LIVER DISEASE

Denatured H-ferritin subunit is a major constituent of haemosiderin in the liver of patients with iron overload

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Background and aims: Iron is stored in hepatocytes in the form of ferritin and haemosiderin. There is a marked increase in iron rich haemosiderin in iron overloaded livers, and ferric iron in amounts exceeding the ferritin and haemosiderin binding capacity may promote free radical generation, causing cellular damage. The aim of this study was to characterise hepatic haemosiderin using four antibodies specific for either native or denatured H/L-ferritin subunits.
Methods: Ferritin and haemosiderin were prepared from the livers of three patients with post-transfusional iron overload. The assembled ferritin molecules were analysed by non-denaturing polyacrylamide gel electrophoresis (PAGE)-immunoblotting. Ferritin subunits in the haemosiderin fraction were assessed by denaturing sodium dodecyl sulphate (SDS)-PAGE-immunoblotting. Distribution of native and denatured ferritin subunits in hepatocytes was examined by immunogold electron microscopy.
Results: Non-denaturing PAGE-immunoblot analyses showed that the assembled liver ferritins were recognised by the antibodies for native ferritins and not by those for the denatured subunits. Both SDS-PAGE-immunoblot and immunogold electron microscopic analyses disclosed that haemosiderin of iron overloaded liver reacted predominantly to the monoclonal antibody for the denatured H-ferritin subunit, to a lesser degree to that for denatured L-ferritin, and very weakly, if any, with antibodies for native H-ferritin or L-ferritin.
Conclusions: These results suggest that in iron overloaded liver, haemosiderin consists predominantly of denatured H-ferritin subunits.

The liver is a major site for body iron storage, deposited as ferritin and haemosiderin.[1 2] In normal liver, ferritin accounts for most of the stored iron, and haemosiderin is present in trace amounts.[3–5] However, in iron overloaded liver, even when caused by genetic or secondary haemochromatosis, gross accumulation of ferritin and haemosiderin has been observed according to the progression of cellular iron overload.[5–8] It has been proposed that the function of ferritin is to sequester "free iron" in its large cavity and thus protect cells against oxygen radicals generated by Fenton type reactions.[9] On the other hand, it has been shown that the iron associated with haemosiderin is readily released and may subsequently cause parenchymal cell damage.[10] Previous studies have disclosed that haemosiderin consists of insoluble iron rich granules in which the iron moiety is structurally similar to that of ferritin iron cores and is mainly found as massive electron dense aggregates known as siderosomes.[11–13] Biochemical characterisation of haemosiderin isolated from iron overloaded rat or human liver suggests that it is derived from ferritin molecules whose shells have been digested by lysosomal enzymes.[14–17]

Ferritin consists of H-subunits and L-subunits which assemble to form a shell of 24 subunits with a cavity capable of storing up to 4500 Fe atoms as hydrous ferric oxide polymers.[18] The amino acid sequence of H-ferritin and L-ferritin chains have 55% identity, most of the substitutions being present on the outer surface, in the cavity, or on the hydrophobic channel sequences.[19 20] Analysis of recombinant homopolymers for the H-chain (rHF) and L-chain (rLF) disclosed that the H-subunit has an iron oxidation site which catalyses ferrous to ferric iron and shows faster initial rates of iron incorporation than L-ferritin.[21] In addition, L-ferritin was shown to be more stable under denaturation than the H-chain,[22] suggesting that degradation of H-ferritin may occur faster and more preferentially than that of L-ferritin during the haemosiderin formation process, a hypothesis not confirmed.

In the present study, we examined the biochemical-immunological properties of haemosiderin and ferritin components in human iron overloaded livers using four antibodies specific for either native or denatured H/L-ferritin subunits. We demonstrate that the haemosiderin in iron overloaded liver is mainly derived from denatured H-ferritin subunits.

MATERIALS AND METHODS

Patients

Three male patients (aged 26, 53, and 62 years) admitted to Sapporo Medical University Hospital were diagnosed by liver biopsy as having secondary haemochromatosis. All patients had been given frequent blood transfusions for anaemia, with haematological diseases including aplastic anaemia and two malignant lymphomas. Livers were obtained post mortem at the time of autopsy after informed consent from relatives. As a control, a histologically normal liver was obtained from a patient (aged 58 years) who had died of lung cancer with strict informed consent. No liver involvement or infiltration of malignant cells was observed histologically in any patient.

