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Hepatocyte apoptosis in hepatic iron overload diseases

M. Zhao, J.A. Laissue and A. Zimmermann
Institute of Pathology of the University, Berne, Switzerland

Summary. In this retrospective study, we systematically analyzed hepatocyte apoptosis in three situations of hepatic iron overload (hereditary hemochromatosis; hepatic iron overload of unknown reason; iron overload due to hyperhemolysis or exogenous iron administration). Apoptosis was assessed by use of DNA nick end-labelling. The results suggest that hepatic iron overload is associated with an increased apoptotic rate of hepatocytes, and that iron-laden hepatocytes in hemochromatosis behave, with respect to apoptosis, differently from those in other states of iron overload. The hepatocyte apoptotic rate tended to increase as a function of the degree of iron storage. As in other pathological liver changes studied so far, an elevated apoptotic rate of hepatocytes predominated in the pericentral parts of liver acini in hemochromatosis, but not in the two other groups of hepatic iron overload. Possible mechanisms for this difference are discussed, particularly with respect to a participation of the Kupffer cell system.

Key words: Hemochromatosis, Apoptosis

Introduction

Chronic iron overload to the liver caused by genetic defects of iron metabolism or due to other disorders is associated with liver disease, frequently ending up with hepatic fibrosis and cirrhosis (Deugnier et al., 1992). Hepatocyte injury and the production of liver fibrosis seem to be directly related to the iron content of parenchymal cells and, therefore, the pattern of damage is similar, irrespective of the etiology of iron overload (Sherlock and Dooley, 1993). Mechanisms operational in hepatocyte damage and, finally, hepatocyte death are currently thought to involve alterations of cellular membranes and organelles, including mitochondria and lysosomes (Bacon and Britton, 1990; Myers et al., 1991). Most probably, free radical-mediated processes play a significant role. Iron can induce free radicals in vitro (Halliwell and Gutteridge, 1985), and in particular nucleotide complexes of iron (Tien et al., 1981) and circulating low-molecular weight iron complexes (Gutteridge et al., 1985) seem to be initiators of lipid peroxidation and promotors of hydroxyl radical formation.

Apart from non-specific cell breakdown, these mechanisms may theoretically induce programmed cell death or apoptosis. Whereas simple necrosis is a degenerative phenomenon induced by several environmental or toxic insults, apoptosis is an active suicidal response to various stimuli, involving specific gene activation and protein synthesis (Martin, 1993). In the liver, apoptosis is characterized morphologically by shrinkage of hepatocytes and of biliary epithelial cells, with condensation and increased eosinophilia of cytoplasm, occurrence of nuclear fragments, and formation of so-called apoptotic bodies. In human livers, eosinophilic apoptotic bodies are usually located in the acinar zone close to the terminal hepatic venules (Benedetti et al., 1988). In states of chronic iron overload to the liver, such apoptotic bodies can be visualized by conventional light microscopy, and they may contain iron-positive pigment granules. However, the extent of apoptosis in these situations, and the eventual role of apoptosis in iron-induced liver damage cannot easily be assessed without specific methods, because programmed cell death does not show up with hepatic apoptotic body formation in all instances (Nawaz and Fennell, 1994; Afford et al., 1995).

The classical morphological features associated with apoptosis only occur during advanced stages of the process, and the half-life of apoptotic cells in tissues is thought to be at the most several hours (Searle et al., 1987a). Therefore, it is necessary to identify cells at an earlier stage in the apoptotic pathway, where morphology may be essentially normal (Allen et al., 1993).

An immunohistochemical method has recently become available for the identification of 3'-OH DNA ends generated as a result of intranucleosomal cleavage. In situ DNA end labelling (ISEL; Gavrieli et al., 1992; Ansari et al., 1993) exploits the activation of a

Offprint requests to: Dr. Arthur Zimmermann, M.D., Professor of Pathology, Institute of Pathology of the University, Murtenstrasse 31, CH-3010 Berne, Switzerland

nucleosomal endonuclease that occurs in cells undergoing apoptosis. In the present retrospective investigation, we used this method to study the extent of liver cell apoptosis in three different situations of chronic hepatic iron overload.

Materials and methods

Patients groups

Biopsies from three groups of patients were used in this study: A) patients with hereditary hemochromatosis (HC) showing severe hepatic iron overload (N=34; 10 female, 24 male; age range: 35 to 75 years); hemochromatosis in these patients was clinically and histologically confirmed; 17 patients were in the cirrhotic stage; B) patients with mild to moderate hepatic iron overload not related to homozygous HC (NHC; N=24; 5 female, 19 male; age range: 35 to 86 years); this group included patients with alcoholic liver disease, chronic viral hepatitis, fatty change of the liver, hepatic parenchymal damage of unknown reasons, and possibly heterozygous HC; and C) patients with advanced exogenous hepatic iron overload due to transfusions (EIO; N=5; 3 female, 2 male; age range: 26 to 51 years). Biopsies of grafted livers showing acute rejection and bile duct cell apoptosis, and human tonsil tissue were used as positive controls.

