— **Regular Article** —

Quantitative Analyses of Inhibitory Effects of Bisphenol A on Neural Stem-cell Migration Using a Neurosphere Assay *in vitro*

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The large numbers of environmental chemicals that now exist have made assessing their neurodevelopmental risks challenging. In this study, we quantified neurodevelopmental toxicity of bisphenol A, using a neurosphere assay *in vitro*. Neurosphere was isolated from rat E16 mesencephalone. A neurosphere assay was carried out in the amine-coated plates. Cells emerged from the neurosphere and migrated along the radial axis from the sphere. The migrating populations comprised cells that were positive for nestin, microtubule-associated proteins, and glial fibrillary acidic protein. Exposure to bisphenol A inhibited cell migration and decreased proliferative cells in dose-dependent manner. Quantitative analyses revealed a linear function between the inhibition of migration and the logarithm of bisphenol A concentration (0–100 μM); the percent inhibition by 1 μM bisphenol A of migration was 35% ($p < 0.05$). Thus, we showed for first time that bisphenol A inhibited migration as well as proliferation of neural stem cells *in vitro* and that a neurosphere assay *in vitro* is very useful to rapidly quantify neurodevelopmental toxicity of environmental chemicals.

Key words —— bisphenol A, neural stem cell, neurosphere assay, migration, proliferation

INTRODUCTION

Environmental estrogens are originally defined as a diverse group of synthetic and naturally occurring compounds that mimic the action of steroidal estrogens. These chemicals are called endocrine disruptors or endocrine disrupting chemicals. Bisphenol A is one of the well-known endocrine disruptors. Bisphenol A [2,2-bis(4-hydroxyphenyl)propane] is used as a monomer in the manufacture of polycarbonate plastics, epoxy resins, and composites and consequently has extensive applications in the food packaging industry and dentistry.[1] Bisphenol A is released from polycarbonate flasks during autoclaving or from lacquer coatings in food cans, showing estrogenicity for human breast cancer MCF-7 cells.[2,3] The reproductive effects of this endocrine disruptor have thus been examined.

Recent evidence points to an important effect of exposure to environmental neurotoxicant chemicals on the marked increase seen in neurodevelopmental disorders. The effects of perinatal exposure to bisphenol A have been shown to affect various aspects of behavior such as exploratory, sociosexual,[4] and aggressive behaviors,[5] as well as anxiety level,[6] and nociception,[7] and the response to D-amphetamine.[8] Impairment by bisphenol A of the sexual differentiation of the exploratory behavior and the size of the locus coeruleus was also observed,[9] and depression-like behavior was increased by the chemical.[10] Furthermore, neocortical histogenesis was perturbed by bisphenol A.[11] We have demonstrated that intracisternal administration of some endocrine-disrupting chemicals, such as bisphenol A,[12,13] octylphenol,[14] cyclohexylphthalate,[15] and tributyltin,[16] caused hyperactivity in male Wistar rats, and concluded that endocrine-disrupting chemicals seem to be neurotoxic to the developing rat brain, but it is still unclear whether their effects on the developing brain result from their endocrine disrupting activity or some other as yet uncharacterized process.

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Proliferation and migration ability of neural stem cells is useful for testing the developmental effects of environmental chemicals since neural stem cells play an essential role in the development of the central nervous system.

In this study we have developed a simple and rapid quantitative system to evaluate neurodevelopmental toxicity of environmental chemicals, using cultured rat neurospheres.

MATERIALS AND METHODS

Chemicals —— Bisphenol A was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Rotenone was from Sigma-Aldrich (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was from Nacalai Tesque (Kyoto, Japan).

Antibodies —— Cy3-conjugated antibodies against glial fibrillary acidic protein (GFAP) and antibodies against microtubule-associated proteins (MAPs) were purchased from Sigma-Aldrich. Antibodies against nestin and antibodies against Ki-67 were from BD Biosciences, Tokyo, Japan. Secondary antibodies such as Alexa Fluor 405 and Alexa Fluor 488 were from Invitrogen, Tokyo, Japan.

