A Genetic Defect in Phosphatidylcholine Biosynthesis Triggers Apoptosis in Chinese Hamster Ovary Cells*

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From the Lipid and Lipoprotein Research Group and Department of Biochemistry, ‡Surgical-Medical Research Institute, University of Alberta, Edmonton, Alberta T6G 2Z2, Canada and the INSERM Unité 326, Hôpital Purpan, 31059 Toulouse cedex, France

We have investigated the cell death of a Chinese hamster ovary mutant (MT-58) with a thermo-sensitive CTP: phosphocholine cytidylyltransferase, the rate-limiting enzyme of the CDP-choline pathway for phosphatidylcholine biosynthesis. (Esko, J. D., Wermuth, M. M., and Raetz, C. R. H. (1981) J. Biol. Chem. 256, 7388–7393). After MT-58 cells were shifted to the restrictive temperature of 40°C, the cytidylyltransferase was inactivated immediately leading to a decrease in phosphatidylcholine biosynthesis and cell death. DNA content and number of cells in the S phase decreased significantly in the dying MT-58 cells according to flow cytometrical analyses. The fragmentation of genomic DNA was detected by DNA ladders in agarose gel and release of the prelabeled genomic DNA into cytosolic fractions 14 h after the temperature shift. The dying cells underwent a dramatic reduction of cellular volume while maintaining the membrane containment of cellular contents. These events indicated that the inactivation of cytidylyltransferase triggered apoptosis in Chinese hamster ovary cells. This is the first report that apoptosis was induced in cultured cells, not by an added agent, but by a mutation in phospholipid biosynthesis.

A healthy life requires an intricate balance between cell growth and death. As a part of this cellular balance and a built-in cellular mechanism, apoptosis is controlled precisely by the genetic instructions of its own cell (1–5). Apoptosis has been implicated in many important biological events, such as development, immune defense, differentiation, and growth control (6, 7). Abnormal apoptosis has been linked to many human diseases, including cancer (8), AIDS (9), Ataxia Telangiectasia (10), Alzheimer's disease (11), and diabetes (12). Defective apoptosis could cause uncontrolled cell growth and hyperactive apoptosis may lead to premature cell death.

In multicell organisms, these internal death mechanisms are also influenced by many extracellular signals and external stimuli. Apoptosis is a two-stage process: condemnation and execution (13). Apoptotic cells share many common characteristics of execution: cell shrinkage, chromatin condensation, exposure of phosphatidylserine on cell surface, activation of proteases, activation of endonucleases, and fragmentation of genomic DNA. In the condemnation stage, the events appear more subtle and specific. It is believed that the death signal is recognized by the targeted cell and transmitted along an intrinsic pathway that can only be activated specifically for this event. At the end of this pathway lies the death apparatus that carries out execution upon the arrival of the death signal. It is still not clear how the death message is transduced in mammalian cells.

PC1 is the most abundant phospholipid in mammalian cells. In addition to its structural role in membranes, PC is the precursor of several second messenger molecules (e.g. diacylglyceride, phosphatidic acid) in signal transduction pathways (14). Most PC is synthesized via the CDP-choline pathway (15). MT-58 is a thermo-sensitive mutant of CHO cells generated by chemical mutagenesis (16, 17). Lipid analyses revealed that the level of PC was severely reduced at the restrictive temperature. This lipid reduction was accompanied by a significant accumulation of choline phosphate, a substrate for CT. At 40°C, CT activity and incorporation of choline into PC were completely abolished, leading to cell death. The addition of saturated PCs and lysoPCs rescued MT-58 from cell death, suggesting that the lack of the end product of the CDP-choline pathway was responsible for the lethal phenotype (18). The death of MT-58 revealed a direct link between PC biosynthesis and cell death. This link appears to be a novel mechanism by which PC, the unique phospholipid, controls cell growth and survival.

In the present studies, we found that the death of MT-58 mutant at the restrictive temperature bore many characteristic events of apoptosis. Inactivation of CT and subsequent decrease of PC apparently activated a specific pathway of programmed cell death in CHO cells. Apoptosis of MT-58 was unique since the cell death was induced not by any added agent, rather by a mutation in PC synthesis.

EXPERIMENTAL PROCEDURES

Materials—[methyl-3H]Choline chloride (15 Ci/mmol) and [methyl-3H]thymidine (41 Ci/mmol) were purchased from Amersham Interna- tional. Phospho[methyl-3H]choline was synthesized enzymatically from[methyl-3H]choline and ATP with choline kinase (19). Ham's F-12 nutrient mixture, fetal bovine serum, insulin, calcium-free phosphate-buffered saline, penicillin, streptomycin, trypsin/EDTA solution, and all other chemicals were obtained from Sigma. Silica Gel G60 thin-layer chromatography plates were purchased from Merck (Darmstadt, Germany).