Biopsies and light microscopic methods

Tissue consisted exclusively of needle biopsies. All biopsies were obtained before any therapy had been applied. Fixation was with 4% neutral buffer formalin for less than 30 hours throughout. Samples were embedded in paraffin and processed for 4 µm thick sections. Routine staining included haematoxylin-eosin, PAS, Van Gieson's, reticulin and chromotrope aniline stains. For the detection of stainable iron, Perls' iron stain was used (Perls, 1867).

Histopathological assessment and grading of lesions

Deposition of stainable iron in the form of hemosiderin was graded according to the proposition of Rowe (Rowe et al., 1977), in the modification of Searle and coworkers (1987b). The Rowe grading comprises four grades, depending on the ease of observation and magnification required.

Fibrosis and cirrhosis of the liver were assessed and graded according to the classification system proposed by Knodell and coworkers (1981), with some modifications (Schmid et al., 1994). Grade 0 means no fibrosis; 1: mild, portal tract fibrosis only; 2: portal tract fibrosis plus incomplete fibrous septa; 3: complete septa, bridging portal-portal; 4: complete septa bridging portal-central, and/or focal complete cirrhosis; 5: diffuse complete and/or focal complete cirrhosis; and 6: diffuse complete cirrhosis.

Iron deposition was not chemically quantified in the biopsy material analyzed in this study.

Immunohistochemical methods

In situ DNA end labelling (ISEL)

For the in situ visualization of apoptotic cells, ISEL was employed according to the method published in 1992 (Gavrieli et al., 1992). ISEL is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of DNA through a mechanism of synthesis of a polydeoxynucleotide polymer at chromatin DNA breaks. This method does not require homogenization of the entire cell population and analysis of the pooled DNA extract used in the DNA laddering procedure (Arends and Wyllie, 1991).

Paraffin sections (4-6 µm) were affixed to Super Frost/Plus slides (Menzel-Gläser, Germany). Deparaffinization was done by heating the sections for 4 hours at 58 °C. Hydration was performed by transfering the slides through the following solutions: twice to xylene bath for 10 min each, twice to 100% ethanol for 5 min each, and then for 3 min to 96%, 70% and 35% ethanol, and three times to double-distilled water (DDW). Fresh solvents were used, as traces might have interfered with the enzyme reaction.

Prepared paraffin sections were digested by incubation with 5 µg/ml proteinase K (PK, Sigma Chemical Company) for 20 min at room temperature, and then washed in DDW for 5 min, three times. Endogenous peroxidase and DNAses were inactivated by incubating the sections in 2% H_2O_2 for 5 min at room temperature. The sections were rinsed with DDW three times. TdT was from Boehringer, Mannheim. The TdT reaction mix (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride, TdT enzyme 0.2 e.u./µl, and digoxygenin-conjugated or biotinylated dUTP in buffer was used for the reactions) was added to cover the sections and then they were incubated in a humid atmosphere at 37 °C for 60 min. The reaction was terminated by transfer of the slides to 2xSSC buffer (300 mM sodium chloride, 30 mM sodium citrate, Merck) for 10 minutes twice and TBS buffer for 10 minutes twice. The sections were then incubated for 30 min in 1% blocking reagent (Boehringer, Mannheim) at room temperature followed by washing in phosphate-buffered saline twice for 5 min. Sections were covered with extra-avidin alkaline phosphatase (Boehringer, Mannheim; at a dilution of 1:100) to biotinylated dUTP, or sheep antidigoxygenin-alkaline phosphatase (Fab fragments; Boehringer Mannheim; at a dilution of 1:1000) to alkaline phosphatase-conjugated-dUTP in the reaction mix for 30 min at room temperature. The latter was followed by a 30 min incubation with APAAP complex (Dako) at a dilution of 1:50. The New Fuchsin substrate solution was used to visualize the reaction followed three times by washing in phosphate buffer.

The substrate reaction was stopped by rinsing the slides in cold tap water and the sections were finally counterstained with haematoxylin and mounted with water soluble mounting medium (Aquadex).

All the TdT tailing reaction reagents were purchased from Boehringer, Mannheim.

