Role of non-transferrin bound iron in iron overload and liver dysfunction in long term survivors of acute leukaemia and bone marrow transplantation

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Abstract

Aims—To determine whether non-transferrin bound iron is present in the serum of long term survivors of acute leukaemia and bone marrow transplantation who have liver dysfunction as indicated by consistently raised serum aspartate aminotransferase (AST) activities.

Methods—Thirty eight patients, who were at least three years from the end of treatment, were studied. Serum samples were analysed for hepatitis C, hepatitis B, AST, ferritin, and non-transferrin bound iron. A bleomycin based assay was used to detect non-transferrin bound iron. Patient and blood bank records were examined to determine the number of units of transfused blood received by each patient.

Results—Ten patients had consistently raised serum AST activities. Of these, two had evidence of hepatitis C infection, one had chronic hepatitis B infection and one had chronic graft versus host disease affecting the liver. None of these four patients had detectable non-transferrin bound iron. The remaining six patients had no obvious reason for raised AST activities, but four had non-transferrin bound iron detectable in their serum as compared with only two out of 28 patients with normal AST activities. Patients with abnormal AST activities had higher serum ferritin concentrations than those with normal AST, though serum ferritin was raised in 21 of 28 patients without liver dysfunction.

Conclusion—Non-transferrin bound iron may be found in this group of patients, suggesting that iron overload is the cause of the observed liver dysfunction. Non-transferrin bound iron may also be a more specific indicator of iron overload than the serum ferritin concentrations.

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Keywords: liver dysfunction, non-transferrin bound iron, acute leukaemia, bone marrow transplantation.

Liver dysfunction is a recognised finding following treatment of haematological malignancy and has a variety of possible causes including: hepatitis B virus (HBV),¹ hepatitis C (HCV),² graft versus host disease (GvHD),³ and veno-occlusive disease (VOD).⁴ The latter

is generally only seen soon after transplantation; however, the others can be responsible for continuing liver damage years after the cessation of treatment. In addition, many chemotherapeutic agents used in the treatment of haematological malignancies are known to be hepatotoxic.⁵

Recently, it has been suggested that iron overload is responsible for abnormal liver blood tests in some patients surviving at least one year after bone marrow transplantation (BMT).⁶ It is possible that this may also be the case in patients who have received chemotherapy alone for haematological malignancy.

It is known that in situations of iron overload serum iron binding capacity becomes fully saturated and that non-transferrin bound iron complexes appear in the serum. Non-transferrin bound iron is also described as free iron. Serum ferritin is increased in this situation, but ferritin functions as an intracellular iron storage protein. Only trace amounts of ferritin are present in the serum and this is glycosylated and contains little or no iron; it does not seem to function as an iron scavenger.^{7,8} Free iron or non-transferrin bound iron has been reported in several conditions including idiopathic haemochromatosis⁹ and following cytotoxic chemotherapy.¹⁰ Non-transferrin bound iron may mediate tissue damage by the formation of highly reactive hydroxyl radicals from superoxide and hydrogen peroxide.¹¹

The presence of non-transferrin bound iron can be detected using a bleomycin based assay.¹² Iron is required to bind to bleomycin in the presence of ascorbate before it can cleave DNA at G-C (5'→3') and G-T (5'→3') sequences. If bleomycin, ascorbate and DNA are in excess, the amount of DNA degradation is proportional to the concentration of non-transferrin bound iron.¹²

In cases where iron overload is the cause of abnormal liver blood tests following treatment of haematological malignancy, we postulate that serum non-transferrin bound iron should be present. Additionally, in a population of multitransfused patients non-transferrin bound iron should be a better indicator of iron mediated liver dysfunction than serum ferritin. In such a population increased ferritin concentrations may be found in many subjects without liver dysfunction. Liver dysfunction can also result in an increase in serum ferritin concentrations.¹³

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Table 1 Details of patient diagnosis and treatment

Diagnosis	Total	Treatment		
		Chemotherapy	Autologous BMT	Allogeneic BMT
AML	18	12	6	—
ALL	7	3	4	—
CML	2	—	—	2
Hodgkin's disease	6	—	6	—
Non-Hodgkin's lymphoma	4	—	4	—
Plasmacytoma	1	—	1	—

AML = acute myeloid leukaemia; ALL = acute lymphoblastic leukaemia; CML = chronic myeloid leukaemia.

Table 2 Median (95% confidence interval) AST activities (units/l) in patients with and without non-transferrin bound iron

	Number of patients	Median AST activities
Non-transferrin bound iron	6	47.5 (25.7–69.3)*
No non-transferrin bound iron	28	23.0 (14.7–31.3)

*p = 0.017.

