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TUNEL-positive hepatocytes in alcoholic liver disease

A retrospective biopsy study using DNA nick end-labelling

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Abstract Alcohol-induced damage to the liver results in a wide array of typical alterations. Whereas the mechanisms involved in the pathogenesis of fatty change, hepatocyte ballooning, Mallory body formation and fibrosis have been studied in detail, little is known about hepatocyte apoptosis in alcoholic liver disease (ALD). In this retrospective study we analysed parenchymal cell death in ALD systematically by the use of in situ DNA nick-end labelling (ISEL/TUNEL). We show that increased hepatocyte TdT labelling occurs in ALD. Labelling is observed more frequently in parenchymal areas exhibiting advanced damage (ballooning degeneration with or without Mallory bodies, cholestasis and perisinusoidal fibrosis). In addition, hepatocyte TdT labelling is higher where there is septal fibrosis and nodular remodelling. Conversely, it is not elevated in ballooning hepatocytes themselves, but rather in the apparently normal hepatocytes in their vicinity.

Key words Apoptosis · TUNEL · Hepatocytes · Alcoholic liver disease

Introduction

Alcoholic liver disease (ALD) is characterized histologically by the combination of macrovesicular fatty change of hepatocytes, hepatocyte ballooning with or without formation of Mallory bodies, granulocytic infiltrates around damaged parenchymal cells, a distinct type of perisinusoidal fibrosis, and hepatic cirrhosis in advanced stages of the disease [4, 10, 11, 16, 19, 25, 33, 37, 38, 42, 44, 48, 50]. Alcohol toxicity to the liver is mediated by several pathogenic pathways, including cellular damage by acetaldehyde [23, 31, 32] and effects of cytotoxic reactive oxygen species [8, 9, 12–14, 15, 22, 24, 27, 29, 40, 41]. Mechanisms involved in the production of Mallory bodies and of ballooned hepatocytes have been studied by many authors, but the pathogenesis of these changes has not been clarified [18, 51]. Similarly, the pathogenesis of hepatocyte loss caused by cell death in response to alcohol toxicity is not well known, even though it has been suggested that apoptosis might be involved in alcoholic hepatitis [26]. There is increasing evidence that necrosis is not the only mechanism responsible for cell death, and that target cells can be induced to undergo apoptosis. This latter phenomenon is distinguished from necrosis chiefly because apoptosis involves active processes needing specific gene activation and protein synthesis.

Apoptosis, as based on morphological criteria, has been shown to occur in liver disease [6, 35, 43, 46], and particularly in allograft rejection [1, 39]. However, assessment of apoptotic cells in liver tissue sections on the basis of cytomorphological criteria alone is difficult, owing to disruption of tissue architecture, the presence of many different cell types and the paucity of so-called apoptotic bodies in many types of liver disease (with the notable exception of acute viral hepatitis). The detection and quantification of apoptosis is hampered by the fact that cell shrinkage, nuclear condensation, and chromatin fragmentation also occur in necrosis, indicating that at least a morphological dualism, “apoptosis” versus “necrosis”, may be questionable. In addition, signs of apoptosis may be seen during the advanced stages of this process only as the half-life of apoptotic cells in tissues is thought to be in the region of several hours at most [2, 34, 36]. Therefore, it is necessary to identify apoptotic cells at an earlier stage of this active pathway. One method proposed as a way of overcoming these problems is the use of in situ DNA nick end-labelling (ISEL; TUNEL) [3, 20], which exploits the activation of a nucleosome endonuclease that occurs in cells undergoing apoptosis. This approach may result in the detection of early phases of an apoptotic pathway, even though Ansari et al. [3] stated, in their basic article, that the DNA nick end-labelling method is not specific for pro-

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grammed cell death and that results must be interpreted with caution and correlated with morphological criteria of apoptosis.

In the present investigation we assessed, in a systematic retrospective study on liver biopsies, the frequency and spatial distribution of TUNEL-positive hepatocytes in ALD. We further analysed TUNEL-positivity with respect to ballooning hepatocyte degeneration (with or without Mallory body formation), and correlated the results with the stage of ALD.

