

Biochemical and genetic defects underlying human congenital hypotransferrinemia

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Introduction: Human congenital hypotransferrinemia is a rare disorder characterized by the virtual absence of transferrin in the serum. No information on the causes of the disease is known.

Materials and methods: Here we describe the identification of a new case, its treatment and the biochemical and genetic defects underlying the disorder.

Results: At diagnosis the patient had serum Tf levels equal to about 1% of the normal values. The treatment with plasma infusions each month allowed a good erythropoiesis and the prevention of iron overload with no need of red blood cell transfusions or iron chelators. In order to define the genetic basis of the disease, we performed a haplotype analysis of the Tf gene region in the 26 individuals forming the proband's family, and demonstrated that the genetic defect is located in the Tf gene and that it is inherited as a recessive trait. Protein analyses indicate that the proband serum contains two transferrin forms: one of 80 kD analogous to the normal one, and a smaller one of 50 kD, which may arise from a specific degradation or be the gene product of a modified allele.

Conclusion: These data suggest the presence of two Tf alleles carrying genetic defects that cause two distinct abnormalities. One allele causes low expression of an apparently normal protein that probably allowed the survival of the patient in the first years of age. The other allele produces a modified Tf with different biochemical characteristics compared to the normal one.

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Introduction

Transferrin (Tf) is one of the key proteins in iron metabolism together with Transferrin Receptor (TfR) and ferritin. These proteins are responsible for serum transport, cellular uptake and storage of the metal, respectively.¹ Studies on iron have extended its significance beyond the traditional area of erythropoiesis and nutrition, so that it is now recognized as a key element in fields such as oncology, pathology and infectious disease.² Since iron at physiological pH and ionic conditions is mainly insoluble, biological systems

have evolved iron binding properties to facilitate transport and storage.

Transferrin is a highly specialized ~80 kD, monomeric glycoprotein which binds two iron atoms with high affinity. Its physiological role is to sequester circulating iron in a safe non-toxic form and to deliver it to the tissues. The diferric-Tf binds TfR exposed to the cell surface, the complex is internalized by endocytosis, and, in the acidic endosomes, the iron is made available to transfer to the cytosol. Apotransferrin is then recycled back to the cell surface and released into the blood stream.³ Transferrin is synthesized at high levels in the liver and at lower levels in other organs such as brain, testis, lactating mammary glands, some fetal tissues during development and lymphomyeloid cells.^{4–6} Extra-hepatic Tf may be required as an iron binding protein in tissues such as brain and testis,

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which are sheltered from circulating Tf, or it may serve to scavenge iron in the milk and inhibit bacterial growth in the lactating mammary gland. Data indicate that Tf produced by lymphomyeloid cells plays an important role in the regulation of immune responses.⁷

Congenital hypotransferrinemia (OMIM n. 209300) is a very rare human disorder characterized by very low levels of Tf which are insufficient to support adequate erythropoiesis resulting in the development of anemia. Ten cases have been reported so far.⁸⁻¹⁶ In some cases the disease appears to be inherited as an autosomal recessive trait. Only in one case did analysis of serum Tf in the proband and his family show that the disease is related to the presence of a combined Tf defect and that the proband and two siblings were compound heterozygous for a 'null' and a 'variant' allele.¹⁷ A mouse model of hypotransferrinemia, in which the Tf gene has an alternative splicing site that determines ineffective expression of the protein, has been extensively studied and used to better understand iron metabolism and transport, as well as mechanisms of iron overload.^{18,19} The heterozygous animals appear asymptomatic while the homozygotes need transferrin injection to survive.

In this study we describe the clinical course of a case of human congenital hypotransferrinemia and its treatment. Molecular studies performed on the proband and his family provided indications of the mode of inheritance and the biochemical defect underlying the disorder.

Materials and methods

Clinical laboratory tests

Serum Tf values of the proband, his parents and older brother were determined with standard immunonephelometric method. They were confirmed by an ELISA test (The Human Transferrin ELISA Quantitation Kit, Bethyl Laboratories Inc, Montgomery, TX, USA) in the proband. For the other family members serum Tf was calculated from the Total Iron Binding Capacity (TIBC). The soluble TfR was determined by an immuno-nephelometric method (Dade Behring, Liederbach, Germany). Other routine clinical laboratory tests were carried out using standard techniques.

