Choline deficiency induces apoptosis in SV40-immortalized CWSV-1 rat hepatocytes in culture[1]

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ABSTRACT **Immortalized CWSV-1 rat hepatocytes, in which p53 protein is inactivated by SV40 large T antigen, had increased numbers of cells with strand breaks in genomic DNA (terminal dUTP end labeling) when grown in 0 μM choline (67–73% of cells) than when grown in 70 μM choline (2–3% of cells). Internucleosomal fragmentation of DNA (DNA ladders) was detected in cells grown with 5 μM and 0 μM choline for 72 h. Cells treated with 0 or 5 μM choline for 72 h detached from the substrate in high numbers (58% of choline deficient cells vs. 1.4% of choline sufficient cells detached) exhibited a high incidence of apoptosis (apoptotic bodies were seen in 55–75% of cells; 67–73% had DNA strand breaks), and an absence of mitosis and proliferating cell nuclear antigen (PCNA) expression. Cells undergoing DNA fragmentation had functioning mitochondria. At 24 h, cells grown in 0 or 5 μM choline synthesized DNA more rapidly than those grown in 70 μM choline. By 72 h, the cells grown in 0 or 5 μM choline were forming DNA much more slowly than control cells (assessed by thymidine incorporation, PCNA expression, and mitotic index). Western blot analysis showed that p53 in the nucleus of cells was detected in direct association with SV40 T-antigen, and was therefore likely to be inactive. We conclude that choline deficiency kills CWSV-1 hepatocytes in culture by inducing apoptosis via what may be a p53-independent process, and that this process begins in viable cells before they detach from the culture dish.—Albright, C. D., Liu, R., Bethea, T. C., da Costa, K.-A., Salganik, R. I., Zeisel, S. H. Choline deficiency induces apoptosis in SV40-immortalized CWSV-1 rat hepatocytes in culture. *FASEBJ.* 10,510–516 (1996)**

Key Words: p53 · hepatocytes · PCNA expression · cell proliferation

CHOLINE IS IMPORTANT IN MAINTAINING the structural integrity of the plasma membrane, in transmembrane signal transduction, and is an important methyl donor (reviewed in refs 1–3). When rats are fed a choline-deficient diet, the liver undergoes repeated rounds of cell death and proliferation (4–10). Because choline deficiency impairs triglyceride secretion from the liver (11–13), investigators have assumed that massive accumulation of lipid in the liver causes hepatocyte rupture with ensuing necrosis and release of intracellular enzymes (9). However, DNA damage is also an early consequence of choline deficiency (14, 15). Since DNA damage is an important factor in the induction of morphologic changes associated with apoptosis (16), we hypothesized that the induction of apoptosis could contribute to the liver cell death associated with choline deficiency.

Apoptosis is a physiological form of cell death that occurs in single cells. The morphologic changes classically associated with apoptosis include: *1)* chromatin condensation and nuclear pyknosis, *2)* fragmentation of the nucleus, and *3)* formation of apoptotic bodies consisting of membrane-enclosed pieces of condensed chromatin and well-preserved organelles (16, 17). Another characteristic change often occurring in apoptosis results from endonuclease activity that cleaves transcriptionally active nuclear DNA (16), and not mitochondrial DNA (18), into internucleosomal fragments. This DNA breakage is induced before actual apoptosis is detectable by changes in cell morphology (19).

It is well documented that an intact p53 gene and protein are prerequisites for apoptosis by many known triggers (20, 21). We determined whether choline deficiency induced DNA strand breaks with subsequent morphologic appearance of apoptosis in cultured CWSV-1 hepatocytes in which p53 protein was inactivated by simian virus 40 (SV40)[3] large T antigen.

METHODS

Cell culture

SV40 T-antigen immortalized CWSV-1 adult rat hepatocytes were provided by Dr. Harriet C. Isom (Department of Microbiology, The Pennsylvania

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[3] Abbreviations: SV40, simian virus 40; TUNEL, terminal dUTP nucleotide DNA end-labeling technique; PCNA, proliferating cell nuclear antigen; MI, mitotic index; PBS, phosphate-buffered saline; CS, choline-sufficient; CD, choline-deficient.