Preparation of Neurospheres from Embryonic Mesencephalons —— Rat mesencephalic neural stem cells were prepared essentially according to the method of Reynolds and Weiss.[17] Pregnant Wistar rats at embryonic day 14 (E14) were obtained from Clea (Tokyo, Japan). The animals were maintained in home cages at 22°C with a 12-hr light-dark cycle. They received the MF diet (Oriental Yeast Corp., Tokyo, Japan) and distilled water *ad libitum*. All animal care procedures were in accordance with National Institute for Environmental Studies (NIES) guidelines. The rats were sacrificed by diethyl ether overdose on E16. The embryos were removed and transferred to minimal essential medium (MEM; Sigma-Aldrich). Subsequently, the mesencephalons were dissected from the embryos, and were enzymatically digested with 50 U deoxyribonuclease I (Takara Corp., Kyoto, Japan) and 0.8 U papain (Sigma-Aldrich) at 32°C for 12 min. After stirring, the digestion mixture was passed through a 70-μm cell strainer (BD Biosciences). The run-through containing the neural stem cells was centrifuged at 800 × *g* for 10 min. It was then resuspended in 6-ml of Dulbecco's Modified Eagle's Medium (DMEM) and F12 medium (1 : 1; Invitrogen) supplemented with B27 (Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Inc., Minneapolis, MN, U.S.A.) and 10 ng/ml epidermal growth factor (EGF; Roche Applied Science, Tokyo, Japan), and cultured in uncoated 60-mm dishes for 12 hr to remove the neural stem cells which were adhesive to the dish surface. Thereafter, the floating neural stem cells were transferred to a 60-mm Ultra-low attachment dish (Corning, Tokyo, Japan) without serum at 20–70% of confluency for 1–4 months. Fresh culture medium (3 ml) containing EGF and bFGF was added after 3–4 days.

A Neurosphere Assay —— Three to five neurospheres (100–300 μm in diameter) were seeded in an amine-coated 24-well plate (BD Biosciences) in the medium (500 μl), containing 20 ng/ml bFGF and 10 ng/ml EGF for 3 hr, allow cells to adhere. The cells were then exposed to a variety of concentration of bisphenol A or rotenone which was suspended in 50% DMSO. The migrating distance of the cells was statistically measured from the edge of the sphere, using National Institute of Health (NIH) ImageJ 1.38x software (public domain software).

Immunocytochemistry —— The stem cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.5% Triton X-100 and labeled as described previously,[12] using primary antibodies diluted at 1 : 100. Secondary antibodies were properly used, diluting at 1 : 200. Specimens were then observed with an inverted microscope (IX-70; Olympus, Tokyo, Japan) and images were captured using Viewfinder Lite version 1.0 camera software and a DP-50 digital (Olympus) or Leica TCS SP5 confocal microscope system equipped with an AF6000 inverted microscope (Leica Microsystems, Tokyo, Japan).

Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) —— TUNEL labeling was carried out with fluorescein dUTP (Roche Applied Science) in the presence of terminal deoxynucleotidyl transferase for 1 hr at 37°C. Following labeling, the cells were washed with phosphate-buffered saline (PBS) twice and then directly surveyed under a fluorescence microscope. Images were captured as above.

Statistics —— Statistical analyses were carried out with Student's *t*-test using JMP version 6.03 (SAS Institute, Cary, NC, U.S.A.).