Materials and methods

Liver biopsies were obtained from 43 patients with alcoholic liver disease (ALD; 39 male, 4 female; age range: 21–79 years). The diagnosis of ALD was based on the patient's history, on clinical and biochemical findings, and on histological patterns, in accordance with published criteria [4, 11, 16]. The cases studied had no signs of chronic hepatitis. Biopsies of livers showing no relevant histological change (tumour staging biopsies without evidence of malignant disease) were obtained from 13 patients covering a similar age range, and these biopsies served as normal controls. As controls for advanced fibrosing liver disease not related to ALD or inflammatory liver disease, biopsies from 17 patients with established haemochromatosis in the fibrotic/cirrhotic stage were employed (age range 38–75 years).

For light microscopy and ISEL/TUNEL, biopsies were fixed in 4% neutral buffered formalin for 2–4 h and embedded in paraffin. Sections 4 μm thick were processed for routine staining (haematoxylin-eosin, PAS, iron, Van Gieson's, reticulin and chromotrope anilin stains) and for ISEL/TUNEL (see below).

Macrovesicular fatty change was estimated as the percentage of parenchymal surface involved (three classes: <10%; 10–50%; >50%). Ballooning degeneration of hepatocytes, Mallory bodies (MB), granulocyte reaction around Mallory-body-containing hepatocytes and megamitochondria were recorded as either present or absent. Hepatic fibrosis was evaluated as previously described [28], with some modifications [45]. Perisinusoidal fibrosis in the pericentral (perivenular) acinar zone was registered as either present or absent. For grading of portal tract and septal fibrosis, the system recently proposed by Schmid et al. [45] was employed (grade 0, none; grade 1, mild, only portal tract fibrosis; grade 2, portal tract fibrosis plus incomplete septa; grade 3, fibrous septa bridging portal-portal; grade 4; fibrous septa bridging portal-central, and/or focal incomplete cirrhosis; grade 5, diffuse incomplete and/or focal complete cirrhosis; and grade 6, diffuse complete cirrhosis).

For the in situ visualization of apoptotic cells, ISEL according to the method published by Gavrieli et al. [20] was employed. Paraffin sections (4 μm thick) were affixed to Super Frost/plastic slides (Menzel-Gläser, Braunschweig, Germany). Deparaffination was performed by heating the sections for 4 h at 58°C. Hydration was executed by transfering the slides through the following solutions: twice in xylene bath for 10 min each, twice in 100% ethanol for 5 min each, and then for 3 min in 96%, 70% and 35% ethanol, and three times in double-distilled water. Fresh solvents were used in each case. The prepared paraffin sections were then digested by incubation with 5 μg/ml proteinase K (Sigma Chemical Company, Buchs, Switzerland) for 20 min at room temperature, and washed three times in double-distilled water for 5 min each. Peroxidase and DNAses were inactivated by incubating the sections in 2% H_2O_2 for 5 min at room temperature. Afterwards, sections were rinsed three times with double-distilled water. The TdT (terminal deoxynucleotidyl transferase; E.C. 2.7.7.31) reaction mix (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride, TdT enzyme 0.2 U/ml, and digoxygenin-conjugated or biotinylated dUTP in ENTP buffer; all TdT tailing reaction reagents were purchased from Boehringer Mannheim, Germany) was added to cover the sections and then incubated in humidified atmosphere at 37°C for 60 min. The reaction was stopped by transferring the slides to 2 × SSC buffer for 10 min (twice) and TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 10 min (twice) and sections were incubated for 30 min in 1% blocking reagent (Boehringer, Mannheim, Germany) at room temperature, followed by washing in phosphate-buffered saline twice for 5 min. Sections were covered with Extra-avidin alkaline phosphatase (Boehringer, Mannheim, Germany) at a dilution of 1:100 to biotinylated-dUTP, or sheep anti-digoxygenin-alkaline phosphatase, FAB fragments (Boehringer, Mannheim, Germany) 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, Glostrup, Denmark) 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, Merck, Darmstadt, Germany).