Quantitation of apoptotic liver epithelial cells based on TdT labelling indices

Assessment of reactive nuclei based on the ISEL method was restricted to hepatocytes in the present study. For the determination of a «TdT labelling index», the number of positive nuclei per 100 hepatocyte nuclei analyzed was counted, and this ratio used as labelling index. In the first part of this procedure, overall labelling of hepatocytes within the parenchyma was assessed. For this purpose, areas of interest were randomly chosen. In a second phase, the apoptotic rate was analyzed for specific areas within the liver acinus, in particular the perivenular area (see below).

Statistical methods

All results are expressed as mean ± SD. Kruskal-Wallis and Mann-Whitney U tests were employed. $P<0.05$ was considered to be statistically significant.

Results

Histopathological characterization of disease groups

Histopathological features observed in biopsies of the three disease groups are compiled in Tables 1 and 2.

Table 1. Hepatic iron overload due to hemochromatosis (HC): histopathology and TdT labeling indices.

CLINICAL DATA		HISTOPATHOLOGY GRADES			TdT LABELING INDEX (%)				
Patient	Sex/Age	Rowe	Fibrosis	Cirrhosis	L-1	Z-3	Z-2	Z-1	T-I
1	F/36	4	0	0	8	6	2	1	17
2	M/43	4	4	1	2	0	0	0	2
3	M/43	4	5	1	5	3	1	2	11
4	F/50	4	3	1	2	0	1	0	3
5	M/43	4	2	0	5	0	2	1	17
6	M/60	4	4	1	0	0	0	0	0
7	M/50	4	5	1	0	0	0	0	0
8	M/45	4	1	0	3	5	2	2	12
9	F/75	4	4	1	0	0	0	0	0
10	M/71	4	6	2	0	0	0	0	0
11	M/38	4	4	1	0	0	0	0	0
12	F/64	4	4	1	0	0	0	0	0
13	M/47	4	0	0	0	2	2	1	5
14	F/53	4	4	1	0	0	0	0	0
15	M/48	4	2	0	6	2	1	2	11
16	M/64	3	5	1	3	2	1	1	7
17	M/52	4	1	0	3	1	1	1	6
18	M/52	4	0	0	0	0	0	0	0
19	M/35	3	0	0	3	3	1	1	8
20	M/51	3	0	0	2	1	1	1	5
21	F/49	4	0	0	3	1	1	2	7
22	M/61	4	4	1	0	0	0	0	0
23	M/53	4	4	1	0	0	1	1	2
24	F/35	3	2	0	0	0	0	0	0
25	M/56	4	4	1	0	1	1	2	3
26	M/36	4	4	1	0	0	0	0	0
27	F/62	4	0	0	4	1	2	1	8
28	M/40	4	4	1	3	1	1	2	7
29	F/41	4	0	0	**0**	**1**	**1**	**1**	**3**
30	M/43	3	0	0	0	1	1	1	3
31	M/61	3	2	0	0	0	1	1	2
32	F/54	4	2	0	3	0	1	1	5
33	M/67	4	6	2	0	0	0	0	0
34	M/51	4	2	0	0	0	0	0	0

Rowe: estimation of hepatocyte iron overload according to Rowe's grading. For grading of fibrosis, see Materials and methods. Grading of cirrhosis: 0, no cirrhosis; 1, incomplete cirrhosis; 2, complete cirrhosis. TdT labeling index: L-1, pericentral (perivenular) layer of hepatocytes; Z-3: hepatocytes in acinar zone 3; Z-2, hepatocytes in acinar zone 2; Z-1, hepatocytes in acinar zone 1; T-I: total TdT labeling index.

Table 2. Hepatic iron overload in NHC and EIO: histopathology and TdT labeling indices.

CLINICAL DATA		HISTOPATHOLOGY GRADES			TdT LABELING INDEX (%)				
Patient	Sex/Age	Rowe	Fibrosis	Cirrhosis	L-1	Z-3	Z-2	Z-1	T-I
A. Hepatic iron overload not related to homozygous hemochromatosis (NHC)									
1	M/65	2	2	0	0	0	1	0	1
2	M/41	2	1	0	0	2	1	0	3
3	M/78	2	0	0	0	1	0	0	1
4	M/60	1	0	0	0	0	0	0	0
5	F/47	1	0	0	0	0	0	0	0
6	M/53	1	0	0	1	2	2	1	6
7	M/42	2	0	0	0	0	1	0	1
8	M/35	2	0	0	0	0	0	0	0
9	M/46	2	0	0	0	0	1	0	1
10	M/77	2	0	0	3	1	1	1	6
11	M/40	3	0	0	6	2	1	1	10
12	M/41	2	0	0	0	0	0	0	0
13	M/50	2	5	1	0	0	1	1	2
14	F/71	2	0	0	9	6	5	4	24
15	M/85	2	0	0	0	0	1	0	1
16	M/46	1	0	0	0	3	2	0	5
17	M/48	2	0	0	0	0	4	0	4
18	M/46	2	0	0	0	0	1	0	1
19	M/53	2	0	0	0	0	0	0	0
20	M/38	3	0	0	0	0	1	0	1
21	M/54	1	2	0	0	0	0	1	1
22	**M/58**	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
23	M/61	1	5	0	0	1	0	0	1
24	F/59	1	4	1	0	0	0	0	0
B. Exogenous hepatic iron overload (EIO)									
1	F/51	2	0	0	0	0	0	0	0
2	M/47	4	1	0	0	4	2	3	9
3	F/26	4	0	0	0	0	0	0	0
4	M/42	3	1	0	0	0	0	0	0
5	F/50	4	2	0	0	0	0	0	0