RESULTS

For the assay, the cultured neurospheres were

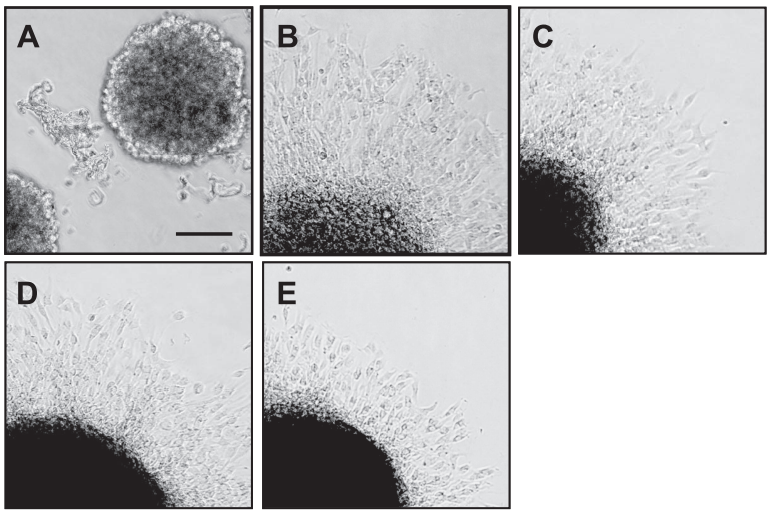

Fig. 1. Typical Photographs of Inhibition by Bisphenol A of Cell Migration from Neurosphere *in vitro*

A neurosphere assay was carried out by plating the cultured neurosphere on the amine-coated dish, allowing to adhere. For first 3 hr, there were no migrating cells (A). During the subsequent 24 hr, cells emerged and migrated when bisphenol A was added at concentrations of 0 μM (B), 1 μM (C), 10 μM (D), and 100 μM (E). Scale bar: 100 μm.

seeded in the amine-coated plates in the presence of 20 ng/ml bFGF and 10 ng/ml EGF. During the culture, cells emerged from the plated neurospheres and migrated along the radial axis (Fig. 1A and 1B). After 3 hr, the plated cells were treated with various concentrations of bisphenol A (0–100 μM) for 24 hr (Fig. 1B–1E). The migration distance of the cells was measured from the edge of the sphere using NIH ImageJ 1.38x public domain software. Bisphenol A prevented the cells from migrating from the sphere in a semilogarithmic-linear, dose-dependent manner (Fig. 2). Thirty five percent inhibition was seen by the treatment with bisphenol A (1 μM; 24 hr). Higher concentrations of bisphenol A such as 10 μM and 100 μM inhibited cell migration 48% and 60%, respectively.

Since cell migration is often associated with cell proliferation, we then identified proliferative cells using anti-Ki-67 antibody. The antibody recognizes Ki-67 protein, a nuclear cell proliferation-associated antigen expressed in all active stages of the cell cycle. The neurosphere assay and the bisphenol A treatment were carried out as described above. The treated cells were then fixed and permeabilized. The specimens were labeled with primary antibody for Ki-67. The relative number of positive cells was calculated based on the ratio of cell counts from Ki-67-specific staining and 4′,6-diamino-2-phenylindole (DAPI) nuclear staining. The number of Ki-67-positive cells in the migrating population was also reduced by bisphenol A in dose-dependent manner (Figs. 3A–3D and 4). For the controls, the

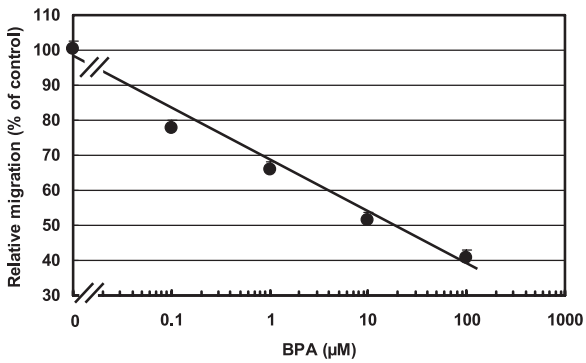

Fig. 2. Quantification of Inhibitory Effects of Bisphenol A on Cell Migration from Neurosphere *in vitro*

A neurosphere assay for bisphenol A (BPA) was carried out as legend in Fig. 1. Migration distance was then quantitatively measured with NIH ImageJ 1.38x software. Results were represented as a percentage of the value of migration distance obtained without bisphenol A. Please note logarithmic x-axis. Data are indicated as mean ± S. E. (n = 10).

number of Ki-67-positive cells was 65% of the proliferative population, whereas the value in the stationary population was 35%. By contrast, treatment with 1 μM and 10 μM bisphenol A for 24 hr significantly reduced the number of Ki-67-positive cells to 47% and 28% of the proliferative population, respectively ($p < 0.05$ and $p < 0.0001$).

