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Induction of apoptosis in cultured hepatocytes and in regressing liver by transforming growth factor β1

(mature and latent transforming growth factor β1)

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ABSTRACT **In previous studies hepatocytes undergoing cell death by apoptosis but not normal hepatocytes in rat liver showed immunostaining for transforming growth factor β1 (TGF-β1). Staining was much stronger with antibodies recognizing the pro-region of TGF-β1 than the mature peptide itself. Therefore we investigated the ability of both forms of TGF-β1 to induce apoptosis in primary cultures of rat hepatocytes. Mature TGF-β1 induced rounding up of the cells and fragmentation into multiple vesicles. As revealed by the DNA-specific stain H33258, the chromatin of these cells condensed and segregated into masses at the nuclear membrane; this was obviously followed by fragmentation of the nucleus. Ultrastructurally the cytoplasm was well preserved, as demonstrated by the presence of intact cell organelles. These features strongly suggest the occurrence of apoptosis. Quantification of nuclei with condensed chromatin revealed that mature TGF-β1 was 30-fold more effective than the TGF-β1 latency-associated protein complex. Finally, we administered TGF-β1 *in vivo* using an experimental model in which regression of rat liver was initiated by a short preceding treatment with the hepatomitogen cyproterone acetate. Two doses of TGF-β1, each 1 nM/kg, augmented the incidence of apoptotic hepatocytes 5-fold. Equimolar doses of TGF-β1 latency-associated protein complex were ineffective. These studies suggest that TGF-β1 is involved in the initiation of apoptosis in the liver and that the mature form of TGF-β1 is the active principle.**

Elucidation of the regulation of liver growth has made significant progress in recent years. Specific factors have been identified that positively or negatively regulate liver cell proliferation (1, 2). Among these are hepatocyte growth factor (3, 4), epidermal growth factor (5), and transforming growth factor α (6), which stimulate cell proliferation. Interleukin 1β (7) and transforming growth factor β1 (TGF-β1; refs. 8–12) inhibit hepatocyte DNA synthesis *in vitro*. TGF-β1 has been shown to inhibit *in vivo* DNA synthesis taking place after partial hepatectomy (13). Cell proliferation, however, is not the only determinant of liver size. Cell death by apoptosis was found to be an important determinant in the control of organ size under a number of different conditions. Apoptosis is a type of cell death that serves to eliminate excessive or unwanted cells during remodeling of embryonic tissues, during organ involution, and in regression of tumors (14, 15). Evidence suggests that apoptosis is under the control of growth-regulatory signals, such as hormones (16–19). In the liver apoptosis is involved in normal and preneoplastic cell turnover (20–22). Hepatomitogens and tumor promoters inhibit apoptosis. Their withdrawal can induce apoptosis (16–18). Identification of endogenous factors involved in the control of apoptosis in the liver therefore appears of considerable interest.

TGF-β1 seems to be a negative regulator of liver growth, and proteins of the TGF-β family are associated with regression of other epithelial tissues in the embryo (23) or adult (18) and of certain cancers (24). Recently it was reported that cell proliferation and apoptosis are coordinately regulated by TGF-β1 in cultured uterine epithelial cells (25). We hypothesized that this factor might be a signal for apoptosis in the liver (26, 27).

In agreement with this hypothesis, studies *in vivo* showed that apoptotic hepatocytes in normal and preneoplastic liver exhibited immunostaining for TGF-β1 (26). Somewhat unexpectedly, staining was much more pronounced for an epitope of the pro-region (pre-266–278) than for an epitope of the mature region of TGF-β1. In cultured hepatocytes TGF-β1 was found to induce cell death but its mode (apoptotic vs. necrotic death) could not be identified unequivocally (27). In the present work, by using improved culture conditions and rigorous criteria, we provide evidence that hepatocyte death induced by TGF-β1 *in vitro* is indeed apoptosis. Furthermore, we tested whether TGF-β1 precursor would also induce apoptosis and compared the activity of TGF-β1 latency-associated protein complex (LAP) with that of mature TGF-β1. Finally, we have injected TGF-β1 into animals and found that it also triggered apoptosis in the liver *in vivo*. These studies strongly suggest that TGF-β1 is a signal factor of apoptosis in the liver.