TdT-reactive nuclei of hepatocytes were counted. The number of positive nuclei per 100 nuclei analysed was used as a "TdT labelling index" (TdT-LI). For the determination of TdT-LI, two strategies were employed. First, areas of interest within the hepatic parenchyma were chosen randomly, whereas in a second step the pericentral acinar zone was separately analysed, because florid signs of ALD mostly start in this area and because it had recently been shown that the prevalence of apoptotic bodies is higher in this area [5].

All results are expressed as mean±SD. T-Test, Kruskal-Wallis one-way analysis, Pearson's correlation analysis, and Chi-square analysis were employed. $P < 0.05$ was considered to be statistically significant.

Results

Histopathological changes and their grading are compiled in Table 1. It is seen that macrovesicular fatty change was present in 41 of the 43 biopsies, involving 50% or more of parenchymal surface in about a third of cases. Based on haematoxylin- and eosin-stained sections, we noted a microvesicular component of fatty change in 6 of the 43 biopsies only. No foamy degeneration of hepatocytes [49] was observed. Mallory bodies (MBs) were detected in 55.5% of preparations. They were frequently associated with neutrophil granulocytes in the vicinity, with neutrophil satellitosis in 10 of the 25 cases showing MBs (data not shown). Most biopsies showing MBs also disclosed ballooning degeneration of hepatocytes.

Perisinusoidal/pericellular fibrosis was present in all but 2 biopsies. It presented the lattice-like pattern typical for ALD in 71.1%, involving more than the pericentral area in 35.5% of cases. Incomplete and complete fibrous septa not associated with nodular change were present in 26.6%, whereas liver cirrhosis (either incomplete or complete [25]) was noted in 44.4% of biopsies.

No relevant histological changes were detected in control biopsies obtained during staging procedures. In the control group with haemochromatosis ($n = 17$), 15 patients showed incomplete cirrhosis and 2 patients, complete cirrhosis.

TdT labelling was visualized in the form of a strong reaction product in hepatocyte nuclei (Fig. 1), and it was

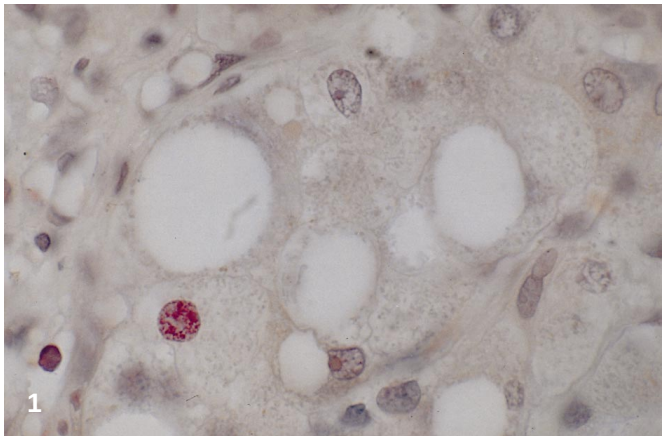
Table 1 Histopathology in 43 cases of human alcoholic liver disease (*FC* fatty change (% of surface), *MA* macrovesicular, *MI* microvesicular, *MB* Mallory bodies, *MM* megamitochondria (I MM type I, II MM type II), *BD* ballooning degeneration, *CH* cholestasis, *DP* ductular proliferations, *SF* septal fibrosis, *PS* perisinusoidal fibrosis, *CF* central vein fibrosis/sclerosis)