To identify cell death, TUNEL staining was carried out. However, bisphenol A (0–100 μM) failed to induce TUNEL-positive cell death (data not shown).

To characterize the migrating cells, we stained these with primary antibodies against nestin (a marker protein for neural stem cell), GFAP (an as-

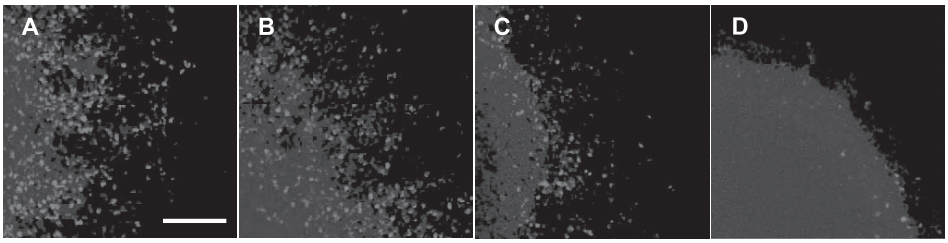

Fig. 3. Bisphenol A Inhibition of Proliferation of Migrating Cells from Neurospheres *in vitro*

A neurosphere assay was performed as described for Fig. 1. Bisphenol A was added at concentrations of 0 μM (A), 1 μM (B), 10 μM (C), and 100 μM (D). The treated cells were stained with anti-Ki-67 antibody (gray). Scale bar: 100 μm.

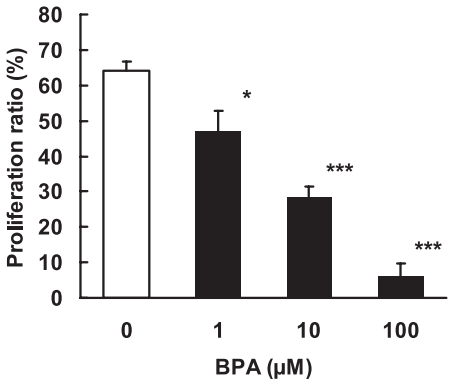

Fig. 4. Quantification of Inhibitory Effects of Bisphenol A on Proliferation of Migrating Cells from Neurosphere *in vitro*

A neurosphere assay was carried out as legend in Fig. 3 with various concentrations of bisphenol A (BPA) as indicated. Results were represented as a percentage of cell counts from Ki-67-specific staining and DAPI nuclear staining. Data are indicated as mean ± S. E. (n = 10). $^{*}p < 0.05$, $^{***}p < 0.0001$.

trocytic marker), or MAPs (marker proteins for neuron). In untreated control cells, nestin immunoreactivty was seen in the migrating cells (Fig. 5, green), indicating that the cell did not reach a fully differentiated phenotype under the experimental conditions used. It was also notable that the migrating cells in the untreated controls were also positive for anti-GFAP antibodies (Fig. 5, red) and anti-MAPs antibodies (Fig. 5, blue). They all migrated from the sphere along the radial axis. Their migration was inhibited by bisphenol A exposure in dose-dependent manner (0–100 μM).

To compare to neurodevelopmental toxicity of bisphenol A, using a neurosphere assay, we tested the potency of rotenone, an environmental dopaminergic toxin. Neurosphere assay was carried out as in the case of bisphenol A. After plating the spheres on the amine-coated plate, a various concentrations of rotenone (0–10 μM) were added for 24 hr. Then, migration distance was measured. It was also inhibited by rotenone treatment in a semilogarithmic-linear, dose-dependent manner (Fig. 6). The percent inhibition by 1 μM rotenone of migration was 70%.

