Congenital Erythropoietic Porphyria: Characterization of Murine Models of the Severe Common (C73R/C73R) and Later-Onset Genotypes

David F Bishop,[1] Sonia Clavero,[1*] Narla Mohandas,[2] and Robert J Desnick[1]*

[1]Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, New York, New York, United States of America; and [2]The Red Cell Physiology Laboratory, New York Blood Center, New York, New York, United States of America

Congenital erythropoietic porphyria (CEP) is an autosomal recessive disorder due to the deficient activity of uroporphyrinogen III synthase (UROS). Knock-in mouse models were generated for the common, hematologically severe human genotype, C73R/C73R, and milder genotypes (C73R/V99L and V99L/V99L). The specific activities of the UROS enzyme in the livers and erythrocytes of these mice averaged approximately 1.2%, 11% and 19% of normal, respectively. C73R/C73R mice that survived fetal life to weaning age (~12%) had a severe microcytic hypochromic anemia (hemoglobin 7.9 g/dL, mean cellular volume 26.6 fL, mean cellular hemoglobin content 27.4 g/dL, red cell distribution width 37.7%, reticulocytes 19%) and massively accumulated isomer I porphyrins (95, 183 and 44 μmol/L in erythrocytes, spleen and liver, respectively), but a nearly normal lifespan. In adult C73R/C73R mice, spleen and liver weights were 8.2- and 1.5-fold increased, respectively. C73R/V99L mice were mildly anemic (hemoglobin was 14.0 g/dL and mean cellular hemoglobin was 13.3), with minimally accumulated porphyrins (0.10, 5.54 and 0.58 μmol/L in erythrocytes, spleen and liver, respectively), whereas adult V99L/V99L mice were normal. Of note, even the mildest genotype, V99L/V99L, exhibited porphyria *in utero*, which disappeared by 2 months of age. These severe and mild mouse models inform therapeutic interventions and permit further investigation of the porphyrin-induced hematopathology, which leads to photoinduced cutaneous lesions. Of significance for therapeutic intervention, these mouse models suggest that only 11% of wild-type activity might be needed to reverse the pathology in CEP patients.

Online address: http://www.molmed.org
doi: 10.2119/molmed.2010.00258

INTRODUCTION

Congenital erythropoietic porphyria (CEP; MIM 263700) is an autosomal recessive disorder of heme biosynthesis due to the markedly deficient, but not absent, activity of uroporphyrinogen III synthase (UROS; EC 4.2.1.75) and the accumulation of nonphysiological porphyrinogen I isomers (1–3). Normally, UROS catalyzes the conversion of hydroxymethylbilane to uroporphyrinogen III. In CEP, the enzymatic deficiency leads to the nonenzymatic conversion of hydroxymethylbilane to uroporphyrinogen I, which is then converted to coproporphyrinogen I with oxidation to uroporphyrin I (URO I) and coproporphyrin I (COPRO I), which cannot be further metabolized. The URO I and COPRO I isomers primarily accumulate in the erythron, where heme biosynthesis is most active, and erythroid cell lysis and/or fragmentation results in high levels of these porphyrins in the circulation, from which they are accumulated in the skin, tissues and bones and are excreted in the urine and feces.

The clinical manifestations of human CEP range in severity from nonimmune hydrops fetalis to transfusion-dependency with secondary hypersplenism, to mild, later-onset phenotypes that develop only cutaneous lesions in adult life (1,3,4). The variation in clinical severity depends on the level of residual UROS activity, and genotype/phenotype correlations have been reported (5). The most common severe human phenotype results from homozygosity for c.217T>C (p.C73R) (1). Prokaryotic expression of the C73R allele resulted in <1% of wild-type activity, and C73R/C73R patients had <1% UROS activity in bone marrow cells and massive amounts of urinary URO and COPRO I isomers (6). The mutation mainly compromises enzyme stability, since the pure enzyme has about 30% of wild-type activity (7). Patients heteroallelic for C73R and other low-residual activity mutants (for example, p.P53L and p.T228M) also had severe phenotypes; patients heteroallelic for C73R and alleles that prokaryotically expressed 5–8% residual activity (for example, p.A104V and p.V99A) had

**DFB and SC contributed equally to this work.*

Address correspondence and reprint requests to *David F. Bishop, Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. Phone: 212-659-6795; Fax: 212-849-2508; E-mail: david.bishop@mssm.edu.*
Submitted December 10, 2010; Accepted for publication February 24, 2011; Epub (www.molmed.org) ahead of print February 25, 2011.

moderately severe disease, and heteroallelic patients with those alleles that expressed 14–35% residual activity (p.A66V and p.V82F) had only mild cutaneous phenotypes (3,5,8). In addition, CEP-like cutaneous symptoms and urinary excretion of URO I and COPRO I have been reported in patients who were diagnosed with myelodysplastic syndrome (9,10). To date, the etiology of this acquired form of CEP has not been determined, nor have there been any reports of *UROS* or *GATA1* gene sequence mutations in these patients (11).

Hematologically, severe patients have significant hemolysis accompanied by anisocytosis, poikilocytosis and markedly increased reticulocytosis. Hemolysis presumably results from the accumulated URO I in erythrocytes. Erythroid hyperplasia usually accompanies the hemolytic anemia manifesting ultimately as a massive secondary splenomegaly. The erythroid abnormality has been attributed to ineffective erythropoiesis, with the most likely cause being porphyrin extrusion from marrow erythroid precursors (12–14).

