Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

# Neonatal bone marrow transplantation prevents liver disease in a murine model of erythropoietic protoporphyria

Yann Duchartre[1,2,#], Nicolas Petit[1,2,#], Corrine Moya[1,2], Magalie Lalanne[1,2], Pierre Dubus[3], Hubert de Verneuil[1,2], François Moreau-Gaudry[1,2], Emmanuel Richard[1,2,*]

[1]*Univ. de Bordeaux Biothérapies des maladies génétiques et cancers, U1035, F-33000 Bordeaux, France;* [2]*INSERM, Biothérapies des maladies génétiques et cancers, U1035, F-33000 Bordeaux, France;* [3]*EA 2406 Histology and Molecular Pathology of Tumors, Univ. Victor Segalen Bordeaux 2, Bordeaux F33076, France*

**Background & Aims**: Erythropoietic protoporphyria (EPP) is an inherited disorder of heme biosynthesis caused by partial ferrochelatase deficiency, resulting in protoporphyrin IX (PPIX) accumulation in erythrocytes, responsible for skin photosensitivity. In some EPP patients, the development of cholestatic liver injury due to PPIX accumulation can lead to hepatic failure. In adult EPP mice, bone marrow transplantation (BMT) leads to skin photosensitivity correction but fails to reverse liver damages, probably because of the irreversible nature of liver fibrosis. Our aim was to determine the time course of liver disease progression in EPP mice and to evaluate the protective effect of BMT into neonates.
**Methods**: We studied the development of liver disease from birth in EPP mice, in relation with erythroid and hepatic PPIX accumulation.

To prevent the development of liver disease, BMT was performed into newborn mice using a novel busulfan-mediated preconditioning assay.
**Results**: We showed that hepatic PPIX accumulates during the first 2 weeks and correlates with the onset of a progressive liver fibrosis in 12-day-old EPP mice. Transplantation of normal congenic hematopoietic stem cells into EPP neonates led to long-term donor hematopoiesis recovery. A full correction of erythroid PPIX accumulation and skin photosensitivity was obtained. Furthermore, five months after neonatal BMT, liver damage was almost completely prevented.
**Conclusions**: We demonstrated for the first time that BMT could be successfully used to prevent liver disease in EPP mice and suggested that BMT would be an attractive therapeutic option to prevent severe liver dysfunction in EPP patients.

Keywords: Porphyria; Liver fibrosis; Cirrhosis; Cellular therapy; Newborn transplantation.
*Received 26 July 2010; received in revised form 2 September 2010; accepted 19 September 2010; available online 23 October 2010*
* Corresponding author. Address: INSERM U1035, Université Segalen Bordeaux, Bordeaux F-33000, France. Tel.: +33 5 57 57 13 73; fax: +33 1 44 41 24 46.
*E-mail address:* Emmanuel.Richard@u-bordeaux2.fr (E. Richard).
[#] These authors contributed equally to this work.
*Abbreviations:* BMNC, bone marrow nucleated cells; BMT, bone marrow transplantation; BM, bone marrow; BU, busulfan; EPP, erythropoietic protoporphyria; FECH, ferrochelatase; HSC, hematopoietic stem cells; MFI, mean fluorescence intensity; PPIX, protoporphyrin IX; RBCs, Red blood cells.



## Introduction

EPP is an inborn error of heme biosynthesis, inherited with both recessive and dominant patterns, caused by a partial deficiency of the mitochondrial enzyme ferrochelatase (FECH, EC 4.99.1.1), which catalyzes the insertion of ferrous iron into protoporphyrin IX (PPIX) [1]. Reduced FECH enzymatic activity results in the accumulation of PPIX in erythrocytes, plasma, feces, and the liver. PPIX is highly toxic and induces tissue damages through reactions with free radicals, especially in the skin after light exposure [1]. EPP is characterized clinically by the childhood onset of lifelong cutaneous photosensitivity and is commonly associated with mild microcytic and hypochromic anemia [1,2]. Ferrochelatase is highly active in cells that produce heme including erythroid precursors in the bone marrow (BM) and hepatocytes. However, in EPP patients, the majority of PPIX (80% or more) originates from the BM and especially the erythroid lineage, with most of the remainder generated by the liver [3]. PPIX is excreted by the liver and is insoluble in the bile where it exerts cholestatic effects leading to architectural changes in the hepatobiliary system, ranging from mild inflammation to fibrosis and cirrhosis. There is a wide variation in the severity of liver disease in EPP patients, but PPIX deposition, liver damage, as well as abnormal biochemical parameters of liver function are relatively common. Severe PPIX-induced hepatotoxicity is a rare complication occurring in 1–16% of patients for whom liver transplantation is often required [3,4]. However, liver transplantation fails to correct the underlying metabolic deficiency, and PPIX damage to the transplanted liver is likely.