Microsatellites analysis

Peripheral blood samples were collected from the proband and all his family members after informed consent and genomic DNA was extracted according to standard procedures.²⁰ Genome location, primer sequences, PCR conditions, and PCR product sizes of microsatellites D3S1551, D3S3569, D3S3584, D3S1238, D3S1589, D3S3606, D3S3607, D3S1484 have been obtained from the Genome DataBase site of the Johns Hopkins University. All PCRs comprised 30 cycles and were performed in 20 µl reaction volumes containing 20 pmol of each primer (one of which was

5'-end labeled with Cy5-fluorochrome), 0.2 mM dNTPs, 1 mM MgCl₂, 1 U of Taq Polymerase, and 20 ng of genomic DNA. PCR products were run on a polyacrylamide gel using the ALFred DNA Sequencer Automated System (Amersham Pharmacia Biotech, Little Chalfont, UK) under standard conditions. The Allele Link software (Amersham Pharmacia Biotech) was used for data evaluation.

Southern analysis

Genomic DNA, obtained from blood samples of the proband, his parents and three normal controls, was digested separately with *EcoRI* and *BamHI*, electrophoresed and blotted according to standard methods.²⁰ Membranes were hybridized with the Tf full-length cDNA (purified from commercial plasmid TFR27A: ATCC cat. no. 53106) and with genomic probes synthesized by Long PCR (Expand High Fidelity PCR System, Boehringer Mannheim, Mannheim, Germany). Three genomic probes were amplified as follows: (1) primer on exon 1 (p1: GTCCGAC-TGTGCTCGCTGCTCA) and primer on exon 8 (p4: GAGGAGAGCTGAATAGTTGGAAT); (2) exon 9 (p19: AGAAGCCCCAACAGATGAATGC) and exon 13 (P39: CCCTCTTTGTTGTTGGGTTTAC); and (3) exon 13 (P40: GCTGTGTATGGGCTCAGGC) and exon 17 (P10: GCTGTGAAGACAGACG-TGGTTAG). Probes were ³²P-labeled and repetitive elements of genomic clones were blocked by competition with sonicated human placental DNA before hybridization.²¹ Membranes were washed under stringent conditions to a final stringency of 0.1 × SSC, 0.1% SDS at 65°C for 20 min.

Detection of hemochromatosis gene mutations

To identify the two hemochromatosis gene (HFE) mutations, Cys282Tyr and His63Asp, DNA samples of the family members were PCR amplified with proper primers and digested with *RsaI* and *BclI/MboI* enzymes, respectively, as previously described.²²

Analysis of transferrin protein

Serum samples of the proband and family members were frozen immediately after separation and thawed just before analysis. An aliquot of proband serum was obtained before the beginning of plasmapheresis therapy, and other aliquots were obtained during therapy and before plasma transfusion when the level of transferrin was the lowest (6 mg/dl) and composed by comparable proportion of the endogenous and exogenous transferrins. Serum samples were untreated, to avoid possible protein degradation. Non-denaturing PAGE were performed on 7.5% polyacrylamide slab gels, the samples were diluted in 125 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, with or without 1% 2-mercaptoethanol, heated at 100°C for 5 min, and loaded on the gel. Proteins were electrophoretically transferred onto nitrocellulose filters using a semi-dry

blotting apparatus (Hoefer, California). The filters were incubated for 1 h at room temperature with goat anti human Tf antisera (Sigma, St Louis, MO, USA) diluted 1:1500 in TBS with 10% defatted dry milk, followed by Horseradish Peroxidase-labeled rabbit anti goat IgG (Sigma) diluted 1:3000, the peroxidase activity was developed by the chemoluminescence method (ECL, Amersham, Little Chalfont, UK). Under these conditions a single band was detected in control serum samples, co-running with the purified human Tf (Sigma) both iron free and iron loaded.

Cellular studies

Transferrin expression was analysed on various cell types: (1) Stable CD4⁺ and CD8⁺ cell lines derived from the proband's T-lymphocytes were prepared by immortalization of peripheral blood mononuclear cells (PBMC) with human T-lymphotropic virus type I (HTLV-I) or Herpesvirus Saimiri, respectively, according to published protocols,^{23,24} and were cultivated in RPMI medium with IL2 50 U/ml; (2) PBMC from the proband and three healthy blood donors were separated on a Ficoll gradient and then expanded for 5–7 days in RPMI plus PHA and IL2 100 U/ml. Part of the cell populations were then CD8⁺-depleted by incubation with lymphoid-specific magnetic microbeads, as previously described.²⁵ Immunophenotype of T-lymphocytes was established by flow cytometry with monoclonal antibodies against CD4, CD8, CD3, CD16-56, CD14, CD45, CD19 antigens; and (3) HepG2 cell line (hepatocellular carcinoma cell line used as positive control expressing Tf) was cultivated in D-MEM 10% FCS.

