Effects of dioxin and nutrition on cellular proliferation and dioxin- and estrogen-linked genes in ovarian cancer cell lines

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Summay

This study was undertaken to examine the effects of dioxin (TCDD) and nutrition on cellular proliferation and dioxin- and estrogen-linked gene expression in ovarian cancer cell lines. Caov-3 and SK-OV-3 cells were incubated in a medium supplemented with 0.5-10% fetal bovine serum (FBS). Cell proliferation was assayed with an MTT assay. Dioxin- and estrogen-linked genes (AhR, ERα, ERβ, CYP1A1 and ARNT) expressed were determined with the RT-PCR method. Caov-3 cells, but not SK-OV-3 cells, were proliferated with TCDD alone with increased AhR and ERa mRNA expressions when incubated in the low FBS concentration. CYP1A1 and ARNT mRNA expressions of SK-OV-3, but not that of Caov-3, were suppressed in the low FBS (under 1.0%) concentration.

In the low FBS concentration medium with dioxin, AhR and ERa expression were increased with the proliferation of Caov-3 cells; CYP1A1 and ARNT were stable. Each ovarian cancer cell line may have its own distinct responsiveness to dioxin depending on the nutritional state.

Key words: Dioxin; Aryl hydrocarbon receptor; Estrogen receptor; CYP1A1; Fetal bovine serum.

Introduction

Chemical pollutants, including a halogenated aromatic hydrocarbon, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), have been recognized as disruptors to the well-being of all living organisms. The incidence of pelvic endometriosis in rhesus monkeys has increased with dioxin exposure [1], and the blood concentrations of dioxin are higher in women with pelvic endometriosis [2]. This suggests the adverse effects of environmental compounds on endometriosis growth. A great deal of attention has focused on the possibility that TCDD could disrupt estrogen homeostasis and be carcinogenic.

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor and forms a fuctional heterodimer with AhR nuclear translocator (ARNT). TCDD binds AhR with high affinity and AhR-mediated biochemical and the toxic responses of TCDD have been extensively investigated [3].

Cytochrome P450 (CYP) is one of the superfamily of enzymes which catalyze various endogenous and exogenous substances including toxicants, carcinogens and drugs. Also the CYP1 family catalyzes carcinogens and is induced by polycyclic aromatic hydrocarbons, including TCDD, mainly mediated by AhR and ARNT. There are three genes encoding the enzymes; CYP1A1, CYP1A2, and CYP1B1 [4], some of which are related to estrogen metabolism.

There is evidence for cross-talk of signaling between ER- and AhR-regulation. ER-AhR crosstalk alters estrogen metabolism [5] and has inhibitory effects on estrogen-related gene expression [3].

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While estrogen regulates TCDD responsiveness in cell lines [6, 7], the effects of the culture medium concentration, a supplier of nutrition, were determined on the cellular proliferation and dioxin- and estrogen-linked regulatory gene expression of ovarian cancer cell lines.

Materials and Methods

Materials

2,3,7,8-tetrachlorodibenzo-p dioxin (lot# A8100095) was purchased from AccuStandard Inc. (New Haven, CT). SK-OV-3 (from adenocarcinoma of ovary) was purchased from ATCC (Manassas, VA) and maintained in McCoy's 5a medium with 1.5 mM L-glutamine, 90%; fetal bovine serum (FBS), 10%. Caov-3 (from adenocarcinoma of ovary) was purchased from ATCC and maintenanced in Dulbecco's modified Eagle's medium with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 90% and fetal bovine serum, 10%.

MTT assay for cell proliferation

Quantification of cell proliferation was performed via the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (blue product), which indicates mitochondrial succinate-dehydrogenase activity. The cell concentration was 5,000 cells/ml. The cell suspension (100 µl per well) was inoculated and cultured in 96 well plates. Various concentrations of TCDD and a solvent vehicle with 0.1% (v/v) DMSO and FBS were added to each well and incubated for 72 hours. Cells were washed twice with medium. One hundred µl of MTT solution (1 mg/ml 1M PBS, pH 7.6) was added to the wells and allowed to incubate for 3h at 37°C in 5% CO_2/95% air. The cells were washed and 100 µl of isopropanol added. The plates were shaken for 10 min at room temperature to thoroughly dissolved the formazan. The density was measured using a microplate reader at 570 nm.

