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Tumor Necrosis Factor-α-Induced Apoptosis in Hepatocytes in Long-Term Culture

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Apoptosis occurs naturally in the liver and increases in specific pathogenic processes. We previously described the use of a chemically defined medium supplemented with epidermal growth factor and dimethylsulfoxide to maintain rat hepatocytes in a highly differentiated state for more than 30 days (long-term culture). In this study, we showed that hepatocytes in long-term dimethylsulfoxide culture have definite advantages over using cells in short-term culture (cells in culture for 2 to 4 days) to study apoptosis. We demonstrated that treatment with tumor necrosis factor (TNF)-α induced apoptosis (detected morphologically and by formation of an oligonucleosomal DNA ladder) only in hepatocytes that had been subjected to dimethylsulfoxide removal. Neither treatment with TNF-α alone or dimethylsulfoxide removal alone induced apoptosis. Apoptosis could be induced by concentrations as low as 500 U of TNF-α/ml. Although a DNA ladder was not detected by 12 hours after TNF-α treatment, it was easily identified by 24 hours. We conclude that this system can be used 1) to examine the underlying mechanism by which TNF-α causes apoptosis in hepatocytes and 2) to study induction of apoptosis in hepatocytes by other agents. (Am J Pathol 1996, 148:485–495)

Cell death occurs as a result of either necrosis or apoptosis. Apoptosis, or programmed cell death, is of central importance in normal development, homeostasis, and pathogenic processes[1–5] and is an important defense mechanism for removal of potentially dangerous cells such as self-reactive lymphocytes, cells that have been infected by viruses, and tumor cells.[6–9] Apoptosis occurs naturally in the liver, but at the low rate of 2 to 4 per 10,000 hepatocytes in the adult rat,[10,11] making identification of apoptotic bodies in untreated liver somewhat difficult. It has been demonstrated that induction of mitogenesis in the liver by several agents including environmental pollutants (DDT and hexachlorocyclohexane), estrogenic or progestational steroids, the sex steroid cyproterone acetate, lead nitrate, or drugs such as phenobarbital leads to pronounced cell multiplication in the liver normally followed by apoptosis.[12,13] Interruption of portal flow in rats also leads to considerable cell loss by apoptosis.[14,15] Immunologically induced apoptosis is observed in the liver and may be caused by drugs or viruses. Cytotoxic lymphocytes attack foreign antigens on the hepatocyte membrane and can lead to necrosis and/or apoptosis in acute hepatitis, chronic hepatitis, primary biliary cirrhosis, etc.[16] Interestingly, the Councilman bodies first described in yellow fever were most likely apoptotic bodies.[17]

Tumor necrosis factor (TNF)-α, a 17-kd mammalian cell macrophage/monocyte-derived lymphokine, originally defined for its anti-tumor activity, binds to specific receptors on most mammalian cells and has many different effects on target cells.[18] TNF-α can affect growth, induce differentiation, and alter gene expression. TNF-α has been shown to initiate apoptotic cell death and DNA fragmentation in several mammalian cell lines including human leukemia cell lines and murine fibrosarcoma cell lines.[19] Kupffer cells (liver macrophages), the largest population of fixed macrophages in the body, are thought to be a major source of TNF-α.[20] Therefore, it is highly possible that TNF-α may play a role in apoptosis that

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occurs spontaneously in liver or during disease processes. Indeed, it has been demonstrated previously that TNF-α can kill primary hepatocytes in short-term culture[21,22] and more recently that injection *in vivo* of TNF-α induces apoptosis in hepatocytes in D-galactosamine-sensitized mice.[23]

We previously developed a method in our laboratory for long-term culture of differentiated hepatocytes that involves plating primary rat hepatocytes on a plate coated with rat tail collagen and feeding the cells a chemically defined medium (CDM) supplemented with 2% dimethylsulfoxide (DMSO).[24] We have successfully used this long-term DMSO-treated hepatocyte culture system to study molecular mechanisms of albumin expression,[25,26] immortalization and transformation of hepatocytes,[19,27,28] and DNA synthesis.[29] Other laboratories have used DMSO-treated hepatocytes in long-term culture to study differentiated hepatocyte function at the level of maintenance of cytochrome P450 content,[30–32] maintenance of responsiveness to the peroxisome proliferator nafenopin,[30,32] increased synthesis of several different cytochrome P450s,[33,34] and enhanced lipid synthesis and secretion.[35] The purpose of the present study was to determine 1) whether long-term DMSO-treated hepatocytes can be used to study apoptosis and, if so, 2) whether TNF-α can induce apoptosis in long-term DMSO-treated hepatocytes.

Materials and Methods

Hepatocyte Cultures

Primary adult rat hepatocytes were isolated by collagenase perfusion of male Fischer 344 rats (180 to 200 g) as previously described[36] and modified.[37,38] Hepatocytes were washed in a CDM (RPCD medium[25,27]) supplemented with 3% fetal calf serum and plated at a density of approximately 10^6 cells/60-mm tissue culture dish (Becton Dickinson Labware, Bedford, MA) coated with rat tail collagen.[39] At 5 to 6 hours after plating, hepatocyte monolayers were fed fresh serum-supplemented RPCD, and at 24 hours after plating the cells were fed fresh serum-free CDM supplemented with 2% DMSO and 25 ng of epidermal growth factor (EGF)/ml (Sigma Chemical Co., St. Louis, MO). Long-term hepatocytes were fed fresh serum-free CDM plus DMSO plus EGF every 2 days. Long-term hepatocytes were exposed to serum only during the first 24 hours after plating. For DMSO removal experiments, cells were fed fresh CDM plus DMSO plus EGF or CDM plus EGF (DMSO-deficient medium) when the experiment was initiated and every 48 hours thereafter. For TNF-α (recombinant murine TNF-α; Boehringer Mannheim, Gaithersburg, MD) treatment experiments, cells were fed CDM plus DMSO plus EGF plus TNF-α or CDM plus EGF plus TNF-α (DMSO-deficient medium) when TNF-α treatment was initiated. Control cultures not treated with TNF-α were fed fresh CDM plus DMSO plus EGF or fresh CDM plus EGF when parallel experimental cultures were fed fresh medium supplemented with TNF-α.

