[2] vom Saal FS, Bronson FH. In utero proximity to female mouse fetuses to males: effects on reproductive performance during later life. Biol Reprod 1978;19:843–53.
[3] Clark MM, Galef Jr BG. Effect of uterine position on rate of sexual development in female Mongolian gerbils. Physiol Behav 1988;42:15–8.
[4] vom Saal FS. Sexual differentiation in litter bearing mammals: influence of sex of adjacent fetuses in utero. J Anim Sci 1989;67: 1824–40.
[5] Clark MM, Galef Jr BG. Prenatal influences on reproductive life history strategies. Trends Ecol Evol 1995;10:151–3.
[6] Jubilan BM, Nyby JG. The intrauterine position phenomenon and precopulatory behaviors of house mice. Physiol Behav 1992;51:857–72.
[7] Hernandes-Tristan R, Arevalo C, Canals S. Effect of prenatal uterine position on male and female rats sexual behavior. Physiol Behav 1999;67:401–8.
[8] Sherry DF, Galef BG, Clark MM. Sex and intrauterine position influence the size of the gerbil hippocampus. Physiol Behav 1996;60: 1491–4.
[9] Tarraf CG, Knight JW. Effect of intrauterine position on conceptus development, placental and endometrial release of progesterone and estrone in vitro, and concentration of steroid hormones in fetal fluids throughout gestation in swine. Domest Anim Endocrinol 1995;12:179–87.
[10] vom Saal FS, Timms BG. The role of natural and manmade estrogens in prostate development. In: Naz RK, editor. Endocrine disruptors: effects on male and female reproductive systems. Boca Raton, FL: CRC Press; 1999. p. 307–27.
[11] vom Saal FS, Bronson FH. Variation in length of the estrous cycle in mice due to former intrauterine proximity to male fetuses. Biol Reprod 1980;22:777–80.
[12] vom Saal FS. Variation in infanticide and parental behavior in male mice due to prior intrauterine proximity to female fetuses. Elimination by prenatal stress. Physiol Behav 1983;30:675–81.
[13] Clark MM, Karpiuk P, Galef BG. Hormonally mediated inheritance of acquired characteristics in Mongolian gerbils. Nature (London) 1993;364:712.
[14] Howdeshell KL, Hotchkiss AK, Thayer KA, Vandenberg JG, vom Saal FS. Exposure to bisphenol A advances puberty. Nature (London) 1999;401:763–4.
[15] Welshons WV, Nagel SC, Thayer KA, Judy BM, vom Saal FS. Low-dose bioactivity of xeno17β estradiols in animals: fetal exposure to low doses of methoxychlor and other xeno17β estradiols increases adult prostate size in mice. Toxicol Ind Health 1999;15:12–25.
[16] Howdeshell KL, vom Saal FS. Developmental exposure to bisphenol A: interaction with endogenous estradiol during pregnancy in mice. Am Zool 2000;40:429–37.
[17] Timms BG, Peterson RE, vom Saal FS. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin interacts with endogenous estradiol to disrupt prostate gland morphogenesis in male rat fetuses. Toxicol Sci 2002;67:264–74.
[18] Guide for the care and use of laboratory animals. NIH Publication 86–23, Bethesda, MD: National Institutes of Health; 1985.
[19] Everett JW. Neurobiology of reproduction in the female rat: a fifty-year perspective. New York: Springer-Verlag; 1989.
[20] Haseman JK, Hogan MD. Selection of the experimental unit in teratology studies. Teratology 1975;12:165–72.
[21] Holson RR, Pearce B. Principels and pitfalls in the analysis of prenatal treatment effects in multiparous species. Neurotoxicol Teratol 1992;14:221–8.
[22] Bliss CI. Statistics in biology, vol. I. New York: McGraw-Hill; 1967. p. 116–7.
[23] Wise LD, Vetter CM, Anderson CA, Antonello JM, Clark RL. Reversible effects of triamcinolone and lack of effects with aspirin or L-656224 on external genitalia of male Sprague–Dawley rats exposed in utero. Teratology 1991;44:507–20.
[24] Clark RL. Endpoints of reproductive system development. An evaluation and interpretation of reproductive endpoints for human health risk assessment. Washington, DC: ILSI Press; 1999. p. 10–27.
[25] Gallavan RH, Holson JF, Stump DG, Knapp JF, Reynolds VL. Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. Reprod Toxicol 1999;13:383–90.
[26] Heinrichs WL. Current laboratory approaches for assessing female reproductive toxicity. In: Dixon RL, editor. Reproductive toxicology. New York: Raven Press; 1985. p. 95–108.
[27] vom Saal FS. Variation in phenotype due to random intrauterine positioning of male and female fetuses in rodents. J Reprod Fertil 1981;62:633–50.
[28] Vandenbergh JG, Huggett CL. The anogenital distance index, a predictor of the intrauterine position effects on reproduction in female house mice. Lab Anim Sci 1995;45:567–73.
[29] Nonneman DJ, Ganjam VK, Welshons WV, vom Saal FS. Intrauterine position effects on steroid metabolism and steroid receptors of reproductive organs in male mice. Biol Reprod 1992;47:723–9.
[30] Even MD, Dhar MG, vom Saal FS. Transport of steroids between fetuses via amniotic fluid in relation to the intrauterine position phenomenon in rats. J Reprod Fertil 1992;96:709–16.
[31] vom Saal FS, Dhar MG. Blood flow in the uterine loop artery and loop vein is bidirectional in the mouse: implications for transport of steroids between fetuses. Physiol Behav 1992;52:163–71.
[32] Gandelman R, vom Saal FS, Reinisch JM. Contiguity to male fetuses affects morphology and behavior of female mice. Nature (London) 1977;266:722–4.
[33] McDermott NJ, Gandelman R, Reinisch JM. Contiguity to male fetuses influences ano-genital distance and time of vaginal opening in mice. Physiol Behav 1978;20:661–3.
[34] Zielinski WJ, Vandenbergh JG, Montano MM. Effects of social stress and intrauterine position on sexual phenotype in wild-type house mice (*Mus musculus*). Physiol Behav 1991;49:117–23.
[35] Simon NG, Cologer-Clifford A. In utero contiguity to males does not influence morphology, behavioral sensitivity to testosterone, or hypothalamic androgen binding in CF-1 female mice. Horm Behav 1991;25:518–30.
[36] Brown-Grant K, Sherwood MR. "The early androgen syndrome" in the guinea pig. J Endcrinol 1971;49:277–91.
[37] Goy RW. Experimental control of psychosexuality. Phil Trans R Soc London 1970;259:149–62.
[38] Stiff ME, Bronson FH, Stetson MH. Plasma gonadotropins in prenatal and prepubertal female mice: disorganization of pubertal cycles in the absence of a male. Endocrinology 1974;94:492–6.
[39] Perrigo GH, Bronson FH. Foraging effort, food intake, fat deposition and puberty in female mice. Biol Reprod 1992;29:455–63.
[40] Kinsley C, Miele J, Konen C, Ghiraldi L, Svare B. Intrauterine contiguity influences regulatory activity in adult female and male mice. Horm Behav 1986;20:7–12.
[41] Hitchcock FA. Studies in vigor: V. The comparative activity of male and female albino rats. Am J Physiol 1925;75:205–10.
[42] Gentry RT, Wade GN. Sex differences in sensitivity of food intake body weight and running wheel activity to ovarian steroids in rats. J Comp Physiol Psychol 1976;90:747–54.
[43] Broida J, Svare B. Sex differences in the activity of mice: modulation by postnatal gonadal hormones. Horm Behav 1984;18:65–78.
[44] Hurst JL. Urine marking in populations of wild house mice (*Mus domesticus* Rutty). II. Communications between females. Anim Behav 1990;40:223–32.
[45] Berman DM, Russell DW. Cell-type-specific expression of rat steroid 5α-reductase isozymes. Proc Natl Acad Sci USA 1993;90:9359–63.
[46] vom Saal FS, Timms BG, Montano MM, Palanza P, Thayer KA, Nagel SC, et al. Prostate enlargement in mice due to fetal exposure

