CLASSIFICATION OF ESTROGENIC COMPOUNDS BY HUMAN cDNA MICROARRAY GENE EXPRESSION PATTERNS

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Introduction

Endocrine disrupting chemicals have been intensively studied in order to understand the molecular mode of action, various effects and endpoints, and in vitro, in vivo, and in silico test/screening methods[1)]. National endocrine disruptor screening programs are mainly focusing on estrogenic, androgenic, and thyroid hormone effects. In this study, a human cDNA microarray, consisting of 203 genes including estrogen-responsive genes, was tested in order to examine whether this technology enables us to classify estrogenic chemicals. Screening the estrogenic chemicals is feasible using this technology.

Methods and Materials

Several persistent organic and estrogenic compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxins (TCDD), 17β-estradiol (E2), diethylstilbestrol (DES), and 4-n-nonylphenol (NP) were chosen as test chemicals. TCDD, E2, DES, and NP were purchased from Cambridge Isotope Laboratory (USA), Kanto Chemical (Japan), ICN Biomedical (USA), and Dr.Ehrenstorfer GmbH (Germany), respectively.

Most chemicals were tested for cell proliferation before DNA microarray analysis in order to determine concentrations added to cell dishes. MCF-7 cells were distributed to each well (3,000 cells/well) of 96-well plates and allowed to adhere for several hours before the test chemicals were added. TetraColor ONE (Seikagaku, Japan) or Cell Counting Kit-8 (Donjindo, Japan) was used to count the numbers of cells three days after the test chemicals were added. At least 6 wells in a 96-well plate were used for each concentration of the test chemicals in order to check reproducibility.

EstrArray, a human cDNA microarray, was purchased from InfoGenes Co., Ltd. (Japan) The cDNA microarray contains 175 estrogen-responsive genes including and 28 control genes. The estrogen-responsive genes were mainly selected as a result of gene expression profiles using a

Human UniGEM v 2.0 microarray (IncyteGenomics, USA) consisting of 9128 human cDNA clones. Details are published elsewhere[2]. All experiments were performed using the stock cells cultured within 15 passages. The cells were maintained in RPMI 1640 medium 1 (Nissui, Japan) with phenol red and 10% fetal bovine serum (FBS) for ten days, and further maintained in phenol red free RPMI 1640 medium (Sigma, R-8755) containing 10% FBS treated with dextran-coated charcoal (DCC-FBS) for ten days. L-Glutamine (300 wt-ppm) and sodium bicarbonate (750 wt-ppm) were added to both media. The cells were kept in a CO_2 incubator at 37 ºC and 5.0% CO_2. Each chemical dissolved in dimethyl sulfoxide (DMSO) or DMSO only as a control was added to three to ten cell dishes depending on the cell's response to chemicals, and mRNA was isolated using a commercially available mRNA isolation kit (PolyAtract, Promega). The isolated mRNA was further purified by isopropyl alcohol precipitation. Estrogen response was confirmed by gel electrophoresis after reverse transcription-polymerase chain reaction (RT-PCR) for trefoil factor 1 or pS2 (up-regulated), estrogen receptor 1 or ERα (down-regulated), and β-actin (control). In order to obtain microarray expression patterns, Cyanine 3 (Cy3) and Cyanine 5 (Cy5)-labaled cDNA were prepared and hybridized to EstrArray overnight at 65 ºC. The procedure is summarized in Fig.1.