MATERIALS AND METHODS

Materials. TGF-β1 purified from human platelets was obtained from R & D Systems (Minneapolis). Recombinant mature TGF-β1 and TGF-β1 LAP synthesized by Chinese hamster ovary transfectants was purified as described (28, 29). H33258 was obtained from Riedel-De Haen (Hannover, F.R.G.). Cyproterone acetate (CPA) was a gift from Schering.

Animals and Treatment. Induction of liver hyperplasia in female Wistar rats (155 g) was induced as described (21). CPA suspended in maize oil was administered by gavage daily at 9:00 a.m. (100 mg/kg per day for 3 days, followed by 130 mg/kg per day for 4 days). Mature TGF-β1 or TGF-β1 LAP was injected into the tail vein 20 and 43 hr after the last application of CPA at a dose of 1 nM/kg (= 25 μg of mature TGF-β1 per kg) of body weight. Mature TGF-β1 was dissolved in 4 mM HCl in phosphate-buffered saline (PBS) containing 1 mg of bovine serum albumin (BSA) per ml and TGF-β1 LAP was dissolved in PBS containing 1 mg of BSA

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Abbreviations: CPA, cyproterone acetate; BSA, bovine serum albumin; LAP, latency-associated protein complex; TGF-β1, transforming growth factor β1.

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per ml, both proteins at 5 mM. As controls, 4 mM HCl in PBS containing 1 mg of BSA per ml was injected at the same time points in CPA-treated and sham-treated animals. Five animals were used for each treatment. At the peak of apoptosis incidence, 49 hr after the last application of CPA (21), animals were weighed and decapitated and the livers were removed. Livers were weighed and slices were fixed in Carnoy's solution.

The incidence of apoptotic bodies was evaluated by scoring 55–65 fields corresponding to ≈1500 cells of one hematoxylin/eosin-stained liver section under the light microscope. The numbers of normal hepatocytes and of apoptotic bodies with and without chromatin were counted per field as described elsewhere (21).

Cell Culture. Hepatocytes were isolated by perfusion of rat livers (F344, female, 140–160 g) with collagenase according to Seglen (30). Collagen gel (hydrated collagen lattice) was prepared exactly according to Chambard (31), and plastic dishes and glass slides were covered with 130 μl of gel per cm^2. The isolated hepatocytes were allowed to attach to the collagen substratum at a density of 25,000 or 87,500 cells per cm^2 (designated "low" and "high" cell density) in William's medium supplemented with 100 nM dexamethasone, 10 nM 3,3',5-triiodo-L-thyronine, 6.6 nM insulin, 0.4 nM glucagon, 20 mM Hepes, and 1 mg of BSA per ml. After 1 hr the cultures were rigorously washed with medium to remove dead cell aggregates only loosely attached to the collagen gel. After an additional 2–3 hr medium containing TGF-β1 was added. Time of factor addition was designated "0". In the time course experiment medium was replaced after 48 hr by medium without TGF-β1. In the dose–response experiment hepatocytes were treated for 48 hr with various concentrations of TGF-β1 and then harvested for morphological evaluation. All assays were performed in triplicate.

Morphological Evaluations. To prevent detachment of apoptotic cells from monolayers, medium changes and removal at the end of incubation were done very gently. For decantation of the supernatant, dishes were inverted and put onto filter paper to remove the medium as completely as possible. Immediately thereafter, cultures were fixed for 5 min with 3% paraformaldehyde in PBS (pH 7.4) at 4°C, washed with distilled water, and dried at room temperature. Cells were stained with H33258 (8 μg/ml) for 5 min, washed, and mounted in Moviol. The slides could be stored at 4°C up to 2 months.