to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. Proc Natl Acad Sci USA 1997;94:2056–61.
[47] Zuckerman S. The endocrine control of the prostate. Proc R Soc Med 1936;29:1557–68.
[48] Glenister TW. The development of the utricle and of the so-called 'middle' or 'median' lobe of the human prostate. J Anat 1962;96:443–55.
[49] Blacklock NJ. The development and morphology of the prostate. In: Ghanadian R, editor. The endocrinology of prostate tumours. Lancaster, England: MTP Press; 1983. p. 1–13.
[50] Timms BG, Petersen SL, vom Saal FS. Prostate gland growth during development is stimulated in both male and female rat fetuses by intrauterine proximity to female fetuses. J Urol 1999;161:1694–701.
[51] Baum MJ, Woutersen PJA, Slob K. Sex difference in whole-body androgen content in rats on fetal days 18 and 19 without evidence that androgen passes from males to females. Biol Reprod 1991;44:747–51.
[52] Tobet SA, Dunlap JL, Gerall AA. Influence of fetal position on neonatal androgen-induced sterility and sexual behavior in female rats. Horm Behav 1982;16:251–8.
[53] Houtsmuller EJ, de Jong FH, Rowland DL, Slob AK. Plasma testosterone in fetal rats and their mothers on day 19 of gestation. Physiol Behav 1995;57:495–9.
[54] Richmond G, Sachs BD. Further evidence for masculinization of female rats by males located caudally in utero. Horm Behav 1984;18:484–90.
[55] Hotchkiss A, Parks LG, Ostby J, Lambright C, Wolf C, Wilson VS. A quantitative determination of the environmental sources of variability in androgens of fetal Sprague–Dawley rats. Biol Reprod (Abstract) 2000;62:183.
[56] Meisel RL, Ward IL. Fetal female rats are masculinized by male littermates located caudally in the uterus. Science 1981;213:239–42.
[57] Slob AK, Van der Schoot P. Testosterone induced mounting behavior in adult female rats born in litters of different female to male ratios. Physiol Behav 1982;28:1007–10.
[58] NIEHS, National Toxicology Program (NTP). Endocrine disruptors low dose peer review report; 2001. p. 467.
[59] Witorsch RJ. Low-dose in utero effects of xenoestrogens in mice and their relevance to humans: an analytical review of the literature. Food Chem Toxicol 2002;40:905–12.

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Exacerbation of benzene pneumotoxicity in connexin 32 knockout mice: enhanced proliferation of CYP2E1-immunoreactive alveolar epithelial cells

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Abstract

The pulmonary pathogenesis triggered by benzene exposure was studied. Since the role of the connexin 32 (Cx32) gap junction protein in mouse pulmonary pathogenesis has been suggested, in the present study, we explored a possible role of Cx32 in benzene-induced pulmonary pathogenesis using the wild-type (WT) and Cx32 knockout (KO) mice. The mice were exposed to 300 ppm benzene by inhalation for 6 h per day, 5 days per week for a total of 26 weeks, and then sacrificed to evaluate the pneumotoxicity or allowed to live out their life span to evaluate the reversibility of the lesions and tumor incidence. Our results clearly revealed exacerbated pneumotoxicity in the benzene-exposed Cx32 KO mice, characterized by diffuse granulomatous interstitial pneumonia, markedly increased mucin secretion of bronchial/bronchiolar and alveolar epithelial cells, and hyperplastic alveolar epithelial cells positive for CYP2E1. But the results did not indicate any enhancement of pulmonary tumorigenesis in the Cx32 KO mice though the number of animals was small.

Keywords: Benzene; Connexin 32; Cx32 knockout mice; CYP2E1; Interstitial pneumonia; Pneumotoxicity

1. Introduction

Benzene has been reported to be a carcinogen capable of producing not only hemopoietic malignancies but also various solid tumors including lung cancers in mice, chronically exposed to it by ingestion or inhalation (Snyder et al., 1988; Huff et al., 1989; Farris et al., 1993).

Benzene toxicity and benzene-induced tumor development in the lung should be taken into consideration for the risk assessment in humans, since the lung is one of the benzene target organs and inhalation is the most common route through which humans are exposed to benzene. Furthermore, a strong relationship

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between benzene exposure and lung cancer development in humans has been assumed for the past decades (Aksoy, 1985, 1989). In addition, benzene metabolites such as benzene oxide, benzene dihydrodiol and diolepoxide have been shown to induce lung tumorigenesis in mouse neonates (Busby et al., 1990). However, little information is available on the pulmonary pathogenesis triggered by benzene exposure.

Intercellular communication through gap junction proteins (GJICs) plays an important role in cellular homeostasis by regulating cell growth, cell differentiation, and apoptosis (Yamasaki, 1996). Based on this concept, alteration in GJICs has been demonstrated to be closely associated with the pathogenesis and carcinogenesis induced by chemicals, particularly by nongenotoxic agents (Yamasaki et al., 1995; Kolaja et al., 2000). Furthermore, down modulation of GJICs is known to induce cytochrome P450s by other chemicals that may be involved in benzene metabolism (Neveu et al., 1994; Snyder and Hedli, 1996; Shoda et al., 2000). We, therefore, hypothesized that GJICs may contribute also to the processes of benzene-induced pneumotoxicity and lung carcinogenesis.

As the presence and the functional role of connexin 32 (C32) gap junction protein in the mouse lung tissue have been suggested in previous in vitro and in vivo studies (Albright et al., 1990; Lee et al., 1997; Ruch et al., 1998; Abraham et al., 1999, 2001), in the present study, we explored a possible role of Cx32 in the lung pathogenesis induced by chronic exposure to benzene, using Cx32 knockout (KO) mice. For this purpose, wild-type (WT) and Cx32 KO mice were exposed to 300 ppm benzene by inhalation for 6 h per day, 5 days per week for 26 weeks. Then the pathological changes were determined based on the results of histopathology, histochemistry for detecting mucin secretion, and immunohistochemistry for detecting CYP2E1 and proliferating cell nuclear antigen (PCNA). The tumor incidence in the pulmonary tissue was also compared between the benzene-exposed WT and Cx32 KO mice.

2. Materials and methods

2.1. Animals

Cx32 KO mice, from the Institut für Genetik, Universität, Bonn, Germany (Moennikes et al., 2000), were maintained as heterozygous KO mice at the animal facility of National Institute of Health Sciences (NIHS), Japan. Because the *Cx32* gene is linked to the X-chromosome, we generated Cx32 WT ($Cx32^{+/Y}$) and KO male ($Cx32^{-/Y}$) mice for this study by cross breeding female $Cx32^{+/-}$ heterozygous mice and male C57BL/6 wild type mice. The Cx32 genotypes of the neonates were identified by the standard PCR assay (Moennikes et al., 2000). The WT and Cx32 KO mice aged 8–9 weeks were used in the study. During the study, the mice were housed within stainless-steel wire cages in inhalation chambers that were maintained on a 12-h light-dark cycle. The basal pellet diet (CRF-1; Funabashi Farm, Tokyo, Japan) was provided ad libitum, except during the 6-h daily inhalation of benzene when the food was withdrawn. Water was automatically supplied throughout the study.

