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Apoptosis in hepatocellular carcinomas with neuroendocrine differentiation

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Summary. We have studied apoptosis in a subset of thirty hepatocellular carcinomas (HCCs) exhibiting neuroendocrine differentiation (ND). Apoptosis was assessed by use of in situ DNA end labelling, and was quantified employing a TdT labelling index. It turned out that apoptosis occurred in HCCs with ND, albeit at different rates. Apoptosis was visualized as a clearly detectable reaction product mostly localized in tumor cell nuclei and in apoptotic bodies. Almost no staining was observed in nuclei of multinuclear tumor giant cells. In HCCs with ND, apoptosis was not related to type or grade, but tumors mainly consisting of hepatocyte-like cells and/or clear cells showed a significantly higher apoptotic rate. This was also the cell type most frequently disclosing neuroendocrine features. The apoptotic rate was significantly higher in HCCs with ND than in a control group of HCCs not showing ND. The findings suggest that neuroendocrine differentiation in liver cell tumors is associated with an altered pattern of programmed cell death, and that this phenomenon may therefore have an influence on clinical behavior.

Key words: Hepatocellular carcinoma, Apoptosis, Liver, Neuroendocrine cells

Introduction

There is increasing evidence that neuroendocrine differentiation does not only occur in tumors originating in neuroendocrine tissues, but also in neoplasms without an evident neuroendocrine phenotype in conventional histological sections (Bosman, 1989; Wright, 1990). This also holds true for neoplasms of the hepatocyte lineage. It has been demonstrated that hepatocellular carcinomas (HCCs) and hepatoblastomas may contain cellular components with carcinoid features (Barsky et al., 1984; Ruck et al., 1990). Furthermore, several studies have uncovered signs of neuroendocrine differentiation in hepatocellular tumors not showing a carcinoid-like phenotype (Payne et al., 1986; Wang et al., 1991; Zhao et al., 1993). The detection of neuroendocrine features in hepatocellular carcinomas is of interest insofar as tumors with neuroendocrine differentiation may generally behave more aggressively than counterparts without such features (Arends et al., 1986; Sarsfield and Anthony, 1990; Hsu et al., 1991). As in many renewing normal tissues and in other neoplasms, the growth of HCCs with or without neuroendocrine differentiation is not only determined by cell proliferation, but also by cell death. Among factors operational in tissue cell loss, apoptosis as a form of programmed cell death holds a particularly important place. Apoptosis is now recognized to represent a widespread phenomenon that counterbalances cell proliferation to preserve tissue homeostasis and to determine the overall growth of neoplastic processes. Apart from factors stimulating the apoptotic pathway in tumors, protection against apoptosis through the action of bcl-2 has also been suggested to play a significant role in HCCs (Zhao et al., 1994). The detection and quantification of apoptosis based on conventional preparations is difficult owing to the fact that cell shrinkage, nuclear condensation, and chromatin fragmentation mostly occur during advanced stages of the process only, the halflife of apoptotic cells in tissues thought to be in the region of several hours (Searle et al., 1987). It is, therefore, necessary to be capable of identifying apoptotic cells in an early stage of this active pathway (Allen et al., 1993). This can be achieved by use of in situ DNA end-labelling (ISEL; Gavrieli et al., 1992; Ansari et al., 1993), which exploits the presence of DNA breaks occurring in nuclei of cells undergoing apoptosis. ISEL has previously been employed for studying several liver diseases, including allograft rejection and end-stage liver disease (Afford et al., 1995), and HCC (Hino et al., 1996). In order to assess the apoptotic rate in a distinct subset of HCCs with neuroendocrine differentiation, we performed a systematic retrospective study using ISEL on 30 HCCs with previously confirmed neuroendocrine features (Zhao et al., 1993).

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Material and methods

Material for histolgy

The study was based on 30 biopsies or resection specimens of HCCs with neuroendocrine differentiation. Typing and subtyping of these tumors was performed according to published criteria (Gibson and Sobin, 1978; Nakashima and Kojiro, 1987). Grading was based on a four-grade procedure (Edmondson and Steiner, 1954). Age and sex of the patients, and types and grades of HCCs are listed in Table 1. 20 HCCs not showing neuroendocrine features were used as tumor controls. Biopsies of human tonsils and of 13 livers showing no relevant histological change (staging biopsies) served as normal controls.

Formalin-fixed and paraffin-embedded material was used for conventional light microscopy, immunohistochemistry and ISEL. Tissue samples were fixed in 10% formaldehyde solution. Formalin fixation before paraffin embedding was less than 30 hours throughout, which is important for both immunohistochemistry and ISEL. For typing and grading of the lesions, paraffin sections were stained with hematoxylin-eosin, PAS, van Gieson's, and Gomori reticulin stains.