The cutaneous photosensitivity of CEP results from the release of photocatalytic porphyrins from the porphyrin-loaded erythroid cells and is manifested by increased friability and blistering of the epidermis on the hands and face and other sun-exposed areas. Recurrent vesicles and secondary infection can lead to cutaneous scarring and deformities, as well as to loss of digits and facial features such as the eyelids, nose and ears. Later-onset patients with ~3–10% residual UROS activity may have a compensated anemia with reticulocytosis, but otherwise near-normal hematologic values, and often exhibit only the photo-induced cutaneous lesions due to decades of porphyrin accumulation in the skin (1,3,15). Additional manifestations often include hypertrichosis, alopecia and erythrodontia. Successful bone marrow or hematopoietic stem cell transplantation is essentially curative but is associated with increased morbidity and mortality (16–19).

Therefore, efforts have been directed to develop murine models of CEP to investigate the disease pathophysiology and evaluate various therapeutic strategies. Homozygous *uros* knockout mice were early fetal lethals (20). Knock-in mice with sufficient residual activity for survival had hemolytic anemia and moderate skin photosensitivity (21,22). Our previous knock-in CEP mouse had ~2% of normal hepatic UROS activity, demonstrating that extremely low levels of nonerythroid UROS permitted sufficient heme synthesis for normal energy metabolism and other functions involving housekeeping hemoproteins.

To investigate the hematologic bases for the severe and milder human phenotypes, knock-in mice were generated to mimic (a) the most common, human genotype, C73R/C73R, which results in nonimmune hydrops fetalis in humans, or, if the fetus survives, a severe, transfusion-dependent phenotype; and (b) the C73R/V99L and V99L/V99L genotypes that have hematologically milder phenotypes mimicking the human genotypes with minimal disease. Here, the clinical, biochemical and hematologic findings in these murine models are described. The hematologic severity in these mice was directly related to their residual UROS activity, with lifelong severe anemia in the C73R/C73R mice, nonpathogenic mild anemia in the C73R/V99L mice and essentially normal erythrocyte indices and hemograms in the adult V99L/V99L mice, with ~1%, 11%, and 19% of wild-type UROS activities, respectively, in erythrocytes and liver. Extrapolating from the mouse model to humans, these studies suggest that only about 10–15% of wild-type activity, expressed systemically as in the C73R/V99L mice, may be sufficient to reverse the pathology in human CEP patients.

MATERIALS AND METHODS

Generation of Knock-In Models of CEP

Heterozygous C73R and V99L mouse lines were established by homologous recombination in 129/OLA ES cells using knock-in point mutations as previously described (21). Chimeras were mated with C57BL/6 and CD1 mice, with littermates, and subsequently C73R and V99L heterozygous mice were mated with each other to maintain the lines. The LoxP-flanked *neo* resistance marker cassette between exons 4 and 5 was floxed out by mating mice with the ubiquitously expressing *cre* line B6.FVB-Tg(EIIa-cre)C5379Lmgd/J (The Jackson Laboratory, Bar Harbor, ME, USA) (23). After floxing of the neo^R marker, V99L homozygotes were conceived with 100% viability, as were the V99L/C73R compound heterozygotes. C73R homozygotes were conceived with ~29% viability (for example, 32/109 expected live births) after multiple generations of selection for viable offspring. Of the live births, ~41% survived (for example, 13/32) or ~12% of expected overall. Control mice were established from wild-type offspring of chimeras. Thus, both control and mutant mice were mostly C57BL/6, with some 129/OLA and CD1 genetic background. Animal procedures were approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee.

Genotype/Phenotype Analyses

Genotyping of tail tip DNA was performed as previously described (21). With experience, monitoring fluorescence intensity of bones, teeth and urine with a handheld LED Wood's lamp (405 nm; Vector 4, Cliplight Manufacturing, Toronto, ON) permitted accurate genotype identification when breeding V99L/C73R mice with each other. The newborn to 21-day-old C73R/C73R mice fluoresced uniformly and the C73R/V99L mice fluoresced moderately in the skull, bones and urine, whereas the V99L/V99L mice fluoresced primarily in their teeth.

Enzyme Activity and Porphyrin Quantitation Assays

Blood was obtained from the retro-orbital plexus under anesthesia using 7 μL/g body weight of a 1:1 mixture of ketamine-xylazine solutions composed of 100 mg/mL ketamine and 20 mg/mL xylazine. Blood was drained through a he-

parinized capillary tube into an ethylene diamine tetraacetic acid (EDTA)-containing storage tube and mixed well immediately after collection. Lethally anesthetized mice were perfused with phosphate-buffered saline (PBS), and tissues were collected for histology and porphyrin determinations. For histology preparations, tissues were embedded in 10% neutral-buffered formalin and processed as described below. For porphyrin determinations, tissues were homogenized in 50 mmol/L HEPES, pH 7.5, 150 mmol/L potassium chloride, 1 mmol/L EDTA , 1 mmol/L dithiothreitol and 1% phenylmethylsulfonyl fluoride, and the supernatants were collected after centrifugation at 10,000*g* for 30 min. Blood was centrifuged at 2,000*g* for 10 min, and the red blood cells were washed once in three volumes of PBS. The cell pellets were lysed by adding three volumes of 100 mmol/L HEPES, pH 7.5, 0.02% sodium azide and 0.1% Triton X-100.

