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Gene Expression Profiles of Dibutyl Phthalate and 17β-Estradiol using cDNA microarray in MCF7 Human Breast Cancer Cell Line

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ABSTRACT: **Phthalates, suspected endocrine disruptor, are plasticizer and solvent used in industry, and some phthalates are known as potential carcinogen. Most common human exposure to this compounds may occur with contaminated food. It may migrate into food from plastic wrap or may enter food from general environmental contamination, and it has become widespread environmental pollutants, thus leading to a variety of phthalates that possibly threaten the public health. Dibutyl phthalate (DBP) may play a part of cell proliferator, which mediates changes in gene expression and the metabolism of xenobiotics. An understanding of the role of DBP in modulating gene regulation should provide insight regarding mechanisms of DBP induced xenoestrogenic impact. To elucidate the type of genes that are associated with estrogenic activity induced by DBP at the dose (10^{-8} M) appeared proliferating effects, the pattern of gene expression in MCF7 cells was compared between 17β-estradiol and DBP exposure in the cDNA microarray. From the results, it showed some differences of gene expression patterns between MCF7 cells treated with 17β-estradiol and DBP, and also DBP shows estrogenic potential with changes in estrogen-related gene expression levels.**

Keywords : **cDNA microarray, dibutyl phthalate, 17â-estradiol, gene expression, endocrine disruptor**

Introduction

Dibutyl phthalate (DBP) is a kind of phthalate analogues widely used as plasticizer to increase the flexibility and workability of high-molecular-weight polymers. The world wide production of phthalates approximates 2.7 million metric tons a year (Bauer and Herrmann, 1997). Phthalates are used in plastic goods (e.g., in children's toys, paints, lacquers, cosmetics, as well as food wrappings). Furthermore, many medical items, such as blood bags, tubes, and filtering membranes, may contain a considerable amount of phthalates. Since these substances are not limited to the original products, and they may enter the environment and have become widespread environmental pollutants, thus leading to a variety of phthalates that possibly threaten the public health. Concern about their use has been mounting.

It has been suggested that substances present in the environment may contribute to the development of hormone-dependent cancers and comprise reproductive capacity in humans and wildlife (Colborn *et al.*, 1993; Davis *et al.*, 1993; Eubanks, 1997). Phthalates are often mentioned as suspected endocrine disruptors, i.e., some phthalates are blamed for causing damage to the testes and decreasing sperm production (Gray *et al.*, 1982; Hardell *et al.*, 1997) and are reported to be a potential carcinogen. Among many phthalate compounds, butyl-benzyl-phthalate and di-n-butyl-phthalate (DBP) may act as xenoestrogens or antiandrogens (Bradbury, 1996; Mylchreest *et al.*, 1998). Estrogenic activity of phthalate esters was investigated *in vitro* assay (Harris *et al.*, 1997; Ryu *et al.*, 2002). Di-(2-ethylhexyl)-phthalate and DBP were demonstrated to increase the frequency of proliferating cells in a human breast cancer cell line (Blom *et al.*, 1998). Also, DBP was reported to be genotoxic on human mucosa of the upper aerodigestive tract (Kleinsasser *et al.*, 2000).

To clarify the mechanisms of DBP-induced xenoestrogenic effects, we investigated the pattern of gene expression in MCF7 cells using cDNA microarray. Microarray containing cDNA clone have been used to compare patterns of gene expression in which thousands of genes can be examined in a single hybridization (Schena *et al.*, 1996; Derisi *et al.*, 1997). In this study, we try to identify genes that associated with estrogenic activity induced by DBP.

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Materials and methods

Materials

Dibutyl phthalate (CAS No. 84-74-2) and 17-estradiol (CAS No. 50-28-2) were obtained from Sigma-Aldrich Co. (St. Louis, USA). They were dissolved in ethanol immediately before use. The final concentration of ethanol used in the medium was below 1%.

Cell culture

Human breast cancer estrogen-sensitive MCF7 cell line was kindly donated by Dr. Soto in Tufts University School of Medicine. For routine maintenance, cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum at 5% CO_2 under saturating humidity (Soto and Sonnenschein, 1985).

Cell Morphology

MCF7 cell line was tested with slight modification described by Soto *et al.* (1995). Cells were trypsinized and plated in 12-well plates at an initial concentration of 3×10^4 cells per well in 5% FBS in DMEM. The cells were allowed to attachment for 24 hr, then 5% charcoal dextran-treated fetal bovine serum (CDFBS) supplemented phenol red-free DMEM was substituted for the seeding medium. The concentration of test compounds, 10^{-8} M 17β-estradiol, 10^{-7} M DBP and 1% ethanol control were added and incubated for 48 hr. Morphology of cells was observed at a magnification of ×400 with microscope.