Methods

All surviving patients treated within a single unit for acute leukaemia or who underwent BMT (autologous or allogeneic), and who were at least three years from the end of treatment, were identified and studied. Serum samples were assayed for aspartate aminotransferase (AST) activity. Ferritin was assayed by microparticle enzyme immunoassay (Abbott Diagnostics).

All samples were screened for HCV infection by a third generation enzyme linked immunosorbant assay (ELISA) (Ortho Diagnostics). Positive results in the screening ELISA were confirmed using a second EIA (Murex Diagnostics) and a recombinant immunoblot assay (Ortho Diagnostics). All samples were also assessed for HCV RNA by a reverse transcription polymerase chain reaction (RT-PCR) assay as described by Garson *et al.*¹⁴ Screening for HBV was performed by EIA (Organon Teknika) for HBV surface antigen and by enhanced luminescent immunoassay (Johnson and Johnson) for HBV core antigen.

Assays for non-transferrin bound iron were performed blind in a different institution (City

Figure 1 Correlation between serum ferritin concentrations and number of red cell transfusions.

Hospital, Birmingham). The bleomycin assay used to measure non-transferrin bound iron has been described elsewhere.¹⁰ Briefly, 0.4 ml of DNA at 1 mg/ml (Sigma, Poole, Dorset, UK), 50 µl bleomycin 0.6 mM (Lundbeck), 0.1 ml magnesium chloride 50 mM (BDH, Poole, Dorset, UK), 0.1 ml Tris buffer, pH 7.4 (BDH), 50 µl ascorbate 7.5 mM (BDH), and 20 µl of sample or control were incubated in clean plastic test tubes for 30 minutes at 37°C. Then, 0.1 ml EDTA 0.1 M (BDH), 0.5 ml thiobarbituric acid 1% w/v in 50 mM sodium hydroxide (Sigma), and 0.5 ml hydrochloric acid 25% w/v in distilled water (BDH) were added and the mixture heated for five minutes at 100°C to develop TBA (thiobarbituric acid) reactivity. After cooling, the absorbance was measured at 532 nm using a PU 8610 kinetics spectrophotometer (Pye Unicam) and compared with the appropriate standards and blanks. The bleomycin assay used to measure serum non-transferrin bound iron does not measure iron bound to transferrin or ferritin.^{12 15}

Individual medical and blood bank records were examined to determine the number of units of blood transfused during treatment and to record all elevated AST activities. For the purposes of this study an AST activity was considered consistently raised if it was > 35 U/l on two samples at least six months apart (upper end of normal range 30 U/l).

Statistical analysis was performed using the Minitab for Windows release 10 statistics package. The Mann-Whitney test and the Pearson correlation test were used.

Results

A total of 38 patients was studied. Table 1 shows details of patient diagnosis and treatment.

Ten patients were found to have consistently raised AST activities and four of these had a clear cause: two had evidence of HCV infection, being positive by both the EIAs, RIBA and RT-PCR; one had chronic HBV infection and chronic active hepatitis on liver biopsy; and a further patient had biopsy confirmed chronic GvHD affecting the liver. Tests for HBV and HCV were negative in all other patients. None of the four patients with a clear cause for an increased AST activity had detectable non-transferrin bound iron in their serum, and they have been excluded from the following statistical analysis. Six patients had raised AST activities without an obvious explanation. Four of these had detectable non-transferrin bound iron in their serum compared with only two of 28 patients with normal AST activities (p = 0.0003). Table 2 shows AST activities found in patients with and without non-transferrin bound iron in their serum. Table 3 shows characteristics of patients found to have serum non-transferrin bound iron.

In all six patients with no obvious cause for their raised AST activities the ferritin concentration was greatly raised (range 980–4214 ng/ml; normal ranges: men 30–233 ng/ml, women 6–81 ng/ml). Although these ferritin concentrations were significantly higher than

Table 3 Characteristics of patients found to have serum non-transferrin bound iron (patients 1–4 have consistently raised AST activities)

Patient number	Diagnosis	Age (years)	Sex	Years from diagnosis	Number of red cell units transfused	Non-transferrin bound iron concentration (µmol/l)	Serum ferritin concentration (ng/ml)
1	AML	58	F	10.8	75	0.8	4214
2	AML	33	F	3.0	38	0.7	1740
3	AML	68	M	3.0	38	0.5	3644
4	ALL	19	F	3.6	61	0.5	1011
5	ALL	23	F	7.2	15	0.4	9
6	AML	61	M	10.5	24	0.2	1020

AML = acute myeloid leukaemia; ALL = acute lymphoblastic leukaemia.

those seen in patients with normal AST activities ($p = 0.0055$), 21 of the 28 patients with normal AST activities also had a raised serum ferritin concentration (range 103–2815 ng/ml). We found no correlation between ferritin and the presence of non-transferrin bound iron. However, all patients with raised AST and non-transferrin bound iron had ferritin concentrations of over 1000 ng/ml compared with nine of the 28 patients with normal AST activities. There was a moderate correlation between ferritin concentrations and the number of units of blood transfused (fig 1).