Antibodies

The murine monoclonal antibody (MoAb) LF03 for native human L-ferritin,[23] the MoAb rH02 for native H-ferritin,[24] the MoAb HS-59 for denatured H-ferritin,[25] and the murine

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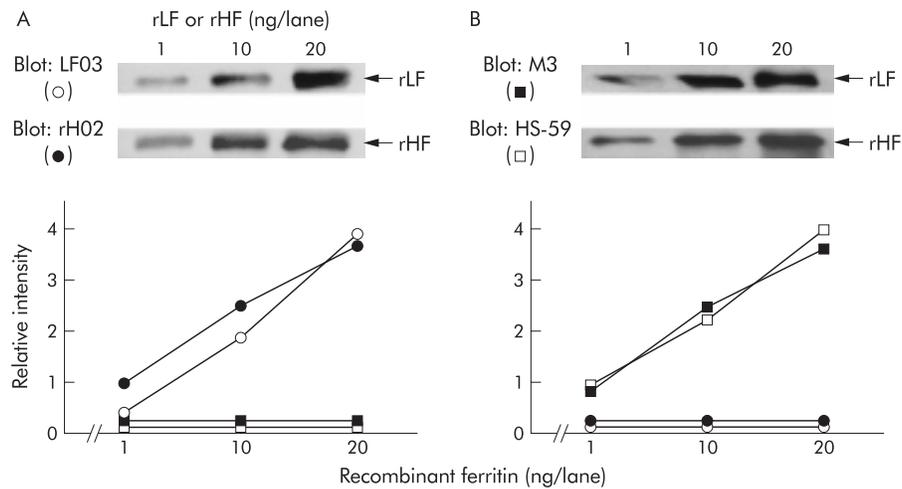
Figure 1   Analysis of the reactivity of antiferritin antibodies against native or denatured recombinant H-ferritin (rHF) and recombinant L-ferritin (rLF). (A) Different amounts of rHF or rLF (1, 10, and 20 ng/lane) were sodium dodecyl sulphate (SDS)-denatured and subjected to 4–20% SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were blotted on Immobilon-P membranes and incubated with LF03 or rH02 antibodies, followed by peroxidase labelled anti-Ig. (B) Same as (A) except that rHF and rLF were SDS denatured and subjected to 15–25% PAGE. The proteins were blotted and incubated with M3 or HS-59 antibodies. The bands were semiquantified by densitometric scanning, as described in materials and methods. The intensity of each band is represented as a ratio to the band of 1 ng of rHF.

polyclonal antibodies M3 for denatured L-ferritin[25] were used for both immunogold electron microscopy and immunoblot analyses.

Isolation of liver ferritin and haemosiderin
The liver ferritin and haemosiderin fractions were prepared as described previously.[26] Briefly, each liver sample (0.5 g) was homogenised in four volumes (w/v) of distilled water using a Potter homogeniser, followed by sonication at 100 W for three minutes. The homogenates were then incubated in a water bath at 80°C for 10 minutes and centrifuged for 15 minutes at 14 000 g. To evaluate the proportion of ferritin subunits, 10 μl of each supernatant containing a ferritin rich fraction was subjected to 4–20% polyacrylamide gradient gel electrophoresis (PAGE) or to 1% sodium dodecyl sulphate (SDS)-15–25% PAGE (SDS-PAGE) under reducing conditions. Gels were stained with Coomassie Brilliant Blue R-250 (CBB) and each ferritin band was semiquantified by scanning densitometry using NIH Image 1.61 software (National Institutes of Health, Bethesda, Maryland, USA). Purity of the ferritin protein in the supernatant (ferritin rich fraction) was 72–75% in all livers. To further enrich haemosiderin, precipitates were resuspended in 1 ml of 1 M NaCl and sonicated at 100 W for one minute. The suspensions were centrifuged for 10 minutes at 25 000 g, obtaining precipitates of three layers; the top and middle yellow-brown layers containing the haemosiderin fraction were transferred to another tube and resuspended in 1 ml of 1 M NaCl. The suspension was then centrifuged for 10 minutes at 25 000 g and the precipitates were successively treated with detergents: 1 ml of 4 M LiCl, 8 M urea, and 5 M guanidinium chloride. The final haemosiderin preparation was solubilised from the pellet with 0.2 ml Laemmli sample buffer containing 1% SDS.[27]

