Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC *et al.* (2001) Minimum information about a microarray experiment (MIAME) – toward standards for microarray data. *Nat Genet* **29**, 365–371.

Brenner S, Jacob F, Meselson M (1961) An unstable intermediate carrying information from genes to ribosomes for protein synthesis. *Nature* **190**, 576–581.

Brown PO, Botstein D (1999) Exploring the new world of the genome with DNA microarrays. *Nat Genet* **21**, 33–37.

C. elegans Sequencing Consortium (1998) Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* **282**, 2012–2018.

Chang R, Wang E (2007) Mouse translation elongation factor eEF1A-2 interacts with Prdx-I to protect cells against apoptotic death induced by oxidative stress. *J Cell Biochem* **100**, 267–278.

Churchill GA (2002) Fundamentals of experimental design for cDNA microarrays. *Nat Genet* **32** (Suppl.), 490–495.

Crick FH (1958) On protein synthesis. *Symp Soc Exp Biol* **12**, 138–163.

Crick FH, Barnett L, Brenner S, Watts-Tobin RJ (1961) General nature of the genetic code for proteins. *Nature* **192**, 1227–1232.

Cruse JM, Lewis RE, Pierce S, Lam J, Tadros Y (2005) Aberrant expression of CD7, CD56, and CD79a antigens in acute myeloid leukemias. *Exp Mol Pathol* **79**, 39–41.

Czapski G, Peled E (1973) Reaction rates of electrons at short times. *J Phys Chem* **77**, 893.

Czapski G, Peled E (1975) Reaction rates of electrons at short times. In *Radiation Research: Biomedical, Chemical and Physical Perspectives*, OF Nygaard, HI Adler, WK Sinclair (eds). Academic Press: New York.

Fodor SP, Rava RP, Huang XC, Pease AC, Holmes CP, Adams CL (1993) Multiplexed biochemical assays with biological chips. *Nature* **364**, 555–556.

Hamadeh HK, Bushel P, Paules R, Afshari CA (2001) Discovery in toxicology: mediation by gene expression array technology. *J Biochem Mol Toxicol* **15**, 231–242.

Hao L, El Shamy WM (2007) BRCA1-IRIS activates cyclin D1 expression in breast cancer cells by downregulating the JNK phosphatase DUSP3/VHR. *Int J Cancer* **121**, 39–46.

Harris J, Lowden M, Clejan I, Tzoneva M, Thomas JH, Hodgkin J, Ahmed S (2006) Mutator phenotype of *Caenorhabditis elegans* DNA damage checkpoint mutants. *Genetics* **174**, 601–616.

Hastie ND, Allshire RC (1989) Human telomeres: fusion and interstitial sites. *Trends Genet* **5**, 326–331.

Henke FU, Lubarsch O (1924–1952). *Handbuch des speziellen pathologischen Anatomie und Histologie*, vols **1–12**. Springer: Berlin.

Hill AA, Brown EL, Whitley MZ, Tucker-Kellogg G, Hunter CP, Slonim DK (2001) Evaluation of normalization procedures for oligonucleotide array data based on spiked cRNA controls. *Genome Biol* **2**, RESEARCH0055.

Hourihan RN, O'Sullivan GC, Morgan JG (2003) Transcriptional gene expression profiles of oesophageal adenocarcinoma and normal oesophageal tissues. *Anticancer Res* **23**, 161–165.

Hu T, Gibson DP, Carr GJ, Torontali SM, Tiesman JP, Chaney JG, Aardema MJ (2004) Identification of a gene expression profile that discriminates indirect-acting genotoxins from direct-acting genotoxins. *Mutat Res* **549**, 5–27.

Hunt JW (1976) Early events in radiation chemistry. In *Advances in Radiation Chemistry*, vol. **5**, M Burton, JL Magee (eds). John Wiley and Sons, Ltd: New York, p. 185.

Inoue T (2003) Introduction: Toxicogenomics – a New Paradigm of Toxocology. In *Toxicogenomics*, T Inoue, WD Pennie (eds). Springer-Verlag: Tokyo, pp. 3–11.

Kong M, Fox CJ, Mu J, Solt L, Xu A, Cinalli RM, Birnbaum MJ, Lindsten T, Thompson CB (2004) The PP2A-associated protein alpha4 is an essential inhibitor of apoptosis. *Science* **306**, 695–698.

Kroll TC, Wolfl S (2002) Ranking: a closer look on globalisation methods for normalisation of gene expression arrays. *Nucleic Acids Res* **30**, e50.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W *et al.* (2001) Initial sequencing and analysis of the human genome. *Nature* **409**, 860–921.

Laursen LV, Bjergbaek L, Murray JM, Andersen AH (2003) RecQ helicases and topoisomerase III in cancer and aging. *Biogerontology* **4**, 275–87.

Lewin, B (2004) *Genes VIII*. Pearson Prentice Hall, Upper Saddle River, NJ.

Liu Q, Zhao X, Frissora F, Ma Y, Santhanam R, Jarjoura D, Lehman A, Perrotti D, Chen CS, Dalton JT, Muthusamy N, Byrd JC (2007) FTY720 demonstrates promising pre-clinical activity for chronic lymphocytic leukemia and lymphoblastic leukemia/lymphoma. *Blood* **111**, 275–284.

Lodish H, Berk A, Matsudaira P, Kaiser C, Krieger M, Scott M, Zipursky SL, Darnell J (2004) *Molecular Cell Biology*. WH Freeman: New York.

Lovett RA (2000) Toxicogenomics. Toxicologists brace for genomics revolution. *Science* **289**, 536–537.

Manohar A, Shome SG, Lamar J, Stirling L, Iyer V, Pumiglia K, DiPersio CM (2004) Alpha 3 beta 1 integrin promotes keratinocyte cell survival through activation of a MEK/ERK signaling pathway. *J Cell Sci* **117**, 4043–4054.

Nakayama T, Yoshizaki A, Izumida S, Suehiro T, Miura S, Uemura T, Yakata Y, Shichijo K, Yamashita S, Sekin I (2007) Expression of interleukin-11 (IL-11) and IL-11 receptor alpha in human gastric carcinoma and IL-11 upregulates the invasive activity of human gastric carcinoma cells. *Int J Oncol* **30**, 825–833.

Neviani P, Santhanam R, Oaks JJ, Eiring AM, Notari M, Blaser BW, Liu S, Trotta R, Muthusamy N, Gambacorti-Passerini C, Druker BJ, Cortes J, Marcucci G, Chen CS, Verrills NM, Roy DC, Caligiuri MA, Bloomfield CD, Byrd JC, Perrotti D (2007) FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia. *J Clin Invest* **117**, 2408–2421.

Oh M, Choi IS, Park SD (2002) Topoisomerase III is required for accurate DNA replication and chromosome segregation in *Schizosaccharomyces pombe*. *Nucleic Acids Res* **30**, 4022–4031.

Petersen D, Chandramouli GV, Geoghegan J, Hilburn J, Paarlberg J, Kim CH, Munroe D, Gangi L, Han J, Puri R, Staudt L, Weinstein J, Barrett JC, Green J, Kawasaki ES (2005) Three microarray platforms: an analysis of their concordance in profiling gene expression. *BMC Genomics* **6**, 63.

Rahmouni S, Cerignoli F, Alonso A, Tsutji T, Henkens R, Zhu C, Louis-dit-Sully C, Moutschen M, Jiang W, Mustelin T (2006) Loss of the VHR dual-specific phosphatase causes cell-cycle arrest and senescence. *Nat Cell Biol* **8**, 524–531.

Schadt EE, Li C, Ellis B, Wong WH (2001) Feature extraction and normalization algorithms for high-density oligonucleotide gene expression array data. *J Cell Biochem Suppl* **37**, 120–125.

Seki M, Yoshida K, Nishimura M, Nemoto K (1991) Radiation-induced myeloid leukemia in C3H/He mice and the effect of prednisolone acetate on leukemogenesis. *Radiat Res* **127**, 146–149.

Singer M, Berg P (1991) *Genes & Genomes: A Changing Perspective*. University Science Books: Mill Valley, CA.

Storck T, von Brevern MC, Behrens CK, Scheel J, Bach A (2002) Transcriptomics in predictive toxicology. *Curr Opin Drug Discov Dev* **5**, 90–97.

Stoyanova R, Querec TD, Brown TR, Patriotis C (2004) Normalization of single-channel DNA array data by principal component analysis. *Bioinformatics* **20**, 1772–1784.

Tomlinson VA, Newbery HJ, Bergmann JH, Boyd J, Scott D, Wray NR, Sellar GC, Gabra H, Graham A, Williams AR, Abbott CM (2007) Expression of eEF1A2 is associated with clear cell histology in ovarian carcinomas: overexpression of the gene is not dependent on modifications at the EEF1A2 locus. *Br J Cancer* **96**, 1613–1620.

Tsai HJ, Huang WH, Li TK, Tsai YL, Wu KJ, Tseng SF, Teng SC (2006) Involvement of topoisomerase III in telomere–telomere recombination. *J Biol Chem* **281**, 13717–13723.

Tsimanis A, Shvidel L, Klepfish A, Shtalrid M, Kalinkovich A, Berrebi A (2001) Over-expression of the functional interleukin-11 alpha receptor in the development of B-cell chronic lymphocytic leukemia. *Leuk Lymphoma* **42**, 195–205.

Varis A, Wolf M, Monni O, Vakkari ML, Kokkola A, Moskaluk C, Frierson Jr H, Powell SM, Knuutila S, Kallioniemi A, El-Rifai W (2002) Targets of gene amplification and overexpression at 17q in gastric cancer. *Cancer Res* **62**, 2625–2629.

Wassenhove-McCarthy DJ, McCarthy KJ (1999) Molecular characterization of a novel basement membrane-associated proteoglycan, leprecan. *J Biol Chem* **274**, 25004–25017.

Waterston RH, Lindblad-Toh K, Birney E, Rogers J, Abril JF, Agarwal P, Agarwala R, Ainscough R, Alexandersson M, An P *et al.* (2002) Initial sequencing and comparative analysis of the mouse genome. *Nature* **420**, 520–562.

Watson JD, Baker TA, Bell SP, Gann A, Levine M, Losick T (2007) *Molecular Biology of the Gene*. Pearson/Benjamin Cummings: San Francisco, CA.

Watson JD, Crick FH (1953) Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* **171**, 737–738.

Yoshida K, Inoue T, Nojima K, Hirabayashi Y, Sado T (1997) Calorie restriction reduces the incidence of myeloid leukemia induced by a single whole-body radiation in C3H/He mice. *Proc Natl Acad Sci U S A* **94**, 2615–2619.

Yoshizaki A, Nakayama T, Yamazumi K, Yakata Y, Taba M, Sekine I (2006). Expression of interleukin (IL)-11 and IL-11 receptor in human colorectal adenocarcinoma: IL-11 up-regulation of the invasive and proliferative activity of human colorectal carcinoma cells. *Int J Oncol* **29**, 869–876.

Yu L, Liu C, Vandeusen J, Becknell B, Dai Z, Wu YZ, Raval A, Liu TH, Ding W, Mao C, Liu S, Smith LT, Lee S, Rassenti L, Marcucci G, Byrd J, Caligiuri MA, Plass C (2005) Global assessment of promoter methylation in a mouse model of cancer identifies ID4 as a putative tumor-suppressor gene in human leukemia. *Nat Genet* **37**, 265–274.