UROS enzyme activities in tissues and red blood cells were determined according to the previously described coupled assay (24). Tissue homogenate supernatants for porphyrin determinations were deproteinized by addition of an equal volume of 1:1 20% trichloroacetic acid/dimethyl sulfoxide for a final concentration of 5% trichloroacetic acid. After precipitation for 10 min, the supernatants were collected after centrifugation at 10,000*g* for 10 min and diluted in 1 N HCl as needed. Porphyrin isomer content was analyzed using an Acquity® UPLC System (Waters Corporation, Milford, MA, USA) as previously described (25).

Hematologic Analyses

Retro-orbital blood was collected as above in EDTA tubes. Blood was diluted 1:10 with saline before standard blood and reticulocyte analyses on an Advia 120 Hematology system (Siemens Healthcare Diagnostics, Deerfield, IL, USA).

Histology and Photography

Liver, spleen and kidney tissues were obtained from perfused mice, fixed in formalin, paraffin embedded and stained with hematoxylin/eosin and Perl's iron stains. Peripheral blood and bone marrow smears were stained with Wright-Giemsa. Unstained marrow and peripheral blood smears were imaged for fluorescence microscopy with a Nikon Eclipse 80i equipped with a custom filter cube containing Semrock BrightLine 405 ± 10 nm excitation, 510 nm dichroic

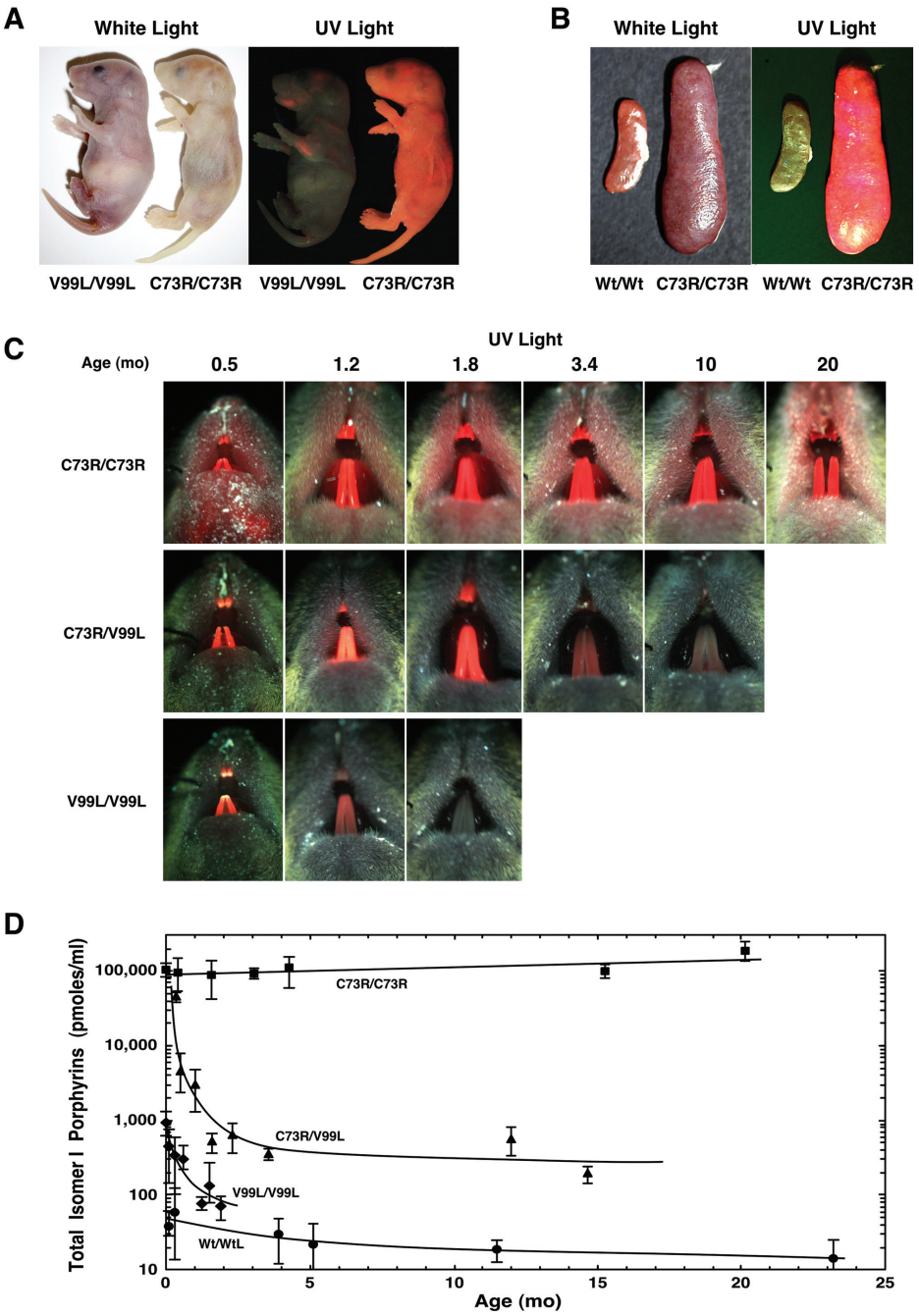

Figure 1. Phenotypes of the CEP mice. (A) Newborn V99L/V99L and C73R/C73R mice photographed under white light and UV light. (B) Wild-type and C73R/C73R (age 3 months) spleens photographed under white light and UV light. (C) C73R/C73R, C73R/V99L and V99L/V99L mice of various ages photographed under UV light under identical conditions. (D) Urinary total porphyrin concentration versus age.

Table 1. UROS enzyme activities in Wt/Wt and knock-in CEP mice.

