Choline deficiency induces apoptosis in primary cultures of fetal neurons

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ABSTRACT **Treatment of rats with choline during brain development results in long-lasting enhancement of spatial memory whereas choline deficiency has the opposite effect. Changes in rates of apoptosis may be responsible. We previously demonstrated that choline deficiency induced apoptosis in PC12 cells and suggested that interruption of cell cycling due to a decrease in membrane phosphatidylcholine concentration was the critical mechanism. We now examine whether choline deprivation induces apoptosis in nondividing primary neuronal cultures of fetal rat cortex and hippocampus. Choline deficiency induced widespread apoptosis in primary neuronal cells, indicating that cells do not have to be dividing to be sensitive to choline deficiency. When switched to a choline-deficient medium, both types of cells became depleted of choline, phosphocholine and phosphatidylcholine, and in primary neurons neurite outgrowth was dramatically attenuated. Primary cells could be rescued from apoptosis by treatment with phosphocholine or lysophosphatidylcholine. As described previously for PC12 cells, an increase in ceramide (Cer) was associated with choline deficiency-induced apoptosis in primary neurons. The primary neuronal culture appears to be an excellent model to explore the mechanism whereby maternal dietary choline intake modulates apoptosis in the fetal brain.—Yen, C.-L. E., Mar, M.-H., Meeker, R. B., Fernandes, A., Zeisel, S. H. Choline deficiency induces apoptosis in primary cultures of fetal neurons. *FASEB J.* 15, 1704–1710 (2001)**

Key Words: · *primary neurons* · *hippocampus* · *PC12 cells*

Choline is a nutrient essential for normal function of all cells (1). In addition to being the precursor for acetylcholine synthesis, it is a major methyl group donor (1, 2). Phospholipids containing choline, such as phosphatidylcholine (PtdCho) and sphingomyelin, are not only major structural components of cell membranes (3), but also reservoirs for lipid second messengers (diacylglycerol and ceramide, or Cer) that modulate cell growth and apoptosis (4, 5).

Studies in rodents suggest that dietary intake of choline early in life can diminish the severity of memory deficits in aged animals. Memory function of adult rats can be permanently altered by choline supplementation in utero, even though there is no supplementation throughout the rest of their lives (6–9). The choline-related memory changes are correlated with altered distribution and morphology of septal neurons in the adult animals (10) and with changes in the electrophysiological properties of the hippocampi in supplemented animals (11, 12). The memory changes observed in choline-supplemented rats are also correlated with changes in the neurotransmitter-related enzymes present in the adult brains (13, 14). The likely mechanism explaining these differences in memory involves choline-mediated alterations in the birth, migration, and death of cells in the memory centers of brain during critical periods in their development (15, 16).

We previously reported that choline deficiency induced apoptosis in fetal rat hippocampus (day 18 gestation) (15, 17) and in a variety of cell types in culture (18, 19). We proposed that induction of apoptosis was caused by a decrease in membrane PtdCho concentration (19, 20) because this choline ester is needed for normal progression through the cell cycle (21). To test this hypothesis we wanted to study the effects of choline deficiency in cells that were not dividing. For this reason, and because we seek a model system that more closely describes events that might occur in fetal brain, we now characterize the effects of choline deficiency in primary culture of postmitotic fetal rat brain neurons derived from hippocampus and cortex.

MATERIALS AND METHODS

Cell culture

PC12 cells

PC12 cells were a kind gift from Dr. J. P. O'Bryan at the University of North Carolina at Chapel Hill. Cells were maintained in DMEM/F12 medium (Atlanta Biologicals,

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Norcross, GA) containing 10% fetal bovine serum (Life Technologies, Grand Island, NY). Because choline and PtdCho are present in sera, a chemically defined medium (DMEM/F12 with modified N2 supplement) was used for both experimental and control groups of cells. The components of the defined medium and the experimental procedure have been reported previously (19).