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State University College of Medicine, Hershey, Pa.). It is known that p53 protein is inactivated by complexing with SV40 large T antigen (22, 23). These cells were derived from normal hepatocytes (24); they express normal levels of liver-specific proteins, grow in serum-free, chemically defined medium (25), and in syngeneic animals are tumorigenic at high, but not at the low, passages we used (26). CWSV-1 cells were grown in serum-free RPMI 1640 medium (American Biorganics, Inc., Niagara Falls, N.Y.) containing 2 mM L-glutamine and supplemented with 70 μM choline chloride, transferrin (0.4 mg/l) (Sigma Chemical Co., St. Louis, Mo.), insulin (12 μg/l) (Sigma), glucagon (35 mg/l) (Sigma), dexamethasone (0.4 mg/l) (Sigma), and pen-strep (penicillin-streptomycin; GIBCO/BRL, Grand Island, N.Y.) at 37°C in a humidified atmosphere containing 5% CO_2.

Choline treatment

Cells were seeded at 1.0×10^4 cells/cm^2 in 6-well plates or in 100 mm dishes and grown in 70 μM choline-sufficient (CS) RPMI 1640 medium for 4 days before rinsing with phosphate-buffered saline (PBS) and growing them in fresh, CS or choline-deficient (CD: 5 μM, 2 μM, or 0 μM choline, as indicated in the figures), RPMI 1640 medium for 3 days.

Measurement of mitochondrial function

Mitochondrial function was assayed by the ability of cells to reduce MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) to an insoluble formazan reaction product that was measured using spectrophotometry (Hitachi U-2000 spectrophotometer) at 570 nm (27, 28).

Morphological analysis of apoptosis

Cells that remained attached to the substrate were fixed in 95% alcohol, stained using hematoxylin and eosin, mounted in Gel/Mount (Biomedia Corp., Foster City, Calif.), and covered with a no. 1 thickness coverglass (29). Cells that had sloughed into the medium were deposited onto glass slides using a cytocentrifuge, then fixed and stained as described for attached cells. Morphologically, apoptosis was defined as the fragmentation of the nucleus into multiple, small hematoxylinophilic bodies (i.e., apoptotic bodies) (17, 30). The percentage of cells with a fragmented nucleus was determined from the hematoxylin- and eosin-stained slides by counting at least 250 cells in 2–4 replicate cultures per treatment.

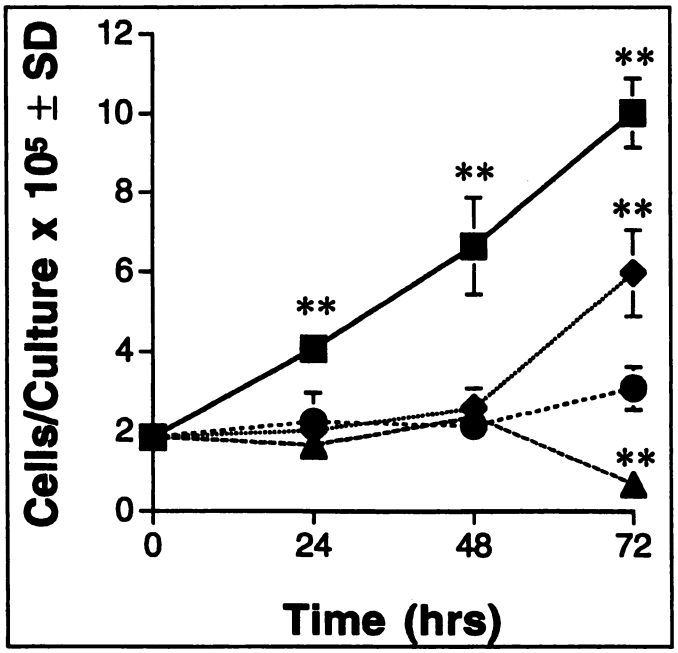

Figure 1. Effect of choline deficiency on cell growth. CWSV-1 liver cells grown in 70 μM choline medium for 4 days were switched at time 0 to choline-sufficient (CS) (70 μM: square) or -deficient (CD) (5 μM: diamond, 2 μM: circle or 0 μM: triangle) medium and maintained for 3 days. Data are expressed as mean ± SD; n = 3/point. (** = $P < 0.01$ different from time zero by 1-way ANOVA).