Cell Culture—CHO WT-K1, MT-58, and CT-transfected MT-58 (MT-CT) were described previously (20). Cells were grown in 5% CO2 and Ham's F-12 medium with bovine pancreatic insulin (20 μg/ml) and 10% FCS.

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Relationship between CT activity, PC biosynthesis, cell growth, and DNA fragmentation in CHO cells. After growing at 33°C for 24 h, cells were shifted to 40°C for various times. A, CT activity: cells were harvested by scraping, homogenized, and measured for the enzyme activities (see "Experimental Procedures"). B, MT biosynthesis: 1 μCi/ml [3H]choline was added to medium and incubated for 2 h. The radioactivities incorporated into PC were determined and plotted as the percentage of WT at 33°C. C, cell growth: cell number was determined as described under "Experimental Procedures." D, the release of the prelabeled soluble DNA: cells were prelabeled with [3H]thymidine, then shifted to 40°C for various times. The soluble contents were released from cells by Triton X-100 treatment. The radioactivities associated with the medium, soluble, or particulate fractions were determined. The percentage of radioactivities in insoluble fraction was plotted against the time at 40°C.

Isotopic Quantification of DNA Fragmentation—Cells were prelabeled at 33°C for 24 h with [3H]thymidine (0.5 μCi/dish), washed, resuspended in fresh medium, and then shifted to 40°C. At each time point, media were collected. The cells were lysed in 15 mM Tris HCl, pH 8.0, 20 mM EDTA, 0.5% Triton X-100 for 30 min at 4°C. The lysed cells were scraped and separated into soluble and insoluble fractions by 50% centrifugation at 12,000 rpm. The soluble contents were released from cells by Triton X-100 treatment. The radioactivities associated with the medium, soluble, or particulate fractions were determined. The percentage of radioactivities in soluble fraction was plotted against the time at 40°C.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis—The fragmented DNA was preferentially isolated from the apoptotic cells (22). The isolated DNA from approximately 5 × 10⁶ cells of each sample was separated on a 1.2% agarose gel and stained with ethidium bromide. λ DNA digested by HindIII was the size standard.

Flow Cytometry Analysis—In the single label system, cell cycle parameters were determined by the propidium iodide (PI) labeling of nuclear DNA. The cells were trypsinized and resuspended in 70% cold ethanol. The membranes were disrupted and the nuclear DNA was labeled with PI (22). The labeled cells were then stained with DNA dye. The nonpermant PI (10 μg/ml) was added directly to the culture medium and incubated for 15 min. The cells were then trypsinized and fixed in 70% cold ethanol. The permanent Hoechst 33342 (5 μg/ml) was then used with the cells to label total cellular DNA. The labeled cells were analyzed by flow cytometry at 630 nm for PI and 450 nm for Hoechst.

Scanning Electron Microscopy—The cells, grown on coverslips, were prefixed in 2.5% glutaraldehyde in Millonig's buffer, pH 7.2, at room temperature for 1 h, postfixed in 1% osmium tetroxide for 1 h, and dehydrated through an ascending series of ethanol concentrations up to 100%. They were critical point-dried, mounted, and sputter-coated with gold. Micrographs were taken with a Hitachi Electron Microscope S-2500.

Phospholipid Analysis—Phospholipids were separated by thin-layer chromatography and quantitated by the measurement of their phosphorus content (23). To determine the radioactivity in the soluble fraction, cells were incubated with 1 μCi/ml [3H]choline in regular medium for 2 h and total lipids were extracted. After separation by thin-layer chromatography, radioactivities in PC spots were measured by liquid scintillation counter.

