Differential effect of cadmium on cholinephosphotransferase activity in normal and cancerous human mammary epithelial cell lines

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Abstract
Cadmium (Cd) is an ubiquitous environmental carcinogen. Membrane phospholipids as well as fatty acid profile of membrane phospholipids are known to be altered in tumorigenicity and malignancy. Synthesis of cellular phosphatidylcholine (PC) has been used as a marker for membrane proliferation in the neoplastic mammary gland tissue. Cholinephosphotransferase (CPT), the terminal enzyme in de novo synthesis of PC, has an important role in regulating the acyl group of PC in mammalian cells. Our previous studies have shown that CPT is expressed differentially in the normal and cancerous mammary epithelial cell lines. In this study, we examined the effect of cadmium on CPT activity using normal (MCF-12A and MCF-12F) and cancerous (MCF-7, BT-549, and 11-9-1-4) human mammary epithelial cell lines. There was no consistent pattern of CPT activity in response to different doses of cadmium. The activity did not show a time-dependent variation at 5 μM concentration, except in MCF-7 and 11-9-1-4. CPT gene expression increased with cadmium as evident from slot blots. Mutation in the nucleotide sequence was also observed as the result of cadmium but this did not result into amino acid sequence changes. [Mol Cancer Ther. 2004;3(2):199–204]

Introduction
Alterations in membrane phospholipids are associated with malignant transformation (1), tumorigenicity (2), and metastasis (3, 4). Phosphatidylcholine (PC) is one of the most important phospholipids of eukaryotic membrane along with phosphatidylethanolamine (PE). CDP-choline pathway is primarily responsible for the de novo PC biosynthesis in all eukaryotic cell types thus studied except the liver where the transmethylation pathway involving phosphatidylethanolamine is predicted to contribute 30% of the net PC synthesis. It has been recently suggested that phospholipid signaling plays an important role in cancer cell-endothelial cell interaction (5). Cholinephosphotransferase (CPT) is the terminal enzyme in the biosynthesis of PC, and has an important role in regulating the acyl group of PC in the mammalian cells. Synthesis of total cellular PC has been suggested to be a marker for membrane proliferation in neoplastic mammary gland tissues in C3H mice (6). Human breast cancer cells have higher levels of PC than human normal mammary epithelial cells and phospholipid metabolism is shown to be modulated in breast cancer cell lines (7). Both MCF-7 and T47D human breast cancer cell lines show augmented synthesis of PC and PC has been suggested as a metabolic marker for breast cancer (8). We have also shown in our laboratory that there is an inherent difference in the expression and nucleotide sequence of the CPT gene between the normal (MCF-12A) and aggressive (11-9-1-4) breast cancer cell lines (9). In general, we expect a higher level of CPT gene expression in breast cancer cell lines. Therefore, it is important to know whether any mutagen, such as Cadmium (Cd), can increase the expression of the CPT gene in breast epithelial cells.

Cadmium is one of the most toxic transition metal pollutants and is associated with air and water pollution as well as cigarette smoking and its potential harm has increased with increasing industrial usage of the element (10). It is shown to have a wide physiological function and it activates the expression of several mammalian genes. Cadmium has been shown to have toxic effects on human neuroblastoma cells (11), porcine and rat kidney cells (12), and human prostate epithelial cells (13). Correlation of cadmium with estrogen receptors in breast cancer has been found suggestive (14). Cadmium has been shown to attribute to carcinogenicity by enhancing DNA mutation rates and to stimulate mitogenic signaling pathways and expression of oncoproteins that control cellular proliferation (15).

Breast carcinoma is the third most common cancer worldwide (16). A mechanism by which cadmium may be involved in the initiation of the breast cancer is not known. Since CPT activity can be used as a marker for membrane proliferation associated with cancer and cadmium has been shown to be a potential carcinogen, therefore, the present study was undertaken with the objective to find out if cadmium has any putative role in the etiology of cancer development and associated membrane proliferation using breast carcinoma as the model. Present work focuses on studying the differential effect of cadmium on CPT in normal and cancerous human mammary epitheli- um cells and elucidating the putative mechanism of action of cadmium on CPT gene of these cell lines.
Materials and Methods

Maintenance of Human Mammary Epithelial Cell Lines

Five cell lines representing normal (MCF-12A and MCF-12F), nonaggressive, (MCF-7) and aggressive (11-9-1-4 and BT-549) human mammary epithelial cell lines were used in the study. All cell lines except 11-9-1-4 were obtained from American Type Culture Collection, Manassas, VA. 11-9-1-4 cell line was obtained from the Meharry Tissue Procurement Facility, Meharry Medical College, Nashville, TN. It originated from a human breast epithelial cell line BT-549 at ATCC and transfected with galectin-3, a β-galactoside binding protein (a cell adhesion molecule).