RNA extraction and reverse transcription-polymerase chain reaction amplification

AhR mRNA, ERα mRNA, ERβ mRNA, CYP1A1 mRNA and ARNT mRNA expressions at various FBS concentrations were measured with RT-PCR. After cells were incubated for 72 hours at 0.5, 1.0, and 10% FBS concentrations, total RNA was extracted from cell lines using the guanidine isothiocyanate/acid phenol method [8]. Each mRNA density was quantified as previously described [9]. A random primed complementary DNA (cDNA) library was made from 5 μg total RNA using Moloney murine leukemia reverse transcriptase under the conditions recommended by the supplier. The reaction product was heat-denatured at 95°C for 5 min, and quickly chilled on ice. cDNA (1 μl) was amplified in a 50 μl reaction volume containing 100 mmol/l Tris-HCl (pH 8.3), 500 mmol/l KCl, 15 mmol/l $MgCl_2$, and 2.5 mmol/l each deoxynucleotide triphosphate, 5 U/μl Taq polymerase, and 0.5 μmol/l primers. In each step, linearity of amplifications was checked with a dilution series.

The sequences of oligonucleotide primers are designed according to the published sequences of human AhR, ERa, ERb, CYP1A1, ARNT and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) which used as an internal standard.

AhR primer (180 bp), 5'-ATACTGAAGCAGAGCTGTGC-3' (sense); 5'-AAAGCAGGCGTGCATTAGAC-3' (antisense): ERa primer (309bp), 5'-ACAAGGGAAGTATGGCTATG-3'(sense); 5'-CATCTCTCTGGCGCTTGTGT-3' (antisense): ERb primer (293bp), 5'-TGTTACTGGTCCAGGTTCAA-3' (sense); 5'-TTCTCTGTCTCCGCACAAGG-3' (antisense): CYP1A1 (367bp), 5'-TTCCGACACTCTTCCTTCGT-3'(sense), 5'-ATGGTTAGCCCATAGATGGG-3'(antisense): ARNT (238bp), 5'-CCCTAGTCTCACCAATCGTGGATC-3'(sense); 5'-GTAGCTGTTTTGCTCTGATCTCCCAG-3'(antisense): GAPDH primer (983 bp), 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' (sense); 5'-CATGTGGGCCATGAGGTCCACCAC-3' (antisense).

Thirty-five cycles of amplification were carried out, with each cycle consisting of denaturation at 90°C for 45 sec, annealing at 55°C for 45 sec, and extension at 72°C for 90 sec, followed by a final extension for 5 min at 72°C. The DNA product (10 μl) was electrophoresed on 2% agarose gel, and bands were visualized using an UV transilluminator after ethidium bromide staining. Expression levels were corrected by GAPDH. The sequence of the PCR products was analyzed and confirmed manually by the dideoxy chain termination method [10] using Sequenase II, according to the manufacturer's protocol.

Statistics

For statistical analysis, the Student's t-test was used. Differences were considered to be significant if p was less than 0.05.

Result

Effects of TCDD on cellular proliferation at various FBS concentrations

In the low FBS (0.5%) condition, cellular proliferation of Caov-3 cells was increased by TCDD in a range from 20 to 200 pM, but not at the 10% concentration. SK-OV-3 cells did not show any effects from the TCDD doses determined (Figure 1).

AhR mRNA expression at various FBS concentrations

Cells were incubated with three concentrations of FBS (0.5 - 10%) for 72 hours. AhR mRNA expression at the low FBS concentration was increased in Caov-3 cells, but not in SK-OV-3 cells (Figure 2).

ERα mRNA expression at various FBS concentrations

ERα mRNA expression in Caov-3 cells was increased at the low FBS concentrations after 72 hours' incubation, while that in SK-OV-3 decreased (Figure 3).

ERβ mRNA expression at various FBS concentrations

ERβ mRNA expression in Caov-3 and SK-OV-3 cells was unchanged in the low FBS conditions after 72 hours' incubation (Figure 4).