Assessment of distinct cell types occuring in HCCs

In order to test whether apoptosis would preferentially occur in distinct cell types occuring in HCCs, several epithelial cell types were assessed in each tumor. These included medium- to large-sized granular cells microscopically resembling hepatocytes (GR), eosinophilic cells (EO), and large and oxyphilic cells (OX). In addition, cells deviating from the usual hepatocyte morphology were registered separately (clear cells, CL; cells with fatty change, FA; giant cells, GI; spindle cells, SP; and polymorphous cells, PO). These different cell types may form the majority of a given tumor and are known to be related to type and grade, but they may also occur in heterogenous distribution in HCCs (Nakashima and Kojiro, 1987; Zimmermann, 1994).

Immunohistochemical detection of neuroendocrine features

Methodological details for the immunohistochemical detection of neuroendocrine features in the tumors presently analyzed have previously been published (Zhao et al., 1993). Antibodies for the detection of neuroendocrine features included neuron-specific enolase (NSE), serotonin (5HT), S100 protein (S100), somatostatin (SOM), synaptophysin (SYN), chromogranin A (CG; all these 6 antibodies from DAKO), neurotensin (NEU), vasointestinal peptide (VIP), bombesin (BOM; these 3 antibodies from Biogenex), Leu-7 (LEU; Becton Dickinson), pancreatic polypeptide (PAP; DAKO) and PGP 9.5 (PGP; Ultraclone). Immunohistochemistry itself was performed by use of modified ABC and APAAP procedures (Zhao et al., 1993). Positive control sections expressing respective immunodeterminants were processed in parallel. Positive normal control tissues for the assessment of neuroendocrine markers included brain, spinal cord, duodenal mucosa, skin, pancreas, hypothalamus, adrenal gland, gastric mucosa, lung and prostate. With the exception of bombesin, all antisera directed against neuroendocrine determinants used in the present study led to a clearly detectable reaction product in the tissues in which expression of the corresponding marker was anticipated (Zhao et al., 1993).

In situ DNA-end-labelling (ISEL)

For the in situ visualization of apoptotic cells, ISEL, according to a method previously published (Gavrieli et al., 1992), was employed.

Paraffin sections (4 µm thick) were affixed to Super Frost/plastic slides (Menzel-Gläser, Germany). Deparaffinization was performed by heating the sections for 4 hours at 58 °C. Hydration was executed by transferring 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 as even traces of impurities may interfere with the reactions. The prepared paraffin sections were then digested by incubation with 5 µg/ml proteinase K (Sigma Chemical Company) 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 with double-distilled water three times. The TdT (terminal deoxynucleotidyl transferase; nucleoside-triphosphate: DNA deoxynucleotidyl exotransferase; EC 2.7.7.31) reaction mix (30 mM Trizma base, pH 7.2, 140mM sodium cacodylate, 1mM cobalt chloride, TdT enzyme 0.2 µ/ml, and digoxygenin-conjugated or biotinylated dUTP in ENTP buffer; Boehringer, Mannheim) 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 2x SSC buffer for 10 min (twice) and TB buffer (300mM sodium chloride, 30mM sodium citrate) for 10 min (twice) and sections were incubated 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 anti-digoxygenin-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, Merck). All TdT tailing reaction reagents were purchased from Boehringer, Mannheim.

Assessment of apoptotic rate (based on ISEL)

TdT-reactive nuclei of HCC cells were counted within the areas of interest. The number of positive nuclei per 1000 nuclei analyzed was used as a TdT labelling index (TdT-LI; permille). Within HCCs, five areas of interest were randomly chosen, and in each area 1000 cells were analyzed. For control biopsies, the same strategy was employed.

Statistical analysis

For the statistical analysis of differences in apoptosis labelling between groups, one-way Kruskal-Wallis and Kolmogorov-Smirnov analyses were employed. $p<0.05$ was considered as statistically significant.

Results

Morphological and immunohistochemical characterization of HCCs

The pertinent findings are summarized in Table 1. It is seen that tumors with a trabecular or mixed (i.e. trabecular and pseudoglandular) growth pattern predominated. Three tumors contained considerable amounts of neoplastic cells with fatty change and were, therefore, classified as so-called fatty HCCs. Two HCCs were of the fibrolamellar variant. Most HCCs were of grade 2 or 3 according to Edmondson and Steiner (1954), a grade 1 occurring per definition in the two FL-HCCs, whereas grade 1 was found only as small components in the other tumors.