	Wt/Wt, U/mg protein ± SD (n)	C73R/C73R, mean ± SD (n); % of Wt	C73R/V99L, mean ± SD (n); % of Wt	V99L/V99L, mean ± SD (n); % of Wt
Erythrocytes	8.35 ± 1.07 (7)	0.09 ± 0.01 (4); 1.1	1.08 ± 0.20 (4); 12.9	2.01 ± 0.28 (3); 24.1
Spleen	18.4 ± 8.8 (6)	0.65 ± 0.29 (9); 3.5	0.69 ± 0.28 (4); 3.8	3.79 ± 1.38 (3); 20.6
Liver	9.9 ± 5.6 (6)	0.14 ± 0.07 (11); 1.4	0.86 ± 0.07 (4); 8.7	1.40 ± 0.21 (3); 14.1

Adult male and female BL/6 mice were 3–22 months of age.

and 620 ± 10 nm emission filters. Visible light photography of mice and tissues were taken with a tripod-mounted Canon EOS 50D camera. Fluorescence photographs were made in a darkroom with a Wratten #4 ultraviolet-blocking filter using 405 nm LED illumination.

RESULTS

Establishment and Viability of C73R/C73R, C73R/V99L and V99L/V99L CEP Mice

The C73R/C73R and V99L/V99L mice were initially fetal lethals. After floxing out the strongly inhibiting *neo*^{R} gene, the V99L homozygous mice were viable, but the C73R/C73R mice remained fetal lethals. Because compound heterozygous C73R/V99L mice were viable, they were bred to each other, and occasionally live C73R/C73R mice were born but were often severely runted and rarely survived. The variable survivability may be because the introduced mutations were on a mixed background that was predominantly BL/6, but also contained contributions from the SV129, CD1 and *cre*-expressing strains (see "Materials and Methods"). C73R/V99L breeding pairs that produced live C73R/C73R births were then selected, and eventually a colony was established that produced C73R homozygotes (at reduced numbers) that had a nearly normal lifespan (>600 d versus wild-type >700 d). When mating the C73R/V99L mice to each other, the genotype frequencies at birth were 8.9% (32/360) for C73R/C73R, 61% (219/360) for V99L/C73R and 30% (109/360) for V99L/V99L. At weaning, these frequencies were 3.8% (13/341), 64% (219/341) and 32% (109/341), respectively. Newborn C73R/C73R mice were typically runted (Figure 1A), with body weights about 75% of C73R/V99L mice at weaning and 90% and 85% at 3 months of age for males and females, respectively. By 4–5 months of age, male C73R/C73R mice had attained body weights that were ~98% of wild-type. Male C73R/C73R mice were fertile and were routinely mated with C73R/V99L females because female C73R/C73R mice were infertile. Phenotyping of the mice by the fluorescent intensity of their teeth and bones was diagnostic (Figure 1C); DNA genotyping was confirmed on occasion but was not usually necessary for these lines.

UROS Activities and Porphyrin Levels in the CEP Mice

In vitro prokaryotic expression of the murine UROS C73R and V99L cDNAs resulted in recombinant proteins with residual activities of 0.24% and 14.8% of wild-type activity, respectively, when measured previously in crude bacterial lysates (21). The UROS activities in the erythrocytes, spleen and liver for C73R/C73R, C73R/V99L and V99L/V99L genotypes are shown in Table 1. The enzymatic activities in erythrocytes were ~1%, 13% and 24% of wild-type, respectively (see Table 1).

Table 2 shows the porphyrin isomer levels in erythrocytes, plasma, urine, liver and spleen of 3- to 4-month-old adult mice with the three genotypes. Notably, the highest concentrations of URO I and COPRO I were in the C73R/C73R mice. For example, the I isomer levels in their erythrocytes and spleen, the major site of extramedullary erythropoiesis, were ~18,000- and 7,400-fold greater than their respective wild-type concentrations. Consistent with the low level of residual UROS activity, the porphyrin I/III isomer ratio was very high (40 to 840) in C73R/C73R erythrocytes, spleen and liver, compared with the respective wild-type values (1.7 to 4.1; see Table 2).

Adult C73R/V99L and V99L/V99L mice accumulated markedly less porphyrin (see Table 2). C73R/V99L mice had only 0.1–3% of the URO I levels in the C73R/C73R mice, whereas the V99L/V99L mice had porphyrin levels that were similar to those in wild-type mice.

Of note, there was a strong temporal effect on the porphyrin levels in the C73R/V99L and V99L/V99L CEP mice. In both murine genotypes, an increased transient fetal porphyria resulted in the urinary porphyrin accumulation being highest at birth, as evidenced by the intense erythrodontia under UV light (Figure 1C) and the markedly elevated urinary porphyrins in the first week of life (Figure 1D). Although the C73R/C73R mice retained intense erythrodontia and high urinary porphyrin excretion throughout life, the porphyrin excretion and then the erythrodontia decreased with time in both the C73R/V99L and V99L/V99L mice. In the C73R/V99L mice, the urinary porphyrins rapidly decreased during the first 2 months of life and the erythrodontia faded by 1 year of age, whereas the V99L/V99L urinary porphyrins subsided in the first month and the teeth lost their fluorescence by 2 months of age (Figure 1C, D).