Rowe: estimation of hepatocyte iron overload according to Rowe's grading. For grading of fibrosis, see Materials and methods. Grading of cirrhosis: 0, no cirrhosis; 1, incomplete cirrhosis; 2, complete cirrhosis. TdT labeling index: L-1, pericentral (perivenular) layer of hepatocytes; Z-3: hepatocytes in acinar zone 3; Z-2, hepatocytes in acinar zone 2; Z-1, hepatocytes in acinar zone 1; T-I: total TdT labeling index.

Among the 34 cases of HC, 28 biopsies (82.4%) showed severe iron overload, corresponding to Rowe grade 4, whereas 6 biopsies only were grade 3 (17.6%), and no biopsy showed a grade less than 3. Predominance of iron pigment in the periportal acinar zone 1 was observed in all cases. The 24 biopsies of patients with NHC (Table 2A) showed lesser degrees of iron overload (grade 1: 33.3%; grade 2: 58.3%; grade 3: 8.3%), and no biopsy exhibited a Rowe grade 4 lesion. The third patient group (EIO; Table 2b) showed grade 4 iron in 3/5 grade 3 in 1/5, and grade 2 in 1/5, with stainable iron involving both hepatocytes and Kupffer cells. In these cases, the periportal predominance of iron overload was lacking. 50% of HC biopsies showed a cirrhotic stage already, albeit only in 2/17 with diffuse complete cirrhosis. Fibrosis, however, was noted in79.4% of biopsies. In contrast, only 25% of cases of the second group showed fibrosis, in two cases associated with incomplete cirrhosis, and 3/5 biopsies of the third group exhibited a fibrosis of low degree.

Distribution of TdT-labelled hepatocytes

As shown in Fig. 1, parenchymal TdT staining was confined to the nuclei of hepatocytes, and no cytoplasmic staining was observed. A similar nuclear reaction product was observed in bile duct cells of biopsies from rejecting allografted livers (data not shown). Reactivity mostly occurred in parenchymal cells not showing morphological features of late-stage apoptosis, i.e. without cell shrinkage, chromatin condensation or formation of apoptotic bodies. A higher rate of staining was observed in the centri-lobular regions, in particular directly around the central vein, both in individual hepatocytes and in small groups of cells (Fig. 2). Positive nuclei could be detected in