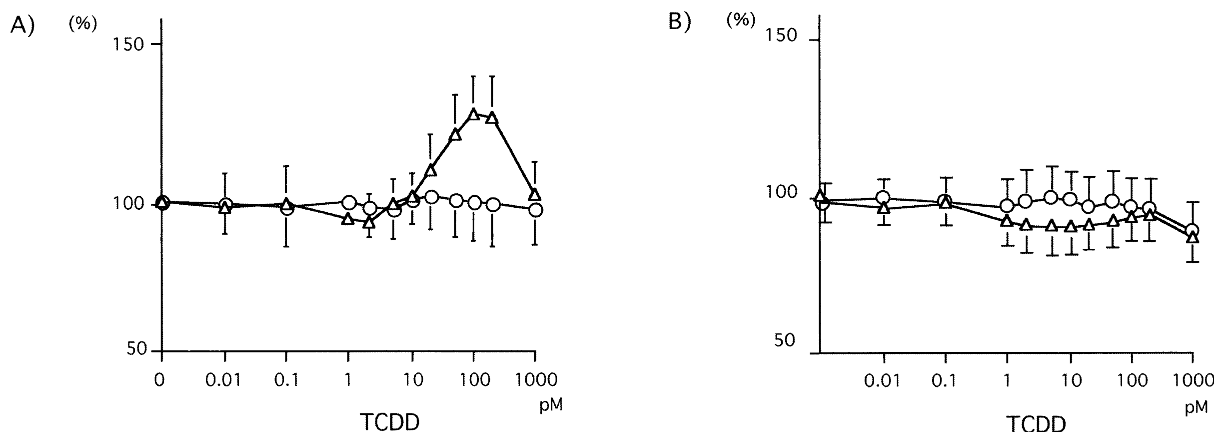

Figure 1. — Effects of dioxin (TCDD) on the proliferation of A) Caov-3 and B) SK-OV-3 in various FBS concentrations assayed with MTTassay. Cells were incubated in two FBS concentration groups (10% FBS; open circles and 0.5% FBS; open triangles) for 72 hours prior to TCDD inoculation. After treatment for 72 hours with various concentrations of TCDD or with the solvent vehicle, 0.1% (v/v) DMSO, each well was inoculated with MTT solution and incubated for three hours. Formazan formation was quantitated with a microplate reader at 570 nm.
MMT,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TCDD,2,3,7,8-tetrachlorodibenzo-p-dioxin; FBS, fetal bovine serum; DMSO,dimethyl sulfoxide.

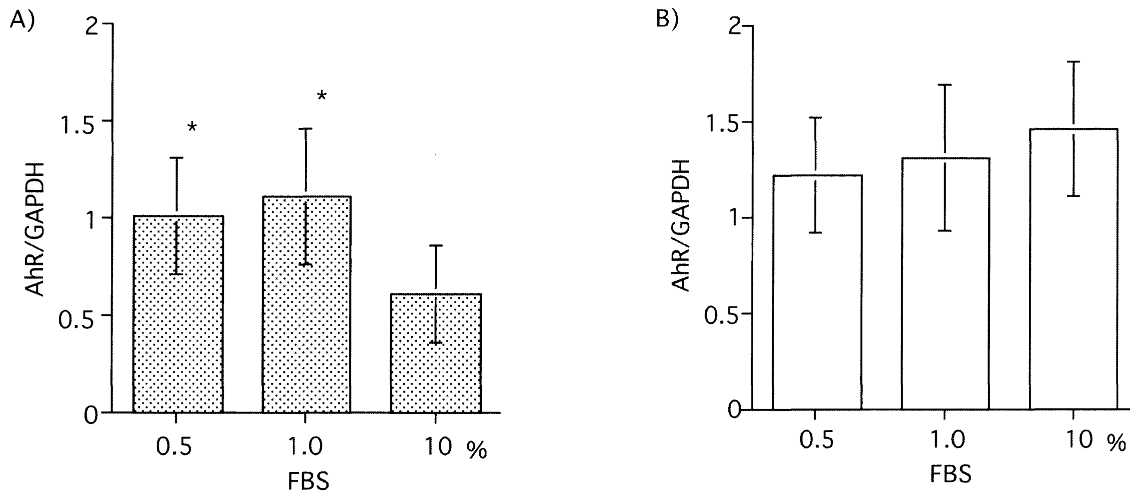

Figure 2. — Effects of FBS concentrations added to the medium on AhR mRNA expression in A) Caov-3 and B) SK-OV-3. Cells were incubated in the medium with various concentrations of FBS (0.5-10%) for 72 hours. Total RNA was extracted from cells and mRNA was quantitated with the RT-PCR method. Expression levels were corrected by GAPDH. Significant differences from 10% FBS, *$p < 0.05$. AhR, aryl hydrocarbon receptor.