Primary neuronal culture

Fetal brains were obtained from timed pregnant Sprague-Dawley rats (Charles River, Raleigh, NC) at embryonic day 18. Both sexes of fetuses were used. After removing dura-arachnoid membranes, the cortexes and hippocampi were isolated and incubated in 5 ml calcium, magnesium-free Hank's balanced salt solution containing 2.5 U/ml dispase and 2 U/ml DNase. Dissociated cells were counted and seeded on poly-D-lysine-coated coverslips at a density of ~150,000 cells/cm^2 in minimal essential medium containing 10% fetal bovine serum and 20 μg/ml gentamicin (Sigma, St. Louis, MO). Cells were then shifted into the experimental media (same as those used for PC12 cells except for the addition of B-27 supplement; Life Technologies) 3 h after seeding.

Immunohistochemistry

Neurofilament

Primary cells in culture for 4 days were prepared for immunocytochemical staining of neurofilaments. Cells were fixed in ice-cold methanol:acetone (1:1) and washed with phosphate-buffered saline (PBS) before incubating in 0.6% H_2O_2 for 20 min. After blocking with 3% normal goat serum prepared in PBS for 1 h, cells were incubated overnight at 4°C with a mouse monoclonal anti-neurofilament antibody (68 kDa, 1:5000; Boehringer Mannheim, Indianapolis, IN). The antibody was washed away the following day with PBS and the cells were incubated in biotinylated goat anti-mouse IgG (1:200) at room temperature for 1 h. After washing with PBS, the cells were reacted with ABC reagent (Vector Labs, Burlingame, CA) and the antibody was visualized with diaminobenzidine (DAB, 0.5 mg/ml) in the presence of 0.01% H_2O_2. The cells were then counterstained with methyl green.

MAP-2

Cells fixed in 4% paraformaldehyde for 15 min at room temperature were stained as described for neurofilament staining except that cells were incubated with a 1:5000 dilution of mouse monoclonal anti-MAP-2 antibody (Boehringer Mannheim). DAB was used as a chromogen as described above.

Flow cytometry for cell cycle analysis

Cells were trypsinized, dislodged, and washed with PBS twice. Cell numbers were determined and 2×10^6 cells were fixed by slowly adding them to ethanol (final concentration 70%) for at least 18 h; 30 min before analysis, cells were stained with propidium iodide (50 μg/ml) in PBS containing 100 U/ml RNase A. The flow cytometer FACscan (Becton-Dickinson, San Jose, CA) and the software Mofit (Verity Software House, Topsham, ME) were used to analyze the percentage of cells in each phase of the cell cycle.

Determination of apoptosis

Morphological analysis

Apoptosis was assessed in both attached and detached PC12 cells, which were collected and deposited onto glass slides using a cytocentrifuge. Cells were then fixed with methanol, stained with hematoxylin (Fisher, Fair Lawn, NJ), and mounted with Permount (Fisher). Slides were examined under a light microscope and the percentage of apoptotic cells was determined by counting at least 200 cells in four replicate cultures per treatment. Cells with fragmented nuclei (multiple, small hematoxylinophilic bodies) were defined as apoptotic. Primary cells grown on poly-D-lysine-coated glass chamber slides were fixed in 4% paraformaldehyde and then incubated with the nuclear stain 4,6-diamidino-2-phenylindole (1 μg/ml DAPI, Sigma). The percentages of apoptotic cells were determined under fluorescent microscope. Detached cells could be lost during the staining process. We found that the majority of primary cells remained attached after 48 h of nutrient deprivation and that the majority of detached cells were apoptotic. Therefore, the observed differences between control and choline-deficient cells could have been underestimated. Alternatively, we identified apoptotic cells using TUNEL staining, in which DNA fragments in the nuclei were labeled with FITC-coupled nucleotides and normal nuclei were counterstained with ethidium homodimer. Fluorescein-FragEL™ DNA fragmentation detection kit (Oncogene Research Products, Cambridge, MA) was used and the manufacturer's protocol was followed, with the addition of ethidium homodimer as counterstaining.