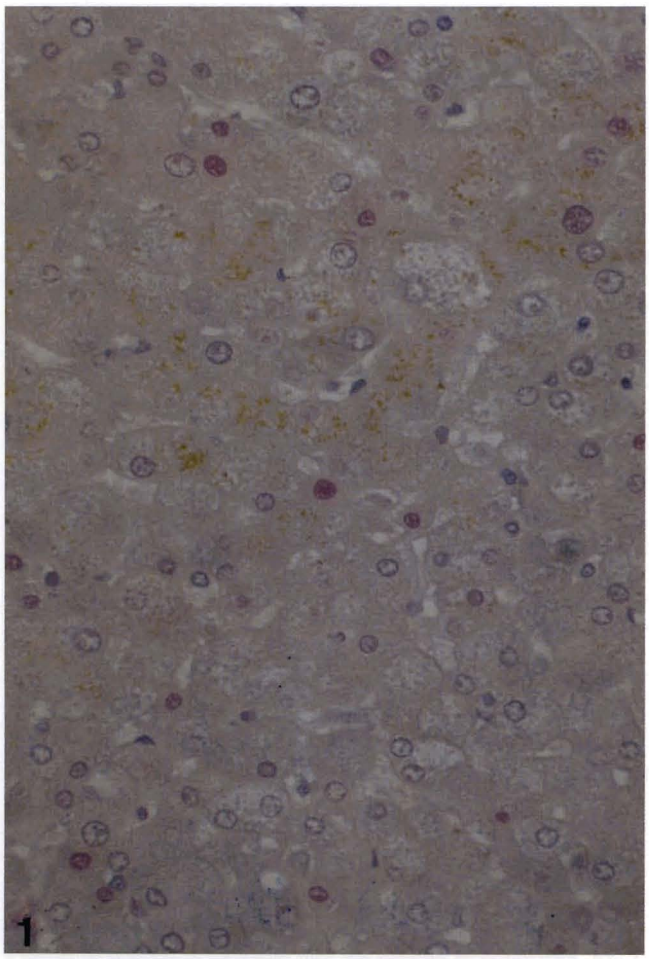

Fig. 1. Pigment-laden liver parenchyma in hemochromatosis. Several hepatocyte nuclei exhibit red reaction product after TdT-labelling. Hepatocytes involved do not show shrinkage, nuclear condensation, or other morphological signs of apoptosis. ISEL procedure, counterstain with haematoxylin. x 180

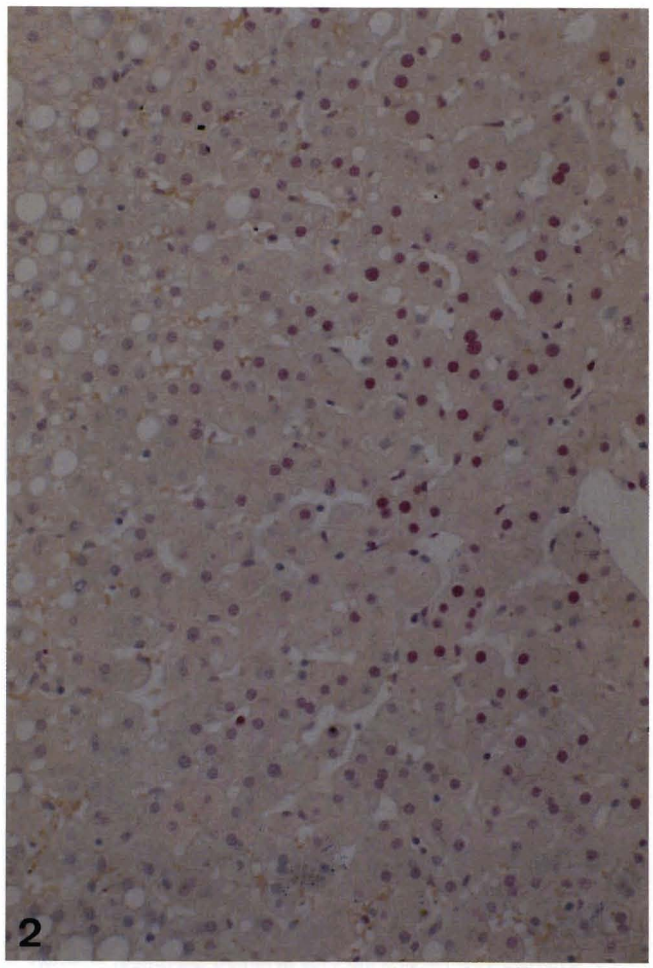

Fig. 2. Pericentral zonal and midzonal areas of a liver lobule in HC. The central vein is seen at the right border. Typically for HC, no pigment is visualized in the pericentral zone. Note the preferential TdT reactivity in hepatocyte nuclei in the pericentral area. ISEL procedure, counterstain with haematoxylin. x 180

morphologically normal-looking hepatocytes, in those with a low degree of iron pigment deposition, and in cells with a heavy iron load (Fig. 3). In a few situations, condensed parenchymal cells, probably representing apoptotic bodies and apparently having dropped out of liver cell plates into sinusoids, were TdT-reaction-positive (Fig. 4).

Mean values of TdT labelling indices for the three disease groups, for the different Rowe grades, and for the fibrosis/cirrhosis status are listed in Tables 1 and 2. It is seen that the apoptotic rate in the entire hepatocyte population was somewhat higher in the HC group, and was lower in the small EIO group than in the NHC group but without reaching a statistically significant difference. Overall, TdT labelling indices tended to be lower in hepatocytes without or with only slight deposition of stainable iron than in hepatocyte populations with a high iron overload (NS). No correlation was found between the TdT labelling indices and grades of fibrosis of the cirrhosis status, respectively (Table 3).

A different pattern surfaced when looking at the area where apoptosis of hepatocytes was more regularly seen, i.e. the innermost part of acinar zone 3, and in particular the subpopulation of hepatocytes surrounding central veins (in cases where acinar structures were still preserved). As seen in Table 4, the TdT labelling index was significantly higher for immediately pericentral hepatocytes in the HC group than in the patient groups with NHC and EIO. Again, TdT labelling indices tended to be higher in hepatocyte populations showing a higher degree of iron overload (Rowe grades 3 and 4 vs. grades 1 and 2). For the whole of lobular zone 3, and for zones 1 and 2, no such relationship was observed in biopsies where a lobular zonation could still be visualized.