All cell lines were cultured in DMEM/F-12 media supplemented with 100 μg/ml penicillin-streptomycin, 2.5 μg/ml fungizone, 20 ng/ml epidermal growth factor, 98 ng/ml cholera toxin, 10% heat-deactivated fetal bovine serum, 2 mM glutamine, and nonessential amino acids. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. All cell culture supplies were purchased from Sigma, St. Louis, MO.

Cadmium was added to the culture medium as the CdCl2 solution. Coogan et al. (17) showed that in cultured rat liver cells (TRL 1215), LC₅₀ for a 2-h cadmium exposure was approximately 660 μM (for high-passage cells) to 1060 μM (for low-passage cells) and the genotoxic concentration was 500 μM cadmium. Therefore, the cadmium concentrations (0–50 μM) used in this study are within the physiological limit, and can be considered low dose with respect to its toxicity. Different concentrations (1–50 μM) were made in DMEM/F-12 medium and cells were incubated for different time periods (1–48 h).

Assay of CPT Activity

CPT activity was measured as described earlier (9). CPT activity was measured by monitoring the incorporation of CDP-[methyl-¹⁴C]choline into PC. The final reaction mixture contained the following: 10 mM MgCl₂; 5 mM reduced glutathione; 50 mM Tris-HCl (pH 8.5); 80 μM CDP-[methyl-¹⁴C]choline (specific activity 52.5 Ci/mol); 6 mM of 1,2-dioleoyl-glycerol; and the protein in the total volume of 100 μl. The reaction was started by adding 20 μl of samples and incubated at 37°C for 2 min. The reaction was stopped by adding 50 μl of n-butanol. Lipids were extracted by adding 500 μl of butanol/water (1:1, v/v). The mixture was allowed to equilibrate for 10 min and centrifuged at 3000 rpm for 10 min. Approximately 300-μl butanol layer was removed and carefully placed in counting vial. The radioactivity was determined after adding 5 ml hydrofluor and counted in a Beckman LS-355 scintillation counter.

Enzyme activity showed a cell line-specific pattern; therefore, all further experiments on CPT gene expression were performed on optimum dose and time for each cell line where it showed maximum activity. Since this itself varied between the cell lines, therefore, all further studies were with different time and concentrations of cadmium for different cell lines.

Isolation of RNA

RNA was isolated from 10⁶ cells using Trizol (Invitrogen, Frederick, MD). Cells were first washed with sterile PBS three times and resuspended in 1 ml Trizol and cells were disrupted by repeated pipetting and incubated at RT for 5 min. Then 200 μl of chloroform were added and mixture was vigorously shaken and phases were separated by centrifuging at 12,000 rpm for 15 min at 4°C. The RNA was then precipitated from the aqueous phase with isopropanol. The pellet was washed with 1 ml of sterile 70% ethanol. The concentration and purity of the RNA were analyzed in a UV spectrophotometer.

Reverse Transcriptase-PCR

RT-PCR was performed using 5 μg of RNA from all the samples, using one-step RT-PCR kit (Invitrogen). The primers were synthesized to amplify approximately 200 amino acids in the carboxyl end of the protein and based on the sequences from the GenBank accession no. NM_020244. The primer sequences were 5’-TTGCCGCTCATGGCACACTTG-3’ (forward) and 5’-TCTCTTCAAATCCATCCATGTTATCTGA-3’ (reverse). RT-PCR products were electrophoresed on a 1% agarose gel and were purified (QIAquick PCR purification kit, Qiagen, Chatsworth, CA) and sequenced using BidDye-terminators kit (Applied Biosystems, Foster City, CA). The sequences were analyzed using Applied Biosystems Automated sequencer (ABI 3700 model). An alpha imager (Alpha Innotech Corporation, San Leandro, CA) quantitation of the band intensities was also obtained.