To study *in vitro* Tf production by T-lymphocytes, 2 × 10⁵ PBMC were plated in a 96-well plate in 200 µl of RPMI 10% FCS, or RPMI 10% FCS plus PHA 4 µg and IL2 10 U/ml, or RPMI 10% FCS plus PHA 4 µg and IL2 100 U/ml. The supernatants were tested for Tf concentration with the ELISA test at 24, 48, and 72 h in triplicate for each condition. Lymphocyte activation induced by PHA and IL2 was verified by ³H-thymidine incorporation at 72 h.

Analysis of Tf transcripts

Total RNA was extracted from cultivated cells using the phenol chloroform method²⁶ and polyA mRNAs obtained using the mRNA Isolation Kit (Boehringer Mannheim, Mannheim, Germany) according to the

manufacturer's procedure. RNA was electrophoresed, blotted and hybridized with ³²P-labeled Tf full-length cDNA according to standard procedures.²⁶ Blots were washed to a final stringency of 0.5 × SSC, 0.1% SDS at 65°C for 20 min. Membranes were exposed to autoradiography for 10 days at -70°C. For RT-PCR studies, cDNA was synthesized with random examers using Superscript RNase H Reverse Transcriptase (GIBCO BRL Life Technologies, Paisley, UK) according to the manufacturer's instructions. Samples of 3 µl were diluted to a 50 µl total volume of the PCR mixture containing 2 mM MgCl₂, 0.2 mM dNTP, 1 U of Taq Polymerase and 15 pmoles of Tf specific primers. PCR conditions were: 94°C for 2 min, then 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 15 s. PCR primers were on exon 7 (p5: AACCAGGCCAGGAACATTTTG) and exon 9 (p18: GCACACCACTTCACAGGCTTGC) and generated a 236 bp product which was visualized on ethidium bromide-stained 2.5% agarose gel.

Results

Clinical description

The patient under study is a male child that was born after a regular pregnancy in 1993 at the 42nd week. Caesarean surgery was necessary due to fetal distress. The newborn child was small (2650 g, 48 cm) with bilaterally twisted feet, penoscrotal hypospadias and ambiguous genitalia. A karyotype was performed for the latter, which resulted normal (46, XY). At birth he had severe anemia (Hb 5.9 g/dl, Hct 19.5%, RBC 2 320 000/µl), hypovolemic shock, severe metabolic acidosis and persistency of fetal circulation (that lasted for four days). He was admitted to a neonatal intensive care unit and was dismissed after two months in general good health with oral iron supplementation (which he took for 12 months) due to persistency of hypochromic and microcytic anemia and supplement of vitamin A and D2.

He was then followed as an outpatient by the same clinic and a control at six months found Hb 12 g/dl, RBC 4 690 000/µl, MCV 73.4 fl. The patient remained in good health until 22 months when for a strong pallor he was admitted in the same hospital and severe anemia was diagnosed (Hb 3.7 g/dl, RBC 2 290 000/µl, MCV 50.5 fl). Promptly transfused, he was sent to our hospital referral centre for hematological disorders.

Table 1 Hematological findings in proband's family at diagnosis

	Hb g/dl	Hct %	MCV fl	Tf mg/dl	Serum iron mg/dl	Tf saturation %	Ferritin µg/l
Proband	5.6	19.5	57	3	10	>100	317
Father	17	51	87	151	94	49	319
Mother	14	42	84	249	75	23	7
Brother	13	39	79	185	59	25	25
Normal values	11.8–17.8	37–52	79–97	200–360	60–140	22–46	10–200

Laboratory findings in the proband's family at the time of diagnosis.

The absence of the β -1 globulin band at electrophoresis, serum iron levels of 10 $\mu\text{g}/\text{dl}$, serum Tf levels of 3 mg/dl together with the persistency of hypochromic microcytic anemia supported the diagnosis of congenital hypotransferrinemia (Table 1). No pathological findings were detected at the physical examination except for a strong pallor. A ferritin level ranging from 317–365 $\mu\text{g}/\text{l}$ suggested a slight hemosiderosis.

At first, the patient received monthly transfusions of red blood cells to maintain a sufficient concentration of hemoglobin, and of Desferrioxamine to prevent iron overload. *In vivo* grade human Tf was not available and plasma was substituted for transfusion. An initial plasmapheresis raised the patient's serum Tf concentration from 3 mg/dl baseline level to 120 mg/dl and produced a good erythropoietic response with normal Tf kinetics (half-life of about 15 days) (Figure 1). At present, the patient is regularly infused with plasma at approximately one-month intervals to keep Hb levels above 12 mg/dl , and red blood cell transfusions and iron chelators are no longer required. To minimize the risk of virus infection from blood products, we selected a small group of donors with high levels of serum Tf. After the beginning of the treatment the patient showed a normal growth rate, with reticulocyte count

and serum ferritin levels returning within the normal range. The patient is now in apparently good health.