RESULTS AND DISCUSSION

Inactivation of PC Synthesis in MT-58 Cells at 40°C Triggers DNA Fragmentation—To correlate the events of cell death and defective CT, we analyzed the time courses of DNA fragmentation, PC synthesis, and cell growth of three cell lines. K1 is the original wild type cell line from which MT-58 was generated. MT-58 CT is a cell line of the mutant with the stable expression of the functional recombinant CT of rat liver, driven by the constitutive cytomegalovirus promoter (20). At 33°C, a 70% drop of CT activity (Fig. 1A) and a 20% decrease of [3H]choline incorporation into PC (Fig. 1B) had already occurred in MT-58 cells. When MT-58 was shifted to 40°C, the CT activity was inactivated immediately (Fig. 1A) and the incorporation of [3H]choline into PC was shut-down almost completely (Fig. 1B). The concentration of PC was reduced gradually to 60% of wild type level after 24 h at 40°C (data not shown) when cells began to die. Cell count by the Trypan blue exclusion showed growth arrest of MT-58 after 24 h (Fig. 1C) and cell death after 48 h. The expressed wild-type CT was able to recover the synthesis of PC from choline, restore the level of PC and rescue the mutant from cell death (Fig. 1, A–C). Prelabeled with [3H]thymidine at 33°C and then shifted to 40°C, the cells were analyzed for the generation of soluble [3H]DNA. Not found in the wild type cells, DNA release in MT-58 cells began 14 h after the temperature shift to 40°C and peaked at 48 h (Fig. 1D), indicating that about 80% of MT-58 cells underwent apoptosis. The expressed CT in MT-58 prevented the release of DNA fragments, suggesting that defective CT was responsible for the subsequent DNA fragmentation. Since the release of prelabeled DNA could not distinguish DNA fragmentation from random DNA degradation often observed in necrotic or lysed cells, the DNA fragments were also analyzed by an agarose gel electrophoresis. The DNA ladder typical of apoptosis occurred in 18 h and peaked about 42 h (Fig. 2). The appearance of the DNA ladder was consistent with the detection of the DNA release.

Decrease of S Phase Cells and Maintenance of Membrane Integrity during the Cell Death of MT-58—DNA content and cells in each phase of the cell cycle were examined by flow cytometry. In the single label experiments, cell cycle parameters were determined by labeling nuclear DNA with PI (Fig. 3). WT cells at 40°C displayed a typical repartition of growing...
cells between the different phases of the cell cycle (Fig. 3, left column). Of total cells, 40% was in the G1 phase, 20% in the G2/M phase, and 40% in the S phase. In contrast, MT-58 cells with DNA content lower than 2n appeared after 25 h at 40 °C and peaked at 72 h (Fig. 3, middle column). Meanwhile the mutant cells in the S phase decreased from 45 to 20% in 24 h (not shown). The recombinant CT rescued MT-58 from these changes efficiently (Fig. 3, right column). The low content of DNA in apoptotic MT-58 was consistent with the fragmentation of DNA detected by the DNA release and DNA ladder. Since necrotic or lysed cells also display lower DNA content in the single label assays, we performed double label experiments to distinguish disrupted cells from apoptotic cells. Unable to penetrate intact membranes, PI only labels the DNA of cells with permeabilized plasma membrane. As a membrane-penetrating fluorescent dye, on the other hand, Hoechst 33342 binds to the DNA of the cells that are undergoing cell cycle. In double labeling with PI and Hoechst 33342, WT and MT-58 cells were incubated with the dyes before and after the temperature shift. In contrast to WT cells at both temperatures and MT-58 at 33 °C (Fig. 4a–c), MT-58 cells at 40 °C exhibited low content of Hoechst dye (Fig. 4d) indicating the loss of DNA. However, the low level of PI suggests that the cellular membrane remained intact during cell death of MT-58. Necrotic or lysed cells should have displayed a high PI fluorescence. The patterns of MT-58-CT at both 33 °C and 40 °C were similar to that of WT cells (not shown). Moreover, a significant decrease of S phase MT-58 cells at 40 °C was also detected (Fig. 4d).

Blockage of apoptotic MT-58 cells at G1 phase is consistent with that choline-deficient medium arrested fibroblasts in G1 phase (25). CT activity is also important at the G1/S transition in a macrophage cell line (26). It appears that G1 is an active phase participating in the initiation of apoptosis (7). Thus, PC is likely an important component of a normal progression in the G1 phase of cell cycle.

Condensation of the Dying MT-58 Cells—The intact cellular membrane ensures a containment of apoptotic cell debris. Condensation of cellular volume facilitates the process in which the dead cells and apoptotic bodies are engulfed by adjacent cells (27). The morphological changes of MT-58 cells were observed by scanning electron microscopy. At 40 °C, the condensation of MT-58 cells became visible at 36 h (Fig. 5b) and prominent at 72 h (Fig. 5E). The apoptotic contraction of MT-58 cells left the visible trails marking the edges of cells before shrinkage (Fig. 5, C and D). Once the condensation was complete, cells became much smaller and round with intact membrane (Fig. 5E). The

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**Fig. 3.** Cell cycle and DNA content analyses of MT-58 cells determined by the single label flow cytometry. WT, MT-58, or MT-CT cells were incubated in 100-mm dishes at 33 °C, then shifted to 40 °C. At each time point, cells were collected and fixed in ethanol as described under “Experimental Procedures.” Nuclear DNA was labeled with PI, and cells were analyzed by flow cytometry. Cell cycle curves were plotted as a function of time at 40 °C. Arrows indicate the position of G0/G1, S, and G2/M cells as well as apoptotic cells (lower fluorescent intensity).

