Hepatic stellate cell activation in genetic haemochromatosis

Lobular distribution, effect of increasing hepatic iron and response to phlebotomy

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Background/Aims: Activated hepatic stellate cells produce increased levels of collagen in animal models of chronic iron overload; however, their role in human genetic haemochromatosis is unknown. This study examined the relationship between hepatic iron concentration and hepatic stellate cell activation in genetic haemochromatosis.

Methods: Liver biopsies from 75 patients (55 with haemochromatosis, 14 haemochromatosis patients both pre- and post-phlebotomy and six non iron-loaded disease control subjects) were stained for iron using Perls' Prussian Blue. Thirty biopsies in which there was no evidence of either steatosis or inflammation were subjected to immunohistochemistry for a-smooth muscle actin and desmin and counterstained for iron. Forty-five biopsies demonstrated either steatosis or inflammation, in addition to excess iron.

Results: Stellate cells were identified by light microscopy as perisinusoidal cells containing numerous intracellular fat droplets. a-Smooth muscle actin was detected in biopsies with an hepatic iron concentration >60 μmol/g dry weight. Increasing hepatic iron concentration and hepatic iron index correlated with an increase in a-smooth muscle actin expression (r=0.81 and 0.72, respectively). Phlebotomy resulted in a significant decrease in a-smooth muscle actin expression. In early disease prior to histological evidence of collagen deposition, whilst activated stellate cells were located in Zone 1, greater numbers were found in Zones 2 and 3 distal to the region of heaviest iron overload.

Conclusions: This study has demonstrated for the first time in humans a correlation between hepatic iron concentration and stellate cell activation in haemochromatosis, which is reversed by iron removal. Humoral factors from either iron-loaded hepatocytes or activated Kupffer cells may be responsible for early stellate cell activation in areas of the liver remote from heavy iron loading.

Key words: Fat-storing cell; Hemochromatosis; Hepatic fibrosis; Iron; Ito cell; Lipocyte; Liver.
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Hepatic stellate cells, which are also known as lipocytes, Ito cells, or fat-storing cells, have been proven to be the principal collagen-producing cells in pathological conditions of the liver (68). These cells are located in the perisinusoidal space of Disse, beneath the endothelial cells and in close contact with hepatocytes (9). HSC have traditionally been characterized as non-proliferative, quiescent cells that express desmin, store vitamin A and are capable of synthesizing small amounts of type III collagen. In experimental models of liver injury, HSC are transformed into a highly proliferative, "myofibroblast-like" cell (reviewed in 7). This transformation process, termed "HSC activation", is associated with a reduction of intracellular vitamin A stores, increased production of type I collagen and the expression of α-smooth muscle actin (α-SMA) microfilaments (7).

HSC activation has been demonstrated in a variety of different models of liver injury including alcohol toxicity (10,11), iron overload (12,13), carbon tetrachloride (CCI₄)-induced necrosis (8,14-16) and bile duct ligation (8,10,12,17). Recent investigations using an animal model of iron overload have demonstrated that chronic iron administration results in HSC activation in vivo (12,13). Ramm and colleagues demonstrated that HSC isolated from carbonyl iron-loaded rats express increased levels of the HSC activation marker, α-SMA (12). Furthermore, these cells produce increased levels of collagen and non-collagen protein as compared to HSC isolated from control rats (12).

Although these recent studies implicate activated HSC in the production of increased levels of collagen in an animal model of chronic iron overload, the role of HSC in the development of hepatic fibrogenesis in humans with haemochromatosis is unknown. Therefore, the aim of this study was to examine the relationship between increasing HIC and HSC activation, as determined by the expression of the HSC activation marker, α-SMA, in human subjects with genetic haemochromatosis.

Methods

Patients

Liver biopsies were obtained from 75 patients (56 males, 19 females) with a suspected diagnosis of genetic haemochromatosis. The mean age of patients was 42±14 years (males 41±13, females 46±19). Haemochromatosis was suspected in patients with a serum ferritin concentration >250 μg/l for males, or >200 μg/l for females and/or a persistently elevated serum transferrin saturation (i.e. >50%). The diagnosis of haemochromatosis was confirmed by the histological or chemical demonstration of increased hepatic iron in the absence of other causes and an hepatic iron index (HII) >2.0 (5).

