

# Apoptosis Induced by Transforming Growth Factor- $\beta$ in Fetal Hepatocyte Primary Cultures

INVOLVEMENT OF REACTIVE OXYGEN INTERMEDIATES\*

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**Transforming growth factor- $\beta$  (TGF- $\beta$ ), a growth regulator of fetal hepatocytes in primary culture, also regulates death of these cells. Dose-response analysis showed that the TGF- $\beta$  concentration needed to induce hepatocyte death (2.5 ng/ml) was 5 times that needed to inhibit growth in these cells (0.5 ng/ml). In response to TGF- $\beta$ , hepatocytes induced DNA fragmentation and the appearance of nuclei with a DNA content lower than 2C (diploid content), typical of a programmed cell death model. TGF- $\beta$ -induced apoptosis in fetal hepatocytes was preceded by an induction of reactive oxygen species production and a decrease in the glutathione intracellular content, indicating that this factor induces oxidative stress in fetal hepatocytes. Studies performed to analyze levels of *c-fos* mRNA, a gene whose expression is modulated by redox state, demonstrated that only high, apoptotic concentrations of TGF- $\beta$  (2.5 ng/ml) produced an increase in the mRNA levels of this gene, the level of induction being similar to that found when cells were incubated in the presence of *tert*-butyl hydroperoxide. Gel mobility shift assays showed that the *c-fos*-induced expression was coincident with an increase in AP-1 activity. Finally, cell death induced by TGF- $\beta$  in fetal hepatocytes was partially blocked by radical scavengers, which decreased the percentage of apoptotic cells, whereas these agents did not modify the growth-inhibitory effect elicited by TGF- $\beta$  in these cells. In summary, the results presented in this paper provide evidence for the involvement of an oxidative process in the apoptosis elicited by TGF- $\beta$  in fetal hepatocytes.**

Mechanisms that regulate cell death are essential for normal development and maintenance of homeostasis. Cell death can be developmentally controlled, apoptosis being the most common morphologic expression of such programmed cell death (1). Lethal cellular programs that lead to apoptosis may be triggered by a variety of exogenous and environmental stimuli. Transforming growth factor- $\beta$  (TGF- $\beta$ )<sup>1</sup> is one of the best

known physiological inhibitors of epithelial cell proliferation. It is a member of a large family of structurally related factors that play a critical role during embryogenesis in mammals, frogs, and flies (2, 3). TGF- $\beta$  is particularly multifunctional, being able to regulate cell proliferation, differentiation, and morphogenesis, and recently attention has turned to its possible role in cell death. Localized cell death by apoptosis was described in *Drosophila* by mutation in a gene coding for a TGF- $\beta$  homolog (4). TGF- $\beta$ 1 is one of the genes activated by the initiation of apoptosis during prostate regression (5); it has been found to stimulate apoptotic cell death in cultured human gastric carcinoma cells (6) and in endometrial stromal cells (7); and, furthermore, it both inhibits proliferation and increases apoptosis in cultured uterine epithelial cells (8), in rat hepatocytes (9), and in hepatoma cells (10).

The mechanisms by which TGF- $\beta$ 1 exerts its effect are still only partly understood. Cross-linking experiments have revealed that most cells contain three types of receptors (for a review, see Ref. 11). The type III receptor (or betaglycan) is a proteoglycan with a short cytoplasmic domain and is not likely to mediate any of the known biological activities of TGF- $\beta$ . The type I (65–70-kDa) and type II (85–110-kDa) receptors have recently been cloned and identified as transmembrane serine/threonine (Ser/Thr) kinases, both receptors being essential for signaling. Recent advances in the characterization of the cell cycle machinery have stimulated studies aimed at understanding how TGF- $\beta$  signaling leads to growth arrest in the late G<sub>1</sub> phase of the cell cycle (12). In spite of these findings, the question of the signaling pathways from the receptor to the nuclei remains to be elucidated. Some reports have related the action of TGF- $\beta$  to the production of hydrogen peroxide (13, 14). Reactive oxygen intermediates are, in general, considered to be cytotoxic and are implicated in the progression of cancer, inflammation, radiation injury, and aging (for a review see Ref. 15). Peroxides and highly reactive free radicals can also trigger cell death, and although some investigators believe that oxidation is merely another metabolic disturbance that leads cells to respond to external stimuli, others propose a more central role for reactive oxygen species in cell death. In this context, overexpression of *bcl-2*, a protooncogene that is unique among cellular genes in its ability to block apoptotic death in different cell types (16) decreases lipid peroxidation and can increase resistance to apoptotic killing by hydrogen peroxide, menadione, and depletion of glutathione (17). It has recently been shown that the Bcl-2 oncoprotein functions as a prooxidant and presumably protects cells from oxidative stress by activating the cellular antioxidant defenses (18). To molecularly dissect growth arrest and apoptosis induced by TGF- $\beta$ , Selvakumaran *et al.* (19) performed a number of elegant studies showing that elevated expression of Bcl-2 blocked the TGF- $\beta$ 1-induced apo-

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<sup>1</sup> The abbreviations used are: TGF- $\beta$ , transforming growth factor- $\beta$ ; EGF, epidermal growth factor; DCFH-DA, 2',7'-dichlorofluorescein diacetate; MOPS, 4-morpholinepropanesulfonic acid; PDTC, pyrrolidine carbodithioic acid; NAC, *N*-acetyl-L-cysteine; NDGA, nordihydroguaiaretic acid; TBH, *tert*-butyl hydroperoxide.

ptotic pathway but did not prevent the growth arrest induced by this factor in myeloid leukemia cells.

