-linked immunosorbent assay (ELISA; Biotang Inc., Waltham, MA; R&D Systems, Minneapolis, MN; and Elab, Wuhan, China, respectively). For both the ischemic and cryoinjured mice, the heart was first weighed, the atria were excised, and only the ventricular region containing the injured area was retained. Proteins were then extracted from the ventricular region using the same protocol as for Western blot analysis and adjusted to 1 mg/mL total protein before analysis.

Colorimetric Calcium Measurement

The level of mineralization in the hearts was quantified with a colorimetric assay58 that measures directly the amount of calcium within excised tissue, which is then normalized to the weight of the excised tissues, as previously described. 50 Briefly, for cryoinjured hearts, wet tissues corresponding to the ventral area comprising the injured side of the heart were separated from the uninjured dorsal region. Both sections were minced and incubated at room temperature for 48 hours in 0.15N HCl. For ischemic injuries, the same wet tissue procedure was applied but to the whole ventricular area of the hearts minus the atria. Samples were centrifuged at 16,100 × g, and the total calcium content of the HCl supernatant was assessed using the Calcium Liquicolor kit (Stanbio, Boerne, TX). Calcium content was normalized to

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total tissue weight before mincing. The calcium values obtained were expressed in μg/dL and per milligram of tissue, and the ratio of calcium content in injured/uninjured (background level) tissue was calculated.

Quantitative RT-PCR

We used real-time PCR to determine the level of mRNA expression for the mouse Abcc6. Total RNA was extracted from approximately 20 mg of liver sample using the RNeasy kit (Qiagen Inc., Valencia, VA). The RNA was converted into first-strand cDNA using a first-strand synthesis kit with random hexamers (GE Healthcare, Buckinghamshire, UK). The levels of expression of the mouse Abcc6 and human ABCC6 and corresponding endogenous GAPDH were detected by quantitative RT-PCR using commercially available TaqMan probes (Applied Biosystems, Foster City, CA).

Apoptosis Cell Death Detection

Cell death through apoptosis was detected using an in situ cell death detection kit (Roche Applied Science, Penzberg, Germany). The protocol was followed according to the manufacturer’s instructions, with the final step being the detection of apoptosis by fluorescence. For comparison, paraffin-embedded sections from Abcc6+/− and WT mice were taken from the injured area of cryoinjured heart at 1, 3, and 7 days after treatment. After treatment with the detection kit, slides were mounted in DAPI-containing mounting medium (Vector Laboratories), covered with a coverslip, and viewed on an Axioscope 2 fluorescent microscope, as previously described.

Western Blot Analysis

Proteins were extracted by homogenizing several whole lobes of frozen liver tissue on ice in 10 mL of radioimmunoprecipitation assay buffer containing 1 × protease inhibitor minicomplete cocktail (Roche Applied Science, Indianapolis, IN) and 5 mmol/L EDTA. Homogenates were centrifuged at 16,000 × g, and the protein concentration in the supernatant was determined by absorbance using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). A total of 50 μg was combined with reducing Novex sample buffer (Invitrogen) and loaded into wells of 4% to 12% polyacrylamide gel (Invitrogen). Proteins were transferred to nitrocellulose membrane and blotted with the polyclonal antibody K-14 that was raised against the C-terminal end of the rat ABCC6 for the Western blot detection and quantification of the mouse ABCC6 protein. Appropriate secondary antibodies were detected using the Odyssey infrared imaging system (Li-cor, Lincoln, NE). For densitometry, the net intensity of the target bands was normalized to that of β-actin (Abcam, Cambridge, MA) to obtain a relative level of the protein of interest on an unsaturated exposure using ImageJ software version 1.45k (NIH, Bethesda, MD).

Results

Cardiac Dystrophic Mineralization in Abcc6−/− Mice

Cryoinjury to the myocardium of Abcc6−/− mice resulted in significant and extensive dystrophic calcification in the region of injured tissue. The calcification within the injured