Fluorescent H33258-stained nuclei were scored and categorized according to the condensation and staining characteristics of the chromatin as follows: (*i*) normal nucleus, uncondensed chromatin dispersed over the whole nucleus; (*ii*) nucleus with condensed chromatin, masses of condensed chromatin located at the nuclear membrane; (*iii*) fragmented nucleus, groups of isolated pieces of condensed chromatin after nuclear fragmentation. An illustration is shown in Fig. 1 *C–E*. Fluorescence was observed using a Leitz orthoplan microscope equipped with a Leitz PL Fluotar 40/0.70 objective. Ten fields per dish in two cultures were counted. The average number of nuclei per field was calculated.

Electron Microscopy. Cells were seeded at high cell density on 100 × 20 mm plates (5 × 10^6 cells per plate) covered with collagen gel and treated with 3 ng of mature TGF-β1 per ml. After 48 hr of incubation the supernatant was rapidly decanted. To ensure preservation of the *in situ* situation, the collagen gel was rolled up and put onto a filter paper on top of absorbent paper. At the end of the dehydration process, drops of fixative (2.5% sodium phosphate-buffered glutaraldehyde, pH 7.4) were pipetted onto the pellet, which was then transferred to cold fixative. After 1 hr the pellet was transferred to 0.2 M sodium phosphate buffer (pH 7.4) and postfixed for 1 hr in 1% veronal acetate-buffered OsO_4. Ultrathin sections were stained in alcoholic uranyl acetate and alkaline lead citrate and viewed in a Phillips 400 electron microscope.

Statistical Analysis. If not reported otherwise, each experiment was performed four times. The means ± SEM of four experiments are shown in Figs. 3 and 4; the means ± SD are given in Tables 1 and 2. Pairwise testing was performed with Student's *t* test.

RESULTS

Light Microscopy of Hepatocytes Treated with Mature TGF-β1. The typical morphological appearance of a high-density hepatocyte monolayer treated with 120 pM of mature TGF-β1 is shown in Fig. 1. Obviously, fragmentation of some cells has occurred (Fig. 1*B*). This probably reflects the end stage of TGF-β1-induced retraction from neighboring cells and of cytoplasmatic shrinkage, as observed previously by time-lapse micrography (27). To characterize the processes taking place in the nucleus we used the DNA-specific dye H33258.

The most prominent feature seen in the nuclei of some TGF-β1-treated hepatocytes was the condensation of chromatin masses at the nuclear membrane (Fig. 1 *C* and *D*) and subsequent nuclear fragmentation (Fig. 1*E*). These two features were often detectable in clusters of cells (Fig. 1*D*). Condensation of the chromatin and nuclear fragmentation of a similar or identical morphology are known to reflect sequential stages of apoptosis. Thus, fragmentation of cytoplasm and of nuclei suggests that TGF-β1 induces an apoptotic type of cell death.

Electron Microscopy of Hepatocytes Treated with Mature TGF-β1. The ultrastructural features of a representative dead hepatocyte are shown in Fig. 2. The cell is highly condensed with parts of the cytoplasm being separated and pinched off (Fig. 2*A*) to form vesicles containing cell organelles (Fig. 2*B*) and masses of chromatin. The organelles are well preserved, as demonstrated by the existence of intact lysosomes, rough endoplasmatic reticulum, and mitochondria (Fig. 2). This preservation of organelles is typical of the early stages of apoptotic cell death and corresponds to the light microscopical examination of the apoptotic hepatocytes described above. Thus morphological data shown in Figs. 1 and 2

