Effects of the Flame Retardants, Polybrominated Diphenyl Ethers, on Human Umbilical Vein Endothelial Cells using DNA microarray analysis

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Abstract

Polybrominated diphenyl ethers (PBDEs) are used as flame retardants in consumer products. The level of PBDEs has increased in humans and wildlife. Since fetuses and infants are more sensitive to a variety of environmental toxicants than adults, it is feared that the effect of exposure to PBDEs on fetuses will be more severe. In this study, the effects of penta-BDE (DE71), octa-BDE (DE79), and deca-BDE (DE83) on human umbilical vein endothelial cells (HUVECs) were investigated using DNA microarray analysis and real-time RT-PCR analysis. Hierarchical cluster analysis of gene expression revealed that the specific gene expression pattern in HUVECs was similar on exposure to both DE71 (40 μM) and DE79 (40 μM). Moreover, exposure to DE71 and DE79 up-regulated the breast cancer resistance protein (*ABCG2*) expression. α-NF partially antagonized the *ABCG2* expression induced by DE71 and DE 79. Therefore, it was suggested that DE71 and DE79 had similar effects on gene expression in HUVECs, and that exposure to PBDEs, particularly DE71 and DE79, might cause detrimental effects similar to those caused by tetrachlorodibenzo-*p*-dioxin (TCDD) on the growth and development of fetuses.

Introduction

Polybrominated diphenyl ethers (PBDEs) are used as flame retardants in consumer products, such as electronics, construction materials, and textiles. The use of PBDEs has increased over the years[1]. PBDEs have been marketed as penta-, octa-, and deca- brominated mixtures[1]. They are structurally similar to polychlorinated biphenyls (PCBs) and thyroid hormones, PBDEs have the propensity to disrupt thyroid hormones and cause neurobehavioral deficits[2]. The level of PBDEs has increased in human and wildlife. In humans in particular, PBDEs were detected in blood serum, adipose tissue, breast milk, and umbilical cord serum[3,4,5]. It was suggested that fetuses might be exposed to PBDEs. Fetuses and infants are significantly more sensitive to a variety of environmental toxicants than adults[6,7]. Hence, we are investigating the effects of exposure to chemicals on fetuses by using umbilical cord tissues that were part of fetus tissues[8].

The purpose of this study is to identify the effect of PBDEs on human umbilical vein endothelial cells (HUVECs) by using DNA microarray analysis and real-time RT-PCR. Cultured HUVECs were exposed to penta- BDE (DE71), octa-BDE (DE79), and deca-BDE (DE83). The results of DNA microarray analysis indicated a difference in the expression pattern of genes on exposure to DE71, DE79, and DE83. The expression of the breast cancer resistance protein (*ABCG2*), which was confirmed by real-time RT-PCR analysis, was increased by DE71 and DE79 through the aryl hydrocarbon receptor (AhR)-mediated pathway.

Materials and Methods

Materials

Commercial mixtures of PBDEs (DE71[lot 05500F16P], DE79[lot 0525D108A], and DE83[lot 9480DA14A]) were purchased from Wellington Laboratories Inc. (Ontario, Canada). According to the manufacturer, the

percentage composition of homologous groups in DE71 is tri-congener 0.4%, tetra-congener 34.8%, penta-congener 55.9%, hexa-congener 8.9%, and hepta-congener 0.2%; that in DE79 is penta-congener 0.1%, hexa-congener 7.9%, hepta-congener 39.9%, octa-congener 35.7%, nona-congener 14.7%, and deca-congener 1.8%; and that in DE83 is nona-congener 3.1%, and deca-congener 96.9%. Dimethyl sulfoxide (DMSO) and α-naphthoflavone (α-NF) were purchased from Wako (Osaka, Japan).

Cell culture and treatment.
HUVECs were purchased from Cell Applications, Inc. (San Diego, USA). Cells were grown in medium 199, which was a phenol red-free medium, containing 20% charcoal/dextran-treated fetal bovine serum (HyClone, USA) at 37℃ in air containing 5% CO_2. HUVECs were grown to become subconfluence and incubated for 24, 48, and 72 h in a medium containing 4 and 40 μM DE71, DE79, and DE83, respectively. PBDEs were dissolved in DMSO and then added to the medium. The final concentration of DMSO in each sample didn't exceed 0.5% (vol/vol). In experiments using an antagonist, cultured cells were pre-incubated with 10 μM α-NF for 1 h and the incubation medium was changed to a medium containing 40 μM of PBDEs and 10 μM α-NF for 48 h.

DNA microarray analysis.
The cultured HUVECs were exposed to 4 and 40 μM PBDEs for 48 h. RNA was prepared using RNeasy Mini Kit (QIAGEN KK, Tokyo Japan) according to the manufacturer's protocol. Triplicate samples were assayed with using a Gene Chip® (HG-U133 plus 2.0 array, Affymetrix) containing 54687 probes. Data analyses were carried out using ArrayAssist® (MediBIC Tokyo Japan). The expression values were normalized and log-transformed. We compared the average values of the triplicate data of PBDEs and DMSO. Only probes with an expression level of more than 100 were selected; future, probes with expression levels more than 2-fold or 0.5-fold of the control samples were selected.