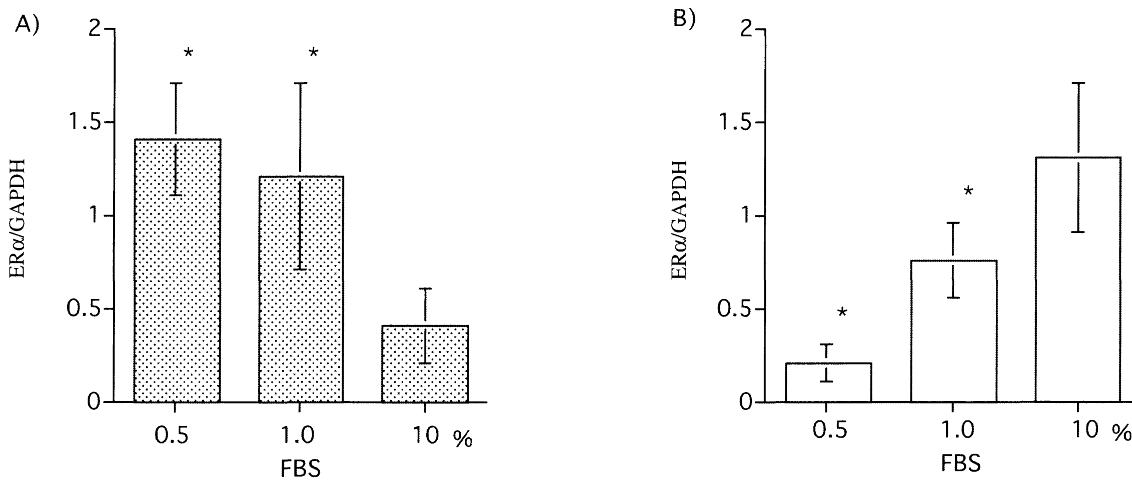

Figure 3. — Effects of FBS concentrations added to the medium on ERa mRNA expression in A) Caov-3 and B) SK-OV-3. Cells were incubated in the medium with various concentrations of FBS (0.5-10%) for 72 hours. Total RNA was extracted from cells and mRNA was quantitated with the RT-PCR method. Expression levels were corrected by GAPDH. Significant differences from 10% FBS, *$p < 0.05$. ER, estrogen receptor.

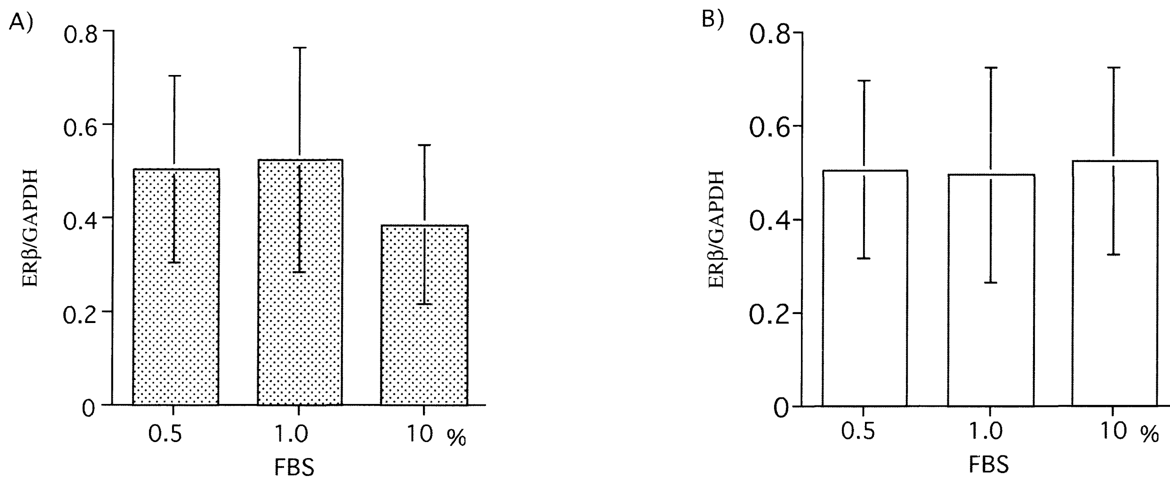

Figure 4. — Effects of FBS concentrations added to the medium on ERβ mRNA expression in A) Caov-3 and B) SK-OV-3. Cells were incubated in the medium with various concentrations of FBS (0.5-10%) for 72 hours. Total RNA was extracted from cells and mRNA was quantitated with the RT-PCR method. Expression levels were corrected by GAPDH. ER, estrogen receptor.