Immunoblot
Human recombinant homopolymers for L-ferritin (rLF) and H-ferritin (rHF)[21] were subjected to non-denaturing PAGE on 4–20% gradient gels or SDS-PAGE on 15–25% gradient gels and transferred onto an Immobilon-P membrane (Millipore) at 100 mA for one hour in Bjerrum and Sehafer-Nielsen transfer buffer[28] (48 mM Tris, 39 mM glycine, 20% methanol, 0.01% SDS, pH 9.2) using a semidry transfer apparatus (Bio-Rad Laboratories, Richmond, California, USA). To examine ferritin subunits in the ferritin rich and haemosiderin rich

fractions, each 5 μ of sample was heated at 100°C for five minutes in Laemmli sample buffer containing 1% SDS and 5% 2-mercaptoethanol, and subjected to SDS-PAGE-immunoblot as described above. Assembled ferritin in the liver was analysed by native PAGE-immunoblotting. Each 5 μ of the ferritin rich extract was diluted with Laemmli sample buffer without 2-mercaptoethanol (1:1) and separated on 4–20% PAGE. Proteins in the gel were transferred onto a nitrocellulose membrane (BA85, Schleicher & Schuell, Dassel, Germany) in 0.7% acetic acid using a transfer cell unit (Bio-Rad) according to the method of Towbin and colleagues.[29] The membranes were soaked in 5% non-fat dry milk phosphate buffered saline (PBS) for one hour at room temperature to block non-specific absorption sites. They were then probed with antiferritin antibodies, washed, and further incubated with peroxidase labelled antimouse Ig rabbit antibodies (1:2000). The antigen-antibody complex was visualised using the Vectastain ABC-kit (Vector Laboratories Inc., Burlingame, California, USA). Amounts of protein were semiquantified by scanning densitometry using NIH Image 1.61 software (National Institutes of Health).

Immunoelectron microscopic analysis
Liver samples not exceeding 1 mm³ in volume were fixed in 0.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M sodium phosphate buffer, pH 7.4, for one hour at room temperature. Fixed tissue samples were then washed three times in the same phosphate buffer and embedded in LR White acrylic resin (Polysciences Inc., Warrington, Pennsylvania, USA) using the method of Egea and colleagues.[30] Ultrathin sections (80 nm) mounted on carbon coated nickel grids were floated for 30 minutes on 0.5% bovine serum albumin (BSA)-Tris buffer (20 mM Tris-HCl (pH 7.4), 0.9% NaCl, 0.1% BSA), supplemented with 5% heat inactivated fetal calf serum (Gibco-BRL, Gaithesburg, Maryland, USA), and transferred to droplets of 0.1% BSA-Tris buffer containing each antiferritin antibody (at working concentrations of 1 μg/ml for the MoAbs and at 20 μg/ml for the M3 polyclonal antibodies). After one hour of incubation in a moist chamber at 37°C, sections were washed with 0.1% BSA-Tris buffer and incubated with 0.1% BSA-Tris buffer containing 0.05% Tween-20, 5% fetal calf serum, and a rabbit antimouse IgG coupled with colloidal gold particles (10 nm diameter, 1/10 dilution) (BioCell, Cardiff, UK). After one hour of incubation at room temperature,
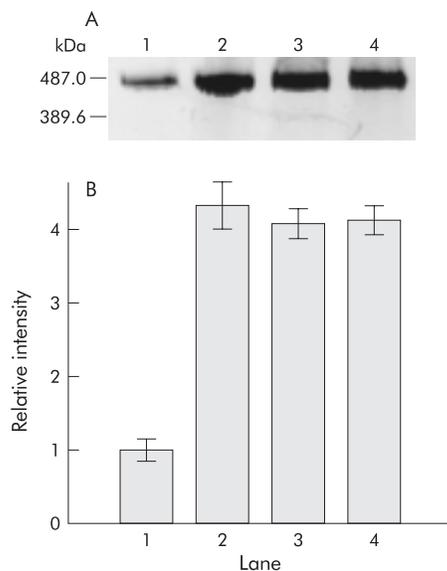
Figure 2 (A) Analysis of assembled ferritin in the liver extracts. Each 10 µl of ferritin enriched fractions obtained from the liver was loaded onto 4–20% non-denaturing polyacrylamide gel electrophoresis, as described in materials and methods. The proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, normal control liver; lanes 2–4, livers from patients (patient Nos 1–3) with iron overload. The protein bands were semiquantified (B) by densitometric scanning, as described in materials and methods. Values are given as mean (SD).