Yun K, Mantani A, Garel S, Rubenstein J, Israel MA (2004) Id4 regulates neural progenitor proliferation and differentiation *in vivo*. *Development* **131**, 5441–5448.

Yunis JJ, Soreng AL, Bowe AE (1987) Fragile sites are targets of diverse mutagens and carcinogens. *Oncogene* **1**, 59–69.

Zhang W, Shmulevich I (2006) *Computational and Statistical Approaches to Genomics*. Springer Science+Business Media: New York.

Zurita AJ, Troncoso P, Cardo-Vila M, Logothetis CJ, Pasqualini R, Arap W (2004) Combinatorial screenings in patients: the interleukin-11 receptor alpha as a candidate target in the progression of human prostate cancer. *Cancer Res* **64**, 435–439.

The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.33, No.4, 389-403, 2008

Review

Low-dose effects of bisphenol A: a serious threat to human health?

Jun Sekizawa

Faculty of Integrated Arts and Sciences, University of Tokushima, 1-1 Minami-josanjimamachi, Tokushima-shi, Tokushima 770-8502, Japan

(Received June 30, 2008)

ABSTRACT — The author tried to review and summarize low-dose effects of endocrine disrupting chemicals (EDCs) through an extensive literature survey of toxicological studies with bisphenol A (BPA), taking BPA as an example for which many studies were published. Data on low-dose effects with BPA, especially on neurobehavioral effects after fetal or early postnatal exposures, suggested that there would be new aspects to be considered. Specific mention for future tasks was made. Firstly, toxicity tests should be designed with more elaboration to ensure a sufficient number of animals with careful handling of litters to allow adequate statistical analysis and appropriate selection of dosages to obtain insight in dose-response relationship. Secondly, precise measurement of plasma levels in both humans and rodents and construction of relevant physiologically-based pharmacokinetic models would help obtain quantitative estimates of intake and target-organ exposure relationship. Thirdly, biological backgrounds, particularly differences and similarities in endocrinological, neurological and immunological aspects among species, should be revisited. Fourthly, mechanistic deliberations on the possibilities of epigenetic mechanism and examinations of putative neurobehavioral effects or a presumptive link of miscarriage with BPA exposures are requested. Finally, general public concerns must be addressed in a thoughtful way so that a simple precautionary approach is not pursued, but uncertainties of the new toxicological aspects should be carefully explained. Further researches and internationally concerted efforts on elucidating risk of low-dose effects by integrating knowledge will contribute to setting new directions in toxicology and improving chemical risk assessments.

Key words: Low-dose effects, Endocrine disrupting chemicals, Bisphenol A, Human health risk, Exposure estimate

INTRODUCTION

Early descriptions of low-dose effects by endocrine-disrupting chemicals

From inception of early warning by Rachel Carson in 1962 depicted in "Silent Spring" of the potential adverse effects of chemicals to human health and wildlife, many researches have been conducted to answer questions and resolve concerns in this direction. The term, endocrine-disrupting chemicals (EDCs), was first introduced at the Wingspread conference in 1991 as a new concept in toxicology for the changes which are induced by chemicals through disturbance in regulation of endocrinological actions. One of the intriguing claims is that EDCs, like hormones, exert their effects at very low dose levels but those effects may not be observed at relatively high dose because of a homeostatic mechanism. EDCs may cover a variety of chemical classes including hormones, plant constituents, pesticides, compounds used in the plastics industry and other industrial by-products and pollutants. Bisphenol A (BPA) was named as one of the EDCs for its weak estrogenic action shown in *in vitro* assays, and putative low-dose effects of BPA raised much concern, for its wide use in consumer products and in dental therapy, etc.

In line with the notion of EDC, vom Saal *et al.* (1997) reported that fetal exposure to low doses of estradiol or diethylstilbestrol caused prostate enlargement in mice, while they had opposite effects at high doses. Furthermore, they reported that BPA, when dosed to female mice for gestation day 11-17, permanently increased preputial gland size, but reduced epididymides size at 2 μg/kg body weight, and significantly decreased sperm production at

Correspondence: Jun Sekizawa (E-mail: sekizawa@ias.tokushima-u.ac.jp)

20 μg/kg body weight, presumably through its weakly estrogenic activity (vom Saal *et al.*, 1998).

Endocrine disruptors low-dose peer review by National Toxicology Program

In October 2000, National Toxicology Program (NTP)/ National Institute of Environmental Health Sciences (NIEHS) organized a workshop and conducted a scientific peer review of the reported low-dose reproductive and developmental effects at the request of the USA Environmental Protection Agency (EPA). Briefly, they concluded that low-dose effects were demonstrated in laboratory animals exposed to certain endocrine active agents, although the effects were dependent on the compounds studied and the endpoint measured, and in some cases the findings could not be replicated (NTP, 2001). Since the traditional multigeneration reproduction study protocol has not revealed major reproductive or developmental effects in rodents at doses approaching lowest-observed-adverse-effect level (LOAEL, see below) observed by the standard testing paradigm, they indicated that the classical testing paradigm used for reproductive and developmental toxicity should be revisited to see if changes are needed regarding dose selection, animal model selection and age of the animals for the exposure to endocrine active agents.

They further reported that several studies provided credible evidence for low-dose effects of BPA, such as the increased prostate weight in male mice at six months of age after *in utero* exposure to 2 μg/kg/day, as compared to the LOAEL estimated by EPA for oral exposure to BPA of 50 mg/kg/day based on the NTP chronic toxicity study in rats (US EPA, 2008). A clear difference, however, was observed in sensitivity between strains of rats, e.g., F344 and Sprague-Dawley in occurrence of low-dose effects in uterine growth and in serum prolactin levels, and specific comments were made that not only differences in genetic backgrounds of the strains, but also different diets with different background contamination by phytoestrogens etc. might have contributed to the differences in hormonal levels in animals resulting in the discrepancies in experimental outcomes, and that additional research should clarify uncertainties related to the low-dose effects of BPA (NTP, 2001).

Global assessment by International Programme on Chemical Safety

In 1997, International Programme on Chemical Safety (IPCS) of the World Health Organization convened a group of international scientists to assess the state-of-the-science of EDCs and prepared a global assessment report (IPCS, 2002). They discussed issues of mechanism of action, dose-response relationships, effects in wildlife and to humane health, exposure to selected potential EDCs, and causal criteria and weight- of-evidence framework for the effects resulting from exposure to EDCs. They considered that endocrine disruption is not a toxicological endpoint per se but a functional change that may lead to adverse effects, and defined EDC as "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organisms, or in progeny, or (sub)populations", and discriminated a potential EDC from EDC, as "an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in intact organisms, or in progeny, or (sub)populations."

A framework was proposed based on the aspects of temporality, strength of the association, consistency of the observations, biological plausibility and evidence of recovery. Five areas of the further efforts and researches, namely biology underlying endocrine-mediated effects, methodology, monitoring, identification of EDCs and database development, were considered of high priority. The IPCS group stressed the importance of understanding endocrine systems and mechanistic considerations in evaluating effects of EDCs. For example, (i) normal homeostatic mechanisms (e.g., down-regulation of receptor expression) may compensate possible effects in adulthood exposures, (ii) however, exposure during the period of programming of the endocrine system in progress (critical windows of exposure) may result in permanent change of function or sensitivity to stimulatory/inhibitory signals, (iii) exposure to the same level of an endocrine signal at different stages in life history may produce different effects, (iv) because of cross talk between different endocrine systems, effects may occur unpredictably, (v) and therefore, considerable caution should be exerted in extrapolating *in vitro* measures of hormonal activity to the situation *in vivo*. IPCS further mentioned that several field observations in wildlife, such as reproductive and immune dysfunction in Baltic seals, or eggshell thinning and altered gonadal development in birds, might be related to exposure to organochlorines (polychlorinated biphenyls: PCBs, 2,2-di-(p-chlorophenyl)-1,1-dichloro-ethylene: DDE) and 1,1,1-trichloro-2,2-di-(p-chlorophenyl)ethane (DDT), although precise mechanisms remained unclear, and that masculinization and decline of populations of marine gastropods was more clearly related to exposure to organotin compounds such as tributyltin compounds (TBT) used in antifouling paints for boats.

Development of testing guidelines by Organisation for Economic Co-operation and Development

Organisation for Economic Co-operation and Development (OECD) developed a series of guidelines, which are relevant to testing chemicals that may have a potential to exert endocrine disrupting effects as follows: Test No. 415 for one-generation reproduction toxicity study (1983); No. 424 for neurotoxicity study (1997); No. 416 for two-generation reproduction toxicity study (2001); No. 414 for prenatal developmental toxicity study (2001); and No. 426 for developmental neurotoxicity study (2007). These guidelines constitute harmonized testing protocols and data reporting requirements to support meaningful comparisons of the data produced by various laboratories, especially where discrepancies were observed in the outcomes of tests with different experimental designs utilized in neuropathology or in behavioral studies.

TOXICOLOGICAL REVIEW OF LOW-DOSE EFFECTS OF BISPHENOL A

Literature survey of toxicological studies on bisphenol A

Bisphenol A (CAS No. 80-05-7) is a component of epoxy resins used to make hard plastic polycarbonate bottles and containers. Its production volume in Japan was 445 thousand tons in 2002 with 162 and 41 thousand tons for export and import, respectively (MOE, 2004).

There have been many studies on BPA focusing mostly related to presumed its endocrine disrupting potential. The total number of literature searched with the CAS No. 80-05-7 in PubMed till June 23, 2008 was 1,137, of which 1,029 were published in the period after 2000, indicating increasing concerns and active researches of this compound in recent years. As a part of the research project supported by the Ministry of Health, Labour and Welfare (MHLW) Japan, the author surveyed literature on BPA, especially with special interest on the potential low-dose effects of the compound, and developed a series of reports with the data books (in Japanese) on low-dose effects of BPA (Sekizawa, 2005-2009). Literature was searched using the CAS No. 80-05-7 in PubMed. Toxicological studies in animals and epidemiological studies for humans were searched, excluding those of analytical methods, effects to organisms in the environment, elution from dental resin formulations, etc. Human exposure data, and toxicokinetics and metabolism studies with humans and animals were searched, too. Also, major reviews by international organizations and national scientific bodies were referred to. Original literature was collected and the data were examined carefully with respect to study design, experimental conditions, data presentation and statistical analysis.