In light of all these observations, the aim of our work has been to evaluate the possible implications of reactive oxygen intermediates in the mechanisms by which TGF- $\beta$  induces apoptotic death in the liver. Our experimental model uses fetal hepatocytes in primary culture. These cells are able to carry out both proliferation and differentiation processes simultaneously. We have shown that some growth factors such as epidermal growth factor (EGF), hepatocyte growth factor, or transforming growth factor- $\alpha$  (20, 21) are able to induce DNA replication in these cells, whereas some hormones (glucagon, noradrenaline, glucocorticoids), by themselves or in synergism with the positive growth factors, regulate the expression of some liver-specific genes (22–24). TGF- $\beta$  inhibits the EGF and/or hepatocyte growth factor-induced DNA synthesis and modulates protooncogene and liver-specific gene expression in these cells (20, 21, 25, 26). Furthermore, high concentrations of this factor induce fetal hepatocyte death (26). All of these results suggest that primary cultured fetal hepatocytes are a good model in which to study the possible role of TGF- $\beta$  in the regulation of liver apoptosis during fetal life and the implication of oxygen radicals in its molecular mechanism.

#### EXPERIMENTAL PROCEDURES

**Materials**—TGF- $\beta$  was from Austral Biologicals (San Ramon, CA). Collagenase was from Boehringer Mannheim. Glutathione, glutathione reductase, crystal violet, the antioxidant agents, and the 2',7'-dichlorofluoresceine diacetate (DCFH-DA) were from Sigma. Fetal and neonatal calf serum and culture media were from Imperial Laboratories (Hampshire, United Kingdom). Radiochemicals were from ICN (Irvine, CA). Multiprimer DNA labeling system was from Amersham Corp. The nick-translation labeling system was from Life Technologies, Inc.

**cDNA Probes**—Mouse *v-fos* cDNA was from Oncor (Gaithersburg, MD) (27). *Ha-ras* cDNA was kindly provided by Dr. Santos (Bethesda, MD) (28).  $\alpha$ -Fetoprotein cDNA was a gift of Dr. Danan (Paris, France) (29). 18 S ribosomal probe was a gift of Dr. Rozengurt (London, UK).

**Isolation and Culture of Fetal Rat Hepatocytes**—Hepatocytes from 20-day-old fetal Wistar rats were isolated as described previously by collagenase disruption (30) and plated on dishes in arginine-free medium 199, supplemented with ornithine (200  $\mu$ M), fetal calf serum (10%), penicillin (120  $\mu$ g/ml), and streptomycin (100  $\mu$ g/ml). Cells were incubated in 5% CO<sub>2</sub> at 37 °C for 4 h, allowing cell attachment to plates. Medium was changed at that time and replaced by one of the same composition except that the 10% fetal calf serum was replaced with 2% newborn bovine serum. After 18–20 h, the medium was again replaced for one of identical composition but in the absence of serum. Two hours later, growth factors and/or the different agents were added. When antioxidants were used to protect against cell death, these agents were added 15 min after the TGF- $\beta$  treatment in order to avoid interferences in the factor-receptor binding.

**TGF- $\beta$ -mediated Cytotoxicity Assay**—After cell incubation in the absence or presence of the different factors, the medium was discarded, and the remaining viable adherent cells were stained, as described previously (31), with crystal violet (0.2% in 2% ethanol) for 20 min. After this time, plates were rinsed with tap water and allowed to dry, and 1% sodium dodecyl sulfate was added to solubilize them. The absorbance of each plate was read photometrically at 560 nm. Dishes without cells were processed in parallel to correct the nonspecific adhesion of crystal violet to the plastic. Remaining viable cells were calculated as percentage of absorbance with respect to control cells (incubated in the absence of growth factors).

**Assay of DNA Synthesis**—For this assay, cells were cultured in the absence or presence of growth factors, and DNA synthesis was determined by [<sup>3</sup>H]thymidine incorporation (0.5  $\mu$ Ci/ml, 1  $\mu$ M thymidine) into DNA. Radioactivity in acid-precipitable material was determined with a liquid scintillation counter as described previously (20).

**Analysis of Nuclear DNA Content by Flow Cytometry**—The ploidy determination of hepatocyte nuclei was estimated by flow cytometry DNA analysis as described previously (20). Cells were detached from dishes by the addition of 0.25% trypsin, 0.02% EDTA. After 2–3 min, trypsinization was stopped by adding 10% fetal calf serum to the culture medium. The DNA content per nucleus was evaluated in a FAC-SCAN flow cytometer (Becton-Dickinson, San Jose, CA) after using the

Cycle TEST™ DNA reagent kit (Becton-Dickinson) to stain nuclei with propidium iodide. This analysis was performed using a double discriminator module, which permits a distinction to be made between the signals coming from a single nucleus and the ones produced by two or more aggregated nuclei. For the computer analysis, only signals from single nuclei were considered (10,000 nuclei/assay).

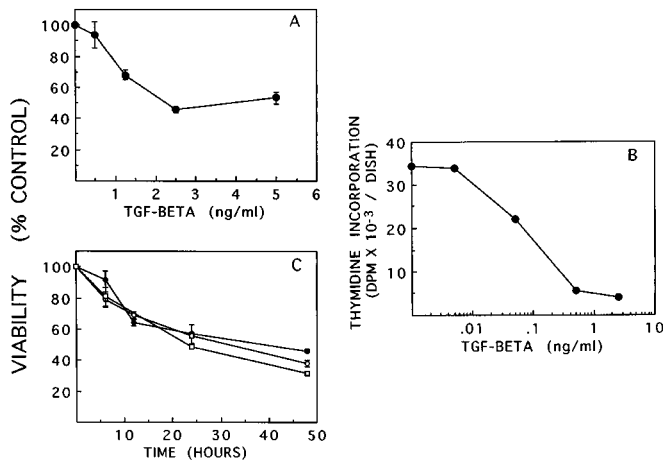
**Analysis of DNA Fragmentation**—A modified version of the method of Lyons *et al.* (32) was used to assess the fragmentation of hepatocyte DNA. Rat fetal hepatocytes (40–50  $\times 10^3$ /cm<sup>2</sup>), incubated in the absence or presence of TGF- $\beta$ , were washed twice with phosphate-buffered saline and then scraped and pelleted at 4 °C. Cells were resuspended in 500  $\mu$ l of buffer containing 10 mM EDTA, 0.25% Triton X-100, 2.5 mM Tris-HCl, pH 8.0, and stored at 4 °C for 15 min. Intact nuclei were pelleted and eliminated by centrifugation at 500  $\times g$  for 10 min, and the supernatant was centrifuged at 25,000  $\times g$  at 4 °C for 30 min. DNA in the supernatant was precipitated at –80 °C after the addition of 2 volumes of ethanol (70% final concentration), pelleted by microcentrifugation at 4 °C for 15 min, dried, resuspended in 200  $\mu$ l of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0 (TE buffer), and incubated at 37 °C for 30 min with 0.1 mg/ml RNase A and for 2–3 h with 0.25 mg/ml proteinase K. DNA was purified by phenol-chloroform extraction and precipitated at –70 °C after adding (1/10 volume of) 3 M sodium acetate, pH 5.3, and (2 volumes of) ethanol. Precipitated DNA was dissolved in TE buffer containing 30% glycerol, 1  $\mu$ g/ml ethidium bromide and electrophoresed in a 1.5% agarose gel. Gel was visualized and photographed under transmitted UV light with a Polaroid camera.