2.2. Benzene exposure

Benzene was purchased from Wako Chemical Company (Osaka, Japan). The mice were randomized and exposed to benzene in 1.3 m^3 inhalation chambers, as described elsewhere (Yoon et al., 2001). Briefly, the benzene vapor was generated by heating liquid benzene to 16 °C and directed into the inhalation chambers (Sibata Scientific Technology Ltd., Tokyo, Japan) with a room temperature of 24 ± 1 °C. The flow rate of benzene was about 650 l/min, and the benzene concentration in the chambers was measured at 30-min intervals during the daily exposures using a gas chromatograph (Shimadzu Co., Kyoto, Japan). The temperature and humidity in the chambers were automatically controlled at 24 ± 1 °C and 55 ± 10%, respectively. As described in the previous Section 2.1, mice were supplied water ad libitum but withdrew the food pellets during the exposure.

The WT and Cx32 KO mice were, respectively, divided into the sham-exposed control group and the benzene-exposed groups; each group was composed of ten to twelve mice. The experimental group was exposed to 300 ppm benzene for 6 h per day, 5 days per week, for 26 weeks and the sham-exposed control group was maintained under the same conditions but without benzene inhalation. Five to six mice from each group were first sacrificed after the 26-week exposure to evaluate pneumotoxicity and the remaining five to seven mice from each group were allowed to

live out their lives to further evaluate their recovery from pulmonary lesions and the incidence of the pulmonary tumor.

2.3. Measurement of food consumption and body weight

Food consumption and body weight were measured every Friday throughout the 26-week benzene exposure.

2.4. Autopsy, organ weight measurement and histopathology

After the 26-week benzene exposure, five to six mice from each group were sacrificed under ethyl ether anesthesia for autopsy. Gross morphological examination of the mice was performed and the major visceral organs were weighed and analyzed. For the histopathological examination, tissues from both lungs were fixed in 10% neutral buffered formaldehyde for 24 h. Pulmonary tissues were sliced and immediately immersed in the fixative. After routine processing, the paraffin-embedded sections were stained with hematoxylin and eosin and then examined histopathologically under a light microscope.

2.5. Immunohistochemistry and histochemistry

The avidin–biotin–peroxidase complex (ABC) method was used for immunohistochemistry to detect the expression of the P450 CYP2E1 enzyme and PCNA. After the lung tissue sections mounted on poly-L-lysine-coated slides were deparaffinized and hydrated, endogenous peroxidase activity was blocked with methanol containing 0.3% hydrogen peroxide for 15 min. The lung tissue sections in a Caplin jar containing 1 mM citric acid (pH 6.0) were microwaved for 10 min for retrieval of PCNA. After washing in phosphate-buffered saline (PBS, pH 7.4) for 15 min, the tissue sections were incubated with 10% normal serum at room temperature for 60 min to block nonspecific binding sites. The sections were then incubated with a mouse anti-PCNA monoclonal antibody (1:300, Sigma–Aldrich, Amherst, NY, USA) for 50 min at room temperature and a goat anti-rat CYP2E1 polyclonal antibody (1:1000, Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) overnight at 4 °C. The tissue sections were washed three times in PBS, incubated with the corresponding biotinylated secondary antibodies for 40 min at room temperature, and subsequently incubated with the ABC reagent for 30 min at room temperature. As a chromogen, 0.5% 3,3′-diaminobenzidine tetrahydrochloride was used, and the sections were counterstained with methylene blue. As a positive control for PCNA and CYP2E1, normal testis and kidney sections were used, respectively, and as a negative control, PBS instead of the primary antibodies was applied to the sections.

Periodic acid–Schiff (PAS) reaction was performed to detect mucus secretion. After deparaffinization, the tissue sections were immersed in 0.5% periodic acid solution. After washing with distilled water, the sections were incubated with the Schiff reagent for 15 min, washed with warm tap water for 10 min, and then counterstained with hematoxylin.

2.6. Statistical analysis

ANOVA was performed to evaluate the significant differences in food consumption and body weight between the nonexposed sham exposed control and benzene-exposed groups of WT and Cx32 KO mice as well as between WT and Cx32 KO mice of each group.

3. Results

3.1. Changes in body weight during the 26-week benzene exposure

No significant difference was observed between WT and Cx32 KO mice of the nonexposed sham-control group throughout the study, even when the mean body weight of Cx32 KO mice was slightly less than that of WT mice at the late stage of this study (Fig. 1). Benzene exposure induced a significant decrease in the body weight of the benzene-exposed group of both WT and Cx32 KO mice compared with the nonexposed sham-control mice. The reduction was much more marked in Cx32 KO mice (Fig. 1), which was observed after seven weeks of exposure ($P < 0.05$). On the other hand, in WT mice, a significant difference in body weight was observed after the fourteenth week of exposure (Fig. 1). Furthermore, after the twelfth week

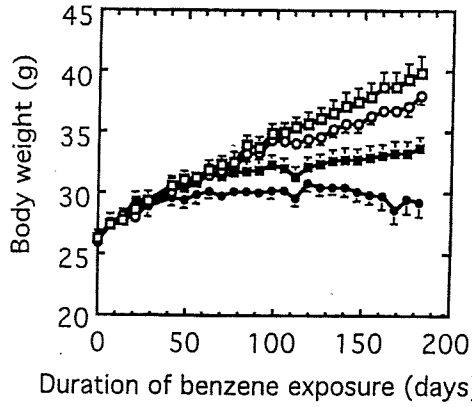

Fig. 1. Changes in body weights of WT and Cx32 KO mice during benzene exposure. Benzene (300 ppm) was inhaled for 6 h per day, 5 days per week for 26 weeks. Eleven to 12 mice per group were used. (□) WT-sham group; (○) Cx32 KO-sham group; (■) WT-benzene-exposed group; (●) Cx32KO-benzene-exposed group. There is significant difference between benzene-exposed group from the corresponding sham-control group after 10 weeks exposure for the Cx32 KO and 14 weeks exposure for WT. Vertical bars mean standard errors.

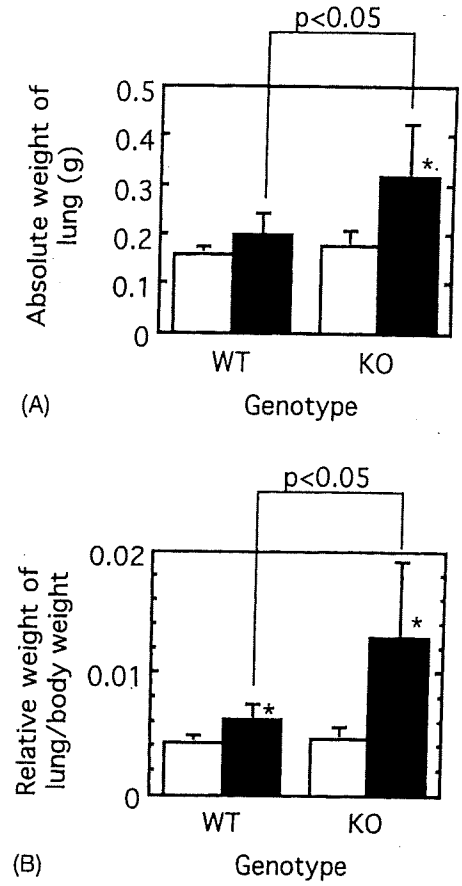

Fig. 2. Changes in weights of the lungs of mice exposed to 300 ppm benzene for 26 weeks. Open column; sham-control group, closed column; benzene-exposed group. Vertical bars mean standard deviations. Symbol (*) indicates significantly different from the corresponding sham-control group at $P < 0.05$.

of exposure, the mean body weight was significantly different between benzene-exposed WT and Cx32 KO mice ($P < 0.05$) (Fig. 1).