Slot Blot Hybridization

The forward primer 5’-TTGCCGCTCATGGCACACTTG-3’ was digoxigenin-labeled using Terminal DIG labeling kit (Roche Biochemicals, Indianapolis, IN). Different amounts of RNA (0, 1, 4, and 8 μg) from control cells (without cadmium treatment) and experimental cells (with cadmium treatment) were loaded onto a positively charged nylon membrane (MSI, Atlanta, GA) using a turboblotter (Bio-Rad, Richmond, CA). Both the control and experimental cells were of same passage. The samples were then linked using a UV cross-linker (Stratagene, La Jolla, CA). A nonradioactive detection method using digoxigenin (Roche Biochemicals) was used to detect any hybridization. The band intensities were quantitated using alpha imager (Alpha Innotech).

All the experiments were done with five replicates and each repeated for a total of three times. Results obtained were subjected to standard statistical procedures and significance of the difference in the result between the control and the experimental was calculated for each set of experiment.

Statistical Analysis

Differences between cadmium-treated and untreated cells were assessed by using ANOVA, and the significance level was set for P ≤ 0.05.

Results

Cell Morphology and Viability

All the cell lines used in the present study showed distinct differences in their morphology and growth
pattern. Cadmium modified cellular morphology only at concentrations exceeding 10 μM (photograph not shown). We did not find any significant changes in the dose-response study until we used 10–50 μM cadmium, which showed total loss of viability in MCF-12A (Fig. 1A). Furthermore, the time response study also did not show any measurable difference in viability at 5 μM dose (Fig. 1B).

CPT Activity

Figure 2A represents the dose-response study on CPT activity. Even though there was no consistent pattern of CPT activity in response to the dose, there was a clear dose-dependent increase in CPT activity at high concentrations of cadmium in normal cell lines, MCF-12A and MCF 12-F, for which this increase in activity was significant (P ≤ 0.05). Figure 2B represents the time response study on CPT activity. Data reveal that significant (P ≤ 0.05) difference in activity over time was observed in only two cancer cell lines (MCF-7 and 11-9-1-4). In both these cell lines, this initial increase was followed by a decrease in the activity.

Expression of CPT Gene

CPT gene expression, as evident from the total RNA slot blots, showed that there was increased gene expression with cadmium in all cell lines (Fig. 3; Table 1) when compared to their corresponding controls. Significant (P ≤ 0.05) increase was observed only in case of 11-9-1-4 and MCF-12F. RT-PCR product obtained from both control and cadmium-treated cells was 0.7 kb in size (Fig. 4). The nucleotide sequence analyses of the cDNA revealed similarity in nucleotide sequence of various cell lines and that from human CPT gene from NCBI database (NM_020244). All cell lines except MCF-12A showed mutations in their nucleotide sequence as the result of cadmium treatment when compared with the corresponding controls without cadmium treatment. The effect of cadmium was highest in MCF-12F cell line that showed a total of six mutations (Table 2) when compared to control MCF-12F cells without cadmium treatment.

Figure 1. Dose-dependent effect of cadmium on cell viability after 24 h of exposure (A) and time-dependent effect of a fixed (5 μM) concentration of cadmium on cell viability for various exposure times (B).

Figure 2. A, dose-dependent effect of cadmium on CPT activity in human breast cancer cell lines incubated for 24 h. B, time-dependent effect of 5 μM cadmium on CPT activity in various human breast cancer cell lines.

Figure 3. Slot blot hybridization. Increasing amounts of RNA (0, 1, 4, 8 μg) were taken from both control (A) and cadmium-treated (B) breast epithelial cells and hybridized with probe. Control is without cadmium treatment and the dose and the time of individual cadmium treatment is as mentioned in Table 1.
However, translation of these nucleotide sequences revealed possible amino acid substitutions in the CPT gene product only in MCF-12F and BT-549 as the result of cadmium (Table 2).

