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A p53-dependent G1 checkpoint function is not required for induction of apoptosis by acute choline deficiency in immortalized rat hepatocytes in culture

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Apoptosis is an intrinsic cell program that causes unwanted or damaged cells to commit suicide. It is not well appreciated that nutrients can modulate this significant process. Choline is a nutrient that is crucial for the normal function of all cells, and its absence induces apoptosis. We examined whether p53 or p21$^{Waf/Cip1}$ were required for induction of apoptosis by choline deficiency in CWSV-1 rat hepatocytes immortalized with SV40 large T antigen. When grown in a defined, serum-free, choline-sufficient (70 μM) medium, CWSV-1 cells failed to undergo apoptosis in response to γ-irradiation, a well-known p53-dependent trigger of apoptosis. However, primary hepatocytes with an intact p53 gene expressed typical morphologic features of apoptosis and underwent G1 cell cycle arrest in response to γ-irradiation. CWSV-1 cells continued to proliferate following γ-irradiation, which is consistent with the loss of the G1 checkpoint response. These cells also failed to undergo apoptosis in response to cisplatin, whereas the p53-competent primary hepatocytes underwent apoptosis in response to this drug. When maintained for 48 hours in choline-deficient (CD) medium (5 μM choline), CWSV-1 cells exhibited terminal dUTP nucleotide DNA end-labeling assay (TUNEL)-positivity, a DNA ladder typical of internucleosomal DNA fragmentation, and classical cellular features of apoptosis. CD also induced apoptosis in Hep3B hepatocytes, a p53 deletion-mutant. Thus, it is reasonable to suggest that choline deficiency is capable of overcoming a block in the p53 pathway, activating an alternative apoptosis signaling pathway. (J. Nutr. Biochem. 9:476–481, 1998) © *Elsevier Science Inc. 1998*

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Introduction

Apoptosis is an intrinsic cell suicide process that eliminates unwanted or damaged cells.[1] It has an essential role in embryogenesis, and abnormally regulated apoptosis contributes to a number of human diseases, including cancer, neurodegenerative diseases, and acquired immunodeficiency syndrome.[2] Little attention has been given to the role of nutrients in regulating apoptosis. Choline is an essential component of cell membrane phospholipid.[3] Choline and its metabolites play an important role in maintaining membrane integrity and in signal transduction.[4] Dietary depletion of choline alters membrane structure and function,[3,5] and animals maintained on a choline-deficient (CD) diet develop hepatocellular carcinomas in the absence of known carcinogens.[6–9] We previously showed that acute deprivation of choline causes apoptosis in CWSV-1 rat hepatocytes[10] and that chronic deprivation of choline selects for

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resistance to this form of apoptosis, resulting in malignant transformation in these cells.[11] At the time of our first report, we speculated that choline deficiency apoptosis might be induced independent of activation of p53 or $p21^{Waf/Cip1}$. To more completely understand the mechanisms of choline deficiency apoptosis, we examined whether these two gene products were required.

Expression of the wild-type p53 gene is an essential component of the G1 checkpoint response to DNA-damaging agents, leading to cell cycle arrest or apoptosis.[12,13] Typically, p53-dependent apoptosis occurs in response to agents that cause nonrepairable DNA damage.[14] When expression of wild-type p53 is induced, the protein is translocated from the cytosol to the nucleus, where it increases the expression of $p21^{Waf1/Cip1}$[15,16] and other growth arrest gene products (e.g., GADD45) that bind to cyclin-cdk complexes, inhibit G1-S phase cell cycle transition, and stimulate DNA repair.[17] Additionally, p53-independent expression of $p21^{Waf/Cip1}$ can result in G1 checkpoint arrest and apoptosis.[18] The actual inducers of apoptosis downstream of a functioning p53 gene product are not clear but may involve independent expression of a nuclear lamin protease (e.g., ICE) and activity of a DNA fragmentation endonuclease.[19]

We show, for the first time, that the form of apoptosis induced by acute withdrawal of the nutrient choline does not require an intact G1 checkpoint response, and thus, occurs independent of the p53- or $p21^{Waf/Cip1}$-dependent apoptosis pathways.