During the benzene exposure for 26 weeks, there had been no significant difference in food consumption between the nonexposed group and the benzene-exposed group of both WT and Cx32 KO mice and between WT and Cx32 KO mice of both groups (data not shown).

3.2. Weight of the lung

Significant increases were noted in the absolute lung weight of Cx32 KO mice and in the relative lung weights of both the WT ($P < 0.05$) and Cx32 KO mice ($P < 0.05$) after the twenty-sixth week of exposure to 300 ppm benzene (Fig. 2).

3.3. Histopathology and histochemistry

Severe diffuse interstitial pneumonia was observed in the lungs of the benzene-exposed Cx32 KO mice, which was comparable with that in the lungs of the WT mice showing much milder pulmonary lesions (Table 1, Fig. 3B and E). The alveolar walls were thickened by heavy infiltration of macrophages, the presence of a small number of lymphocytes and neutrophils, and a considerably increased number of type II alveolar epithelial cells (Fig. 3C). The proliferation of basophilic epithelial cells in the terminal bronchioles and alveolar ducts was frequently noted in the lungs of benzene-exposed Cx32 KO mice (Fig. 3F), while the lungs of benzene-exposed WT mice had mild and a few basophilic proliferating epithelial cell-proliferating foci. The numbers of mucus-secreting epithelial cells increased in the bronchi and bronchioli of both WT and Cx32 KO mice exposed to benzene for 26 weeks (Fig. 4C and D). In particular, in the benzene-exposed Cx32 KO mouse lungs, aggregates composed of mucin-secreting alveolar epithelial cells were occasionally detected (Fig. 4D).

Table 1
Pathological findings in the lungs of the wild-type (WT) and Cx32 knockout (KO) mice exposed to 300 ppm benzene for 26 weeks

Group (with or without benzene treatment)	Genotype			
	WT		Cx32 KO	
	Sham-exposed	300 ppm	Sham-exposed	300 ppm
Histopathology/no. of animals examined	6	5	5	5
Interstitial pneumonia granulomatous, diffuse	0 (0.0)	4 (80.0)	0 (0.0)	5 (100.0)
Moderate		4 (80.0)		1 (20.0)
Severe		0 (0.0)		4 (80.0)
Hyperplastic basophilic cell foci	0 (0.0)	1 (20.0)	0 (0.0)	4 (80.0)
Alveolar and bronchiolar epithelial cells		1 (20.0)		4 (80.0)
Mucin-secreting cells	0 (0.0)	5 (100.0)	1 (20.0)	5 (100.0)
Bronchial/bronchiolar epithelial cells		5 (100.0)	1 (20.0)	5 (100.0)
Alveolar epithelial cells		0 (0.0)	0 (0.0)	3 (60.0)

Number in parentheses represents the percentage (%) of the lesions.

3.4. Immunohistochemistry for PCNA and CYP2E1

The labeling indices for PCNA, compared with those of the corresponding control groups, significantly increased in both benzene-exposed WT and Cx32 KO mice; from 79.9 to 162.3‰ ($P < 0.005$) and 92.7 to 533.0‰ ($P < 0.002$), respectively (Fig. 5).

A few bronchial and bronchiolar epithelial cells of sham-control WT and Cx32 KO mice were positive for the CYP2E1 enzyme (Fig. 6A). The numbers of

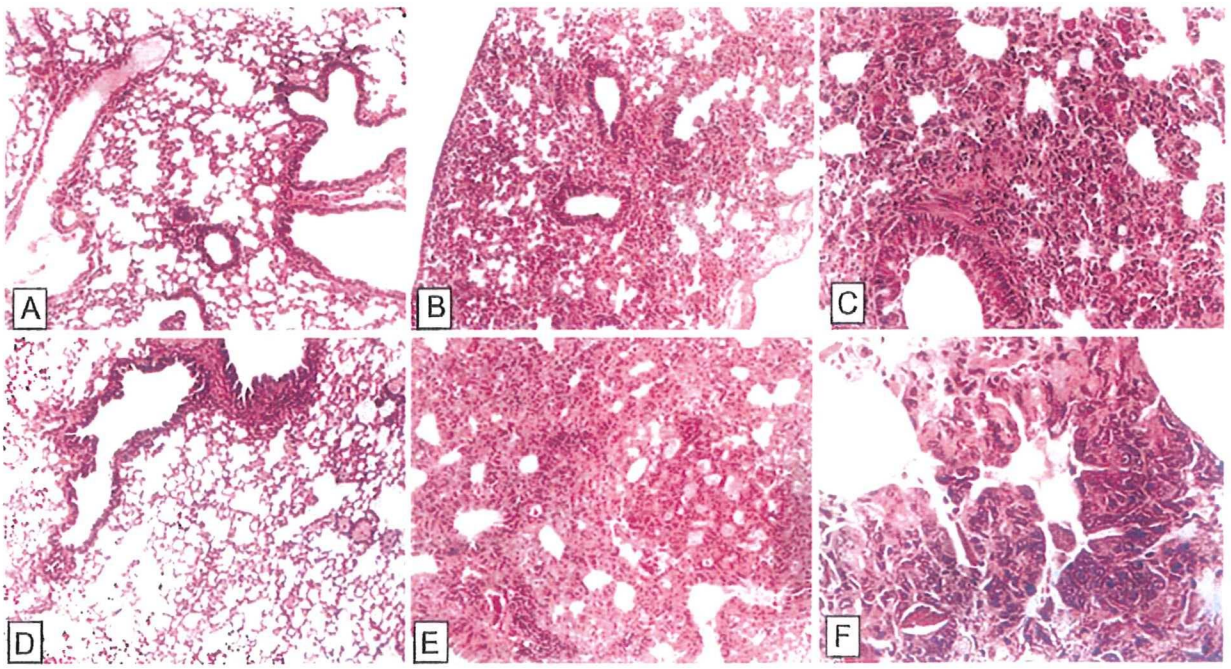

Fig. 3. Histopathological changes of the lungs of benzene-exposed WT and Cx32 KO mice exposed to 300 ppm benzene for 26 weeks. (A) sham-control WT mice, (B and C) benzene-exposed WT mice, (D) sham-control Cx32 KO mice, (E and F) benzene-exposed Cx32 KO mice. Note the granulomatous interstitial pneumonia in the lungs of benzene-exposed WT and Cx32 KO mice, and basophilic epithelial cell-proliferating foci frequently observed in the lungs of benzene-exposed Cx32 KO mice (F). Original magnification: (A) ×100; (B) ×100; (C) ×200; (D) ×100; (E) ×100; (F) ×400. Hematoxylin- and eosin-stained.