Figure 1 Surface lesions and calcification of Abcc6−/− and control mice. Cardiac lesions were obtained through a single transdiaphragm cryoinjury. Mice were sacrificed 7 days after surgery, and hearts were exposed by opening the chest cavity and quickly imaged before excision. A: A representative image obtained with an Abcc6−/− mouse illustrates the macroscopic appearances of the surface lesions outlined with a dashed circle. The calcification is visible as white deposits. B and C: The lesions of a heterozygous Abcc6+/− mouse and a sham—operated on Abcc6−/− mouse devoid of any obvious white deposits indicating the lack of calcification. D: The discoloration of the myocardial tissue after ischemic injury (4 weeks, permanent occlusion) is delineated by the dashed circle. The arrowhead points to the suture ligating the left coronary artery.
tissue was detectable by eye before excision (Figure 1) and confirmed histologically (Figure 2). The calcification was contained within the site of injury and appeared as a well-defined necrotic region on the left ventricle wall (Figures 1 and 2). This allowed the precise quantification of mineralization in the injured section, which was expressed as a ratio between the injured ventral and uninjured dorsal sections. Interestingly, the level of calcification does not appear to significantly abate within 4 weeks (Figure 3A), indicating the pathological condition is not reversible within this time frame. Because calcification quickly follows induced cardiac necrosis when ABCC6 function is impaired, we also tested whether Abcc6+/− mice responded to the ligation of the left descending coronary artery as a method to represent severe cardiac ischemia. This type of injury resulted in calcification of the myocardium, although the injured area was more diffuse and less defined than with cryoinjury (Figure 2). Calcium deposits were sporadically located throughout the left ventricle wall from the region of ligation to the apex of the heart (data not shown). Because of the diffuse nature of the ischemic injury, the calcification phenotype could not be reliably expressed as a ratio between the injured and uninjured regions and was, therefore, evaluated using total tissue calcium content, as previously described. The mortality resulting from the ligation of the left descending coronary artery was low (2 of 18 Abcc6+/− mice), whereas none of the nine WT animals died after surgery and within the 4-week study period.

The ratio between the calcium content in the injured and uninjured heart sections after cryoinjury was markedly higher in Abcc6+/− mice, at 6.93 ± 1.14 (n = 22), than those of the control mice (eg, the sham—operated mouse, WT mice, which had lower ratios of 1.08 ± 0.30 (n = 7), 0.92 ± 0.34 (n = 5), and 1.10 ± 0.28 (n = 4), respectively) (Figure 3A). An illustration of the visible ectopic calcification on the injury site is shown in Figure 3B. We did not find evidence of mineralization by histochemical staining of the myocardium of the control mice (data not shown). For ischemic injury, the total heart calcium content was also significantly elevated in the experimental mice (n = 8), with values of 69.6 ± 9.2 μg/mL/mg compared with 36.4 ± 4.6 μg/mL/mg, 22.6 ± 3.6 μg/mL/mg, and 28.4 ± 4.4 μg/mL/g for the Abcc6+/− mice.

**Figure 2** Histological characterization of myocardium of Abcc6+/− mice and mice after cryoinjury or sham treatment. After excision, hearts were fixed in formalin and paraffin embedded. Sections were stained with H&E or Alizarin Red S and imaged. A and B: Images of heart cross sections of Abcc6+/− mice show the mineralization occurring after cryoinjury but not in the sham—operated mouse. C and D: The consequence of the ischemic injury after left coronary artery ligation (H&E staining). Arrows point to the left coronary artery upstream (C) and downstream of the ligation and the affected portion of the myocardium, which is outlined (D). E: An image of a serial section from D, stained with Alizarin Red S to reveal the calcification. Scale bar = 500 μm (A and C).
Prepared for X-gal staining to examine the distribution of expression. After excision, the whole liver and both kidneys were lightly stained for X-gal. No detectable expression was found in either kidney, demonstrating the tissue-specific distribution pattern throughout the lobes of the liver, although on close examination. The method revealed an extensive distribution throughout all lobes of the liver, with a significant reduction in expression with no detectable expression in kidneys (Figure 4, E and F).

Consequently, HTVI into Abcc6−/− mice using a human ABC6 cDNA vector encoding a functional ABC6 protein and targeted for hepatic expression through the pLIVE vector resulted in a liver-specific expression with no detectable expression in kidneys (Figure 4, E and F).