Selection of toxicological studies

No human studies were available which adequately related the observed effects with BPA exposures. Studies of rats and mice, with which most of studies were conducted and plenty of background information was available, were examined, but studies with other experimental animals were excluded. Table 1 summarizes the low-dose effects data in the order of the LOAELs from low to high up to 2.5 mg/kg/day (the reason for cutoff for higher doses will be found below). Although there are studies which showed no effects at low-dose exposure levels from various reasons, they are not named specifically in this review. For example, when two generations of rats were gavaged with 0, 0.0002, 0.002, 0.020, or 0.200 mg/kg/day BPA prior to and during mating and throughout the gestation and lactation period, none of the observed effects covering wide ranges of developmental or reproductive endpoints were related to BPA treatment despite careful experimental design and data treatment (Ema *et al.*, 2001). For details and discussion of each study, readers are recommended to refer to either original literature or reviews of governmental and international bodies cited in this review, and later sections, respectively. Since human exposure to BPA is known to be overwhelmingly through the oral route (NTP, 2008), those studies which used gavage or oral exposure via food or drinking water were considered relevant in evaluating human health implications; and only data of gavage or oral exposure studies were examined for low-dose effects and those from other routes of exposure were excluded. Endpoints considered here are structural or functional disorders which have implications to health, and biochemical changes in hormone levels or effects to gene expressions which are difficult to be interpreted as disorders are not included as hazards observed at low doses. For statistical analysis, the number of animals per group etc. was checked; however the author relied in general on the recent judgment made by the expert group of Center for the Evaluation of Risks to Human Health Reproduction (CERHR) in this aspect (CERHR, 2007). The adequacy of the studies in examining low-dose effects determined by the group of CERHR experts is also indicated in the table.

Criteria of low-dose effects in this review

European Union (EU) established a Tolerable Daily Intake (TDI) of 0.05 mg/kg/day applying an uncertainty factor of 100 for the no-observed-adverse-effect

Table 1. Summary of Low Dose Effects

	Toxicity profile *1	Animal species (strain, sex)	Dose: μg/kg/day, (number/group)	Exposure route and period	Observed Effects	LOAEL (NOAEL) μg/kg/day	Note: *2 (comment)	Statistical analysis	Reference
1	D-M	rats (Wistar, male)	0, 0.2, 2, 20 (4/group)	gavage, PND 45-90	Reduced absolute and relative (to body weight) weights of testis and epididymis, increased ventral prostate weights at all dose levels. Decreased sperm motility. Dose-dependent decrease of antioxidant enzyme activities,	0.2	Oxidative stress was hypothesized as a factor to cause effects on male reproduction function: L (small group size)	ANOVA with student t-test	Chitra *et al.*, 2003
2	D-B	rats (Spurague-Dawley, male)	0, 0.4 (12/group)	gavage, PND 23-30	Juvenile behaviors directed to the object (biting, sniffing, climbing) at lower frequency. Decreased intromission latency and the lower plasma testosterone level on PND 37 and PND 105.	0.4	Single dose, Behavioral effects interpreted as consistent with estrogenic mediation: H	ANOVA and Fisher least significant difference test	Della Seta *et al.*, 2006
3	D	mice (C57BL/6N, female)	0, 2, 20, 200 (25/group)	gavage, GD 11-17	Decrease in absolute seminal vesicle weight at 2 μg/kg/day only.	2	Exposures at juvenile or adult stage did not induce effects, and significant effects seen only at 2 μg/kg/day only: H	Bartlett's test with ANOVA when homogeneous	Nagao *et al.*, 2002
4	D-M	mice (CF-1, female)	0, 2, 20 (7/group)	gavage, GD 11-17	Prostate weights 30% higher in 6-month-old males	2	Soy phytoestrogens in the diet and no positive control, small sample size, no histopatholgical analysis: H	ANCOVA with body weight as the covariate	Nagel *et al.*, 1997
5	D	rats (Long Evans, female & male)	0, 2.4, 10, 10^5, $2x10^5$ (Exp#1), 0, 2.4 (Exp#3) (10-12/group)	gavage, PND 21-35 (Exp#1), PND 21-90 (Exp#3)	Reduced serum 17β-estradiol level (Exp#1). Reduced seminal vesicle weight and testicular testosterone level (Exp#3).	2.4	Effects observed by exposures at early postnatal days: H	ANOVA with multiple comparisons conducted by Duncan multiple range test	Akingbemi *et al.*, 2004
6	R	mice (CF-1, female)	0, 2.4 (21/group)	feeding, GD 11-17	Increase in body weight at weaning in pups. A significant decrease in time to first estrous according to intrauterine position. Reduction in pup survival between birth and weaning.	2.4	Single dose: L	Analyzed on a litter basis to control for maternal effects, statistical method not clear	Howdeshell *et al.*, 1999
7	D-B	mice (CD-1, female)	0, 10 (8/group)	gavage, GD 14-18 as fetus and/or GD 14-18	Decreases of the time dams spent for nursing and increases of the time dams spent in the nest for resting.	10	Single dose. Not clear why exposure during fetal development or as a dam only affected maternal nursing behavior, but did not by exposures in both period: H	ANOVA, Holms t-test, and/or Fisher protected least-squared difference test.	Palanza *et al.*, 2002

Table 1. (Continued)

	Toxicity profile *1	Animal species (strain, sex)	Dose: μg/kg/day, (number/group)	Exposure route and period	Observed Effects	LOAEL (NOAEL) μg/kg/day	Note: *2 (comment)	Statistical analysis	Reference
8	D-B (SD)	mice (CD-1, female)	0,10 (5/sex/group)	gavage, GD 11-18	Decrease in d-amphetamine-inforced conditioned place-preference in females specifically.	10	Single dose. Effect was sexually dimorphic and male was not affected: H	ANOVA (litter treated as block variable), Tukey HSD test.	Laviola *et al.*, 2004
9	D	mice (CD-1, female)	0, 10 (6/group)	gavage, GD 14-18	Increase in the numbers of prostate ducts, volume, and proliferation in prostate regions.	10	Single dose: H (small sample size)	ANOVA, followed by Fisher least-squares mean test	Timms *et al.*, 2005
10	D-B (SD)	rats (Wistar, male and female)	0, 15 (6/group)	drinking water, GD 13-PND 0	Impairment in sexual differentiation in rearing and struggling behavior.	15	Single dose. Observation in disruption in sexually dimorphic behaviors: I (small sample size and statistical treatment of litter not clear)	ANOVA, Fisher protected least significant difference test, and Mann-Whitney U test.	Fujimoto *et al.*, 2006
11	D-B/M (SD)	rats (Wistar, female)	0, 15, 150 (6 dams/group and 7-8/test group):	drinking water, GD 0-PND 21	Sexually dimorphic behavior in frequency of rearing and moved distance disappeared and also sexually dimorphic volume of locus ceruleus was reversed by the treatment.	15	Dose method not stated and actual dose not accurately estimated: I doses re-estimated from the above data of the same laboratory, (small sample size and statistical treatment of litters not clear)	Behavior and brain structure data by ANOVA and differences between sexes by Student t-test	Kubo *et al.*, 2003
12	D-B (SD)	rats (Sprague-Dawley, female)	0, 15, 750 (8-9/group)	drinking water, GD 11-PND 21	Hyperactivity in open field test (at 6 week) and impaired spatial learning/memory in Morris water maze test (at 10 week) in males pups with significant up-regulation of mRNA and protein expression of SRC-1, but not in female pups.	15	Open field test performed with 13-17/group. Number of animals in Morris water maze test not shown	one-way ANOVA for each sex, and Student's t-test between males and females then Fisher's protected least significant difference test for each sex	Xu *et al.*, 2007
13	R	rats (Sprague Dawley, male)	0, 20, 200, $2x10^3$, $2x10^4$, $2x10^5$ (5 or 8/group)	gavage, 13-week old dosed for 6 days	Decrease in daily sperm production/g tissue 24-32% at 20 and above two doses.	20	Short treatment period compared as to sperm production variation: L (small sample size)	Student *t*-test, ANOVA, and Fisher protected least significant difference test	Sakaue *et al.*, 2001
14	R-M	mice (Swiss, 60-day-old male)	0, 5, 25, 100 (10/group)	gavage, for 30 days	Increase in relative testis weight, and decrease in seminal vesicle weight, testicular sperm counts and daily sperm production. Percentage of pregnant females reduced when mated with treated males	25 (5)	Small sample sizes for fertility assessments, inadequate coverage of spermatogenesis cycle, and inadequacy in statistical analysis: L	Student t-test	Al-Hiyasat *et al.*, 2002

Table 1. (Continued)

	Toxicity profile *1	Animal species (strain, sex)	Dose: μg/kg/day, (number/group)	Exposure route and period	Observed Effects	LOAEL (NOAEL) μg/kg/day	Note: *2 (comment)	Statistical analysis	Reference
15	D-B	rats (Sprague Dawley, female)	0, 40 (17/group)	gavage, GD 0-PND 21	Decrease in licking and grooming of pups.	40	Single dose: H	Analysis by general linear model, Duncan multiple range test, and/or Mann- Whitney U test.	Della Seta *et al.*, 2005
16	D-M	mice (CD-1, female)	0, 50 (15/group)	feeding, GD 16-18	Increase in relative prostate weight, and anogenital distance, androgen receptor binding activity at PND 60, and decrease in relative epididymis weight.	50	Single dose. Litter effects not controlled among PND examinations: H	ANOVA	Gupta, 2000
17	D-B	rats (Fisher 344, female)	0, 100 (10-11/group)	gavage, GD 3-PND 20.	Increase in failure of active avoidance, and no increase in locomotion following a challenge with trans-2-phenylcyclopropylamine hydrochloride.	100	Single dose. Irreversible defect in perception of fear-provoking stimuli and monoaminergic neural pathways in male offspring: H	ANOVA, and Fisher protected least significant difference test	Negishi *et al.*, 2004
18	D	rats (Sprague Dawley, female)	0, 100, $5x10^5$ (6/test group)	gavage, GD 6-21	Increase in epithelial cell nuclei, epithelial nuclei with condensed chromatin, and epithelial cells with cavities. Reduced ERβ-positive cells in uterine tissue.	100	Female offspring examined at 4 months of age on the day of estrus: H	Mann-Whitney test	Schönfeld-er *et al.*, 2002a
19	D-B (SD)	mice (C57BL/6, female)	0, 2, 200 (14/group)	gavage, GD 13-PND 21	Early puberty, and increase in anxiety based on time in open arms of plus maze and in light part of light/dark preference box in female at high dose.	200 (2)	Measurements in the established sexually dimorphic behavioral test: H	ANOVA with post-hoc Student t-test (adequate sample size)	Ryan and Vandenbergh, 2006
20	D-M (SD)	rats (Wistar, female)	0, $2.5x10^3$ (8-11/group)	drinking water, GD 0-PND 21	A loss in sex difference in corticotropin-releasing hormone neurons in the anterior and posterior bed nuclei of the stria terminalis of the stria terminalis.	$2.5x10^3$	Single dose. One rat/sex/litter appeared to be examined: L	ANOVA with Fisher protected least significant difference post-hoc test	Funabashi *et al.*, 2004

*1 D: Developmental effect, M: Morphological effect, B: Behavioral, SD: Change in sexual dimorphism or sexual difference in the effects

*2 CERHR evaluation of utility (adequacy) of studies with the reasons stated in the "Note" column. H: High L: Low I: Inadequate

level (NOAEL) which was derived from a comprehensive three-generation study in rats in which the lowest dose with the observed effects was 50 mg/kg/day (Tyl, 2002) and the NOAEL was considered to be 5 mg/kg/day (EFSA, 2006). A recent two-generation reproductive toxicity study in mice conducted following the OECD test guideline 416 (OECD, 2001a) and in compliance with Good Laboratory Practice (GLP) gave the same LOAEL of 50 mg/kg/day for the increased incidence of centrilobular hepatocyte hypertrophy (Tyl, 2008; first published in 2006 as a draft final report). In the past, EU established a temporary TDI of 0.01 mg/kg/day (SCF, 2002) applying a 500-fold uncertainty factor, comprised of 10 for inter-species differences, 10 for inter-individual differences and 5 for uncertainties in the database on reproductive and developmental toxicity. The last factor of 5 is considered no longer required because the database has been significantly strengthened, and the European Food Safety Authority (EFSA) has revised the TDI to 0.05 mg/kg/day (EFSA, 2006). US EPA set oral reference dose (RfD) or called as TDI in other organizations, of 0.05 mg/kg/day in 1993, based on a LOAEL of 50 mg/kg/day for the reduced mean body weight in early weeks of exposure in a rat chronic toxicity study conducted by NTP (1982), applying an uncertainty factor of 1,000 (10 for inter-species differences, 10 for inter-individual differences and another 10 for uncertainty in the effects of duration on toxicity), and this RfD has been maintained (US EPA, 2008). Overall, a NOAEL of 5 mg/kg/day has been adopted in the evaluations by different agencies, and thus this dose level is used as a tentative cutoff dose for the low-dose effects in this review.