5-Bromo-2'-Deoxyuridine (BrdU) Labeling of DNA Synthesis

Primary hepatocytes were labeled with 10 μmol/L BrdU (Boehringer Mannheim Corp., Indianapolis, IN) diluted in sterile cell culture medium for 16 hours at the indicated times. The labeling medium was removed by aspiration and the samples were washed three times with a phosphate-buffered saline (PBS) solution. Cells were fixed in ethanol (70% ethanol in glycine buffer, 50 mmol/L, pH 2.0) overnight at −20°C. Cells were washed three times with PBS and treated with 30% hydrogen peroxide for 20 minutes. Cells were washed three times with PBS and covered with an anti-BrdU working solution for 30 minutes at 37°C. Cells were washed three times with PBS and covered with anti-mouse-Ig-alkaline phosphatase solution and incubated for 30 minutes at 37°C. Cells were washed three times with PBS, covered with freshly prepared color substrate solution containing 13 μl of nitroblue tetrazolium solution, 10 μl of X-phosphate solution, and 3 ml of substrate buffer (100 mmol/L Tris-HCl buffer, 100 mmol/L NaCl, 50 mmol/L $MgCl_2$, pH 9.5), and incubated for 15 to 30 minutes at room temperature. Cells were washed three times with PBS and a coverslip was applied with mounting medium (Shandon Lipshaw, Pittsburgh, PA). The samples were evaluated by light microscopy. Photographs were taken of random fields on the culture plates and used to determine the percentage of labeled nuclei.

DNA Fragmentation Analysis

Low molecular weight DNA was isolated as described.[40–42] For each experimental condition, six 60-mm plates of hepatocytes (initially plated at 10^6 cells/plate) were washed with PBS and trypsinized (GIBCO BRL, Gaithersburg, MD), and the pooled cells were pelleted by centrifugation at 2000 × *g* for 10 minutes. The cell pellet was resuspended in PBS and centrifuged at 8000 × *g* for 3 minutes. The

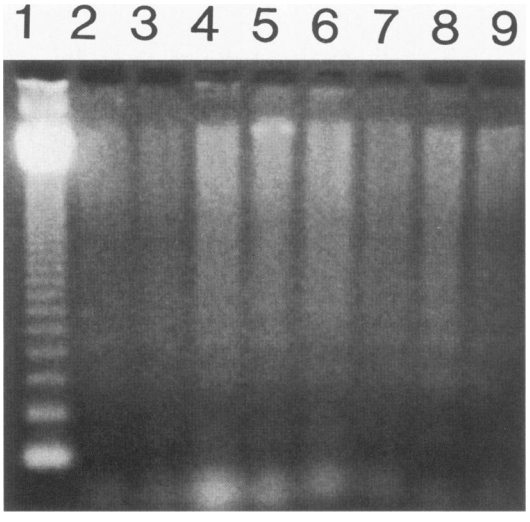

Figure 1. *Effect of TNF-α on hepatocytes in short-term culture. Day 1 hepatocytes were fed CDM supplemented with 2% DMSO and 25 ng EGF/ml* (lanes 2 *and* 3), *CDM with EGF and no DMSO* (lanes 4 *and* 5), *CDM alone* (lanes 6 *and* 7), *or CDM with DMSO and no EGF* (lanes 8 *and* 9). *At day 2 after plating, cells were re-fed culture medium alone* (lanes 2, 4, 6, *and* 8) *or supplemented with 1000 U of TNF-α/ml* (lanes 3, 5, 7, *and* 9) *and harvested 48 hours later, or 96 hours after plating. Low molecular weight DNA was prepared from cell pellets and analyzed on a 1.8% agarose gel.* Lane 1 *contains a commercially purchased 123-bp DNA ladder (GIBCO BRL) as a control. A faint oligonucleosomal DNA ladder against a background smear was observed for all conditions.*

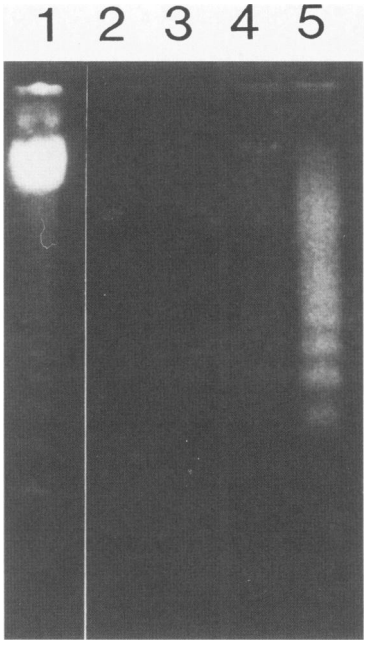

Figure 2. *Effect of TNF-α on hepatocytes in long-term culture. Day 34 hepatocytes were re-fed complete media alone* (lanes 2 *and* 3) *or media deficient in DMSO* (lanes 4 *and* 5) *for 48 hours at which time the cells were re-fed complete media alone* (lane 2), *complete media supplemented with 5000 U of TNF-α/ml* (lane 3), *DMSO-deficient media alone* (lane 4), *or DMSO-deficient media supplemented with 5000 of U TNF-α/ml* (lane 5). *Cells were harvested 48 hours after TNF-α treatment (38 days after plating). Low molecular weight DNA was prepared from cell pellets and analyzed on a 1.8% agarose gel.* Lane 1 *contains a commercially purchased 123-bp DNA ladder (GIBCO BRL) as a control. A strong oligonucleosomal DNA ladder was observed in* lane 5.

