Note

Retinoic Acid Inhibits Uterotrophic Activity of Bisphenol A in Adult Ovariectomized Rats

Tomoko KODA^{1,2}, Masatoshi MORITA^{2,3} and Hideki IMAI^{1,2}

¹Division of Environmental Health Sciences, Department of Social Medicine, Faculty of Medicine, University of Miyazaki, 5200 Kihara, Kiyotake, Miyazaki 889–1692, Japan

²National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba, Ibaraki 305–8506, Japan

³Department of Bioresources, Faculty of Agriculture, Ehime University, 3–5–7 Tarumi, Matsuyama, Ehime 790–8566, Japan

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Summary Bisphenol A (BPA) is used in the production of polycarbonate and epoxy resins. The weak estrogenic activity of BPA has been confirmed by both in vitro and in vivo assays. Retinal acetate has been reported to inhibit the adverse effects of BPA on male mice reproduction. All-*trans*-retinoic acid (ATRA) is a potent natural derivative of vitamin A and is reported to inhibit the estrogen-induced proliferation of human breast carcinoma cells. In this study, we investigated the possible inhibitory effects of ATRA on the estrogenic activity of BPA by a standard in vivo uterotrophic assay. Proliferated and apoptotic uterine cells were identified by 5-bromo-2′-deoxyuridine (BrdU) incorporation and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay. We observed that ATRA supplementation significantly inhibits a BPA-induced uterine weight increase in adult ovariectomized rats. However, there were no significant differences in the increases in the numbers of BrdU-positive cells and TUNEL-positive cells between the BPA and BPA+ATRA groups. These results show that ATRA may have an inhibitory effect on the estrogenic activity of BPA in an in vivo assay.

Key Words retinoic acid, bisphenol A, uterotrophic assay, in vivo, rat

Bisphenol A (BPA) has been widely used in the manufacture of polycarbonate, epoxy resins (1), fungicides, antioxidants, dyes, flame retardants and several types of dental sealant (2). Bisphenol A can be released from polycarbonate flasks during autoclaving (3) and from lacquer coatings inside food cans (4). Bisphenol A has a weak estrogenicity (5). It was found to bind to the human estrogen receptor (ER) (6) and to induce the proliferation of MCF-7 cells (3, 4) and the transcriptional activation of the ER in a yeast-based assay (7).

Vitamin A and its retinoid derivatives have profound effects on cellular proliferation and differentiation (8). All-*trans*-retinoic acid (ATRA) is the primary active metabolite of vitamin A (9, 10) and also the most biologically active retinoid (11). Previous reports showed that ATRA inhibits the estrogen-induced proliferation of several cell types in in vitro and in vivo assays (12–14). In addition, ATRA expresses receptor-mediated antiestrogenic activity and interferes with estrogenic activity at the estrogen-responsive-element (ERE) level (15, 16).

The subcutaneous injection of BPA into neonatal male mice resulted in a decrease in their sperm motility and an increase in the incidence of malformed sperm after the mice became adults, and this BPA effect was cancelled by cotreatment with retinol acetate, a natu-

rally occurring metabolite of vitamin A (17). However, the effects of ATRA on the BPA-induced biological effects in adult female rats have not been investigated. In this study, we examined the inhibitory effect of ATRA on the estrogenic activity of BPA by a standard in vivo uterotrophic assay that was authorized by the Organization for Economic Cooperation and Development (OECD) (18). Exogenous estrogens have been shown to induce hypertrophy, predominantly of the luminal epithelium but also of the glandular epithelium (19–21), and the proliferation of the stroma (22) and myometrium (23, 24) in rodents. Bisphenol A-induced increases in the thicknesses of both stromal and myometrial layers are not mediated through ER (25). Therefore, we examined the degrees of proliferation and apoptosis of the uterine epithelial cells by 5-bromo-2′-deoxyuridine (BrdU) incorporation and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, respectively.

Materials and Methods

Chemicals. Bisphenol A (CAS No. 80-05-7) was obtained from Sigma-Aldrich Corporation (MO, USA). All-*trans*-retinoic acid (CAS No. 302-70-4) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Olive oil (for dissolving ATRA) was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Pen-

E-mail: imahide@med.miyazaki-u.ac.jp

tobarbital (Nembutal®, Abbott Laboratories, IL, USA) was purchased from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Bisphenol A or ATRA was dissolved in olive oil and injected subcutaneously at a volume of 1 mL/kg body weight (b.w.).