**Discussion**

Cadmium is a type D heavy metal of great environmental and human health concern (18). It is a widespread environmental pollutant that is also present in cigarette smoke, and smoking along with occupation are major sources of human exposure (19). Cadmium is taken up by the body by inhalation and ingestion and has a very long biological half-life (>25 years). The mechanisms of cadmium carcinogenesis are poorly understood. *In vitro*, cadmium is cytotoxic in concentrations between 0.1 and 10 mM and induces free radical-dependent DNA damage (20, 21). Takiguchi et al. (22) studied the cellular tolerance of cadmium in rat liver epithelial cells using 0, 50, or 100 \( \mu \)M \( \text{CdCl}_2 \). *In vivo*, it has also been demonstrated in our laboratory that cadmium modulates male reproduction in mice model at a concentration of 1 mg/kg body weight (23). In this study, up to 0–50 \( \mu \)M cadmium concentration used do not show any significance change in cell viability except in MCF-12A, showing that at low doses, cadmium is not toxic to the cells. However, cadmium is a weak mutagen when compared with other carcinogenic metals (24). Cadmium can affect signal transduction pathways, for example, cadmium has been shown to induce inositol polyphosphate formation and to increase cytosolic free calcium levels in various cell types (25). Cadmium has been shown to block calcium channels (26–29). In lower concentrations (1–100 \( \mu \)M), cadmium binds to proteins; decreases DNA repair (30, 31); activates protein degradation; up-regulates cytokines and proto-oncogenes like c-fos,

<table>
<thead>
<tr>
<th>Cell Line and Cadmium Treatment</th>
<th>RNA Concentration (( \mu )g)</th>
<th>IDV Values*</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-12A (10 ( \mu )M; 24 h)</td>
<td>0</td>
<td>0</td>
<td>1.17</td>
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<tr>
<td></td>
<td>1</td>
<td>6,480</td>
<td>7,590</td>
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<td></td>
<td>4</td>
<td>22,320</td>
<td>28,080</td>
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<td>26,565</td>
<td>41,745</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>11,385</td>
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<td></td>
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<td></td>
<td>8</td>
<td>19,440</td>
<td>49,335</td>
</tr>
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<td>BT-549 (5 ( \mu )M; 48 h)</td>
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</tr>
<tr>
<td></td>
<td>1</td>
<td>6,615</td>
<td>7,250</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8,085</td>
<td>8,820</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>11,760</td>
<td>15,435</td>
</tr>
<tr>
<td>11-9-1-4 (5 ( \mu )M; 6 h)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8,820</td>
<td>15,435</td>
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<td></td>
<td>4</td>
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<td>19,845</td>
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<td></td>
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<td>11,025</td>
<td>23,520</td>
</tr>
<tr>
<td>MCF-7 (5 ( \mu )M; 1 h)</td>
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<td>0</td>
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<td></td>
<td>1</td>
<td>9,108</td>
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</tr>
<tr>
<td></td>
<td>4</td>
<td>15,120</td>
<td>20,493</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>18,000</td>
<td>23,529</td>
</tr>
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</table>

*Note: Control is without cadmium treatment and the dose and the time of individual cadmium treatment is mentioned in the parenthesis.

*IDV, integrated density values.
c-jun, and c-myc (32, 33); and induces expression of several genes, such as metallothioneins (34), heme oxygenases, glutathione transferases, heat-shock proteins, acute-phase reactants, and DNA polymerase β (35). It has also been shown that cadmium in subtoxic concentrations (10–30 μM) perturbs the protein folding of p53 (36). Alam et al. (37) observed induction of heme oxygenase-1 gene in MCF-7 mammary epithelial cells by cadmium. Other workers have shown activation of three MAPK subfamilies by cadmium. Other workers have shown that cadmium in subtoxic concentrations (10–30 μM) perturbs the protein folding of p53 (36). Alam et al. (37) observed induction of heme oxygenase-1 gene in MCF-7 mammary epithelial cells by cadmium. Other workers have shown activation of three MAPK subfamilies by cadmium.

Ramirez and Gimenez (41) have observed profound changes in the lipid composition of the peritoneal macrophages when exposed to cadmium in mice. Since one of the earliest metabolic events that occurs simultaneously with the induction of cell growth and proliferation by tumor promoter is increased synthesis of PC, therefore, in the present study, we have focused on the short-term (0–48 h) effect of cadmium on the enzyme CPT, the terminal enzyme of the PC biosynthesis using low doses (0–50 μM) of cadmium. We expect that any change in activity and gene expression of this enzyme will give us an insight of the mechanism of the initial action of cadmium on mammary epithelial cell lines. Our results demonstrate that effect of cadmium on CPT enzyme activity is cell line specific and did not follow any particular trend. For example, only MCF-7 and 11-9-1-4 (cancer cell lines) showed any significant change (P ≤ 0.05) in CPT activity at 5 μM cadmium incubated for different time periods, whereas, MCF-12A and MCF-12F (normal cell lines) and BT-549 did not show any noticeable difference in their CPT activity. There was no consistent pattern of CPT activity in response to the dose and only MCF-12A and MCF-12F (normal cell lines) showed significantly high induction of CPT activity with cadmium (10 and 25 μM, respectively) (P ≤ 0.05). Earlier, Amanuma and Suzuki (42) have demonstrated an increase in the phospholipid content in alveolar wash fluid after exposure to very low doses of cadmium.