A mouse model of EPP was obtained by chemical mutagenesis using ethylnitrosourea [5]. The $Fech^{m1Pas}/Fech^{m1Pas}$ mouse accumulates massive amounts of PPIX in the BM, erythrocytes, and the liver and displays features of normocytic and hypochromic hemolytic anemia, photosensitivity, and severe hepatobiliary disease. The obstruction and damage of large and small bile ducts by PPIX deposits cause PPIX accumulation in the parenchyma and cytotoxic damage of hepatocytes [6,7]. The $Fech^{m1Pas}$ mutation has been introduced into three different congenic mouse strains:

the originally described BALB/cByJCrl, C57BL/6JCrl, and SJL/JOrlCrl [8]. These three strains present with different BM and liver PPIX accumulation patterns, liver disease severity, and biliary PPIX excretion. This demonstrates that the EPP phenotype in mice is modulated by the genetic background, suggesting that one or several modifying genes are involved.

Since most of the accumulated-PPIX originates from the BM, hematopoietic stem cell (HSC) replacement therapy could be viewed as a therapeutic option for EPP [9,10]. BM transplantation (BMT) was successfully used in conjunction with liver transplantation to prevent recurrent liver failure [11,12]. We and others demonstrated a dramatic decrease in plasma and erythrocyte PPIX content, associated with the complete cure of skin photosensitivity after BMT in EPP mice [13–17]. These experiments highlight the absence of natural selective advantage of normal hematopoietic cells in this model and demonstrate the need for efficient HSC gene delivery to cure EPP in mice. However, the liver damage that is invariably present in EPP mice was not or only moderately improved by this treatment, especially if BMT was performed in adult mice (>2 months) with already developed severe hepatitis [13–17]. These results suggest that, when severe liver damage is installed (fibrosis and cirrhosis), a metabolic correction limited to the hematopoietic compartment is insufficient to reverse the liver pathology in EPP mice.

In this paper, we determined the time course of liver disease progression in EPP mice. We demonstrated that, in 2-day-old EPP mice, most of the erythrocytes accumulate excessive amounts of PPIX whereas the liver contains normal PPIX concentration. We showed that PPIX content increases during the first 2 weeks in both erythrocytes and the liver, which correlates with the onset of liver fibrosis in 12-day-old EPP mice. Furthermore, we demonstrated that early BMT into EPP neonates could totally prevent liver disease as compared with a late-onset treatment in EPP adult mice. These results demonstrate for the first time that a complete hepatic, erythropoietic, and skin photosensitivity correction could be obtained in EPP mice by early BMT into neonates. These data highlighted the potential benefit of BMT in preventing liver disease in EPP patients.

## Materials and methods

### *Mice colonies*

B6.SJL-Ptprca Pep3/BoyJ mice (Ly5.1) were obtained from Charles River laboratory and were maintained in the conventional animal facility core of the University Bordeaux 2 under pathogen free conditions. C57BL/6JCrl (Ly5.2) congenic EPP mice ($Fech^{m1Pas}/Fech^{m1Pas}$) were previously described [8]. All procedures were performed in compliance with the local ethical committee of the University Bordeaux 2.

### *Bone marrow transplantation*

BM was harvested from Ly5.1 adult (6–10 weeks old mice) donor mice and bone marrow nucleated cells (BMNC) were separated from RBCs using a Ficoll gradient. Lineage-negative cells (lin neg) were depleted by incubation with a cocktail of rat anti-mouse antibodies against CD5, CD11b, CD45R, Ly-6G (Gr-1), TER119, 7–4, as described by the manufacturer (Mouse Hematopoietic Progenitor Enrichment kit, Stemcell Technologies, Grenoble, France).