With respect to predominant cell types in HCCs, Table 1 shows that granular and eosinophilic, i.e. hepatocyte-like cells were the most frequent cell type encountered. One HCC was entirely of the clear cell variant, whereas clear cell components were observed together with other cell types in 8 other tumors. Giant cells, which appear to occur most frequently in grade 3

Table 1. Hepatocellular carcinomas (HCCs): type, grade, cellular composition, neuroendocrine features, and apoptotic rate.

CASE	AGE	SEX	MICROSCOPY			NEUROENDOCRINE FEATURES		APOPTOTIC RATE
			Type	Grade	Cell types	Marker	Intensity	(TdT-Li, ‰)
1	69	M	T+P	2	CL+GR	LEU+S100	± - ++	1.0
2	74	M	T+P	2	GR-CL	NEU	+	2.0
3	43	M	T	2	CL	NEU	+	0
4	85	M	T	2+3	GR	SOM	+	0.0
5	69	F	T+P	2	CL+GR+GI	LEU	+	3.0
6	39	M	T	2	GR+SP	SYN	±	2.0
7	79	M	T+P	3	GR+EO	LEU+S100	+-++	0.0
8	55	M	T	2	GR	LEU	++	86.0
9	67	M	T	1	GR	LEU	++	2.5
10	64	M	T	3	GR+CL	LEU	++	1.0
11	55	M	T	2+3	GR	LEU+NSE+CG	+-+++	0.0
12	70	M	T+P	3+4	PO	LEU+S100	++-+++	55.0
13	65	M	T	2	GR	SYN+NEU	±-+	0.0
14	51	M	T	2	GR+EO	S100	++	0.0
15	69	M	T	2	GR+EO	LEU+SYN+NSE	±-+++	2.0
16	33	M	T+P	1+2	EO+GR	SYN+NSE+SOM	±-++	1.0
17	69	M	T	1+2	CL+G	S100+NSE	+-++	2.0
18	1	M	T+P	3	GR+CL	S100	++	20.0
19	41	M	T	3	GR+GI	S100	+++	36.0
20	43	F	T	3	GR+GI	LEU	++	7.0
21	47	M	T+P	2	GR+FA	LEU+S100	±	6.0
22	55	M	T	1+2	GR	CG	++	0.0
23	63	M	T+P	2+3	EO+PO+GI	SOM	+	50.0
24	11	M	T	1+2	GR	S100	++	0.1
25	6	M	T+C	3	EO+GR	LEU+S100+NSE+CG	+-++	250.0
26	26	M	T	2	EO+GR	SYN	++	0.0
27	45	F	T+C	3+4	FA+GR+CL+OX+PO	S100+SO+M	+-++	9.0
28	42	F	T+P	3	FA+GR+CL+GI	S100		13.0
29	26	M	T	1	OX(FL-HCC)	S100	+++	11.0
30	34	M	T	1	OX(FL-HCC)	LEU+VIP	+-++	12.2

Abbreviations: HCC type: T, trabecular (sinusoidal); P, pseudoglandular (acinar); C: compact (solid). HCC grade (Edmondson and Steiner, 1954); grades 1 to 4. Cell type: GR, granular cell; CL, clear cell; EO, eosinophilic cell; FA, fatty cell; GI, giant cell; SP, spindle cell; PO, polymorphous cell; OX, oxyphilic cell (FL-HCC; fibrolamellar HCC). Abs (antibodies): NSE, neuron-specific enolase; S100, S100 protein; SOM, somatostatin; SYN, synaptophysin; CG, chromogranin A; NEU, neurotensin; VIP vasointestinal peptide; LEU, Leu-7. Intensity (immunostaining: +++, marked; ++, moderate; +, weak; ±, very weak.

lesions (Nakashima and Kojiro, 1987) were found in five cases, polymorphous cells in three cases, and spindle cells in 1 case.

With respect to neuroendocrine differentiation, 30/50 HCCs displayed one or more neuroendocrine markers, but of the antisera tested, only eight gave positive results in HCC sections (Table 1), as previously reported (Zhao et al., 1993). Positivity for LEU and S100 was observed most frequently (26%), followed by NSE and SYN (10% each), SOM (8%), CG and NEU (6% each), and by VIP in one case. Reactivity for several neuroendocrine markers was observed in 24% of the cases.