DNA fragmentation (DNA ladders)

Samples (2×10^6 cells) were lysed in 200 μl lysis buffer containing 50 mM Tris (pH 7.4), 10 mM EDTA, 0.5% N-laurosarcosine (Sigma) and incubated for 3 h with 0.5 mg/ml protease K (Boehringer Mannheim) at 65°C. Cells were then incubated for 1 h with 24 U/ml DNase-free RNase (Boehringer Mannheim) at 50°C. After extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and precipitation with 2 volumes of absolute ethanol, the DNA was resuspended in 100 μl 10 mM Tris buffer (pH 7.4) containing 1 mM EDTA. The DNA concentration was determined by measurement of optical density at 260 nm. Ten micrograms of DNA were subjected to electrophoresis on a 1.2% agarose gel at 100 V for 2 h. DNA was visualized and photographed under UV light after ethidium bromide staining.

Biochemical determinations

Samples were collected at various time intervals after cells had been treated with experimental media. To ensure an equal number of cells in both the experimental and control groups, DNA was measured as a basis for normalization using a fluorometric method (22). After addition of ^{14}C-labeled internal standards, choline, phosphocholine, and glycerophosphocholine in the aqueous phase of cell extracts (23) were separated using high-performance liquid chromatography (HPLC) (24); phosphatidylcholine in the organic phase was separated using thin-layer chromatography (24). A [^{2}H-methyl]-labeled internal standard for each metabolite was added to permit correction for recovery during analysis of choline moiety by a gas chromatography/mass spectrometry assay (24).

To determine the effect of choline deprivation on intracellular levels of Cer, lipid was extracted and assayed using the

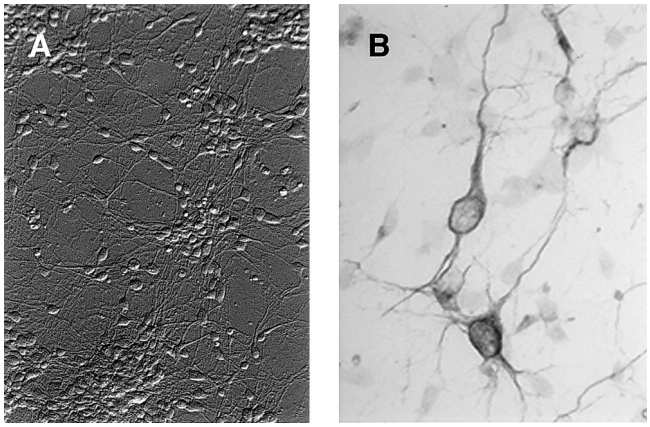

Figure 1. Primary neurons cultured in a chemically defined medium with added choline for 4 days after being isolated from E-18 rat fetal brains. Brain cells were isolated and primary neuronal cultures were established as described in Materials and Methods. *A)* Hoffmann modulation contrast image showing neurons with healthy cell bodies and processes (200×). *B)* Neurons stained with a neurofilament antibody (400×).

HPLC method described by Previati et al. (25). Cells were first fixed on the culture dish with 1.5 ml methanol, scraped off the plate, and transferred into a 15 ml centrifuge tube (Falcon). After adding 3 ml chloroform, the samples were mixed and incubated overnight at −20°C. Cell debris was pelleted by centrifugation, further extracted twice with 1 ml chloroform/methanol (1:1 and 1:2 sequentially), and extracts were combined. The organic phases were separated after adding 1 ml water and the aqueous phases were re-extracted with 2 ml chloroform. The organic phases were then combined and dried in a concentrator (Savant). The residues were dissolved in 100 μl anhydrous chloroform and derivatized with 10 μl of 100 mM (S)-6 methoxy-α-methyl-2-naphthaleneacetic acid (Sigma), 10 μl of 100 mM N, N′ dicyclohexylcarbodiimide (Sigma), and 10 μl of 100 mM 4-dimethylaminopyridine at −20°C for at least 3 h. After incubation, the samples were dried to stop the reaction. The samples were resuspended in 15 μl chloroform and extracted with 2 ml hexane. The supernatants were transferred to new screw-capped tubes containing 5 ml MeOH/water (4:1) and mixed vigorously. After centrifugation, the upper phase was collected and the extraction procedure was repeated. The upper phases were combined and dried. The dried samples were dissolved in 1 ml of hexane and 50 μl of the samples was injected onto HPLC. Derivatized ceramides were resolved on an Econosphere CN, 250 × 4.6 mm column (Alltech, Deerfield, IL) equipped with a guard column with Discovery Cyano cartridge (Supelco, Bellefonte, PA). The mobile phase was delivered at the flow rate of 2 ml/min. The column was initially equilibrated in 97% mobile phase A (hexane) and 3% mobile phase B (3% isopropanol in hexane). It was first run with a linear gradient to 10% B from 0 to 4 min after injection and then an isocratic run at 10% B for 5 min, followed by a linear gradient to 100% B from 9 to 18 min. Cer concentration was quantified using a standard curve of known amounts of Cer.