The efficiency of HTVI was first tested with a pLIVE vector carrying the β-galactosidase (LacZ) gene. The results demonstrated that the transfection resulting from the HTVI was distributed throughout the liver, with a significant presence in all of the individual hepatic lobes 7 days after injection (Figure 4, A–C). Furthermore, this transfection was heterogeneously distributed within the lobes themselves, with patchy clumps of highly transfected cells adjacent to areas with relatively low transfection efficiency (Figure 4D). We also verified that the pLIVE vector resulted in a liver-specific expression pattern (Figure 4D). These immunofluorescent images and Western blot analysis with the K-14 antibody highlighted the sparse, but significant, expression of the human ABC6 protein in mouse livers in patterns similar to the β-galactosidase-transfected mice (Figure 4). Indeed, we detected the ABC6 protein in groups of heterogeneously distributed cells throughout the liver, comparable to the results of a previous study. As expected, no human or mouse Abcc6 mRNA (data not shown) or protein (Figure 5F) was detected in noninjected control Abcc6−/− animals.

Despite the expression level of the human protein being low, as estimated by the number of positively transfected hepatocytes, the amount of ABC6 protein produced was sufficient to significantly reduce the calcification of cryoinjured cardiac tissue in Abcc6−/− mice (Figure 3, A and C). The human ABC6-transfected mice had a calcium ratio of 2.55 ± 0.57 (n = 10), which represented a 2.7-fold reduction in mineralization when compared with an Abcc6-null mouse at 6.93 ± 1.14 (Figure 3A). There was no statistically significant difference between the levels of calcification after cryoinjury in the human ABC6-transfected mice and the sham control animals. To firmly attribute the reduction of calcification to ABC6 function, we repeated the cryoinjury procedure with Abcc6−/− mice subjected to HTVI with a plasmid carrying the human ABC6 cDNA with the PXE-causing p.V1298F mutation. We have previously found this mutant to be transport inactive but fully integrated to the plasma membrane. As expected, the high calcification ratio was similar to the non-treated Abcc6−/− mice (Figure 3A), indicating that the calcification rescue was, in fact, due to a functional human ABC6.

**Figure 4** Verification of the efficiency of HTVI and the overall distribution of the transfected hepatocytes. The commercial pLIVE vector carrying liver-specific promoter, enhancer, and β-galactosidase was used. After excision, the whole liver and both kidneys were lightly fixed and prepared for X-gal staining to examine the distribution of expression. A–C: The method revealed an extensive distribution throughout all lobes of the liver, although on close examination. The distribution of the individual cells was found to be heterogeneous. E and F: No visible expression was detected in either kidney, demonstrating the tissue-specific expression and the efficient delivery achieved by the HTVI expression of the pLIVE vector.

**ABCC6 Deficiency Leads to Changes in the Levels of Several Mineralization Regulators**

We detected an increased expression or a change in the distribution pattern of OPN and ucMGP in the cryoinjury and ischemic injury areas of Abcc6−/− mice. OC showed no significant difference with ELISA measurements or immunofluorescence (Figures 6 and 7). In cryoinjured Abcc6−/− mice, we found a large increase in OPN levels (Figure 6), which were distributed throughout the area of necrotic tissue. There was a large increase in the detectable amount of OPN in the experimental animals when compared with that in control tissues (Figure 7). More interestingly, we found significantly increased amounts of ucMGP tightly localized in the areas of mineralization. Serial sections on
experimental mice and control animals are noted in Figure 7. A similar pattern was observed in the ischemic heart areas. However, because of the more diffuse nature of the damage in this model, colocalizing the immunostainings with the areas of calcium deposits was less evident (Figure 7). The control Abcc6+/−/C0 heterozygous mice exhibited little OC or ucMGP expression in the injured cardiac tissue, but we still detected small amounts of OPN distributed throughout the injured area, despite the noticeable absence of mineralization in cardiac tissues (Figure 7).

**Transient Expression of Human ABCC6 Normalizes the Expression Pattern of MGP**

Along with reduced calcification, the cryoinjured area of cardiac tissue of Abcc6−/− mice expressing the human
ABCC6 showed limited changes in OC and OPN staining when compared with *Abcc6*+/− animals (Figure 7). No changes could be observed between treated and untreated mice using the OC ELISA quantification (Figure 6). The ELISA quantification of OPN showed a small (15%), yet significant, reduction in protein level in the cryoinjured region (Figure 6). As for ucMGP, we found that there was still some positive staining associated with those areas of calcium deposition identified with Alizarin Red S staining (Figure 7); however, the overall levels in the injured site were almost completely normalized (Figure 6).