resulting cell pellet was resuspended in lysis buffer (10 mmol/L Tris-HCl, pH 8.0, 10 mmol/L EDTA, 0.5% Triton X-100) and centrifuged at 13,000 × *g* for 20 minutes at 4°C. The supernatant was treated with DNAse-free, RNAse, (Boehringer Mannheim) at a concentration of 0.1 mg/ml for 1 hour at 37°C. Proteinase K (0.2 mg/ml; Boehringer Mannheim) and sodium dodecyl sulfate (1% final volume) were added for 2 hours at 50°C. The samples were then extracted with a phenol/chloroform (1:1) preparation for 10 minutes. The DNA was precipitated overnight with 100% ethanol and 0.1 volume of 5 mol/L NaCl at −20°C. The DNA samples were analyzed with a 1.8% agarose gel.

Acridine Orange Staining

Cells were fixed in 10% acetic acid in ethanol for 1 hour followed by ethanol for 2 hours and kept in PBS at 4°C until used. Cells fixed in acetic acid in ethanol were stained for 3 minutes in 0.1% acridine orange solution in 67 mmol/L phosphate buffer (67 mmol/L Na_2HPO_4, 67 mmol/L KH_2PO_4, pH 6.0), washed with phosphate buffer for 1 minute followed by 0.1 mol/L calcium chloride. Under UV illumination, acridine-orange-stained double-stranded nucleic acids are yellow/green and single-stranded nucleic acids are orange/red.

Results

Effect of TNF-α on Hepatocytes in Short-Term Culture

We attempted to determine whether TNF-α treatment of primary hepatocytes in short-term culture caused programmed cell death as measured by the presence of an oligonucleosomal DNA ladder. Rat hepatocytes isolated by collagenase perfusion were plated on cell culture dishes coated with rat tail collagen and fed CDM plus DMSO plus EGF, CDM plus EGF, CDM plus DMSO, or CDM. At day 2 after perfusion, cells were re-fed and harvested 48 hours later, or a total of 96 hours after plating (Figure 1). Microscopic observation revealed some cell death under all four cell culture conditions. Analysis of DNA from these cultures revealed the presence of a faint ladder against a background smear indicating that some low molecular weight apoptosis and some necrosis was ongoing in the short-term cultures. Parallel cultures of hepatocytes maintained in the four different culture media were fed 1000 U of TNF-α/ml on day 2 and harvested 48 hours later. Background

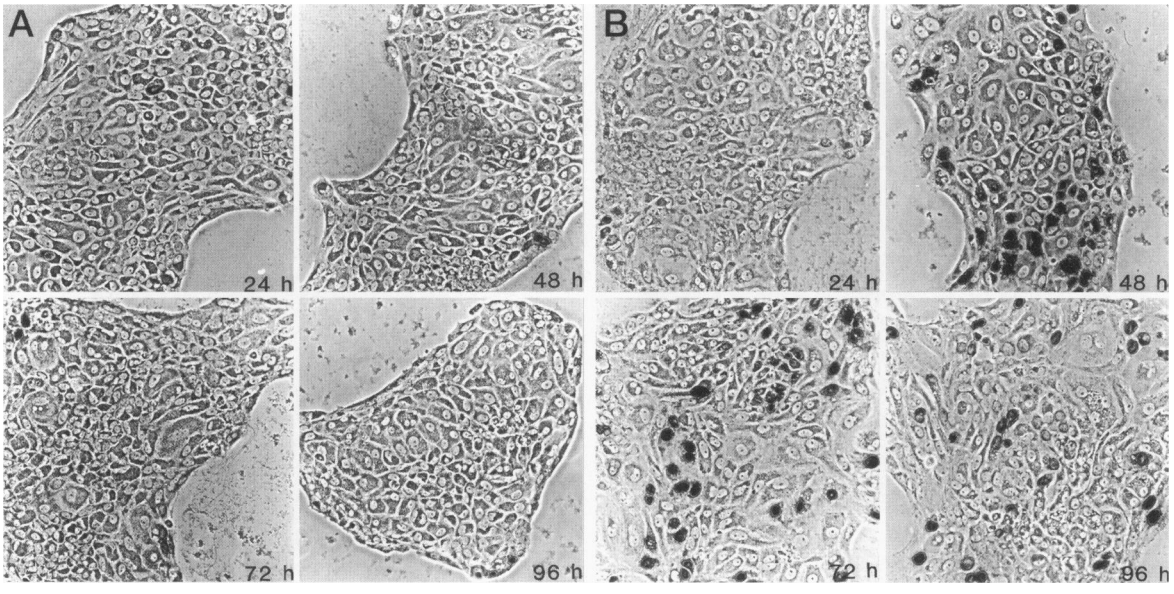

Figure 3. *Photomicrographs of DNA synthesis in hepatocytes subjected to DMSO removal. Hepatocytes maintained in CDM plus EGF plus DMSO for 45 days were fed* (A) *CDM plus DMSO plus EGF or* (B) *CDM plus EGF (DMSO removal) and harvested 24, 48, 72, and 96 hours later. Control cultures and cultures subjected to DMSO removal were labeled with BrdU for 16 hours before harvest. Magnification, ×150. The dark nuclei indicate BrdU-labeled cells.*

levels of cell death similar to those observed in untreated short-term hepatocytes were observed in TNF-α-treated cultures.

