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SHORT REPORT

Soluble transferrin receptor as a potential determinant of iron loading in congenital anaemias due to ineffective erythropoiesis

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Summary. Congenital anaemias due to ineffective erythropoiesis may be associated with excessive iron absorption and progressive iron loading. We investigated whether the soluble transferrin receptor (TfR) level was related to the degree of iron overload in 20 patients with thalassaemia intermedia, six patients with congenital dyserythropoietic anaemia type II (CDA II) and four patients with X-linked congenital sideroblastic anaemia (XLSA). All but two patients had increased serum ferritin levels (median 601 μg/l, range 105–2855 μg/l). Multiple regression analysis showed that 62% ($P<0{\cdot}0001$) of the variation in serum ferritin was explained by age and by changes in soluble TfR.

Keywords: anaemia, erythropoiesis, erythropoietin, iron overload, soluble transferrin receptor.

Congenital anaemias due to ineffective erythropoiesis can be associated with excessive iron absorption and progressive iron loading (Finch, 1994). Life-threatening iron overload may occur in non-transfused patients with thalassaemia intermedia (Pippard *et al*, 1979), congenital dyserythropoietic anaemia type II (CDA II) (Cazzola *et al*, 1983) and X-linked congenital sideroblastic anaemia (XLSA) (Cazzola *et al*, 1983; Peto *et al*, 1983). The degree of anaemia has been shown to be a poor predictor of iron loading, which correlates better with erythroid marrow activity. In fact studies of erythropoiesis and iron balance in anaemic patients have shown that expanded erythropoiesis is associated with increased iron absorption (Pootrakul *et al*, 1988).

The mechanism by which the erythroid marrow expansion induces a positive iron balance is unknown (Finch, 1994). Recent observations suggest that HFE, an MCH-related protein which is mutated in genetic haemochromatosis (Feder *et al*, 1996), may regulate iron homeostasis by interacting with transferrin receptor (TfR). HFE protein, in fact, binds to TfR with high affinity and decreases binding of diferric transferrin (Feder *et al*, 1998). In addition, soluble TfR and HFE bind tightly at the basic pH of the cell surface, but not at the acidic pH of intracellular vesicles (Lebron *et al*, 1998).

A soluble form of TfR, probably representing a truncated form of tissue receptor, is present in human plasma (Huebers *et al*, 1990). The erythroid marrow is its main source and the soluble TfR concentration is increased in conditions with erythroid hyperplasia. We investigated whether the level of soluble TfR was related to the degree of iron overload in a group of patients with congenital anaemia due to ineffective erythropoiesis.

PATIENTS AND METHODS

We studied 20 patients with thalassaemia intermedia, six patients with CDA II and four patients with XLSA who had received no or only occasional blood transfusions; their ages ranged from 7 to 66 years. The 20 patients with thalassaemia intermedia were followed at the Haematology Department of the Istituto di Clinica e Biologia dell'Età Evolutiva, University of Cagliari, Sardinia, Italy. All were of Sardinian descent and fulfilled accepted criteria for a diagnosis of thalassaemia intermedia. The six patients with CDA II and the four males with XLSA were followed at the Department of Internal Medicine and Medical Therapy, University of Pavia Medical School and IRCCS Policlinico S. Matteo, Pavia, Italy. Diagnosis of CDA II was based on

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the following criteria: (a) presence of >10% binucleated erythroblasts in the bone marrow; (b) positivity of acidified serum test with some normal sera and negativity with patient's own serum. Patients with XLSA had a microcytic congenital anaemia associated with the presence of ringed sideroblasts in the bone marrow. In all of them, missense mutations in the erythroid-specific ALA synthase gene (ALAS2) were demonstrated (unpublished observations).

Informed consent was obtained from each subject or from the parents of patients under the age of 18 to draw blood for the study while they were under routine care. Blood counts were obtained with a Coulter Counter Model S. Body iron status was assessed by estimation of serum ferritin through a radioimmunoassay method (Ramco Lab, Houston, Texas, U.S.A.). Circulating erythropoietin levels were measured by a radioimmunoassay method (Incstar Corp., Sillwater, Min., U.S.A.) that utilizes recombinant human erythropoietin for tracer and standards.

The amount of serum TfR was estimated by an enzyme-linked polyclonal antibody assay, using purified placental receptor–transferrin complexes as a reference standard and rabbit antibodies. TfR levels in 165 normal control subjects were 5·0 ± 1·1 mg/l, with 95% confidence limits ranging from 2·9 to 7·1 mg/l.

For molecular characterization of thalassaemia defects, DNA was extracted from peripheral blood leucocytes, and α-globin genotype determination and β-thalassaemia defect characterization were performed.

For PCR-RFLP detection of the two HFE mutations (C282Y and H63D) separate PCR reactions were conducted for the two mutations using the primers described by Feder *et al* (1996). Restriction digests were performed directly in the PCR mixes by addition of 5 U Rsa I (codon 282 reactions) or Bcl I (codon 63 reactions) and incubating for 2 h at 37°C or 50°C, respectively. The products were electrophoresed on a 2% agarose gel.