Mineralization Is Not Due to an Alteration in Cell Death

At 1, 3, and 7 days after cryoinjury, the *Abcc6*−/− and WT hearts showed similar levels and patterns of apoptosis (Figure 8). At 24 hours after cryoinjury, apoptosis was extensive in and around the injured areas, with a concentration at the surface of the heart lessening in intensity farther into the tissue. After 3 days, the level of apoptosis detected by the TUNEL method was considerably reduced and showed a similar sporadic pattern in both *Abcc6*−/− and WT mice. By day 7 after cryoinjury, little apoptosis was seen in either mouse strain. TUNEL staining did not specifically colocalize with areas of calcium deposition in the *Abcc6*−/− mice and, in fact, appeared to involve a larger region, which further indicates the lack of contribution of cell death to the mineralization phenotype (Figure 8).

**Discussion**

The DCC phenotype (in mice) is one of the four similar pathological calcification phenotypes linking ABCC6 deficiency to diseases such as PXE, GACI, and β-thalassemia.6,8,10,13,22,25 In this study, we investigated the effect of variable ABCC6 expression in the calcification response to cardiac injury using two models of cardiac injuries, non-ischemic cryoinjury and the pathologically relevant coronary ligation. Furthermore, we examined the recruitment of calcification-related proteins (OC, OPN, and MGP) in cardiac tissues after cryoinjury and ischemic injury.

The mouse *Abcc6* gene encodes an ABC-transporter protein primarily expressed in the liver and, to a lesser extent, in the kidney.17 We used Western blot analysis and immunofluorescence imaging to estimate the expression of the ABCC6 protein in the liver of WT and *Abcc6*−/− mice. We found the expression in the heterozygous *Abcc6*+/− mice to be at approximately 38% of the WT levels. Because this level of expression was sufficient to prevent the DCC phenotype, we tested whether a gene therapy approach could improve
We have established that both superficial necrosis-inducing cryolesions and ischemic events can trigger DCC in mice lacking ABCC6. After cryoinjury, calcification occurs quickly between days 3 and 7. The ischemia method, on the other hand, requires 4 weeks for a reasonable calcifying response. Because HTVI results in the expression of a recombinant protein for a maximum of 1 to 2 weeks, we have not assessed the possibility of transient gene therapy to rescue cardiac mineralization under ischemic conditions. Repeated tail vein injection could be an option to overcome this obstacle; however, the risk of HTVI to an already compromised heart in these mice is significant.

A recent study by Mungrue et al demonstrated the relationship between ABCC6 function and infarct size under short-term ischemia-reperfusion conditions (< 1 hour). In their studies, the authors noted the absence of calcification in the myocardium of both of the Abcc6-deficient mice tested after 30 minutes of ischemia, followed by 48 hours of reperfusion. This suggests that only a sustained cardiac injury will lead to significant calcification in the absence of ABCC6. The authors also hypothesized a link between ABCC6 and the inhibition of the anti-apoptotic bone morphogenetic protein 4/9 signaling pathway. It would be valuable to investigate this mechanism further with respect to apoptosis and mineralization.

Dystrophic calcification shares similarities with bone mineralization. In this study, we also focused on how the level of ABCC6 affected relevant mineralization-related proteins that might be downstream effectors. We observed this acute calcification phenotype. We applied HTVI to express a functional human ABCC6 cDNA under the control of a liver-specific promoter in Abcc6−/− mice or to deliver control vectors, either empty or carrying an inactivated ABCC6 mutant. The number of hepatocytes showing a detectable expression of human ABCC6 (approximately 13% of total) was counted and found significant 7 days after HTVI.