Effect of TNF-α Treatment of Hepatocytes in Long-Term Culture

We then attempted to determine whether TNF-α would induce an oligonucleosomal ladder in hepatocytes in long-term culture. When hepatocytes maintained in CDM plus EGF plus DMSO medium for 36 days were treated with TNF-α (5000 U/ml) for 48 hours, no DNA ladder was observed (Figure 2, lane 3). It is also important to note that the background cell death observed in short-term cultures (Figure 1) was absent or markedly reduced in long-term cultures in both the absence and presence of TNF-α (Figure 2, lanes 2 and 3).

Effect of DMSO Removal on DNA Synthesis and TNF-α Sensitivity in Hepatocytes in Long-Term Culture

We have previously observed that hepatocytes maintained in CDM plus EGF plus DMSO have very low labeling indices, ranging from 0.8 to 3.0% measured over a range of time from day 1 to day 70 (H. C. Isom, unpublished data). We attempted to determine whether hepatocytes in long-term culture would become sensitive to TNF-α-induced ladder formation if the hepatocytes were induced to synthesize DNA. To achieve this goal, it was necessary to define conditions that would lead to induction of DNA synthesis in a significant percentage of the cell population without causing cell death. Hepatocytes maintained in CDM plus EGF plus DMSO for 48 days were washed and fed medium deficient in DMSO (DMSO removal). The percentage of cells in the population synthesizing DNA at 0, 24, 48, 72, and 96 hours after DMSO removal was determined by BrdU labeling (Figures 3 and 4). Maximal DNA synthesis was ob-

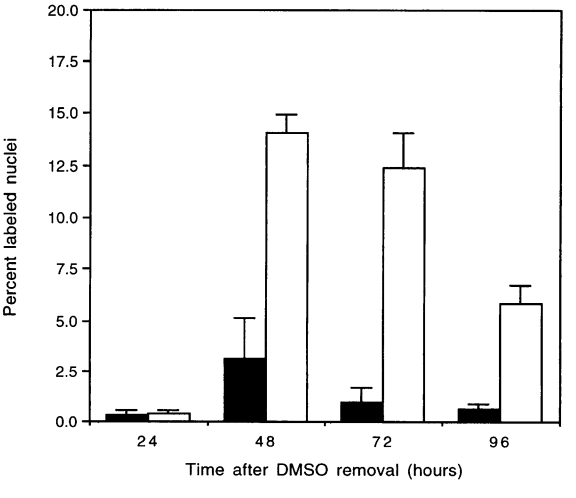

Figure 4. *Quantitative analysis of DNA synthesis in hepatocytes subjected to DMSO removal. Hepatocytes maintained in CDM plus EGF plus DMSO for 45 days were fed CDM plus DMSO plus EGF (■) or CDM plus EGF (DMSO removal, □). Conditions were the same as described in Figure 3. The number of nuclei counted for each experiment ranged from 379 to greater than 900. The data presented are an average of two experiments.*

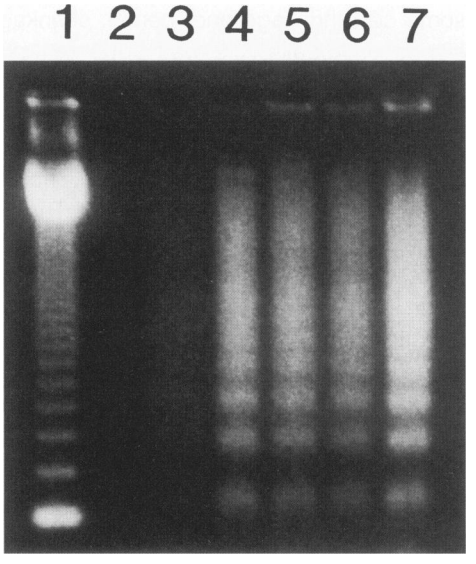

Figure 5. *Effect of concentration on TNF-α-induced apoptosis. Day 34 hepatocytes were subjected to 48 hours of DMSO removal, treated for 48 hours with 0* (lane 3), *500* (lane 4), *750* (lane 5), *1000* (lane 6), *or 5000* (lane 7) *U of TNF-α/ml and then harvested.* Lane 2 *contains DNA extracted from day 38 hepatocytes not subjected to DMSO removal. Low molecular weight DNA was prepared from cell pellets and analyzed on a 1.8% agarose gel.* Lane 1 *contains a commercially purchased 123-bp DNA ladder (GIBCO BRL) as a control. Oligonucleosomal DNA ladders were observed in* lanes 4 *to* 7.

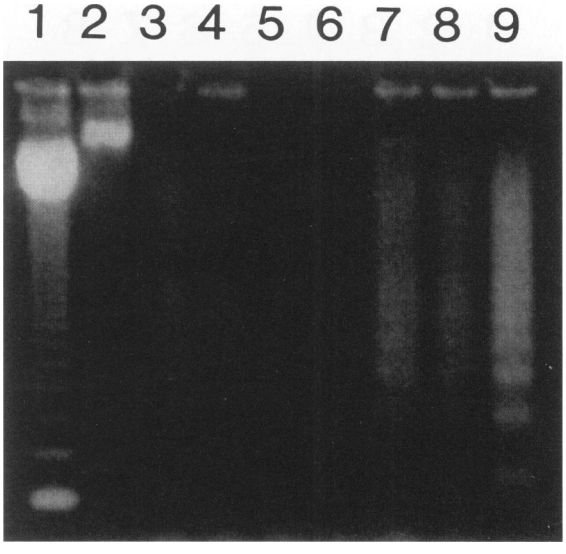

Figure 6. *Effect of time on TNF-α-induced apoptosis. Hepatocytes were subjected to DMSO removal for 48 hours and treated with control medium for 6* (lane 2), *12* (lane 4), *24* (lane 6), *or 48* (lane 8) *hours or medium supplemented with 1000 U of TNF-α for 6* (lane 3), *12* (lane 5), *24* (lane 7), *or 48* (lane 9) *hours. Hepatocytes had been in culture for 50 days when they were harvested. Low molecular weight DNA was prepared from cell pellets and analyzed on a 1.8% agarose gel.* Lane 1 *contains a commercially purchased 123-bp DNA ladder (GIBCO BRL) as a control. A strong oligonucleosomal DNA ladder was observed in* lanes 7 *and* 9. *A fainter ladder was also detected in* lane 8.