Apoptosis in HCCs with neuroendocrine features

Apoptosis as based on TdT-labelling was visualized as a clearly detectable reaction product preferentially localized in tumor cell nuclei (Fig. 1). In several cases, small condensed bodies morphologically corresponding to apoptotic bodies were found to be strongly immunoreactive (Fig. 2), the more intense reaction product being most probably due to densely packed chromatin (karyopyknosis) exhibiting reactive DNA sites. Furthermore, few HCC cells exhibited TdT-staining not only restricted to the nucleus, but also in the cytoplasm (Fig. 3). This phenomenon may be causally related to entry of TdT-reactive DNA fragments from a damaged and permeabilized nucleus into the cytoplasm. Tumor cells with reactive nuclei and TdT-stained apoptotic bodies were detected in some instances in areas showing classical tumor necrosis (Fig. 4). In necrotic foci, TdT-reactivity frequently involved the debris probably rich in reactive DNA fragments (Fig. 5). Apoptosis as based on ISEL was observed in both trabecular and pseudoglandular/acinar types and was not related to tumor grade. Positive nuclei were observed in polymorphous

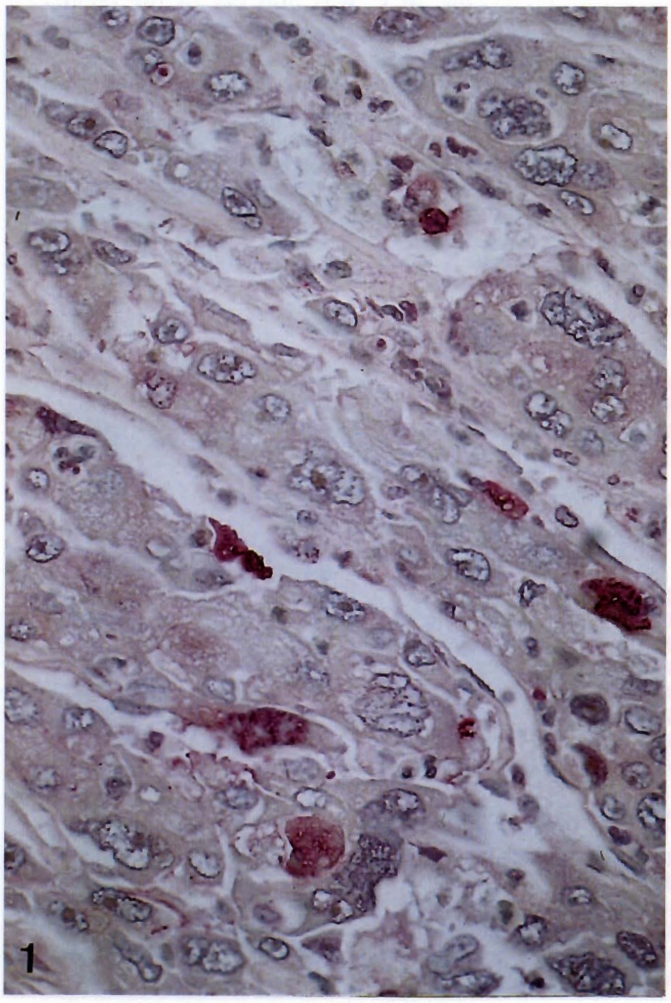

Fig. 1. Hepatocellular carcinoma, trabecular type, grade 3 according to Edmondson and Steiner (1954). Note that part of the neoplastic cells are large and contain polymorphous nuclei. Few tumor cell nuclei are TdT-labelled. In addition there is an apoptotic body exhibiting staining of the nuclear remnant (top half of figure; ISEL procedure, hematoxylin counterstain). x 320