Although the estimated protein levels were markedly lower, with an immunofluorescent signal/DAPI ratio of approximately 0.9 when compared with the WT ratio of approximately 19.4, this relatively modest level of human ABCC6 expression is probably an underestimation of the initial amount because HTVI expression peaks within 24 hours before slowly decreasing. However, this small amount of ABCC6 expression in liver was sufficient to significantly decrease the DCC phenotype to a level comparable to controls. Therefore, we estimated that the minimum level of ABCC6 protein necessary to achieve complete inhibition of mineralization in a C57BL/6J background lies well below 38%, which is found in heterozygous mice. Our findings demonstrate that the method of transient delivery of the human ABCC6 results in a physiologically functional protein that requires few hepatocytes expressing ABCC6 (in mice) to positively affect the DCC response. Furthermore, in agreement with our previous results, we suggest that the liver should be the target organ for possible future gene therapy intervention in diseases characterized by reduced ABCC6 activity.
variation in the level or distribution of OPN and MGP, notably in its undercarboxylated form (ucMGP) in the injured cardiac tissue of Abcc6^−/−/ mice in both the cryoinjured and ischemic groups, but not for OC. Of particular interest was the fact that OPN and ucMGP were not affected equally by both the injuries and the partial restoration of the ABCC6 function in liver. Interestingly, we found that MGP, an inhibitor of calcification, was tightly colocalized with the calcification nodules in the injured myocardium, and its elevated levels were essentially normalized in HTVI-rescued and in heterozygous Abcc6^+/−/ mice. Therefore, one may suggest that the tissue surrounding the sites of calcification reacts to contain the developing mineralization by producing this inhibitor of calcification, albeit in an inactive undercarboxylated form. (uc)MGP was shown to be associated with the chronic extracellular calcification in patients with PXE.36,37 Our results show that ucMGP also plays a role in the intracellular calcification of the acute DCC phenotype. However, it is still unclear if the MGP response is related to the calcification, the ABCC6 status, or both. One would need to look for intermediary elements controlling MGP expression, such as related bone morphogenetic protein levels.48

OC is a key regulator of calcification and is differentially expressed in areas of calcified lesions in vascular tissues.40,41,49 Herein, we only observed a modest redistribution, and nonsignificant changes, of OC levels in the injured myocardium of Abcc6^−/−/ mice, indicating a limited role, if any, for OC in the DCC phenotype. OPN also plays a critical role in vascular mineralization and inflammation,50 but more interestingly, it is up-regulated in cardiac tissues in DCC-susceptible B6.C3H Dyscalc1 mice but not in the DCC-resistant C57BL/6J mice.23,28,38 Both B6.C3H Dyscalc1 animals and the C57BL/6J mice share the same genetic background and only differ on the Dyscalc1 locus on chromosome 7 that contains the mutated Abcc6 gene. Consequently, it was no surprise that OPN level and distribution were dramatically affected in both cryoinjured and ischemic cardiac tissues in Abcc6^−/−/ mice. However, the novelty of our result is that the modulation of ABCC6 expression in the liver positively affected OPN levels in heart injury. Taken together with the fact that one of the authors (Z.A.) has recently identified one of the transcription factors responsible for the Abcc6-dependent OPN transactivation in B6.C3H Dyscalc1 mice,38 it appears that ABCC6 function directly influences OPN expression. Furthermore, Hendig et al.12 have reported an association of polymorphisms within the promoter region of OPN and calcification in patients with PXE.

Our study has clearly developed further the notion that ABCC6 function is not limited to the extracellular calcification of elastic fiber in rare diseases (PXE and GACI) but is more likely to have wider ramifications toward a variety of cellular and molecular processes. Both PXE and DCC are autosomal recessive traits, and the expression of a small amount or a single allele of ABCC6 is sufficient to dramatically decrease the susceptibility to calcification.20,21 We have not observed obvious signs of mineralization after cardiac cryoinjury in heterozygous animals. However, the mice used in these studies are inbred strains with genetic characteristics not fully superimposable to that of humans because it seems that, in older human subjects with reduced ABCC6 function, some show mild PXE manifestations (A.A.B.B., unpublished data).13,52 Other studies have also suggested that human heterozygous carriers of ABCC6 mutations are more likely to develop complications resulting from cardiovascular incidents,53,54 although this is not without some controversy.55 Because the estimated frequency of heterozygous carriers of ABCC6 mutations in the general population is about 1 in 80,56 a significant portion of the general population is likely at an increased risk of cardiovascular calcification, especially if modifying loci are present. In mice, the ABCC6 anti-mineralization potential is modulated by three other loci of unknown characteristics on mouse chromosomes 4, 12, and 14,29 and probably more.57,58 Similar loci could exist in humans as well.

In summary, we have shown that both cryoinjury and ischemic injury lead to acute calcification within the myocardium of Abcc6^−/−/ mice. This phenotype and the levels of local inhibitors of calcification were modulated by the successful expression of a functional human ABCC6 protein in the liver of Abcc6^−/−/ mice. Our results showed that ABCC6 is, therefore, a critical determinant of acute calcification in nonelastic tissues, in addition to being a modulator of chronic mineralization of connective tissues, as previously described.6,9,20,21

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References


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