With regard to metal toxicity, two modes of action have been identified, that is: the induction of oxidative DNA damage and interaction with DNA repair processes and cadmium has been implicated in both (18). Ishido and Kunimoto (12) discussed the apoptogenic nature of cadmium involving DNA fragmentation and chromatin condensation in porcine renal cultured cells. It has been suggested that one possible mechanism of mutagenicity of cadmium is that it displaces the zinc in XPA, a Mr 31,000 protein involved in nucleotide excision repair (NER), and that results in its nonfunctionality in nucleotide excision repair (43) and also by inhibiting mismatch repair (44). In the present study, we compared the DNA sequences of the control cells with that of cadmium-treated cell for the cadmium concentration where it shows the maximum activity to look for corresponding increase in expression of CPT gene and possible mutations. CPT gene expression, as observed in slot blots, showed a consistent increase with the cadmium treatment though profound effect and was observed only in case of MCF-12F and 11-9-1-4 (P ≤ 0.05).

We observed nucleotide mutations as the result of cadmium treatment in almost all the cell lines studied when compared with their corresponding controls (i.e., without cadmium treatment). Therefore, it can be concluded that even in short-term exposures and low doses, cadmium is able to bring about DNA damage in breast cell lines, though again these mutations were also cell line specific. Surprisingly, MCF-12A, which is a control cell line, did not show any mutation even at 10 μM cadmium concentration, whereas, MCF-12F, which is the floating form of MCF-12A, shows a large number of mutations. This kind of variation is expected because these cell lines have been immortalized and may have variations caused by many factors. Most of these mutations did not translate into corresponding amino acid sequence changes, but these results give us an insight into possible mechanism of action of cadmium on cell cycle of human mammary epithelial cell lines.

### Table 2. Position and type of nucleotide and amino acid sequence changes as a result of cadmium treatment as compared to control cells in different human breast cell lines

<table>
<thead>
<tr>
<th>Cell Lines and Cadmium Treatments</th>
<th># Bases Sequenced</th>
<th>Alignment with NM_020244 (CPT Gene)</th>
<th>Mutation Observed with Cadmium (Compared to Control Cells)</th>
<th># Amino Acid Sequenced</th>
<th>Alignment with NM_020244 (CPT Gene)</th>
<th>Putative Mutation in the AA Sequence (Compared to Control Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-12A (10 μM; 24 h)</td>
<td>450</td>
<td>795–1244</td>
<td>None</td>
<td>150</td>
<td>209–358</td>
<td>None</td>
</tr>
<tr>
<td>MCF-12F (25 μM; 24 h)</td>
<td>705</td>
<td>696–1393</td>
<td>Substitution: a933c, a1387g; Insertion: a after 1306, a after 1316, a after 1363, g after 1375</td>
<td>229</td>
<td>176–379</td>
<td>Substitution: N255H</td>
</tr>
<tr>
<td>MCF-7 (5 μM; 1 h)</td>
<td>699</td>
<td>698–1396</td>
<td>Substitution: g1386a, a1387t, t1389g</td>
<td>232</td>
<td>177–405</td>
<td>None</td>
</tr>
<tr>
<td>BT-549 (5 μM; 48 h)</td>
<td>681</td>
<td>700–1380</td>
<td>Substitution: g759a, t781g</td>
<td>226</td>
<td>178–403</td>
<td>Substitution: A197T, L204W</td>
</tr>
<tr>
<td>11-9-1-4 (5 μM; 6 h)</td>
<td>707</td>
<td>691–1396</td>
<td>Insertion: g after 1368</td>
<td>219</td>
<td>175–393</td>
<td>None</td>
</tr>
</tbody>
</table>
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References

41. Ramirez DC, Gimenez MS. Lipid modification in mouse peritoneal macrophages after chronic cadmium exposure. Toxicology, 2002;172:1–12.
Molecular Cancer Therapeutics

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