**Measurement of Intracellular Reactive Oxygen Species by Flow Cytometry**—The oxidation-sensitive fluorescent probe DCFH-DA (33) was used to analyze the net intracellular generation of reactive oxygen species. Fetal hepatocytes were incubated in the presence or absence of the different factors, and at different times cells were detached by trypsinization as described above and incubated with 5  $\mu$ M DCFH-DA. The cellular fluorescence intensity was measured after 20–30 min of DCFH-DA oxidation by using a FACSCAN flow cytometer (Becton Dickinson). For each analysis, 10,000 events were recorded.

**Glutathione Determination**—Rat fetal hepatocytes (40–50  $\times 10^3$ /cm<sup>2</sup>), incubated in the absence or presence of TGF- $\beta$ , were washed twice with phosphate-buffered saline, scraped, and pelleted at 4 °C. Cellular glutathione was extracted in buffer containing 0.2% Triton X-100, 2.5% sulfosalicylic acid. After centrifugation at 15,000  $\times g$  for 15 min, the supernatant was used for the determination of total (GSSG + GSH) and oxidized (GSSG) glutathione, whereas the pellet was resuspended in phosphate-buffered saline, 0.1% Triton X-100 to protein concentration analysis. Total glutathione (GSH + GSSG) was assayed by a modified version of Griffith's method (34), monitoring the change in absorbance at 412 nm in the presence of 0.1 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.15 mM NADPH, and 1 unit of glutathione reductase/ml of assay mixture in 1 mM EDTA, 0.05 M phosphate buffer, pH 7.5. Glutathione disulfide (GSSG) content was determined after derivatization of 500  $\mu$ l of the above extract with 10  $\mu$ l of 2-vinylpyridine. Both GSH + GSSG and GSSG concentrations were calculated by comparison with an internal GSH standard for each assay and are expressed as nmol of GSH equivalents/ $\mu$ g of protein. Protein concentration was determined by the method of Bradford (35), using albumin as a standard.

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated as described by Chomczynski and Sacchi (36). For each assay, RNA was extracted from the pooled cells of two 92-mm diameter dishes, denatured in 50% formamide, 2.2 M formaldehyde, 20 mM MOPS, pH 7.0, 6% glycerol at 65 °C for 15 min, separated by size on gels containing 0.9% agarose and 0.66 M formaldehyde, and blotted on GeneScreen™ membranes (DuPont NEN). RNA was cross-linked to the membrane by UV light. The membrane was then prehybridized for 5 h and hybridized for 24–40 h at 42 °C in a buffer containing 0.25 M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, 0.25 M NaCl, 1 mM EDTA, 100  $\mu$ g/ml denatured salmon sperm DNA, 7% SDS, 50% deionized formamide, and the labeled probe. cDNA probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP either by nick-translation ( $\alpha$ -fetoprotein and 18 S ribosomal cDNA) or by random priming reaction (*v-fos*, *Ha-ras*). After hybridization, filters were washed twice with 2  $\times$  SSC, 0.1% SDS and exposed to Kodak X-Omat AR films. The foregoing cDNA was stripped from the blots, and serial hybridizations with the different probes were performed successively.

**Nuclear Extract Preparation and Gel Mobility Shift Assay**—DNA-binding protein extracts from fetal hepatocytes were prepared as described by Andrews and Faller (37), starting with 5  $\times 10^6$  cells. Cell suspension was resuspended in 10 mM HEPES-KOH, pH 7.9, at 4 °C, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride (Buffer A), allowed to swell on ice for 10 min, and then vortexed for 10 s. Samples were centrifuged, and the supernatant



**FIG. 1. Effects of TGF- $\beta$  on growth and cell viability of cultured rat fetal hepatocytes.** *A*, cells were cultured in the absence or presence of different concentrations of TGF- $\beta$  for 48 h. After this time the number of viable cells was analyzed by staining with crystal violet. Results are expressed as percentage of absorbance, at 560 nm, with respect to control (untreated) cells. Values are the mean  $\pm$  S.E. of three separate experiments of triplicate dishes; *B*, fetal hepatocytes were cultured in the presence of EGF (20 ng/ml) and in the absence or presence of different concentrations of TGF- $\beta$  for 48 h. After this time, DNA synthesis was analyzed by measuring [ $^3$ H]thymidine incorporation into acid-precipitable material over the last 40 h of culture (0.5  $\mu$ Ci/ml, 1  $\mu$ M thymidine). A representative experiment of four is shown. *C*, cells were cultured in the presence of 2.5 (●), 5 (○), and 10 (□) ng/ml TGF- $\beta$  for different times (8, 12, 24, and 48 h), and cell viability was calculated as described for *A*.