Discussion

Iron overload states of the liver have long been

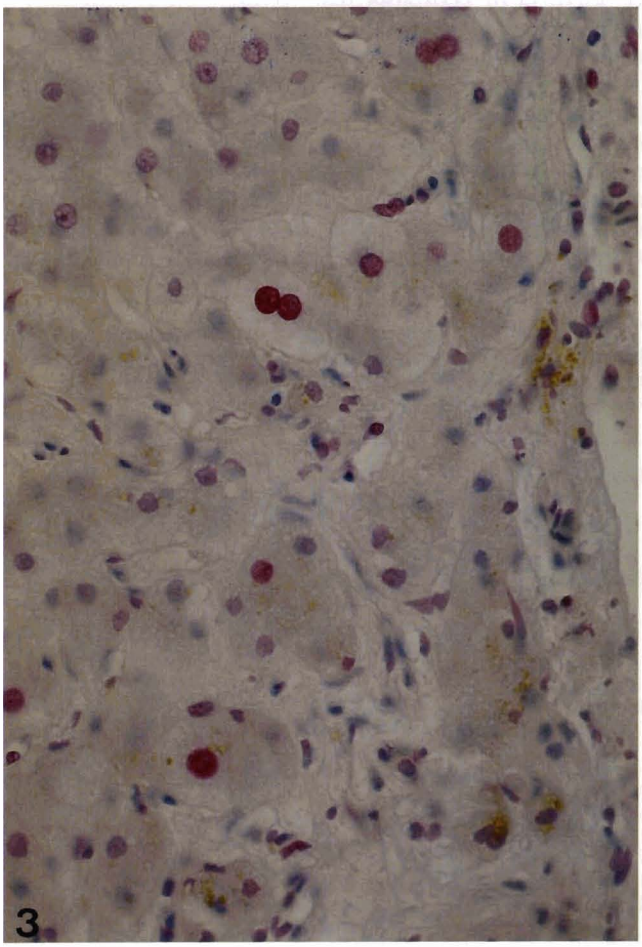

Fig. 3. Liver biopsy from a patient with HC, periportal zone. TdT-reactive nuclei are seen in hepatocytes containing hemosiderin granules, but also in pigment-free hepatocytes, e.g. the cell showing two strongly stained nuclei. ISEL procedure, counterstain with haematoxylin. x 240

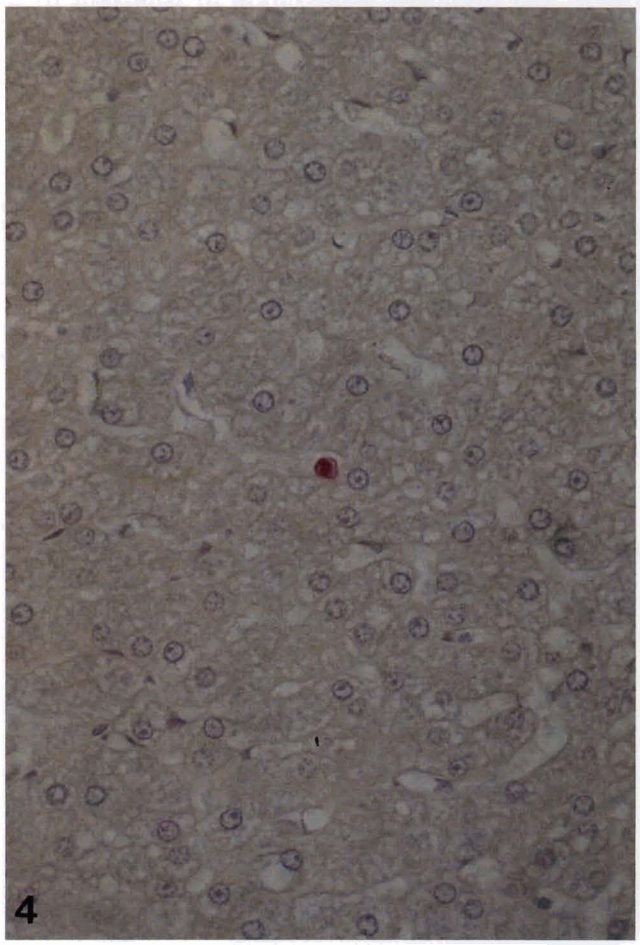

Fig. 4. A condensed hepatocyte shows nuclear TdT staining. This apparently intrasinusoidal cell probably represents an apoptotic body. ISEL procedure, counterstain with haematoxylin. x 180