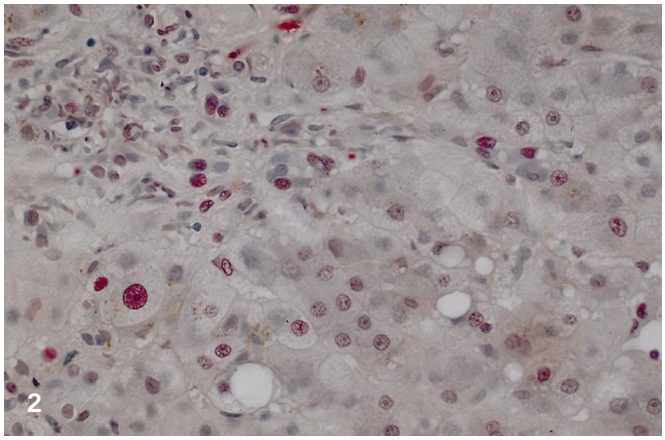
Case	Age/Sex	FC,%	Changes of hepatocytes				Nonparenchymal changes			
			MB	MM	BD	CH	DP	SF	PS	CF
1	52/M	Ma/>50	+	+				2	2	–
2	61/M	Ma/>50	+		+	+	+	4	3	+
3	51/M	Ma/>50	+			+	+	5	2	+
4	63/M	Ma/ 25	+	+;I	+			3	1	+
5	60/M	Ma/>50	+		+	+	+	4	2	+
6	56/M	Ma/<10	+	+;I			+	5	2	+
7	62/M	Ma/>50	+		+		+	6	3	–
8	50/M	Ma/>50	+		+			3	3	–
9	46/M	Ma/>50	+					0	1	+
10	48/M	Ma/10–15	+		+			3	3	+
11	31/M	Ma/>50	+	+;I	+	+		4	3	+
12	44/M	Ma/>50	+		+	+		5	3	+
13	68/F	Ma/ 10	+		+		+	5	3	+
14	58/M	Ma/>20	+		+		+	3	3	+
15	64/M	Ma/>10	+		+		+	5	3	+
16	59/M	Ma/>10	+	+;II	+		+	5	3	+
17	64/M	Ma/>10	+				+	5	2	+
18	56/M	Ma/>50	+				+	5	3	+
19	45/M	Ma/>10	+	+			+	6	3	–
20	55/M	Ma/>10	+	+	+		+	3	2	+
21	56/M	Ma/>10	+	+;I				0	1	+
22	47/M	Ma/>20	+	+	+			0	1	+
23	56/M	Ma/>20	+		+	+	+	5	2	+
24	49/M	Ma/>50	+	+;I	+			4	2	+
25	47/F	Ma/>20	+	+;I				3	2	+
26	33/M	Ma/>50		+;II			+	3	1	–
27	46/M	Mi+Ma/>50			+	+	+	6	2	–
28	63/M	Ma/>50			+		+	5	3	+
29	43/M	Mi+Ma/>30						3	2	+
30	31/M	Ma/>30		+		+	+	4	3	+
31	44/M	Ma/ 50			+		+	5	3	+
32	43/F	Ma/>50						0	0	–
33	59/M	Ma/>50		+;I				3	1	–
34	70/M	Ma/>50						2	2	+
35	60/M	Ma/ 20		+;I+II		+		2	3	+
36	38/M	Mi+Ma/>30		+;I				0	1	–
37	49/F	(–)						1	0	–
38	58/M	Ma/ 25					+	0	1	–
39	45/M	Ma/<10		+;I+II		+		5	2	–
40	79/M	Mi</10					+	0	2	+
41	21/M	Mi/<10						0	2	+
42	72/M	Mi/<10						0	2	–
43	70/M	(–)						0	1	–