The mean erythrocyte porphyrin levels in the C73R/C73R mice were ~100 μmol/L at 3 months of age (see Table 2) and increased to ~180 μmol/L at 19 months (data not shown), whereas the urinary mean total porphyrin concentrations were constant at ~100 μmol/L throughout life. In contrast, the mean

Table 2. Porphyrin isomer concentrations in various tissues of the severe and milder CEP mice.[a]

Genotype/tissue	Erythrocytes (pmol/mL)	Plasma (pmol/mL)	Spleen (pmol/g)	Liver (pmol/g)	Urine (pmol/mL)[b]
Wt/Wt					
Total porphyrin[c]	8.61 ± 2.27	5.68 ± 0.58	34.2 ± 31.4	118 ± 42	111 ± 86
URO I	3.41 ± 0.87	0.28 ± 0.06	17.5 ± 13.6	53.1 ± 29.4	11.6 ± 7.2
URO III	1.70 ± 0.78	0.93 ± 0.16	5.6 ± 6.1	40.3 ± 3.7	3.2 ± 1.8
COPRO I	1.99 ± 0.80	0.76 ± 0.22	3.1 ± 2.2	7.1 ± 2.7	9.3 ± 8.8
COPRO III	1.51 ± 0.23	1.69 ± 0.59	1.0 ± 1.0	ND[d]	76.6 ± 72.1
C73R/C73R					
Total porphyrin[c]	97,500 ± 43,200	2,940 ± 459	208,000 ± 20,800	50,200 ± 4,480	99,900 ± 15,000
URO I	87,000 ± 39,900	1,940 ± 237	179,000 ± 15,700	42,800 ± 3,260	73,000 ± 11,000
URO III	114 ± 151	36.2 ± 8.8	4,620 ± 1,110	1,230 ± 178	3,820 ± 217
COPRO I	7,500 ± 4,900	779 ± 272	13,000 ± 2,530	1,960 ± 443	15,100 ± 4,670
COPRO III	ND	ND	ND	ND	1,610 ± 1,260
C73R/V99L					
Total porphyrin[c]	102 ± 13	24.9 ± 4.6	5,690 ± 1,220	714 ± 81	359 ± 61.9
URO I	63.2 ± 7.3	10.4 ± 0.7	5,460 ± 1,220	575 ± 65	196 ± 20.1
URO III	1.3 ± 0.3	0.47 ± 0.2	21.7 ± 12.7	68.6 ± 13.0	18.0 ± 2.7
COPRO I	33.7 ± 6.3	8.35 ± 2.9	75.3 ± 29.2	9.0 ± 6.5	91.8 ± 27.1
COPRO III	0.1 ± 0.3	1.18 ± 0.5	ND	18.1 ± 5.3	23.1 ± 8.4
V99L/V99L					
Total porphyrin[c]	2.29 ± 0.64	3.06 ± 1.70	6.98 ± 2.15	17.4 ± 4.5	70.8 ± 23.7
URO I	0.69 ± 0.67	0.13 ± 0.14	4.57 ± 0.99	11.0 ± 5.0	11.4 ± 2.2
URO III	0.27 ± 0.07	0.11 ± 0.10	0.00 ± 0.00	0 ± 0	2.8 ± 1.6
COPRO I	0.70 ± 0.62	1.52 ± 1.10	2.02 ± 0.80	1.45 ± 0.83	16.5 ± 7.1
COPRO III	0.61 ± 0.35	1.19 ± 0.49	ND	ND	31.9 ± 24.6

[a]C57BL/6-SV129-DB1 strain. V99L/V99L mice were about 1.5 months of age; all other mice were 3–4 months of age. Means are the average of 3–9 mice (median 4).
[b]The urine dilution is normalized to the average urine creatinine concentration of 4.86 ± 2.37 mmol/L for 122 mice (all genotypes). The average creatinine concentrations for Wt/Wt, C73R/C73R, C73R/V99L and V99L/V99L mice were 5.92 ± 2.88 (33), 3.72 ± 1.79 (29), 5.42 ± 2.02 (37) and 3.91 ± 1.69 (23), respectively (mean ± SD in mmol/L (n)).
[c]Total mean porphyrin concentration = I + III isomers of URO and COPRO and the I isomers of HEPTA, HEXA and PENTA.
[d]ND, not detectable. The limit of detection is around 1 pmol/mL.

total urinary porphyrin levels in the V99L/C73R mice, while relatively high (~50 μmol/L) at birth, decreased by 99%, to ~0.6 μmol/L by 2 months of age; and the mean total urinary porphyrins in the V99L/V99L mice decreased from ~1 μmol/L at birth to ~0.4 μmol/L by 1 month of age (Figure 1D).

Hematologic Findings

Table 3 summarizes the hematologic values in the three lines of CEP mice. The C73R/C73R mice had severe hypochromic, microcytic anemia with 18% reticulocytosis and were unable to compensate for the anemia by erythroid expansion in the spleen. Compared with the wild-type mice at 4 months of age, they had marked microcytosis (27 versus 44 fL), a broad (38% versus 13%) red cell distribution width indicating the presence of both larger and smaller cells, a markedly reduced hemoglobin concentration (7.9 versus 16 g/dL) and an even lower concentration in old age (for example, 5 g/dL at 15 months). Their mean cellular hemoglobin was markedly reduced (7 versus 15 pg). However, because of the microcytosis, the mean cellular hemoglobin content, the actual hemoglobin concentration in each erythrocyte, was only reduced by 23% (27 versus 35 g/dL of packed erythrocytes), accounting for the cell's moderate hypochromia. The erythrocyte count was completely normal at 4 months, but was reduced by ~25% in old (15-month-old) mice, presumably because of hematopoietic exhaustion.