Animals. Female Crj:CD (SD) rats (10–11 wk old) were purchased from Charles River Japan (Ibaraki, Japan). The rats were housed in metal cages with a 12-h light/dark cycle (beginning at 7:00 a.m.), and given free access to commercial food (CE-2, CLEA Japan Inc., Tokyo, Japan) and tap water. The rats were ovariectomized under pentobarbital anesthesia (50 mg/kg b.w.) and allowed to recover for 2 wk.

All experiments were conducted in accordance with the guidelines of the Ethics Committee for Experimental Animals of the National Institute for Environmental Studies, Japan.

Uterotrophic assay. The rats were treated with 100 mg/kg b.w./d BPA with or without 5 mg/kg b.w./d ATRA subcutaneously between 9:00 and 10:00 a.m. once a day for 3 successive days. Twenty-four hours after the last treatment, the rats were sacrificed and uteri were removed immediately. After being cut into small pieces, the uteri were placed on a Kimwipe® and gently pressed to blot the fluid. Blotted-uterus weight was recorded and expressed as mg/100 g b.w. All the rats were injected with 40 mg/kg b.w. BrdU (Sigma-Aldrich) intraperitoneally 8-h before being sacrificed. This adopted procedure was performed in accordance with the OECD protocol (18).

Tissue preparation. The blotted uteri were placed in a neutral 10% formaldehyde buffer solution (Nacalai Tesque, Inc.) for 12-h at room temperature and then dehydrated and embedded in paraffin. Transverse sections were cut into 5- μ m-thick sections using a microtome (LEICA Microsystems Japan, Tokyo) and mounted on MAS-coated slides.

Determination of cell proliferation. To detect uterine cell proliferation, the cellular incorporation of the injected BrdU was determined using a DAKO LSAB® 2 kit (Dako Corporation, CA, USA) according to the manufacturer's instruction. Briefly, the BrdU incorporated into the uterine cells was immunostained and finally visualized with 3,3'-diaminobenzidine (DAB) (Roche Diagnostics Corporation, IN, USA). The preparations were counterstained with hematoxylin after immunostaining. For a quantitative evaluation, the number of BrdU-positive cells and the total numbers of luminal and glandular epithelial cells were counted under a microscope (400 $\times$), and the percentage of BrdU-positive cells was calculated.

Determination of cell death. Apoptotic uterine cells were analyzed by TUNEL assay using an ApopTag® Peroxidase In Situ Apoptosis Detection kit (Chemicon, CA, USA). Briefly, digoxigenin-nucleotide residues were catalytically added to the 3'-OH ends of double- or single-stranded DNAs by utilizing TdT. The labeled cells were visualized using DAB. The slides were counterstained with methyl green after immunostaining. For a quantitative evaluation, the number of TUNEL-positive cells

and the total numbers of luminal and glandular epithelial cells were counted under a microscope (400 $\times$) and the percentage of TUNEL-positive cells was calculated.

Statistical analysis. Dunnett's test was employed to analyze blotted-uterus weight, and the percentages of BrdU- and TUNEL-positive cells.

Results and Discussion

Bisphenol A treatment significantly increased blotted-uterus weight/100 g b.w. (vehicle: 39.9 mg/100 g b.w.; BPA: 81.0 mg/100 g b.w., $p < 0.01$). The coinjection of ATRA significantly decreased the BPA-induced increase in blotted-uterus weight/100 g b.w. (BPA + ATRA: 69.3 mg/100 g b.w., $p < 0.05$). There was no significant difference in blotted-uterus weight/100 g b.w. between the vehicle and ATRA (ATRA: 44.2 mg/100 g b.w.) groups (Fig. 1). We showed here that ATRA treatment significantly inhibits the BPA-induced uterotrophic effect. Several animal experimental studies have shown the effects of exogenous estrogens on the uterus including hypertrophy, predominantly of the luminal epithelium but also of the glandular epithelium (19–21), and the proliferation of the stroma (22) and myometrium (23, 24). It is likely that the changes in the weight of stromal and myometrial cells contribute to the increase in uterine weight. In one study, BPA was shown to induce increases in uterine weight, luminal epithelial cell height and the thicknesses of both the stroma and myometrium (25). In the same study, it was shown that the coadministration of the antiestrogen ICI 182,780 reverses the effect on epithelial cell height; however, the thicknesses of the stromal and myometrial layers were not influenced (25). Nephew et al. reported that estradiol 17 β -induced changes in ER α protein synthesis occurred in the luminal and glandular epithelia, but did not occur in either the stromal cells or the myometrial cells in adult ovariectomized rats (26). Therefore, the enlargements of the stroma and myo-

Fig. 1. Effects of bisphenol A (BPA) and all-*trans*-retinoic acid (ATRA) on blotted-uterus weight/100 g b.w. in ovariectomized rats. The vertical axis shows the blotted-uterus weight/100 g b.w. The horizontal axis shows treatment chemicals. Columns indicate mean values and vertical lines denote the standard errors of the mean (SE). Each column represents five or six rats. * $p < 0.05$, ** $p < 0.01$ versus BPA group by Dunnett's test.