sections were washed with 0.1% BSA-Tris buffer and distilled water, air dried, and finally counterstained with lead citrate and examined on a Hitachi 7000 transmission electron microscope (Hitachi Kohki Co., Tokyo, Japan) at 725 kV. Controls were incubated without the first antibody. Quantitative analysis of micrographs was performed in a similar fashion, as described by Egea and colleagues.[30] In our study, the number of positively stained gold particles was measured per 0.25 µm^2 in the siderosome area (see fig 7 insets) using 10 pictures for each sample. Results are expressed as mean (SD).

Statistical analysis
Results are expressed as mean (SD). Statistical analyses were performed using one way ANOVA and values were considered significantly different at p<0.05.

RESULTS
Specificity of the antiferritin antibodies for rHF and rLF
To analyse the specificity of the four antibodies elicited by native and denatured H/L-ferritin, we used immunoblotting with different amounts of rHF and rLF (1, 10, and 20 ng/lane). Samples were either untreated and electrophoresed on non-denaturing PAGE or denatured by boiling in 1% SDS, electrophoresed on denaturing SDS-PAGE, and then overlaid
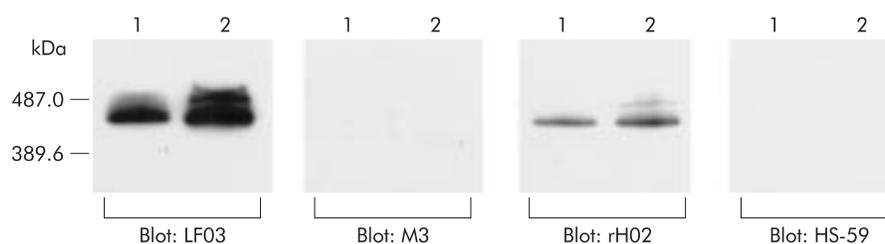

Figure 4 Analysis of ferritin subunits in the liver extracts. Ferritin enriched fractions were loaded onto 1% sodium dodecyl sulphate-15–25% polyacrylamide gel electrophoresis. The proteins were stained with Coomassie Brilliant Blue R-250. Lane M, molecular marker; lane 1, normal control liver; lanes 2–4, samples of livers from three patients with iron overload. H-ferritin (21 kDa) and L-ferritin (19 kDa) were detected (arrows). Faster migrating bands (~10 kD) were also stained (arrowhead).

with the four antibodies. The intensity of the bands obtained was semiquantified by densitometric analysis and plotted. HS-59 and M3 antibodies specifically recognised denatured rHF and rLF subunits, respectively, in a dose dependent fashion, and at working concentrations of 1 µg/ml for HS-59 and 20 µg/ml for M3 they showed an almost equal reactivity for the two antigens (fig 1B) while they did not recognise the native and assembled ferritins. Similarly, rH02 and LF03 antibodies, at working concentrations of 1 µg/ml, specifically recognised with similar intensity assembled rHF and rLF, respectively, in the native PAGE-immunoblot (fig 1A) while they did not produce detectable signals with the denatured subunits. These antibody concentrations were used in the following experiments.

Detection of assembled ferritin in iron overloaded and normal livers
To examine accumulation of the assembled ferritin in iron overloaded and normal livers, ferritin rich fractions were subjected to native PAGE followed by immunoblot analyses. As shown in fig 2, assembled ferritin was detected by CBB staining as a ~440 kDa band in all patient samples. The intensity of ferritin bands in the iron overloaded livers (lanes 2–4) was approximately four times higher (lane 2, patient No 1, 4.4-fold; lane 3, patient No 2, 4.1-fold; lane 4, patient No 3, 4.1-fold) than that in the control liver (lane 1). As each lane is representative of equal weights of tissue and extracts, the results indicate that there is an increased amount of assembled ferritin in iron overloaded livers. As shown in fig 3, non-denaturing PAGE-immunoblot analysis of ferritin fractions of a normal liver (lane 1) and of a patient's liver (patient No 1, lane 2) disclosed that the assembled ferritin reacted with both LF03 and rH02 antibodies, but not with M3 or HS-59, confirming the specificity of the antibodies.