In contrast, the 4-month-old V99L/C73R mice had a slight decrease in mean cellular hemoglobin and in mean cellular volume, but all other values were within normal limits, consistent with the relatively lower porphyrin levels in the erythrocytes, tissues and fluids of these adult mice. The hematologic values for the V99L/V99L mice were essentially normal, consistent with the ~24% residual erythrocyte UROS activity and absence of erythrocytic porphyrin accumulation (see Table 2). Interestingly, the leukocyte counts in the CEP mice were consistently elevated, and a differential count of the C73R/C73R mice indicated that the polymorphonuclear cells were markedly increased (data not shown).

Hepatosplenomegaly

The C73R/C73R mice had marked hepatosplenomegaly. Compared with age-

Table 3. Hematologic parameters for Wt and knock-in CEP mice.[a]

	Wt/Wt	C73R/C73R	C73R/C73R	V99L/C73R	V99L/V99L
Age (months)	3.7 ± 0.7	3.7 ± 0.7	14.9 ± 0.1	3.3 ± 0.5	7.3
n	8	7	4	8	1
Hemoglobin (g/dL)	16 ± 1	**7.9 ± 0.9**	**5.0 ± 0.8**	14 ± 1	17
Hematocrit (%)	45 ± 2	**28 ± 3**	**21 ± 4**	42 ± 1	49
Mean cellular hemoglobin (pg)	15.1 ± 0.6	**7.3 ± 0.6**	**6.9 ± 0.5**	**13.3 ± 0.5**	14.3
Mean cellular hemoglobin content (g/dL)	34.6 ± 1.0	**27.4 ± 2.4**	**24.2 ± 1.8**	32.9 ± 1.0	34.2
Mean cellular volume (fl)	43.6 ± 1.0	**26.6 ± 2.2**	**28.5 ± 0.8**	**40.5 ± 1.6**	41.8
Red cell distribution width (%)	13.4 ± 0.4	**37.7 ± 3.1**	**40.1 ± 1.4**	13.5 ± 0.4	13.9
Red blood cells (10^9/mL)	10 ± 0.5	11 ± 1.0	**7.4 ± 1.0**	10 ± 0.4	11.7
White blood cells (10^6/mL)	3.6 ± 2.5	**22 ± 16**	**24 ± 7**	5.2 ± 2.4	8.2
Reticulocytes (%)	2.4 ± 0.7	**18.7 ± 5.6**	**17.7 ± 5.2**	2.0 ± 0.5	2.2

[a]Adult male and female mice; means ± SD; abnormal data are in bold. Note that there is reduced precision in the hemoglobin, hematocrit, red blood cell and white blood cell values, since the analysis was done on a 10× dilution of the mouse blood.

matched wild-type mice, their spleen and liver weights at 4 months of age were increased about 8- and 1.5-fold, respectively (Figure 1B, Table 4).

Histology

Peripheral blood smears from the C73R/C73R mice had many small, pale and fragmented cells; some anisocytes, including poikilocytes, stomatocytes and cigar cells, with frequent darker-staining reticulocytes and increased leukocytes (Figure 2A). The spleen had massively increased red pulp and decreased white pulp because of the extensive extramedullary erythropoiesis (Figure 2B). The liver sections showed increased numbers of infiltrating phagocytic cells and the hepatocytes contained brown deposits that stained for iron with Perl's stain (not shown). In the kidney, the proximal renal tubules contained heavy iron deposits by Perl's stain (Figure 2C). Fluorescence microscopy of unstained tissues showed 10–20% fluorocytes in peripheral blood, bone marrow (Figure 2D, E) and spleen (not shown). The bone marrow smear showed occasional large porphyrin-containing cells but was not significantly more erythroid than wild-type marrow. Fluorocytes were abundant in the C73R/C73R marrow and absent in wild-type marrow. V99L/C73R and V99L/V99L mice had essentially normal histology with only rare fluorocytes seen in V99L/C73R mice (data not shown).

DISCUSSION

Human CEP presents a range of clinical severity on the basis of the amount of residual UROS activity in patients, as documented by previous genotype/phenotype studies (1,3,5,8). This human inborn error of heme biosynthesis is primarily an erythroid disease, since successful bone marrow or hematopoietic stem cell transplantation is curative (18) and since a hepatic-specific UROS deficiency did not compromise hemoprotein function (21). Because of the rarity of the disease, the extremely variable phenotypic expression and the limits of human experimentation, few hematologic studies of severe and later-onset CEP patients were reported. Thus, the C73R/C73R knock-in mice mimic the most common severe human transfusion-dependent genotype (1); the C73R/V99L mice are hematologically compensated and resemble the human later-onset phenotypes; and the adult V99L/V99L mice are essentially normal. Together, these mouse models provide the opportunity to investigate the pathogenic mechanisms underlying this erythropoietic porphyria.

The generation of a viable C73R/C73R mouse model provides the first murine model of the most common and severe human CEP genotype. Previously, two CEP knock-in mouse models were reported (21,22). Compared with wild-type

Table 4. Splenic and hepatic enlargement in CEP knock-in mice.[a]

Genotype	Wt/Wt	C73R/C73R	Fold[b]	C73R/V99L	Fold[b]	V99L/V99L	Fold[b]
Age (months)	4.3 ± 0.7	4.0 ± 0.8		3.6 ± 0.0		1.5 ± 0.0	
n	8	7		4		3	
Mouse weight (g ± SD)	28.0 ± 4.4	25.3 ± 2.6	1.0	34.3 ± 4.5	1.0	18.4 ± 1.8	1.0
Spleen weight (mg ± SD)	82.7 ± 19.8	605 ± 98	8.1	100 ± 14	1.0	86.7 ± 11.6	1.6
Liver weight (g ± SD)	1.15 ± 0.16	1.67 ± 0.22	1.6	1.43 ± 0.1	1.0	1.00 ± 0.08	1.3

[a]Male and female adult mice.
[b]Fold change relative to wild-type normalized to body weight by comparing tissue weights as percentages of body weight.

