Research Article

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Neonatal iron overload and tissue siderosis due to gestational alloimmune liver disease

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Background & Aims: Gestational alloimmune liver disease is the main cause of the neonatal hemochromatosis phenotype, wherein severe neonatal liver disease is associated with iron overload and extrahepatic tissue siderosis. How fetal liver disease produces extrahepatic siderosis is not known. We hypothesized that fetal liver injury causes deficient hepcidin production and poor regulation of placental iron flux. Under the resulting conditions of iron overload, the tissue pattern of extrahepatic siderosis is determined by the normal expression of proteins involved in the import of non-transferrin-bound iron and the export of cellular iron.

Methods: Liver and extrahepatic tissues from infants with gestational alloimmune liver disease were examined and compared to normal age-appropriate tissues.

Results: Serum iron indices indicate iron overload and excess non-transferrin bound iron in gestational alloimmune liver disease. The diseased liver showed significantly reduced hepcidin, hemojuvulin, and transferrin gene expression compared to the normal fetal and neonatal liver. Those extrahepatic tissues that are typically involved in pathological siderosis in neonatal hemochromatosis, whether from normal or diseased newborns, consistently expressed solute carrier family 39 (zinc transporter), member 14 (ZIP14) for non-transferrin-bound iron uptake and expressed little ferroportin for iron export.

Conclusions: Excess non-transferrin-bound iron in gestational alloimmune liver disease may result from fetal liver injury that causes reduced synthesis of key iron regulatory and transport proteins. Whereas, the pattern of extrahepatic siderosis appears to be determined by the normal capacity of various tissues to import non-transferrin-bound iron and not export cellular iron.

Keywords: Neonatal hemochromatosis; Extrahepatic siderosis; Non-transferrin-bound iron; ZIP14; Ferroportin.

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Abbreviations: NH, neonatal hemochromatosis phenotype; GALD, gestational alloimmune liver disease; NTBI, non-transferrin-bound iron; TFR1, transferrin receptor-1; TFR2, transferrin receptor-2; DMT1, divalent metal transporter-1; ZIP14, solute carrier family 39 (zinc transporter), member 14; HAMP, hepcidin gene; HFE2, hemojuvulin gene; TF, transferrin gene.

Introduction

Neonatal hemochromatosis (NH) is defined as the association of severe neonatal liver disease with siderosis of extrahepatic tissues in a distribution similar to that observed in hereditary hemochromatosis [1–5]. Though initially described as an inborn error of iron metabolism, NH is now considered to be a phenotype that follows severe fetal liver injury. We have recently determined that gestational alloimmunity directed against the fetal liver causes most cases of NH [6,7]. The defining feature of gestational alloimmune liver disease (GALD) is complement-mediated hepatocyte injury, the evidence for which comes from detection of the C5b-9 complex (membrane attack complex) by immunohistochemistry [6]. All fetal immunoglobulin consists of IgG transported from the mother, whereas all fetal complement is synthesized by the fetus. In the fetal setting, the classical pathway activation of the terminal complement cascade must, therefore, take place through binding of the maternal IgG (subclass IgG1 or IgG3) to a fetal alloantigen [8]. Thus, by demonstrating complement-mediated fetal liver injury, one can be sure that it is due to materno–fetal alloimmunity. GALD is now considered to be the disease entity causing the "severe neonatal liver disease" component of the phenotype in most cases of NH. This leads to the question of how fetal liver disease produces the iron overload/siderosis component of the phenotype.

The placenta tightly controls the movement of iron from mother to fetus by mechanisms that are similar to those controlling the absorption of dietary iron from the intestine [9]. Ferroportin is highly expressed in placental cells, and hepcidin produced by the fetal liver is thought to be the principal regulator of iron efflux from the placenta via its interaction with ferroportin [10,11]. Transgenic mice that overexpress liver hepcidin are born severely iron deficient [12]. We hypothesized the opposite to be the case in NH, proposing that GALD-related liver injury results in deficient

Table 1. Plasma/serum iron indices in GALD cases in comparison to newborn reference values.

	GALD cases	Reference values	
	Mean ± SD (n)	Range or mean ± SD	[Ref.]
Ferritin (ng/ml)	2174 ± 1699 (20)	40-775 35-309	[23] [22]
Iron (μg/dl)	160 ± 63 (8)	72-203 118 ± 19	[23] [21]
Iron binding capacity (μg/dl)	174 ± 54 (7)	155-330 245 ± 50	[23] [21]
Binding saturation (%)	90 ± 13 (13)	49.9 ± 15.6	[21]

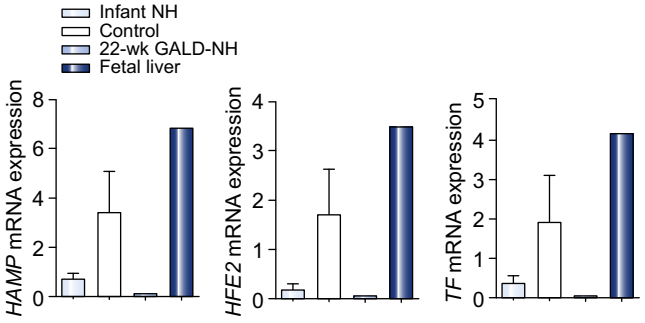

Fig. 1. Liver gene expression in GALD-NH compared to control. Gene expression as determined by real-time PCR and normalized to the housekeeping gene 18S is shown for *HAMP* (hepcidin), *HFE2* (hemojuvulin) and *TF* (transferrin). The first bar in each panel represents the mean expression in the 6 livers from neonates with GALD-NH undergoing liver transplantation with the bars showing standard error. The 2nd bar shows the mean expression of the 3 control samples. All genes showed lower expression in the GALD-NH cases than in the control samples (p <0.05 by Student's t-test). The third bar (barely visible) shows gene expressions in the 22-week gestation GALD case, and the fourth bar shows the value obtained from the pooled fetal liver for visual comparison. All genes showed markedly reduced expression in the GALD case.