Outcome of toxicological study review

At least 20 studies (11 in rats and 9 in mice) which were regarded reporting low-dose effects are found (Table 1). Of those, 17 studies reported developmental effects and 3 studies reported reproductive effects. It is striking that 18 reports of low-dose effects out of 20, were observations made on pups from dams exposed during pregnancy and/or early post-natal days, which indicates that there might be critical windows of exposure for low-dose effects in developmental or reproductive effects. Out of 17 studies on developmental effects, 9 studies reported behavioral effects in pups that resulted from maternal exposures during pregnancy and/or early post natal exposures. Three studies noted impairment in sexual differentiations either morphologically in organs or in behavior, or both. Regrettably, 10 low-dose studies were conducted using only a single dose level and sometimes with a small number of animals, and therefore, it was difficult to assess dose-response relationship with the effects, and also statistical significance was challenged. According to CERHR (2007), twelve, five and two studies in the table were regarded as adequate and of high utility, of limited utility, and inadequate for the evaluation of low-dose effects, respectively (see Note column in Table 1). It appears there is not much specificity in low-dose effect expressions among species or strains tested.

Fig. 1 shows the distribution of dose levels with regard to effects in the order of effect levels from low to high, with observed effects and animal species in the horizontal axis and the dose level in the ordinate axis. In 9 out of 20 studies, effects were observed at doses lower than 10 μg/kg/day. In summary, although there are still controversies with the low-dose effects, it can be said at least that many evidences suggesting low-dose effects, especially of developmental effects after perinatal exposures, have been compiling recently.

EXPOSURE ESTIMATION

Exposure estimate by international or national organizations

The primary source of human exposure to BPA is considered via diet through contamination of the plastic monomer eluted from various sources (NTP, 2008). Human exposure has been estimated by direct measurement of human tissue samples, such as serum, breast milk, urine, or from dietary studies where concentrations in food and beverages were measured and an aggregate exposure was calculated by multiplying food and drinking water consumption data. Exposure estimates by several organizations are summarized in Table 2.

Exposures for infants, children and adults were estimated to be 1, 0.04-0.07, and 0.026 μg/kg/day, respectively as an aggregate of exposures from various sources through the studies performed in the USA, Europe and Japan (CERHR, 2007). When the intake was back calculated from urine data based on toxicokinetic studies (see below) on BPA, the average intake values for females and males were 0.044 and 0.057, respectively (NTP, 2008).

Scientific panel on "food additives, flavourings, processing aids and materials in contact with food" of the EFSA assumed conservative scenarios in estimating the aggregate exposure from various sources, and thus, relatively high values are presented in the table (EFSA, 2006). They estimated that the potential exposure of breastfed babies would be 0.1 μg/kg/day, considering a daily intake of 174 ml/kg bw of human breast milk and mean concentrations of 0.61 μg/l in the breast milk of lactating women in Japan (Sun *et al.*, 2004). The dietary exposure of

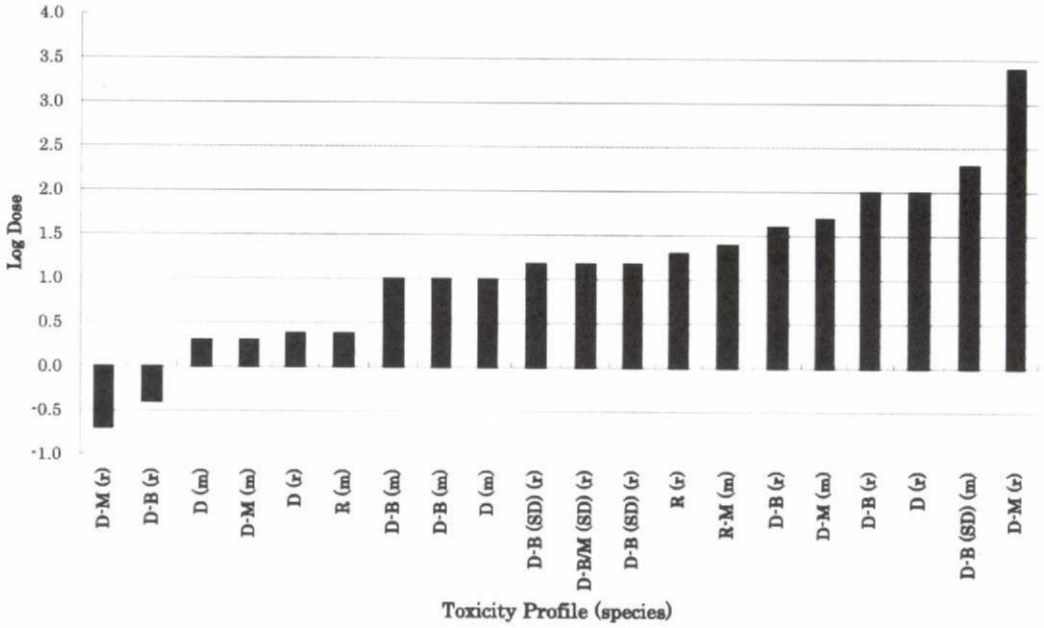

Fig. 1. D: Developmental effects, R: Reproductive effects, M: Morphological, B: Behavioral, SD: Effects on sexual dimorphism r: rats, m: mice

3-month infants weighing on average 6.1 kg and consuming 174 ml/kg bw/day of infant formula, was estimated to be 1.7 μg/kg/day (based on a migration of 10 μg/l from epoxy-phenolic coated cans), and this with 2.3 μg/kg/day from canned powdered infant formulae based on German DONALD study (Kersting, 1998), is summed up to be 4 μg/kg/day. The potential exposure in infants of 6-12 months (average weight 7.8 kg) was estimated to be 8.3 μg/kg/day based on 10μg/l migration from the infant formulae with 13μg/kg/day for the highest leaching estimate from polycarbonate (PC) bottles, and that in 1.5-year-old children consuming 2 kg commercial foods/beverages was 5.3 μg/kg/day (4.4 μg/kg/day from canned foods and beverages with 0.9 μg/kg/day from PC tableware and food storage container), while that in adults consuming 3 kg commercial foods/beverages was much lower, i.e., 1.5 μg/kg/day (1.2 μg/kg/day from 1 kg of canned foods and 2 liters of canned beverages, and 0.25 μg/kg/day from PC tableware and food storage container).

NEDO/CRM-AIST (2005) made an estimation of potential human exposure, applying Monte Carlo simulation to the urine data in two Japanese studies with 36 young male adults or 22 adults (11 each of males and females). The exposures were estimated to be 0.028-0.049 μg/kg/day for male adults and 0.034-0.059 for female adults based on the assumption that the amount of BPA daily excretion in urine was considered equivalent to that of daily uptake. This assumption was based on the data that 97-118% of orally dosed deuterium-labeled BPA was recovered in urine as either free BPA or glucuronide. BPA glucuronide was cleared from human blood and excreted in urine with terminal half life of less than 6 hr (Völkel *et al.*, 2002).

Other exposure estimates

Concentrations of free-form (i.e., not conjugated) BPA were measured in the serum samples, placental tissues and umbilical cord from 37 pregnant mothers (32-41 weeks of gestation and after births) by gas chromatography (GC)/mass spectrometry (MS) after specific derivatization (Schönfelder *et al.*, 2002b). Median concentrations and ranges (in the bracket) were 2.3 ng/ml (0.2-9.2 ng/ml), 3.1 ng/ml (0.3-18.9 ng/ml), and 12.7 ng/g (1.0-104.9 ng/g) for fetal plasma, maternal plasma and placental tissue, respectively. BPA concentrations in fetal plasma were higher in males than in females. The data showed that placenta did not work as a barrier to BPA contamination from mothers, and male fetuses may be exposed to higher concentrations than females. Vandenberg *et al.* (2007) reviewed human serum concentration data of BPA measured by

Table 2. Summary of exposure estimates by several organizations

		Average body weight	CERHR [a], NTP [b]	EFSA [c]	NEDO/CRM-AIST [d]
			Average	Average	Average
		kg		μg/kg/day	
Breastfed babies			1 [a]	0.1 [e]	
Infants		4.5	1 [a]		
	0-4, 0-5 or 0-6 months	6.1 (3 months)		4 = 1.7 [f] + 2.3 [g]	0.055, 0.062 [d1]
	6-11 or 6-12 months	7.8		8.3 [g]	0.18, 0.20 [d1]
	1.5 years			5.3 = 4.4 [h] + 0.9 [i]	
Children					
	1.5-5 or 1-6 years		0.04-0.07 [a]		1.2, 1.2 [d2]
Adults			0.026 [c]	1.5 = 1.2 [h] + 0.25 [i]	0.19, 0.23 [d2]
	Female		0.044 [b]		0.034-0.059 [d2]
	Male		0.057 [b]		0.028-0.049 [d2]

a: Estimates from intake data
b: Back calculation from urinary concentrations; NTP (2008)
c: Based on typical simulation scenarios EFSA (2006)
d: Based on typical simulation scenarios NEDO/CRM-AIST (2005)
d1: Estimates from aggregate exposures, male and female
d2: 95-percentile value of back calculation from urinary concentrations
Sources of contamination
e: breast milk, f: epoxy-phenolic coated cans, g: canned powdered infant formulae, h: canned foods + beverages,
i: polycarbonate tableware and food containers

GC after derivatization, GC/MS, high performance liquid chromatography (HPLC) or enzyme-linked immunosorbent assay (ELISA) to be in the range of 0-4.4 ng/ml (the highest value observed by derivatization-GC/MS) and the central measure of the distribution in the range of 0.3-4.4 ng/ml. Dekant and Völkel (2008) reviewed biomonitoring methods and discussed values of free BPA in serum were much higher than expected from the toxicokinetic study in which 54-90 μg/kg bodyweight (10-fold higher than worst case estimates for daily indirect human exposures to BPA), were dosed (Völkel *et al.*, 2002). When d_{16}-BPA was orally administered to 9 human subjects (3 female and 6 male ages from 24 to 57), elimination was complete within 24-hr with a terminal half-life in blood of 5.3 hr. Peak blood concentrations of BPA-glucuronide measured either by GC/MS or Liquid chromatography (LC)/MS reached a concentration of approximately 150-200 ng/ml at 81 min after administration, while free BPA concentrations were below 2.3 ng/ml (Völkel *et al.*, 2002). In a case-control study comparing women with recurrent spontaneous abortions (n = 45) to healthy nulligravid women (n = 32) from the same city in Japan, mean values for BPA in patients and control, were 2.59 ng/ml and 0.77 ng/ml, respectively, however median BPA concentrations were identical for cases and control (0.71 ng/ml) (Sugiura-Ogasawara *et al.*, 2005).