**Fig. 4.** Cell cycle and membrane integrity analyses of MT-58 cells determined by the two labels flow cytometry. WT or MT-58 cells were incubated at 33 °C or 40 °C for 36 h. At each time point, cells were prelabeled with PI for 15 min, then collected and fixed in ethanol. Total DNA was labeled with the permeant dye Hoechst 33342. The cells were analyzed by flow cytometry at the wavelengths of 630 and 450 nm. a, WT at 33 °C; b, WT at 40 °C; c, MT-58 at 33 °C; d, MT-58 at 40 °C. Density of the spots corresponds to a logarithm of the cell number. Normal cycling cells are in panels 1, apoptotic cells are in panels 3, while necrotic or lysed cells are in panels 2 and 4. Position of G0/G1, S and G2/M cells is indicated by the arrows.

**Fig. 5.** Shrinkage of MT-58 cells observed by scanning electron microscopy. WT and MT-58 cells were incubated at 33 °C on coverslips, then shifted to 40 °C. Cells were treated as described under “Experimental Procedures” and analyzed by scanning electron microscopy. A, MT-58 cells at 33 °C; B–D, MT-58 cells at 40 °C between 36 to 48 h; E, MT-58 cells at 40 °C for 72 h; F, WT cells at 40 °C.
morphological changes of dying MT-58 cells were also typical of apoptosis. This study is the first demonstration that apoptosis is induced by a mutation in phospholipid biosynthesis. The events associated with the cell death of MT-58 mutant at the restrictive temperature are characteristic of apoptosis. Inactivation of CT and subsequent decrease of PC apparently activated a specific pathway of programmed cell death in CHO cells. Rescue of the mutant by wild type CT (20), exogenous PC or lysoPC (18) indicated that life and death of CHO cells could be regulated directly by PC via at least one apoptotic pathway. The rescue of cell death by exogenous lipid is also selective for the PC molecular species with saturated acyl chains while polyunsaturated PCs are ineffective (18). In contrast, PC generated up to the wild type level by the expressed phosphatidylethanolamine-N-methyltransferase-2 in MT-58 could not rescue the mutant (20), suggesting that the initial component of the apoptotic pathway in MT-58 may recognize specific PC molecules according to their localization or composition. Moreover, membrane integrity was maintained throughout apoptosis of MT-58 despite the severe decrease in PC and reduction of cellular volume. These results also indicate that the functional involvement of PC in cells may be more prominent than its structural role in providing lipid mass for membranes. The function of PC on the regulation of cell viability may have been achieved via its direct interaction with the mitogenic and apoptotic pathways. It is still not clear how mitogenic pathways recognize PC and how apoptotic pathways detect the depletion of PC. Molecular and biochemical analyses of the apoptotic components will allow us to address these questions in the future. It is also intriguing that a defect in phosphatidylethanolamine (PE) biosynthesis resulting in the slight depletion of PE in Drosophila does not induce the death of the mutant animal (28). However, it is not known if defective synthesis of PE causes death in a few of selected cells. More direct comparisons between the genetic defects of PE and PC biosynthesis in cultured cells and in animals may reveal some unique functions of each phospholipid.

Without the addition of a foreign agent, MT-58 provides a clearly defined system to study apoptosis. Although some inhibitors of the CDP-choline pathway were found to induce DNA fragmentation (29, 30), these compounds lack specificity. For instance, dietherphosphatidylcholine, a PC analog, also inhibits phosphatidylinositol-specific phospholipase C and protein kinase C (31, 32). Therefore, apoptosis induced by these agents might compromise the specificity of the apoptotic pathways under investigation.

MT-58 also suggests that apoptosis is a defense mechanism against mutations in animals. DNA damage happens in cells frequently because of the close association of animals with the mutagens in food, drinking water, air, and sunlight. The initial DNA damage can be corrected by the intrinsic repair mechanism. However, if the initial damage in an essential gene escapes repair, apoptosis, similar to that in MT-58, will be activated to eliminate the defective cells and to ensure the overall health of animals.

Apoptosis in MT-58 is an event secondary to the defect in PC biosynthesis. In other words, programmed cell death can be triggered by defects outside the apoptotic pathway. Early studies of random mutagenesis (33) estimated that about 50% of genes in each genome are “essential.” Inactivation of these essential genes leads to cell death. We predict that most, if not all of these cell deaths, like MT-58, could be apoptotic. Apoptotic death may be a widely employed response of cells to mutations. Therefore, in the diseases of abnormal apoptosis, the initial defect may not necessarily occur in the apoptotic pathway but may provoke a defensive response of apoptosis.

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