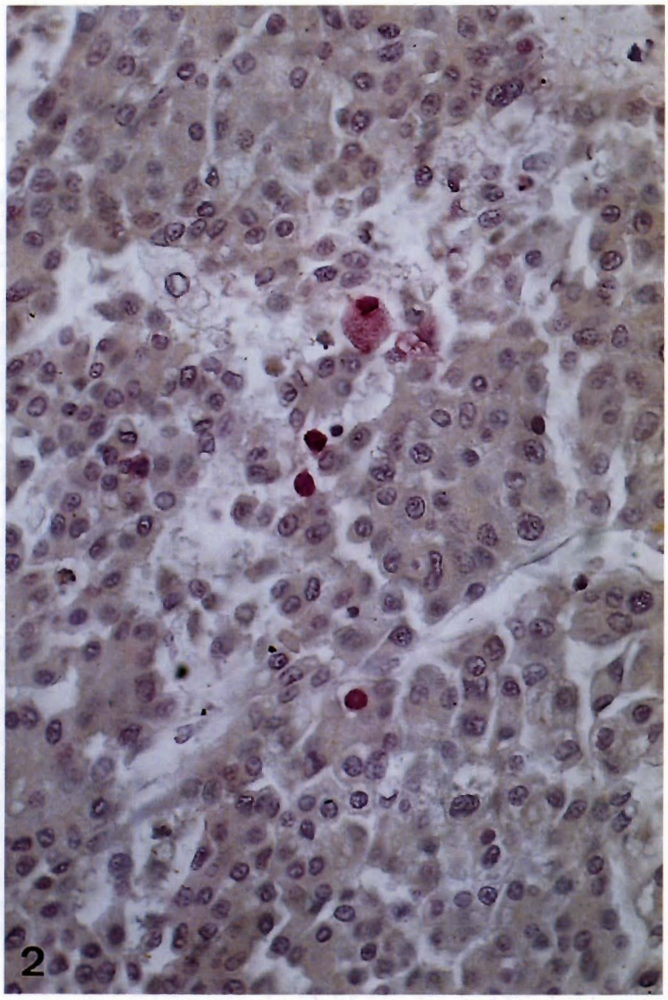

Fig. 2. In this trabecular, grade 2 hepatocellular carcinoma, three apoptotic bodies are seen. They are easily detectable owing to their dense structure and the strong TdT-staining. (ISEL procedure, hematoxylin counterstain). x 320

tumor cells, but not in nuclei of tumor giant cells (Fig. 6).

Apoptotic rate of HCCs

Apoptotic rates of HCCs with neuroendocrine features expressed as TdT labelling indices (TdT-LI; permille) are listed in Table 1. It is seen that TdT labelling of tumor cells was detected in 22/30 cases, TdT-LIs ranging from 0.0 to 250 permille (mean: 19.1 ±12.2). In comparison with tumors, only very few nuclei of normal-looking hepatocytes were TdT-positive, similar to the normal liver tissue samples used as controls. In HCCs without neuroendocrine differentiation, the mean TdT-LI was 5.8 (±0.7) permille (data not shown). Table 2 shows the correlation of TdT-LIs of HCCs with neuroendocrine features with respect to the non-neuroendocrine HCC control group, HCC growth pattern (trabecular vs. pseudoglandular vs. compact), tumor grade, and major HCC cell types encountered in the tumors. The apoptotic rate of HCCs with neuroendocrine differentiation was significantly higher than that of HCCs without neuroendocrine features. There was no correlation with respect to growth pattern or to tumor grade. With regard to major cell types involved, tumors chiefly consisting of granular (i.e., hepatocyte-like) and/or clear cells exhibited a significantly higher TdT-LI than the other tumors, whereas tumors containing morphologically highly differentiated eosinophilic cells revealed a lower TdT-LI.

Discussion

By use of in situ DNA labelling about two thirds of hepatocellular carcinomas (HCCs) with neuroendocrine differentiation disclosed signs of apoptosis, and the apoptotic rate expressed as a TdT labelling index was significantly higher in this subset of tumors than in a control group of HCCs not showing neuroendocrine

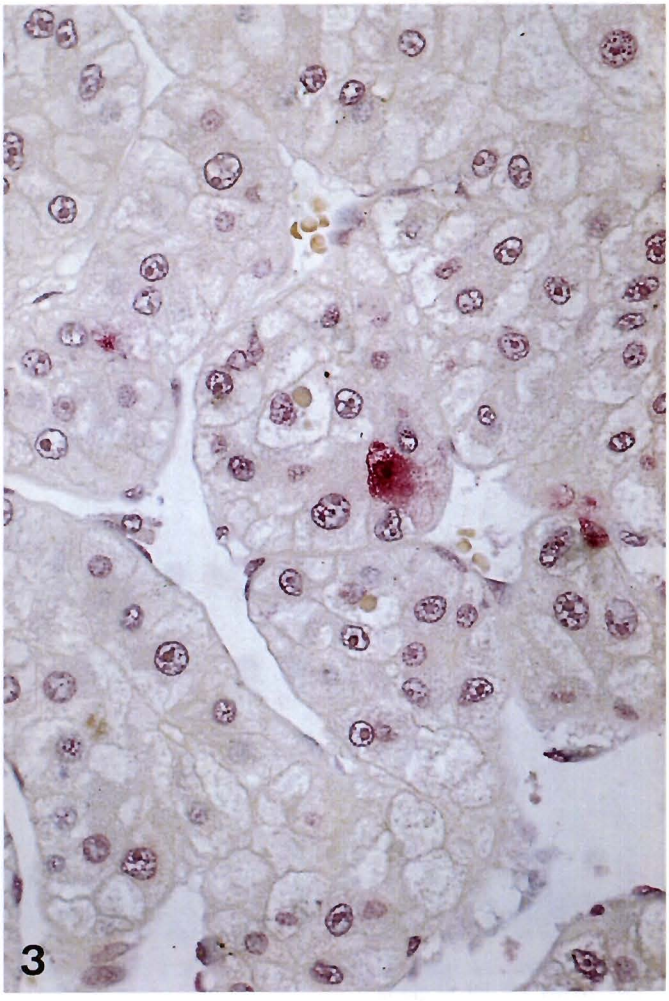

Fig. 3. Hepatocellular carcinoma, trabecular type, grade 2. One tumor cell exhibits TdT staining of both the nucleus and cytoplasm (ISEL procedure, hematoxylin counterstain). x 400