Figure 3 Immunoblot of assembled ferritin in the liver extracts of a normal subject (lane 1) and of a patient with iron overload (patient No 1, lane 2). Ferritin enriched fractions were loaded onto 4–20% non-denaturing polyacrylamide gel electrophoresis and subjected to immunoblotting using the four antiferritin antibodies indicated.
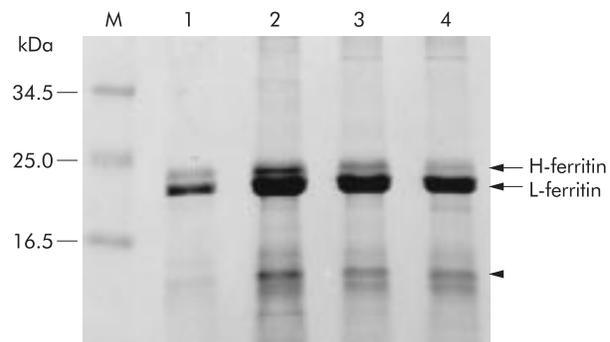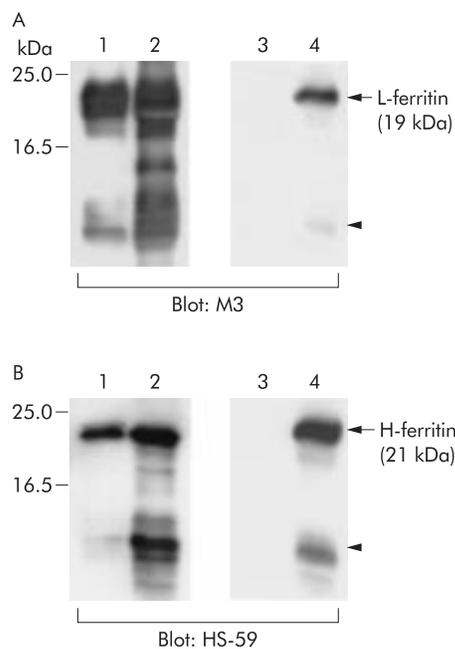
Figure 5 Immunoblot analysis of ferritin subunits in the ferritin rich fractions (lanes 1, 2) and haemosiderin fractions (lanes 3, 4) of a normal control liver (lanes 1, 3) and of a liver of an iron overloaded patient (patient No 1, lanes 2, 4). Ferritin enriched fractions and sodium dodecyl sulphate (SDS) extracted haemosiderin fractions were loaded onto 1% SDS-15–25% polyacrylamide gel electrophoresis. The proteins were blotted on Immobilon-P membranes and incubated with M3 (A) or HS-59 (B) antibodies.

Proportion of H/L-ferritin subunits in the ferritin rich fraction and haemosiderin from iron overloaded and normal liver

As it has been shown that each ferritin subunit (H and L) can be separated by SDS-PAGE under reducing conditions,[31] we next examined the molecular proportion of the two subunits in the ferritin rich fraction of the liver extracts. As shown in fig

4, H-subunits (21 kDa) and L-subunits (19 kDa) were found in all samples. The intensity of each band was compared with that of the H-subunit in the control liver (lane 1, upper band) and we obtained the following L:H subunit ratios: 4:1 in the control liver (lane 1), and 17.5:3.7 (lane 2), 16.4:1.7 (lane 3), and 14.6:1.2 (lane 4) in the three iron overloaded livers. This indicated that the relative proportion of L- to H-subunits was higher in the iron overloaded liver than in the control liver, which was consistent with previous reports demonstrating that L- and H- subunits in normal liver are present in approximately a 3–4:1 ratio and that iron loading causes an increase in the amount of L-subunits giving rise to a shift in the isoferritin distribution.[31 32]

The haemosiderin enriched fractions were solubilised by Laemmli sample buffer containing SDS and subjected to SDS-PAGE-immunoblot analyses using M3 and HS-59 antibodies (fig 5). The sample normal liver showed protein bands of about 20 kDa which however were not recognised by the antiferritin antibodies (lanes 1, 3) while both ferritin subunits were detected in the iron overloaded samples (lanes 2, 4). In addition to the 19–21 kDa bands, faster bands of ~10 kDa and some faint bands were observed, suggesting that the haemosiderin preparations contained partially degraded ferritin subunits. SDS-PAGE-immunoblot analysis confirmed that the ~10 kDa bands were recognised by both HS-59 and M3 antibodies, suggesting that in haemosiderin, the ferritin subunits are unfolded or certain epitopes buried in ferritin polymers were exposed. Furthermore, densitometry showed that the L-subunits and H-subunits were in a ratio of approximately 1:5, a reverse distribution compared with the 4:1 ratio found in the ferritin rich fractions (fig 4).

