Apoptosis Triggered by 1-O-Octadecyl-2-O-methyl-rac-glycero-3-phosphocholine Is Prevented by Increased Expression of CTP:Phosphocholine Cytidylyltransferase*

A HeLa cell line was constructed for the regulation of CTP:phosphocholine cytidylyltransferase (CCT) expression via a tetracycline-responsive promoter to test the role of CCT in apoptosis triggered by exposure of cells to the antineoplastic phospholipid 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (ET-18-OCH₃). Basal CCT expression in the engineered HeLa cell line was the same as in control HeLa cells lines, and CCT activity and protein were elevated 25-fold following 48 h of induction with doxycycline. Increased CCT expression prevented ET-18-OCH₃-induced apoptosis. Acylation of exogenous lysophosphatidylcholine circumvented the requirement for CCT activity by providing an alternate route to phosphatidylcholine, and heightened CCT expression and lysophosphatidylcholine supplementation were equally effective in reversing the cytotoxic effect of ET-18-OCH₃. Neither CCT overexpression nor lysophosphatidylcholine supplementation allowed the HeLa cells to proliferate in the presence of ET-18-OCH₃, indicating that the cytosstatic property of ET-18-OCH₃ was independent of its effect on membrane phospholipid synthesis. These data provide compelling genetic evidence to support the conclusion that the interruption of phosphatidylcholine synthesis at the CCT step by ET-18-OCH₃ is the primary physiological imbalance that accounts for the cytotoxic action of the drug.

ET-18-OCH₃¹ is a nonmetabolizable analog of LPC and belongs to the first generation of ether lipids tested as growth inhibitors (1). These compounds do not directly target DNA, and numerous studies have demonstrated a selective cytotoxic action of ET-18-OCH₃ against transformed cells in whole animals and tissue culture (2–8). Recent work has established that the cytotoxic effect of ET-18-OCH₃ is due to the ability of the antineoplastic phospholipids to induce apoptosis in sensitive cells (9–12). A plethora of biological processes have been suggested as primary targets for the antineoplastic ether-linked phospholipids (for reviews, see Refs. 13–16). The long list of physiological imbalances includes the inhibition of phosphatidylinositol phospholipase C and calcium movements (17–19), protein kinase C-regulated functions (20–24), lysophospholipid metabolism (25, 26), and PtdCho synthesis (12, 27–39). Although some of the results are contradictory, it is clear that there are multiple targets for ET-18-OCH₃, and it is not yet possible to distinguish the physiological imbalances that are causative from those that are either derivative or unrelated to the main event. Thus, a major contemporary focus in the field is to identify the critical cellular target(s) that are responsible for the cytotoxic and cytosstatic actions of ET-18-OCH₃.

Our work has focused on the role of the inhibition of PtdCho synthesis in the mechanism of antineoplastic phospholipid action. PtdCho is essential for the survival of cultured cells because it is a major structural building block of biological membranes and the precursor to the other two most abundant membrane phospholipids, phosphatidylethanolamine (40) and sphingomyelin (41). Thus, the cessation of PtdCho synthesis has a global effect on membrane structure and function. CCT catalyzes the formation of CDP-choline and is a key enzyme controlling the PtdCho biosynthetic pathway (42, 43). We proposed that the inhibition of PtdCho synthesis was the underlying cause for the cytotoxicity of ET-18-OCH₃ and hexadecylphosphocholine due to their ability to limit the formation of CDP-choline. These compounds effectively mimic LPC, a physiological regulator of CCT activity (12, 38, 39). Like LPC, both ET-18-OCH₃ and hexadecylphosphocholine reduce the CDP-choline formation in intact cells and inhibit purified CCT in an in vitro assay (12, 38, 39).