served at 48 hours after DMSO removal. Day 34 hepatocytes were incubated in media deficient in DMSO for 48 hours, at which time the cells were re-fed DMSO-deficient media alone or DMSO-deficient media supplemented with TNF-α (5000 U/ml). Cells were harvested 48 hours after TNF-α treatment, and low molecular weight DNA was extracted and analyzed. It should be noted that DMSO removal continues during TNF-α treatment; that is, cells were harvested 48 hours after TNF-α treatment, which was also 96 hours after DMSO removal. DMSO removal for 96 hours alone did not induce apoptosis (Figure 2, lane 4), but DMSO removal followed by TNF-α treatment led to the presence of a distinct oligonucleosomal DNA ladder (Figure 2, lane 5).

Effect of Concentration on TNF-α-Induced Apoptosis

Initial experiments on the effects of TNF-α on hepatocytes were carried out arbitrarily using 5000 U of TNF-α/ml. To determine the effect of concentration of TNF-α, hepatocytes in long-term culture were subjected to 48 hours of DMSO removal and then treated for 48 hours with 500, 750, 1000, or 5000 U of TNF-α/ml. An oligonucleosomal ladder was easily detectable when cells were treated with TNF-α at a concentration of 500 U/ml, but the intensity of the ladder significantly increased when treatment was with 5000 U (Figure 5).

Effect of Time on TNF-α-Induced Apoptosis

We initially observed apoptosis by treating hepatocytes exposed to 48 hours of DMSO removal to TNF-α for 48 hours. To determine the length of time of TNF-α treatment required to be able to detect DNA ladder formation, long-term cultures of hepatocytes were subjected to DMSO removal for 48 hours and treated with control medium or medium supplemented with 1000 U of TNF-α/ml for 6, 12, 24, or 48 hours (Figure 6). As was expected from our previous results (Figure 2, lane 5) ladder formation was easily detectable after 48 hours of treatment. Ladder formation was not detectable after 6 or 12 hours of TNF-α treatment but was detectable after 24 hours of TNF-α treatment.

Effect of Length of Time of DMSO Removal Before Exposure to TNF-α-Induced Apoptosis

Hepatocytes in long-term culture were subjected to DMSO removal for 16, 24, or 48 hours and then treated with TNF-α for 48 hours (1000 U/ml; Figure 7). Ladder formation was equally strong whether

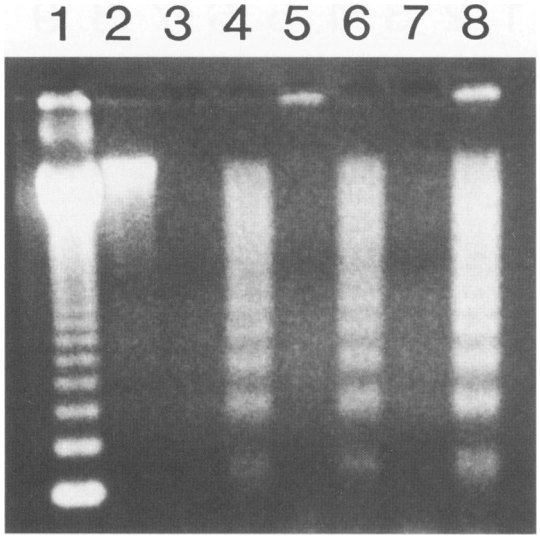

Figure 7. *Effect of length of time of DMSO removal on TNF-α-induced apoptosis. Hepatocytes were subjected to DMSO removal for 16* (lanes 3 *and* 4), *24* (lanes 5 *and* 6), *or 48* (lanes 7 *and* 8) *hours, treated with control medium* (lanes 3, 5, *and* 7) *or 1000 U of TNF-α/ml for 48 hours* (lanes 4, 6, *and* 8), *and harvested.* Lane 2 *contains DNA extracted from control long-term cells not subjected to DMSO removal. Hepatocytes had been in culture for 36 days when they were harvested. Low molecular weight DNA was prepared from cell pellets and analyzed on a 1.8% agarose gel.* Lane 1 *contains a commercially purchased 123-bp DNA ladder (GIBCO BRL) as a control. Strong oligonucleosomal DNA ladders were observed in* lanes 4, 6, *and* 8.

DMSO removal was for 16, 24, or 48 hours. No ladders were observed when cells were subjected to DMSO removal but were not treated with TNF-α.

