Cadmium inhibits forskolin-induced differentiation of human placental BeWo cells

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ABSTRACT — Cadmium (Cd) is an environmental pollutant. Blood Cd levels in pregnant women have been associated with premature births, infant birth size, placenta previa, and placenta accreta. There have been concerns on the reproductive developmental toxicity of Cd. The choriocarcinoma cell line BeWo, a cellular in vitro model for studying syncytial fusion, has been widely used to study the reproductive and developmental toxic effects of pollutants. Here, we examine the inhibitory effect of Cd against forskolin (FSK)-induced BeWo differentiation. Results showed that Cd exposure inhibited the FSK-induced expression of syncytiotrophoblast-related genes LGALS13, ERVFRD1, SDC1, and CGB3. Inhibition of LGALS13 expression was due to the inhibition of the PKA pathway, whereas the inhibition of the other three genes could be due to the inhibition of the other pathways. These findings could help clarify the reproductive and developmental toxicity of Cd.

Key words: Cadmium, Placenta, PKA pathway

INTRODUCTION

Cadmium (Cd) is a naturally occurring metal and environmental pollutant (Tokar et al., 2012). It is toxic to humans, with the major routes of exposure being tobacco smoking and consumption of rice that has taken up Cd from the soil. Prolonged consumption of foods containing high concentrations of Cd can cause renal dysfunction due to reabsorption impairment of the proximal tubule. Itai-itai disease is an example of Cd poisoning, which is caused by various factors (pregnancy, lactation, aging, nutritional deficiency, etc.) in addition to long-term intake of high concentrations of Cd. The provisional tolerable weekly intake has been set at 7 µg/kg body weight/week (Joint FAO/WHO Expert Committee on Food Additives, 2006). Cd intake is estimated to be around 35% of TDI in Japan (Ministry of the Environment, 2011) and 40%–60% in JACFA’s report (Alexander et al., 2009). Based on this information, harmful effects of Cd are not observed in people. However, the Japan Environment and Children’s Study (JECS) showed the association between whole blood concentrations of Cd in pregnant women and premature births (Tsuji et al., 2018). It was found that early preterm birth is 1.9 times more likely to occur in the high concentration group (> 0.902-ng Cd/g blood) than in the very low concentration group (< 0.497-ng Cd/g blood). The median Cd concentration in the Japanese population is 1.0–1.23 µg/L, which corresponds to 0.95–1.17 ng/g when 1.05 is used as the specific gravity of blood (Ikeda et al., 2011; Ministry of the Environment, 2011; Trudnowski and Rico, 1974). The JECS has also reported an association between blood Cd levels in pregnant women and gestational infant birth size, placenta previa, and placenta accreta (Inadera et al., 2020; Tsuji et al., 2019). Thus, there are concerns about the reproductive developmental toxicity of Cd (Kumar and Sharma, 2019). One possible reason for premature births and small infant birth size is placental hypoplasia.

The placenta, the organ that links the mother with the fetus during pregnancy, acts as the fetal respiratory, digestive, and endocrine system and protects it from the maternal immune system. It supports foetation and is formed through the proliferation and differentiation of cyto-
trophoblast cells into extravillous trophoblast (EVT) and syncytiotrophoblast (ST) cells. There are concerns that exposure to environmental pollutants may inhibit differentiation into EVT and ST, resulting in impaired placental function. The choriocarcinoma cell line BeWo, a cellular in vitro model to study syncytial fusion, has been used to clarify the effects of pollutants (Pattillo et al., 1968). BeWo cells show a low spontaneous fusion rate, which can be boosted by the addition of cAMP, its analog 8-Bromo-cAMP, or forskolin (FSK) (Wice et al., 1990). Syncytialization can be evaluated by microscopic observation of fusion, detection of released human chorionic gonadotropin (hCG), or expression of ST-related gene (Pattillo et al., 1968; Wice et al., 1990). In this study, we evaluated syncytialization by the expression of LGALS13, ERVFRD1, syndecan-1 (SDC1), and CGB3. It has been reported that LGALS13 is associated with severe preeclampsia (Balogh et al., 2011; Gadde et al., 2018). ERVFRD1, an endogenous retroviral envelope protein, is part of a human endogenous retrovirus provirus (Toufaily et al., 2015), has retained its original fusogenic properties, and participates in trophoblast fusion and syncytium formation during placenta morphogenesis. ERVFRD1 is expressed in normal placental tissue at high levels. SDC1 is a transmembrane heparan sulfate proteoglycan (Malhotra et al., 2015) that mediates cell binding, cell signaling, and cytoskeletal organization. CGB3 is a member of the glycoprotein hormone beta chain family and encodes the beta 3 subunit of CG. Glycoprotein hormones are heterodimers consisting of a common alpha subunit and a unique beta subunit that confers biological specificity. CG is produced by the trophoblastic cells of the placenta and stimulates the ovaries to synthesize the steroids that are essential for maintaining the pregnancy. Placental toxicity by numerous chemicals, such as methylmercury, bisphenol A, 2,3,7,8-tetrachlorodibenzo-p-dioxin, p-nonylphenol, perfluorobutane sulfonate, perchlorate, has been reported that the effects of Cd on BeWo include toxicology. In this study, we examined the inhibitory effect of Cd against FSK-induced BeWo differentiation.