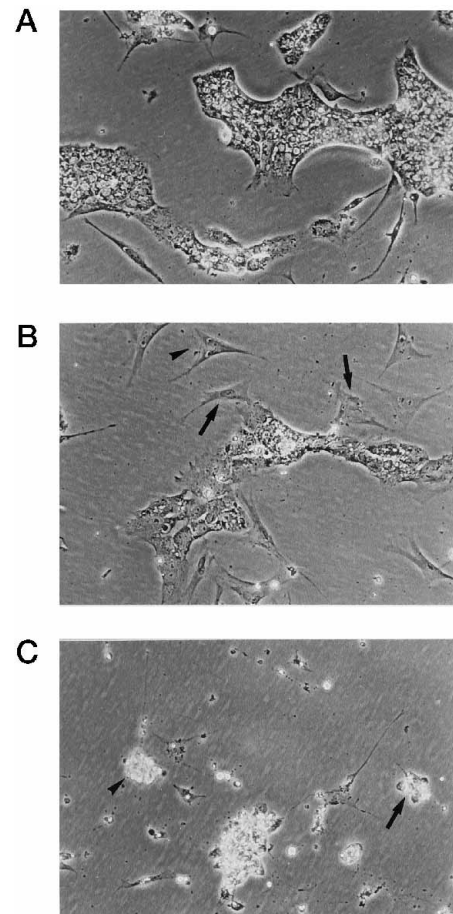
was discarded. The pellet was resuspended in cold Buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 0.75  $\mu$ g/ml each of leupeptin, antipain, and aprotinin) and incubated on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation for 2 min at 4 °C, and the supernatant fraction was stored at -70 °C. The gel mobility shift assay was performed essentially as described previously (38). The double-stranded oligonucleotide used as AP-1 probe was composed of the sequence AGCTTGATGAGTCAGCCGATC, and labeling was performed by using Klenow polymerase and  $\alpha$ -<sup>32</sup>P-labeled deoxyadenosine triphosphate. The binding reaction mixture contained 0.5 ng of double-stranded oligonucleotide probe, 2  $\mu$ g of poly(dI-dC)·poly(dI-dC), and 2  $\mu$ g of protein in Buffer C supplemented with 35 mM MgCl<sub>2</sub>. After a 20-min incubation at 4 °C, the mixture was electrophoresed through a 6% polyacrylamide gel in 0.25  $\times$  Tris borate-EDTA running buffer for 2 h. The dried gel was then autoradiographed.

## RESULTS

**Characterization of the Apoptotic Death Induced by TGF- $\beta$  in Fetal Hepatocyte Primary Cultures**—When fetal hepatocytes from 20-day gestation rats were isolated, plated on dishes, and incubated in the presence of different concentrations of TGF- $\beta$ , a dose-dependent cytotoxicity effect was always observed (Fig. 1A). Maximum effect was obtained at a dose of 2.5–5 ng/ml, where 50% of the cells died. This concentration was 5-fold higher than that needed to completely inhibit the EGF-induced DNA synthesis in fetal hepatocytes in culture (Fig. 1B). The time dependence study of cell death process showed that most of the cells died during the first 24 h after adding the factor (Fig. 1C). Moreover, regardless of the TGF- $\beta$  concentration, the final percentage of dead cells was never higher than 60%.

TGF- $\beta$  induced changes in cell morphology (Fig. 2). 8–10 h after the addition of the factor, cells lose cell contacts and migrate on the plate (Fig. 2B), and 5–10 h later (12–20 h in the presence of TGF- $\beta$ ), cellular blebbing and detachment of degenerated hepatocytes were always observed (Fig. 2C).

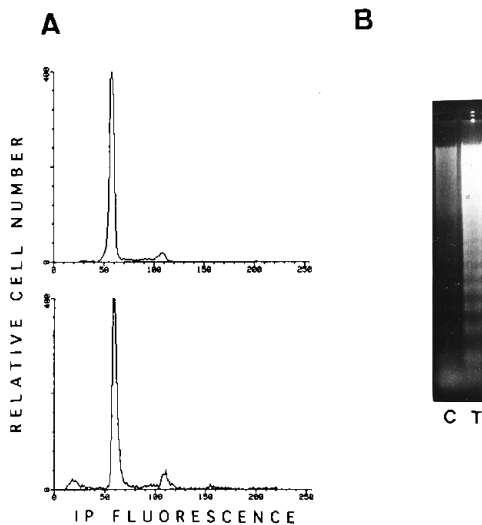
To assess if fetal hepatocyte death involved a process of apoptosis, we first analyzed the normal nuclear DNA content



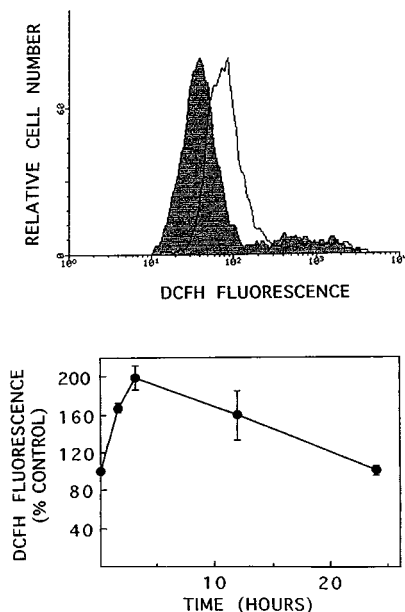
**FIG. 2. Morphological changes induced by TGF- $\beta$  in rat fetal hepatocytes in primary culture.** Light microscopy photographs of cultured fetal hepatocytes in the absence (*A*) or presence of TGF- $\beta$  (2.5 ng/ml) for about 8 and 16 h (*B* and *C*, respectively). TGF- $\beta$  induces losing in cell contacts (*B*, long arrow), and cells migrate on the plate (*B*, short arrow). At longer times, cell blebbing (*C*, long arrow) and detachment of the hepatocytes (*C*, short arrow) is observed. Magnification: 200  $\times$ .

by flow cytometry, staining nuclei with propidium iodide (Fig. 3A, upper panel). Cells that had been incubated in the presence of TGF- $\beta$  (2.5 ng/ml) for 12 h showed between 12 and 15% of the nuclei with a DNA content lower than 2C (Fig. 3A, lower panel). Then, in order to know if these cells showed fragmentation in their DNA, we isolated the extranuclear DNA from parallel dishes that had been incubated in the absence or presence of TGF- $\beta$  (2.5 ng/ml). A representative experiment is shown in Fig. 3B. DNA from TGF- $\beta$ -treated cells appeared cleaved into a "ladder," which is generally associated with the internucleosomal site cleavage occurring in the apoptotic process. This effect was first observed after 6–8 h of treatment and continued throughout for the next 12–16 h (20–24 h in the presence of the factor; data not shown).