Morphological Analysis of Apoptotic Cell Death in Hepatocytes Subjected to DMSO Removal and Treated with TNF-α

Experiments were next carried out to analyze apoptotic cell death at the morphological level in hepatocytes in long-term DMSO culture subjected to DMSO removal and treated with TNF-α. Hepatocytes in long-term culture were fed medium deficient in DMSO for 24 hours, at which time the cells were re-fed DMSO-deficient medium supplemented with TNF-α (5000 U/ml) and harvested 24, (Figure 8, B and G) 48 (Figure 8C), and 72 (Figure 8, D and H) hours after TNF-α treatment. Control cultures were fed complete medium (Figure 8, A and E) or complete medium supplemented with TNF-α (Figure 8F). Cells were fixed, stained with acridine orange, observed under UV illumination, and photographed or fixed, stained with hematoxylin and eosin (H&E), observed under light microscopy, and photographed. The overall morphology of the hepatocyte monolayer changed markedly with time after TNF-α treatment. By 24 hours after treatment, apoptotic bodies and fragmentation of nuclear material were detectable and some cell shrinkage and, hence, shrinkage of the islands of hepatocytes was apparent, but, in general, the monolayer remained intact. By 48 hours, cell shrinkage was more apparent and, by 72 hours, many edges of the hepatocyte islands had curled. The number of apoptotic bodies steadily increased at 48 and 72 hours after TNF-α treatment. The use of two different staining procedures, H&E and acridine orange, to examine apoptosis in hepatocytes at the morphological level was advantageous. The overall effects on the hepatocyte monolayer were easily observed in H&E-stained cells whereas the condensation of DNA and formation of apoptotic bodies were more apparent in acridine-orange-stained cells.

Comparison of Apoptotsis and Necrosis in Hepatocytes in Long-Term Culture

Experiments were carried out to compare apoptosis and necrosis in hepatocytes in long-term DMSO culture. Rotenone, an inhibitor of electron transport, was selected as the necrosis-inducing agent. Hepatocytes maintained in long-term DMSO culture for approximately 60 days were 1) fed fresh complete medium (control cells), 2) subjected to DMSO removal for 24 hours followed by 48 hours of treatment with 5000 U of TNF-α/ml, and 3) fed fresh complete medium supplemented with rotenone (100 μmol/L) for 16 hours. As expected from previous experiments, no oligonucleosomal ladder was induced in the control cells and a strong oligonucleosomal ladder was induced in cells subjected to DMSO removal and TNF-α treatment (Figure 9). In rotenone-treated cells, no ladder was present. Microscopic analyses of fixed and stained rotenone-treated cells revealed the presence of many large holes within the hepatocyte islands (Figure 8, I and J). Hepatocytes that remained attached to the collagen-coated dish appeared to be no longer viable and had small irregularly shaped nuclei and an abnormally stained cytoplasm.

Discussion

We conclude the following from this report: 1) short-term rat hepatocyte cultures (cells in culture for 2 to 4 days) are not a good system for studying apoptosis because of background levels of cell death; 2) rat hepatocytes maintained in the long-term culture conditions used in this study (30 to 60 days in CDM plus EGF plus DMSO) represent a good system for studying apoptosis because background cell death is essentially absent; 3) TNF-α treatment alone does