Supplementation of the medium with LPC provides a pathway to PtdCho that is independent of CCT and prevents apoptosis induced by either ET-18-OCH₃ (12) or hexadecylphosphocholine (39), consistent with the idea that CCT inhibition is causative in initiating apoptosis. This idea is corroborated by experiments with a mutant CHO cell line, mutant 58, that has a temperature-sensitive defect in CCT activity (44). The mutant 58 cell line undergoes apoptosis when shifted to the non-permissive temperature (45), and LPC supplementation rescues the mutant cells from programmed cell death (45, 46). LPC supplementation does not allow the continued proliferation of cells in the presence of ET-18-OCH₃, indicating that the drug also has cytostatic properties that are not related to its interference with PtdCho biosynthesis (12, 39). These experiments are completely concordant with the hypothesis that CCT is the critical cellular target for ET-18-OCH₃-induced apoptosis. However, LPC could possibly rescue cells by competitively reversing the inhibitory effect of ET-18-OCH₃ on another important cellular target in addition to restoring PtdCho synthesis. Thus, additional experiments are required to verify a specific role for CCT in ET-18-OCH₃ action. In this report we provide genetic evidence for the CCT hypothesis by demon-
strating that the specific elevation of cellular CCT content confers resistance to ET-18-OCH₃ cytotoxicity.

EXPERIMENTAL PROCEDURES

Materials—Sources of supplies were: CLONTECH, pTRE, and pTK-Hyg vectors, and HeLa Tet-On<sup>™</sup> cells; Sigma, doxycycline, protein A-Sepharose, and chromatography standards; Life Technologies, cell culture media; Calbiochem, ET-18-OCH₃, Avanti Polar Lipids, lyso-phosphatidylcholine; American Radiolabeled Chemicals, phospho-

**Cytotoxicity Studies**—Cells were seeded at densities 2.5 × 10⁵ cells/10-cm tissue culture plate and treated with 2 μg/ml doxycycline and/or 50 μM LPC 4 h prior to the addition of 4 μM ET-18-OCH₃. At 24-, 48-, and 72-h intervals, the cells were washed twice with phosphate-buffered saline, and the washes were collected. Adherent cells were removed by trypsinization for 1 min. Trypsinization was stopped by addition of 1 ml of complete medium. The trypsinized cells were combined with saline washes, centrifuged, and resuspended in 1–2 ml of complete medium. One drop of trypan blue was added to each sample, and the numbers of total and viable cells were counted.

**RESULTS**

Regulation of CCT Expression in HeLa Cells—HeLa cells were selected for this study for two reasons. First, HeLa cells are sensitive to ET-18-OCH₃ and rapidly undergo apoptosis when exposed to the drug (9). Second, a HeLa cell line is available that stably expresses a transcriptional regulator consisting of the DNA binding domain of a mutant Tet repressor fused to the transcriptional activation domain of VP16 (53). This cell line affords tight control over the expression of introduced genes cloned downstream of the promoter elements for the tetracycline-responsive transcriptional repressor of Esche-

**CCT Assay**—HeLa cells were washed twice with phosphate-buffered saline on ice and harvested by scraping into 1 ml of the same buffer followed by centrifugation. The cell pellets were resuspended in lysis buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 1% phenylmethanesulfonyl fluoride, 2% sodium deoxycholate, 1 μg/ml leupeptin, 50 mM NaF, 100 μM Na<sub>3</sub>VO<sub>4</sub>) and sonicated three times for 30 s. The standard CCT activity assay contained an aliquot of the HeLa cell lysate mixed with 125 mM bis-Tris-HCl, pH 6.5, 0.5 μM of phospho[methyl-<sup>14</sup>C]choline, 1 mM phosphocholine, 2 mM CTP, 20 mM MgCl<sub>2</sub>, 50 μM PtdCho/18:1 (1:1) in a final volume of 40 μl. The incubations were for 10 min at 37 °C and were stopped by placing the samples on ice for 3 min. The samples were added to 1 ml of 0.5 x EDTA, 0.05 x EDTA. CTT activity was determined by thin layer chromatography (49). CCT-specific activity was calculated from a series of assays that were linear with time and protein. Protein was determined according to the method of Bradford (50) using γ-globulin as a standard.