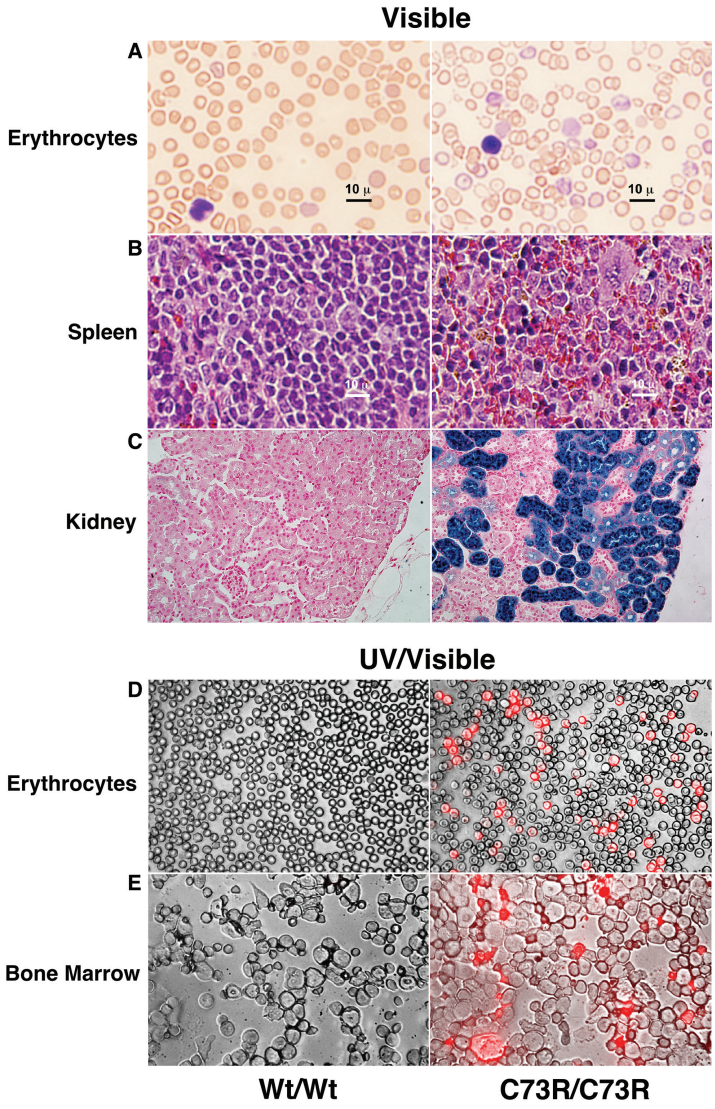

Figure 2. Histology of wild-type and C73R/C73R erythrocytes, spleen and kidney. All images were photographed at 400× magnification. (A) After fluorocyte photography of air-dried blood smears, these peripheral erythrocytes were acetone-fixed and stained with Wright-Geimsa. The C73R/C73R erythrocytes showed anisocytosis, microcytosis and polychromasia and included poikilocytes and target cells. Formalin-fixed splenic (B) and renal (C) sections were stained with hematoxylin and eosin and with Perls. Wild-type and mutant splenic sections were similar and showed occasional iron staining, whereas abundant iron staining was observed only in the proximal tubules of the C73R/C73R kidneys. Bright-field micrographs of erythrocytes (D) and bone marrow (E) with overlaid fluorescence images obtained with a 405-nm excitation filter and a 625-nm emission filter. No fluorocytes were observed in wild-type mice, whereas C73R/C73R erythrocytes and bone marrow contained about 10% fluorocytes.

mice, the C73R/V99AT mouse model (21) had ~1% liver and ~12% erythroid UROS activity, suggesting that the very low residual activity in CEP patients was sufficient for normal hepatic hemoprotein function. The P248Q/P248Q and C73R/C73R mice (22) had similar levels of residual erythrocyte UROS enzyme activity (<0.1%) and a similar percentage of circulating fluorocytes (~20%), but the P248Q/P248Q mice had four-fold greater urinary URO I than the C73R/C73R mice. On the other hand, the C73R/C73R mice had a lower fetal viability and generally more severe hematologic indices. Of note, the C73R/C73R genotype occurred in about 10–20% of reported CEP patients (5,26), permitting genotype/phenotype comparisons between mice and humans, whereas the P248Q/P248Q genotype is markedly less common.

The C73R/C73R mice had ~1% erythrocyte UROS activity, massive erythrocyte URO I and COPRO I accumulation (~10,000-fold greater than wild-type), 88% fetal/neonatal lethality and severe anemia. Their hematologic indices revealed a marked microcytic hypochromic anemia, with half-normal hemoglobin concentration, abnormal erythrocyte morphology and 19% reticulocytosis with massive secondary splenomegaly. These murine findings closely resembled those in human patients with the C73R/C73R or other severe, transfusion-dependent genotypes, who present with hydrops fetalis or a transfusion-dependent phenotype and a severe microcytic hypochromic anemia with marked reticulocytosis, abnormal erythrocyte morphology and medullary and/or extramedullary expansion (1,3,4). To date, nine C73R/C73R probands have been reported (6,26–31); porphyrin levels were provided for four patients. Of these, the mean urinary total URO and COPRO I and III isomers was 40 μmol/L, with a range of 14–78 μmol/L (6,28–30), compared to a mean of 100 μmol/L for the C73R/C73R mice. For one patient, 95% of the total urinary URO isomers was URO I (28), identical to that found in the mice. Both human and murine probands exhibited erythrodontia at tooth eruption.