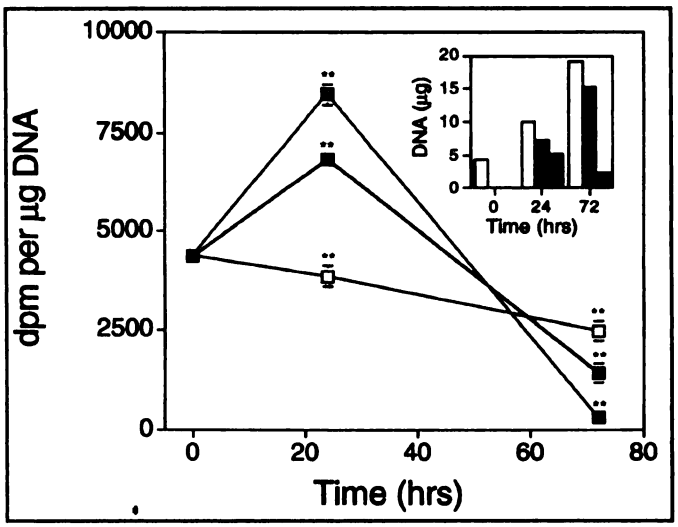

Figure 2. Effect of choline deficiency on DNA synthesis. CWSV-1 liver cells were grown as described in legend to Fig. 1. [^{3}H]Thymidine incorporation into DNA was measured at timed intervals in cells growing in choline-sufficient (CS) (70 μM: white box) or -deficient (CD) (5 μM: black box or 0 μM: hatched box) medium. Data are expressed as mean ± SD; n = 3/point. Inset: DNA mass measured at timed intervals in cells growing in choline-sufficient (CS) (70 μM: white bar) or -deficient (CD) (5 μM: black bar, or 0 μM: hatched bar) medium. (** = $P < 0.01$ by 1-way ANOVA).

Electron microscopy

Detached cells concentrated by centrifugation, as well as cells remaining attached to the substrate, were rinsed two to three times with serum-free RPMI, fixed in 3% glutaraldehyde in serum-free RPMI, and then processed for transmission electron microscopy. Grid-mounted sections were observed on a Zeiss EM10 transmission electron microscope (Carl Zeiss, Oberkochen, Germany).

Analyses of DNA changes

CWSV-1 cells were plated in plastic chamber slides (LabTek, NUNC Inc., Naperville, Ill.) as described above. The cells were fixed in 1% formaldehyde in PBS, pH 7.4, then rinsed twice with PBS.

TUNEL technique

DNA fragmentation was detected using the terminal dUTP nucleotide DNA end-labeling technique (TUNEL), a direct immunoperoxidase method (ApopTag, Oncor, Inc., Gaithersburg, Md,) which visualizes the incorporation of digoxigenin-labeled nucleotide into free 3′-OH ends of DNA, a biochemical change associated with the morphological appearance of apoptosis (31). The TUNEL technique will also detect strand breaks that occur in necrosis and autolysis due to postmortem DNA changes (32). When DNA degradation occurs in necrosis, it follows membrane rupture, and shutdown of mitochondria and other organelles, and thus occurs after a loss of viability. During necrosis, DNA is nonspecifically degraded into a conspicuous smear of nonuniform sizes, as opposed to the characteristic DNA ladder pattern often observed in apoptosis. For this reason we used a combination of the TUNEL method, morphology, and DNA ladders to establish the presence of apoptosis.

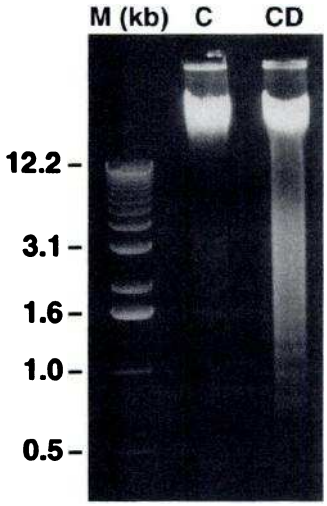

Figure 3. DNA ladder. DNA fragmentation in cells undergoing choline deficiency-mediated cell death. Gel electrophoresis of: lane 1, DNA marker; lane 2, DNA extracted from cells in 70 μM choline medium (C); lane 3, DNA extracted from cells in 0 μM choline medium. A DNA ladder consistent with apoptosis is seen in CD cells but not in CS cells.

Terminal dUTP end-labeling occurred in CWSV-1 cells that were viable and not as a result of postmortem processes. For negative controls, deionized distilled water was substituted for Tdt enzyme in the reaction mixture. Negative controls showed no incorporation of labeled nucleotide.