DISCUSSION

In this study, it was demonstrated for first time that an environmental chemical, bisphenol A, inhibited migration as well as proliferation of neural stem cells *in vitro*, using undifferentiated neurosphere. Bisphenol A suppressed proliferation. Consequently, the failure to migrate might have been an epiphenomenon. The mechanism of cell migration *in vitro* might be coupled to those of cell proliferation. As there has been reported that bisphenol A can pass through the placenta into embryos,[4–6] our current findings suggest that exposure to bisphenol A *in utero* disturbs the development of central nervous system derived from neural stem cell.

There have been reported that reproductive effects of bisphenol A were the nonlinear relationship between the effects and concentration of the chemical, resulting in hampering the risk assessment.[18,19] However, quantitative analyses in this study revealed the linearity in function as migration inhibition versus the logarithm of chemical concentration. Owing to the linearity of the functional relationship between the migration inhibition and the concentration of test chemicals, this approach could be employed as a reliable quantitative assay system, excluding the issue of nonlinearity in low dose of bisphenol A. As an example, we compared the potency of neurodevelopmental toxicity of two dopaminergic toxicants,[20] bisphenol A[12,13,21] and rotenone.[22] The percent inhibition of migration by bisphenol A or rotenone at 1 μM was 35% and 70%, respectively. Thus, the rank order of potency of chemicals was: bisphenol A < rotenone.

There has been limited to report neurotoxicity of bisphenol A *in vitro*. Kim *et al.* demonstrated that at a high concentration (> 400 μM), bisphenol A was

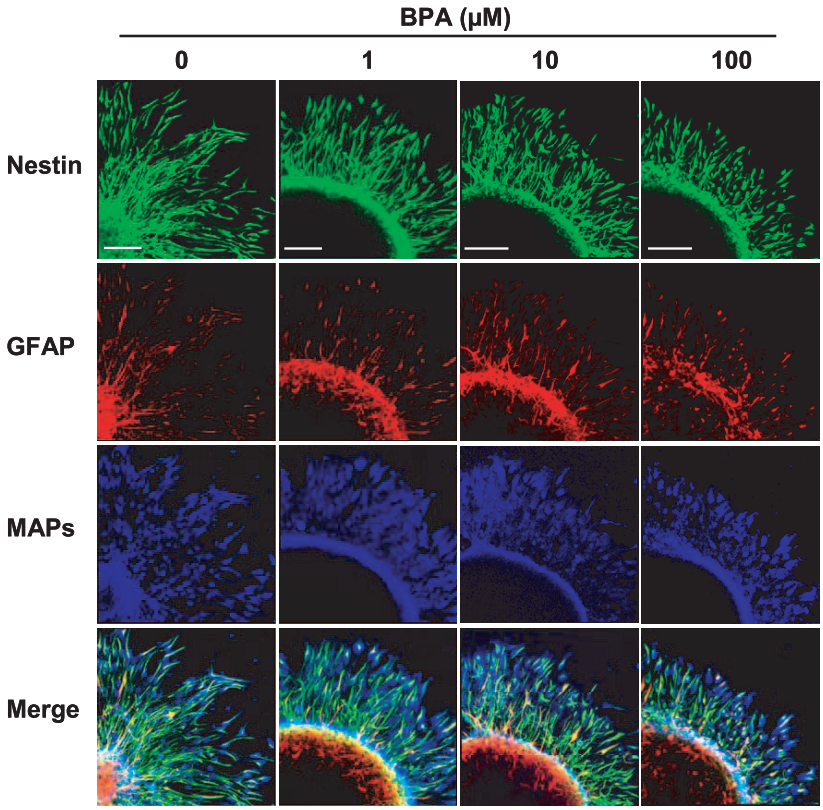

Fig. 5. Effect of Bisphenol A (BPA) on Differentiation of Migrating Cells from Neurospheres *in vitro*

A neurosphere assay was carried out with 0 μM bisphenol A, with 1 μM bisphenol A, with 10 μM bisphenol A, or with 100 μM bisphenol A, as indicated. The treated cells were then stained with primary antibodies for nestin (green), GFAP (red), or MAPs (blue). Merged images are also shown. Scale bar = 100 μm.