Materials and methods

Cell isolation and culture

Primary rat hepatocytes were isolated from male Fisher 344 rats using a collagenase digestion of perfused liver[20] with the following modifications: The liver was perfused in situ through the portal vein with a calcium-free buffer (Buffer A) at a rate of 3 to 4 mL/min for 10 minutes. This buffer solution contained NaCl (115 mM), KCl (5 mM), KH_2PO_4 (1 mM), EGTA (0.5 mM), and HEPES (25 mM), pH 7.4. After this perfusion, the liver was perfused for 20 minutes with Buffer A, to which was added $CaCl_2$ (1 mM) and 0.3 mg/mL collagenase A (Worthington Biochemicals, Freehold, NJ USA) (Buffer B). Following this second perfusion, the liver was removed and the cells were dispersed in 10 mL of ice-cold Buffer B, filtered through two layers of sterile gauze and washed twice by centrifugation at 500 rpm for 5 minutes at 4°C through Buffer B and once with cell attachment medium (see below). Isolated cells obtained by this method are 95% or greater cytochemically and morphologically identifiable hepatocytes.[21–23] Isolated trypan blue-negative hepatocytes were plated on uncoated dishes at 3×10^5 cells/100 mm dish or at 2×10^4 cells/well in four-well tissue culture slides (LabTek, NUNC, Inc., Naperville, IL USA) in MEM that was supplemented with 10% fetal bovine serum (FBS), 1% Pen-Strep, 50 μg/mL gentamicin, and 25 mM HEPES and allowed to attach for 6 hours. The cells were then switched to serum-free MEM that was supplemented with 10 μM norepinephrine (Sigma, St. Louis, MO USA) and 10 ng/mL EGF (Sigma).[24] All experiments were performed on subconfluent cell cultures.

SV40 large T-antigen immortalized CWSV-1 rat hepatocytes were obtained from Dr. Harriet C. Isom (Department of Microbiology, The Pennsylvania State University College of Medicine, Hershey, PA USA). CWSV-1 cells were derived from normal male Fisher 344 rat hepatocytes; they are routinely grown in serum-free medium and express liver-specific proteins.[21–23,25] Cells that are transfected with SV40 large T antigen have an inactivated p53.[25] CWSV1 cells are nontumorigenic at the low passages used in these studies.[21] CWSV-1 cells were maintained in serum-free RPMI 1640 medium (American Biorganics, Inc., Niagara Falls, NY USA) that was supplemented with 70 μM choline as described previously.[10,11]

Hep3B human hepatocytes (ATCC, Rockville, MD USA) were plated in MEM that was supplemented with 1% nonessential amino acids, 1% sodium pyruvate, 1% Pen-Strep, and 10% FBS. After 3 days in culture, the cells were switched to serum-free RPMI 1640 that was supplemented with choline as indicated. Hep3B cells express a deletion mutation in their p53 gene and thus, are p53 knockout cells.[26]

p53-Dependent apoptosis

Primary and CWSV1 hepatocytes were plated in plastic chamber slides as described. After 4 days in culture, hepatocytes in control medium were sham-irradiated or received a lethal dose of γ-irradiation (8 Gy) and then were incubated at 37°C for an additional 6 hours. At that time they were assayed for the induction of apoptosis by the terminal dUTP nucleotide DNA end-labeling (TUNEL) assay (ApoTag, Oncor, Inc., Gaithersburg, MD USA),[27,28] morphology, and cell cycle analysis as described below. Nuclear localization of $p21^{Waf1/Cip1}$ was determined as described below. In a separate experiment, primary hepatocytes in control medium were treated for 2 hours with 10 μM cisplatin [cis-diamine dichloride platinum (II); Sigma], rinsed with phosphate-buffered saline (PBS), returned to control medium for an additional 22 hours, and then assayed for the induction of apoptosis. CWSV-1 cells were plated in choline-sufficient (CS) medium until approximately 50% confluent, at which time the cells were treated for 2 hours with 10 μM cisplatin, and then switched to experimental medium (70 μM or 5 μM choline) for an additional 22 hours.

