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Modeling hepatic osteodystrophy in *Abcb4* deficient mice

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ABSTRACT

Hepatic osteodystrophy (HOD) denotes the alterations in bone morphology and metabolism frequently observed in patients with chronic liver diseases, in particular in case of cholestatic conditions. The molecular mechanisms underlying HOD are only partially understood. In the present study, we characterized the bone phenotypes of the ATP-binding cassette transporter B4 knockout mouse (*Abcb4*$^{-/-}$), a well-established mouse model of chronic cholestatic liver disease, with the aim of identifying and characterizing a mouse model for HOD. Furthermore, we investigated the influence of vitamin D on bone quality in this model. The bone morphology analyses revealed reduced bone mineral contents as well as changes in trabecular bone architecture and decreased cortical bone densities in *Abcb4*$^{-/-}$ mice with severe liver fibrosis. We observed dysregulation of genes involved in bone remodeling (osteoprotegerin, osteocalcin, osteopontin) and vitamin D metabolism (7-dehydrocholesterol reductase, Gc-globulin, *Cyp2r1*, *Cyp27a1*) as well as alterations in calcium and vitamin D homeostasis. In addition, serum RANKL and TGF-β levels were increased in *Abcb4*$^{-/-}$ mice. Vitamin D dietary intervention did not restore the bone phenotypes of *Abcb4*$^{-/-}$ animals. We conclude that the *Abcb4*$^{-/-}$ mouse provides an experimental framework and a preclinical model to gain further insights into the molecular pathobiology of HOD and to study the systemic effects of therapeutic interventions.

Introduction

Patients with chronic liver disease frequently demonstrate alterations in bone mineral metabolism, leading to osteopenia and osteoporosis. The metabolic bone disease that causes defective bone remodeling in the setting of chronic liver diseases is termed hepatic osteodystrophy (HOD) [1,2]. To date, the exact prevalence of HOD is unknown. However previous studies estimated that up to 75% of all patients with chronic liver disease suffer from severe osteoporosis [3,4].

Depending on the etiology of the liver disease, different patterns of progressive bone diseases can be detected. Specifically, chronic cholestasis appears to affect bone metabolism and structure. Indeed, the reported prevalence of osteopenia and osteoporosis in chronic viral hepatitis is approximately 20%, whereas up to 60% of patients with chronic cholestatic diseases display decreases in bone mass [2,5]. Low bone mass and diseases associated structural deterioration in patients with chronic cholestasis results in increased frequency of fractures of spine, hip and femoral neck as well as other peripheral fractures [1]. However, it is well understood that in addition to low bone mass, other factors such as bone geometry and altered states

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of bone turnover, contribute to the risk of fracture [6]. In patients with chronic liver diseases fractures do severely affect the quality of life and result in increased morbidity, which in turn compromises long-term prognosis.

HOD, once it has developed, is difficult to treat and special care is required to support the healing of existing fractures [7]. Better understanding of the pathogenesis of HOD is essential to develop adequate treatment strategies. To date several factors have been identified to be associated with HOD, but the pathobiological mechanisms have yet to be fully defined. In particular, bilirubin, insulin-like growth factor 1 (IGF-1) deficiency and the receptor activator of nuclear factor κB ligand (RANKL) — osteoprotegerin (OPG) system have been investigated [6]. Distorted calcium and vitamin D homeostasis seem to play a prominent role in cholestasis-induced bone disease. In short, vitamin D represents a key regulator of calcium homeostasis and is therefore essential for bone formation and metabolism. It can be absorbed from food or synthesized endogenously from cholesterol derivatives by ultraviolet irradiation in the skin. Here, 7-dehydrocholesterol is converted to cholecalciferol, which then undergoes 25-hydroxylation in hepatocytes, a process mediated primarily by cytochrome P450 enzyme CYP2R1 as well as CYP27A1, CYP2J2 and CYP3A4 [8]. The hydroxylation product, 25-hydroxyvitamin D (25(OH)-vitamin D, also termed calcidiol), enters the systemic circulation, where it is transported by vitamin D binding-protein (also known as Gc, group-specific component or Gc-globulin) [9]. Calcidiol undergoes further hydroxylation by CYP27B1 mainly, but not exclusively, in the kidney. The resulting hormonal metabolite of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2$-vitamin D) is, known to act on genes involved in bone metabolism, such as *BGLAP* (osteocalcin), *RANKL* (a.k.a. tumor necrosis factor ligand superfamily member 11, *TNFSFF11*) and *SPP1* (osteopontin) [10].

The ATP-binding cassette transporter B4 knockout mouse (*Abcb4*$^{-/-}$) is a well-established mouse model of chronic liver diseases with a distinct and well-characterized hepatic phenotype. Due to the lack of the hepatobiliary phosphatidylcholine floppase ABCB4, the mice develop bile acid-induced liver damage, leading to sclerosing cholangitis and biliary fibrosis [11–13]. Since, to our knowledge, there are no data outlining the bone phenotype of these mice, we sought to characterize *Abcb4*$^{-/-}$ mice with regard to bone mass, structure and metabolism, with the goal of ascertaining the suitability of the *Abcb4*$^{-/-}$ mouse as a model for HOD. Second, we investigated the influence of vitamin D treatment on bone quality in this new model.