MATERIALS AND METHODS

Cell culture and treatment
BeWo cells were purchased from the RIKEN BioResource Center Cell Bank (Tsukuba, Japan) and cultured in Ham’s F12 medium (Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France) and a mixture of antibiotic–antimycotic solution (Nacalai Tesque, Inc.) at 37°C under a 5% CO2 atmosphere. The cells were passaged every 2 or 3 days. Before cell seeding, cell viability and density were measured with trypan blue exclusion assays (Strober, 2015). Cells were seeded in a 12-well plate at a density of 5.0 × 10^4 cells/well and treated with 1.0-µM FSK (prepared from Coleus forskohlii , Calbiochem, Gibbstown, NJ, USA) and/or Cd (treated as CdCl2) at indicated concentrations after 24 hr. For the cAMP-dependent Protein Kinase (PKA) inhibition tests, the cells were treated with H89 from LKT Laboratories, Inc. (St. Paul, MN, USA).

Cell viability assay
BeWo cells (4.75 × 10^4 cells/well) were cultured in a 96-well plate containing 100-µL culture medium per well. Twenty-four hours after harvest, the cells were treated with different concentrations of Cd. Cell Counting Kit-8 (CCK-8, 10 µL/well; Dojindo, Kumamoto, Japan) was added after 24 hr, followed by incubation for 4 hr. A microplate reader (Infinite 200 PRO, Tecan Group Ltd., Männedorf, Switzerland) was used to determine the cell viability by monitoring optical density at 450 nm.

Quantitative reverse transcription PCR
RNA was isolated from the cells using Isogen RNA extraction reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer’s protocol. Extracted total RNA was reverse transcribed into complementary DNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) and a random primer. For quantitative PCR (qPCR), we used TB Green Premix Ex Taq (Tli RNaseH Plus, Takara Bio, Kusatsu, Japan) reagents and an Eco Real-Time PCR System (Illumina, San Diego, CA, USA). Primers for amplifying differentiated cell-specific genes were designed using the PrimerQuest tool provided by IDT Integrated DNA Technologies, Inc. (Coralville, IA, USA). Primer sequences are shown in Supplementary Table 1. Expression levels were normalized using GAPDH.

Re-analysis of RNA-seq data
Raw RNA-sequencing reads of FSK-treated BeWo cells published at the NCBI website as BioProject Access-
sion No. PRJNA397241 were obtained as SRA files and reanalyzed. Reads were pseudoaligned using kallisto-0.46.2 (Bray et al., 2016). The cDNA sequence of hGRC38 was used as the reference sequence, and the output file was visualized and analyzed using sleuth (Pimentel et al., 2017). Transcript expression levels were shown as transcripts per kilobase million (TPM).

**Statistical analysis**

Data were analyzed with Tukey’s test using PASW Statistics 18 software (IBM, Armonk, NY, USA). Differences between groups were considered significant at \( P < 0.05 \).

**RESULTS AND DISCUSSION**

We first examined the time course of BeWo differentiation after FSK treatment by measuring differentiated cell-specific gene expression. The expression levels of ERVFRD1 and SDC1 were highest 2 days after FSK treatment. In LGALS13, it was the highest after 3 days. In CGB3, 2 days were required for gene expression, and there was no difference in the expression level between the second and third days (Fig. 1). Although SDC1 expression was slightly induced, its time course is consistent with reports that SDC1 is upstream of CGB (Prakash et al., 2011). Similar time-course changes were reported in the RNA-seq data published on the NCBI website as BioProject Accession No. PRJNA397241. The reanalyzed expression levels of the ST and normalization markers are shown in Table 1. Expression levels were normalized using GAPDH. Since ACTB and B2M were used as internal controls for qPCR, their expression levels

| FSK (µM) | Culture (hr) | LGALS13 | ERVFRD1 | SDC1 | CGB3 | GAPDH | ACTB | B2M |
|----------|--------------|---------|---------|------|------|-------|------|-----|
| 100      | 0            | 1.46    | 19.1    | 132  | 73.3 | 5772  | 4551 | 1051|
| 100      | 24           | 1.36    | 51.4    | 184  | 252.7| 6782  | 3729 | 572 |
| 100      | 48           | 1.52    | 202.3   | 1205 | 1777.5| 10082 | 2425 | 506 |
| 100      | 72           | 2.96    | 145.9   | 1863 | 6147.5| 10797 | 2105 | 489 |
| 0        | 72           | 0.90    | 17.6    | 181  | 125.4| 5669  | 2749 | 1556|