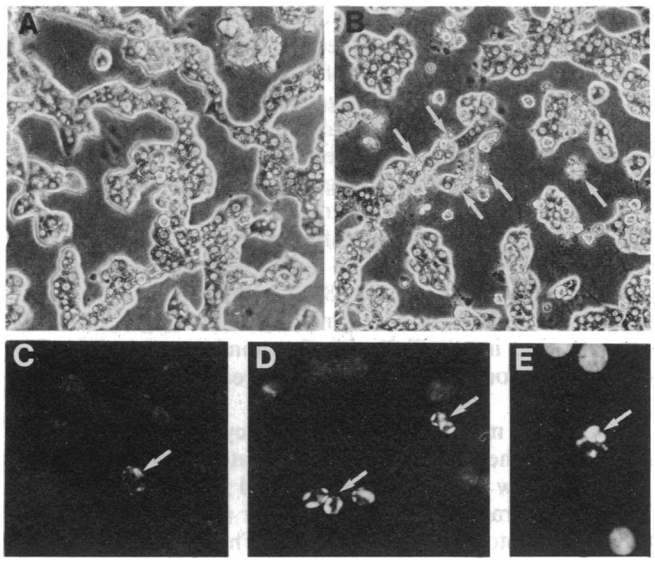

FIG. 1. Morphology of hepatocytes treated with mature TGF-β1. (*A* and *B*) Phase-contrast. (×60.) (*C–E*) Fluorescence microscopy of H33258 staining. (×280.) (*A*) Control. (*B–E*) TGF-β1 (120 pM). Arrows indicate fragmentation of the cell (*B*), presumptive beginning of nuclear chromatin condensation (*C*), condensation of the chromatin and aggregation at the nuclear membrane (*D*), designated "nucleus with condensed chromatin," and fragmented nucleus (*E*).

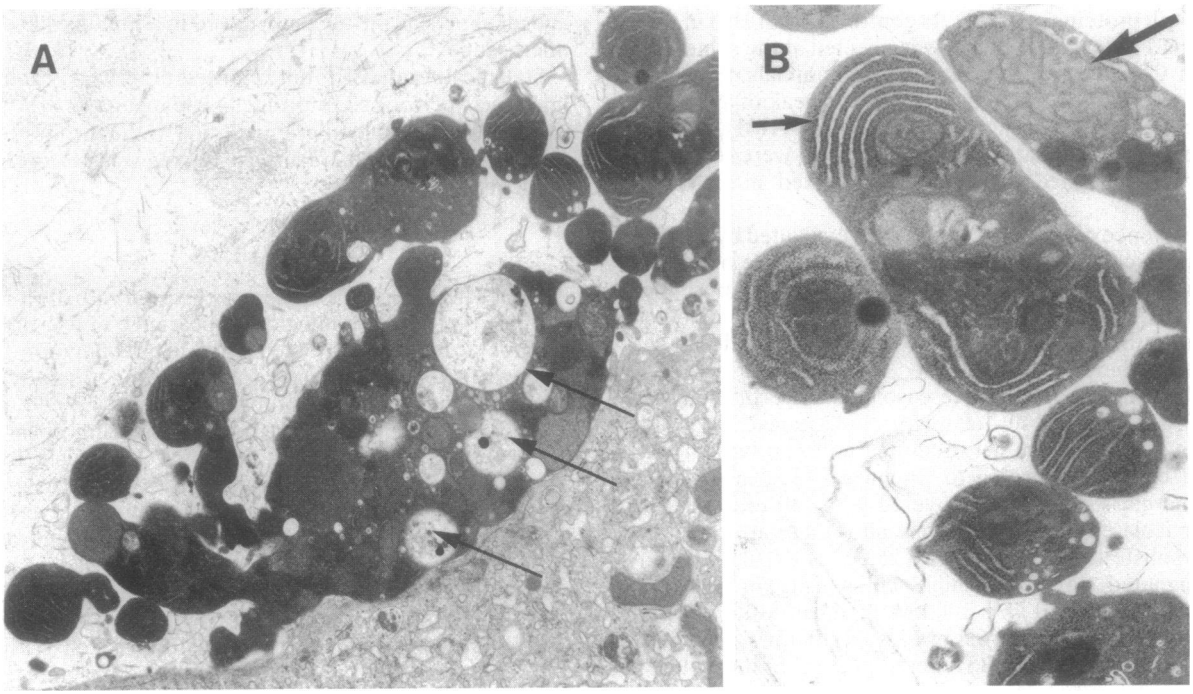

FIG. 2. Ultrastructure of an apoptotic hepatocyte. (*A*) Apoptotic cell. (×865.) (*B*) Detail of cytoplasmatic fragments. (×14,100.) Long arrows, lysosomes; middle arrow, mitochondria; short arrow, rough endoplasmic reticulum.

strongly suggest the occurrence of apoptosis in hepatocyte cultures treated with mature TGF-β1.