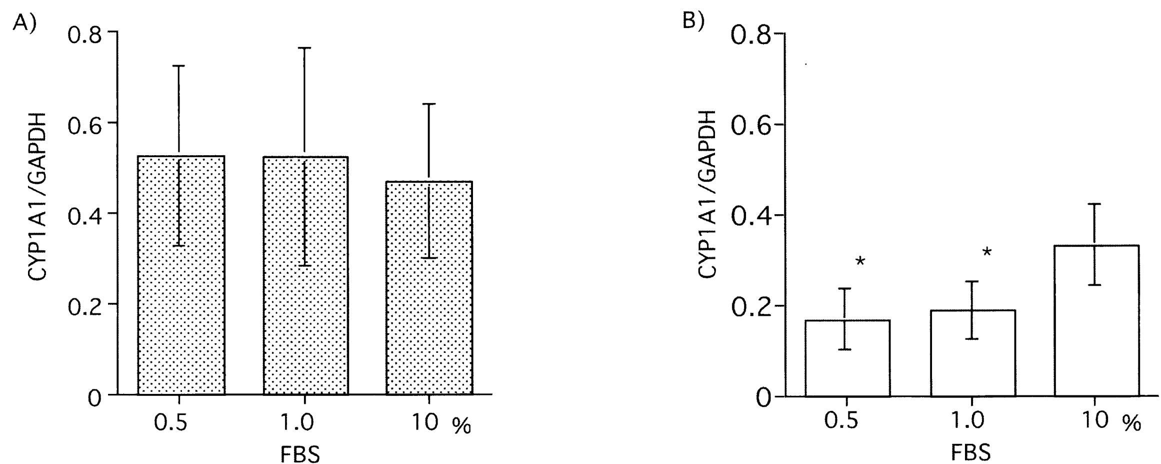

Figure 5. — Effects of FBS concentrations added to the medium on CYP1A1 mRNA expression in A) Caov-3 and B) SK-OV-3. Cells were incubated in the medium with various concentrations of FBS (0.5-10%) for 72 hours. Total RNA was extracted from cells and mRNA was quantitated with the RT-PCR method. Expression levels were corrected by GAPDH.
Significant differences from 10% FBS, *$p < 0.05$. CYP, Cytochrome P450.

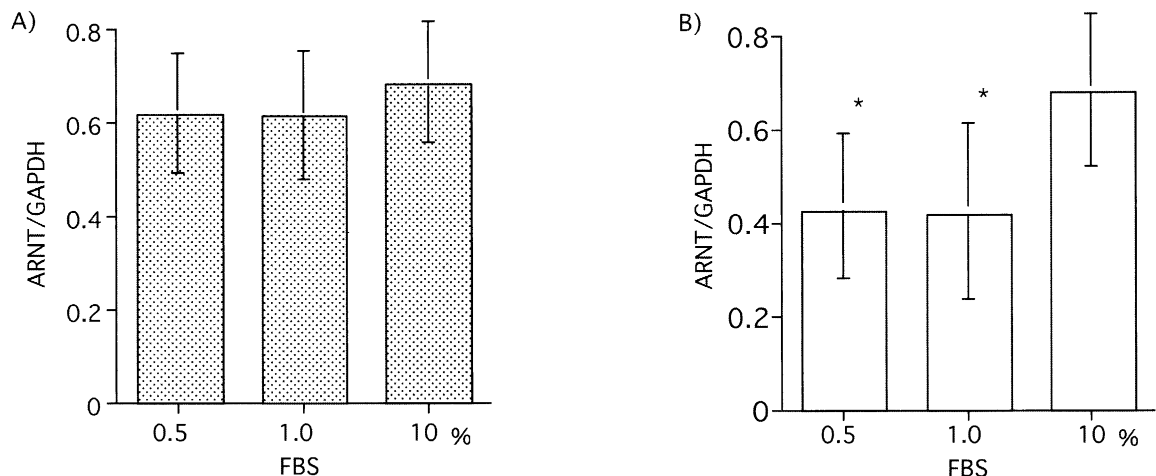

Figure 6. — Effects of FBS concentrations added to the medium on ARNT mRNA expression in A) Caov-3 and B) SK-OV-3. Cells were incubated in medium with various concentrations of FBS (0.5-10%) for 72 hours. Total RNA was extracted from cells and mRNA was quantitated with the RT-PCR method. Expression levels were corrected by GAPDH.
Significant differences from 10% FBS, *$p < 0.05$. ARNT, AhR nuclear translocator.