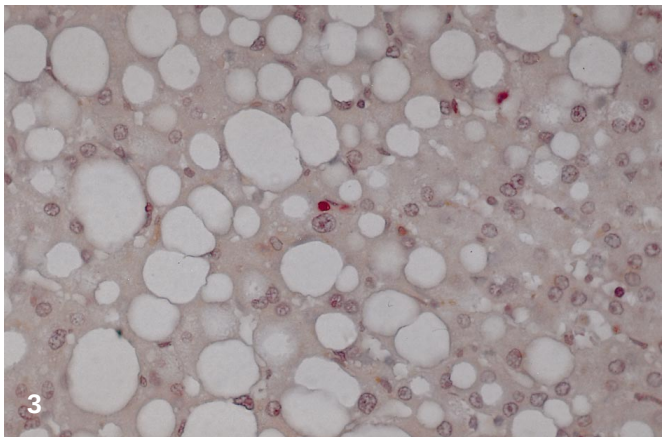
of similar intensity to the labelling observed in nuclei of bile duct and ductular cells (Fig. 2) and non-parenchymal cells (Fig. 3). With the staining protocol used, the localization of reaction product was specific, that is to say only nuclei were stained and not the cytoplasm. In some situations morphologically detectable apoptotic bodies occurring with low frequency disclosed a very strong reaction product within a condensed nucleus (Fig. 4). Interestingly, TdT labelling was not observed in nuclei of ballooned hepatocytes with or without MBs (Fig. 5).

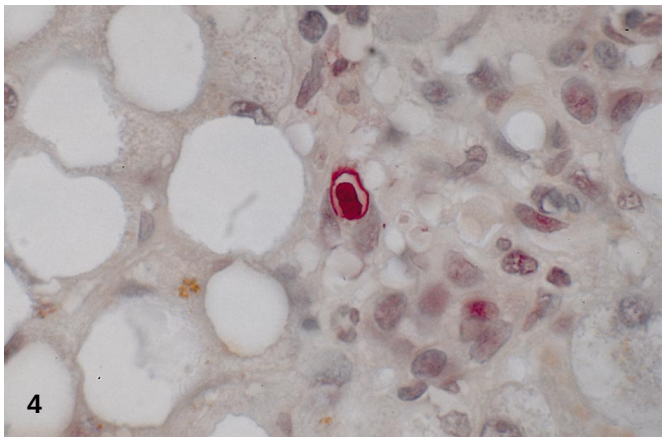
In normal control biopsies, very few, mostly non-parenchymal, cells showed nuclear reactivity (data not shown). Biopsies from patients with haemochromatosis (prefibrotic and fibrotic stages) exhibited nuclear reactivity in lymphocytes forming portal tract infiltrates, in a few bile duct cells (both in small interlobular bile ducts and in ductules), and in hepatocytes, the latter mainly in the pericentral zone of parenchyma (data not shown).

Hepatocyte TdT labelling indices (LIs) for the 43 biopsies as a function of the cell position in lobules or in the nodules are listed in Table 2, and their relationship with major morphological changes associated with ALD is given. For all 43 biopsies, the total mean TdT-LI in acinar or nodule central halves was 4.0%, whereas the mean TdT-LI for the acinar or nodular peripheral halves was 5.3% (NS). In biopsies showing preserved lobules (fibrosis classes 0–4), the mean TdT-LI for pericentral hepatocytes was 2.8% and that for periportal hepatocytes, 5.2% (NS). With respect to lobular centres, it is









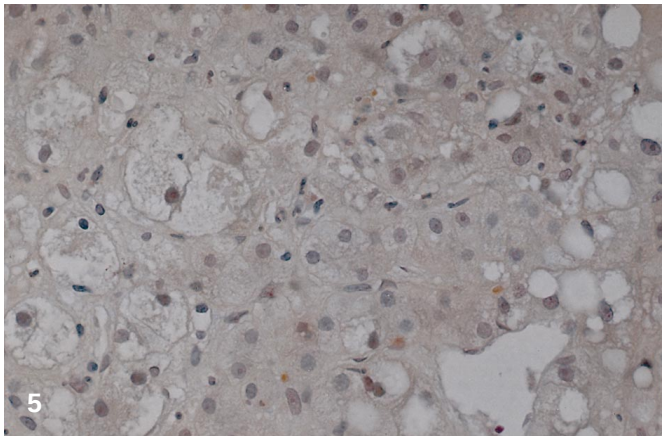


Fig. 1 Liver tissue in alcoholic liver disease (ALD), showing macrovesicular fatty change of some hepatocytes (*central part*) and incipient distortion of liver cell plate architecture related to perisinusoidal fibrosis. Note that one hepatocyte nucleus is strongly stained after the TdT reaction. ISEL procedure, haematoxylin counterstain, ×320