Time Course of Induction of Apoptosis in Hepatocyte Cultures by Mature TGF-β1. The changes in nuclear morphology revealed by H33258 staining provided a means to quantify apoptosis *in vitro*. For this purpose we distinguished three kinds of nuclei—namely, normal nuclei, nuclei with condensed chromatin, and fragmented nuclei (Fig. 3 *A–E*).

At both cell densities investigated no significant loss of normal nuclei was observed in control cultures. After 96 hr of culture the monolayer still contained 90% of the cells present after plating. A few fragmented nuclei were detectable at both cell densities; nuclei with condensed chromatin were only in high cell density cultures.

In the time course experiment TGF-β1 induced a loss of normal nuclei over the period of 96 hr (Fig. 3 *A* and *B*), similar for both cell densities investigated (about 50% of control). This process became detectable at about 40–48 hr and continued as a linear decline thereafter. The number of nuclei with condensed chromatin (Fig. 3 *C* and *D*) began to rise at 20–24 hr, peaked around 48 hr in low cell density cultures (Fig. 3*C*), and dropped thereafter. In high cell density cultures (Fig. 3*D*) the number of nuclei with condensed chromatin showed a plateau from 48–88 hr and decreased rapidly afterward. The number of fragmented nuclei (Fig. 3 *E* and *F*) began to rise in parallel with the number of nuclei with condensed chromatin yet tended to increase till the end of the experiment.

Apparently most apoptotic hepatocytes eventually detached from the substratum, as indicated by the low incidence and slow increase of fragmented nuclei (Fig. 3 *E* and *F*). Nuclear fragmentation apparently represents the final stage of apoptosis in cell culture. This detachment was directly observed by phase-contrast microscopy of the cultures and has been shown previously by determining the DNA content in the supernatants of TGF-β1-treated cultures (27). No major difference in the percentage of apoptosis in low and high cell density cultures could be detected, as the kinetics of the decrease of normal nuclei under both conditions were similar.

Dose Dependency of Induction of Apoptosis by Mature TGF-β1. As shown in Fig. 4 *C* and *D*, mature TGF-β1 induced the formation of apoptotic nuclei at doses above 12 pM ($P < 0.05$ versus control at both cell densities). The