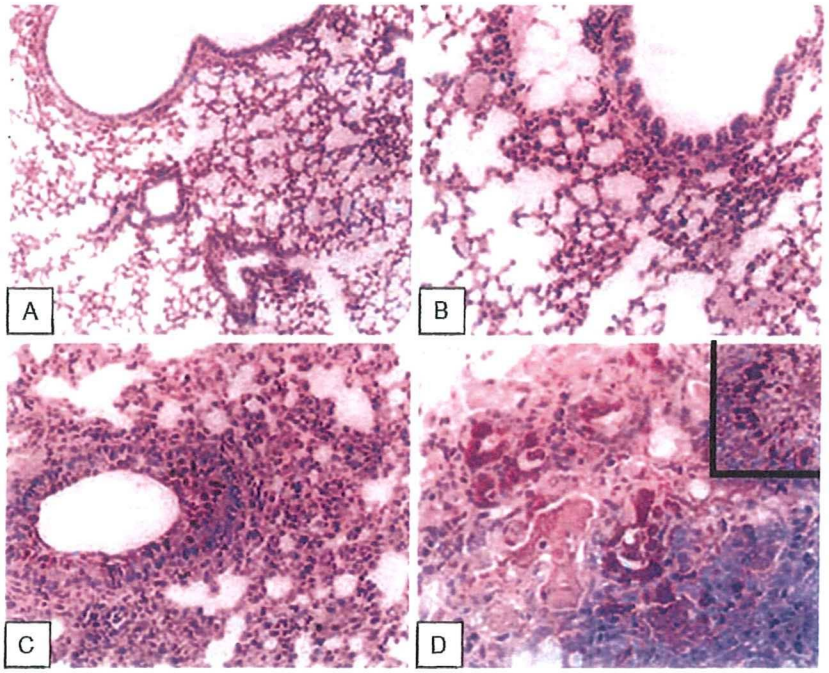

Fig. 4. Histochemistry for detection mucin secretion. (A) Sham-control WT mice, (B) sham-control Cx32 KO mice, (C) benzene-exposed WT mice, (D) benzene-exposed Cx32 KO mice. Note the enhanced mucin secretion from bronchiolar epithelial cells of WT mice (C) and Cx32 KO mice (Inset in D), and the aggregated cells releasing mucin occasionally observed in the benzene-exposed Cx32 KO mice (D). Original magnification: (A) ×200; (B) ×200; (C) ×200; (D) ×400.

CYP2E1-positive bronchial and bronchiolar epithelial cells considerably increased following long-term benzene exposure in both WT and Cx32 KO mice (Fig. 6B). The proliferating basophilic alveolar epithelial cells frequently observed in the benzene-exposed Cx32 KO mice were strongly positive for CYP2E1 (Fig. 6D), which was significantly comparable with the WT mice in which these findings were rarely observed.

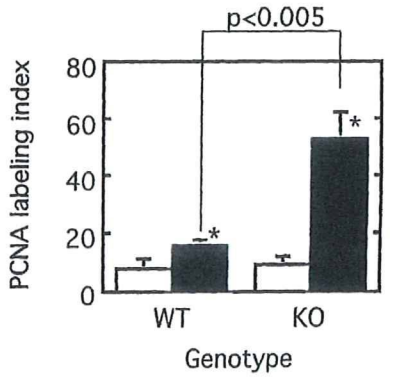

Fig. 5. PCNA labeling indices in the mouse lung tissues exposed to 300 ppm benzene for 26 weeks. Values represent the number of PCNA-positive cell per 1000 cells. More than 3,000 alveolar epithelial cells were counted under a light microscope at a high magnification (×400).

3.5. Survival curves for life time observation

Five to seven mice were randomly selected and allowed to live their life span to evaluate their recovery from pulmonary lesions and the incidence of pulmonary tumor. Survival curves for each group are shown in Fig. 7. In each group the number of mice were limited to about five to seven mice per group. There was no intermittent death during the exposure time up to 182 days (26 weeks). The sham-exposed control group indicated by open symbols, circles for WT mice and squares for Cx32 KO mice, show a longer life span than the benzene-exposed group indicated by closed symbols, circles for WT mice and squares for Cx32 KO mice. Interestingly, in the exposed group, Cx32 KO mice showed a longer life span than the wild-type mice, although the sham-exposed group does not show much difference between wild-type mice and Cx32 KO mice. During the observation period, all the mice that

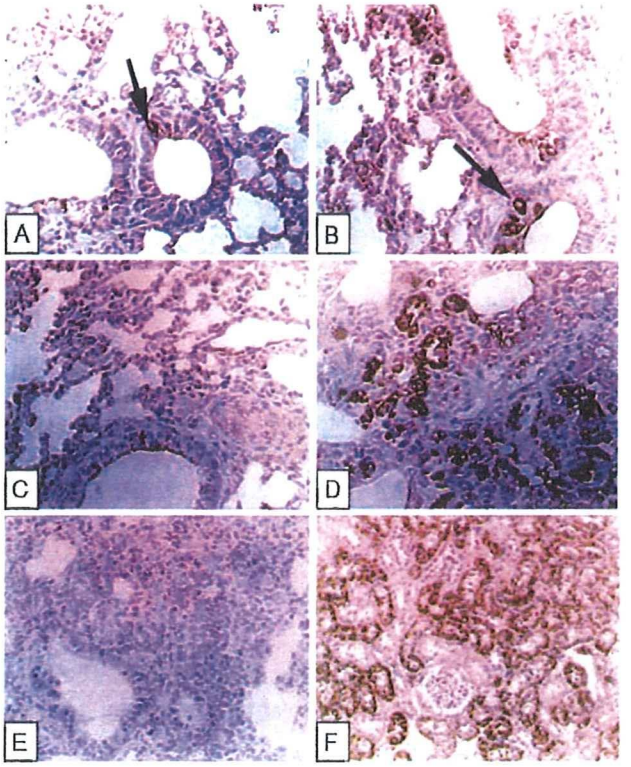

Fig. 6. Immunohistochemistry for P450 CYP2E1 in the lungs of WT and Cx32 KO mice exposed to 300 ppm benzene for 26 weeks. (A) Sham-control WT mice, (B) benzene-exposed WT mice, (C) sham-control Cx32 KO mice, (D) benzene-exposed Cx32 KO mice, and (E and F) negative and positive control, respectively. A few bronchiolar epithelial cells of sham-control WT and Cx32 KO mice were positive for CYP2E1 (arrows). Benzene exposure induced increases in the numbers of the CYP2E1-producing bronchial/bronchiolar and alveolar epithelial cells in WT and Cx32 KO mice. Note the proliferating basophilic alveolar epithelial cells positive for CYP2E1 in the benzene-exposed Cx32 KO mice (D). Original magnification: (A) ×200; (B) ×200; (C) ×200; (D) ×400; (E) ×200; (F) ×200.

died were immediately autopsied, whenever possible, and histopathological examinations were performed.