Fourteen of the 75 patients had liver biopsies performed both before and after phlebotomy-treatment for their disease. Of these 14 pairs, eight demonstrated significant steatosis or portal inflammation. In the remaining six biopsy pairs, the only abnormality observed was excess iron and therefore these biopsies were examined for the expression of α-SMA.

Control subjects

Six of the 75 patients, while demonstrating increased serum ferritin and/or a persistently elevated serum transferrin saturation, did not show increased hepatic iron and were therefore used as control subjects for this study. The protocols used in this investigation were approved by the Ethics Committees of the Royal Brisbane Hospital and The Queensland Institute of Medical Research and informed consent was obtained from each patient.

Light microscopic examination of liver biopsies

Liver biopsies were divided and used for both routine histological examination and the determination of HIC. For histology, sections were fixed in 10% buffered formalin and embedded in paraffin. Thin sections were cut and stained with haematoxylin and eosin and for the presence of iron, collagen and reticulin, using Perls' Prussian Blue, haematoxylin van Gieson or Masson's trichrome and reticulin stains, respectively.

Determination of hepatic iron concentration

Small sections of the liver biopsies were wrapped in aluminium foil and placed in a drying oven for 3 days at 110°C. The HIC was determined by atomic absorption spectrometry following wet ashing of specimens as previously described (18).

Immunohistochemistry

Immunohistochemistry was performed on sections of formalin-fixed, paraffin-embedded liver using mono-
clonal antibodies to the HSC marker, desmin and to the HSC activation marker, α-SMA. This procedure was performed using a Dako StreptAB Complex/horseradish peroxidase kit (Dako, Santa Barbara, CA), with either a mouse monoclonal anti-desmin (1:50, D33; Dako, Santa Barbara, CA), or an anti-α-SMA primary antibody (1:400, clone 1A4; Sigma Chemical Co., St. Louis, MO). The secondary antibody was a biotinylated rabbit anti-mouse immunoglobulin complex (1:400; Dako, Santa Barbara, CA) and the chromogenic substrate was diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co., St. Louis, MO).

The number, size and staining intensity of α-SMA-positive cells were scored semi-quantitatively, from 0 to 3+, using a Video Pro 32 Image analysis operating software package for computerised morphometric analysis (Faulding Instruments, Sydney, Australia). Immunohistochemistry for α-SMA was quantitated and represented as a percentage of the field of view (×200 magnification) between the portal tract and central vein region of the acinus, at two different sites on the same biopsy. These analyses were carried out by two investigators working independently of each other (G.A.R., N.I.W.) and without prior knowledge of the hepatic iron concentration of each biopsy. The sections in which the morphometric score for immunohistochemical analysis was (a) from 0 to 0.3%, were graded 0; (b) from 0.31% to 1.1%, were graded 1+; (c) from 1.2% to 2.6%, were graded 2+; and (d) >2.7%, were graded 3+.

Counterstaining techniques
In non-counterstained sections, iron-loaded cells will appear brown, as do HSC with DAB-pigmentation. Therefore, following immunohistochemistry for α-SMA or desmin, liver sections were counterstained with Perls’ Prussian Blue to demonstrate intracellular iron and eosin to define cellular outline. This counterstaining technique clearly distinguished α-SMA-positive HSC from iron-laden hepatocytes and Kupffer cells.

### Table 1

<table>
<thead>
<tr>
<th>Biochemical data</th>
<th>α-Smooth muscle actin expression (Immunohistochemical grading)</th>
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<tbody>
<tr>
<td></td>
<td>0 (n=6)</td>
</tr>
<tr>
<td>SF (μg/l)</td>
<td>333±77</td>
</tr>
<tr>
<td>TS (%)</td>
<td>55±17</td>
</tr>
<tr>
<td>HIC (μmol/g)</td>
<td>29±5</td>
</tr>
<tr>
<td>HII</td>
<td>1.1±0.3</td>
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SF, TS, HIC and HII denote serum ferritin, transferrin saturation, hepatic iron concentration and hepatic iron index, respectively. Results are expressed as the mean±SEM. Patients with α-SMA negative immunohistochemistry constitute the six non iron-loaded human disease control livers. There was a significant relationship between increasing grades of α-SMA expression and both the HIC and HII (ANOVA, p <0.0001).

**Mathematical and statistical analysis of data**
The correlation between increasing HIC and the intensity of α-SMA immunostaining was determined by linear regression. All other results are expressed as the mean±standard error of the mean and the statistical difference between groups was determined using ANOVA and Student’s t-test.