Those patients with raised AST activities received a median of 49 units of blood (range 11–75) compared with a median of 25 (range 4–71) in those with normal AST activities ($p = 0.037$). The four patients with both non-transferrin bound iron and raised AST had received a median of 50 units (38, 38, 61, 75).

Discussion

It is well known that intensive treatment for haematological malignancy can have significant long term adverse effects. Well described examples include cardiomyopathy due to anthracyclines,¹⁶ lung fibrosis due to bleomycin,¹⁷ cataracts following total body irradiation,¹⁸ and endocrine dysfunction, including growth retardation, in children.¹⁹ Liver damage is also a recognised finding and, as already discussed, has several well described causes. Drugs, especially cytotoxic agents, may cause abnormal liver function by a variety of mechanisms and this has been reviewed extensively.⁵ Chronic viral hepatitis is also a well recognised complication and has been investigated extensively.

In 1988 Hetherington and Buchanan²⁰ noted a correlation between raised serum aminotransferase values and number of prior blood transfusions in 59 children with acute lymphoblastic leukaemia. They proposed non-A non-B hepatitis as the likely cause rather than, or in addition to, chemotherapy induced hepatic injury. Iron overload was not considered as an explanation for the observed raised aminotransferase activities.

Since the identification of HCV as the cause of transfusion related non-A non-B hepatitis, its impact in patients treated for haematological malignancy has been explored in several centres. In one study a group of 50 Italian children with leukaemia (in long term remission) and chronic liver disease was examined.²¹ Twenty three (46%) of the children were found to be HCV antibody positive. However, this

figure may be an underestimate as a result of the use of a first generation EIA which may have given false negative results. Iron overload was not considered as a possible cause for the chronic liver disease in the remaining 27 (54%) patients. In a more recent Italian study² investigating the prevalence of HCV infection in patients receiving treatment for acute lymphoblastic leukaemia, 21 of 102 children had raised aminotransferase activities. Of these, 16 had evidence of HCV infection (using a RIBA III and RT-PCR) and five did not; again iron overload was not regarded as a possible explanation for the raised aminotransferases in those without HCV infection. In our study the rate of HCV infection was low (5%) and reflects the low prevalence in the local donor population (0.028% in 1991; unpublished data) as compared with other areas—for example, 1.3% in Italy²² and 0.11% in Finland.²³

Iron overload is a well recognised cause of liver damage. Indeed, hepatic dysfunction is one of the most frequent findings in idiopathic haemochromatosis. Iron overload due to both haemochromatosis and multiple blood transfusion results in the saturation of transferrin and the appearance of non-transferrin bound iron in the serum.²⁴ Non-transferrin bound iron is toxic to living systems because it can act as a catalyst in the formation of highly reactive hydroxyl radicals, which in turn stimulate lipid peroxidation in membranes.²⁴ Iron bound to ferritin and transferrin is not reactive in this way.²⁵ It has been shown previously that in patients with leukaemia non-transferrin bound iron is only detected when transferrin is fully saturated, and that there is no significant association between serum ferritin and the presence of non-transferrin bound iron.¹⁰

Finding non-transferrin bound iron in four out of six patients with an unexplained increase in AST activity leads us to suggest that not only is iron overload responsible for the liver dysfunction, but that non-transferrin bound iron is a more specific indicator of this than serum ferritin. The latter was raised in most of our patients following treatment for haematological malignancy, whilst only 16% had unexplained increases in AST. The presence of non-transferrin bound iron in patient 5 (table 3) is difficult to explain, but may be a false positive result due to contamination. This patient, with a normal serum ferritin concentration, received relatively few red cell transfusions and has never had a raised AST activity.

Our findings support those of McKay *et al*,⁶ that iron overload has a causal role in liver dys-

function following BMT. We have also shown that this may apply to patients treated with standard chemotherapy for acute leukaemia. We also agree with McKay *et al*⁶ that the role of venesection in these patients needs assessing, and that iron overload should be considered in any patient with persistently abnormal liver blood tests following treatment for haematological malignancy. We are currently assessing the role of venesection in such patients. In a population of patients with raised ferritin concentrations, the bleomycin assay for non-transferrin bound iron may offer a relatively simple method of identifying patients with iron induced liver damage.

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