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An immunohistochemical study of placental syncytiotrophoblasts in neonatal hemochromatosis

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ABSTRACT

Introduction: Neonatal hemochromatosis (NH) is a rare neonatal disorder that results in liver cirrhosis with hemosiderin deposition in the liver and other organs, similarly to hereditary hemochromatosis. Excess iron is transferred from the mother to fetus through the placenta in NH. We examined the expression of iron metabolism-related substances in placental syncytiotrophoblasts (STB) by immunostaining to clarify how the transfer of iron through STB increases in NH.
Methods: Immunostaining was performed using formalin-fixed, paraffin-embedded sections of placentae from three NH cases, four gestational age-matched controls, and, depending on the antibody examined, five to seven full-term controls. The reactivity of immunostaining was assessed by averages of scores assigned by 3 researchers.
Results: On the microvillar surface of STB, the reactions of the antibodies against transferrin receptor 1 (TFR1), transferrin, ferritin, hepcidin, ferroportin, divalent metal transporter-1 (DMT1), hephaestin, and HFE were stronger in NH than in controls. In the cytoplasm, the reactions of antibodies against TFR1, transferrin, ferritin, hepcidin, DMT1, hephaestin, HFE, and ZIP 14 were stronger in NH than in gestational age-matched controls. Among these reactions, those of anti-TFR1 antibody on the surface of STB in NH was especially marked.
Discussion: In the placenta of NH, increases in expressions of TFR1, transferrin, and ferritin of which those of TFR1 were especially marked, reflect increased iron influx from the mother to fetus. The hepcidin observed on the surface and in the cytoplasm of STB of NH is suggested to be from the mother, possibly to compensate for the decreased fetal liver-derived hepcidin.

1. Introduction

Neonatal hemochromatosis (NH) is a severe liver disease in neonates that results in liver cirrhosis with the deposition of hemosiderin in the liver and other organs, similarly to hereditary hemochromatosis in adults [1–3] After an index case, the recurrence rate of NH in subsequent siblings is very high (80%). As prenatal high-dose immunoglobulin treatment of the mother is a very effective preventive therapy for NH, it has been considered to be one of the gestational alloimmune liver diseases [4] and [5]. It has been postulated that IgG from the mother damages the fetal liver, so the production of hepcidin in the fetal liver is reduced. In the duodenal mucosa and placental syncytiotrophoblasts (STB), hepcidin prevents ferroportin from taking iron into the tissues. Therefore in this disorder, ferroportin is considered to continue transporting iron from the mother to fetus through the placental

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STB [6]. The purpose of this study was to examine the expression of substances concerning iron metabolism in the placenta of NH by immunostaining and clarify the mechanisms of how iron transfer through the placenta increases.

2. Methods

2.1. Patients and materials

Formalin-fixed, paraffin-embedded sections of placentae from three NH cases (gestation period, 30–35 weeks), four gestational age-matched controls (Matched Co) (gestation period, 32–33 weeks), and seven full-term controls (Term Co) (gestation period, 36–42 weeks) were used. In Term Co, three infants were growth-restricted (2,016, 2058, and 2296 g at birth), but they grew satisfactorily although comparatively small (2810 g on the 33rd day, 4172 g on the 51st day, and 3770 g on the 60th day after birth, respectively). Cases of pre-eclampsia were avoided as controls because it is a disease involving impaired transferrin receptor expression in STB [7]. Two of the 3 NH cases (cases 1 and 2) were siblings, and one of the 7 Term Co had the same parents as the 2 NH siblings and was born after preventive therapy with high-dose immunoglobulin administration [8]. One of the NH cases had an older sibling with NH who is alive after medical treatment. The cases studied are summarized in Table 1.

2.2. Immunostaining

Conventionally, formalin-fixed, paraffin-embedded sections (4-μm thickness) were prepared.