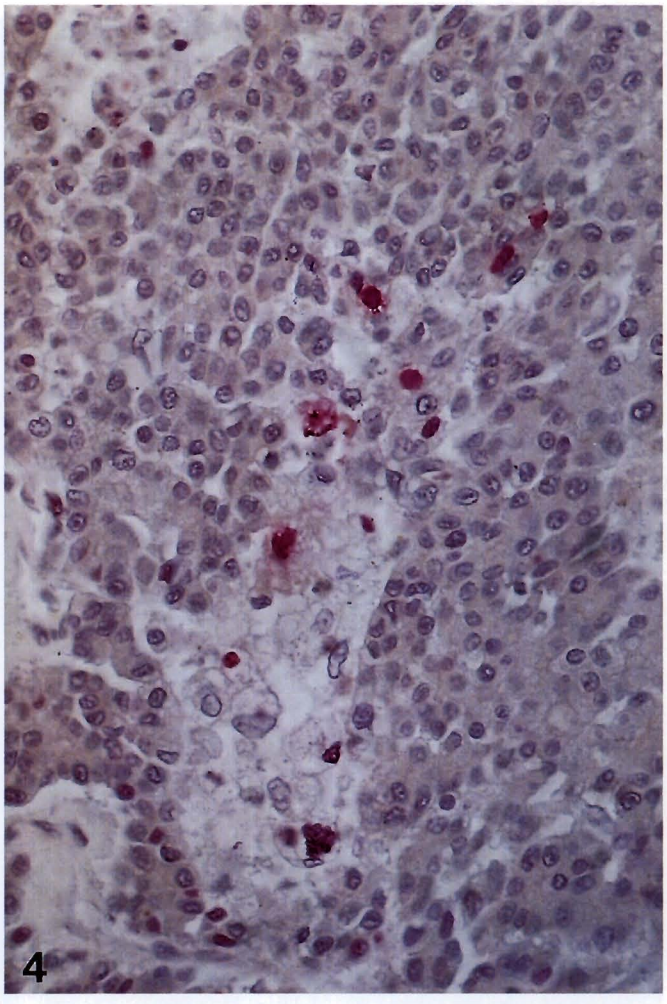

Fig. 4. A small area of necrosis is seen in this hepatocellular carcinoma (center of the figure). Note that a few tumor cells close to the necrosis show TdT-reactivity, whereas more remote cells do not (ISEL procedure, hematoxylin counterstain). x 320

Table 2. Hepatocellular carcinomas (HCCs) with neuroendocrine differentiation: correlation of TdT-labelling indices (TdT-LI) with respect to non-neuroendocrine HCCs, growth pattern, tumor grade, and cell type.

PARAMETER	p
Neuroendocrine vs. non-neuroendocrine HCC	0.016
Growth pattern	
(T+P vs. T vs. T+C)	0.104
Tumor grade	
(1 vs. 2 vs. 3 vs. 4)	0.105
Cell type	
CL vs GR	0.778
CL vs. MX	0.001
CL vs. EO	0.778
GR vs. MX	0.000
GR vs. EO	0.186
MX vs. EO	0.001

P: level of significance. Significant correlations are underlined. For abbreviations, see Table 1. MX: Group containing cell types FA, GI, SP and OX (see Table 1).

features. Apoptosis was visualized in the form of a clearly detectable reaction product chiefly localized in the tumor cell nuclei and in condensed apoptotic bodies, but some cells exhibited cytoplasmic staining as well probably owing to entry into the cytoplasm of TdT-reactive DNA fragments from damaged nuclei. Almost no staining was detected in nuclei of multinuclear tumor giant cells. Apoptosis was not related to HCC type or grade, but tumors mainly consisting of hepatocyte-like cells and/or clear cells showed a significantly higher apoptotic rate, whereas HCCs rich in morphologically well differentiated eosinophilic cells revealed a lower apoptotic rate.