3.6. Tumor incidence and recovery of pulmonary lesions

Results of histopathological observation are shown in Table 2. No pulmonary tumors were observed in WT and Cx32 KO C57BL/6 mice sacrificed after the 26th week of exposure to 300 ppm benzene. Pulmonary adenoma developed in one Cx32 sham-control mouse. Pulmonary adenoma and adenocarcinoma developed only in two out of the seven benzene-exposed WT

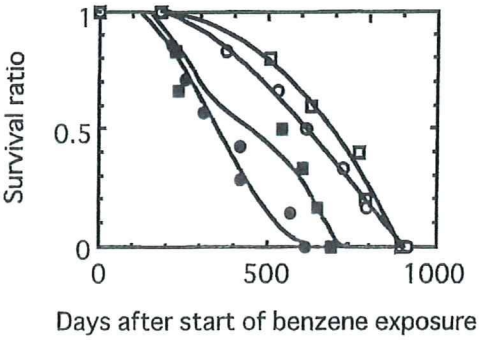

Fig. 7. Survival curves of groups for lifetime observation. The number of mice was limited about five to seven mice per group. There was no intermittent death during the exposure time up to 182 days (26 weeks). The sham-exposed group, indicated by open symbols, circles for wild-type mice and squares for Cx32 KO mice, show a longer life span than the 300-ppm benzene-exposed group indicated by closed symbols, circles for wild-type mice and squares for Cx32 KO mice.

mice, at 59.7 weeks and 87.3 weeks of the study, respectively (Table 2). The pulmonary lesions observed in the WT and Cx32 KO mice exposed to benzene for 26 weeks were considerably attenuated and regressed with time after cessation of the exposure (data not shown).

Most of the benzene-exposed WT and Cx32 KO mice, which were allowed to live out their lives after termination of benzene exposure, died far earlier than the sham-control mice of each genotype due to malignant lymphomas, squamous cell carcinomas, spindle cell sarcoma and hepatomas or a combination of these tumors (Table 2).

The incidence of hemopoietic neoplasia in C57BL/6 mice was enhanced by 300 ppm benzene exposure as previously reported elsewhere (Snyder et al., 1980; Cronkite et al., 1985; Kawasaki et al., unpublished observation). In the Cx32 KO group, incidences of hemopoietic neoplasia were identical for both the benzene-exposed and sham-exposed control mice, although peak incidences were earlier in the benzene-exposed mice than in the sham-exposed control mice. (see Fig. 7).

4. Discussion

Benzene has been suspected for years as an agent that induces human pulmonary cancer (Aksoy, 1989)

Table 2
Tumor development in the wild-type (WT) and Cx32 knockout (KO) mice that were allowed to live out their life span after termination of 26-week exposure to 300 ppm benzene

Tumor/group (with or without benzene treatment)	Genotype			
	WT		Cx32 KO	
	Sham-exposed	300 ppm	Sham-exposed	300 ppm
No. of animals examined	6	7	5	6
Animals bearing tumor(s)	3 (50.0)	6 (85.7)	4 (80.0)	6 (100.0)
Pulmonary adenoma/adenocarcinoma	0 (0.00)	2 (28.6)	0 (0.0)	0 (0.0)
Hemopoietic neoplasia	2 (33.3)	5 (71.4)	4 (80.0)	5 (83.3)
Hepatoma	2 (33.3)[a]	0 (0.0)	1 (20.0)[a]	1 (16.7)
Squamous cell carcinoma	0 (0.0)	2 (28.6)[b]	0 (0.0)	4 (66.7)[c]
Spindle cell sarcoma	0 (0.0)	1 (14.3)	0 (0.0)	1 (16.7)
Animals without tumor(s)[d]	3 (50.0)	1 (14.3)	1 (20.0)	0 (0.0)

Number in parentheses represents the percentage (%) of the lesions.
[a] Concomitant with malignant lymphomas.
[b] Concomitant with pulmonary adenocarcinoma and spindle cell sarcoma.
[c] Concomitant with spindle cell sarcoma for one mouse, granulocytic leukemia for two mice and hepatoma for one mouse.
[d] Mice without tumor in the WT sham-control and the 300-ppm benzene-exposed groups had auricular thrombosis and one mouse without tumor in the Cx32 KO-control group died of ascending nephritis and renal infarction.

and the long-term exposure of mice to benzene had been shown to notably increase the incidence of pulmonary adenoma and adenocarcinoma (Huff et al., 1989; Maltoni et al., 1989; Farris et al., 1993). However, little information is available on the mechanism by which benzene exerts its pneumotoxicity and induces lung cancer.

Experimental conditions of benzene exposure and the incidence of hemopoietic neoplasia occurring in groups for lifetime observation were identical to those previously reported by Snyder et al. (1980, Fig. 4, p. 326 in their article) and also to our separate large-scale study (Kawasaki et al., unpublished observation). In the present study, we specifically focused on a possible role of Cx32 in benzene-induced pneumotoxicity and the pathogenesis of pulmonary tumor using bioengineered Cx32 KO and the WT mice.

Although Cx32-deficient mice have a late-onset peripheral neuropathy, a condition with features similar to those of Charcot–Marie–Tooth disease in humans, their gross morphology had been reported to be normal independent of age (7–28 weeks) and gender, except for a slightly lower body weight than the wild-type mice of the same genetic background (Nelles et al., 1996; Anzini et al., 1997). In agreement with a previous report, the body weight of Cx32 KO mice was lower than that of WT mice at the late stage of this study, although this difference was not significantly different (Fig. 1). The organ weight and histological findings consistently indicated that the decrease in body weight observed during long-term exposure to benzene closely correlated with the development of pulmonary lesions, characterized by diffuse granulomatous interstitial pneumonia, regenerating alveolar epithelial cell proliferation, and increased mucus secretion (Table 1, Figs. 2–5). The pulmonary lesions were far severer in Cx32 KO mice than in WT mice, strongly suggesting that Cx32 prevents the benzene-induced lung pathogenesis.

It has generally been accepted that the metabolism of benzene by the CYP2E1 enzyme to phenolic metabolites is a critical event in its toxic and carcinogenic mechanisms. A noteworthy finding of our study was the active proliferation of bronchiolar–alveolar epithelial cells expressing the CYP2E1 enzyme in the lungs of benzene-exposed Cx32 KO mice (Figs. 4D and 6F). This suggests that benzene exposure stimulates CYP2E1-producing epithelial cells in the lungs through a pathway that is regulated by the Cx32 gap junction protein. The activation of CYP2E1-producing epithelial cells may enhance the metabolism of benzene to metabolites that are potentially pneumotoxic such as benzene oxide, phenol and hydroquinone, resulting in exacerbation of

benzene-induced pneumotoxicity. CYP2E1 has been detected in the lungs of humans and rats (Tindberg and Ingelman-Sundberg, 1989; Carlson and Day, 1992; Wheeler et al., 1992). Moreover, recent studies have shown the important role of the enzyme in benzene metabolism resulting in its pneumotoxicity (Powley and Carlson, 2000, 2001), which is also supported by the finding of benzene metabolism inhibition by a CYP2E1 inhibitor, diethyldithiocarbamate (Chaney and Carlson, 1995). The metabolic level of benzene in the pulmonary tissue has not been evaluated yet. However, a previous study showed that pulmonary microsomes can metabolize benzene at similar rates to those of hepatic microsomes, and that they are likely more efficient in generating hydroquinone (Chaney and Carlson, 1995). Recently, with regards to CYP2E1-mediated 1,1-dichlroethylene-induced lung toxicity, Forkert et al. (2001) reported good correlations among the amount of the enzyme, metabolism of 1,1-dichlroethylene to a toxic metabolite, and lung cytotoxicity.