Fig. 2 Peripheral lobular areas and portal tract of an ALD biopsy. Hepatocyte fatty change is only slight in degree in this field, but stronger in centrilobular parts. One hepatocyte nucleus (*left half*) discloses strong nuclear TdT reaction staining similar in intensity to that of a few bile duct/ductular cell nuclei (*top left of centre*). Some hepatocyte nuclei are pink, that is they exhibit questionable staining ISEL procedure, haematoxylin counterstain, ×180

Fig. 3 Liver tissue in ALD, showing major macrovesicular fatty change of hepatocytes. In the centre, a strongly reactive nucleus of a non-parenchymal cell is seen (probably an intrasinusoidal lymphocyte). Hepatocyte nuclei are not TdT-reactive in this area. ISEL procedure, haematoxylin counterstain, ×180

Fig. 4 Liver biopsy in ALD. Hepatocytes show macrovesicular fatty change (*left half*). In the centre, a condensed body with strong TdT-reaction product is seen. Most probably this alteration represents an apoptotic body. ISEL procedure, haematoxylin counterstain, ×320

Fig. 5 Pericentral area of an ALD liver biopsy; the central vein is seen in the *bottom right corner*, where some fatty change is also present. The left half of the figure shows several enlarged hepatocytes exhibiting ballooning degeneration. The rightmost of these cells harbours a Mallory body (the nucleus is not seen). Ballooned hepatocytes with or without Mallory bodies did not disclose nuclear TdT reaction staining. ISEL procedure, haematoxylin counterstain, ×180

seen that the mean TdT-LI was significantly higher in areas showing heptocytes with ballooning degeneration, MBs and cholestasis, even though ballooned hepatocytes themselves (with or without MBs) were not labelled. No significant correlation was found for the parameters, fatty change, pericellular granulocytic reaction, presence or absence of megamitochondria, haemosiderosis and intralobular or portal tract lymphocytic inflammatory reaction. The mean hepatocyte TdT-LI was significantly higher in biopsies showing normal central veins than in those with central vein sclerosis (perisclerosis), and there was a trend for a lower TdT-LI in biopsies without peri-

Table 2 TdT labelling indices of hepatocytes and bile ductular cells: correlation with major histopathological changes (*TdT-LI* TdT-labelling index)

Histopathology		*n*	TdT – LI (%, mean ± SD)	*P*
Central area of lobules/nodules				
Fatty Change	>50%	23	5.9±1.0	0.775
	10–50%	10	5.0±1.5	
	<10%	10	4.1±1.5	
Ballooning degeneration	Present	19	7.7±1.0	**0.001**
	Absent	24	3.3±0.9	
Mallory Bodies	Present	25	6.4±0.9	**0.050**
	Absent	18	3.7±1.1	
Megamitochondria	Present	17	5.0±1.2	0.501
	Absent	26	5.5±0.9	
Cholestasis	Present	12	8.2±1.3	**0.050**
	Absent	31	4.2±0.8	
Fibrosis	Grade-0	10	3.7±1.3	0.100
	Grade-2	3	2.3±2.4	
	Grade-3	9	4.4±1.4	
	Grade-4	5	12.2±1.90	
	Grade-5	12	5.5±1.2	
	Grade-6	3	5.3±2.4	
Central vein fibrosis	Present	29	3.4±1.2	**0.035**
	Absent	14	6.2±0.9	
Peripheral area of lobules/nodules				
Fatty Change	>50%	23	4.2±0.8	0.622
	10–50%	10	2.9±1.2	
	<10%	10	4.7±1.2	
Ballooning degeneration	Present	19	6.0±0.8	**0.001**
	Absent	24	2.5±0.7	
Mallory Bodies	Present	25	5.0±0.7	**0.006**
	Absent	18	2.6±0.8	
Megamitochondria	Present	17	4.4±0.9	0.844
	Absent	26	3.8±0.7	
Cholestasis	Present	12	5.8±1.0	**0.018**
	Absent	31	3.3±0.6	
Ductular proliferation	Present	21	5.7±0.7	**0.001**
	Absent	22	2.4±0.7	
Fibrosis	Grade-0	10	1.2±0.8	**0.000**
	Grade-2	3	3.0±1.4	Grade-6 vs 0, 2, 3
	Grade-3	9	2.2±0.8	Grade-5 vs 0
	Grade-4	5	7.4±1.1	Grade-4 vs 3
	Grade-5	12	5.0±0.7	
	Grade-6	3	11.7±1.4	