**Flow Cytometric Analysis of Intracellular Reactive Oxygen Species Produced by TGF- $\beta$** —Using flow cytometry, we next examined the generation of reactive oxygen species in fetal rat hepatocytes that were treated with TGF- $\beta$ . For this, we used DCFH-DA, an oxidation-sensitive fluorescent probe (33). When cells were incubated in the presence of 0.5 ng/ml TGF- $\beta$ , DCFH fluorescence was identical in control (nontreated) and TGF- $\beta$ -treated cells (results not shown). However, when cells were treated with higher doses of this factor (2.5 ng/ml), they showed an important increase in DCFH fluorescence (Fig. 4, upper panel). This effect was time-dependent, with a maximum at 3 h. After this time, reactive oxygen species decreased, returning



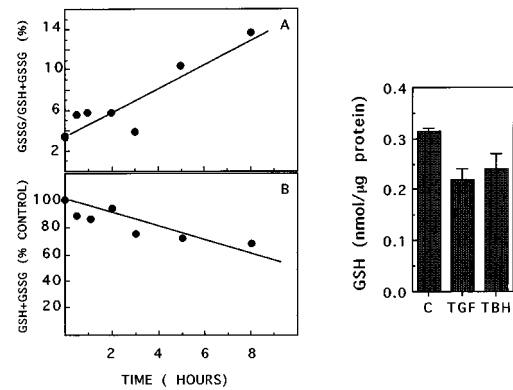
**FIG. 3. Effect of TGF- $\beta$  on nuclear DNA content and DNA fragmentation.** A, DNA content histograms of untreated hepatocytes (*top panel*) and hepatocytes treated with TGF- $\beta$  (2.5 ng/ml) (*bottom panel*) as measured by flow cytometry. Fetal hepatocytes cultured in the absence or presence of TGF- $\beta$  for 12 h were collected and treated for analysis of DNA content as described under "Materials and Methods." A representative experiment of four is shown. B, after an 8-h incubation of fetal hepatocytes in the absence (C) or in the presence of TGF- $\beta$  (T), cells were scraped and subjected to cytoplasmic DNA extraction. Purified DNA, from parallel 10-cm diameter dishes, was electrophoresed in a 1.5% agarose gel and visualized by UV fluorescence after staining with ethidium bromide. A representative experiment of five is shown.



**FIG. 4. TGF- $\beta$  induction of intracellular reactive oxygen species content.** Fetal hepatocytes cultured in the absence or presence of TGF- $\beta$  were detached and incubated for 20–30 min with 5  $\mu$ M of an oxidation-sensitive fluorescent probe (DCFH-DA), measuring the fluorescence intensity by flow cytometry. The *top panel* shows a representative histogram of cells untreated (*shaded area*) or treated for 3 h with 2.5 ng/ml TGF- $\beta$  (*white area*). The *bottom panel* represents a time course analysis of intracellular reactive oxygen species content. Cells were incubated for different times (1.5, 3, 12, and 24 h) in the presence of TGF- $\beta$  (2.5 ng/ml), and the production of reactive oxygen species was analyzed as described previously. Data are expressed as a percentage of DCFH fluorescence with respect to control value and are means  $\pm$  S.E. from three independent experiments.

to control values at 24 h (Fig. 4, *lower panel*).

**Effect of TGF- $\beta$  on Glutathione Intracellular Levels in Fetal Hepatocyte Primary Cultures**—Since the oxidative stress and

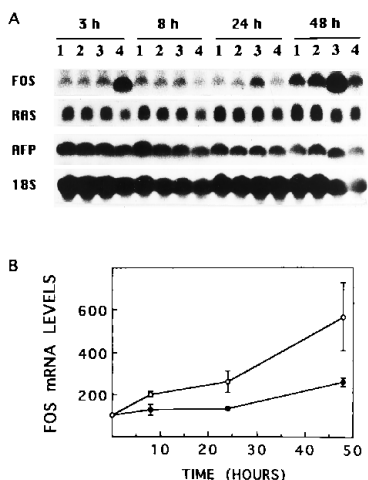


**FIG. 5. Effect of TGF- $\beta$  on glutathione intracellular levels.** *Left*, after incubating fetal hepatocytes in the absence or presence of 2.5 ng/ml TGF- $\beta$ , cells were collected, and cellular glutathione was extracted and determined spectrophotometrically as described under "Materials and Methods." The means of triplicate dishes of a representative experiment are shown (A). The GSSG/(GSH + GSSG) ratio (%) in TGF- $\beta$ -treated cells is presented as a function of time in the presence of the factor. Control cells (in the absence of TGF- $\beta$ ) did not show changes in this ratio (data not shown). B, in this same experiment, the glutathione (GSH + GSSG) intracellular concentration is presented *versus* time of TGF- $\beta$  treatment. In this case, data are calculated, at each time, as percentage with respect to control (untreated) cells. *Right*, cells were incubated for 3 h in the absence (C) or presence of 2.5 ng/ml TGF- $\beta$  (TGF) or 0.25 mM TBH. GSH intracellular concentrations are expressed as nmol/ $\mu$ g protein and are the mean of duplicate dishes of three independent experiments. Bars represents S.E.

the presence of peroxides produce changes in the GSSG/(GSH + GSSG) ratio and in the glutathione concentration of the hepatocytes (39), we have measured these parameters in fetal hepatocytes incubated in the absence or presence of TGF- $\beta$  (2.5 ng/ml) (Fig. 5). This factor produced an increase in the GSSG/(GSH + GSSG) ratio (Fig. 5A, *left panel*). Simultaneously, a decrease in the glutathione intracellular concentration was always observed (Fig. 5B, *left panel*). We wanted to know if the decrease in glutathione levels was similar to that found when cells were incubated in the presence of 0.25 mM *tert*-butyl hydroperoxide (TBH), a compound that produces oxidative stress in hepatocytes (39). For this, cells were incubated for 3 h in the absence or presence of these factors. We choose this time because at longer times TBH-treated cells had died. As can be seen in Fig. 5 (*right panel*) the decrease in GSH concentration is similar for TGF- and TBH-treated cells.