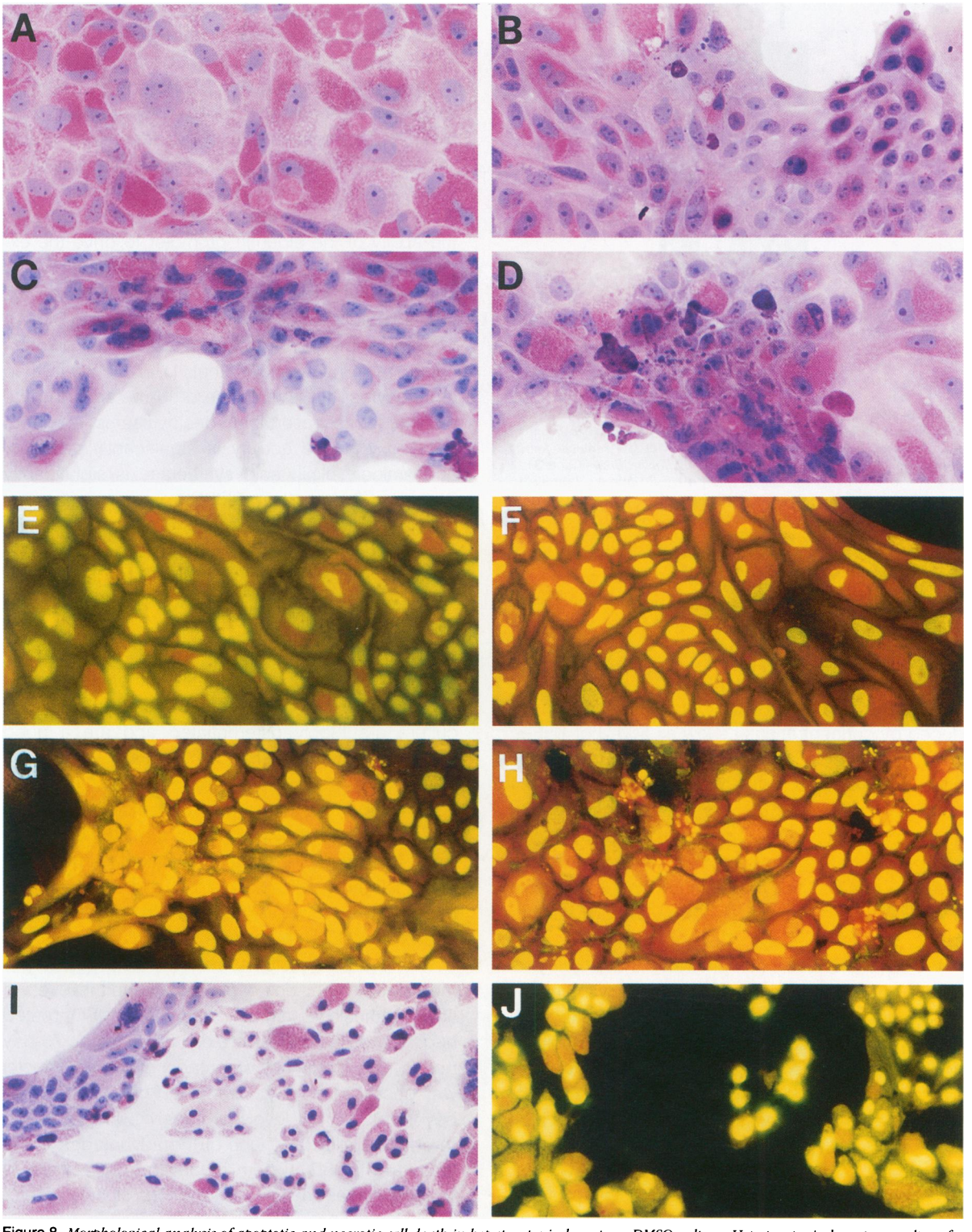

Figure 8. *Morphological analysis of apoptotic and necrotic cell death in hepatocytes in long-term DMSO culture. Hepatocytes in long-term culture for 56 days were either fed complete medium* (A *and* E) *or complete medium supplemented with TNF-α for 72 hours* (F) *or subjected to DMSO removal for 24 hours and treated with TNF-α (5000 U/ml) for 24* (B *and* G), *48* (C), *or 72* (D *and* H) *hours. Hepatocytes in long-term culture that were not subjected to DMSO removal were treated with rotenone (100 μmol/L) for 16 hours* (I *and* J). *Cells were fixed, stained with H&E, observed under light microscopy, and photographed* (A *to* D *and* I) *or fixed, stained with acridine orange, observed under UV illumination, and photographed* (E *to* H *and* J). *Magnification, ×240.*

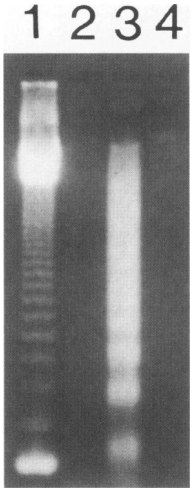

Figure 9. *Absence of oligonucleosomal ladder in hepatocytes treated with rotenone. Hepatocytes in long-term culture for 62 days were either fed complete medium* (lane 2), *subjected to DMSO removal for 24 hours, and treated with TNF-α (5000 U/ml) for 48 hours* (lane 3) *or treated with rotenone (100 μmol/L) for 16 hours* (lane 4). *Low molecular weight DNA was prepared from cell pellets and analyzed on a 1.8% agarose gel.* Lane 1 *contains a commercially purchased 123-bp DNA ladder (GIBCO BRL) as a control. A strong oligonucleosomal DNA ladder was observed in* lane 2.

not induce apoptosis in hepatocytes in long-term culture; and 4) TNF-α induces apoptosis in hepatocytes in long-term culture that have been subjected to DMSO removal, and this process is both time and concentration dependent. Apoptosis could be induced by concentrations as low as 500 U of TNF-α/ml. Although apoptosis was not detected at 12 hours after TNF-α treatment, it was easily identified by 24 hours after treatment. In the process of carrying out this study, we also determined that DNA synthesis can be induced in long-term DMSO-treated hepatocytes by removal of DMSO alone without downshift and without simultaneous removal of dexamethasone.

We have previously demonstrated that primary rat hepatocytes plated on plates coated with rat tail collagen and maintained in CDM supplemented with 2% DMSO survive, retain hepatocyte morphology, and continue to secrete albumin at high levels for over a year.[24,25] Supplementation of the medium with EGF was not necessary for maintenance of high levels of albumin production per cell but did increase the number of cells per dish by approximately 50%. We have also demonstrated that 1) DMSO-treated hepatocytes in culture for 40 days retain the ability to express albumin RNA at 45% of the level of normal liver, and five other liver-specific genes (A_1AT, ligandin, phenylalanine hydroxylase, phosphoenolpyruvate carboxykinase, and transferrin) at levels ranging from 21 to 72%[25]; 2) long-term DMSO-treated hepatocytes express the mRNAs for seven acute-phase proteins at levels ranging from 50 to 150% of the levels in liver; 3) transcription of albumin is 22% of the level in liver and accounts for approximately 43% of the albumin RNA made by the cells; 4) the half-life for albumin mRNA of approximately 26 hours is in agreement with values determined *in vivo*, indicating that there is no significant stabilization of albumin message in long-term DMSO-treated hepatocytes; and 5) the albumin enhancer can increase the ability of the albumin promoter to drive the expression of a CAT reporter gene in long-term DMSO-treated hepatocytes.[26] Other laboratories have examined maintenance of liver differentiation in DMSO-treated primary hepatocytes by quite different criteria and have shown that 1) in DMSO-treated hepatocytes, the level of the drug metabolizing the enzyme ethoxyresorufin-*O*-deethylase is elevated, heme oxygenase activity is significantly reduced, and aminolevulinic acid synthetase is significantly increased[34] and 2) in DMSO-treated hepatocytes exposed to the peroxisome proliferator nafenopin, cytochrome P450 content is increased, the cyanide-insensitive B-oxidation of palmitoyl-CoA is induced, the peroxisomal compartment is markedly expanded, and proliferation of smooth endoplasmic reticulum membranes is observed.[30] Therefore, based on our studies and those of other laboratories we conclude that hepatocytes in long-term DMSO culture are highly differentiated and are a good model system for understanding hepatocyte function *in vivo*. The findings from this study provide additional support for the model. Specifically, we have shown that hepatocytes in long-term DMSO culture resemble *in vivo* liver in that the level of spontaneous apoptosis and cell death is low, but the cells, like hepatocytes in liver, retain the capacity to be induced to undergo apoptosis.