Vandenberg *et al.* (2007) estimated blood levels of free BPA of rats and mice after a single oral administration at 50 μg/kg (US EPA RfD level) from 11 acute metabolic studies of higher dose exposures in which measurement of free BPA was reported. Their estimation indicated that peak concentrations in the first 30 min, and at 1 hr after dosing, would be 0.11 ng/ml and 0.047 ng/ml (median), respectively, which were lower than the BPA blood levels measured in humans above. This is apparently contradicting to the report of more rapid metabolism and clearance of BPA in humans than in rodents (Teegurden *et al.*, 2005); and the estimated human exposure could become well above 500 μg/kg/day, if the human serum levels can be a few ng/ml level.

IMPLICATIONS TO HUMAN HEALTH

Recent reviews by governmental and international bodies, etc.

Government of Canada initiated on April 19, 2008,

a 60-day public comment period on whether to ban the importation, sale and advertisement of baby bottles which contain BPA (Canada, 2008). Recently, US CERHR/NTP issued a report on reproductive and developmental toxicity of BPA based on the review by the CERHR expert panel (CERHR, 2007). They summarized that no data on human developmental exposure to BPA are available, but rodent studies suggest that BPA causes neural and behavioral alterations related to disruptions in normal sex differences in rats and mice at doses of 0.01-0.2 mg/kg/day. There are insufficient data to evaluate whether BPA causes male or female reproductive toxicity in humans. However there is sufficient evidence in rats and mice that BPA causes female and male reproductive toxicity with subchronic or chronic exposures at relatively higher doses, i.e., with a NOAEL of 47.5 mg/kg/day in females and 4.75 mg/kg/day in males. NTP (2008) has drafted NTP brief on BPA based on the report of CERHR (2007) for a review at the Meeting of NTP Board of Scientific Counselors in June, and the final brief will be released as a part of the NTP-CERHR Monograph on BPA in late summer 2008. Based on human observations and laboratory animal study, they concluded that there is some concern for neural and behavioral effects in fetuses, infants, and children at current human exposures. The NTP (2008) also has some concern for BPA exposure in these populations based on effects in the prostate gland, mammary gland and an earlier age for puberty in females (Table 3). *1,2 (See NOTE)

What are the critical data gaps?

Most low-dose studies were conducted with a single exposure only, which makes it difficult to analyze dose-response relationship. Since the testing guideline for developmental neurotoxicity was developed by OECD (2007), it is critical to examine reproducibility and/or credibility of the data in accordance with the internationally harmonized protocol. Though there are criticisms on the study design, experimental conditions and statistical analysis of the existing studies, it may be useful to carefully examine the possibility of effects in neonates or risk of developmental effects which may be detected in the later stages of life, as NTP (2008) noted in their draft.

It is very important to confirm human serum concentrations of BPA as well as the serum concentrations in rodents when effects are observed at low doses, because the human serum data are apparently contradicting the estimated values from human intake data. The serum levels in humans have been assumed to be much lower than in rodents since it has been claimed that the effects would not be significant even if humans are exposed to the same amount from food, because of more efficient clearance of BPA in humans than in rodents. More precise understanding is required of pharmacokinetics and metabolism of BPA in humans and rodents as well as the key factors, if any, which may differentiate toxic effects between humans and rodents.

Since it was claimed that BPA can be a potent meiotic aneugen to elicit detectable meiotic effects in the female mouse after a short-term, low-dose exposure during the final stages of oocyte growth (Hunt *et al.*, 2003), and aneuploidy is the leading cause of spontaneous abortion in humans (accounting for 30-60% of spontaneous abortions), the presumptive link between BPA exposure and miscarriage as mentioned above, must be examined by a well-designed study.

Importance of mechanistic consideration

Up to the present day, numerous data have been compiled suggesting at least some effects in development and/or reproduction through exposure during the critical window of perinatal period which may persist to later periods of life. The toxicological significance of those observations, however, must be examined and considered by not only assuring reproducibility of data, but also integrating evidences from mechanistic studies, e.g., studies *in vitro* or on molecular levels. In this regard, although not much mention was made to the studies of signal transduction and gene expression, they might offer some clues to shed lights to the background mechanism of effects through BPA exposure during the critical window of perinatal period.

For example, tributyltin (TBT) compounds, a notorious EDC, was known to cause imposex (masculinization of female gastropods) with a no-observed-effect level (NOEL) of less than 1 ng/l, and suppression of thymus-dependent immune responses in rats with the LOAEL of 0.25 mg/kg/day levels (IPCS, 1999). It was, however, not easy to understand whether there was some link between these effects until the discovery that TBT was a dual, nanomolar affinity ligand of the retinoid X receptor (RXR) and peroxisome proliferators-activated receptor γ (PPARγ) (Saitoh *et al.*, 2001).

There is good evidence that the membrane-limited actions of hormones, particularly estrogens, involve the rapid activation of kinases and the release of calcium and that these are linked to physiologically relevant scenarios in the brain (Vasudevan and Pfaff, 2008). Membrane actions of estrogens activate rapid signaling cascades, and also potentiate nuclear transcription in both the central nervous system and in non-neuronal cell lines. Vasudevan and Pfaff (2008) presented a theoretical scenario to under-

Table 3. Conclusions in the draft NTP brief on Bisphenol A (prepared from NTP, 2008)

NTP conclusions on effects of Bisphenol A (BPA)
In concurrence with the CERHR Panel Report, there is some concern for neural and behavioral effects in fetuses, infants and children at current human exposures. NTP has some concern for BPA exposure in these populations based on effects in the prostate gland, mammary gland, and an earlier age for puberty in females. *1,2
NTP has negligible concern that exposure of pregnant women to BPA will result in fetal or neonatal mortality, birth defects or reduced birth weight and growth in their offspring.
In concurrence with the CERHR Panel Report, there is negligible concern that exposure to BPA causes reproductive effects in non-occupationally exposed adults and minimal concern for workers exposed to higher levels in occupational settings
*1,2: see **NOTE** in the main text on the recommendation for changes

Weight of evidence approach for respective effects from human observations and animal studies
There is insufficient evidence for a conclusion that BPA causes developmental and reproductive effects in humans.
There is clear evidence that BPA causes adverse developmental effects in laboratory animals for "high" dose developmental toxicity[1].
There is some evidence that BPA causes adverse reproductive effects in laboratory animals[2].
There is limited evidence that BPA causes adverse "low" dose developmental toxicity in laboratory animals[3].

1 Based on reduced survival in fetuses or newborns (> 500 mg/kg/day)[#1-5], reduced fetal or birth weight or growth of offspring early in life (> 300 mg/kg/day)[#1,2,6] and delayed puberty in female rats (> 50 mg/kg/day and male rats and mice (>50 mg/kg/day)[#2,6-8]

2 Based on possible decreased fertility in mice (> 875 mg/kg/day)[#5]; altered estrous cycling in female rats (> 600 mg/kg/day)[#9] and cellular effects on the testis of male rats (235 mg/kg/day)[#10].

3 Based on a variety of effects related to neural and behavioral alterations (> 10 µg/kg/day)[#11-17], precancerous lesions in the prostate (10 µg/kg/day)[#18] and mammary glands (25 µg/kg/day- 1 mg/kg/day)[#19,20]; altered prostate gland and urinary tract development (10 g/kg/day)[#21], and early onset of puberty (2.4 and 200 µg/kg/day)[#15,22].

References

#1. Kim, J.C., Shin, H.C., Cha, S.W., Koh, W.S., Chung, M.K., Han, S.S. (2001): Life Sci., **69**, 2611-2625.
#2. Tyl, R.W., Myers, C.B., Marr, M.C., Thomas, B.F., Keimowitz, A.R., Brine, D.R., Veselica, M.M., Fail, P.A., Chang, T.Y., Seely, J.C., Joiner, R.L., Butala, J.H., Dimond, S.S., Cagen, S.Z., Shiotsuka, R.N., Stropp, G.D., Waechter, J.M. (2002): Toxicol. Sci., **68**, 121-146.
#3. Morrissey, R.E., George, J.D., Price, C.J., Tyl, R.W., Marr, M.C., Kimmel, C.A. (1987): Fund. Appl. Toxicol., **8**, 571-582.
#4. Tyl, R.W., Myers, C.B., Marr, M.C., Abbreviated one-generation study of dietary bisphenol A (BPA) in CD-1® (Swiss) mice. (2002): In. Research Triangle Park, NC: RTI (sponsored by the Society of the Plastics Industry, Inc.).
#5. NTP (1985) Bisphenol A: reproduction and fertility assessment in CD-1 mice when administered in the feed. NTP-85-192. Research Triangle Park, NC.
#6. Tyl, R.W., Myers, C.B., Marr, M.C. (2006): Two-Generation Reproductive Toxicity Evaluation of Bisphenol A (BPA; CAS No. 80-05-7) Administered in the Feed to CD-1® Swiss Mice (Modified OECD 416). Sponsored by the American Plastics Council. audited draft revised final report.
#7. Tan, B.L., Kassim, N.M., Mohd, M.A. (2003): Toxicol. Lett., **143**, 261-270.
#8. Tinwell, H., Haseman, J., Lefevre, P.A., Wallis, N., Ashby, J. (2002): Toxicol. Sci., **68**, 339-348.
#9. Yamasaki, K., Sawaki, M., Noda, S., Imatanaka, N., Takatsuki, M. (2002): Arch. Toxicol., **76**, 65-74.
#10. Takahashi, O. and Oishi, S. (2001): Arch. Toxicol., **75**, 42-51.
#11. Palanza, P.L., Howdeshell, K.L., Parmigiani, S., vom Saal, F.S. (2002): Environ. Health Perspect., **110**(Suppl 3), 415-422.
#12. Laviola, G., Gioiosa, L., Adriani, W., Palanza, P. (2005): Brain Res. Bull., **65**, 235-240.
#13. Gioiosa, L., Fissore, E., Ghirardelli, G., Parmigiani, S., Palanza, P. (2007): Horm. Behav., **52**, 307-316.
#14. Ceccarelli, I., Della Seta, D., Fiorenzani, P., Farabollini, F., Aloisi, A.M. (2007): Neurotoxicol. Teratol., **29**, 108-115.
#15. Ryan, B.C., Vandenbergh, J.G. (2006): Horm. Behav., **50**, 85-93.
#16. Della Seta, D., Minder, I., Belloni, V., Aloisi, A.M., Dessi-Fulgheri, F., Farabollini, F. (2006): Horm. Behav., **50**, 301-307.
#17. Negishi, T., Kawasaki, K., Suzaki, S., Maeda, H., Ishii, Y., Kyuwa, S., Kuroda, Y., Yoshikawa, Y. (2004): Environ. Health Perspect., **112**, 1159-1164.
#18. Ho, S.M., Tang, W.Y., Belmonte de Frausto, J., Prins, G.S. (2006): Cancer Res., **66**, 5624-5632.
#19. Durando, M.L.K., Piva, J., Sonnenschein, C., Soto, A.M., Luque, E., Muñoz-de-Toro, M. (2007): Environ. Health Perspect., **115**, 80-86.
#20. Murray, T.J., Maffini, M.V., Ucci, A.A., Sonnenschein, C., Soto, A.M. (2007): Reprod. Toxicol., **23**, 383-390.
#21. Timms, B.G., Howdeshell, K.L., Barton, L., Bradley, S., Richter, C.A., vom Saal, F.S. (2005): PNAS, **102**, 7014-7019.
#22. Howdeshell, K.L., Hotchkiss, A.K., Thayer, K.A., Vandenbergh, J.G., vom Saal, F.S. (1999): Nature, **401**, 763-764

stand this phenomenon that these signaling cascades may occur in parallel or in series but subsequently, converge at the modification of transcriptionally relevant molecules such as nuclear receptors and/or coactivators. Although estrogenic activity of BPA was claimed to be fairly low as compared to estradiol (E_2), recently, BPA was found to be able to interact with membrane estrogen receptor (ER) α and could cause rapid estrogen-mediated signal-regulated kinase signaling in developing rat cerebellar neurons as a potent nongenomic agonist at 10^{-12} M concentration as comparable to E_2 (Zsarnivsky *et al.*, 2005).