Choline deficiency and apoptosis

Cells were seeded at 1.0×10^4 cells/cm^2 in plastic chamber slides (LabTek) in 70 μM CS RPMI 1640 medium for 4 days before rinsing with PBS and switching them to fresh, 70 μM CS or CD (5 μM choline, as described in figures) RPMI 1640 for an additional 2 days. DNA strand breaks were detected using the TUNEL method. TUNEL labeling was not observed when terminal deoxynucleotidyl transferase was omitted from the labeling reaction mixture. Positive controls (regressing rat mammary gland undergoing apoptosis) were TUNEL (+), consistent with previous reports[29] on the specificity of in situ end-labeling methods for the detection of apoptosis. Classical morphologic features of apoptosis, including shrinkage of cell and nuclear size with condensation of nuclear chromatin and fragmentation of the nucleus into multiple small hematoxylinophilic bodies (i.e., apoptotic bodies), were detected in hematoxylin and eosin stained chamber slides using the method recommended by Wyllie,[30,31] as described previously.[10] The percentage of cells with TUNEL (+) nuclei (i.e., DNA strand breaks) was determined by counting at least 300 cells in three to six replicates per experiment. The occurrence of DNA fragmentation into characteristic approximately 200 bp fragments was analyzed by agarose gel electrophoresis as described previously.[10]

Cell cycle analysis

Cells in the log phase of growth were pulsed with 10 μM bromodeoxyuridine (BrdU; Boehringer Mannheim, Indianapolis,

IN) for the final 2 hours of posttreatment incubation, harvested by trypsinization, washed with Hanks balanced salt solution (HBSS), labeled with anti-BrdU-FITC[32] treated with 50 units RNase, and incubated with 25 μg/mL propidium iodide for simultaneous analysis, by flow cytometry, of DNA content and cell cycle as previously described.[33–35] Radiation-induced inhibition of early S-phase fraction was enumerated as a measure of G1 checkpoint function.[35]

Localization of p21$^{Waf1/Cip1}$ protein

Immunocytochemical analysis of p21$^{Waf1/Cip1}$ protein was performed 6 hours after treatment with γ-irradiation. Cells were rinsed with PBS and fixed with ice-cold methanol for 10 minutes at –20°C. Afterward they were rinsed with PBS and then treated with 0.1% hydrogen peroxide in PBS for 10 minutes to block endogenous peroxidase activity. Cells were blocked with nonimmune horse serum in PBS for 10 minutes at room temperature, rinsed with PBS, incubated in primary rabbit antibody specific for p21$^{Waf1/Cip1}$ and not cross-reactive with p27^{Kip1} or other mitotic inhibitors (Santa Cruz Biotech., Santa Cruz, CA USA) at a dilution of 1 μg/mL for 1 hour at room temperature, and then rinsed with PBS. Negative controls were incubated with nonimmune serum for 1 hour at room temperature. Sites of p21$^{Waf1/Cip1}$ protein localization were visualized using an ABC method (Vector Laboratories, Burlingame, CA USA) and hematoxylin counterstaining.

Statistical analyses

Significant differences between treatment groups were assessed with analysis of variance or Student's *t*-test.[36]

Results

We examined whether CWSV-1 hepatocytes were sensitive to p53-dependent inducers of apoptosis. Based on classical morphologic features, an average fourfold increase (range, two- to sixfold over four independent studies) in apoptosis occurred in primary F344 rat hepatocytes treated with 8 Gy of γ-irradiation (*Figure 1*). In parallel sets of cultures, primary hepatocytes that were classified as apoptotic based on morphology were found to be TUNEL (+). In contrast, irradiated CWSV-1 hepatocytes showed no increase in the number of apoptotic cells compared with controls (*Figure 1*).

To confirm that CWSV-1 hepatocytes were insensitive to another p53-dependent apoptotic trigger, we investigated the induction by cisplatin of DNA strand breaks characteristic of apoptosis. Primary hepatocytes were highly sensitive to 10 μM cisplatin, whereas CWSV-1 hepatocytes were unresponsive to this apoptosis inducer (*Table 1*).