Apoptosis has a central function in the cell turnover of normal and neoplastic tissues (Majno and Joris, 1995). It has been demonstrated that programmed cell death is operational in normal liver cells and in several types of hepatobiliary diseases (Alison and Sarraf, 1994;

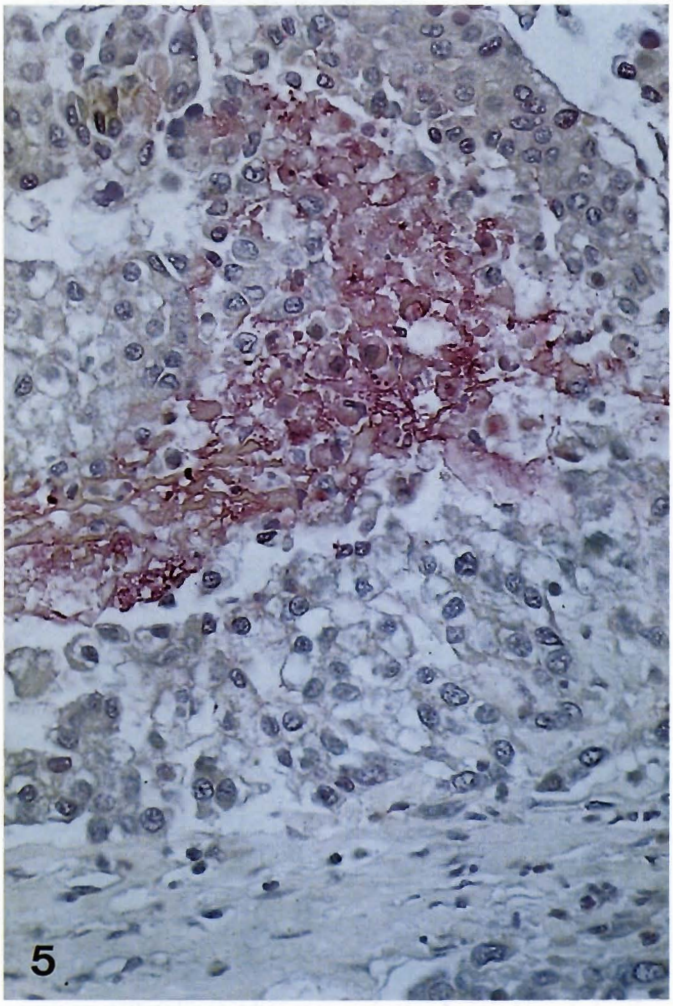

Fig. 5. A larger necrosis is visualized in this hepatocellular carcinoma. Most of the TdT-staining is due to reactivity of debris probably rich in reactive DNA fragments (ISEL procedure, hematoxylin counterstain). x 320

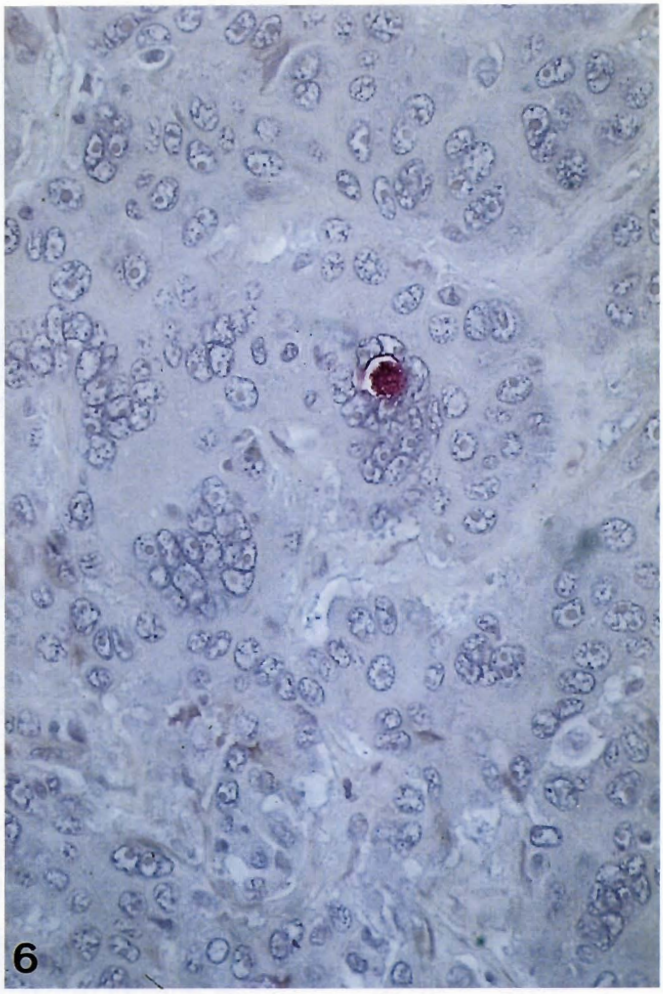

Fig. 6. Multinuclear giant cells occurring in a subset of hepatocellular carcinomas (mostly of grade 3 according to Edmondson and Steiner (1954)) are shown in this figure. Note that the numerous nuclei of these cells are TdT-negative. The stained spherical structure represents an apoptotic body apparently engulfed by a multinuclear tumor cell (ISEL procedure, hematoxylin counterstain). x 320