Activation of alveolar pneumocytes by benzene was considered to be another possible important event for the pneumotoxicity of benzene observed in the present study, as shown in the lungs of benzene-exposed groups of WT and Cx32 KO mice (Fig. 3). Alveolar epithelial cells, containing a large amount of peroxidase, are capable of metabolizing phenolic compounds to genotoxic reactive species that can induce DNA adducts and generate oxygen-free radicals (Brieland et al., 1987; Schlosser et al., 1989; Smith et al., 1989) and of producing nitric oxide by themselves (Laskin et al., 1995). The production of reactive oxygen intermediates has been implicated in cytotoxicity and carcinogenesis, by inhibiting GJICs as well as causing DNA damage (Kuo et al., 1998; Upham et al., 1997, 1998). Several investigators have shown that oxygen radicals from benzene-activated alveolar epithelial cells play an important role in the genotoxic and nongenotoxic mechanisms of benzene-target organs (Subrahmanyam et al., 1991; Kolachana et al., 1993; Laskin et al., 1995). In the lung, Suleiman (1987) showed that benzene induces lipid peroxidation and increases the amount of the lysosomal enzyme released by activating alveolar epithelial cells, contributing to the pathological changes. The formation of oxygen radicals and related reactive oxygen species is highly controlled in a biological system by physiological antioxidant defense mechanisms. In a study by Kojima et al. (1996), a potential role of Cx32 in the regulation of oxygen radical production in cultured hepatocytes has been suggested based on the correlation found between the expression of Cx32 and the effect of oxygen radical scavengers. Therefore, it can also be hypothesized in our present study that the disregulation of reactive oxygen species production by benzene in lung tissues due to a dysfunction of GJICs caused by Cx32 might contribute to the exacerbation of pulmonary lesions in Cx32 KO mice. Further studies will be required to prove this hypothesis.

Despite the finding that the Cx32-mediated disruption of GJICs enhanced the pneumotoxicity of benzene, our present study, though with a small number of animals did not indicate any enhancement of the development of pulmonary tumor in the Cx32 KO mice (Table 2).

Therefore, the pathological lesions exacerbated in Cx32 KO mice may not seem to be critical changes for pulmonary carcinogenesis of benzene. This was supported by their recovery from the pulmonary lesions after removal of benzene and the absence of tumor incidence in benzene-exposed Cx32 KO mice.

In conclusion, our present study indicates that Cx32 attenuates the pneumotoxicity of benzene, particularly in the case of chronic exposure in vivo, most likely by regulating proliferation of CYP2E1-producing lung cells population. However, the role of Cx32 in benzene-induced pulmonary tumorigenesis was not clarified in the present study.

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References

Abraham, V., Chou, M.L., DeBolt, K.M., Koval, M., 1999. Phenotypic control of gap junctional communication by cultured alveolar epithelial cells. Am. J. Physiol. 276, L825–834.

Abraham, V., Chou, M.L., George, P., Pooler, P., Zaman, A., Savani, R.C., Koval, M., 2001. Heterocellular gap junctional

communication between alveolar epithelial cells. Am. J. Physiol. Lung Cell Mol. Physiol. 280, L1085–1093.

Aksoy, M., 1985. Malignancies due to occupational exposure to benzene. Am. J. Ind. Med. 7, 395–402.

Aksoy, M., 1989. Hematotoxicity and carcinogenicity of benzene. Environ. Health Perspect. 82, 193–197.

Albright, C.D., Jones, R.T., Grimley, P.M., Resau, J.H., 1990. Intercellular communication in bronchial epithelial cells: review of evidence for a possible role in lung carcinogenesis. Toxicol. Pathol. 18, 324–341 (Discussion 341–323).

Anzini, P., Neuberg, D.H., Schachner, M., Nelles, E., Willecke, K., Zielasek, J., Toyka, K.V., Suter, U., Martini, R., 1997. Structural abnormalities and deficient maintenance of peripheral nerve myelin in mice lacking the gap junction protein connexin 32. J. Neurosci. 17, 4545–4551.

Brieland, J.K., Kunkel, R.G., Fantone, J.C., 1987. Pulmonary alveolar macrophage function during acute inflammatory lung injury. Am. Rev. Respir. Dis. 135, 1300–1306.

Busby Jr., W.F., Wang, J.S., Stevens, E.K., Padykula, R.E., Aleksejczyk, R.A., Berchtold, G.A., 1990. Lung tumorigenicity of benzene oxide, benzene dihydrodiols and benzene diolepoxides in the BLU:Ha newborn mouse assay. Carcinogenesis 11, 1473–1478.

Carlson, G.P., Day, B.J., 1992. Induction by pyridine of cytochrome P450IIE1 and xenobiotic metabolism in rat lung and liver. Pharmacology 44, 117–123.

Chaney, A.M., Carlson, G.P., 1995. Comparison of rat hepatic and pulmonary microsomal metabolism of benzene and the lack of benzene-induced pneumotoxicity and hepatotoxicity. Toxicology 104, 53–62.

Cronkite, E.P., Drew, R.T., Inoue, T., Bullis, J.E., 1985. Benzene hematotoxicity and leukemogenesis. Am. J. Ind. Med. 7, 447–456.

Farris, G.M., Everitt, J.I., Irons, R.D., Popp, J.A., 1993. Carcinogenicity of inhaled benzene in CBA mice. Fundam. Appl. Toxicol. 20, 503–507.

Forkert, P.G., Boyd, S.M., Ulreich, J.B., 2001. Pulmonary bioactivation of 1,1-dichloroethylene is associated with CYP2E1 levels in A/J, CD-1, and C57BL/6 mice. J. Pharmacol. Exp. Ther. 297, 1193–1200.

Huff, J.E., Haseman, J.K., DeMarini, D.M., Eustis, S., Maronpot, R.R., Peters, A.C., Persing, R.L., Chrisp, C.E., Jacobs, A.C., 1989. Multiple-site carcinogenicity of benzene in Fischer 344 rats and B6C3F1 mice. Environ. Health Perspect. 82, 125–163.

Kojima, T., Mitaka, T., Mizuguchi, T., Mochizuki, Y., 1996. Effects of oxygen radical scavengers on connexins 32 and 26 expression in primary cultures of adult rat hepatocytes. Carcinogenesis 17, 537–544.

Kolachana, P., Subrahmanyam, V.V., Meyer, K.B., Zhang, L., Smith, M.T., 1993. Benzene and its phenolic metabolites produce oxidative DNA damage in HL60 cells in vitro and in the bone marrow in vivo. Cancer Res. 53, 1023–1026.

Kolaja, K.L., Engelken, D.T., Klaassen, C.D., 2000. Inhibition of gap-junctional-intercellular communication in intact rat liver by nongenotoxic hepatocarcinogens. Toxicology 146, 15–22.

Kuo, M.L., Jee, S.H., Chou, M.H., Ueng, T.H., 1998. Involvement of oxidative stress in motorcycle exhaust particle-induced DNA damage and inhibition of intercellular communication. Mutat. Res. 413, 143–150.

Laskin, J.D., Rao, N.R., Punjabi, C.J., Laskin, D.L., Synder, R., 1995. Distinct actions of benzene and its metabolites on nitric oxide production by bone marrow leukocytes. J. Leukoc. Biol. 57, 422–426.

Lee, Y.C., Yellowley, C.E., Li, Z., Donahue, H.J., Rannels, D.E., 1997. Expression of functional gap junctions in cultured pulmonary alveolar epithelial cells. Am. J. Physiol. 272, L1105–1114.