As control populations, we selected 30 normal subjects and 30 β°-thalassaemia heterozygotes with low haemoglobin (Hb) levels, well matched with respect to sex and age. Heterozygous β-thalassaemia was chosen as an anaemic condition that generally is not associated with iron loading.

Data were stored, analysed and reported with the packages STATISTICA/Mac (StatSoft™, Tulsa, Okla.), Exstatix™ (Select Micro Systems Inc., Yorktown Heights, N.Y.) and DeltaGraph™ Pro 3 (DeltaPoint Inc., Monterey, Calif.), all run on a Macintosh Quadra 650 (Apple Computer Inc., Cupertino, Calif.) personal computer. Results were expressed as mean ± 1 standard deviation (SD) unless otherwise stated. The Student's *t*-test and/or the F test (analysis of variance) were used to evaluate the probability of any significant difference between groups. Multiple regression analysis was employed to identify the parameters more closely related to serum ferritin. *P* values <0·05 were considered to be statistically significant.

RESULTS

The main haematological findings are summarized in Table I. The degree of anaemia was highly variable, with the Hb ranging from 6·6 to 12·5 g/dl in the patient population. Soluble TfR levels ranged from 3 to 12 times normal and were inversely related to Hb levels, although the level of significance was borderline ($P = 0{\cdot}05$). Serum erythropoietin values ranged from 40 to 1600 U/l and were inversely related to Hb levels ($r = 0{\cdot}52$, $F = 11{\cdot}99$, $P = 0{\cdot}0017$).

All but two subjects had increased serum ferritin levels and 7/10 individuals aged >30 years had clinical signs of parenchymal organ dysfunction. The proportion of individuals aged >30 years with evidence of organ dysfunction was significantly higher than that of those aged ⩽30 ($\chi^2 = 9{\cdot}07$, $P = 0{\cdot}0026$).

Univariate regression analysis was first performed to identify the parameters more closely related to serum ferritin. As judged by serum ferritin levels, iron loading occurred regardless of the degree of anaemia and also showed no relationship with sex, basic disease and serum erythropoietin. 15/30 patients had never been transfused and the remaining 15 had received only occasional transfusions (<12 and <0·5 per year in any case). Regression analysis showed no relationship between serum ferritin and the number of RBC transfusions received. 8/30 patients (27%) were heterozygous for the HFE H63D

Table I. Haematological and iron status parameters.

Patients and controls	Hb (g/dl)	Serum ferritin (μg/l)	Serum erythropoietin (U/l)	Soluble transferrin receptor (mg/l)
Iron-loading anaemias ($n = 30$)	9·0 ± 1·5 (6·6–12·5)	957 ± 792 (105–2855)	425 ± 429 (40–1600)	39·0 ± 11·5 (15·8–58·7)
Heterozygous β-thalassaemia ($n = 30$)	10·4 ± 0·8 (9·0–12·0)	82 ± 72 (15–264)	19 ± 9 (6–45)	6·7 ± 1·7 (4·0–10·9)
Normal control subjects ($n = 30$)	14·4 ± 1·3 (12·5–16·8)	69 ± 45 (25–178)	16 ± 5 (8–30)	5·3 ± 1·0 (3·7–7·5)

Numbers are mean ± 1 SD with the range in parentheses.

mutation, whereas only one was heterozygous for the HFE C282Y mutation; there was no relationship between HFE mutations and serum ferritin.

Age and soluble TfR were the only two factors related to iron load in univariate regression analysis. In fact, as judged by serum ferritin, iron load was closely related to the patient's age ($P<0{\cdot}0001$) and the soluble TfR level ($P=0{\cdot}0002$). Serum ferritin values in excess of 1000 μg/l were found only in patients with soluble TfR levels > 30 mg/l, i.e. > 6 times normal.

Multiple regression analysis showed that 62% ($P<0{\cdot}0001$) of the variation in serum ferritin was explained by age (representing the length of time of exposure to the risk) and by changes in soluble TfR (the putative determinant of iron loading). As expected, there was a close relationship between the serum ferritin concentration and the product of the above two parameters (Fig 1).

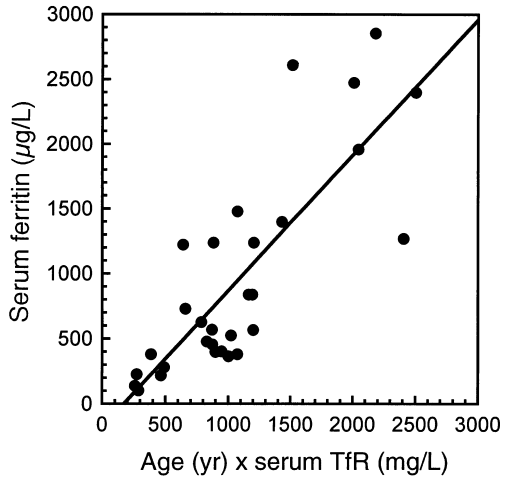

Fig 1. Relationship between the product of the patient's age times the soluble TfR level at the time of study and the serum ferritin concentration. Regression analysis provided the following results: serum ferritin (μg/l) = $-1205 + (41{\cdot}2 \times$ age, yr) + ($26{\cdot}5 \times$ soluble TfR, mg/l) (multiple-R = 0·788, F = 24·783, $P<0{\cdot}0001$).