Apoptotic ladders

Samples (~ 10^6 cells) were lysed in 2 ml of 10 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl, 1.0% SDS, and incubated for 15 min with 1 mg/ml proteinase K at 65°C, and then incubated for 60 min with 24 U/ml DNAse-free RNAse (Sigma) at 37°C. After phenol-chloroform extraction, the DNA was resuspended in 500 μl 10 mM Tris (pH 7.4), 0.1 mM EDTA, and the nucleic acid concentration was determined by measurement of OD260. Nucleic acid (10 μg) was electrophoresed on a 1.8% agarose gel at 25 V for 18 h, stained overnight with ethidium bromide, destained, and then photographed under UV light with Polaroid type 665 film.

Cell proliferation assays

Cells were plated on 6-well plates as described above. At 0, 24, and 72 h, [^{3}H]thymidine (1 μCi/well; 20 mCi/mmol in ethanol, New England Nuclear, Boston, Mass.) was added for a 4 h period. DNA was extracted and radiolabeled DNA was quantitated using scintillation spectrophotometry (LKB rackbeta) (10). Thymidine concentration in the medium was measured using high-pressure liquid chromatography with detection at 267 nm (33); at time 0 media contained approximately 2 μM thymidine, and concentrations did not change over the time period that cells were in culture. After treatment for 72 h with CS or CD medium, the cells were fixed in 1% formaldehyde in PBS, pH 7.4, rinsed once with PBS, then postfixed in ice-cold methanol ×5 min followed by ice-cold acetone ×2 min, and allowed to air-dry. Proliferating cell nuclear antigen (PCNA) was localized in cells using mouse monoclonal antibody clone PC10 (34) (Oncogene Science, Uniondale, N.Y.), at a dilution of 1:200 in PBS, and an immunoperoxidase method. Positively stained cells contained either an intense, dense distribution or a discrete punctate distribution of PCNA, depending on the position of the cells in S-phase (35). PCNA, an auxiliary protein of DNA polymerase-δ (36), associates with replicon clusters, correlating with the bromodeoxyuridine labeling of cells in S-phase, and thus is a marker of cells in S-phase of the cell cycle (35). The mitotic index (MI) was determined by counting the fraction of cells in mitosis in hematoxylin- and eosin-stained cell cultures as described previously (37).

Western blot analysis of p53 and SV40 T-antigen

After 2 days in 70 or 5 μM choline, the cells were washed twice with PBS and then lysed as described by Donaldson and Cohen (38). Proteins were resolved by SDS/10% PAGE and electrophoretically transferred to nitrocellulose, and Western blot analysis was performed using pAb1801 (Ab2; Oncogene Science) to detect p53 (39). Immunoreactive proteins were detected by ECL using X-OMAT/AR film (Kodak). The blots were incubated in stripping buffer (100 mM 2 mercaptoethanol, 2% SDS, 50 mM Tris-HCl, pH 7.5) at 50°C for 30 min with agitation every 10 min. The membranes were then washed in an excess of PBS-0.1% Tween-20 (PBS-T) for 10 min ×2 changes and reblocked by immersion in 5% skim milk in 1X PBS for 1 h at room temperature. The membranes were then rinsed with fresh changes of PBS-T for 2 min, then 15 min, and finally for 5 min with agitation. The membranes were then reprobed with pAb416 (Oncogene Science.) to detect SV40 large T-antigen. Immunoreactive proteins were detected by ECL.

p53 immunostaining

Cells on chamber slides were fixed in methanol for 10 min at -20°C and washed three times in PBS; endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 20 min, and the cells were then rinsed three times in PBS. Slides were incubated in 10% normal horse serum in PBS for 30 min, rinsed three times in PBS, and then incubated at room temperature for 1 h with a 1:100 dilution of a mouse monoclonal anti-p53 antibody (clone PAb 421 and clone PAb 1801, Oncogene Science; clone NCL-p53-D07, Novocastra). The cells were rinsed twice in PBS, incubated for 20 min with a 1:32 dilution of a goat anti-mouse peroxidase-conjugated IgG antibody (Sigma) for 45 min, rinsed three time in PBS, and then incubated for 40 min in a DAB substrate (Vector Laboratories, Burlingame, Calif.). The cells were then rinsed extensively with distilled water, lightly counterstained with hematoxylin, and mounted with GelMount (Fisher Scientific, Pittsburgh, Pa.). Positively stained cells contained a brown reaction product in the nucleus. We obtained consistent staining results using PAb 421 and D07 clones; PAb 1801 produced less consistent results (more false negatives; it is an anti-human-p53 antibody) in our hands, but when positive staining was obtained, the results were similar to those observed using PAb 421 and D07.