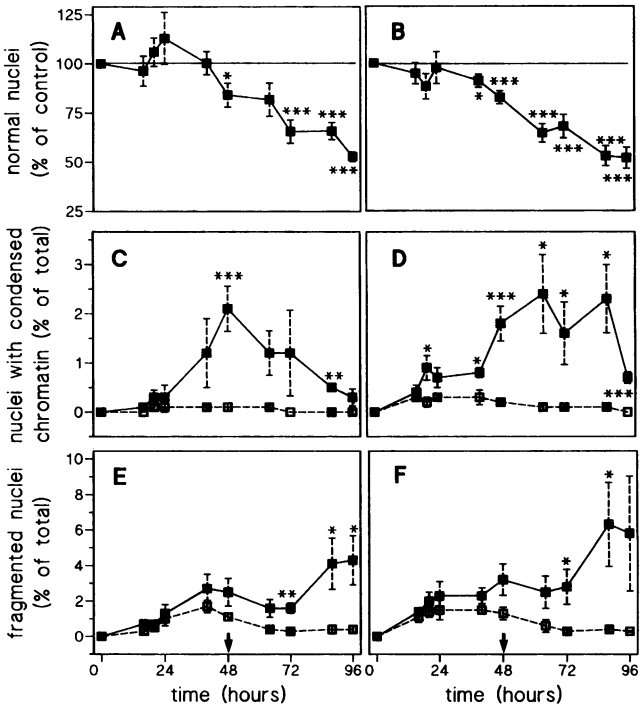

FIG. 3. Time course of induction of apoptosis by mature TGF-β1. (*A*, *C*, and *E*) Low cell density. (*B*, *D*, and *F*) High cell density. (*A* and *B*) Nuclei of TGF-β1-treated cultures are given as percent of nuclei in untreated parallel cultures at each time point. (*C–F*) Features of TGF-β1-treated and untreated cultures are given as percent of the sum of normal and altered nuclei at each time point. □, Control; ■, 120 pM TGF-β1. Arrows indicate medium change to medium without TGF-β1. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ vs. control at each time point.

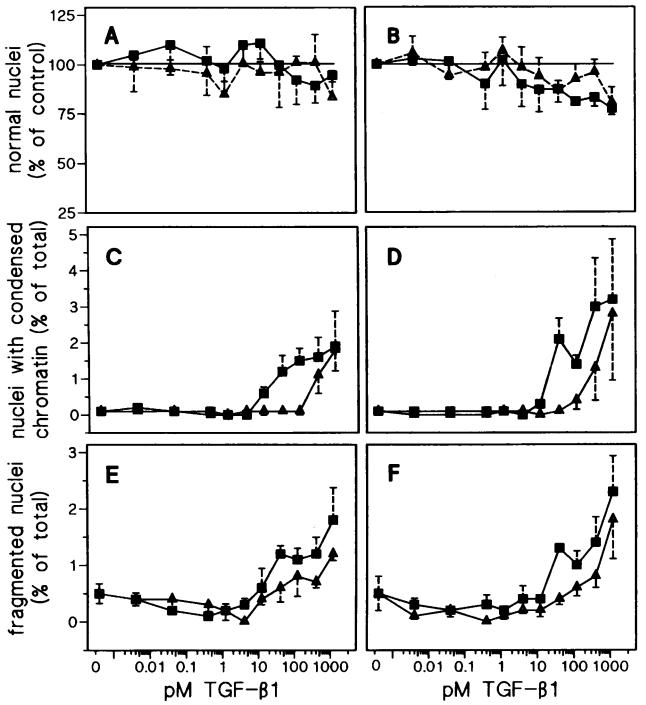

FIG. 4. Dose–response of induction of apoptosis by mature TGF-β1 and TGF-β1 LAP. (*A*, *C*, and *E*) Low cell density. (*B*, *D*, and *F*) High cell density. (*A* and *B*) Nuclei of TGF-β1-treated cultures are given as percent of nuclei in untreated cultures. (*C–F*) Features of TGF-β1-treated cultures are given as percent of total parameters. ■, Mature; △, TGF-β1 LAP.

increase (Fig. 4 *E* and *F*) of fragmented nuclei was parallel to the increase of nuclei with condensed chromatin. The number of normal nuclei (Fig. 4*A*) in low cell density cultures did not decrease significantly, whereas a significant decrease was evident in the high cell density cultures (Fig. 4*B*) from a dose of 40 pM upward ($P < 0.01$ vs. control). This difference is probably due to statistical effects.

Dose Dependency of Induction of Apoptosis by TGF-β1 LAP. *In vivo* studies have suggested that the TGF-β1 precursor may be involved in the induction of apoptosis. We have thus investigated the activity of TGF-β1 LAP in our culture system. As shown in the dose–response experiment in Fig. 4, TGF-β1 LAP seemed to be relatively ineffective in the induction of apoptosis. The incidence of nuclei with condensed chromatin increased from 400 pM TGF-β1 LAP ($P < 0.05$ vs. control at low cell density; $P < 0.01$ at high cell density) upward (Fig. 4 *C* and *D*). The number of fragmented nuclei showed a parallel increase (Fig. 4 *E* and *F*). The number of normal nuclei (Fig. 4 *A* and *B*) showed a significant decline in high cell density cultures (Fig. 4*B*) at 1200 pM TGF-β1 LAP ($P < 0.01$ vs. control). These data reveal a 30-fold greater potency of mature TGF-β1 vs. TGF-β1 LAP.