We have previously shown that DMSO-treated primary hepatocytes do not proliferate and the levels of DNA synthesis remain low.[24,29] When DMSO-treated hepatocytes in culture were examined over a 70-day period, the maximal percentage of labeled nuclei was observed on day 3 (ranging from 2.7 to 3.7%); beyond day 5, the levels averaged 1.0 to 1.5% and rarely exceeded 2%. One of the advantages of the long-term DMSO system is that the percentage of labeled nuclei are low, which more closely resembles what is observed in liver *in vivo*. Direct evidence showing that DMSO inhibited DNA synthesis in hepatocytes was reported by McGowan[43] for adult rat hepatocytes and by Baribault and Marceau[44] for hepatocytes from suckling rats. It was also of interest to determine whether long-term DMSO-treated hepatocytes retained the capacity to be induced to syn-

thesize DNA. McGowan showed that DMSO-mediated inhibition of DNA synthesis was reversible.[43] We previously reported that hepatocytes maintained in CDM plus DMSO plus EGF for 20 days and then shifted down to medium deficient in EGF alone or deficient in insulin, glucagon, and EGF for 7 days synthesized DNA when, at day 27 after plating, they were shifted back up into complete medium.[29] The percentage of labeled nuclei declined to less than 1% at 7 days after the shift down and was elevated to almost 5% after the shift up. A considerable increase to 20% or greater labeled nuclei occurred when both DMSO and dexamethasone were removed at the time the cells were shifted back into complete medium. We also found that removal of both DMSO and dexamethasone from day 20 or day 27 cells without a shift down yielded a high labeling index. The concept that removal of DMSO alone from DMSO-treated hepatocytes results in stimulation of hepatocyte DNA synthesis was reported previously by Chan et al[45]; specifically, when hepatocytes were maintained in media supplemented with DMSO and EGF for 7 days, stimulation of DNA synthesis occurred when EGF was maintained and DMSO was removed at day 7. DNA synthesis peaked at 24 and 48 hours after DMSO removal and then declined. We have not previously analyzed the effects of removing only DMSO (as compared with DMSO and dexamethasone) from our long-term DMSO-treated hepatocyte culture system. The findings we report here differ only slightly from those reported by Chan et al[45] in that we observed a maximal labeling index at 48 to 72 hours after DMSO removal compared with their report of 24 to 48 hours after DMSO removal.[45] This difference could be directly related to the number of days the hepatocytes were in culture or could simply reflect differences in media composition, labeling procedure, etc. The unique contribution reported in the current study is that hepatocytes in culture for more than 30 days can be induced to synthesize DNA when subjected to removal of DMSO alone.

Previous studies have shown that apoptosis can be induced in short-term hepatocytes in culture treated with transforming growth factor-β,[46–48] activin,[49] okadaic acid,[50] vinblastin,[51] colchicine,[51] or TNF-α.[21,22] It was demonstrated that TNF-α alone causes apoptosis in rat hepatocytes, and the effect was potentiated by the addition of interferon-γ.[22] DNA ladder formation was observed for hepatocytes that were treated with a combination of TNF-α and interferon-γ. A small number of dying cells were present in the control cultures, which was not surprising for short-term cultures. In an independent study, it was shown that mouse hepatocytes in short-term culture were not sensitive to TNF-α but that TNF-α caused a concentration-dependent cell death in hepatocytes pretreated with actinomycin D, D-galactosamine, or amanitin but not in cells pretreated with cycloheximide or puromycin.[21] Ladder formation was observed for hepatocytes that were pretreated with actinomycin D and then treated with TNF-α. The results of both of the previously reported TNF-α studies as well as the work we report here indicate that TNF-α does not readily induce apoptosis in normal hepatocytes in culture but can induce apoptosis if the cells are physiologically altered.

The question arises as to why it is necessary to subject hepatocytes to DMSO removal to sensitize the cells to TNF-α-induced apoptosis. One possibility is that TNF-α actually can induce apoptosis in hepatocytes in long-term culture, but TNF-α-induced apoptosis is observed only after DMSO removal because DMSO blocks TNF-α function. We were unable to address this question directly in primary hepatocytes because of the design of the DMSO removal experiments. However, we were able to address the question indirectly. In an independent study, we showed that the SV2di 424 cell line (an SV40-immortalized rat hepatocyte cell line designated CWSV2[28] stably transfected with a dimer of the hepatitis B virus genome) is killed when treated with TNF-α (Guilhot, Miller, Cornman, and Isom, unpublished data). If TNF-α is inactivated or its ability to interact with cellular TNF receptors is altered by the presence of DMSO, then SV2di 424 cells treated with TNF-α in the presence of DMSO should not be killed. In fact, we observed that DMSO had no effect on TNF-α killing of SV2di 424 cells, thereby eliminating this possibility (data not shown). We conclude that DMSO removal triggers a cellular response that enables treatment with TNF-α to initiate programmed cell death. This alteration may involve altered TNF-α receptor expression, initiation of immediate early responses that culminate in DNA synthesis, or altered gene expression triggered by DMSO removal but independent from the events that lead to stimulation of DNA synthesis.

Increased circulating TNF-α has been demonstrated in most patients with chronic liver disease,[52–54] in particular in patients with alcoholic liver cirrhosis[55] and chronic hepatitis B virus infection.[56] These disease processes lead to inflammation and subsequent release of elevated levels of the cytokine TNF-α. It appears that TNF-α kills hepatocytes that have already been damaged. Conditions that arise under certain pathophysiological situations may pre-

dispose hepatocytes to direct TNF-α toxicity *in vivo*. We conclude that the system we describe can be used to examine the underlying mechanisms by which TNF-α causes apoptosis in hepatocytes and why it is necessary to subject hepatocytes to DMSO removal to sensitize the cells to TNF-α-induced apoptosis. In addition, the system we have defined can also be used to study induction of apoptosis in hepatocytes by other agents.

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