RNA isolation

MCF7 cell line was grown up to 80~90% confluence and then 10^{-8} M 17β-estradiol and 10^{-7} M DBP were treated in plates. Approximately 1×10^7 cells were used for each batch. After 48 hr incubation, cells were lysed and mRNA was isolated using the Fast Track 2.0 Kit (Invitrogen, CA, USA) according to the manufacturers instructions. RNA yield was determined by measuring absorbance at 260 nm.

Hybridization and data analysis

The cDNA microarray chip (Micromax direct cDNA microarray system) was purchased from NEN Life Science Products (Boston, USA). According to the manual of Micromax direct cDNA microarray system, experiment was processed. The mRNA in each pool treated 10^{-8} M

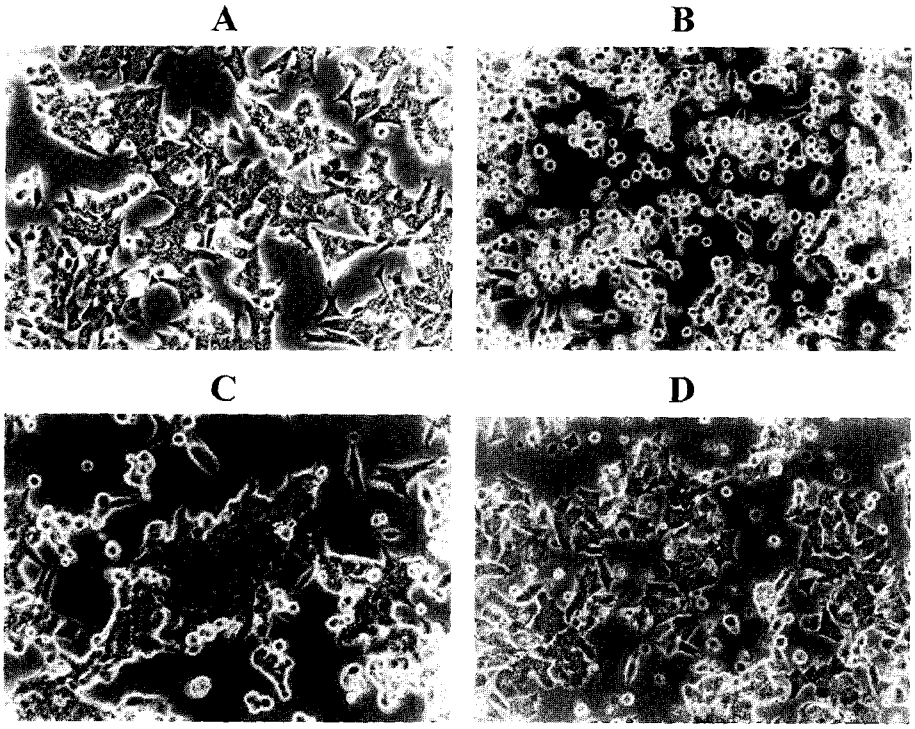

Fig. 1. Morphological changes of MCF7 cells. *A*: Normal MCF7 cells were incubated on the medium with 5% FBS. *B, C* and *D*: Normal MCF7 cells were grown on 5% CDFBS supplemented phenol red-free DMEM for 48 hr. (*C*) treatment with 17β-estradiol (10^{-8} M) and (*D*) treatment with DBP (10^{-7} M) were showed clear proliferation of MCF7 cells compared to control (*B*).

17β-estradiol and 10^{-7} M DBP, without further purification, is then converted into cyanine 3 and cyanine 5-labeled analogues, for use as individually traceable gene targets. These cDNA are pooled together and simultaneously hybridized to a microarray in an overnight incubation, resulting in differential deposition of cyanine 3 and cyanine 5 fluorescent molecules on the cDNA spots. The fluorescence intensity was scanned, and data were analyzed by using data analysis software ImaGene (BioDiscovery, Inc., CA, USA). Results were also analyzed by normalizing fluorescence intensities between experiments using a subset of cDNA clones.