Ultrastructural features and distribution of native and denatured ferritin subunits in hepatocytes of normal and iron overloaded liver

To explore the distribution of native and denatured ferritins in hepatocytes of normal and iron overloaded livers, we immunohistochemically stained ultrathin sections with the four antibodies. In normal hepatocytes, LF03 gave a scattered pattern within the cytosol (fig 6A) while M3 (fig 6B), rH02 (fig 6C), and HS-59 (fig 6D) produced hardly any signal. In the iron overloaded liver, many massive aggregates of electron
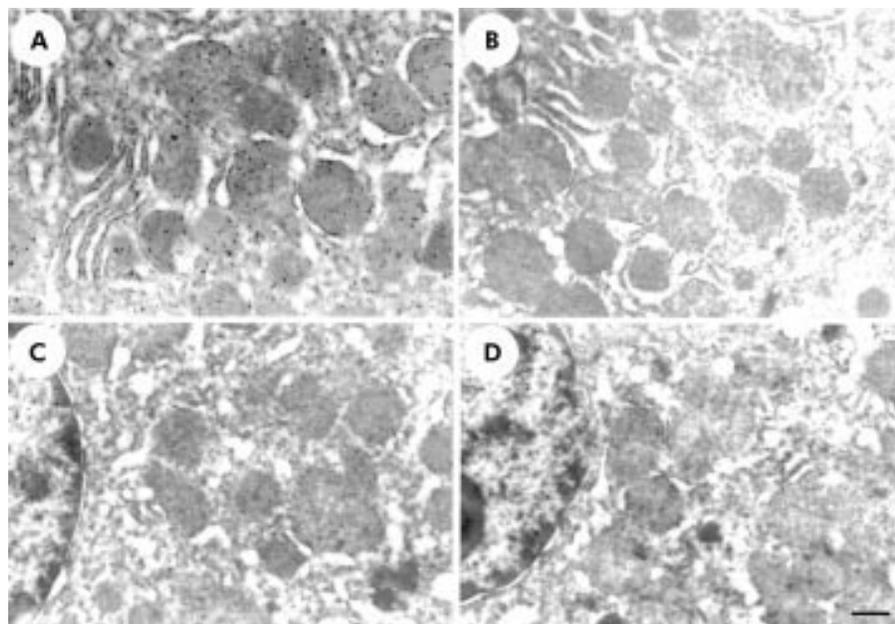

Figure 6 Ultrastructural features and distribution of ferritin subunits in hepatocytes of a normal liver. Ferritin subunits in the ultrathin sections were immunohistochemically stained using antiferritin antibodies LF03 (A), rH02 (B), M3 (C), and HS-59 (D), followed by addition of a rabbit antimouse IgG coupled with colloidal gold particles. Bar represents 0.5 μm.
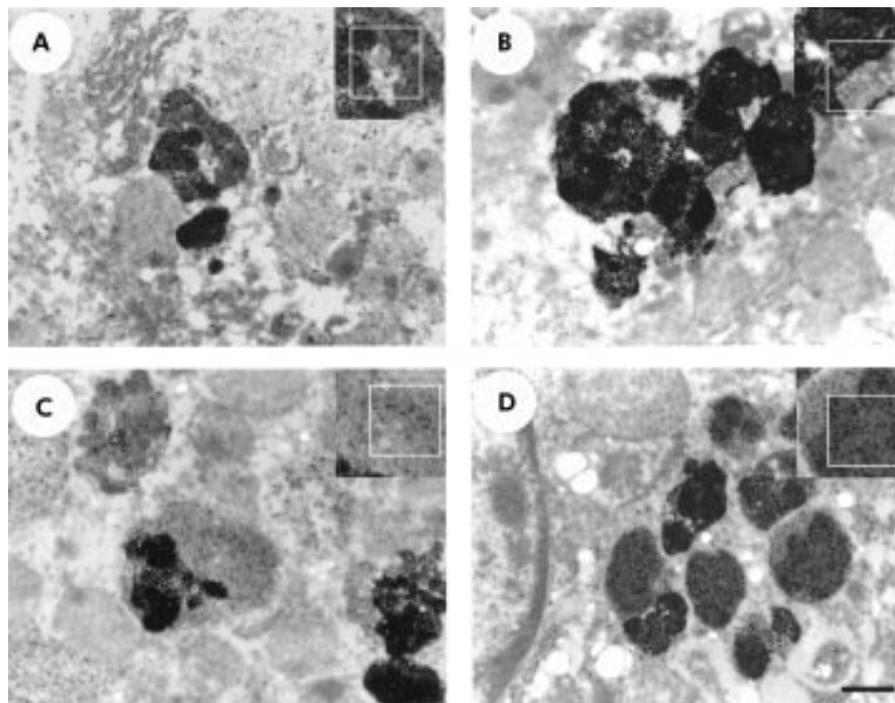
Figure 7 Same analysis as in fig 6 but in an iron overloaded liver. Ferritin subunits in the ultrathin sections were immunohistochemically stained using antiferritin antibodies LF03 (A), rHO2 (B), M3 (C), and HS-59 (D), followed by addition of a rabbit antimouse IgG coupled with colloidal gold particles. Bar represents 0.5 µm. The insets show an example of the 0.5 µm square in the siderosome area used to count the number of positively stained gold particles.