Cross talk between receptors and possibility of epigenetic mechanism

Ohtake *et al.* (2003) found that estrogenic action of arylhydrocarbon receptor (AhR) agonists might be exerted through a direct interaction (cross-talk) between AhR/arylhydrocarbon receptor nuclear translocator (Arnt) and unliganded ER and by the formation of functional units bound to estrogen responsible element (ERE) to activate transcription in uterine gene induction, but AhR agonists exhibit anti-estrogenic activities in the presence of high doses of E_2 in animals and cultured cells by repressing liganded ER. Thus, not only the presence or absence of estrogens, but also the presence of AhR ligands at certain levels might regulate exquisitely the expression of estrogen dependent activity in the target cells. Embryonic exposure to BPA at low dose level (20 ng/kg/day or higher orally to gravid mice in gestation days 6 to 17) markedly increased expressions of mRNA of AhR, retinoic acid receptor (RAR) α, or RXRα in the cerebra, cerebella, testes or ovaries at gestation days 14 or 18 (Nishizawa *et al.*, 2005).

Understanding the potential of epigenetic changes may explain the mechanism of developmental effects of BPA or other EDCs (vom Saal, 2007). Developmental exposure to BPA increases the susceptibility to prostate carcinogenesis and epigenetically regulates phosphodeiestrase type 4 variant 4 (Ho *et al.*, 2006). In this regard, it is interesting that the role of premature leptin surge in obesity resulting from intrauterine undernutrition was reported (Yura *et al.*, 2005). Offspring with fetal undernutrition, when fed a high-fat diet develop pronounced weight gain and adiposity, and therefore, a mother who admires slim body and eats less may affect her child obesity. Penetrance in genetic inheritance may also somehow relate to the different effects between studies. Not only for BPA, but for other EDCs, it is necessary to further study specific designs of protocols to elucidate the issue of critical period of exposure, which may lead to developing new aspects in toxicology.

Species differences in endocrinology and pharmacokinetics

Precise understanding of species differences not only in pharmacokinetics and metabolism, but also basics in endocrinological differences between humans and rodents is required in extrapolating animal data to humans. Witorsch (2002) pointed that there are major differences between humans and rodents in the role of the corpus luteum during pregnancy, hormonal control of the corpus luteum of pregnancy, the source and pathways of sex steroid production throughout gestation, and the specific types of estrogens secreted and the levels within the fetus attained throughout gestation. He noted also that concentrations of E_2 in late stage of gestation in mothers and fetuses are 15-20 ng/ml, and 5-10 ng/ml, respectively, whereas those in rats and mice are 3-60 pg/ml, and 100-150 pg/ml and that rodents are considered more susceptible to xenoestrogens at lower doses compared to humans.

Further research studies and internationally concerted efforts will be necessary to assess risks associated with exposure to BPA. These efforts will further contribute to the development of new aspects in toxicology and ensuring safe use of chemicals in general.

DISCUSSION

Aim of this review

This review is not meant to be comprehensive and exhaustive, or to present a new estimation of risks with BPA exposure, particularly because the risk assessment will require integration of all available knowledge and thoughtful discussions by experts in various fields, as well as involvement of other stakeholders, eg., susceptible populations and/or pertinent industries, when necessary. Rather, the author, having worked as a member of the IPCS chemical risk assessment group for more than 20 years, would try to review and summarize the matter of EDCs taking BPA as a good example for which many studies have been conducted and published, to consider important issues in the field of toxicology and risk assessment.

While there has been much dispute over low-dose effects of BPA partly because of its wide use in polymer production, by reviewing the data on low-dose effects with this compound, especially those on neurobehavioral effects following fetal or early postnatal exposures, it is suggested that there are new aspects to be considered carefully, and that it is imperative to pursue further investigations.

Future tasks and challenges

Specific mention for future tasks and challenges is made as follows. Firstly, animal toxicity tests should be designed with more elaboration, e.g., to ensure a sufficient number of test animals with careful handling of litters to avoid litter effects to allow adequate statistical analysis, and appropriate selection of multiple dose levels to obtain insight in dose-response relationship. Secondly, precise serum/plasma levels both in humans and rodents should be estimated to allow extrapolation of effects observed in rodents to humans, and in this connection, construction of relevant physiologically based pharmacokinetic models, would help obtain quantitative estimates of intake and target-organ exposure relationship. Thirdly, biological backgrounds, particularly differences and similarities in endocrinological, neurological, and immunological aspects among species or strains, should be revisited to explain what were observed in certain species or strains. Fourthly, mechanistic deliberations, e.g., on the possibilities of epigenetic mechanism, etc., based on recent findings in molecular levels and/or examination of putative neurobehavioral effects or a presumptive link between miscarriage and BPA exposure through well-designed epidemiological studies are requested. Finally, general public concerns, particularly of women who are going to bear babies, should be addressed in a thoughtful way so that a simple precautionary approach is not pursued, but uncertainties in the new toxicological aspects should be carefully explained. Further research studies and internationally concerted efforts will be necessary to assess risks from low-dose effects through integrating available knowledge, and such efforts will definitely contribute to setting new directions in toxicology and improving risk assessments of chemicals.

NOTE

Following information pertinent to this review was obtained after submission of the draft.

*1 The NTP Board of Scientific Counselors issued "Action on the draft brief on Bisphenol A" on June 11, 2008 recommending changes in the level of concern in the Draft NTP Brief on Bisphenol A from "some" to "minimal" for effects in the mammary gland and an earlier age for puberty in females taking into consideration of the comments NTP received. (see http://ntp.niehs.nih.gov/files/BSCactionsBPA_508. pdf)

*2 Commentaries on the CERHR review were published together with the original expert panel report in the following scientific journal. (see Birth Defects Research (part B) (2008) **83** 151-395)

ACKNOWLEDGMENTS

Author acknowledges support by the fund from the Ministry of Health, Labour and Welfare, Japan (grant #2004-chem-002, and #2007-chem-general-004). Cooperative efforts of experts in preparing data books on BPA low-dose effects, and stimulating discussions and advice from Dr. Tohru Inoue of the National Institute of Health Sciences, the project leader of the research team, are very much appreciated.

REFERENCES

Akingbemi, B.T., Sottas, C.M., Koulova, A.I., Klinefelter, G.R. and Hardy, M.P. (2004): Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. Endocrinology, **145**, 592-603.

Al-Hiyasat, A.S., Darmani, H. and Elbetieha, A.M. (2002): Effects of bisphenol A on adult male mouse fertility. Eur. J. Oral. Sci., **110**, 163-167.

Arakawa, C., Fujimaki, K., Yoshinaga, J., Imai, H., Serizawa, S. and Shiraishi, H. (2004): Daily urinary excretion of bisphenol A. Environ. Health. Preventive. Med., **9**, 22-26.

Canada (2008): Challenge Advisory Panel Summary Report Government of Canada March 28, 2008, http://www.chemicalsubstanceschimiques.gc.ca/challenge-defi/bisphenol-a_e.html

CERHR (2007): NTP-CERHR Expert panel report on the reproductive and developmental toxicity of bisphenol A, Center for the Evaluation of Risks to Human Health Reproduction/National Toxicology Program, US Department of Health and Human Services NTP-CERHR-XXXX-00

Chitra, K.C., Latchoumycandane, C. and Mathur, P.P. (2003): Induction of oxidative stress by bisphenol A in the epididymal sperm of rats. Toxicology, **185**, 119-127.

Dekant, W. and Völkel, W. (2008): Human exposure to bisphenol A by biomonitoring : Methods, results and assessment of environmental exposures. Toxicol. Appl. Pharmacol., **228**, 114-134.

Della Seta, D., Minder, I., Belloni, V., Aloisi, A.M., Dessì-Fulgheri, F. and Farabollini, F. (2006): Pubertal exposure to estrogenic chemicals affects behavior in juvenile and adult male rats. Hormones and Behavior, **50**, 301-307.

Della Seta, D., Minder, I., Dessi-Fulgheri, F. and Farabollini, F. (2005): Bisphenol-A exposure during pregnancy and lactation affects maternal behavior in rats. Brain Res. Bull., **65**, 255-260.

EFSA (2006): Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from the Commission related to 2,2 - BIS (4-HYDROXYPHENYL) PROPANE (Bisphenol A) Question number EFSA-Q-2005-100, The EFSA Journal, **428**, 1-75.

Ema, M., Fujii, S., Furukawa, M., Kiguchi, M., Ikka, T. and Harazono, A. (2001): Rat two-generation reproductive toxicity study of bisphenol A. Reprod. Toxicol., **15**, 505-523.

Fujimoto, T., Kubo, K. and Aou, S. (2006): Prenatal exposure to bisphenol A impairs sexual differentiation of exploratory behavior and increases depression-like behavior in rats. Brain Res., **1068**, 49-55.

Funabashi, T., Kawaguchi, M., Furuta, M., Fukushima, A. and

Kimura, F. (2004): Exposure to bisphenol A during gestation and lactation causes loss of sex difference in corticotropin-releasing hormone -immunoreactive neurons in the bed nucleus of the stria terminalis of rats. Psychoneuroendocrinology, **29**, 475-485.

Gupta, C. (2000): Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals. Proc. Soc. Exp. Biol. Med., **224**, 61-68.

Ho, S., Tang, W., de Frausto, J.B. and Prins, G.S. (2006): Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphordiesterase type 4 variant 4. Cancer Res., **66**, 5624-5632.

Howdeshell, K.L., Hotchkiss, A.K., Thayer, K.A., Vandenbergh, J.G. and vom Saal, F.S. (1999): Exposure to bisphenol A advances puberty. Nature, **401**, 763-764.

Hunt, P.A., Koehler, K.E., Susiarjo, M., Hodges, C.A., Ilagan, A., Voigt, R.C., Thomas, S., Thomas, B.F. and Hassold, T.J. (2003): Bisphenol A exposure causes meiotic aneuploidy in the female mouse, Curr. Biol., **13**, 546-553.