fetal liver hepcidin expression and thus fetal iron overload. Hepcidin also controls the export of iron from reticuloendothelial cells via its interaction with ferroportin [11]. As a consequence, iron overload with an intact hepcidin system results in iron retention in reticuloendothelial cells [13]. In the NH phenotype, the reticuloendothelial system is typically devoid of iron. Thus, we hypothesized that, in NH, the extrahepatic tissue distribution of siderosis would be determined by the individual tissue's capacity for importing non-transferrin-bound iron (NTBI) in combination with the lack of ferroportin expression, which renders it incapable of eliminating iron even in a state of hepcidin deficiency. Since GALD is a singular, definable cause of the NH phenotype and is a human disease for which there is currently no animal model, we tested our hypotheses by examining tissue gene and protein expressions in cases of proven GALD-associated NH (GALD-NH) in comparison to normal, non-diseased fetal, and neonatal tissues.

Materials and methods

Human subjects

This is a clinical observational study utilizing tissues obtained from newborns at autopsy or liver transplantation and data acquired during clinical care of families of children with NH [14]. Eleven cases of GALD were studied. The clinical context in all cases was neonatal liver failure, and all cases had a maternal sibling with autopsy-proven NH. Review of the prenatal records showed that all of the mothers of these infants received routine care including iron supplementation as needed to prevent anemia. GALD was diagnosed by demonstrating complement-mediated hepatocyte injury using immunohistochemistry for C5b-9 complex [6]. Ten of the subjects studied herein were among the 33 cases of NH previously reported to exhibit complement-mediated hepatocyte injury [6]. The current cases showed pathologic amounts of C5b-9 complex in >90% of residual hepatocytes. These subjects had clinical or autopsy confirmation of extrahepatic siderosis and thus had GALD-NH. The 22-week gestation infant with acute liver failure has been reported as having C5b-9 complex in 100% of hepatocytes and no extrahepatic siderosis, and thus had acute GALD [15].

Immunohistochemistry was performed on autopsy-acquired paraffin embedded tissues. Four cases of GALD-NH whose complete autopsy was available were studied. These infants died at 2–14 days of life (38–41 weeks post-conception) from complications of liver failure. The INR exceeded 5.0 in all four cases. The hepatic histopathology in these cases was consistent with subacute or chronic liver injury, with extensive lobular collapse and fibrosis [4]. As previously reported, quantitative morphology demonstrates prominent hepatocyte loss; the 4 cases studied herein were included in that report and had the hepatocyte area density of <15% [16]. Non-diseased control tissues were from four newborns whose deaths were due to perinatal asphyxia with post-conception age (38–40 weeks) matched to the cases. These control cases have been used in a prior study [16].

In the clinical setting, oral mucosal biopsy provides a source of tissue that may show siderosis in minor salivary glands [17]. In order to determine if this tissue shows the same protein expression pattern as other tissues prone to siderosis, we studied the oral mucosal biopsy samples from two infants (post-conception ages 32 and 38 weeks) with clinically diagnosed NH. No comparable normal control tissue was available for the study.

For the analysis of hepatic gene expression in GALD-NH, snap frozen liver specimens were obtained at the time of liver transplantation from 6 GALD-NH cases (post-conception age 35–43 weeks) with liver failure, and from the immediate post-mortem liver biopsy of one 22-week gestation infant that died within minutes of birth. Snap frozen specimens of the control liver were obtained at immediate post-mortem autopsy from 2 term newborns with congenital heart disease. These liver specimens were histologically normal. Fetal liver RNA from a pool of 68 medical abortions with gestational ages of 22–40 weeks was purchased (Clontech, Mountain View, CA). For the purposes of statistical analysis, the controls (N = 3) for hepatic gene expression studies consisted of the 2 term newborns and the pooled fetal sample.

In order to obtain a representative sample of serum iron indices, our database was searched for cases of proven GALD-associated NH in which serum/plasma iron indices had been recorded during clinical care. Twenty cases were identified in which serum ferritin and 13 where percent saturation of iron binding capacity were recorded, both being recorded in 12 cases. Serum iron concentration was recorded in 8 cases and total iron binding capacity in 7 cases. All cases were live-born infants, in order to have received clinical evaluation, and included the 6 infants whose livers obtained at transplantation were used in gene expression studies. Measurements were made in clinical laboratories, and no attempt was made to correct for differences in laboratory normal values, as they are generally established for adults and not relevant in this setting. Reference values for newborns were obtained from the literature.

The collection and study of these samples and data were approved by the Children's Memorial Hospital Institutional Review Board by exemption and with an approved waiver of privacy of healthcare information in compliance with the Health Insurance Portability and Accountability Act (HIPAA).

Gene expression studies

Liver gene expression of transferrin (*TF*), hepcidin (*HAMP*), and hemojuvulin (*HFE2*) was measured by quantitative real-time polymerase chain reaction (qPCR) and normalized to the expression of the housekeeping gene 18S. Total RNA was isolated from frozen liver samples using Trizol reagent (Invitrogen by Life Technologies, Carlsbad, CA). One microgram of total RNA from each sample was reverse transcribed to obtain cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). QPCR was performed with a 7500 Real Time PCR System (Applied Biosystems by Life Technologies, Carlsbad, CA) using a QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA). Individual primers were created by Integrated DNA Technologies, Inc. (Coralville, IA). Sequences are available on request.