dense particles (2–5 μm in diameter), considered siderosomes, were observed in all patient livers, and the LF03 antibody showed more pronounced staining than in normal liver with a distribution within the entire cytosol but not within siderosomes (fig 7A). Few native gold granules were produced in the cytosol by rHO2 (fig 7B) or normal mouse Ig (data not shown). In contrast, both M3 and HS-59 antibodies produced heavy decoration in the cytosol, particularly concentrated within the siderosome area (fig 7C, D). Counting of gold grain density within the siderosome provided a semiquantitative analysis of antibody responses, and showed that the HS-59 and M3 antibodies for denatured H- and L-subunits,

respectively, gave signals approximately 29-fold and threefold higher than those of the antibodies for the corresponding native ferritins (p<0.01) rHO2 and LF03, respectively (fig 8). This indicates that in siderosomes, the denatured ferritin subunits are largely predominant over the native ferritins.

DISCUSSION
It has been proposed that in severe iron overload, the iron exceeding the iron binding capacity of haemosiderin and ferritin may promote free radical generation to cause lipid peroxidation, DNA breakage, and 8-hydroxy-2'-deoxyguanosine formation, resulting in hepatic injury, hepatic fibrosis, and carcinogenesis.[7 8 33] However, the biochemical nature of the haemosiderin and its relationship with ferritin in iron overloaded liver have not been fully elucidated. The present study shows that haemosiderin contains ferritin subunits mainly in a denatured conformation, and that the H-subunit is predominant over the L-subunit.

Ferritin rich and haemosiderin rich fractions were isolated form iron overloaded livers and analysed with antibodies which specifically recognise H- or L-ferritin subunits in the assembled and denatured conformation. The analyses showed that ferritin was more than fourfold more abundant in the iron overloaded than in the normal liver, and that it was richer (L:H=5:1 to 12:1) in L-subunits (fig 4). The variation in L- and H-subunit ratios among the iron overloaded liver samples might be due to differences in disease progression or in some other individual variation. In contrast, in the haemosiderin rich fraction, samples obtained from iron overloaded livers, the denatured H-subunit was predominant over the L-subunit (fig 5). This indicates that in iron overloaded livers, expression of H- and L-ferritins is higher than in normal liver, as expected, since the translation of the two mRNAs is controlled by common cytosolic proteins, the iron regulatory proteins, which bind to the iron responsive elements (IREs) in the 5' untranslated region of the two transcripts. Expansion of the intracellular iron pool decreases IRE binding activity and
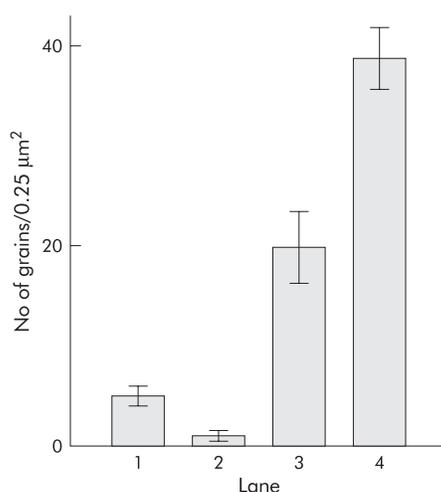