**Effect of TGF- $\beta$  on *c-fos* mRNA Levels and AP-1 Binding Activity in Fetal Hepatocytes in Primary Culture**—There is increasing evidence that reactive oxygen intermediates play an important role in the control of gene expression (40). The activity of transcription factors, such as AP-1, is modulated by the redox state of the cell, *de novo* synthesis of *c-fos* and *c-jun* mRNAs being required. When fetal hepatocytes were incubated in the presence of 0.25 mM TBH, the oxidative stress induced in the cells produced an increase in *c-fos* mRNA levels with a maximum at 3 h (Fig. 6). Similarly, TGF- $\beta$ -treated cells induced *c-fos* expression 24–48 h after adding the factor (Fig. 6). It is important to point out that this increase was only observed when high concentrations of TGF- $\beta$  (2.5 ng/ml) were used (Fig. 6, *lane 3*) but not when cells were incubated in the presence of low, nonapoptotic concentrations (0.5 ng/ml) (Fig. 6, *lane 2*). Moreover, this effect on *c-fos* was specific, since the expression of another protooncogene, such as *Ha-ras*, and a liver-specific gene, such as  $\alpha$ -fetoprotein, were not substantially altered by TGF- $\beta$  treatment (Fig. 6).

To further characterize the cell response to oxidative stress, we looked at AP-1 binding activity. Nuclear extracts from fetal hepatocytes incubated for 24 h, in the absence or presence of

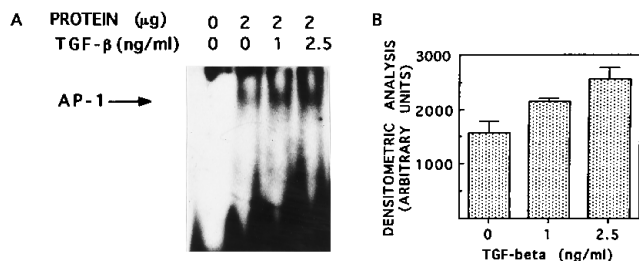


**FIG. 6. Effect of TGF- $\beta$  on *c-fos* mRNA levels in cultured fetal hepatocytes.** *A*, cells were cultured in the absence (*lane 1*) or presence of 0.5 ng/ml TGF- $\beta$  (*lane 2*), 2.5 ng/ml TGF- $\beta$  (*lane 3*), and 0.25 mM TBH (*lane 4*). After 3, 8, 24, and 48 h of treatment, total RNA was isolated, and 20  $\mu$ g were subjected to Northern blot analysis using the cDNA probes specific for *fos*, *ras*, and  $\alpha$ -fetoprotein mRNAs. An 18 S ribosomal probe was used for RNA normalization. A representative experiment of three is shown. *B*, the radioactivity visualized in the Northern blots with *c-fos* probe was measured by scanning densitometry and normalized with the 18 S probe. Data are expressed as percentage of mRNA levels at zero time (before TGF- $\beta$  treatment) and are the mean  $\pm$  S.E. of three independent experiments. ●, control; ○, 2.5 ng/ml TGF- $\beta$ .

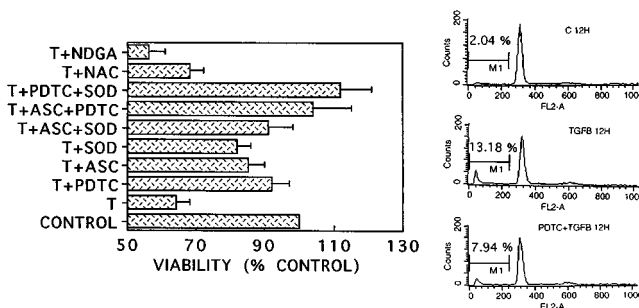
TGF- $\beta$ , were used in a gel mobility shift assay using a consensus AP-1 site oligonucleotide as probe. After 24 h of treatment, TGF- $\beta$  induced AP-1 binding activity in a dose-dependent manner (Fig. 7). These results are consistent with the elevation of *c-fos* mRNA previously seen at apoptotic concentrations of TGF- $\beta$  (2.5 ng/ml).

**Effect of Radical Scavengers on the Induction of Apoptosis by TGF- $\beta$  in Fetal Hepatocytes in Primary Culture**—In order to know whether the increase in reactive oxygen species may be related to the fetal hepatocyte death, we next examined the effects of antioxidants or radical scavengers, *i.e.* ascorbic acid (vitamin C), pyrrolidine carbodithioic acid (PDTC), *N*-acetyl-L-cysteine (NAC), superoxide dismutase, and nordihydroguaiaretic acid (NDGA) on the apoptosis induced by TGF- $\beta$  in the cells. We incubated the cells in the presence of different concentrations of these factors in order to choose efficient but not cytotoxic doses (data not shown). A summary of the results is shown in Fig. 8. The addition of ascorbic acid or PDTC, which did not have any effect on cell survival on their own (results not shown), largely prevented the induction of cell death by TGF- $\beta$  (Fig. 8, *left panel*). Even the addition of superoxide dismutase alone to the culture medium significantly increased cell viability. Combinations of these agents were able to completely prevent the induction of cell death by TGF- $\beta$ . The antioxidant protective effect was observed during the first 24 h of culture in the presence of TGF- $\beta$ . In contrast with these results, NAC, a compound that is able to raise intracellular glutathione levels, or NDGA, a 5-, 12-, and 15-lipoxygenase inhibitor, were ineffective in protecting cells against TGF- $\beta$ -induced cell death (Fig. 8, *left panel*).