To determine whether cells could be rescued from choline deficiency-induced apoptosis, they were cultured as described previously in choline-free medium for 36 h. At this time cells were either left in choline-free medium (deficient) or switched to medium containing either 50 μM lysophosphatidylcholine (palmitoyl; Sigma) in 1 mM BSA, 100 μM phosphocholine (Sigma) in basal medium, or 100 μM phosphatidylcholine (dipalmitoyl; Sigma) prepared by sonicating in basal medium. At 72 h, viable cell numbers were determined by trypan blue exclusion and apoptosis was determined by TUNEL assay.

Statistics

We used one-way analysis of variance, followed by the Dunnett's critical difference test to determine statistical significance between the treatment groups and control at time 0. Comparisons for each pair were determined with Student's *t* test (JMP Version 2, SAS, 1989).

RESULTS

Primary neuronal culture

The majority of the cells isolated from the E18 cortex and hippocampus attached onto poly-D-lysine-coated matrix within an hour. When cultured in the chemically defined medium containing choline, most of the cells exhibited the morphology of healthy neurons (**Fig. 1*A***). Immunocytochemical staining of the neuron-specific marker neurofilament (Fig. 1*B*) or MAP-2 (data not shown) identified most cultured cells as neurons. When assessed by flow cytometry, more than 92% of primary cells were in G0/G1 phases of the cell cycle compared with less than 70% PC12 cells in these phases (**Fig. 2**). Accordingly, only 2% of primary cells were in G2/M phases after 24 h in culture, whereas 8% of PC12 cells were in these phases of the cell cycle at the same time point. Tritiated thymidine incorporation into DNA was assessed. Negligible amounts of label were incorporated into DNA of primary cells in culture (0.02% of added label was incorporated) over a period of 24 h. In contrast, dividing PC12 cells incorporated most of the label. Finally, we measured DNA concentration/plate in our primary cell cultures and found no accumulation of DNA (24 h=37.2 μg; 48 h=39.9 μg; 72 h=36.1 μg). These data support our suggestion that these primary cells are not dividing in culture.

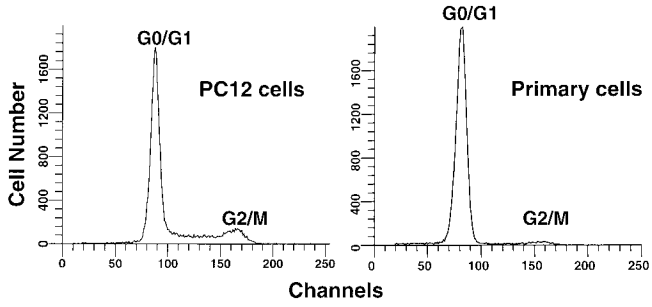

Figure 2. Most primary cells are at the $G0/G_1$ phase of cell cycle while PC12 cells progress through the cell cycle. Cell cycle status of PC12 cells and primary cells in culture was determined by flow cytometry as described in Materials and Methods. 24 h after cells were placed in culture, cells were detached and fixed in 70% ethanol overnight before being stained with propidium iodide.

Apoptosis

When choline was not included in the chemically defined medium, apoptosis was induced. Compared with cells grown in the complete medium (**Fig. 3*A***), more primary neurons exhibited apoptotic morphology (condensed chromatin and fragmented nuclei) when grown in the same medium devoid of choline (Fig. 3*B*). Compared with control cells (Fig. 3*C*), more apoptotic nuclei (assessed by TUNEL staining) were identified in choline-deprived cells (Fig. 3*D*). Primary neuronal cells appeared to be more sensitive to choline deprivation than were PC12 cells since significantly more apoptosis was seen in primary cells at an earlier time point than PC12 cells (48 h vs. 60 h; **Fig. 4**). In both primary neurons and PC12 cells deprived of choline, we observed a laddering pattern characteristic of apoptosis when genomic DNA was resolved with an agarose gel (Fig. 4, insets).