The 30 subjects with heterozygous β-thalassaemia had Hb levels ranging from 9 to 12 g/dl (Table I), markedly lower than those of normal control subjects (F = 215·08, $P<0{\cdot}0001$). There was no associated congenital or acquired condition that could explain the low Hb levels of these individuals. In particular, the following conditions were excluded in each case: excess of alpha genes, haemoglobinopathies or disorders of the red cell membrane, iron deficiency, folate or vitamin B12 deficiency, Soluble TfR levels of these anaemic subjects with heterozygous β-thalassaemia were only slightly higher than those of normal controls ($6{\cdot}7 \pm 1{\cdot}7$ *v* $5{\cdot}3 \pm 1{\cdot}0$ mg/l, F = 13·85, $P=0{\cdot}0004$). There was no difference between β-thalassaemia heterozygotes and normal individuals with respect to serum ferritin levels ($P=0{\cdot}39$).

DISCUSSION

Iron-loading anaemias are primarily characterized by very high degrees of erythroid proliferation that involve parallel increases in iron exchange. In fact, whereas the normal erythroid iron turnover is about 30 mg/d, as much as 300 mg of iron per day may be required in individuals with expanded erythropoiesis. The increased erythron requirement has important effects on the reticuloendothelial cells and intestinal mucosa. Reticuloendothelial cells return a far greater proportion of processed iron than is normally released to transferrin, although they also hold an increased amount of iron. At the same time there is a parallel increase in iron absorption (Pootrakul *et al*, 1988), and the amount of iron absorbed exceeds the body iron losses, so that there is a progressive parenchymal iron loading.

Experimental and clinical evidence suggests that the erythron-induced increase in iron absorption is in some way related to erythropoiesis (Finch, 1994), although different anaemic states differ in the magnitude of the absorptive response. In fact, iron loading is generally more severe in patients with anaemia due to ineffective erythropoiesis than in haemolytic states (Pootrakul *et al*, 1988).

Clearly some components of erythroid marrow must mediate the message between expanded erythropoiesis and increased intestinal iron absorption. It has been previously proposed that the transferrin receptor mass of the bone marrow is the regulator and transferrin the mediator of the message (Finch, 1994). When extremely high rates of erythropoiesis are required, transferrin receptor needs are not met and increased absorption results. It is this state of receptor unsaturation which appears to result in progressive iron loading.

Transferrin receptor density on erythroid cells is regulated by several factors, the most important ones being cellular iron status and erythropoietin stimulation. Both deprivation of iron and erythropoietin stimulation increase the number of receptors (Weiss *et al*, 1997). Soluble TfR is a truncated form of tissue receptor (Shih *et al*, 1990) whose generation is probably due to a proteolytic mechanism. The erythroid marrow is the main source of soluble transferrin receptors and increased levels are found in erythroid hyperplasia (Huebers *et al*, 1990). Evidence has been provided that unsaturation of transferrin receptors on erythroid cells enhances generation of soluble TfR (Baynes, 1995). All these observations suggest that anaemias associated with expanded but ineffective erythropoiesis involve the presence of large amounts of TfR on erythroid cells and the production of large amounts of soluble receptor.

Recent studies have reported evidence of a physical interaction between HFE and TfR (Feder *et al*, 1998; Lebron *et al*, 1998). This interaction appears to result in inhibition of iron absorption, although the underlying mechanisms are not known. In the context of our current understanding of HFE/TfR interactions, the findings of the present study suggest that high-soluble TfR levels may contribute to impairing regulation of iron homeostasis and determining progressive iron loading in patients with congenital anaemias. Our working hypothesis is that soluble TfR inactivates the inhibitory effect of HFE protein on intestinal iron absorption. This is in keeping with the observation that iron absorption is increased both in iron deficiency and in iron-loading anaemias, i.e. two contrasting

conditions whose only common denominator is a high level of soluble TfR. On the other hand, it is also in keeping with the observation that anaemic individuals with heterozygous β-thalassaemia had only marginally elevated soluble TfR levels and nearly normal serum ferritin concentrations.

Based on the data presented in this paper we cannot rule out that soluble TfR may simply represent an indirect indicator of erythroid activity, which is already known to be somehow associated with increased iron absorption. From a clinical point of view, however, the measurement of soluble TfR may be useful in predicting the risk of iron overload in individual patients with congenital anaemia with ineffective erythropoiesis. Our findings suggest that values steadily in excess of 5–6 times normal are associated with a high risk of iron overload in middle age.

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