Family studies

To test the hypothesis that the defect causing hypotransferrinemia was genetic and located on or near the Tf gene, we studied the proband's large family for Tf haplotype, serum Tf and other indices of iron metabolism. Haplotype analysis was performed using five microsatellites covering the 3q21 region around the Tf gene: two (D3S1551 and D3S3569) were proximal, two (D3S3584 and D3S1238) were distal, and D3S1589 localized close to the Tf gene. All subjects were tested for serum iron and TIBC, from which Tf concentration was calculated. In all but two cases we observed an association between one haplotype of the proband and low/intermediate levels of serum Tf (130–200 mg/dl) (Figure 2). The exceptions were the grandmother (I4) with normal Tf level, that was tested only once, and the mother (II6), who showed extremely low levels of serum ferritin (6–7 $\mu\text{g}/\text{l}$ in repeated determinations) indicating the absence of iron stores. Given the inverse relationship between Tf and serum ferritin concentration,²⁷ she appeared to have inappropriately low levels

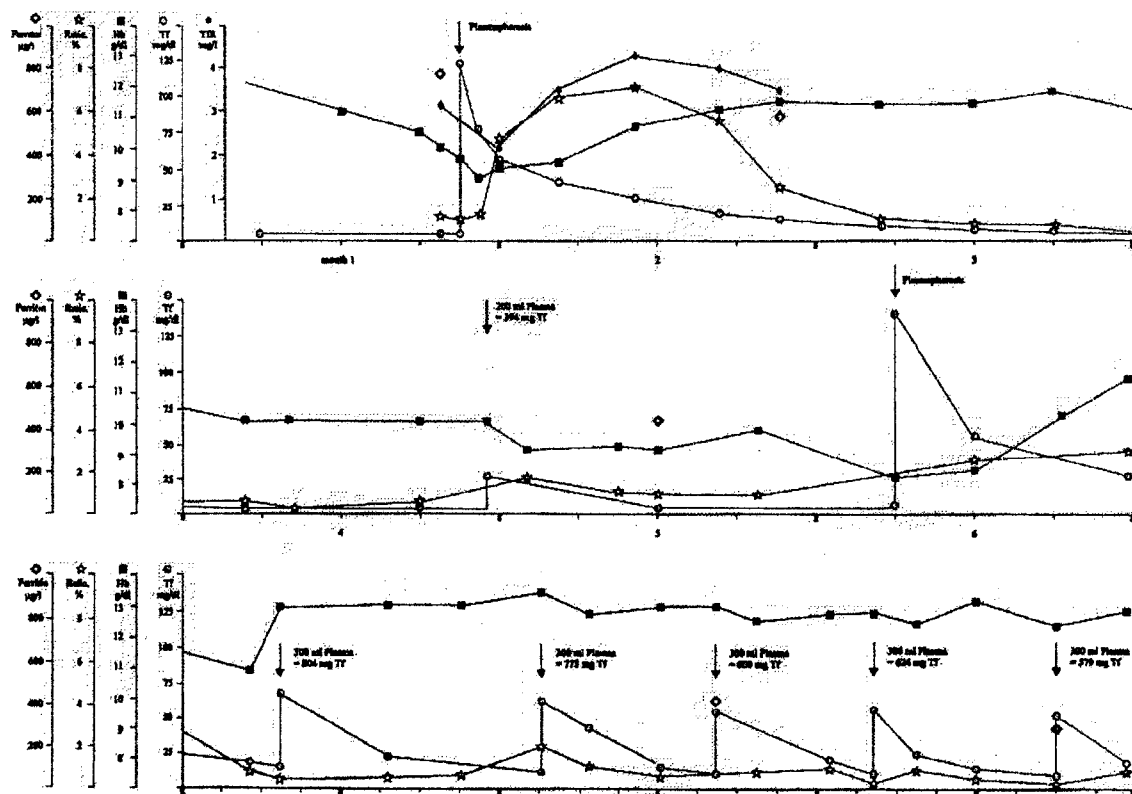


Figure 1 Time course of some hematological parameters of the hypotransferrinemia patient during the one year of plasma transfusion therapy. Ferritin, reticulocyte count (Retic), hemoglobin (Hb), Tf and TfR are indicated