We found that primary cells deprived of choline for 36 h could be rescued from apoptosis by addition of choline (100% viable at 72 h vs. 3% in-deficient medium, $P<0.01$), lysophosphatidylcholine (73% viable at 72 h; $P<0.01$), or phosphocholine (77% viable at 72 h; $P<0.01$). The addition of PtdCho did not rescue cells (6% viable at 72 h).

Choline and metabolites

In both cell types, most of the intracellular choline moiety existed as PtdCho (**Fig. 5**). The next most abundant choline ester was phosphocholine. Unesterified choline concentrations were ~4% of that in PtdCho (Fig. 5). Primary neuronal culture contained lower levels of choline, phosphocholine, and PtdCho when compared with PC12 cells on a per DNA basis (Fig. 5). PC12 cells contained slightly more DNA on average than did the primary cells, but the difference was not statistically significant (14±0.45 vs. 12±1.12 μg DNA/10^6 cell, $P>0.32$). Upon choline deprivation, choline and phosphocholine concentrations dropped dramatically to the barely detectable range (0.1–1 pmol/μg protein) in both cell models. This occurred within 24 h in primary cells and 48 h in PC12 cells. In primary neurons growing in choline-sufficient medium, PtdCho concentrations increased with time in culture; in PC12 cells growing in choline-sufficient medium, PtdCho concentrations stayed relatively constant (Fig. 5). Ptd-

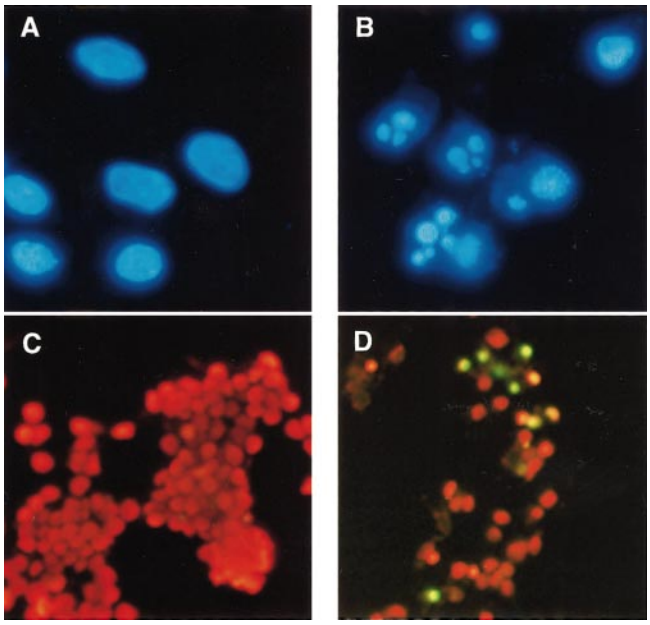

Figure 3. Choline deprivation induces cell death via apoptosis. Primary neuronal cultures were prepared as described in Materials and Methods. At day 3 in culture, apoptotic cells were identified by nuclear morphology (*A, B*) as well as end-labeling of DNA strand breaks (*C, D*) with terminal deoxynucleotidyl transferase (TdT) and FITC-coupled nucleotides (green). Nonapoptotic cells' nuclei were visualized with ethidium homodimer (red). *A, C* = control; *B, D* = choline deficient.