We have evaluated whether the protective effect on cell death elicited by the antioxidants is coincident with a decrease in the percentage of apoptotic cells by measuring nuclear DNA content. When cells were incubated with TGF- $\beta$  but in the presence of ASC, superoxide dismutase, or PDTC, the percentage of nuclei with a DNA content lower than 2C decreased from 13–15% (TGF- $\beta$ -treated cells) to about 7–8% (control values were 2–3%). Thus, it is clearly the case that these radical



**FIG. 7. Effect of TGF- $\beta$  on the specific binding to the AP-1.** *A*, 2  $\mu$ g of nuclear extracts from control or TGF- $\beta$ -treated (24 h) fetal hepatocytes were incubated for 20 min with 0.5 ng of  $^{32}$ P-labeled double-stranded oligonucleotide used as AP-1 probe (see “Experimental Procedures”). After this time, the mixture was electrophoresed through a 6% polyacrylamide gel. The autoradiograph of a representative experiment is shown. *B*, dose-response increase in AP-1 activity. The radioactivity visualized in the gel mobility shift assays with AP-1 probe was measured by scanning densitometry. Results are mean  $\pm$  S.E. of three independent experiments.



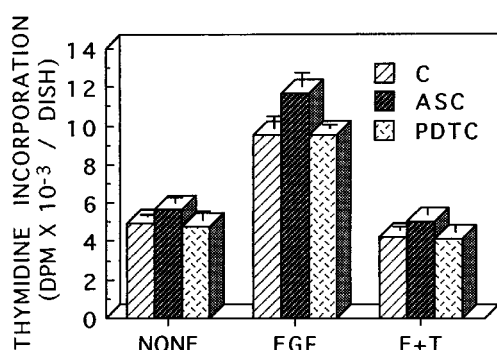
**FIG. 8. Effect of radical scavengers on TGF- $\beta$ -induced apoptosis.** *Left*, cells were left untreated or were treated with 2.5 ng/ml TGF- $\beta$  alone or in the presence of different radical scavengers: 50  $\mu$ M PDTC, 1 mM ascorbic acid (ASC), 300 units/ml superoxide dismutase (SOD), 20 mM NAC, and 5  $\mu$ M NDGA added 15 min after the TGF- $\beta$  treatment. Following a 16-h treatment period, cells were stained with crystal violet, and the viability was quantified as described under “Materials and Methods.” Results are expressed as percentage of control and are means  $\pm$  S.E. of triplicate determinations from 3–5 experiments. *Right*, after 12-h incubation of the cells in the absence (C) or presence of 2.5 ng/ml TGF- $\beta$  (TGF $\beta$ ) and the combination of 2.5 ng/ml TGF- $\beta$  + 50  $\mu$ M PDTC (PDTC + TGF $\beta$ ), nuclear DNA content was analyzed in a flow cytometer by staining nuclei with propidium iodide. Representative histograms together with the proportion of nuclei (%) with a DNA content lower than 2C are shown.

scavengers offer a high degree of protection for fetal hepatocytes against TGF- $\beta$ -induced apoptosis. A representative experiment for PDTC effect is shown in Fig. 8 (*right panel*).

In contrast to these results the inhibitory effect of TGF- $\beta$  on fetal hepatocyte growth was not modified in the presence of radical scavengers. Thus, when these cells were incubated in the presence of EGF (20 ng/ml) and TGF- $\beta$  (2.5 ng/ml), the EGF-induced [ $^3$ H]thymidine incorporation into DNA was completely blocked by TGF- $\beta$ , regardless of whether ascorbate or PDTC were present (Fig. 9). Identical results were observed when 0.5 ng/ml TGF- $\beta$  was used (data not shown). These results seem to indicate that the oxidative stress induced by TGF- $\beta$  does not preclude its well understood inhibitory effect on fetal hepatocyte growth.

#### DISCUSSION

Programmed cell death, or apoptosis, is the process whereby cells are induced to activate their own death or cell suicide. Apoptosis occurs in a wide variety of cell types and is required during the development of many tissues. Failure to negatively regulate apoptosis is associated with degenerative diseases, and failure to positively regulate apoptosis is associated with cancer and autoimmune diseases (1). In many models of apoptosis, cells are induced to die as a result of changes in envi-



**FIG. 9. Effect of radical scavengers on TGF- $\beta$  inhibition of fetal hepatocyte growth.** Cells were left untreated (*NONE*) or treated for 24 h with 20 ng/ml EGF or 20 ng/ml EGF + 2.5 ng/ml TGF- $\beta$  (*E + T*), alone (*C*) or in the presence of different radical scavengers: 1 mM ascorbic acid (*ASC*) or 50  $\mu$ M PDTC added 15 min after the TGF- $\beta$  treatment. DNA synthesis was analyzed as described under "Materials and Methods" after incubating the cells in the presence of [<sup>3</sup>H]thymidine (0.5  $\mu$ Ci/ml, 1  $\mu$ M thymidine) for the previous 20 h. Results are mean  $\pm$  S.E. of three independent experiments.

ronmental stimuli, such as growth factors and hormones. TGF- $\beta$  constitutes one of these apoptotic factors for some types of cells, such as rat hepatocytes (9) and hepatoma cells (10).

We have recently shown that TGF- $\beta$  may be a modulator of both growth and differentiation of fetal hepatocytes in primary culture (26). The results presented here demonstrate that TGF- $\beta$  also regulates death of these cells. Hepatocytes die in response to the factor (Figs. 1 and 2), inducing DNA fragmentation and the appearance of nuclei with a DNA content lower than 2C (Fig. 3), typical of a programmed cell death model. These last observations contrast with those of Oberhammer *et al.* (9, 41, 42), who showed that apoptosis induced by TGF- $\beta$ , both in adult hepatocytes and in regressing liver, did not show DNA fragmentation. These differences are likely due to the method used for DNA fragmentation analysis. In our studies, only cytosolic, fragmented DNA is isolated, as in total DNA isolation experiments it was difficult to observe the typical oligosomal ladder (results not shown). This may be due to the fact that these primary cultures do not respond synchronically to TGF- $\beta$ , and so, at any given time, not more than 12–15% of the cells show nuclei with a DNA content lower than 2C (Fig. 3). However, we cannot rule out the possibility of adult and fetal hepatocytes having different responses to TGF- $\beta$  in terms of apoptosis. In contrast, our results agree with those of Fukuda *et al.* (10) in hepatoma cells, where TGF- $\beta$  induced ladder-pattern DNA cleavage.