IPCS (1999): Concise International Chemical Assessment Document No. 14, Tributyltin oxide, International Programme on Chemical Safety, World Health Organization, Geneva, ISSN 1020-6167.

IPCS (2002): Global assessment of the state-of-the-science of endocrine disruptors, International Programme on Chemical Safety, WHO/PCS/EDC/02. 2, pp. 180.

Kersting, M., Alexy, U., Sichert_Hellert, W., Manz, F. and Schöch, G. (1998): Measured consumption of commercial infant food products in German infants : results from the DONALD study. Dortmund Nutritional and Anthropometrical Longitudinally Designed. J. Pediatr. Gastroeneterol. Nutr., **27**, 547-552.

Kubo, K., Arai, O., Omura, M., Watanabe, R., Ogata, R. and Aou, S. (2003): Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. Neurosci. Res., **45**, 345-356.

Laviola, G., Gioiosa, L., Adriani, W. and Palanza, P. (2005): D-amphetamine-related reinforcing effects are reduced in mice exposed prenatally to estrogenic endocrine disruptors. Brain Res. Bull., **65**, 235-240.

MOE (2004): Initial Environmental Risk Assessment Report vol. 3: bisphenol A, Ministry of the Environment, September 2004, http://www.env.go.jp/chemi/report/h16-01/pdf/chap01/02_2_15.pdf

Nagel, S.C., vom Saal, F.S., Thayer, K.A., Dhar, M.G., Boechler, M. and Welshons, W.V. (1997): Relative Binding Affinity-Serum Modified Access (RBA-SMA) Assay Predicts the Relative *In Vivo* Bioactivity of the Xenoestrogens Bisphenol A and Octylphenol. Environ. Health Perspect., **105**, 70-76.

Nagao, T., Saito, Y., Usumi, K., Yoshimura, S. and Ono, H. (2002): Low-dose bisphenol A does not affect reproductive organs in estrogen-sensitive C57BL/6N mice exposed at the sexually mature, juvenile, or embryonic stage. Reprod. Toxicol., **16**, 123-130.

NEDO/CRM-AIST (2005): Risk Assessment Report Series Vol. 6, Bisphenol A. pp. 267, Maruzen Company, Limited, Tokyo. http://unit.aist.go.jp/riss/crm/index_e.html (English version)

Negishi, T., Kawasaki, K., Suzaki, S., Maeda, H., Ishii, Y., Kyuwa, S., Kuroda, Y. and Yoshikawa, Y. (2004): Behavioral alterations in response to fear-provoking stimuli and tranylcypromine induced by perinatal exposure to bisphenol A and nonylphenol in male rats. Environ. Health Perspect., **112**, 1159-1164.

Nishizawa, H., Morita, M., Sugimoto, M., Imanishi, S. and Manabe, N. (2005): Effects of in utero exposure to bisphenol A on mRNA expression of arylhydrocarbon and retinoid receptors in murine embryos. J. Reprod. Dev., **51**, 315-324.

NTP (1982): NTP Technical report on the carcinogenesis bioassay of bisphenol A in F344 rat and B6C3F1 mice (feed study), NTP-80-35 Publ. No. 82-1711

NTP (2001): NTP Endocrine Disruptors Low Dose Peer Review Final Report, http://ntp-server.niehs.nih.gov/htdocs/liason/FinalRptLowDoseFR.html, pp. 493.

NTP (2008): Draft NTP Brief on Bisphenol A (Peer Review Date June 11), 2008, April 14, 2008, National Toxicology Program, National Institute of Environmental Health Sciences, US Department of Health and Human Services, pp. 68.

OECD (1983): OECD Guideline for the Testing of Chemicals, Test No. 415 one-generation reproduction toxicity study.

OECD (1997): OECD Guideline for the Testing of Chemicals, Test No. 424 neurotoxicity study.

OECD (2001a): OECD Guideline for the Testing of Chemicals, Test No. 416 for two-generation reproduction toxicity study.

OECD (2001b): OECD Guideline for the Testing of Chemicals, Test No. 414 for prenatal developmental toxicity study.

OECD (2007): OECD Guideline for the Testing of Chemicals, Developmental Neurotoxicity study.

Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Tohyama, C., Krust, A., Mimura, J., Cahmbon, P., Yanagisawa, J., Fujii-Kuriyama, Y. and Kato, S. (2003): Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. Nature, **423**, (6939) 545-550.

Palanza, P.L., Howdeshell, K.L., Parmigiani, S. and vom Saal, F.S. (2002): Exposure to a low dose of bisphenol A during fetal life or in adulthood alters maternal behavior in mice. Environ. Health Perspect., **110**, 415-422.

Ryan, B.C. and Vandenbergh, J.G., (2006): Developmental exposure to environmental estrogens alters anxiety and spatial memory in female mice. Horm. Behav., **50**, 85-93.

Saitoh, M., Yanase, T., Morinaga, H., Tanabe, M., Mu, Y.M., Nishi, Y., Nomura, M., Okabe, T., Goto, K., Takayanagi, R. and Nawata, H. (2001): Tributyltin or triphenyltin inhibits aromatase activity in the human granulosa-like tumor cell line KGN, Biochem Biophys. Res. Commun., **289**, 198-204.

Sakaue, M., Ohsako, S., Ishimura, R., Kurosawa, S., Kurohmaru, M., Hayashi, Y., Aoki, Y., Yonemoto, J. and Tohyama, C. (2001): Bisphenol-A affects spermatogenesis in the adult rat even at a low dose. J. Occup. Health., **43**, 185-190.

SCF (2002): Opinion of the scientific committee on food on bisphenol A, Scietific Committee on Food, SCF/CS/PM/3936 Final 3, May 2002

Schönfelder, G., Flick, B., Mayr, E., Talsness, C., Paul, M. and Chahoud, I. (2002a): *In utero* exposure to low doses of bisphenol A lead to long-term deleterious effects in the vagina. Neoplasia, **4**, 98-102.

Schönfelder, G., Wittfoht, W., Hopp, H., Talsness, C.E., Paul, M. and Chahoud I. (2002b): Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environ. Health Persp., **110**, 703-707.

Sekizawa, J. (2005-2009): Study reports on low-dose effects from fetal exposure to bisphenol A and data books on the evaluation of low-dose effects of bisphenol A, submitted to as a part of reports to the Ministry of Health, Labour and Welfare, Japan for the funding period from 2004 to 2007, Grant #2004-Chem-General-002, and Grant # 2007-Chem.- General-004. http://mhlw-grants.niph.go.jp/niph/search/NIDD00.do

Sugiura-Ogasawara, M., Ozaki, Y., Sonta, S., Makino, T. and Suzumori, K. (2005): Exposure to bisphenol A is associated with recurrent miscarriage, Human Reproduction, **20**, 2325-2329.

Teeguarden, J.G., Waechter, J.M.Jr., Clewell, H.J.III., Covington, T.R. and Barton, H.A. (2005): Evaluation of oral and intravenous route pharmacokinetics, plasma protein binding, and uterine tissue dose metrics of bisphenol A : A physiologically based pharmacokinetic approach. Toxicol. Sci., **85**, 823-838.

Timms, B.G., Howdeshell, K.L., Barton, L., Bradley, S., Richter, C.A. and vom Saal, F.S. (2005): Estrogenic chemicals in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra. PNAS, **102**, 7014-7019.

Tyl, R.W., Myers, C.B., Marr, M.C., Thomas, B.F., Keimowitz, A.R., Brine, D.R., Veselica, M.M., Fail, P.A., Chang, T.Y., Seely, J.C., Joiner, R.L., Butala, J.H., Dimond, S.S., Cagen, S.Z., Shiotsuka, R.N., Stropp, G.D. and Waechter, J.M. (2002): Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. Toxicol. Sci., **68**, 121-146.

Tyl, R.W., Myers, C.B., Marr, M.C., Sloan, C.S., Castillo, N.P., Veselica, M.M., Seely, J.C., Dimond, S.S., Van Miller, J.P., Shiotsuka, R.N., Beyer, D., Hentges, S.G. and Waechter, J.M.Jr. (2008): Two-Gen-eration reproductive toxicity study of dietary bisphenol A in CD-1 (Swiss) mice. advance access published May 5, 2008.

US EPA (2008): bisphenol A, Integrated Risk Information System (IRIS), http://toxnet.nlm.nih.gov/cgi-bin/sis/search

Vandenberg, L.N., Hauser, R., Marcus, M., Olea, N. and Welshons, W.V. (2007): Human exposure to bisphenol A (BPA). Reprod. Toxicol., **24**, 139-177.

Vasudevan, N. and Pfaff, D.W. (2008): Non-genomic actions of estrogens and their interaction with genomic actions in the brain. Frontiers in Neuroendocrinology, **29**, 238-257.

Völkel, W., Colnot, T., Csanády, G.A., Filser, J.G. and Dekant, W. (2002): Metabolism and kinetics of bisphenol A in humans at low doses following oral administration. Chem. Res. Toxicol., **15**, 1281-1287.

vom Saal, F.S., Timms, B.G., Montano, M.M., Palanza, P., Thayer, K.A., Nagel, S.C., Dhar, M.D., Ganjam, V.K., Parmigiani, S. and Welshons, W.V. (1997): Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses, PNAS, **94**, 2056-2061.

vom Saal, F.S., Cooke, P.S., Buchanan, D.L., Palanza, P., Thayer, K.A., Nagel, S.C., Parmigiani, S. and Welshons, W.V. (1998): A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior, Toxicol. Ind. Health, **14**, 239-260.

vom Saal, F.S., Akingbemi, B.T., Belcher, S.M., Birnbaum, L.S., Crain, D.A., Eriksen, M., Farabollini, F., Guillette L.J.Jr., Hauser, R., Heindel, J.J., S.-M., H., Hunt, P.A., Iguchi, T., Jobling, S., Kanno, J., Keri, R.A., Knudsen, K.E., Laufer, H., LeBlanc, G.A., Marcus, M., McLachlan, J.A., Myers, J.P., Nadal, A., Newbold, R.R., Olea, N., Prins, G.S., Richter, C.A., Rubin, B.S., Sonnenschein, C., Soto, A.M., Talsness, C.E., Vandenbergh, J.G., Vandenberg, L.N., Walser-Kuntz, D.R., Watson, C.S., Welshons, W.V., Wetherill, Y. and Zoeller R.T. (2007): Chapel Hill bisphenol A expert panel consensus statement: integration of mechanisms, effects in animals and potential to impact human health at current levels of exposure. Reproduct. Toxicol., **24**, 131-138.

Witorsh, R.J. (2002): Low-dose *in utero* effects of xenoestrogens in mice and their relevance to humans; An analytical review of the literature, Food Chem. Toxicol., **40**, 905-912.

Yura, S., Itoh, H., Sagawa, N., Yamamoto, H., Masuzaki, H., Nakao, K., Kawamura, M., Takemura, M., Kakui, K., Ogawa, Y. and Fujii, S. (2005): Role of premature leptin surge in obesity resulting from intrauterine undernutrition, Cell Metabolism, **1**, 371-378.

Zsarnovszky, A., Le, H.H., Wang, H.S. and Belcher, S.M. (2005): Ontogeny of rapid estrogen-mediated extracellular signal-regulated kinase signaling in the rat cerebellar cortex: potent nongenomic agonist and endocrine disrupting activity of the xenoestrogen bisphenol A. Endocrinology, **146**, 5388-5396.