Real-time RT-PCR analysis.
The expression of each gene whose expression level was shown to be altered in DNA microarray analysis was confirmed using real-time RT-PCR (DNA Engine Opticon; MJ Research Inc., Cambridge, MA). Reverse transcription was performed with QuantiTect Reverse Transcription Kit (QIAGEN KK) according to the manufacturer's protocol. Real-time RT-PCR experiments were performed using SYBR® Green Realtime PCR Master Mix (TOYOBO CO., Japan). Real-time RT-PCR was carried out in triplicate. Each mRNA level was normalized to that of the housekeeping gene *β*-actin in each group.

Statistical analysis.
Data are expressed as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Dunnett's test was used to compare the difference between the groups. Statistical significance was achieved at a value of P value < 0.05. Unpaired Student's *t*-test was used to compare the difference between the samples of the α-NF treatment and the non α-NF treatment in the experiment using an antagonist.

Results and Discussion

In DNA microarray analysis, genes that met the abovementioned criteria (Materials and Methods) were used. The gene expressions on exposure to PBDEs were compared with the expressions on exposure to DMSO. In exposure to 40 μM of each PBDE, 5 genes were found to be up-regulated and 33 down-regulated. On the other hand, the expression of no gene was affected in exposure to 4 μM of PBDEs. Moreover, 4 genes among the down-regulated genes were the same as those used for DE71 and DE79. Hierarchical clustering analysis revealed 2 major clusters (Fig. 1). One cluster comprised genes whose expressions were changed by 40 μM DE71 and DE79, and another cluster comprised those whose regulations were affected by 40 μM DE83, 4 μM of each PBDE, and DMSO. Hierarchical cluster analysis revealed that specific gene expression pattern in HUVECs was similar between exposure to DE71 and DE79. Deca-BDE congener is poorly absorbed, rapidly eliminated, and does not bioaccumulate[2]. Additionally, a recent study showed that polybrominated biphenyls (PBBs) concentration of DE83 was clearly lower than that of DE71 and DE79; on the contrary, polybrominated dibenzofurans (PBDFs) concentration of DE83 was higher than that of others[1]. Therefore, it was suggested that

the effects of DE71 and DE79 were similar on gene expression in HUVECs and were different from those of DE83.

Each gene which was altered in DNA microarray analysis was confirmed their expressions by real-time RT-PCR analysis. The results obtained for some genes were similar to those obtained by the DNA microarray analysis. One of the genes confirmed by real-time RT-PCR analysis included the breast cancer resistance protein (*ABCG2*) (AF098951). *ABCG2* belongs to the ATP-binding cassette transporter[9]. It has an important role in barrier protection and transports cytotoxic agents. In normal human tissues, high levels of *ABCG2* expression are found in the colon, small intestine, placenta, liver, and endothelial cells of the vein; *ABCG2* is localized to the apical membrane[10]. In this study, although gene expression was not altered by *ABCG2* at 24 h, it was significantly up-regulated by 40μ M DE71 and DE79 at 48 h (Fig. 2A). *ABCG2* expression was increased to approximately 1.5- and 2-fold on exposure to 40 μM DE71 and DE79, respectively when compared to that in the control. A recent report suggested that overexpressed *ABCG2* may export a substance important for the growth or survival of committed lineages[11]. In this study, with regard to the up-regulation of ABCG2 by DE71 and DE79, it was inferred that DE71 and DE79 might affect the growth and development of fetuses.

To identify the pathway through which DE71 and DE79 increased the mRNA level of *ABCG2*, an antagonist, namely, α-NF (AhR antagonist), was used. Cultured cells were pre-incubated with 10 μM α-NF for 1 h and the incubation medium was changed to a medium containing 40 μM of PBDEs and 10 μM α-NF for 48 h. α-NF antagonized the *ABCG2* expression induced by DE71 but partially antagonized that indicated by DE79 (Fig. 2B). It was reported that the *ABCG2* expression was up-regulated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and the *ABCG2* expression up-regulated by indolo[3,2-*b*]carbazole (ICZ) AhR agonists was inhibited by the AhR antagonist PD98059[12]. In this study, it was identified that DE71 and DE79 up-regulated *ABCG2* expression through the AhR-mediated pathway, and DE71 and DE79 may have effects similar to that of TCDD.

In summary, our results indicate that DE71 and DE79 had similar effects on gene expression in HUVECs, they may up-regulate *ABCG2* expression through the AhR-mediated pathway. Thus, our findings suggest that exposure to PBDEs, particularly DE71 and DE79, might cause detrimental effects similar to those caused by TCDD on the growth and development of fetuses.