CYP1A1 mRNA expression at various FBS concentrations

CYP1A1 mRNA expression in SK-OV-3 cells was decreased at the low FBS concentrations, while that in Caov-3 cells was stable at the various FBS concentrations determined (Figure 5).

ARNT mRNA expression at various FBS concentrations

ARNT mRNA expression in SK-OV-3 cells was decreased in the low FBS concentrations, but that in Caov-3 cells was stable at the various FBS concentrations determined (Figure 6).

Discussion

Ovarian cancer is one of the leading causes of female cancer deaths, and the incidence is increasing [11]. Also endometriosis is commonly detected. The relationship between endometriosis and ovarian cancer is found especially in clear cell carcinoma and endometrioid carcinomas. Most ovarian cancers have estrogen receptors, suggesting that estrogen is one of the risk factors.

TCDD is reported to be toxic and mutagenic for endometriosis [1], but anti-estrogenic. The present study, therefore, investigated the effect of TCDD on the cellular proliferation of some ovarian cancer cell lines, focusing

on the effects of various concentrations of FBS, which is a supplier of nutrition and estrogen. When cells were incubated in 10% FBS, TCDD had almost no effect on the proliferation of either cell line. However, at the low 0.5% FBS concentration, Caov-3 alone significantly proliferated in the concentration range from 20 to 200 pM TCDD. The higher TCDD concentration of 1000 pM seemed to be toxic to the cells.

Some substances, such as phorbal esters [12] and transforming growth factor-β 1 [13] are reported to have cell-specific effects through AhR expression. The AhR is expressed under complicated regulations, although elevated AhR expression is apparently associated with rapid cell growth [14]. In a rat experiment, AhR expression was upregulated in polychloric aromatic hydrocarbons (PAHs)-induced mammary cancer, and in humans, the over-expression of AhR has been reported in pancreatic cancers. Thus, elevated AhR expression might be a marker of the carcinogenic process [15]. Estrogen is a growth factor for many tissues and cells, especially in female genital organs such as the endometrium and also the mammary glands. Also, estrogen is thought to be a major risk factor for uterine endometrial and breast cancers. AhR upregulation was reported in the endometrium of postmenopausal women administered estrogen [16].

AhR mRNA expression of Caov-3 cells was significantly activated at a low FBS concentration (Figure 2), which might be responsive to TCDD. SK-OV-3 cells showed no change in either AhR mRNA expression or cell proliferation.

Cross-talk of signaling between estrogen receptor a (ERa) and AhR in breast and endometrial cells has been reported [17-20]. TCDD has been shown to decrease estrogen-stimulated tumor growth and cell proliferation, as well as secretion of tissue plasminogen activator, in cell cultures [21-23].

Both Caov-3 and SK-OV-3 showed no marked change in expression of ERβ, but the ERα mRNA expression of Caov-3 was increased at the low FBS concentration and decreased in SK-OV-3 in a FBS dose-dependent manner. The increasse in ERα in Caov-6 cells might be related to the TCDD responsiveness to cellular proliferation at the low FBS concentration; at the low FBS condition, the increase in both AhR and ERα was cooperative for cellular proliferation by TCDD under starvation. On the other hand, SK-OV-3 did not have such cooperation under starvation.

The human CYP1 family acts in estradiol catalyzation [24, 25], and is associated with carcinogenesis [26-29]. CYP1A1 and CYP1B1 genes are transcriptionally controlled by AhR, a TCDD-activated transcription factor, which dimerizes its partners, ARNT, AhR repressor, and hypoxia inducible factor 1α [30]. Expression of CYP1A1 and CYP1B1 is subject to regulation of AhR expression [12, 13, 31, 32]. In human breast cancer cells, a relationship between ERa expression and CYP1A1 induction has been reported [17-20]. In the present study, CYP1A1 mRNA expression of SK-OV-3, but not Caov-3, was suppressed at a low FBS concentration. Expressions of ARNT were suppressed in SK-OV-3, but not in Caov-3, at a low FBS concentration.

Cells cannot live a long time at a low FBS concentration (0.5-1.0%), i.e., a starving level. In Caov-3, increased AhR and ERα, and stationary CYP1A1 and ARNT, signal transduction systems at low FBS concentrations are responsive to TCDD, resulting in cellular proliferation.