Figure 8 The number of positively stained ferritin particles in the siderosome area in fig 7. The gold particles per 0.25 µm^2 in the siderosome area were randomly counted using 10 pictures for each sample. Results are expressed as mean (SD). Lane 1, native L-ferritin (LF03); lane 2, native H-ferritin (rHO2); lane 3, denatured L-ferritin (M3); lane 4, denatured H-ferritin (HS-59).

allows translation of pre-existing ferritin mRNAs to proceed.[34] However, the two subunits accumulate in different proportions in the cytosol (L:H ~4:1) and in the haemosiderin fraction (L:H ~1:5), suggesting that they undergo different post translational processes. It was previously indicated that in rat liver, the H rich ferritins have a faster turnover than the L rich ferritins[32] and the L:H ratio is higher at the level of the accumulated protein than at that of the corresponding mRNAs despite a similar translation rate of the two transcripts.[35] In addition, L-ferritin is more stable under denaturation in vitro than H-ferritin.[22] This may suggest a preferential degradation of the H rich ferritins, which is thought to occur inside the lysosome.[36] Previous and present data indicate that haemosiderin is a byproduct of the lysosomal degradation of ferritin, and the finding that haemosiderin contains a high proportion of H subunits supports the hypothesis of a preferential lysosomal degradation of the H rich ferritin. This may imply a ferritin lysosomal translocation system with a higher affinity for the H subunit, at least in iron overloaded livers. Although there is no direct evidence for such a system as the ferritin degradation pathway is rather obscure, it may be noted that a specific receptor for the ferritin H chain has been discovered on the cell membrane.[37] This raises the possibility that the same or similar proteins may be involved in the lysosomal ferritin uptake for ferritin degradation and haemosiderin formation. The concept of preferential lysosomal uptake for the H rich ferritin in liver is consistent with earlier evidence which indicated that iron overload is invariably accompanied by enrichment in L rich ferritins.[36] That the ferritin subunits in haemosiderin are degraded is indicated not only by the specific recognition of the antidenatured subunit antibodies but also by the finding of immunoreactive peptides of about 10 kDa. This observation is consistent with a report in which partially degraded ferritin was found in iron overloaded liver by SDS-PAGE analysis, suggesting that the ferritin protein has a cleavage site susceptible to lysosomal enzymatic digestion.[38]

In immunohistochemical studies using electron microscopy, heavy staining in the siderosome area indicated a reaction between the HS-59 antibody and H subunit epitopes which are hidden in the assembled ferritin, in agreement with the hypothesis of local protein degradation and unfolding. Our finding is partially consistent with a report by Ruggeri et al in which HS-59 demonstrated specific immunohistochemical staining of bone marrow macrophages, but not of erythroblasts, suggesting that denatured H-ferritin may exist in the macrophages of haemosiderin, although this point was not mentioned.[25] The relative amounts of denatured H- and L-ferritin in haemosiderin, as determined by immunocytochemical analysis, were approximately 2:1 (figs 7, 8), which is slightly different from the results of immunoblotting (H:L=5:1, fig 5). This might be due to differences in antibody reactivity in immunoblotting and tissue sections. Nevertheless, it was evident that with both methods, denatured H-ferritin was predominant in haemosiderin.

The present data do not provide evidence as to whether assembled ferritin molecules accumulate in the haemosiderin fraction as it could not be analysed by native PAGE. However, it has been demonstrated that in secondary and genetic haemochromatotic livers, haemosiderin contains iron cores enveloped in ferritin like structures,[9 10] indicating that the denatured H- and L-ferritin subunits may partially co-assemble in the siderosome. The precise mechanisms by which ferritin is transferred to the siderosome and denatured ferritin subunits undergo co-assembly still remain unclear. Furthermore, it is not known whether similar mechanisms in the haemosiderin formation process are involved in cases of genetic haemochromatosis as it is not currently possible to investigate this.

In conclusion, our results show that in iron overloaded liver, haemosiderin consists mainly of denatured H-ferritin subunits. It is unclear whether these subunits retain the ferro-oxidase activity which may contribute to iron toxicity.

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