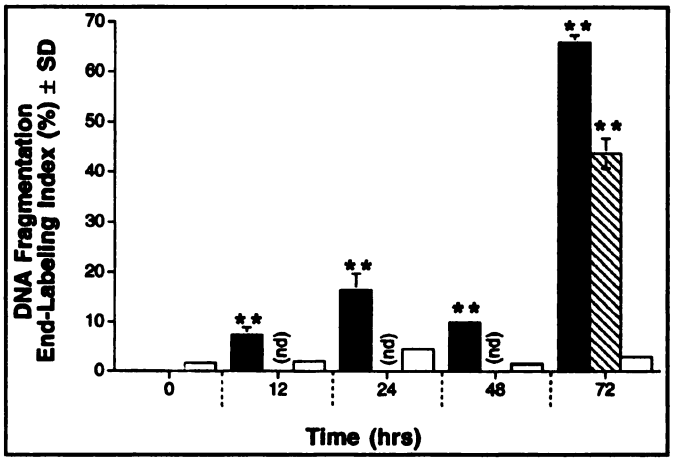

Figure 4. Effect of choline deficiency on DNA strand breakage. Percentage of cells with nuclear incorporation of digoxigenin-11-dUTP in cells growing in choline-sufficient (CS) (70 μM: white bar) or -deficient (CD) (5 μM: black bar, or 0 μM: hatched bar) medium for the times indicated; nd = not done. Data are expressed as mean ± SD; n = 3/treatment. (** = $P < 0.01$ by 1-way ANOVA).

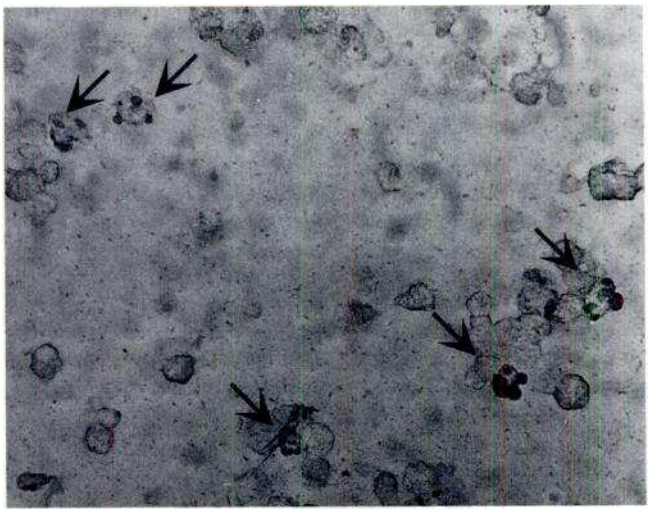

Figure 5. Apoptotic bodies. Classic apoptotic bodies associated with nuclear fragmentation were detected in cells grown in 0 μM choline for 72 h, and stained with digoxigenin-11-dUTP as described in legend to Fig. 4.

RESULTS

Cell proliferation and viability

Time zero was defined as the time when cells, which had been grown in the presence of 70 μM choline for 4 days, were switched to experimental medium (or left in 70 μM choline). Cells grown in 70 μM choline doubled in number approximately every 30 h (**Fig. 1**). At 72 h in 70 μM choline, cells had functioning mitochondria (conversion of MTT to formazan, a measure of mitochondrial function and number, was 0.27 ± 0.01 abs units/cell); of 908,000 ± 7,000 total cells growing in 70 μM choline, only 13,000 ± 6,000 cells had detached from the culture plates. Cells growing in 5 μM choline medium did not replicate until after 48 h in culture (Fig. 1); we speculate that cells may have been synchronized so that they divided during the last day of the studies. In contrast, cell number did not significantly increase when cells were grown in 2 μM choline, and cell numbers declined in 0 μM choline (Fig. 1). At 72 h in 0 ±M choline, cells had functioning mitochondria (conversion of MTT to formazan 0.99 ± 0.18 abs units/cell); of 158,000 ± 18,000 total cells growing in 0 μM choline, 91,000 ± 18,000 cells had detached from the culture plates.

Though cells grown in CD media were not increasing in numbers, they were synthesizing DNA. At 24 h of incubation, thymidine incorporation into DNA was more than twice as great in 0 μM cells than in control cells; 5 μM choline cells had an intermediate rate of thymidine incorporation (**Fig. 2**). By 72 h, thymidine incorporation in all CD groups was lower than in control cells (Fig. 2). This was reflected in diminished expression of PCNA in 0 μM choline (25.9 ± 0.2% of cells) as compared to 70 ±M choline (79.9 ± 6.5% of cells) and in diminished rate of mitosis in cells (mitotic index was 0% in 0 μM choline vs. 2.6 ± 0.7 % in 70 μM choline).