**Immunoprecipitation and Immunoblotting**—Cells were washed with cold phosphate-buffered saline, lysed on the plate with 400 μl of radio-

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Fig. 1. Regulated expression of CCT. Panel A, HeLa cell lines containing the CCT expression vector (CCT.12) were treated with 2 μg/ml doxycycline for 48 h or left untreated for the same length of time. CCT protein was immunoprecipitated from cell extracts with the N-terminal anti-peptide CCT antibody from line CCT.12 or the empty vector control (Vector). The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis, and CCT protein was detected by immunoblotting using an antibody directed against recombinant CCT as described under “Experimental Procedures.” The lane on the left-hand side (Sf9CCT) was a control that contained an extract from Sf9 cells infected with a recombinant baculovirus expressing CCT (49). Panel B, HeLa cell lines containing either the CCT expression vector (CCT.12) were treated with 2 μg/ml doxycycline for 48 h or left untreated for the same length of time. Duplicate dishes of CCT.12 cells or the empty vector control (Vector) were harvested, and the CCT-specific activity was determined as a function of protein concentration as described under “Experimental Procedures.” Doxycycline had no effect on the level of CCT expression or activity in the control cell line (CCT.00) containing the empty vector control (not shown).

Fig. 2. Increased CCT expression prevented ET-18-OCH₃-triggered apoptosis. Morphology of a control HeLa cell line harboring the control vector (CCT.00) and the cell line containing the regulated CCT expression construct (CCT.12) were photographed after treatment with 4 μM ET-18-OCH₃ or 4 μM ET-18-OCH₃ plus 2 μg/ml doxycycline for 48 h. Panel A, the untreated control cell line; panel B, the untreated CCT.12 cell line; panel C, the control cell line treated with 4 μM ET-18-OCH₃; panel D, the CCT.12 cell line treated with 4 μM ET-18-OCH₃; panel E, the control cell line treated with 2 μg/ml doxycycline plus 4 μM ET-18-OCH₃; and panel F, the CCT.12 cell line treated with 2 μg/ml doxycycline plus 4 μM ET-18-OCH₃. static action of ET-18-OCH₃ was directly tested by comparing the sensitivity of the CCT.12 cell line to growth inhibition and cell death in the presence and absence of CCT overexpression. Our previous experiments demonstrated that LPC supplementation prevented ET-18-OCH₃ cytotoxicity but did not permit cell proliferation (12, 39). These data were interpreted to mean that providing an alternate source for PtdCho synthesis via the acylation of exogenous LPC circumvented the requirement for CCT and thus reversed the cytotoxic effects of ET-18-OCH₃.

CCT Overexpression and LPC Supplementation Were Not Additive—Our previous experiments demonstrated that LPC supplementation prevented ET-18-OCH₃ cytotoxicity but did not permit cell proliferation (12, 39). These data were interpreted to mean that providing an alternate source for PtdCho synthesis via the acylation of exogenous LPC circumvented the requirement for CCT and thus reversed the cytotoxic effects of ET-18-OCH₃.
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another cellular target in addition to CCT that was responsi-
bility for these results. Comparing the effectiveness of LPC
CCT overexpression was not additive, suggesting that both
targeted the same process. These data support the
conclusion that LPC supplementation prevents ET-18-OCH₃-
induced cytotoxicity by supplying an alternate route to PtdCho
synthesis that circumvents the CCT step.