TGF- $\beta$ -induced apoptosis in fetal hepatocytes is preceded by an enhancement in reactive oxygen species production (Fig. 4), an increase in the GSSG/(GSH + GSSG) ratio, and a decrease in the glutathione intracellular content (Fig. 5). Reduced glutathione (GSH) is used intracellularly to reduce numerous oxidizing compounds, including reactive oxygen species. Agents that induce oxidative stress in hepatocytes, such as *tert*-butyl hydroperoxide, cause accumulation of GSSG because the capacity of NADP-dependent GSSG-reductase becomes rate-limiting and the NADPH/NADP ratio decreases (39, 43). It has been proposed that efflux of GSSG from the cell then occurs in order to preserve the cellular normal redox state so that a depletion in the glutathione levels is always observed (43). Thus, an increase in the reactive oxygen species production and a decrease in the glutathione concentrations indicate that TGF- $\beta$  induces an oxidative stress in fetal hepatocytes. Support for this idea also comes from the observations that (i) H<sub>2</sub>O<sub>2</sub> production by TGF- $\beta$  has already been found in bovine pulmonary artery endothelial cells (13) and in mouse osteoblastic cells (14), (ii) Kayanoki *et al.* (44) have recently described that

TGF- $\beta$  suppresses the expression of antioxidative enzymes in adult rat hepatocytes, thereby showing that production of peroxides is increased in these cells, and (iii) Abdel-Razzak *et al.* (45) have shown that TGF- $\beta$  down-regulates cytochromes P-450 1A1 and 1A2, two genes whose expression is modulated by oxidative stress (46) in adult hepatocytes. However, at present it is not clear if TGF- $\beta$ -induced peroxide production may cause growth inhibition, apoptosis, or both things. The results presented in this paper clearly relate TGF- $\beta$  reactive oxygen species production to fetal hepatocyte cell death. First, low concentrations of TGF- $\beta$  (0.5 ng/ml) sufficient to completely block fetal hepatocyte growth (Fig. 1) do not induce reactive oxygen intermediate production in these cells (results not shown). Second, studies performed to analyze *c-fos* expression, as a gene modulated by redox state (40, 47), demonstrate that only high, apoptotic concentrations of TGF- $\beta$  (2.5 ng/ml) produce an increase in its mRNA levels; this induction is coincident with an increase in the nuclear AP-1 binding activity (Figs. 6 and 7). Finally, TGF- $\beta$ -induced cell death in fetal hepatocytes may be either partially blocked by single radical scavengers (Fig. 8) or totally blocked by combinations of these. However, these agents do not preclude the TGF- $\beta$  growth-inhibitory effect in these cells (Fig. 9). Antioxidants, such as ascorbic acid (vitamin C) or PDTC, a thiol compound and radical scavenger, have proved to be the most potent inhibitors of apoptosis, although the addition of superoxide dismutase alone to the culture medium also partially blocks cell death (Fig. 8). These results strongly suggest that TGF- $\beta$  may be inducing apoptosis in fetal hepatocytes through the generation of reactive oxygen intermediates.

*N*-Acetyl-L-cysteine or NDGA are unable to prevent apoptosis induced by TGF- $\beta$  in the fetal hepatocytes. The addition of NAC to cells increases intracellular glutathione levels (48), but the utilization of NAC by rat hepatocytes is limited by their rate of uptake and conversion to cysteine (49). Moreover, Garcia-Ruiz *et al.* (50) have recently shown that NAC-treatment does not result in a significant increment of the mitochondrial pool GSH, despite significantly increasing the cytosol GSH pool size, so that this compound is probably unable to counteract a mitochondrial oxidative stress in hepatocytes. NDGA is a 5-, 12-, and 15-lipoxygenase inhibitor. Lipoxygenase metabolites have been implicated in TNF responses such as cytotoxicity, induction of the transcription factor *c-fos*, and the mitochondrial superoxide radical scavenging enzyme, manganous superoxide dismutase (for review, see Ref. 51). The inability of NDGA to protect against TGF- $\beta$ -induced apoptosis in fetal hepatocytes indicates that the lipoxygenase pathway is unlikely to be responsible for the oxidative stress induced by TGF- $\beta$  in these cells.

Elevated expression of *c-fos* has previously been related to programmed cell death (52). However, the time course analysis of *c-fos* mRNA levels shows that its gene expression increases just when cells stop dying (Figs. 1 and 6). So, we can speculate that *c-fos* might be a survival gene in fetal hepatocytes. Moreover, we have found a correlation between *c-fos* mRNA expression and AP-1 activity after 24 h of TGF- $\beta$  treatment (Fig. 7). These results support the idea that functional Fos, in its AP1 form, may be involved in cell response to TGF- $\beta$ -induced oxidative stress and in cell survival.

Reactive oxygen intermediates, such as hydrogen peroxides or oxygen radicals are, in general, cytotoxic. The involvement of oxygen radicals in inflammation or aging processes is well established. In recent years, on the basis of the elegant studies by Kane *et al.* (53) and Hockenbery *et al.* (17) oxidative stress and radical oxygen intermediates have been proposed as integral control elements in the cell's decision to enter apoptosis.

Results presented in this paper provide evidence for the involvement of an oxidative process in the apoptosis elicited by TGF- $\beta$  in fetal hepatocytes. Further work will be necessary to completely understand the molecular mechanism by which high concentrations of TGF- $\beta$  induce oxygen radical production and apoptosis, whereas lower concentrations of this factor (enough to inhibit cell growth) do not.

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