Fig. 2. Effects of bisphenol A (BPA) and all-*trans*-retinoic acid (ATRA) on numbers of BrdU-positive (A, B) and TUNEL-positive (C, D) cells in the luminal and glandular epithelia. The vertical axis shows the percentage of positive cells. The horizontal axis shows treatment chemicals. Columns indicate mean values and vertical lines denote the standard errors of the mean (SE). Each column represents five or six rats. ** $p < 0.01$ versus BPA group by Dunnett's test.

metrium could be attributable to other mechanisms not mediated via ER. Thus, we examined the cell proliferation and cell apoptosis in luminal and glandular epithelial cells.

Bisphenol A treatment with or without ATRA treatment induced cell proliferation, as revealed by BrdU immunostaining both in the luminal and glandular epithelial cells (Fig. 2, A and B). The mean percentages of BrdU-positive cells in the luminal and glandular epithelia significantly increased as a result of BPA treatment (vehicle: luminal epithelium, 0.23% and glandular epithelium, 0.48%; BPA: luminal epithelium, 21.6% and glandular epithelium, 37.4%). There was no significant difference in the mean percentages of BrdU-positive cells in the luminal and glandular epithelia between the BPA and BPA+ATRA (luminal epithelium, 15.5%; glandular epithelium, 39.4%) groups or between the vehicle and ATRA (luminal epithelium, 0.38%; glandular epithelium, 0.85%) groups (Fig. 2, A and B).

Bisphenol A treatment with or without ATRA treatment induced the apoptosis of luminal and glandular epithelial cells, as revealed by TUNEL assay. The mean percentages of TUNEL-positive cells in the luminal and glandular epithelia significantly increased as a result of BPA treatment (vehicle: luminal epithelium, 0.27% and glandular epithelium, 0.32%; BPA: luminal epithelium, 7.7% and glandular epithelium, 1.9%). There was no

significant difference in the mean percentages of TUNEL-positive cells in the luminal and glandular epithelia between the BPA and BPA+ATRA (luminal epithelium, 9.4%; glandular epithelium, 1.4%) groups, or between the vehicle and ATRA (luminal epithelium, 0.11%; glandular epithelium, 0.31%) groups (Fig. 2, C and D).

Previous studies showed that ATRA treatment inhibits estrogen-induced cell proliferation in *in vitro* and *in vivo* assays (12–14). All-*trans*-retinoic acid was suggested to inhibit the growth of human breast cancer cells by promoting apoptosis (27). Our results indicate that the mean percentage of proliferated luminal epithelium cells in the BPA group was significantly larger than that in the vehicle group. The mean percentage of proliferated luminal epithelium cells in the BPA+ATRA group was smaller than that in the BPA group; however, the difference did not reach statistical significance. We performed BrdU incorporation to detect the proliferation of cells. This method can detect S-phase cells, but not G1-, G2- or M-phase cells. Other methods of detecting the proliferation of cells in all phases, such as Ki-67 immunostaining (28), may show a difference in the mean percentage of proliferating cells between the BPA and BPA+ATRA groups. There was no significant difference in the mean percentages of proliferating glandular epithelial cells between the BPA and BPA+ATRA

groups. Because the number of luminal epithelium cells was markedly larger than that of glandular epithelium cells, the number of luminal epithelium cells may have a profound effect on uterine weight. The mean percentage of apoptotic luminal epithelium cells in the BPA group was significantly larger than that in the vehicle group. The mean percentage of apoptotic luminal epithelium cells in the BPA + ATRA group was larger than that in the BPA group; however, the difference did not reach statistical significance.

Bisphenol A was found to bind to ER α (6) and induce the proliferation of MCF-7 cells (29) and the transcriptional activation of the ER in a yeast-based assay (7). However, estrogen and BPA have different effects on ER α , ER β and C3 expressions in the uterus (30). Mechanisms other than the ER-mediated pathway may be involved in these different effects; alternatively, BPA may act via ER α or ER β but may activate in addition to the enhancers or repressors of ER activities in the uterus (30).

Further study is necessary to clarify the inhibitory mechanisms of ATRA on BPA in uteri.

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