**Results**

**Biochemical data**
The biochemical data of patients included in this study including the serum ferritin concentration, serum transferrin saturation, HIC and HII are demonstrated in Table 1. The liver function tests of pre- and post-phlebotomy patients showed a mild elevation in alanine aminotransferase levels (40–60 U/l) prior to phlebotomy therapy, which decreased to normal following therapy. There was no elevation in either aspartate aminotransferase or γ-glutamyl transferase levels in these subjects.

**Immunohistochemical detection of α-SMA expression**
Immunohistochemistry for α-SMA was quantitated by computerised morphometry and represented as a percentage of the field of view (×200 magnification). The results for grades 0, 1+, 2+ and 3+, represented as the

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Fig. 1. Immunohistochemical detection of α-SMA in liver biopsies from haemochromatosis patients. Biopsies were counterstained with Perls’ Prussian Blue to demonstrate intracellular iron and eosin to delineate cell outline. (a), (b), (c) and (d) demonstrate representative liver sections stained for α-SMA and scored as 0, or as grades 1+, 2+ and 3+, respectively. Activated HSC were rarely seen in the hepatic acinus of control livers. The number and staining intensity of α-SMA-positive HSC increased with an increase in the hepatic iron content of liver sections, as demonstrated by Perls’ Prussian Blue (original magnification, ×200).
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mean±standard error, were 0.17±0.03% (range, 0–0.3%); 0.78±0.14% (range, 0.31%–1.1%); 1.47±0.15% (range, 1.2%–2.6%); and 3.55±0.31% (range, >2.7%), respectively.

α-SMA-positive cells were rarely seen in normal human liver tissue, except for smooth muscle cells lining portal vessel walls and an occasional myofibroblast within the hepatic acinus (Fig.1a). In haemochromatosis subjects the number and staining intensity of α-SMA-positive cells in hepatic acini increased as the HIC increased (Fig.1b-d). Using the semi-quantitative scoring system for α-SMA staining, the HIC of biopsies with an α-SMA grading of 0, 1+, 2+ and 3+ is shown in Table 1.

There was a significant correlation between α-SMA staining intensity and HIC (r=0.81, p<0.0001) (Fig. 2a). The results presented in Fig. 2a indicate that HSC activation (as demonstrated by the expression of α-SMA) occurs in liver tissue with an HIC as low as 60 μmol/g dry weight, in the absence of co-existent inflammation and steatosis. Fig. 2 demonstrates a correlation between the HII and α-SMA expression (r=0.72, p<0.0001).

Table 1 also demonstrates the relationship between increasing serum ferritin concentration, transferrin saturation and α-SMA expression, which supports the observations of a positive correlation between HIC, HII and α-SMA expression.

**Immunohistochemical detection of desmin expression**

Desmin expression was not detected in human liver sections. This was despite the use of a reliable monoclonal antibody, i.e. Dako D33 anti-desmin antibody, and the use of appropriate positive controls. These observations support previously published findings which demonstrate that HSC from either normal or pathological human liver may not express desmin (19,20).

**Zonal distribution of α-SMA expression**

This study has utilised a staining approach designed to distinguish activated HSC from iron-laden hepatocytes and Kupffer cells in the same section of human liver tissue. Using this technique, activated HSC were observed in areas distal from heavily iron-loaded cells. Fig. 1b demonstrates the zonal distribution of HSC activation in early iron overload, i.e. in Rappaport...
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Fig. 4. Demonstration of HSC activation in (a) pre- and (b) post-phlebotomy liver biopsies, from a representative patient with haemochromatosis complicated by hepatic fibrosis. Liver biopsies were obtained from a haemochromatosis patient prior to and following phlebotomy-treatment for excess iron overload. (a) Increased numbers of activated HSC were demonstrated throughout the acinus, along with heavily iron-loaded hepatocytes and Kupffer cells. (b) Following phlebotomy, only a small amount of storage iron was present in Kupffer cells and hepatocytes and α-SMA expression was markedly decreased (original magnification, ×200).

Zone 3. With an increase in HIC, activated HSC are localised in Rappaport Zones 2 and 3 (Fig. 1c).