Afford et al., 1995; Patel and Gores, 1995). Loss of cells via apoptosis physiologically takes place in the normal liver, however at a very low rate (Bursch et al., 1984), and increased hepatocyte proliferation upon stimulation is followed by an elevated apoptotic rate (Columbano et al., 1985) in order to preserve tissue homeostasis. As in normal tissues, cell turnover and growth of neoplasms are determined by the difference between cell production through proliferation and cell loss, and it is therefore expected that apoptosis should also take place in liver tumors. It has been demonstrated that liver carcinogens act both by stimulation of cell replication and apoptosis (Schulte-Hermann et al., 1993), and that apoptosis occurs in hepatic preneoplastic lesions (Bursch et al., 1984, 1990, 1995; Columbano et al., 1984; Zerban et al., 1994) and in rat hepatoma cells grown in ascites form (Tessitore et al., 1993). Using conventional methods, detection and quantification of apoptosis is hampered by the fact that cell condensation, karyopyknosis and chromatin fragmentation usually occur during advanced stages of the process only, the halflife of apoptotic cells in tissues thought to be in the region of at most several hours (Searle et al., 1987). It is however, necessary to identify apoptotic cells in an early phase of this pathway (Allen et al., 1993). One efficient way to overcome these constraints is in situ DNA end labelling (Gavrieli et al., 1992; Ansari et al., 1993) which exploits the presence of DNA breaks occuring in cells undergoing apoptosis. Programmed cell death in rat liver tumors has recently been analyzed by use of quantitative methods (Kong et al., 1995a,b) and in human HCCs using TdT-mediated dUTP-biotin nick-end labelling (Hino et al., 1996). The induction and regulation of apoptosis in liver cell tumors are not known so far, whereas apoptosis can be induced in cultured hepatocytes with transforming growth factor-beta (Oberhammer et al., 1992a,b), tumor necrosis factor-alpha (Bour et al., 1996), activin (Schwall et al., 1993) and microtubule-disassembling agents (Tsukidate et al., 1993).

In contrast with a previous report (Hino et al., 1996) demonstrating a correlation in HCCs between increased apoptosis and tumor grade no such relationship was found in the present material when using the grading system proposed by Edmondson and Steiner (Edmondson and Steiner, 1954). Such a difference may, apart from technical constraints, be related to the grading procedure as such and to the typical cellular heterogeneity of HCCs. This is exemplified by our observation that the apoptotic rate was elevated in tumor components rich in granular, hepatocyte-like cells predominating in low-grade HCCs, and lower in morphologically well-differentiated eosinophilic tumor cells, overall tumor grade being strongly influenced by other cell types in both situations. There was, however, a significantly higher apoptotic rate in HCCs exhibiting neuroendocrine differentiation. We have previously reported that neuroendocrine differentiation is frequent in HCCs (Zhao et al., 1993) and that it is not restricted to fibrolamellar hepatocellular carcinoma (Collier et al., 1984) Neuroendocrine multiexpression is seen in a quarter of the cases, and marker coexpression may occur within the same tumor cell. Neuroendocrine differentiation predominates in trabecular and mixed HCCs, but does not appear to be related to grade, and most positive cases show a hepatocyte-like cell morphology. It thus appears that the HCC cell type most likely to show neuroendocrine differentiation is a granular hepatocyte-like one, i.e. differentiated tumor cell rather than a small and poorly differentiated cell. This is also the cell type most likely to exhibit increased apoptosis as shown in the present study.

The reason why HCCs with neuroendocrine differentiation disclose an apoptosis pattern different from that of their non-neuroendocrine counterparts is not known. The same holds true for the question as to why a subset of liver cell tumors, including the fibrolamellar variant of HCCs (Collier et al., 1984), can develop along this particular pathway. The phenomenon is most probably not related to preexisting hepatobiliary neuroendocrine cells, because normal and neoplastic cells of this type have been demonstrated in the bile duct system (Dancygier et al., 1984), but not in the hepatic parenchyma. Conversely, neuroendocrine features have been described for epithelial cells of bile ducts and ductules, and tumors derived thereof (Roskams et al., 1990, 1993; Hsu et al., 1991; O'Hara et al., 1992). One may, therefore, theorize that both bile duct cells and hepatocytes have an inherent capability to enter a neuroendocrine differentiation pathway, and that this feature has, at least in HCCs, an impact on apoptosis. Further investigations are needed to test whether this phenomenon has an influence on the clinical behavior of liver tumors.

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