sinusoidal fibrosis than in those showing this change to a variable degree.

When the hepatocyte TdT-LI was assessed in the lobular or nodular periphery, a similar pattern emerged. Again, hepatocyte TdT-LI was significantly higher in areas showing ballooning degeneration of hepatocytes, MBs (mainly in situations of nodular change, where MBs occur in peripheral parts of parenchyma) and cholestasis.

Mean hepatocyte TdT-LIs increased as a function of the grade of septal fibrosis, significant differences occurring for fibrosis grade 6 vs grades 0, 2, and 3, grade 5 vs grade 0, and grade 4 vs grade 3. Biopsies showing ductular proliferation in portal tracts or septa exhibited a significantly higher TdT-LI of hepatocytes.

TUNEL positivity also occurred in the population of bile duct cells in small interlobular bile ducts and ductules. The TdT-LI in this cell population was significantly higher in biopsies showing ballooning degeneration of hepatocytes, but there was no correlation with MB formation (data not shown). In addition, mean ductular cell TdT-LI was significantly higher in biopsies showing fibrosis of any degree than in those without fibrosis ($P = 0.006$).

Discussion

In situ DNA nick end-labelling (ISEL/TUNEL) has been suggested as a suitable instrument for detecting cells undergoing apoptosis [3, 20], being thought to permit identification of cells that have entered an apoptotic pathway at an early time point while morphological alterations

are absent or limited in extent. Cell injury resulting in cell death with or without formation of apoptotic bodies produces a multitude of new 3'-OH-DNA ends generated by enzyme-mediated DNA cleavage, and TUNEL allows visualization of this phenomenon. However, it has been emphasized that DNA nick end-labelling is not specific for programmed cell death [3], the reason being that other forms of cell death, including those fulfilling the criteria of necrosis (such as ischaemic myocardial necrosis) start with the same phenomenon of DNA fragmentation. Therefore, the conceptual ambiguity with respect to a dualism, "apoptosis" vs "necrosis", has to be taken into account and results obtained by using TUNEL must be interpreted with caution. The consequence for the present work is that we preferred to rely on TdT labelling of nuclei per se, because forms of cell death other than apoptosis may have played a significant part.

The present retrospective study on liver biopsies indicates that increased hepatocyte TUNEL positivity occurs in ALD. Nuclear reactivity (TdT labelling) of parenchymal cells was observed to varying degrees in both central and peripheral parts of lobules and nodules. The finding that there was no significant difference of the hepatocyte TdT-LI in pericentral vs periportal areas is in contrast to previous observations indicating a higher prevalence of apoptotic bodies in the pericentral zone [5]. Theoretically, one would expect a higher magnitude of cell loss in this particular area of the lobule, because liver damage in ALD, as visualized by fatty change, hepatocyte ballooning, disarray of liver cell plates and perisinusoidal fibrosis, typically starts in the parenchyma around the central vein [4, 10, 16, 19, 25, 37, 50]. The absence of evidence for a lobular gradient of TUNEL-positive hepatocyte nuclei in ALD may be compatible with the assumption that pericentral parenchymal damage involves pathways of cell death not observable by a method detecting DNA fragmentation, but cell membrane and organelle breakdown caused by toxicity of ethanol itself and/or its metabolites, in particular acetaldehyde. In fact, during the metabolism of ethanol and the further metabolism of acetaldehyde by cytochrome P450IIE1, toxic free radicals are formed [24, 31]. These highly reactive agents can cause lipid peroxidation in several cellular membrane systems [9, 12, 13, 27]. Furthermore, acetaldehyde itself can alter cellular proteins owing to its capability to form adduction products, thus leading, by poorly elucidated mechanisms, to cell and tissue injury, typically in a distinct zonal distribution [23, 32].