Fig. 1d demonstrates a representative liver section stained for both α-SMA and iron. The HIC of this liver section was 238 μmol/g dry weight and the level of α-SMA expression was grade 3+. Most of the iron stain was concentrated in a periportal distribution (Rappaport Zone 1), with some hepatocellular iron present in Rappaport Zone 2. Although some cells demonstrated α-SMA expression in Zones 1, the majority of activated HSC were found in Zones 2 and 3. These observations were made in liver tissue which demonstrated no evidence of periportal fibrosis.

Morphological identification of hepatic stellate cells
Due to the absence of a suitable marker to distinguish between activated HSC and portal or lobular myofibroblasts, all biopsies used in the histological examination of α-SMA expression were examined morphologically for the presence of intracellular fat droplets. Fig. 3 shows a representative biopsy with grade 3+ α-SMA expression and grade 2+ iron. The perisinusoidal location of these α-SMA-positive fat-storing cells indicates that these cells are HSC.

Evidence of collagen deposition
All biopsies used in the correlation study of α-SMA expression and HIC were examined histochemically for collagen using the Masson's trichrome stain. This examination revealed minor focal subsinusoidal collagen deposition within the acinus, but such staining occurred in few biopsies, was not uniform in affected biopsies and was not recognisably zonal in distribution. There was no significant difference in the appearance of collagen deposition between those biopsies which were α-SMA-positive and those which were α-SMA-negative (results not shown).
Effect of phlebotomy on hepatic stellate cell activation

Of the 14 pairs of pre- and post-phlebotomy haemochromatotic liver biopsies, eight demonstrated significant steatosis or portal inflammation. In the remaining six biopsy pairs, the only abnormality observed was excess iron. These biopsies were examined for the expression of α-SMA and counterstained for iron. Fig. 4 demonstrates representative biopsies from a patient with a pre-phlebotomy HIC of 193 μmol/g dry weight, Perls' stain of grade 3+ and α-SMA stain grade 2+. This biopsy demonstrated morphological evidence of periportal fibrosis and α-SMA expression was localised to Rappaport Zones 2 and 3. In addition, activated HSC were found in Rappaport Zone 1 (Fig. 4a). Following removal of the majority of the storage iron (as evidenced by a decrease in Perls' stain to grade 2+), the liver showed a marked decrease in the number of activated HSC and a marked decrease in the staining intensity of α-SMA within the remaining cells (α-SMA stain grade 0) (Fig. 4b).

Discussion

This study has demonstrated for the first time in humans, a positive correlation between HIC and the number of activated HSC in patients with pre-fibrotic haemochromatosis, in the absence of histological evidence of inflammation or steatosis, and that following phlebotomy, the number of activated HSC was markedly decreased. HSC activation occurred at an HIC as low as 60 μmol/g dry weight. This investigation has also shown that in pre-fibrotic liver tissue obtained from patients with haemochromatosis, whilst activated HSC are located in Rappaport Zone 1, greater numbers are found in Rappaport Zones 2 and 3, distal to the areas of heaviest iron overload.

An improved understanding of iron-related HSC biology is essential to the understanding of the pathophysiological mechanisms involved in hepatic fibrogenesis in haemochromatosis. Previous studies have provided evidence of HSC activation and increased HSC collagen production in an animal model of iron overload (12,13). The present study has demonstrated the presence of activated HSC in human subjects with haemochromatosis. Of particular interest was the observation that HSC activation occurs at an HIC as low as 60 μmol/g dry weight. These results are consistent with the observations of Ramm and colleagues in a rat model of iron overload (12), which suggest that HSC activation can occur at relatively low levels of hepatic iron, in the absence of histological fibrosis. It is of interest that many human studies have shown that fibrosis in haemochromatosis occurs when the HIC is increased to levels of 300–400 μmol/g dry weight (5,21).

The results of the present study suggest that in haemochromatosis, a considerable latent period exists between HSC activation (as determined by α-SMA expression) and the development of hepatic fibrosis.

A number of investigators have suggested that Kupffer cells play a central role in the fibrogenic process in haemochromatosis. It has been proposed that fibrosis occurs after the accumulation of iron in Kupffer cells, following the phagocytosis of necrotic or damaged iron-loaded hepatocytes (22,24), in a process termed sideronecrosis (25). The present study has shown that α-SMA expression is detected at an HIC well below the level at which Kupffer cell iron-loading or sideronecrosis is usually apparent (25). This suggests that in conditions of iron overload, HSC activation may occur independently of Kupffer cell iron-loading.