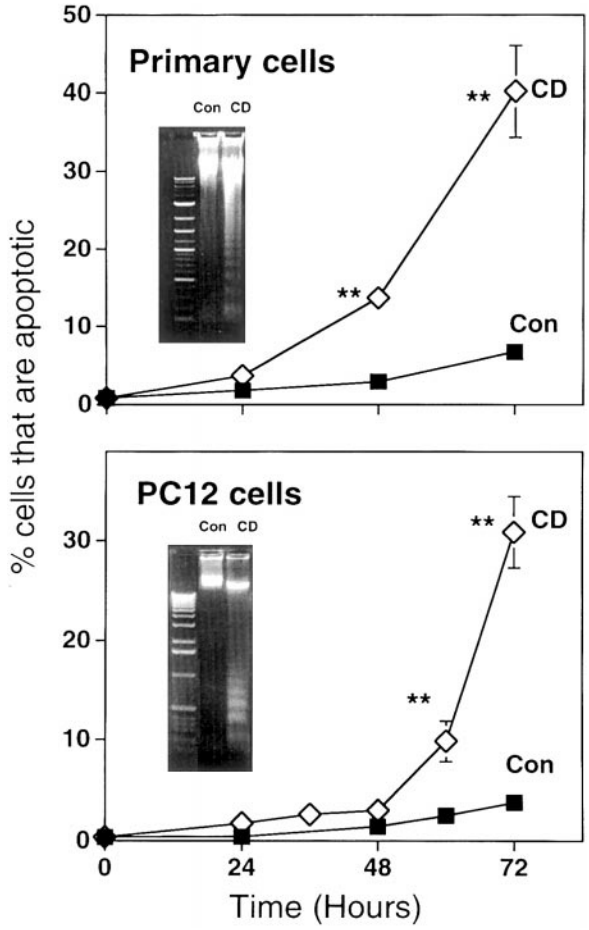

Figure 4. After choline deprivation, primary cells undergo apoptosis at an earlier time than do PC12 cells. Cells were prepared and cultured as described in Materials and Methods. At the times indicated, cells were fixed with 4% paraformaldehyde and stained with 2 μg/ml Hoechst 33258. Percentage of total cells that were apoptotic was determined by dividing the number of cells with fragmented and condensed nuclei by the total cells counted (>200 cells/slide). Con = control, CD = choline deficient. Data represent means ± SE, $n = 5$/point. $^{**}P < 0.01$. Insert: DNA laddering in choline-deprived primary neurons and intact DNA from control neurons. DNA was extracted and separated by electrophoresis as described in Materials and Methods.

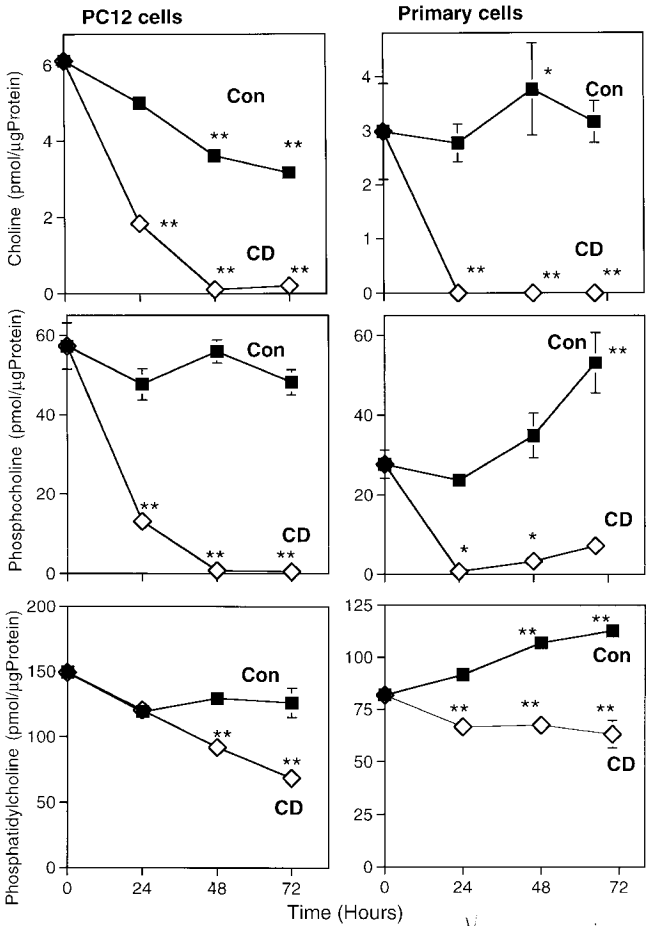

Figure 5. Changes in choline, phosphocholine, and phosphatidylcholine concentrations in primary cells and PC12 cells after choline deprivation. Cells were prepared and cultured as described in Materials and Methods. Choline, phosphocholine, and phosphatidylcholine concentrations were analyzed at the times indicated, as described in Materials and Methods. Con = control, CD = choline deficient. Data represent means ± SE, $n = 8$/point. $^*P < 0.05$, $^{**}P < 0.01$ significantly different from corresponding time 0 control.