To determine whether CWSV-1 hepatocytes have functionally inactivated p53, cell cycle analysis was performed. Primary hepatocytes had a 73 ± 8% ($N = 7$) inhibition of progression from G1 to S phase 8 hours after 8 Gy γ-irradiation indicative of a G1 checkpoint response.[34,35] The percentage of primary hepatocytes with nuclear localization of p21$^{Waf1/Cip1}$, a mediator of the p53-dependent G1 checkpoint response, also was increased 2.2-fold after irradiation to 43% of irradiated cells compared with 19% in controls. Irradiated CWSV-1 cells displayed no inhibition of progression from G1 to S phase and no change in the percentage of cells with nuclear localization of p21$^{Waf1/Cip1}$ postirradiation (14% of control versus 10% of irradiated cells), which is consistent with a loss of p53-dependent G1 checkpoint response.

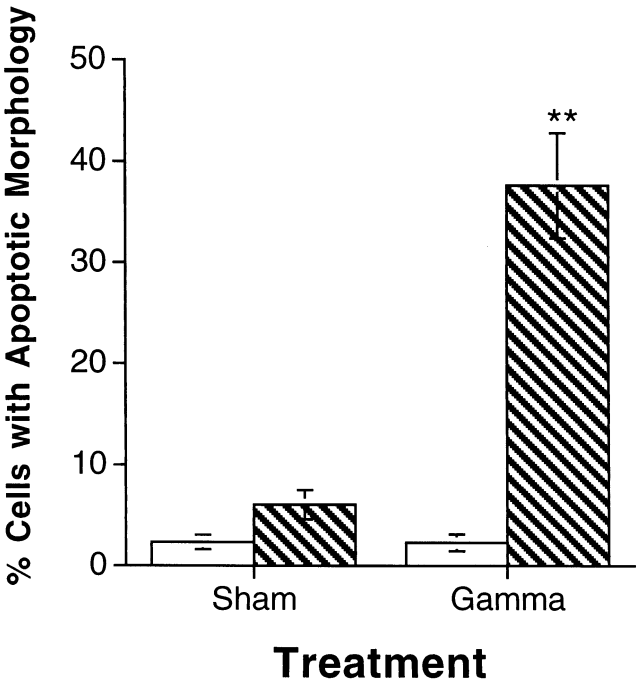

Figure 1 Induction of apoptosis by γ-irradiation, a p53-dependent trigger of apoptosis. Cells in the log phase of growth were assessed for the presence of apoptotic cells using morphometric analysis 6 to 8 hours after exposure to 8 Gy γ-irradiation (Gamma). Increased numbers of apoptotic cells were detected in primary hepatocytes (stippled bars) after irradiation compared with controls (Sham). CWSV1 hepatocytes (white bars) showed no increase in the number of apoptotic cells postirradiation. Mean ± SD; $N = 2–4$ replicates per experiment; ** = $P < 0.01$ versus control by Student's *t*-test.

We investigated the effects of acute choline deficiency on the induction of apoptosis in CWSV-1 cells. We found that these cells had increased DNA fragmentation (TUNEL, DNA ladder) when switched for 2 days to CD medium; internucleosomal DNA fragmentation was not detectable in cells continuously maintained in 70 μM CS medium (*Figure 2*). Hep3B cells, a p53 deletion-mutant line of human hepatocytes, exhibited a greater than threefold increase in cells with apoptotic morphology when made CD (7.8 ± 0.7% of cells apoptotic in CD medium [0 μM choline]

Table 1 Cisplatin induces apoptosis in primary hepatocytes but not in CWSV-1 hepatocytes

	Percent of cells TUNEL (+)	
Cisplatin (μM)	Primary hepatocytes	CWSV-1 hepatocytes
0	9.4 ± 2.1	3.3 ± 1.1
10	37.5 ± 3.2	3.1 ± 1.3

Cells in the log phase of growth were treated for 2 hours with 10 μM cisplatin then assayed 22 hours later for the induction of DNA strand breaks, a marker of apoptosis, using a terminal dUTP nucleotide DNA end-labeling assay (TUNEL) method. Data shown are mean ± SD; $N = 3$; 8 = $P < 0.01$ versus control.