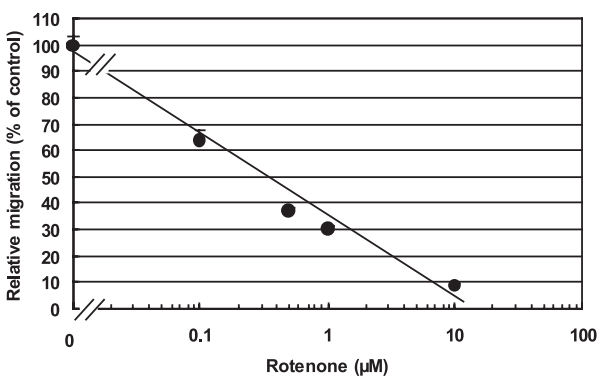

Fig. 6. Inhibition by Rotenone of Cell Migration from Neurosphere *in vitro* in Amine-coated Dishes

A neurosphere assay for rotenone (0–10 μM) was carried out as legend in Fig. 1. Migration distance was then quantitatively measured with NIH ImageJ 1.38x software. Results were represented as a percentage of the value of migration distance obtained without rotenone. The dose of rotenone is expressed on a logarithmic scale and data are indicated as mean ± S. E. (n = 4).

cytotoxic to immortalized mouse neural progenitor C17.2 cells for 24 hr of exposure.[23] High concentrations of bisphenol A (> 100 μM) was also required for cytotoxicity to mouse hippocampal HT22 cells.[24] Thus, it is more sensitive to detect neurotoxicity of the chemical by using a neurosphere assay *in vitro* shown in this study. This is also supported by the case of rotenone: Li *et al.* reported that rotenone at 8 μM decreased cell viability 50% of human neural stem cells after 24 hr of treatment,[25] whereas IC_{50} was 0.32 μM in our system (Fig. 6).

A linear function between the migration and test chemical concentrations was found when dose was expressed on a logarithmic scale (Figs. 2 and 6). This indicates that a large change in cellular inhibition was elicited by a small change in dosage. The cell population might be heterogeneous in sensitivity to the chemical, but it might contain a normal population distribution. Therefore, the semilogarithmic plot yields a straight line.[26]

The development of the nervous system involves the coordinated expression of specific cellular events including proliferation, differentiation, migration, neurite outgrowth, synaptogenesis, myelination, and programmed cell death.[27] Neurons are thought to migrate from their origin near the ventricle to distant territories by detecting environmental cues in the extracellular milieu, sometimes over long distances. Successful migration

is crucial for the establishment of central nervous system cytoarchitectures that are essential for brain functions. Development of dopamine neurons is also the case. During the early stage of midbrain development, neural stem cells at the ventricular zone acquire intrinsic and extrinsic signals that determine the neural identity of their derivatives upon differentiation.[28–30] The positional specification of mesencephalic dopamine neurons is initiated by local inductive signals that impose regional character on neural progenitors. Thus, it is very important to examine if the inhibitory nature of bisphenol A on cell migration is observed *in vivo*.

Recent reports have shown an increased incidence of neurodevelopmental disorders which may result from exposure to chemicals *in utero* and during the early postnatal period.[13, 20, 31–33] Sensitivity to the chemicals is dependent on age and may be greatest *in utero* and in the early postnatal period.[34] Every year a large number of new chemicals are released into the environment, so the establishment of a simple test for developmental neurotoxicity is urgent. Proliferation and migration ability of undifferentiated neural stem cells *in vitro* is the most reasonable approach for such test system. Although our culture system appeared to lack an instructive niche that directed neural stem cells to develop into neural lineage specification, this *in vitro* neurosphere assay could be useful for the primary screening of environmental chemicals that might affect neurodevelopmental programming.

Hopefully, this would develop the risk assessment methods for chemicals based on infant physiology.

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