Cho concentrations decreased in both primary cells and PC12 cells grown in choline-deficient medium (Fig. 5).

Neurite outgrowth

In the primary neurons in culture, most of the cells had extensive neurite outgrowth, forming an elaborate network of processes within 3 days. This outgrowth was dramatically attenuated in cells grown in choline-deficient medium (**Fig. 6**). Under the conditions of these experiments, neurite outgrowth was not induced in PC12 cells (data not shown).

Ceramide

We previously reported that the Cer level in PC12 cells increases with time after choline deprivation whereas it remains relatively constant in cells grown in the complete medium (19). In this study, we found that Cer levels in primary cells deprived of choline were also significantly higher (~twofold that of control) at 72 h despite the observation that Cer levels increased with time in the control cells (**Fig. 7**).

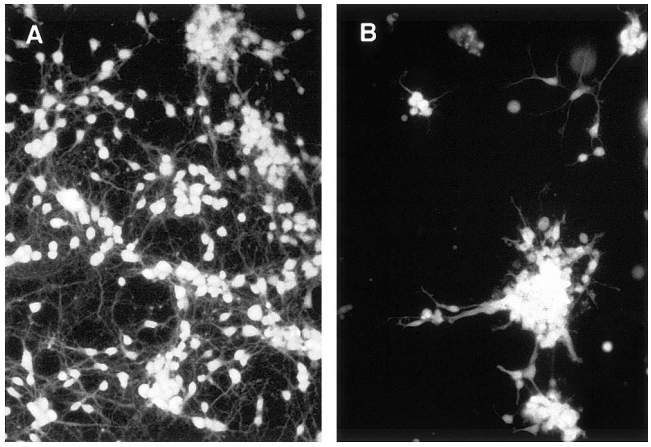

Figure 6. Neurite outgrowth in primary neurons is inhibited in choline-deficient medium. Cells were cultivated in the chemically defined medium with or without added choline for 3 days as described in Materials and Methods. Cells were then incubated with 4 μM calcein-AM (Molecular Probes, Eugene, OR) for 20 min. *A)* In choline-sufficient medium, cells form an extensive outgrowth of neuronal processes. *B)* In choline-deficient medium, cells form fewer neurites (200×).

DISCUSSION

We report that choline deficiency induced apoptosis in postmitotic primary neurons established from fetal rat

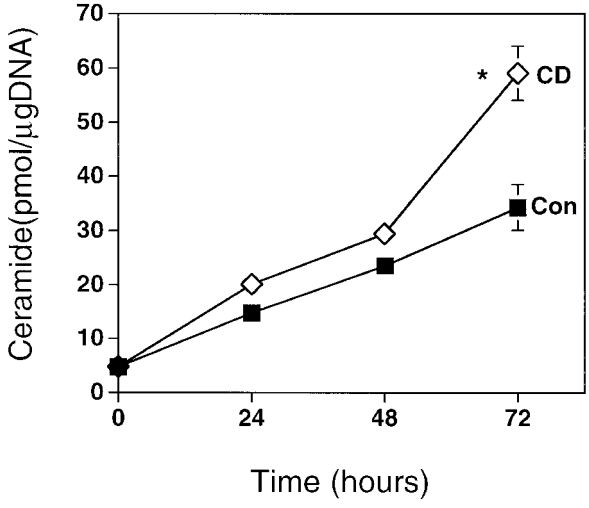

Figure 7. Ceramide concentrations increase in primary neuronal cells after choline deprivation. Cells were cultivated in the chemically defined medium with (Con) or without (CD) added choline for the times indicated as described in Materials and Methods. Cer was measured by HPLC. Data represent means ± SE, $n = 3$/point; $^*P < 0.05$, significantly different from control.