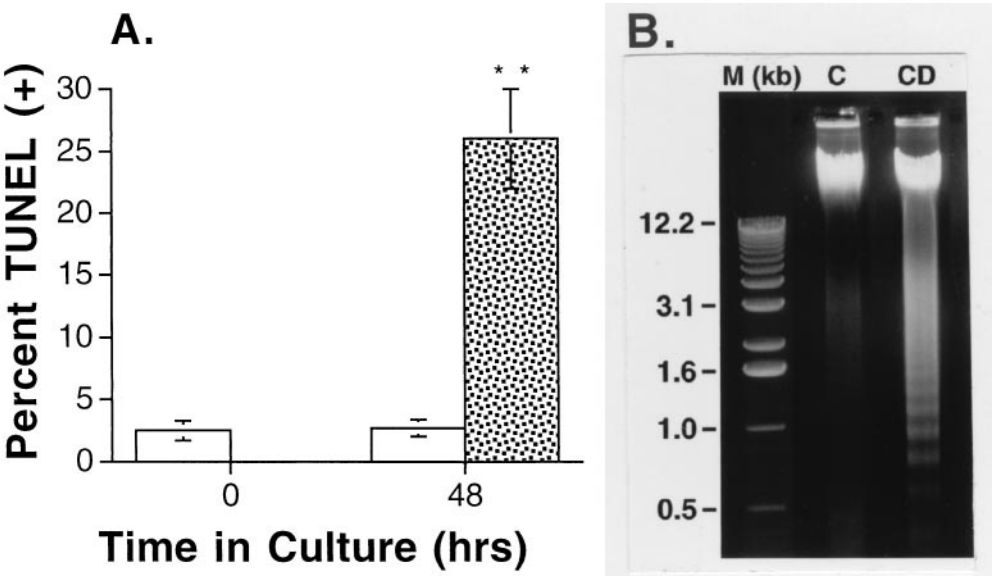

Figure 2 Choline deficiency induces DNA strand breaks in CWSV-1 hepatocytes. Nuclear incorporation of digoxigenin-11-dUTP (TUNEL) was assessed in CWSV-1 cells growing in choline-sufficient (C; 70 μM, white bar) or choline-deficient (CD; 5 μM: stippled bar) medium for the times indicated (*Figure 2A*). Agarose gel electrophoresis of isolated genomic DNA from cells at 48 hours is presented in *Figure 2B*. A significant increase in the number of TUNEL (+) cells (*Figure 2A*) and the occurrence of approximate 200 bp internucleosomal DNA fragmentation (*Figure 2B*), two hallmarks of apoptosis, were seen after 48 hours treatment with CD medium. M = 1 kb size marker. Mean ± SD of ≥ 3 per point; ** = $P < 0.01$ versus control by Student's *t*-test.

compared with 2.4 ± 0.9% of cells apoptotic in CS medium [70 μM choline], $P < 0.01$).

Discussion

In our earlier report[10] we speculated that choline deficiency-induced apoptosis in CWSV-1 hepatocytes occurred independent of p53 activation because SV40 large T antigen binds newly synthesized p53[22,37] and effectively creates a p53 knockout system.[38,39] However, no definitive studies demonstrated that CWSV-1 cells were incapable of partial activation of p53-dependent apoptosis or were incapable of p53-independent activation of $p21^{Waf1/Cip1}$. We now report that this is the case and also that choline deficiency-induced apoptosis occurs in Hep3B hepatocytes, a p53 deletion-mutant cell line.

Lethal doses of γ-irradiation induce p53-dependent G1 arrest and prolonged expression of $p21^{Waf1/Cip1}$ in fibroblasts and lymphocytes,[40,41] and apoptosis is detectable in mice within 6 to 8 hours after irradiation.[42] In normal rat liver increased expression of GADD45, a mediator of cell cycle arrest and DNA repair, occurs within 30 minutes after ionizing radiation in vivo.[43] We found that γ-irradiation induced a depletion of cells in S phase in primary rat hepatocytes but not in CWSV-1 rat hepatocytes, which is consistent with induction of p53-dependent G1 arrest only in primary hepatocytes. This differential response was further reflected in the sensitivity of the two cell classes to the induction of classical apoptotic morphology.