For BMT into adult EPP mice, lineage-negative cells ($1.7–5 \times 10^5$ $Lin^-$ cells) were transplanted by retro-orbital injection into adult recipients (8–10 weeks old mice) that were previously irradiated with two splits of 6 Grays, 24 h apart, as described previously [13,15].

For BMT into newborn mice, 1- to 2-day-old Ly5.2 pups were given single (10, 15, 20 mg/kg) or double ($2 \times 10$ mg/kg, 24 h apart) i.p. injection of busulfan (BU) (Busilvex®, Pierre Fabre, Boulogne, France). BU (6 mg/ml) was diluted in PBS prior to injection and pups were injected with 25–30 µl of the diluted solution. Newborn mice were transplanted 24 h after the last BU injection by intravenous (via temporal vein) injection of $1 \times 10^6$ BMNCs ±$4.5–5 \times 10^5$ $Lin^-$ cells (lin neg).

### *Engraftment and lineage analysis of peripheral blood, BM, spleen, and thymus*

Peripheral blood (PB) was collected via retro-orbital puncture in heparinized capillaries. For analysis of hematopoietic engraftment, CD45.1 positive cells from blood and BM were assessed by flow cytometry using standard protocols. Briefly, cells were incubated with antibodies directed against Ly5.1-fluorescein isothiocyanate (FITC), and granulocytes, monocytes, and lymphocytes were gated by forward and side scatter. BM cells were incubated with antibodies against Ly5.1-FITC and either B220-phycoerythrin (PE) or Gr1-PE for BM engraftment analysis of B lymphoid or myeloid cells (granulocytes/monocytes), respectively. The spleen and the thymus were harvested from transplanted mice, and nucleated cells were extracted by dissection and incubated with antibodies directed against Ly5.1-FITC and either B220-PE (spleen) or CD3-PE (thymus) for engraftment analysis of B lymphoid cells and T lymphoid cells in the spleen and thymus, respectively. Flow cytometric analysis was performed using the FACScalibur™ and CellQuest Pro software (Becton Dickinson, San José, CA, USA).

### *Biochemical parameters, ferrochelatase assay, and porphyrin level*

Total serum bilirubin (TBil), serum alkaline phosphatase (ALP), and serum transaminase (aspartate and alanine aminotransferase, ASAT and ALAT) were determined by standard methods using clinical grade instrumentation (Beckman Coulter Olympus, AU5400). FECH activity and PPIX levels in RBCs, plasma, BM, and the liver were measured as described [13,15].

### *Liver histology*

Histological analysis of the liver was carried out on 4-µm paraffin-embedded sections. Hematoxylin–eosin and Sirius Red coloration were used to determine structure and fibrosis, respectively. Liver sections were digitalized using a Mirax Scan (Zeiss) virtual slides imaging system and analyzed by a trained pathologist, who was blind to the origin of the sections, to evaluate lesions following criteria described elsewhere [8]. Briefly, cholestasis was defined by bile duct retention and canaliculi bile ducts proliferation. Intra-hepatic PPIX deposits were identified as macroscopic brown deposits in hepatocytes, bile canaliculi, and Küpffer cells. Chronic hepatitis consisted of fibrosis and inflammatory infiltrates in portal tracts.

### *Skin photosensitivity assay*

Reversal of skin photosensitivity was assayed 5 months after transplantation using an irradiation protocol as previously described [15]. Skin pictures were taken 5 days after irradiation.

### *Statistical analysis*

Data are presented as the mean ± SEM or median as indicated. The non parametric Mann–Whitney test was used for comparison between two groups. The level of significance was set at $p$ less than 0.05.

## Results

### *Liver pathology develops during the first month in EPP mice*

We analyzed the time course of liver injury development and the relationship with erythrocyte and hepatic PPIX accumulation in EPP and WT mice at 2, 6, and 12 days, 4 weeks, and 4 months after birth. We found that EPP neonates accumulated PPIX in most erythrocytes, as determined by flow cytometry analysis of fluorescing RBCs (named hereafter fluorocytes), (Fig. 1A) and that the intracellular PPIX content progressively increased during the first month (Fig. 1B). Surprisingly, we observed near normal PPIX values in the liver of 2- or 6- day-old EPP mice. Hepatic PPIX