The C73R/V99L mice with ~13% residual erythrocyte UROS activity had a compensated anemia, minimal erythrocyte porphyrin accumulation (0.1% of that in the C73R/C73R mice), no fetal lethality and a normal lifespan. Their erythrocyte counts, hemoglobin concentrations, red cell distribution width values and reticulocyte counts were normal

at 3 months of age. However, their mean corpuscular hemoglobin was slightly reduced (13.3 versus 15.1 g/dL for wild-type), as was their mean cellular volume (40.5 versus 43.6 fL for wild-type), indicating that a mild increase in porphyrins (~3% of C73R/C73R porphyrin levels in the hyper-erythroid spleen tissue) caused some erythroid lysis. The C73R/V99L mice may mimic the mildest later-onset human patients who have essentially normal hematologic values and minimal porphyrin accumulation and who develop photosensitive cutaneous involvement only late in life after decades of porphyrin accumulation.

The essentially normal adult V99L/V99L mice, who had porphyrin accumulation at birth, presumably indicate that about 25% residual UROS activity is required for normal adult hematopoietic indices on this genetic background. They also provide insights into the level of enzyme activity that does not preclude neonatal porphyrin accumulation.

Of note, the erythrocyte and urinary porphyrin concentrations in the three lines of CEP mice were greatest in the newborns. The C73R/C73R mice maintained the high levels throughout life, whereas the levels in the C73R/V99L mice decreased during the first year, and the levels rapidly decreased in the V99L/V99L mice in the first month of life. The cumulative effect of these temporal changes in porphyrin concentrations were clearly evident by the loss of erythrodontia (Figure 1C) and the decreasing urinary porphyrin levels (Figure 1D) with age. Presumably, the increased neonatal porphyrin concentrations are due to an overproduction of porphyrins during erythropoiesis in the fetal liver and account for the early fetal demise of many of the C73R/C73R mice and the hydrops fetalis of C73R/C73R human patients. Even the ~24% residual UROS activity in the mild V99L/V99L mice was unable to prevent fetal porphyrin accumulation, as evidenced by the porphyria of newborns (Figure 1C). Fetal porphyrin accumulation and porphyrin deposition in bones was previously demonstrated, even in normal guinea pigs where the time of maximal amniotic fluid porphyrin concentrations coincided with the time of maximal fetal hepatic erythropoiesis (32,33). In humans, the presence of porphyrins in CEP patient amniotic fluid has facilitated prenatal diagnosis of the disease (30,34), evidencing high porphyrin-induced hemolysis *in utero*.

These findings in the C73R/C73R mice correlated well with the genotype's human counterpart. The decreased fetal survival of the C73R/C73R mice is reflected by the fact that human CEP can present as fatal nonimmune hydrops fetalis or transfusion dependency. Human C73R/C73R newborns are already porphyrin loaded, since these infants have developed cutaneous lesions within 2 weeks of birth (29,30). Another C73R/C73R CEP newborn, who had hyperbilirubinemia, was treated with phototherapy and developed severe blistering lesions due to the high porphyrin concentration present at birth (27). Some C73R/C73R infants may not survive (6) unless they are chronically transfused from birth (29,30), whereas others are candidates for bone marrow or hematopoietic stem cell transplants (26,28). If successful, bone marrow or hematopoietic stem cell transplantation essentially cured the disease, since the recipients were no longer transfusion-dependent or photosensitive (17–19, 29,35). Future studies using these murine models will characterize the hematologic abnormalities including transplantation of CEP hematopoietic stem cells into normal mice and the evaluation of induced pluripotent stem cell transplantation.

Because the mouse models studied here had a full coat of dark hair and dark-pigmented skin and were housed in amber-colored plastic cages that filter out 405 nm light, the dermatologic manifestations of the human disease were not expressed. Future experiments using porphyrin photocatalytic wavelengths (~400–640 nm) will be used in mice with these mutations bred onto a hairless and albino background.

Hematopoietic stem cell transplantation studies into P248Q/P248Q using lentivirally transduced UROS-expressing stem cells indicated that 40% engraftment was required for complete biochemical and phenotypic correction (36). Of note, there was a selective advantage for the corrected cells, due to the poorer survival of the residual endogenous, porphyrin-loaded erythroblasts; hence, less initial engraftment may result in more effective treatment with time.

Previously, the porphyrin-induced hematologic abnormalities and their causative mechanisms were not studied in detail because of the rarity of the disease, the limits of human experimentation and the varying clinical severity of these patients. The murine model of the most common severe form of CEP will now permit these investigations into the sites and causes of hemolysis, the photobiology of the light-induced pathology and various stem cell–based therapies.

ACKNOWLEDGMENTS

The authors thank Nicole Kelly for expert administrative assistance, as well as Xuili An and Xinhual Gou for hematologic measurements and Professor James A Strauchen for histologic consultation. The research was supported in part by a research grant (5 R01 DK026824) from the National Institutes of Health and a research grant (C024404) from the New York State Department of Health.

DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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