brains. The primary cells were more sensitive to choline deficiency than were PC12 cells as apoptosis was induced at an earlier time point. In earlier publications, we proposed that induction of apoptosis in choline deficiency was caused by perturbation of cell cycling due to a decrease in membrane PtdCho concentration (19, 20) because this choline ester is needed for normal progression through the cell cycle (21). This hypothesis was supported by observations that cells incapable of PtdCho synthesis by the CDP–choline pathway died by apoptosis. For example, a genetic defect in PtdCho biosynthesis or exposure to a PtdCho synthesis inhibitor led to cell cycle arrest as well as apoptosis (26–28). Our current studies in primary neuronal culture illustrate that choline deficiency is capable of inducing apoptosis in neurons by a process independent of perturbation in cell cycling, as these cells normally did not progress past G0/G1 and were not dividing (Fig. 2).

In most cell lines we previously examined, choline deficiency was associated with an absolute decline in PtdCho concentrations (19, 20, 29), and this was observed for primary cells and PC12 cells in this study (Fig. 5). We had suggested that when PtdCho concentrations fell below a critical level in some subcellular compartments, apoptosis was induced (19). In the current studies of primary cells in culture, we found that we could rescue cells after 36 h of choline deficiency by adding either phosphocholine or lysophosphatidylcholine (two precursors for PtdCho synthesis), but could not rescue cells with PtdCho itself. We speculate that exogenous PtdCho may not reach some critical subcellular compartment, although endogenously synthesized PtdCho may do so. Alternatively, the critical molecule could be lysophosphatidylcholine rather than PtdCho.

PtdCho is needed for the synthesis of sphingomyelin, which is the reservoir for Cer formation during signaling (30), and its hydrolysis has recently been linked to the characteristic changes of plasma membrane of apoptotic cells (31). We suggested in our previous report that Cer mediates choline deficiency-induced apoptosis based on our observations that Cer concentrations increased before the increase in apoptosis in PC12 cells, that exogenous Cer induced apoptosis, and that inhibition of choline deficiency-induced apoptosis is associated with correction of intracellular Cer levels (19). In several described examples of apoptosis, Cer generated from sphingomyelin hydrolysis mediates apoptosis induced by stimuli such as ultraviolet radiation and activation of the CD95 receptor (30, 32). An increase in de novo Cer synthesis in response to chemotherapy or activation of angiotensin II type 2 receptor may also induce apoptosis (33–35). In our primary cells, we observed an association of Cer accumulation with choline deficiency-induced apoptosis, supporting the suggestion of a causal relationship. However, the increase in Cer was not detected until late in the apoptotic process, suggesting that Cer is not the initiating signal for CD apoptosis. Tepper et al. (31) report that Cer accumulation per se is not required for the progression of apoptosis, but that sphingomyelin hydrolysis and subsequent cholesterol efflux are essential for plasma membrane blebbing/vesiculation during the execution phase of apoptosis. It is possible that Cer accumulation during choline deficiency was not directly linked to apoptosis. Cer mediates the effects of NGF on neurite outgrowth of cultured hippocampal neurons, and treatment with cell-permeable Cer enhances cell survival and dendritic outgrowth of cerebellar Purkinje neurons and hippocampal neurons at immature stages (36, 37). On the other hand, elevation of Cer within distal neurites impedes neurite growth in cultured rat sympathetic neurons (38) and exposure to exogenous Cer causes retraction of dendrites and apoptosis in immature cerebellar granule cells and mature hippocampal neurons (39, 40). Whether the observed Cer increase was responsible for retarded neurite outgrowth (Fig. 7) in our model remains to be determined.

Choline deficiency is of special interest in neurons, as, in the rat, supplemental choline during pregnancy results in life-long enhancement of hippocampal function in offspring (6–10) and the rate of apoptosis in fetal hippocampus is inversely related to the dietary choline intake of the rat dam (17). It has been estimated that more than 50% of cells born during the development of the central nervous system are eliminated (41, 42). Factors modulating apoptosis, such as choline availability, may therefore affect brain development and subsequently brain functions. The primary neuronal culture system appears to be an excellent model to explore the mechanism by which maternal dietary choline intake modulates apoptosis in the fetal brain. FJ

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