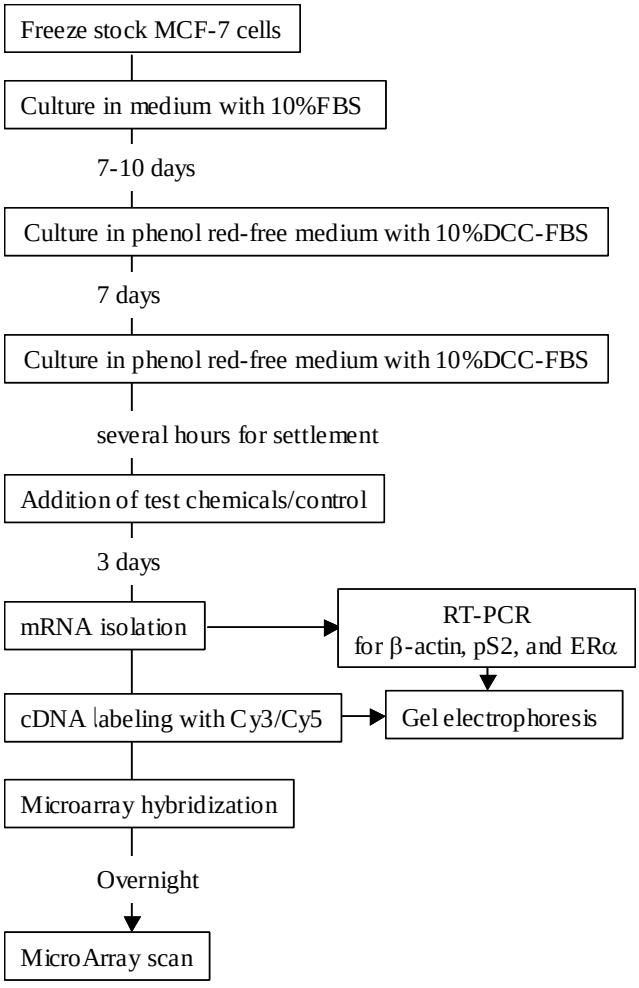

Fig.1 Standard Operating Procedure

Results and Discussion

The cell proliferation assays for E2, DES, and NP were performed at concentrations of 0 (medium), 0 (DMSO), 1, 10, 100, 1,000, 10,000 nM. Growth inhibition curves of E2, DES, and NP are described in Fig.2. DES and NP up to 10nM apparently promoted proliferation of MCF-7 cells. Threshold concentrations of the growth inhibition curves of E2 (10nM), NP (100nM), and DES (100nM) were added to MCF-7 cells, respectively, and mRNA samples were isolated from the cells after three days and used for microarray analysis.