The estrogen dependency of gynecologic tumors is a target for their treatment strategies. ER and AhR signaling pathways are cell-specific and their generation is related to nutrition.

Conclusion

TCDD responsiveness to cellular growth depends upon the cell-specificity and nutritional condition. Generation might occur with an increase in ERa and AhR and the stable condition of ARNT and CYP1A1.

References

[1] Rier S.E., Martin D.C., Bowman R.E., Dmowski W.P., Becker J.L.: "Endometriosis in rhesus monkeys (Macaca mulatta) following chronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin". *Fundam. Appl. Toxicol.*, 1993, *21*, 433.

[2] Mayani A., Barel S., Soback S., Almagor M.: "Dioxin concentrations in women with endometriosis". *Hum. Reprod.*, 1997, *12*, 373.

[3] Safe S., Wormke M., Samudio I.: "Mechanisms of inhibitory aryl hydrocarbon receptor-estrogen receptor crosstalk in human breast cancer cells". *J. Mammary Gland Biol. Neoplasia*, 2000, *5*, 295.

[4] Nelson D.R., Koymans L., Kamataki T., Stegeman J.J., Feyereisen R., Waxman D.J. *et al.*: "P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature". *Pharmacogenetics*, 1996, *6*, 1.

[5] Spink D.C., Lincoln D.W. 2nd., Dickerman H.W., Gierthy J.F.: "2,3,7,8-Tetrachlorodibenzo-p-dioxin causes an extensive alteration of 17 beta-estradiol metabolism in MCF-7 breast tumor cells". *Proc. Natl. Acad. Sci. U S A*, 1990, *87*, 6917.

[6] Spink D.C., Katz B.H., Hussain M.M., Pentecost B.T., Cao Z., Spink B.C.: "Estrogen regulates Ah responsiveness in MCF-7 breast cancer cells". *Carcinogenesis*, 2003, *24*, 1941.

[7] Rogers J.M., Denison M.S.: "Analysis of the antiestrogenic activity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in human ovarian carcinoma BG-1 cells". *Mol. Pharmacol.*, 2002, *61*, 1393.

[8] Chomczynski P., Sacchi N.: "Single-step method of RNA isolation by acid guanidium thiocyanate-chloroform extraction". *Anal. Biochem*, 1987, *162*, 156.

[9] Misao R., Itoh N., Mori H., Fujimoto J., Tamaya T.: "Sex hormone-binding globulin mRNA levels in human uterine endometrium". *Eur. J. Endocrinol.*, 1994, *131*, 623.

[10] Toneguzzo F., Glynn S., Levi E., Mjolseness S., Hayday A.: "Use of a chemically modified T7 DNA polymerase for manual and automated sequencing of supercoiled DNA". *Biotechniques*, 1988, *6*, 460.

[11] Williams S.R., Son D.S., Terranova P.F.: "Protein kinase C delta is activated in mouse ovarian surface epithelial cancer cells by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)". *Toxicology*, 2004, *195*, 1.

[12] Spink B.C., Fasco M.J., Gierthy J.F., Spink D.C.: "12-O-tetradecanoylphorbol-13-acetate upregulates the Ah receptor and differentially alters CYP1B1 and CYP1A1 expression in MCF-7 breast cancer cells". *J. Cell. Biochem.*, 1998, *70*, 289.

[13] Wolff S., Harper P.A., Wong J.M., Mostert V., Wang Y., Abel J.: "Cell-specific regulation of human aryl hydrocarbon receptor expression by transforming growth factor-beta(1)". *Mol. Pharmacol.*, 2001, *59*, 716.