TPM, transcripts per kilobase million.
Next, to clarify the cytotoxicity of Cd against BeWo cells, viability was measured using CCK-8 24 hr after Cd treatment. As shown in Fig. 2, 25-µM Cd drastically decreased A450. So, we examined the effect of 2–20 µM Cd on FSK-induced BeWo cell differentiation. Cd concentration of 0.1–10 µM slightly increased A450. Viability assays using CCK-8 often show an increase in absorbance during such low-concentration treatments, which may indicate a defense reaction of the cells. We have confirmed that there is no significance in the position a group is placed in the 96-well plate at the viability assay. The expression of FSK-induced ST markers was inhibited by 5–10 µM Cd (Fig. 3). The maternal blood Cd concentration, which increased the risk of preterm birth in JECS, was > 0.902-ng Cd/g (> 8 nM) (Tsuji et al., 2018). Placental Cd concentration is about 0.52–14.5 ng/g, which is about four times the blood Cd concentration (Laine et al., 2015; Wang et al., 2018). The concentration of 5-µM Cd in the culture medium is an extremely high concentration compared with this concentration. However, the exposure period for the BeWo cell experiments shorter than that experienced in the mother's body. Measuring the intracellular Cd concentration in BeWo cells after 5-µM Cd treatment may be useful for studying the relationship between preterm birth associated with maternal Cd and the inhibitory effect of Cd in FSK-induced BeWo cell differentiation.

FSK is an adenylyl cyclase activator that activates PKA through increasing intracellular cAMP and induces syncytialization of BeWo cells. In contrast, H89, a PKA inhibitor, partially inhibits the expression of syncytialization markers (Orendi et al., 2010). The molecular mechanisms of FSK-induced BeWo differentiation are com-

Fig. 2. Cell viability after 24 hr treatment. BeWo cells were treated with Cd, and cell viability was determined using the Cell Counting Kit-8 for 4 hr. Data represent the mean ± SD of three independent experiments. *P < 0.05, significantly different from Cd-untreated group.

Fig. 3. Inhibitory effect of Cd on the expression of FSK-induced LGALS13, ERVFRD1, SDC1, and CGB3 in BeWo cells. Cd and FSK were simultaneously added. Three days after Cd and FSK treatment, RNA was extracted and expression levels were measured through RT-qPCR. The values were normalized with endogenous GAPDH expression. Data represent the mean ± SD of three independent experiments. *P < 0.05, significantly different from FSK-untreated group. †P < 0.05, significantly different from Cd-untreated group.
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To clarify whether the inhibitory effect of Cd is mediated by PKA inhibition, we treated BeWo cells with H89 and Cd. If the inhibition by Cd is only caused by PKA inhibition, cotreatment with H89 has an additive effect at low H89 treatments and should not cause further inhibition at high H89 treatments. As shown in Fig. 4, H89 fully inhibited FSK-induced LGALS13 expression, whereas it only partially inhibited FSK-induced CGB3 expression. FSK-induced SDC1 expression was not inhibited by H89. These results suggest that only FSK-induced LGALS13 expression is fully mediated through the PKA pathway. Orendi et al. (2010) reported that CGB protein expression was not altered by H89, whereas LGALS13 protein and mRNA expression decreased significantly to control levels. Our results were consistent with Orendi et al.’s report. Our results suggest that the inhibitory effect of Cd is mediated through the inhibition of the PKA pathway. Moreover, the inhibitory effect of Cd is partial. In contrast, Cd, not H89, inhibited FSK-induced SDC1 expression. This means that FSK-induced SDC1 expression is mediated by the PKA-independent pathway and inhibited by Cd. The MAPK pathway is also involved in the FSK-induced BeWo differentiation (Delidaki et al., 2011). If Cd inhibits the MAPK pathway, it may be the molecular mechanism of the BeWo cell differentiation inhibition. However, many reports indicate that Cd activates the MAPK pathway via oxidative stress (Nemmiche, 2017). Both PKA and PKC are involved in the activation of BeWo differentiation by FSK (Omata et al., 2013). Cd might partially inhibit LGALS13 induction by inhibiting the PKA pathway and SDC1 and CGB expressions through PKA-independent pathway. Further research is needed to verify this possibility.

In conclusion, Cd exposure inhibited FSK-induced BeWo differentiation possibly by inhibiting LGALS13, ERVFRD1, SDC1, and CGB expressions. LGALS13 inhibition was caused by the inhibition of the PKA pathway, whereas the other three could be due to the inhibition of the other pathways. This research could help clarify the reproductive and developmental toxicity of Cd.

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Conflict of interest---- The authors declare that there is no conflict of interest.
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