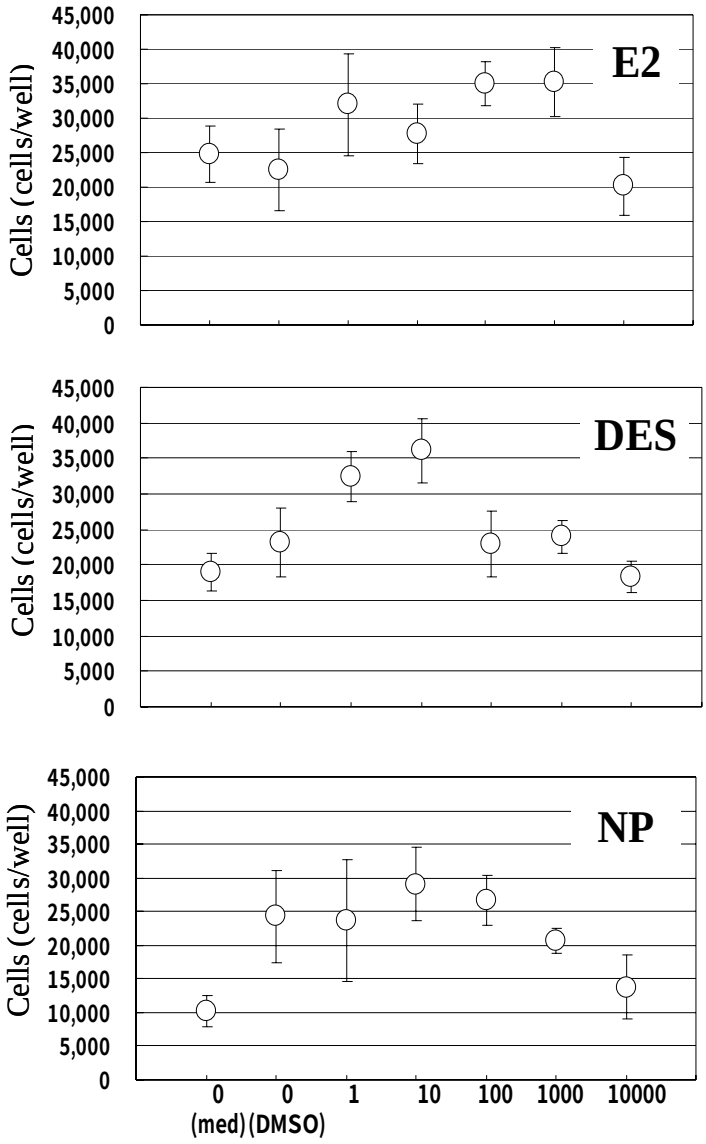

Fig.2 Cell Proliferation Assay of E2, DES, and NP using MCF-7 Cells

The purity of mRNA isolated from MCF-7 cells was confirmed by light absorbance ratios at 260nm and 280nm. The amount of mRNA was calculated by (Absorbance Reading at A260) x (40mg RNA/mL). Each mRNA sample was tested for estrogen responses of up-regulated pS2 and down-regulated ERα by gel electrophoresis after RT-PCR. The isolated mRNA samples (2μg/sample) from the test chemicals and controls were reverse-transcribed with Cy3-dUTP and Cy5-dUTP, respectively. Gel electrophoresis of the resulting solutions indicated the presence of

Cy3- and Cy5-labeled cDNA.

Gene expression profiles were obtained as the ratios of the intensities between Cy3 and Cy5 signals, and log_2(Cy3/Cy5) values were calculated to see the effects of chemicals on gene expression. Figure3 shows a gene expression pattern in response to DES arranged in order of E2-responsiveness. These patterns with other chemicals are obtained for building a database in order to classify chemicals by E2-responsiveness. Environmental samples could be also tested for E2-responsiveness by this method. Cluster analysis of these patterns was performed to classify the effects of the chemicals used in this study. We examined statistical significance and reproducibility of the data.

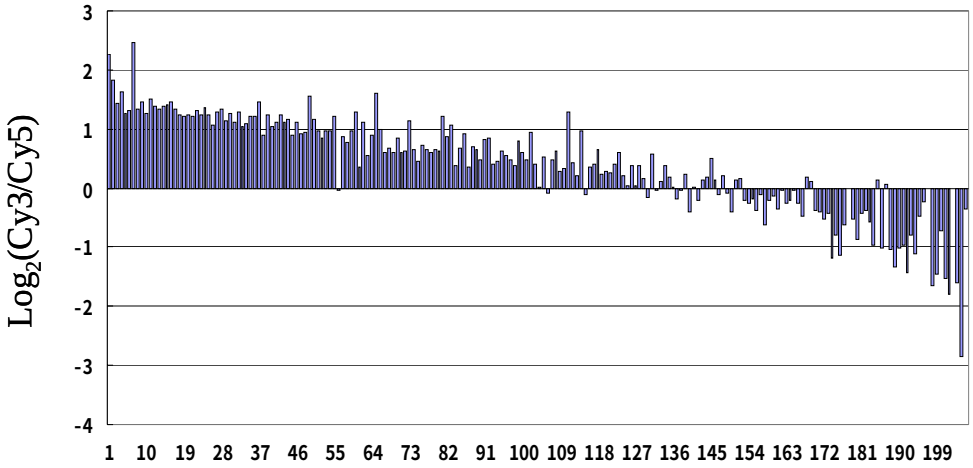

Fig.3 Gene Expression Pattern in MCF-7 Cells Using EstrArray in Response to DES Shown in Order of E2-Responsiveness

Acknowledgements

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References

1) Internatinoal Symposium on Endocrine Active Substances and Supplementary Workshop, SCOPE/IUPAC, Project on Environmental Implications of Endocrine Active Substances: Present State of the Art and Future Research Needs, November 17-21, 2002, Yokohama, Japan.

2) Inoue A., Yoshida N., Omoto Y., Oguchi S., Yamori T., Kiyma R. and Hayashi S. (2002) Journal of Molecular Endocrinology 29, 175-192.