Effect of Mature TGF-β1 and TGF-β1 LAP on Hepatocyte Apoptosis *in Vivo*. Finally, we tested whether mature TGF-β1 and TGF-β1 LAP can induce apoptosis in the liver *in vivo*. To enhance the responsiveness of the organ we investigated a state of involution in which hepatocytes are prone to undergo apoptosis. This state was induced by pretreatment of rats with the hepatomitogen CPA. Mature TGF-β1 and TGF-β1 LAP were injected at the same molarity that has been found to inhibit regenerative DNA synthesis after partial hepatectomy (13). Counting the number of apoptotic bodies revealed a very low incidence in untreated control liver and some increase 49 hr after the last dose of CPA (Table 1), similar to previous findings. Administration of TGF-β1 LAP induced no further increase of the number of apoptotic bodies. In contrast, the equimolar dose of mature TGF-β1 augmented the incidence of apoptotic bodies with and without chromatin 5-fold (Table 1). Thus mature TGF-β1 appears able to induce hepatocyte apoptosis *in vivo*, whereas TGF-β1 LAP had no detectable effect under the present experimental conditions.

Table 1. Induction of apoptosis in regressing liver

Treatment		% of intact hepatocytes	
CPA	TGF-β1	AB with chromatin	AB without chromatin
–	–	0.01 ± 0.02***	0.00 ± 0.00**
+	–	0.37 ± 0.15	1.32 ± 0.64
+	LAP	0.37 ± 0.17†	0.66 ± 0.26†
+	Mature	1.87 ± 0.91**	6.51 ± 2.55**

See text for experimental conditions. A demonstration of apoptotic bodies (AB) with and without chromatin is shown in Fig. 5*B*. **, $P < 0.01$; ***, $P < 0.001$ vs. CPA only.
†Not significant.

DISCUSSION

The present study revealed the occurrence of apoptosis in hepatocyte cultures treated with TGF-β1 according to the following morphological criteria: cells round up and fragment into multiple vesicles. In the nucleus the chromatin shows condensation and aggregation at the periphery of the nuclear envelope. This is obviously followed by nuclear fragmentation. After the incidence of nuclei with condensed chromatin (Fig. 3 *C* and *D*) had dropped, fragmented nuclei still persisted (Fig. 3 *E* and *F*). Furthermore, it appears that the fragmentation of the nucleus occurs simultaneously with the fragmentation of the cytoplasm into multiple vesicles, as observed by light and electron microscopy. Ultrastructural studies of the fragmented hepatocytes revealed that the cytoplasm is extensively condensed with the cellular organelles remaining intact. The preservation of cellular membranes and organelles is one of the most reliable markers of apoptosis (14, 32). It discriminates apoptosis from lytic cell death, such as observed after many toxins or hypoxia, where membrane and organelle damage belong to the earliest morphological changes detectable (14, 32). The morphological evidence presented here is unequivocally consistent with an apoptotic mode of death of cultured hepatocytes treated with TGF-β1.

DNA fragments of oligosomal size have been described as a biochemical marker of apoptosis (33, 34), especially in cells of lymphatic or thymic origin (34). Whether this association applies to all epithelial cells under *in vitro* conditions is not known (15). We have been unable to find extensive DNA fragmentation into nucleosomes in cultured hepatocytes after TGF-β1 treatment. Likewise, in a recent report demonstrating the induction of apoptosis in rat hepatocytes and several other epithelial cell types by okadaic acid (an inhibitor of phosphoprotein phosphatase 1 and 2A), no DNA fragmentation was found (35). On the other hand, activation of endonuclease by Ca^{2+} results in nucleosome formation in hepatocyte nuclei (36) but does not seem to induce the condensation of the chromatin at the nuclear membrane characteristic for the apoptotic process (data not shown). Our observation of unequivocal morphological characteristics of apoptosis in the absence of detectable DNA fragmentation raises the question if the latter process is a necessary early step in all forms of apoptotic cell death.