[14] Vaziri C., Schneider A., Sherr D.H., Faller D.V.: "Expression of the aryl hydrocarbon receptor is regulated by serum and mitogenic growth factors in murine 3T3 fibroblasts". *J. Biol. Chem.*, 1996, *271*, 25921.
[15] Trombino A.F., Near R.I., Matulka R.A., Yang S., Hafer L.J., Toselli P.A. *et al.*: "Expression of the aryl hydrocarbon receptor/transcription factor (AhR) and AhR-regulated CYP1 gene transcripts in a rat model of mammary tumorigenesis". *Breast. Cancer Res. Treat.*, 2000, *63*, 117.
[16] Khorram O., Garthwaite M., Golos T.: "Uterine and ovarian aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT) mRNA expression in benign and malignant gynaecological conditions". *Mol. Hum. Reprod.*, 2002, *8*, 75.
[17] Thomsen J.S., Wang X., Hines R.N., Safe S.: "Restoration of aryl hydrocarbon (Ah) responsiveness in MDA-MB-231 human breast cancer cells by transient expression of the estrogen receptor". *Carcinogenesis*, 1994, *15*, 933.
[18] Spink D.C., Spink B.C., Cao J.Q., DePasquale J.A., Pentecost B.T., Fasco M.J. *et al.*: "Differential expression of CYP1A1 and CYP1B1 in human breast epithelial cells and breast tumor cells". *Carcinogenesis*, 1998, *19*, 291.
[19] Angus W.G., Larsen M.C., Jefcoate C.R.: "Expression of CYP1A1 and CYP1B1 depends on cell-specific factors in human breast cancer cell lines: role of estrogen receptor status". *Carcinogenesis*, 1999, *20*, 947.
[20] Vickers P.J., Dufresne M.J., Cowan K.H.: "Relation between cytochrome P450IA1 expression and estrogen receptor content of human breast cancer cells". *Mol. Endocrinol.*, 1989, *3*, 157.
[21] Holcomb M., Safe S.: "Inhibition of 7,12-dimethylbenzanthracene-induced rat mammary tumor growth by 2,3,7,8-tetrachlorodibenzo-p-dioxin". *Cancer Lett.*, 1994, *82*, 43.
[22] Jana N.R., Sarkar S., Ishizuka M., Yonemoto J., Tohyama C., Sone H.: "Role of estradiol receptor-alpha in differential expression of 2,3,7, 8-tetrachlorodibenzo-p-dioxin-inducible genes in the RL95-2 and KLE human endometrial cancer cell lines". *Arch. Biochem. Biophys.*, 1999, *368*, 31.
[23] Gierthy J.F., Lincoln D.W., Gillespie M.B., Seeger J.I., Martinez H.L., Dickerman H.W. *et al.*: "Suppression of estrogen-regulated extracellular tissue plasminogen activator activity of MCF-7 cells by 2,3,7,8-tetrachlorodibenzo-p-dioxin". *Cancer Res.*, 1987, *47*, 6198.
[24] Spink D.C., Eugster H.P., Lincoln D.W, 2nd., Schuetz J.D., Schuetz E.G., Johnson J.A. *et al.*: "17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1A1: a comparison of the activities induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in MCF-7 cells with those from heterologous expression of the cDNA". *Arch. Biochem. Biophys.*, 1992, *293*, 342.
[25] Hayes C.L., Spink D.C., Spink B.C., Cao J.Q., Walker N.J., Sutter T.R.: "17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1B1". *Proc. Natl. Acad. Sci. U S A*, 1996, *93*, 9776.
[26] Newbold R.R., Liehr J.G.: "Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens". *Cancer Res.*, 2000, *60*, 235.
[27] Devanesan P., Todorovic R., Zhao J., Gross M.L., Rogan E.G., Cavalieri E.L.: "Catechol estrogen conjugates and DNA adducts in the kidney of male Syrian golden hamsters treated with 4-hydroxyestradiol: potential biomarkers for estrogen-initiated cancer". *Carcinogenesis*, 2001, *22*, 489.
[28] Liehr J.G., Ricci M.J.: "4-Hydroxylation of estrogens as marker of human mammary tumors". *Proc. Natl. Acad. Sci. U S A*, 1996, *93*, 3294.
[29] Yager J.D.: "Endogenous estrogens as carcinogens through metabolic activation". *J. Natl. Cancer Inst. Monogr.*, 2000, 67.
[30] Gu Y.Z., Hogenesch J.B., Bradfield C.A.: "The PAS superfamily: sensors of environmental and developmental signals". *Annu. Rev. Pharmacol. Toxicol.*, 2000, *40*, 519.
[31] FitzGerald C.T., Fernandez-Salguero P., Gonzalez F.J., Nebert D.W., Puga A.: "Differential regulation of mouse Ah receptor gene expression in cell lines of different tissue origins". *Arch. Biochem. Biophys.*, 1996, *333*, 170.
[32] Garrison P.M., Rogers J.M., Brackney W.R., Denison M.S.: "Effects of histone deacetylase inhibitors on the Ah receptor gene promoter". *Arch. Biochem. Biophys.*, 2000, *374*, 161.

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