When we focused on sites where important cell and tissue damage was in evidence (areas showing hepatocyte ballooning, MB formation, cholestasis and perisinusoidal fibrosis), however, it turned out that nuclear TdT staining was significantly elevated in these areas in comparison with those not showing these changes. The finding of an accumulation of TUNEL-positive hepatocytes at visibly altered parenchymal sites suggests that, in florid ALD, cell injury characterized by DNA fragmentation plays a significant role in alcohol-induced cell and tissue damage, and some part of these phenomena may be related to apoptosis. Apoptosis has long been proposed as a mechanism for hepatocellular loss in several liver disorders, including acute and chronic hepatitits, and graft rejection [1, 6, 30, 35, 39, 43, 46]. However, the reason why morphologically detectable apoptosis of hepatocytes (particularly apoptotic bodies) in ALD and other types of liver damage is not easily, or frequently, seen may be related to the fact that the half-life of apoptotic cells in tissues is at most several hours [2, 34, 36]. The probability of detection in tissue sections may be high when large numbers of affected cells are present, but much lower in other situations. Alternatively, a low prevalence of apoptosis may exist. That increased apoptotic liver cell loss occurs in alcoholic liver injury has previously been suggested on the basis of rat models [7, 21, 47, 52] and of findings in human liver [26], and ethanol has been shown to increase apoptotic cell death of thymocytes in vivo [17]. In alcohol-fed rats, the degree of changes interpreted as apoptosis evaluated by both immunohistochemistry and flow cytometry was related to the presence of pathological changes, but there was no specific zonal pattern for the distribution of cells involved [52].

The TdT-LI of parenchymal cells increased as a function of increasing septal fibrosis, that is with progression of disease. This phenomenon may either indicate that the severity of hepatocyte damage is higher in the fibrotic stages of ALD, or that increased cell loss accompanies a higher hepatocyte turnover in situations of parenchymal remodelling.

Interestingly, we could not detect nuclear TdT-labelling in hepatocytes showing ballooning degeneration with or without formation of MBs. However, a previous investigation employing the expression of Le^y antigen as a phenotypic marker of apoptosis has demonstrated this apoptosis-related antigen in hepatocytes harbouring MBs in alcoholic hepatitis [26]. These different findings suggest either that cells exhibiting these alterations may not have entered a pathway related to programmed cell death, or that the different methods used by Kawahara et al. [26] and by us detect different phases of apoptosis. Morphometric quantitation of cytoplasmic structures in ballooned hepatocytes has revealed a large increase in nonorganelle cytoplasmic volume associated with an increase in mitochondrial and SER volumes, but it has been shown that ballooning is in fact related to reversible cell swelling owing to failure of membrane cation pumps in ALD (for review, see [19]). Also the observation that cells harbouring MBs can be detected in biopsies for considerable time periods after cessation of ethanol exposure indicates that this type of cell damage may not result in cell death, or at least not necessarily.

We conclude that increased hepatocyte TdT labelling occurs in human ALD; that there is no overall lobular gradient in the hepatocyte TdT-LI; that the highest TdT-LI is present in areas of morphologically maximal tissue damage; that the hepatocyte TdT-LI appears to increase

as a function of liver fibrosis and nodular change; and that ballooned hepatocytes with or without MBs do not exhibit TdT labelling.

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