In our previous work we followed the cellular rounding up and fragmentation in TGF-β1-treated hepatocytes through time-lapse micrography (27). This revealed that the process of rounding up takes about 90 min and the subsequent fragmentation takes about 10–20 min. The stage in which nuclei exhibit chromatin condensation at the nuclear mem-

brane should be of the same duration, as we never observed a nucleus of this type in a cell not rounded up. Thus we correlated the duration of the rounding period with the data of the time course experiment (Fig. 3). From the mean number of nuclei with condensed chromatin at 48–88 hr in the high cell density culture (2.625 per field), we calculate that 2.625 (no.) × 40 (hr)/1.5 (hr) = 70 apoptotic nuclei per field have formed during this period. This estimated number of apoptotic nuclei fits well with the number of 64 nuclei actually lost (from 162 ± 18 to 98 ± 11.5, Fig. 3*B*). These data further support the morphological observation that cell death in TGF-β1-treated hepatocytes is predominantly occurring through apoptosis.

In vivo the stages of apoptosis consisting of condensation of the chromatin, fragmentation and phagocytosis of the apoptotic cell by neighboring cells, followed by intracellular degradation, take about 3 hr (37). The first visible stage, chromatin condensation, has a duration of a few minutes *in vivo* but requires 90 min *in vitro*. Apparently nuclei with condensed chromatin persist longer *in vitro* than *in vivo* before fragmentation of the nucleus occurs.

We investigated the induction of apoptosis at two different cell densities. At low cell density the cells quickly dedifferentiate and synthesize DNA. High cell densities preserve the *in vivo* state of differentiation, and DNA synthesis in such cultures is low. We expected to find a higher response to the induction of apoptosis by TGF-β1 in hepatocytes of a more differentiated state. As the data of the time course and dose–response experiment show, this was not the case. Rather, the induction of apoptosis by TGF-β1 was independent of the cell density under our conditions. This suggests that the effect of TGF-β1 is due to a priming of the cells by the procedure of isolation from the liver. For example, isolation of hepatocytes leads to an expression of c-*myc* preceding the expression of the same oncogene in response of cell density alterations (38).

In summary, three lines of evidence support the regulatory role of TGF-β1 in apoptosis of hepatocytes: (*i*) apoptotic hepatocytes show immunostaining for epitopes of mature and pro-region of TGF-β1; (*ii*) TGF-β1 induces apoptosis in hepatocyte cultures; (*iii*) application of mature TGF-β1 during regression of the liver augmented the induction of apoptosis *in vivo*.

The investigation of TGF-β1 LAP showed that it is also able to induce apoptosis in hepatocyte cultures. There are only a few reports indicating a biological effect of precursor TGF-β1 (39). In cultured hepatocytes TGF-β1 LAP was found to be 30-fold less potent than mature TGF-β1. The induction of apoptosis by TGF-β1 LAP may be brought about through the uptake of TGF-β1 LAP by the mannose-6 phosphate/insulin-like growth factor II receptor (39, 40), which is found on hepatocytes *in vivo* (41) and *in vitro* (42). Substantial evidence for this means of TGF-β1 LAP uptake has been given in the situation of partial hepatectomy (43, 44). Immunostainings performed on regressing livers (26) suggested a more pronounced effect for the LAP protein. This does not necessarily mean that mature TGF-β1 is not present during apoptosis, as the mature protein is far more rapidly degraded than the TGF-β1 LAP (45, 46). Considering the *in vitro* and the *in vivo* results, it now appears that TGF-β1 is either taken up or produced by apoptotic hepatocytes in its latent form. However, the induction of apoptosis is apparently triggered by the mature protein.

Note. While our manuscript was under review, it was reported that TGF-β1 induces apoptosis in cultures of Hep3B cells (47).

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