known (Searle et al., 1987b). The association of cirrhosis with heavy hepatic deposits of iron-containing pigment was first recognized in 1889 by von Recklinghausen, who coined the term hemosiderin for the iron-containing compound, and hemochromatosis for the clinical disease. Hereditary hemochromatosis (or idiopathic hemochromatosis) results from an inborn error of the iron metabolism leading to increased iron absorption from the diet. It is currently thought that most, if not all, primary heavy parenchymal iron overload is due to homozygosity for hemochromatosis. In contrast to homozygous patients, the disease leads to a much less important organ iron accumulation in heterozygous individuals. Similarly, high levels of iron deposition can occur after long-term blood transfusions or in situations of excessive exogenous iron intake (Sherlock and Dooeley, 1993). Chronic iron overload to the liver leads to hepatocyte injury, frequently ending up in the production of liver fibrosis or even cirrhosis of this organ (Deugnier et al., 1992). Even though early activation of hepatic genes in the iron-fed rat occurs in the absence of histological changes (Pietrangelo et al., 1990), and selective localization of iron into liver parenchymal cells is required for the activation of collagen gene during long term iron load (Gualdi et al., 1994), parenchymal cell death may play a significant role for the establishment of chronic liver disease in iron overload. In fact, iron as such induces cell toxicity, probably through a radical-mediated process (Tien et al., 1981; Halliwell and Gutteridge, 1985; Bacon and Britton, 1990; Myers et al., 1991), and iron-induced tissue damage to the liver can be associated by an inflammatory reaction, sometimes mimicking chronic active hepatitis.

In the present study, we were interested to test whether, in addition to simple hepatocyte necrosis, programmed cell death or apoptosis would occur in different states of hepatic iron overload. For this purpose, we applied a new technique for the detection of apoptosis, namely DNA nick end labelling, in three situations of pathological iron deposition in the liver, i.e. HC, hepatic iron overload due to unknown reasons (NHC), and iron overload due to hyperhemolysis and exogenous iron overload (EIO), the last group mainly related to transfusions.

We can show that in comparison to control biopsies, an increased apoptotic rate of hepatocytes occurs in chronic hepatic iron overload. For the entire hepatocyte population, the TdT labelling index tended to be higher in HC than in NHC and EIO biopsies, and tended to be lower in hepatocytes without or with only slight deposition of stainable iron. Biopsies with high iron overload from HC patients were, however, significantly different with respect to apoptotic rate from both, NHC and EIO biopsies, when focusing on the subset of hepatocytes located around the central vein. Pericentral (perivenular) hepatocytes of HC biopsies consistently showed a significantly higher TdT labelling index in comparison with NHC, whereas no labelling was detected in the small group with EIO in this area, even though 4 patients out of 5 in this EIO group showed

Table 3. Mean TdT labeling indices of hepatocyte nuclei as a function of disease group, grade of stainable iron (Rowe grade), grade of fibrosis, and cirrhosis.

	N	ACINAR/NODULAR TdT LABELING INDEX (%; mean±SD)	P
Disease group			
HC	34	4.2±0.8	0.24
NHC	24	2.9±1.0	
EIO	5	1.8±2.2	
Rowe grade			
1	8	1.6±1.7	0.52
2	15	3.0±1.3	
3	9	4.0±1.7	
4	31	4.1±0.9	
Fibrosis			
Grade 0	29	4.0±0.9	0.15
Grade 1	5	6.0±2.2	
Grade 2	9	4.1±1.6	
Grade 3	1	3.0±4.9	
Grade 4	12	1.2±1.4	
Grade 5	5	4.2±2.2	
Grade 6	2	0.0±0.0	
Cirrhosis			
No cirrhosis	44	4.2±0.7	0.09
Incomplete	17	2.2±1.2	
Complete	2	0.0±0.0	

HC: homozygous hemochromatosis; NHC: iron overload not related to homozygous hemochromatosis; EIO: exogenous iron overload.

Table 4. Mean TdT labeling indices of the innermost layer of pericentral (perivenular) hepatocytes as a function of disease group, grade of stainable iron (Rowe grade), grade of fibrosis, and cirrhosis.