Developmental Biology 324 (2008) 310–319

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Role of stromal tenascin-C in mouse prostatic development and epithelial cell differentiation

Kenichiro Ishii [a,*], Kyoko Imanaka-Yoshida [b], Toshimichi Yoshida [b], Yoshiki Sugimura [a]

[a] Department of Nephro-Urologic Surgery and Andrology, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan
[b] Department of Pathology and Matrix Biology, Mie University Graduate School of Medicine, Japan

ARTICLE INFO

Article history:
Received for publication 23 July 2008
Revised 18 September 2008
Accepted 19 September 2008
Available online 10 October 2008

Keywords:
Tenascin-C
Knockout mouse
Prostate
Epithelial–stromal interactions
Epithelial cell differentiation
Epithelial cell cluster
Binucleated cell
Androgen receptor
Tissue recombination
Urogenital sinus mesenchyme

ABSTRACT

Deregulation of epithelial–stromal interactions is considered to play a critical role in the initiation and promotion of benign prostatic hyperplasia (BPH) and prostate carcinoma (PCa). Expression of tenascin-C (TN-C), an extracellular matrix (ECM) glycoprotein, is reportedly higher in BPH and PCa as compared with normal prostate. Remodeling of the ECM alters the homeostatic balance between epithelium and stroma, resulting in physiological changes in cellular functions. To investigate the role of TN-C in prostatic development and differentiation, we evaluated the morphological phenotype of TN-C knockout (KO) mouse prostate (ventral: VP, dorsolateral: DLP, and anterior: AP) and examined tissue recombinants composed of adult mouse DLP epithelium and fetal TN-C KO urogenital sinus mesenchyme (UGM). Histological analysis showed epithelial cell clusters protruding into the ductal lumens in TN-C KO AP and DLP. Interestingly, binucleated cells appeared in epithelium of TN-C KO DLP at 8 weeks. Simultaneously, androgen receptor (AR)-positive cells were decreased in TN-C KO epithelia. Similar to the TN-C KO phenotype, protruded epithelial clusters, binucleated cells, and AR-negative nuclei were induced in DLP epithelium by recombining with TN-C KO UGM. Our results suggest that stromal TN-C might be involved in maintaining epithelial cytodifferentiation, morphogenesis, and androgen receptor expression of normal prostate glands in adult mice.

Introduction

Morphogenesis in tissue development depends on the relationships among cells, the extracellular matrix (ECM), and other support structures (Fata et al., 2004). Stromal morphogenic signals support normal epithelial differentiation and function (Simian et al., 2001). As morphogenic signals for epithelial differentiation, stromal components such as fibroblasts, myofibroblasts, and smooth muscle cells secret a number of growth factors, cytokines, and the ECM molecules. The ECM forms the extracellular environment, in cooperation with cellular ECM-receptors such as integrins and the ECM metabolism by degrading enzymes such as matrix metalloproteinases (MMPs), and their inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) (Weaver et al., 2002; Green and Lund, 2005). The ECM is a heterogeneous substance composed of structural components including collagens and elastin; glycoproteins such as laminin, fibronectin, and tenascin; and proteoglycans. That the ECM environment provides significant influences on epithelial adhesion, proliferation, differentiation, and polarity, as well as apoptosis, during morphogenesis has been well studied (Ingber and Folkman, 1989; Tremblay et al., 1996; Garrison and Kyprianou, 2004).

Tenascin-C (TN-C) is a hexametric glycoprotein of the ECM produced by both epithelial and mesenchymal cells (Chiquet-Ehrismann et al., 1986; Orend and Chiquet-Ehrismann, 2006). During embryogenesis and morphogenesis, TN-C appears at active sites of tissue remodeling and its expression seems to be regulated by reciprocal interactions between epithelial and mesenchymal components via paracrine factors such as transforming growth factor β (TGFβ) (Thompson et al., 2006). TN-C expression is strictly limited in adults but prominently reappears under pathologic conditions, i.e. the expression of TN-C is induced by proliferating epithelia (normal and particularly malignant) and is downregulated with their differentiation (Vollmer et al., 1994b).

The prostate is an organ which is induced by embryonic mesenchyme, fibroblasts and myofibroblasts, during its development and differentiation (Cunha et al., 1980). In mice, prostatic buds develop from the urogenital sinus on 17 days of gestation (Cunha, 1976). TN-C appears in mesenchyme surrounding the urogenital sinus epithelium, which undergoes major morphogenic changes including the formation of prostatic buds (Takeda et al., 1988). The dynamics of TN-C expression are closely associated with the development and maturation of the prostate gland (Shiraishi et al., 1994). However, the precise

* Corresponding author. Fax: +81 59 231 5203.
E-mail address: kenishii@clin.medic.mie-u.ac.jp (K. Ishii).

0012-1606/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2008.09.029

mechanisms of TN-C-directed epithelial cytodifferentiation and morphogenesis in prostate are little understood as yet.

To investigate the role of TN-C as a stromal morphogenic signal in prostatic development and differentiation, we first evaluated the morphological phenotype of TN-C knockout (TN-C KO) mouse prostate. Next, we examined tissue recombinants composed of adult mouse DLP epithelium and fetal TN-C KO mouse urogenital sinus mesenchyme (UGM) to confirm the morphological phenotype observed in TN-C KO mouse prostate was due to stromal TN-C. In regard to the role of TN-C as a stromal morphogenic signal that drives epithelial cell differentiation, we further investigated the effects of stromal TN-C on the adult urothelial transdifferentiation to prostatic epithelia in tissue recombination.

Materials and methods

Antibodies

Rabbit polyclonal anti-androgen receptor (AR) (N-20) and mouse monoclonal anti-Ki-67 (clone MIB-1) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and DakoCytomation, Inc. (Copenhagen, Denmark), respectively. Mouse monoclonal anti-E-cadherin (clone 36) antibody was purchased from BD Transduction Laboratories, Inc. (Lexington, KY, USA). Mouse monoclonal anti-α-actin, smooth muscle (αSMA; A5228) and mouse monoclonal anti-γ-actin (clone B4) antibodies were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and ICN Biomedicals, Inc. (Costa Mesa, CA, USA), respectively. Rabbit polyclonal anti-cleaved caspase-3 (Asp175) and mouse monoclonal anti-uroplakin III (clone AU1) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA) and Research Diagnostics, Inc. (Concord, MA, USA), respectively. Rabbit polyclonal anti-human TN-C antibody was established and characterized as previously reported (Imanaka-Yoshida et al., 2001).

Animals

TN-C null mice, originally generated by Saga et al. (1992) were backcrossed with BALB/c strain mice. WT littermates were used as controls. All animals were maintained in a specific pathogen-free environment under controlled conditions of light and humidity. Food and tap water were provided *ad libitum*.

Termination and prostatic lobe dissection

All animals were sacrificed at specified time points by an overdose of isoflurane followed by cervical dislocation. After measurement of body weight and urogenital weight, prostates were separated into three lobes: anterior prostate (AP), dorsolateral prostate (DLP), and ventral prostate (VP) (Sugimura et al., 1986). Wet weights were determined for each prostatic lobe and the seminal vesicle (SV). For histological and immunohistochemical analyses, tissues were fixed in 10% neutral-buffered formalin (Wako Pure Chemical Industries, Osaka, Japan) at room temperature overnight and then processed and embedded in paraffin in accordance with standard procedures.

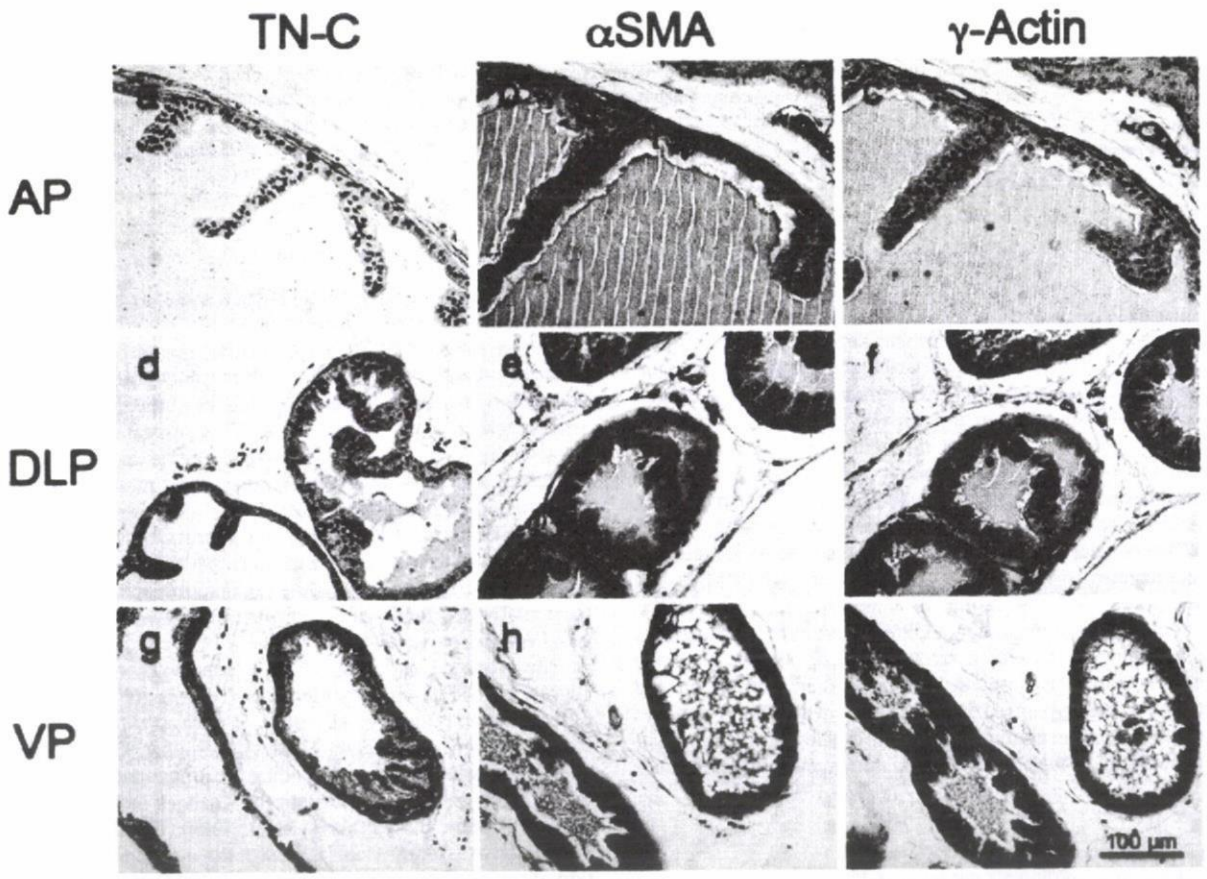

Fig. 1. Localization of TN-C in adult mouse prostate. Localization of TN-C (a, d, and g) was assessed by immunostaining in 17-week-old WT mouse prostate. Immunoreactivities for αSMA (b, e, and h) and γ-actin (c, f, and i) were compared to TN-C expression. The corresponding sections from AP, DLP, and VP are shown in panels a–c, d–f, and g–i, respectively. Scale bar = 100 μm, magnification ×200.