It is established that removal of excess storage iron can either halt or reverse hepatic fibrogenesis and result in normal life expectancy (26,27). Our study has provided an explanation for the cellular basis of this clinical observation as phlebotomy resulted in a decrease in the number of activated HSC pari passu with the decrease in hepatocyte iron.

An important new observation in our study was that early in the disease process, prior to histological evidence of collagen deposition, increased numbers of activated HSC were located in areas of the hepatic acinus where iron deposition is minimal. This is an intriguing, novel observation, particularly when it might be expected that HSC activation would be confined to the area of maximum iron accumulation and subsequent collagen deposition, i.e. in the periportal region of the acinus. However, several authors have observed greater activation of HSC in Zone 3 in other liver diseases. A recent study by Guido and colleagues (28) demonstrated the presence of large numbers of α-SMA-positive HSC in apparently normal perivenular hepatic tissue (Zone 3) obtained from patients with untreated chronic viral hepatitis, i.e. in areas removed from the periportal fibrotic lesions and necro-inflammation (Zone 1). Others have found increased numbers of activated HSC in Zone 3 in experimental (29,30) and human (31) liver diseases. There are several possible explanations for this observation of zonal disparity. Cytokines produced in the periportal areas by activated Kupffer cells or iron-loaded hepatocytes may diffuse down the sinusoid and activate HSC in the centrilobular zone. Activated Kupffer cells are known to secrete transforming growth factors-α and -β (TGF-α, TGF-β) and tumor necrosis factor-α. TGF-β is known to act as a profibrogenic cytokine, while other cytokines such as TGF-α, insulin-like growth factor-1, platelet derived growth factor and interleukin-1 cause increased HSC proliferation (32).
A second possible explanation for this down-stream activation may involve by-products of membrane lipid peroxidation. There is substantial evidence demonstrating that chronic iron overload causes hepatic lipid peroxidation (33–36), which results in elevated levels of malondialdehyde (MDA) (33,35,36) and 4-hydroxynonenal in the liver (34) and the plasma (35). Although the evidence supporting a role for these lipid peroxidation products in the development of hepatic fibrosis is inconclusive, some groups have demonstrated that the antioxidant vitamin E has cytoprotective effects in iron-induced (37) and CCl4-induced (38,39) hepatic fibrosis. Parola and colleagues suggest that these cytoprotective effects may be due to an inhibitory effect of vitamin E on hepatic lipid peroxidation (38,39). A recent study has shown that MDA treatment of quiescent HSC grown on Matrigel®, results in increased HSC proliferation and expression of α-SMA (40). This study also showed that HSC activation by culture on plastic, collagen type I matrix or TGF-α, was blocked by vitamin E and that induction of c-myb and NF-κB appear to be key events in the oxidative stress-induced activation of HSC.

Whilst only a minority of the haemochromatotic liver sections used in our study were fibrotic, the results clearly demonstrate an apparent temporal dissociation of these two markers (α-SMA and collagen production) of the HSC activation process. The reasons for this are unclear; however, one possible explanation may be provided by the reported zonal heterogeneity of both hepatocyte (reviewed in 41,42) and HSC (43–45) function. A number of groups have shown that centrilobular HSC store less vitamin A compared to peri-portal HSC (43–46). Vitamin A is thought to suppress HSC activation (46), thus it is possible that the centrilobular HSC are more sensitive to activating stimuli and therefore may activate in earlier stages of the disease process than do periportal HSC. It is also possible that hepatocytes or Kupffer cells in the centrilobular region, in the presence of the lower oxygen tension which exists in Rappaport Zone 3, may have different metabolic functions with respect to cellular iron processing (i.e. uptake or metabolism of non-transferrin-bound iron, transferrin or ferritin) or cytokine function and the paracrine interaction of these cell types may be different to those in the periportal region.

In summary, this study has demonstrated a positive correlation between increasing HIC and HSC activation in patients with haemochromatosis in the absence of inflammation or steatosis. Furthermore, when iron was removed from the liver by phlebotomy, the number of activated HSC was markedly decreased. In addition, this study has shown that in liver tissue from patients with pre-fibrotic haemochromatosis, activated HSC are located in areas of the hepatic acinus distal to heavily iron-loaded hepatocytes and Kupffer cells. These observations demonstrate that α-SMA expression is an early response to chronic iron overload and that a latent period exists between α-SMA expression and the subsequent production of collagen in iron overload.

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