The rates of apoptosis that we describe in primary hepatocytes after irradiation are higher than have typically been reported for rat hepatocytes in irradiated whole animals. This is likely because most hepatocytes in whole liver are quiescent, whereas cell division is induced in isolated cells in culture. We believe that primary rat hepatocyte cultures are analogous to regenerating liver. Normal liver is not a radiation-resistant organ. Low doses of ionizing irradiation (4.5–9.0 Gy) have been shown to decrease mitotic index and cellularity[44] and to induce DNA damage (e.g., oxidative modification to DNA bases[45] and DNA strand breaks).[46] The extremely low rate of proliferation in normal rat liver[47] may partially explain the dearth of reports of radiation-induced apoptosis in whole liver. The lack of radiation-induced apoptosis in CWSV-1 cells suggests that p53 is required for this type of apoptosis and G1 checkpoint arrest in hepatocytes. However, we cannot exclude the possibility that CWSV-1 cells have additional alterations (e.g., overexpression of autocrine factors, inactivation of additional genes) that could modulate the sensitivity of these cells to p53-dependent apoptotic triggers.

Primary hepatocytes, but not CWSV-1 hepatocytes, underwent apoptosis when treated with cisplatin, an agent that induces the expression of p53 in cells undergoing apoptosis.[48] Downregulation of p53 through overexpression of the MDM2 protein imparts resistance to cisplatin-induced DNA fragmentation and apoptosis.[49,50] The absence of response to both cisplatin and irradiation in CWSV-1 cells implies that these cells lack a p53-dependent DNA damage–apoptosis pathway. Although the present studies demonstrate a requirement for p53 function in radiation-induced apoptosis in hepatocytes, recent studies show that a ceramide-mediated, p53-independent form of apoptosis can occur in other tissues (endothelium, pleura, mesothelium) in response to radiation.[51] A better understanding of the role of membrane-derived and DNA damage-dependent mediators of apoptosis would be important in designing new approaches to manipulate the apoptotic response in clinically important situations.

Interrelationships between choline, folate, methionine, and vitamin B12 can influence the availability of choline.[52] Maintenance of intracellular stores of available methyl groups is frequently cited as the primary functional significance of these molecules. This focus masks the ability to understand the individual contributions of choline and methyl groups to cell survival. We are aware that choline deficiency may cause folate deficiency.[53] Folate deficiency apoptosis is suppressed by the addition of folate;[54] choline deficiency apoptosis is not prevented by the addition of extra folate (or methionine or betaine; providing methyl groups via pathways that bypass the folate requirement).[55] Thus, we are confident that the p53-independent form of apoptosis that we observed is caused by withdrawal of the choline moiety itself.

The sensitivity of cancer cells to anticancer drugs and irradiation is determined to a large extent by the expression of genes that regulate the induction of apoptosis.[56] Gain-of-function alterations in these genes, such as the overexpression of *bcl-xL*, permit transient growth arrest without apoptosis during episodic exposure to cancer therapeutic agents.[57] In contrast, loss-of-function mutations in, for example, the p53-dependent response to DNA damaging forms of therapy, permit continued proliferation without apoptosis.[17,58] Mutations in p53 are the most common genetic alterations in human cancers.[59] Loss of the p53-dependent DNA damage apoptosis pathway is now thought to play a crucial role in tumor development.[60] The high rate of downregulation of the p53 gene (i.e., mutations; overexpression of MDM2 protein) in human cancers is one of the reasons why current anticancer therapeutic approaches may be unsuccessful.[42,61,62] Because choline deficiency induces apoptosis by a p53-independent mechanism, it may prove useful as an adjuvant treatment in cancers that prove unresponsive to anticancer treatment modalities (e.g., cisplatin, irradiation) that exploit p53-dependent apoptosis pathways.

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