**DISCUSSION**

Our genetic experiments provide compelling support for the
conclusion that the interruption of PtdCho synthesis at the
CCT step is essential for the cytotoxic activity of ET-18-OCH₃.
The inhibition of PtdCho synthesis is a universal effect of
ET-18-OCH₃ on cells (27–37), suggesting that the interference
with PtdCho metabolism may be responsible for the biological
effects of the drug. Our previous work identified CCT as the
ET-18-OCH₃ target in the PtdCho biosynthetic pathway based
on the pattern of accumulation of choline-derived pathway
intermediates *in vivo*. ET-18-OCH₃ also was able to inhibit
purified CCT activity *in vitro* by competing for the lipid acti-
varor site on the enzyme (12, 38, 54). The doxycycline-depend-
CCT expression system developed in this report demonstrates
that specifically increasing the cellular concentration of
CCT imparts ET-18-OCH₃ resistance to HeLa cells. These ex-
periments provide a critical genetic test that strongly supports
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ptosis. Acylation of exogenous LPC is a direct route to PtdCho
that circumvents the requirement for cellular CCT activity (45,
46) and, accordingly, also prevents the cytotoxic effects of ET-
18-OCH₃ (12). The possibility that LPC affects the interaction
between ET-18-OCH₃ and a cellular target other than CCT
appears untenable in light of the identical effects of LPC sup-
plementation and CCT overexpression in reversing ET-18-
OCH₃ cytotoxicity and the absence of an additive effect when
the two treatments are used in combination. Taken together,
the biochemical, physiological, and genetic data provide con-
vincing evidence that the inhibition of PtdCho synthesis at the
CCT step is the underlying cause for the programmed cell
death caused by ET-18-OCH₃.

The antiproliferative property of ET-18-OCH₃ is independ-
et of its suppression of PtdCho biosynthesis. A large number
of physiological imbalances are promoted by ET-18-OCH₃ (13–
16), and it is reasonable to assume that the inhibition of one or
more of these potential targets is responsible for the cessation
of cell growth. Our previous work shows that ET-18-OCH₃
arrests cells primarily in the G₁ and G₂ phases of the cell cycle
and that LPC rescue results in G₁ arrest (12). These data
suggest that the relevant targets for the cytostatic action of
ET-18-OCH₃ will be involved in cell cycle progression. Current
research has focused on the ability of ET-18-OCH₃ to inhibit
components of signal transduction pathways involved in
growth stimulation with the idea that blocking one or more of
these events may account for the antiproliferative effects of the
drug (17–19, 55, 56). However, we must also consider the
possibility that ET-18-OCH₃ activates a biochemical cascade
that counteracts the normal transmission of proliferative sig-
nals. In this regard, the reports that ET-18-OCH₃ stimulates
the expression of immediate early genes such as c-fos and
c-junB (57, 58) may be relevant to the activation of a signaling
cascade that results in growth arrest. The problem of identify-
ing a definitive cellular target responsible for the cytostatic
action of ET-18-OCH₃ within this constellation of candidates
will be a major challenge for future research.

The existence of cell lines that are relatively resistant to
ET-18-OCH₃ was recognized early in the investigation of the
action of this drug (6). Our result that elevated CCT expression
bestows ET-18-OCH₃ resistance raises the question of whether
or not the differences between sensitive and resistant cell lines
can be attributed to variations in the cellular concentrations
of CCT. A survey of CCT-specific activities in resistant and sen-
sitive cells is not available, but alterations in the level of CCT
expression may not explain the large range of drug sensitivity
among cell types. Several recent studies indicate that antine-
oplastic ether lipids accumulate to a lesser extent in resistant
cell lines (59–63). ET-18-OCH₃ is a substrate for class I and class II P-glycoproteins and other ABC transporters that mediate pleiotropic drug resistance in a wide range of organisms (64). Resistance to the effects of ET-18-OCH₃ in yeast was dependent on overexpression of a functional efflux pump (64). The interaction of ET-18-OCH₃ with all members of this group of proteins suggests that they are not specific transporters for ET-18-OCH₃ or lysophospholipids. Lack of accumulation of effective dosages of ET-18-OCH₃, together with elevated expression levels of CCT target protein, may each make independent contributions to cellular resistance mechanisms.

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