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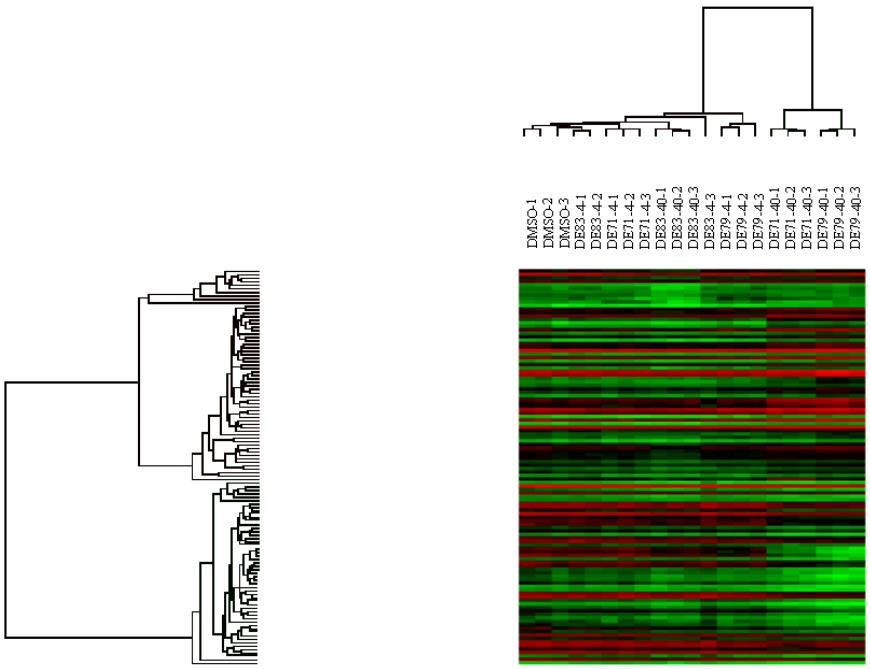

Fig. 1. Hierarchical cluster analysis.
HUVECs were incubated with 4 or 40 μM each PBDE or vehicle (0.5% DMSO) for 48 h (n = 3). Genes found to be significantly different by ANOVA ($p < 0.01$) were analyzed in hierarchical cluster analysis. Each row represents a gene and each column represents a sample. Red and green indicate increased and decreased expression, respectively.

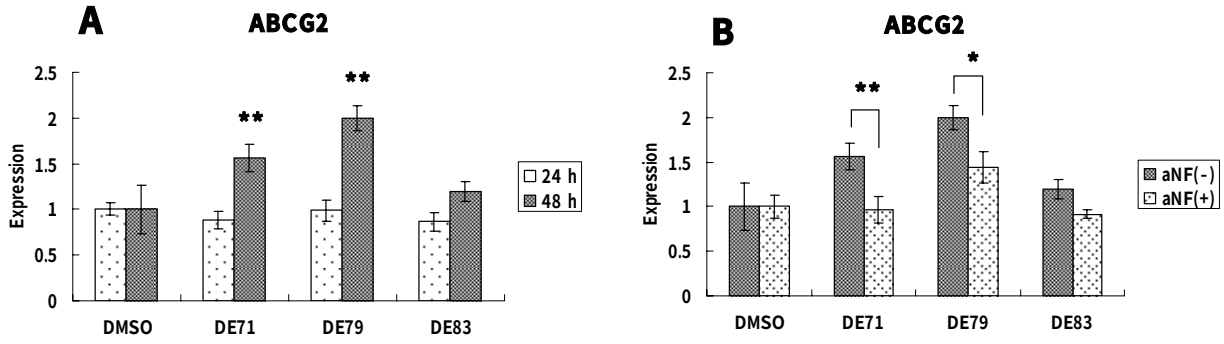

Fig. 2. The expression of ABCG2 by real-time RT-PCR analysis.
Real-time RT-PCR was carried out in triplicate. Each mRNA level was normalized to that of the housekeeping gene *β*-actin in each group. Gene expression ratios were altered on exposure to DMSO. Data are expressed as mean ± standard deviation (S.D.). (A) Gene expression of ABCG2. HUVECs were incubated with 40 μM each PBDE or vehicle (0.5% DMSO) for 24 and 48 h (n = 3). Asterisk indicates statistically significant differences compared with control (**$p < 0.01$; one-way ANOVA, Dunnett's post-test). (B) Antagonism by α-NF of ABCG2 in HUVECs. The cultured cells were preincubated with 10 μM α-NF for 1 h and the incubation medium was changed to a medium containing 40 μM of PBDEs and 10 μM of α-NF for 48 h. Asterisk indicates statistically significant differences between the samples treated with α-NF and those not treated with α-NF (*$p < 0.05$, **$p < 0.01$; unpaired Student's *t*-test).