DNA degradation

The effect of choline on DNA strand breakage was investigated at 12, 24, 48, and 72 h after exposure to fresh CS or CD medium. In cells treated with 0 μM choline, DNA strand breaks were detected after 12 h and increased in amount over 72 h in CD medium (**Fig. 3** and **Fig. 4**). Cells maintained in 70 μM choline-containing medium showed relatively little DNA strand breakage (Fig. 4). At 72 h, total cell numbers were sixfold greater in 70 μM choline than in 0 μM choline (see above); however, there were only 1.8×10^4 cells (2%; in a replicate experiment 3%) with DNA strand breaks in 70 μM choline groups, whereas there were 11×10^4 cells (73%; in a replicate experiment 68%) with DNA strand breaks in 0 μM choline groups. The induction of DNA breakage by choline deficiency occurred in cells that retained mitochondrial function by MTT staining (data not shown). We were able to demonstrate a DNA ladder (Fig. 3), and observed characteristic apoptotic bodies (in 63–65% of at-

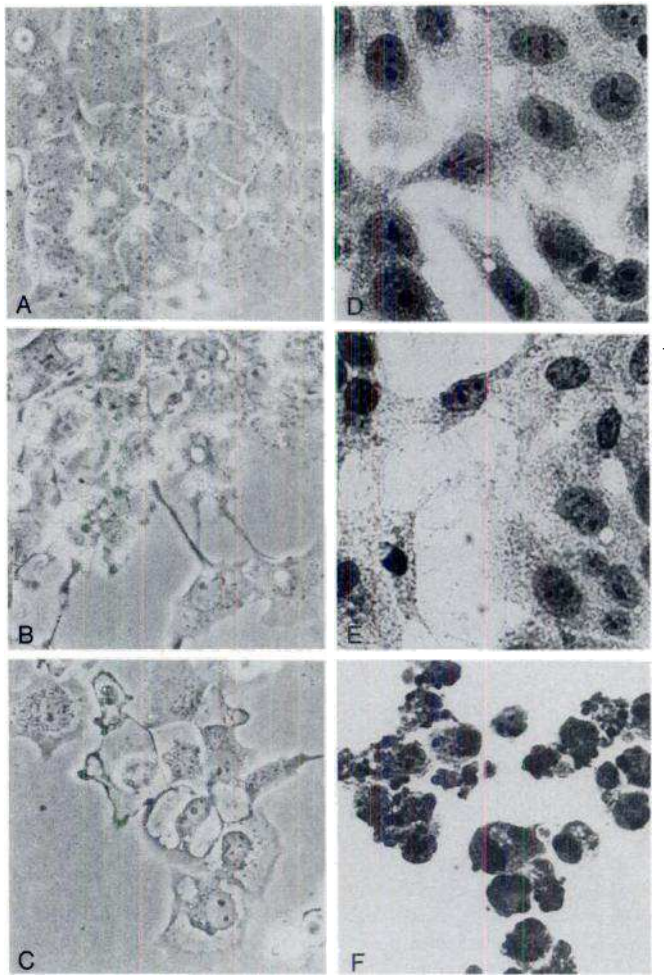

Figure 6. Effect of choline deficiency on light microscopic appearanoe of CWSV-1 cells. Morphology of cells treated with 70 μM (*A–D*), 5 μM (*B–E*), and 0 μM (*C–F*) choline for 72 h. *A–C)* phase contrast; *D–F)* hematoxylin and eosin. Magnification: ×400 (*A–C*); ×600 (*D–F*).

tached cells and 55–75% of detached cells treated with 5 and 0 μM choline, respectively; **Fig. 5**) in our choline-deficient hepatocytes at 72 h.