Experimental procedures

Generation of BALB-Abcb4$^{-/-}$ *mice*

To generate the fibrosis-susceptible BALB-*Abcb4*$^{-/-}$ mouse line, the FVB-*Abcb4*^{tm1Bor} strain was backcrossed into the BALB/cJ background for more than 10 generations. BALB/cJ inbred mice were obtained from Charles River (Sulzfeld, Germany). Mice were kept in 12-h light–dark cycles and were provided with water and standard diet (Altromin 1314, Lage, Germany) ad libitum. Temperature and humidity were regulated to 22 ± 1 °C and 55 ± 5%, respectively.

To confirm the *Abcb4*$^{-/-}$ genotype, we used the polymerase chain reaction (PCR) of tail DNA with *neo* (5′-CTT GGG TGG AGA GGC TAT TC-3′, 5′-AGG TGA GAT GAC AGG AGA TC-3′) and *Abcb4* (5′-CAC TTG GAC CTG AGG CTG TG-3′, 5′-TCA GGA CTC CGC TAT AAC GG-3′) specific primer pairs. The PCR reaction contained 10× PCR buffer (Applied Biosystems, Darmstadt, Germany), 2 mM $MgCl_2$, 10 μM dNTPs, 10 μM primer, 1.25 U *Taq* DNA polymerase (Invitrogen, Darmstadt, Germany), and 20–100 ng DNA in 25 μl-reactions. PCR cycling conditions were 30 s @ 94 °C, 60 s @ 55 °C and 30 s @ 72 °C for 35 cycles, and a final extension step of 10 min @ 72 °C.

The experimental protocols were performed with permission of the federal states of Baden–Württemberg, Bavaria and Saarland according to §8 of the German Law for the Protection of Animals and the Directive 2010/63/EU of the European Parliament.

Phenotypic characterization of hepatic fibrosis

Histopathology and hydroxyproline assay

Liver samples for histopathological evaluation were fixed in 4% neutral buffered formalin at 4 °C for 24 h and embedded in paraffin. Sections (2–5 μm) were stained with hematoxylin-eosin (H&E), Masson Goldner trichrome, and Sirius red.

Liver injury was scored at 5, 15, 20, 30, and 44 weeks of age in groups of 4 animals per genotype and point in time. In detail, slices of the left lateral, the right, the median and the caudate liver lobes were scored (0–20) separately based on the presence of periductal connective tissue, edema, inflammatory infiltrations, periportal fibrosis, spongy or bridging necrosis, connective tissue septa, proliferation, atrophy and diminution of bile canaliculi, and biliary cirrhosis.

Liver fibrosis was quantified in 15-week-old mice using an histomorphometric semi-automatic system for image analysis (Leica microscope, equipped with Leica application suite software; Wetzlar, Germany). The percentage of collagenous area was calculated from 10 microscopic fields (magnification 100×) randomly chosen in each liver section. Hepatic fibrosis was staged according to Batts and Ludwig [14] and the Ishak [15] scoring systems. The F-scores were defined as follows: 0, no fibrosis; 1, scatter periportal and perineoductular fibrosis; 2, periportal, perineoductular fibrosis (complete lamellae with beginning septa); 3, periportal, perineoductular fibrosis with portal–portal septa; and 4, complete cirrhosis.

In addition, hepatic collagen contents were quantified calorimetrically via the collagen specific amino acid hydroxyproline (Hyp), as described by Jamall et al. [16,17].

Clinical chemical and enzyme-linked immunosorbent assays

Blood samples for chemical analyses were obtained from isoflurane-anesthetized mice by puncturing the retro-orbital sinus with capillaries and subsequently collected in heparinized tubes. Plasma alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase (AP) activities as well as calcium and inorganic phosphate concentrations were measured with the Olympus AU 400 autoanalyzer (Olympus, Hamburg, Germany) using adapted reagent kits (Olympus, Hamburg, Germany) or Hitado (Möhnesee, Germany) kits, alternatively.

Serum 25(OH)-vitamin D levels were determined using the chemiluminescence immunoassay LIAISON® 25 OH VitaminD TOTAL assay (DiaSorin, Dietzenbach, Germany). Transforming growth factor-β (TGF-β) levels were measured by TGF-β-receptor cells (MFB-F11), provided by Dr. Ina Tesseur [18] with slight modifications as described [19]. Each sample was measured in triplicate, using active recombinant human TGF-β_1 as control. RANKL and OPG concentrations in serum were measured in duplicates by enzyme-linked immunosorbent assay (ELISA) (Quantikine mouse RANK ligand and OPG immunoassays, R&D Systems, Minneapolis, USA).

Reverse transcription and quantitative real-time PCR

Total mRNA from grinded snap frozen liver tissue specimens was isolated using peqGOLD TriFast™ (Peqlab, Erlangen, Germany) or RNeasy Mini kits (Qiagen, Hilden, Germany). cDNA was synthesized from 1 to 2 μg RNA using cDNA reverse transcription (RT) kits from Applied Biosystems (Carlsbad, USA) or Fermentas (St. Leon-Rot, Germany). Table 1 summarizes primer sequences (5′–3′) and RT-PCR conditions. Products, resolved by gel electrophoresis in a 2% (w/v) agarose gel, were visualized with ethidium bromide. Densitometric analysis of signals was performed using Image J software (NIH, Bethesda, USA).