Results and Discussion

In relation to the estrogenic activity of DBP with gene expression in MCF7 cells, we examined with various concentrations of DBP to select the suitable concentration for cell proliferation of MCF7 cells. A relatively high rate of cell proliferation was observed at the concentration of 10^{-7} M. After treatment of 17β-estradiol (10^{-8} M) and DBP (10^{-7} M) to MCF7 cells for 48 hr, the morphological changes by DBP and 17β-estradiol were observed. In MCF7 cells treated with 10^{-7} M of DBP for 48 hr, DBP (Fig. 1, D) induced the morphological changes of MCF7 cells compared to the control (Fig. 1, B), indicating the proliferation of the cell. Both 17β-estradiol (Fig. 1, C) and DBP (Fig. 1, D) showed that they have influence on cell proliferation of MCF7 cells.

We also further examined to screen the patterns of gene expression using cDNA microarray (Photo not shown) after treatment with 10^{-8} M 17β-estradiol and 10^{-7} M DBP in MCF7 cells. Table 1 shows the expression ratio for expressed gene profile between 17β-estradiol and DBP treated group from two pairs of cDNA microarrays. As shown in Table 1, some variations exist in up-regulated gene expression patterns. Expression of nuclear antigen H731-like protein and X-box binding protein-1 (XBP-

Table 1. Expression ratio for expressed gene profile between 17β-estradiol and dibutyl phthalate treated group from two pairs of cDNA microarrays

GenBank Name	Accession Number	Cy3[a] versus Cy5[b] expression ratio
Up-regulated 17β-estradiol		
Nuclear antigen H731-like protein	U96628	5.774579859
p 18 protein.	J04991	4.182402934
mRNA for Pr22 protein.	Z11566	3.554120078
mRNA for lactate dehydrogenase-A (LDH-A, EC 1.1.1.27)	X02152	2.302655553
tral mRNA forhomologue of murine tumor rejection antigen gp96.	X15187	2.28539041
mRNA for high mobility group-1 protein (HMG-1)	X12597	2.251116938
Alpha-tubulin.	K00558	2.108484267
Alternative splicing factor.	M72709	2.105087604
X box binding protein-1 (XBP-1)	M31627	2.027072037
Up-regulated dibutyl phthalate		4.177388466
X box binding protein-1 (XBP-1)	M31627	2.995909473
Neuropetide Y receptor Y1 (NPYY1) mRNA, exon		2.888560188
2-3 and complete cds.	L07615	2.452146562
Protein tyrosine phosphatase (TEP1)	U96180	2.436033398
Breast cancer, estrogen regulated LIV-1 protein (LIV-1)	U41060	2.31204058
mRNA for KIAA0372 gene.	AB002370	2.29259368
Beta 2-microglobulin.	S82300	2.215518988
Nuclear antigen H731-like protein.	U96628	2.205231412
2-3-oxidosqualene-lanosterol cuclase.	U22526	2.12680461
mRNA for squalene synthase.	X69141	2.042864508
mRNA for glucocerebrosidase.	D13286	
mRNA for lanosterol synthase.	D63807	

[a]Sample treated with DBP and 17β-estradiol was labeled with Cyanine 3 (Cy3) and [b] control sample was labeled with Cyanine 5 (Cy5).

1) gene was induced in both group of cells treated with of 17β-estradiol and DBP. Particularly, breast cancer, estrogen regulated LIV-1 protein (LIV-1) gene which is known to estrogen-regulated gene (el Tanani and Green, 1996a; 1996b) was expressed only in DBP-treated MCF7 human breast cells.

Expression profile and ratios of various genes were not similar between DBP and 17β-estradiol treatment. The function of some up-regulated genes in 17β-estradiol and DBP treatment is related to cell proliferation. Expression of H731-like protein in bladder carcinoma and breast carcinoma tissues was known to be up-regulate or induce in the proliferative cells (Yoshinaga *et al.*, 1999). XBP-1 was known to act as transcriptional factor in B cells (Liou *et al.*, 1990). It was also reported that P18 protein is present in much greater abundance in acute leukemic blast cells (myeloid and lymphoid) than in resting or proliferating nonleukemic lymphoid cells (Zhu *et al.*, 1989). The pr22 protein gene was isolated as a gene, which is expressed in proliferating cells but not in cells which are differentiated or growth-arrested. It was also known to have an additional role other than function associated with microtubules (Hosoya *et al.*, 1996). Particularly, LIV-1 gene which is known as estrogen-regulated gene. (el Tanani and Green, 1996a; 1996b) is expressed only in DBP treated MCF7 human breast cells.

The different gene expression profiles by DBP and 17β-estradiol in this study suggest that DBP may be an endocrine disrupting activity with the different mechanism of gene expression pattern from 17β-estradiol. From the results, it showed some differences of gene expression patterns between MCF7 cells treated with 17β-estradiol and DBP, and also DBP shows estrogenic potential with changes in gene expression levels.

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