Morphologic features

Cells maintained in CD media showed an increase in the accumulation of lipid-like material detectable at 12 h, which persisted over time in culture (**Fig. 6** and **Fig. 7**). During the 24–48 h treatment interval, cells treated with 0 μM choline showed an increase in cell and nuclear size, and usually contained a single large nucleolus per cell. Over time, cells in 0 μM choline rounded up, and the nucleus became phase-dense and fragmented before detaching from the substrate. Ultrastructurally, the detached cells exhibited chromatin condensation and nuclear fragmentation, as well as phagocytosis of membrane-enclosed pieces of condensed chromatin (Fig. 7). These morphological features of apoptosis correlated with the observation by light microscopy of nuclear pyknosis and apparent karyorrhexis in so-called apoptotic bodies (Fig. 6). They contained lipid droplets that stained with Oil-red-O. By electron microscopy, apoptotic cells contained intact mitochondria, rough endoplasmic reticulum, and lysosomes (Fig. 7), consistent with the induction of a nonnecrotic form of cell death. In contrast, a large proportion of the cells treated with 5 μM choline underwent a shape change from flat and polygonal to stellate, with large pseudopod-like extensions. Control cells maintained in 70 μM choline exhibited a flat, polygonal cell shape, contained 1–3 nucleoli per cell, and infrequently contained lipid-like accumulation in the cytoplasm.

Expression of p53

Nuclear staining of p53 protein was seen in nearly all CWSV-1 cells independent of choline concentration; p53 was not detected in the cytoplasm of our cells (not shown). Western blot analysis showed no apparent difference in the accumulation of p53, which was detected in direct association with SV40 T-antigen (**Fig. 8**).

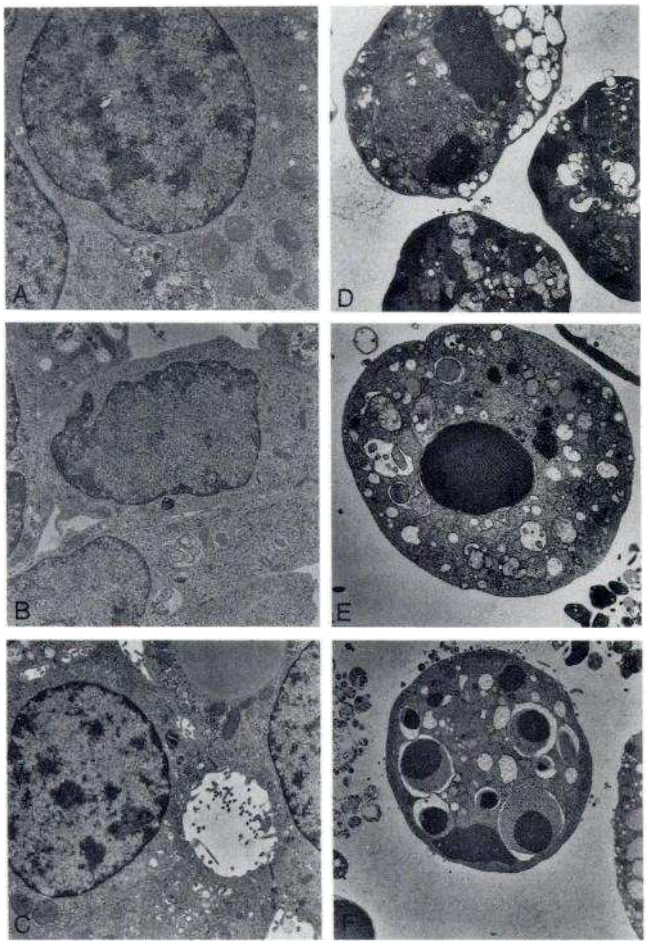

Figure 7. Effect of choline deficiency on electron microscopic appearance of CWSV-1 cells. Morphology of attached (*A–C*) and detached (*D–F*) CWSV-1 cells after treatment for 72 h with 70 μM (*A, D*), 5 μM (*B, E*), and 0 μM choline-supplemented medium (*C, F*) for 72 h. Detached apoptotic cells are found in all treatment groups but increase in number over time, inversely proportional to the choline concentration. Attached and floating cells contain mitochondria. Magnification: ×4000 (*A–C*); ×5000 (*D–F*).