	N	TdT (%; mean±SD)	P
Disease group			
HC	34	1.6±0.4	0.03
NHC	24	0.8±0.4	
EIO	5	0.0±0.0	
Rowe grade			
1	8	0.1±0.8	0.17
2	15	0.8±0.5	
3	9	1.6±0.7	
4	31	1.6±0.4	
Fibrosis			
Grade 0	29	1.3±0.4	0.70
Grade 1	5	1.2±0.9	
Grade 2	9	1.6±0.7	
Grade 3	1	2.0±2.2	
Grade 4	12	0.4±0.6	
Grade 5	5	1.6±0.9	
Grade 6	2	0.0±0.0	
Cirrhosis			
No cirrhosis	44	1.3±0.3	0.60
Incomplete	17	0.9±0.5	
Complete	2	0.0±0.0	

HC: homozygous hemochromatosis; NHC: iron overload not related to homozygous hemochromatosis; EIO: exogenous iron overload.

advanced iron deposition (Rowe grades 3 and 4). No significant correlation was found between the TdT labelling index and the degree of both fibrosis and cirrhosis of the liver.

These findings suggest that hepatic iron overload as such may be associated with an increased apoptotic rate of hepatocytes, particularly concerning the pericentral hepatocyte population, and that iron-laden hepatocytes in HC behave differently from those in NHC and EIO. Several pathways for iron-induced hepatocyte damage in iron overload have been proposed, with particular emphasis placed on the role of membrane lipid peroxidation, probably via active oxygen radicals (Britton, 1996).

We recently showed that the oxygen scavenger, Cu/Zn-superoxide dismutase, was more strongly expressed in HC biopsies than its inducible counterpart, Mn-superoxide dismutase, indicating a role for superoxide dismutases in protection against iron-induced radicals (Zhao et al., 1995). But even though lipid peroxidation in the cell surface membrane and in organelle membranes may result in cell death, this may not necessarily occur through apoptosis. Apoptotic cell death is a distinct pathway needing a distinct stimulus or stimuli and a still functioning cellular machinery (Kerr et al., 1972; for review, see Majno and Joris, 1995), and is aimed at elimination of excessive, unwanted or altered cells (Wyllie et al., 1980; Lockshin and Zakeri, 1990). In the liver, apoptosis of epithelial cells has been demonstrated for both hepatocytes and biliocytes (Patel and Gores, 1995). Recently, it has been shown that in rats and humans hepatocyte apoptosis is more frequently observed in the perivenular zone (zone 3) of the liver acinus (Benedetti et al., 1988). Our findings confirm this phenomenon for situations of iron storage, but the pericentral apoptotic rate was significantly higher in HC than in the two other groups analyzed. In a first approach, this finding may suggest that severe iron overload of hepatocytes in HC enhances, via iron toxicity, the apoptotic rate at a site where apoptosis preferentially occurs, even though iron storage in HC is usually higher in the periportal acinar zone (zone 1). However, this change was not seen in the few cases with exogenous iron overload of similar severity. The reason for this difference is unknown. Several factors have been shown to be capable of inducing hepatocyte apoptosis, including TNF-alpha, TGF-beta1, and growth factor deprivation, and these factors appear to employ common pathways, e.g. increase of oxygen radical formation, upregulating of the pro-apoptotic factors Bax and bcl-X_S, and downregulation of apoptosis-protecting factors bcl-2 and bcl-X_1. In situations of pathological iron storage, a critical role is played by activation of Kupffer cells, as phagocytosis of «sideronecrotic» hepatocytes enhances the release of cytokines involved in an apoptotic signal pathway (Deugnier et al., 1992; Gualdi et al., 1994). In HC, the Kupffer cell system usually does not participate in iron storage, whereas a strong iron overload of Kupffer cells is found in transfusional hepatic siderosis, and this difference may have consequences for the macrophage function, being better preserved in HC. Provided that Kupffer cells play an essential role for mediating apoptosis in hepatic iron storage, this hypothesis may offer a possible explanation for the differences in pericentral apoptotic rate.

No correlation was found between hepatocyte apoptotic rates and grades of fibrosis. We suggest that this may be due to the fact that fibrosis is associated with acinar remodeling and, finally, with cirrhosis, events needing hepatocyte proliferation rather than apoptosis. Mitosis is the complement of apoptosis, and the maintenance, growth or involution of tissues are dependent on the balance between these two processes. In precirrhotic and cirrhotic stages, where collagen synthesis is directly influenced by iron in HC (Gualdi et al., 1994), apoptosis may be downregulated in favour of cell survival and cell proliferation.

In conclusion, the present findigns suggest that 1) hepatocyte apoptosis in iron overload is increased in comparison with controls; 2) the hepatocyte apoptotic rate tends to increase as a function of the degree of iron storage; 3) similar to other pathological liver changes studied so far, an increased apoptotic rate predominates in the innermost (pericentral) part of the acinus, but in HC only, and not in lower level iron storage or in transfusional siderosis; and 4) that the latter phenomenon may be related to differences in the participation of Kupffer cells in the iron storage process.

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