After deparaffinization and dehydration, antigen-retrieval was performed with an appropriate method (Table 2). Then, incubation with 3% H_2O_2 in methanol for 10 min, and immersion in phosphate-buffered saline (PBS)-2% bovine serum albumin-0.1% NaN_3 for 60 min were performed. Then, incubation with the primary antibody was performed at room temperature for 60 min. After rinsing in PBS, the sections were incubated with peroxidase-conjugated goat anti-rabbit IgG Fab', rabbit anti-murine IgG Fab', or rat anti-goat IgG Fab' antibodies (Histofine simple stains, MAX-PO(R), MAX-PO(M), or MAX-PO(G), respectively, Nichirei Biosciences Inc., Tokyo, Japan). After rinsing in PBS, the sections were incubated with the substrate solution (Simple stain DAB solution, Nichirei Biosciences Inc.) and counterstained with hematoxylin.

2.3. Primary antibodies

Commercially available antibodies against divalent metal transporter (DMT) 1, hepcidin, transferrin, transferrin receptor (TFR) 1, Zrt- and Irt-like protein (ZIP) 14, ferritin, ferroportin, HFE (a human hemochromatosis protein), and hephaestin were purchased.

2.4. Assessment of antibody reaction

Images of more than ten medium-power fields which were randomly selected from each section and some representative high-power field images from each medium-power field image were recorded on a personal computer, and antibody reactions were assessed independently by three researchers (AS, YI, and HS) without access to the clinical data according to the following standard: no reaction, zero points; diffuse mild or patchy mild to moderate reaction, one point; diffuse moderate or patchy moderate to marked reaction, two points; and diffuse marked reaction, three points. Averages of all scores for each section given by the three researchers (reaction scores) were calculated (full score: 3 points).

2.5. Statistics

In a preliminary study, evaluations of the immunoreactivity in figures recorded on a DVD were assessed once a day for three consecutive days by three observers, and the stability was evaluated by the standard deviation (s.d.) and coefficient of variation (CV) [9].

To evaluate the bias of three observers in each immunostaining group, the average of CV of all patients and controls was calculated [9].

Reaction scores of sections immunostained by every antibody were evaluated, and significant differences between NH and control groups were assessed by the Mann-Whitney test.

3. Results

Table 3 presents the results of a preliminary study to show the stability of assessment of the immunoreaction by the three observers. CV values were 7.34, 10.17, and 8.50 for the microvillar surface and 12.86, 10.17, and 8.03 for cytoplasm of STB, respectively.

Fig. 1 shows images of immunostaining of the placentae of cases 1 and 2 with NH and the normal sibling of these NH cases. Fig. 2 show the reaction scores involving all cases of NH and controls. Figures in parentheses show the averages of CV.

3.1. Reaction of anti-TFR1 antibody

As shown in Fig. 1, the reaction of anti-TFR1 antibody on the microvillar surface of STB in all cases of NH was marked, and the reaction substance was densely black, whereas that in the normal sibling was weaker and remained brown. As shown in Fig. 2, the average of the reaction scores on the microvillar surface of NH were 3.0, and that of both Matched Co and Term Co was 2.2 (significant differences: $P < 0.01$ between NH and total of Matched Co and Term Co (Total Co); $P < 0.05$ between NH and Term Co). Growth restricted infants involved in Term Co showed a strong reaction of anti-TFR1

Table 1
A summary of three NH cases and control groups.

	#	Gestat period[c,d] (weeks)	Body weight at birth[d] (g)	Placenta[d] (g)	Maternal age[d] (y/o)	Parity[d]	Maternal BMI[d]	Maternal Hb[d] (g/dl)
NH	3	33	1150	313	33	0.67	24.9	10.3
		30–35	822–1358	260–384	30–38	0 or 1	24.4–25.1	8.9–10.5
Matched Co[a]	4	33	2030 g	563	31	1	23.7	11.3
		32–33	1859–2246	550–600	21–36	0 or 2	20.7–28.2	10.4–11.7
Term Co[b]	7	38	2514 g	479	32	1.4	24.0	11.6
		36–42	2016–3119	350–674	27–35	1 or 2	20.7–26.6	10.5–13.2

[a] Gestation period-matched control.
[b] Full-term control.
[c] Gestation period.
[d] Gestation period, body weight at birth, weight of placenta, maternal age, parity, maternal BMI, and Maternal Hb are expressed by averages on the upper lines and ranges on the middle and lower lines.