DISCUSSION

In this study, we developed a model to investigate the effects of choline deficiency on cell death in rat hepatocytes. CWSV-1, SV40 T-antigen immortalized rat hepatocytes maintained in a choline-deficient, serum-free medium underwent DNA fragmentation and expressed morphologic changes consistent with the appearance of end-stage apoptotic cells. Programmed cell death is an energy-dependent process, requiring functioning mitochondria (reviewed in ref 40). In cells undergoing apoptosis, it is known that an increase in mitochondrial potential (~ATP synthesis) (27) and number (41) occurs before the peak of DNA fragmentation. When CWSV-1 cells were exposed to choline-deficient medium they showed an increased labeling of free 3′-OH ends of DNA coincident with an increased ability to convert a tetrazolium salt (MTT) to an insoluble formazan product, a measure of mitochondrial number and/or mitochondrial dehydrogenase activity (42). CWSV-1 cells that formed apoptotic bodies retained intact mitochondria and the ability to convert MTT to formazan. Thus, our findings suggest that choline deficiency induces an energy-dependent fragmentation of DNA in CWSV-1 hepatocytes in culture, a biochemical lesion consistent with death of hepatocytes via apoptosis rather than necrosis. Necrosis is characterized by mitochondrial damage, plasma membrane rupture, ill-defined clumping of nuclear chromatin, increased eosinophilia due to swollen mitochondria, and a loss of microsomal ribonucleoprotein and the presence of a smear pattern on DNA electrophoresis (43, 44). These changes are not seen in our cells, suggesting that choline deficiency induces apoptosis and not necrosis in CWSV-1 cells. In the liver, apoptotic cells are eliminated by phagocytosis (45), whereas in cell culture there may

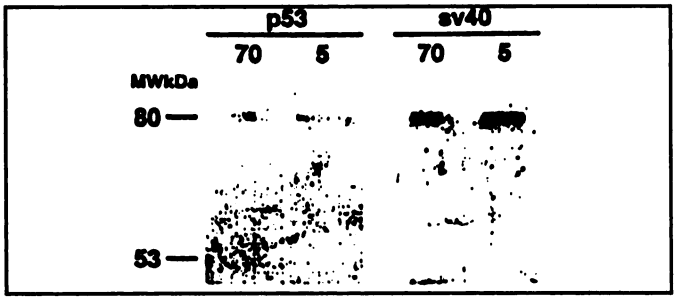

Figure 8. p53 is present as a complex with SV40 T-antigen in CWSV1 hepatocytes. p53 was detected in a complex with SV40 large T antigen in control (70 μM choline) and choline-deficient (5 μM choline) CWSV1 hepatocytes at 48 h in medium. After detection of p53 using monoclonal antibody PAb1801 (Oncogene Science), the membrane was stripped and reprobed using a SV40 large T antigen-specific monoclonal antibody (PAb416; Oncogene Science).

be no mechanism for cell removal, and secondary necrosis may occur.

The nuclear changes occurring in apoptosis are the result of endonuclease activity that cleaves nucleosomal DNA. Fragmentation of DNA into internucleosomal sized fragments was observed by gel electrophoresis in our choline-deficient hepatocytes. Attempts to show such a ladder in previous studies on apoptotic nontumorigenic hepatocytes have been unsuccessful (45, 46). TGF-β1 caused a laddering of DNA in human hepatoma cells (47). In the present study, we found that DNA strand breaks occur early (within 12 h) after exposure to choline-deficient medium, before the morphological appearance of end-stage apoptosis. The percentage of cells with strand breaks in DNA increased over time in culture in parallel with an increase in the number of cells with apoptotic bodies, further evidence that choline deficiency induces the process of apoptosis in CWSV-1 liver cells. p53 in our cells was bound to T-antigen (Fig. 7). p53 interacts with SV40 T-antigen (22), and this complex inactivates p53 (23). Expression of SV40-T antigen increases the stability but knocks out the function of p53 (48). This is consistent with our staining results. We suggest that CD apoptosis may be p53-independent in CWSV-1 cells. Other p53-independent apoptotic pathways have previously been described; for example, the TGF-β1 apoptosis pathway does not involve p53, BCL2, or BAX genes (49). SV40 T-antigen does not interfere with the induction of p53-independent apoptosis in other model systems (47). Although necrosis can occur in the livers of choline deficient rats, our data suggest that apoptosis also contributes to hepatocyte cell death. Retrospective review of an earlier description (10) of liver cell death in choline-deficient rats clearly shows classic apoptotic bodies (referred to at that time as "shrinkage necrosis"). General food restriction can cause apoptosis in liver (50, 51). This is the first report of a single nutrient deficiency inducing apoptosis in hepatocytes.

Nutritional factors account for as much as 35% of all cancer deaths (52). In animal models of hepatocarcinogenesis, long-term dietary choline deficiency has been shown to induce liver cancer in the absence of known carcinogens. Our studies show that choline deficiency is a condition capable of inducing apoptosis in immortalized CWSV-1 hepatocytes despite inactivation of p53 by complexing with SV40 large T antigen. FJ

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