Maltoni, C., Ciliberti, A., Cotti, G., Conti, B., Belpoggi, F., 1989. Benzene, an experimental multipotential carcinogen: results of the long-term bioassays performed at the Bologna Institute of Oncology. Environ. Health Perspect. 82, 109–124.

Moennikes, O., Buchmann, A., Romualdi, A., Ott, T. Werringloer, J., Willecke, K., Schwarz, M., 2000. Lack of phenobarbital-mediated promotion of hepatocarcinogenesis in connexin32-null mice. Cancer Res. 60, 5087–5091.

Nelles, E., Bützler, C., Jung, D., Temme, A., Gabriel, H.D., Dahl, U., Traub, O., Stümpel, F., Jungermann, K., Zielasek, J., Toyka, K.V., Dermietzel, R., Willecke, K., 1996. Defective propagation of signals generated by sympathetic nerve stimulation in the liver of connexin32-deficient mice. Proc. Natl. Acad. Sci. U.S.A. 93, 9565–9570.

Neveu, M.J., Babcock, K.L., Hertzberg, E.L., Paul, D.L., Nicholson, B.J., Pitot, H.C., 1994. Colocalized alterations in connexin32 and cytochrome P450IIB1/2 by phenobarbital and related liver tumor promoters. Cancer Res. 54, 3145–3152.

Powley, M.W., Carlson, G.P., 2000. Cytochromes P450 involved with benzene metabolism in hepatic and pulmonary microsomes. J. Biochem. Mol. Toxicol. 14, 303–309.

Powley, M.W., Carlson, G.P., 2001. Hepatic and pulmonary microsomal benzene metabolism in CYP2E1 knockout mice. Toxicology 169, 187–194.

Ruch, R.J., Cesen-Cummings, K., Malkinson, A.M., 1998. Role of gap junctions in lung neoplasia. Exp. Lung Res. 24, 523–539.

Schlosser, M.J., Shurina, R.D., Kalf, G.F., 1989. Metabolism of phenol and hydroquinone to reactive products by macrophage peroxidase or purified prostaglandin H synthase. Environ. Health Perspect. 82, 229–237.

Shoda, T., Mitsumori, K., Onodera, H., Toyoda, K., Uneyama, C., Takada, K., Hirose, M., 2000. Liver tumor-promoting effect of beta-naphthoflavone, a strong CYP 1A1/2 inducer, and the relationship between CYP 1A1/2 induction and Cx32 decrease in its hepatocarcinogenesis in the rat. Toxicol. Pathol. 28, 540–547.

Smith, M.T., Yager, J.W., Steinmetz, K.L., Eastmond, D.A., 1989. Peroxidase-dependent metabolism of benzene's phenolic metabolites and its potential role in benzene toxicity and carcinogenicity. Environ. Health Perspect. 82, 23–29.

Snyder, C.A., Goldstein, B.D., Sellakumar, A.R., Bromberg, I., Laskin, S., Albert, R.E., 1980. The inhalation toxicology of benzene: incidence of hematopoietic neoplasms and hematotoxicity in ARK/J and C57BL/6J mice. Toxicol. Appl. Pharmacol. 54, 323–331.

Snyder, C.A., Sellakumar, A.R., James, D.J., Albert, R.E., 1988. The carcinogenicity of discontinuous inhaled benzene

exposures in CD-1 and C57Bl/6 mice. Arch. Toxicol. 62, 331–335.

Snyder, R., Hedli, C.C., 1996. An overview of benzene metabolism. Environ. Health Perspect. 104 (Suppl. 6), 1165–1171.

Subrahmanyam, V.V., Ross, D., Eastmond, D.A., Smith, M.T., 1991. Potential role of free radicals in benzene-induced myelotoxicity and leukemia. Free Radic. Biol. Med. 11, 495–515.

Suleiman, S.A., 1987. Petroleum hydrocarbon toxicity in vitro: effect of n-alkanes, benzene and toluene on pulmonary alveolar macrophages and lysosomal enzymes of the lung. Arch. Toxicol. 59, 402–407.

Tindberg, N., Ingelman-Sundberg, M., 1989. Cytochrome P-450 and oxygen toxicity. Oxygen-dependent induction of ethanol-inducible cytochrome P-450 (IIE1) in rat liver and lung. Biochemistry 28, 4499–4504.

Upham, B.L., Kang, K.S., Cho, H.Y., Trosko, J.E., 1997. Hydrogen peroxide inhibits gap junctional intercellular communication in glutathione sufficient but not glutathione deficient cells. Carcinogenesis 18, 37–42.

Upham, B.L., Deocampo, N.D., Wurl, B., Trosko, J.E., 1998. Inhibition of gap junctional intercellular communication by perfluorinated fatty acids is dependent on the chain length of the fluorinated tail. Int. J. Cancer 78, 491–495.

Wheeler, C.W., Wrighton, S.A., Guenthner, T.M., 1992. Detection of human lung cytochromes P450 that are immunochemically related to cytochrome P450IIE1 and cytochrome P450IIIA. Biochem. Pharmacol. 44, 183–187.

Yamasaki, H., Mesnil, M., Omori, Y., Mironov, N., Krutovskikh, V., 1995. Intercellular communication and carcinogenesis. Mutat. Res. 333, 181–188.

Yamasaki, H., 1996. Role of disrupted gap junctional intercellular communication in detection and characterization of carcinogens. Mutat. Res. 365, 91–105.

Yoon, B.I., Hirabayashi, Y., Kawasaki, Y., Kodama, Y., Kaneko, T., Kim, D.Y., Inoue, T., 2001. Mechanism of action of benzene toxicity: cell cycle suppression in hemopoietic progenitor cells (CFU-GM). Exp. Hematol. 29, 278–285.

4. トキシコゲノミクス

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遺伝子発現カスケード解析を目指した形質非依存型トキシコゲノミクスに適用するため，マイクロアレイから細胞1個当たりのmRNA絶対量を得る方法（“Percellome”）を開発した．これにより遺伝子発現量を，ゼロを起点とする均等目盛りで表示し直接比較することができるようになった．対照群も処置群も無理なく同列に表示することができ，さらなる標準化操作が原則的に不必要となったため，測定し得たすべての遺伝子についてマイクロアレイ間はもとより，実験間での直接比較が行えるようになった．この特長は，生物学者が内容を直感的に把握しやすいようなデータの可視化にも役立ち，その後のデータ解析とインフォマティクス形成を促進することが示されつつある．本システムは大型プロジェクトを対象として開発したものであるが，実際には小規模の実験サンプルに対しても有用性が高いことが実証されている．特に変動遺伝子リストの遺伝子数が飛躍的に増大することが多い．それは，変動比率による足切りやハズレ値計算のような統計手法を用いる必要がなく，個々の遺伝子について逐一比較検討ができるためである．異なったプラットフォーム間でのデータ互換にも拡張可能であり，研究規模やプラットフォームの種類にかかわらずデータをもちより，相互にデータを直接比較することが可能なコンソーシアムを構築することに本手法が貢献することが期待される．

はじめに

毒性学は，生物界（biosphere）と化学物質界（chemosphere）との相互作用を解析し，現実に起きてしまった有害作用（薬の副作用，健康被害など）の把握，評価，対策のみならず，そのような事態の未然防止を目指す学問体系である．例えば，PCB（ポリ塩化ビフェニール）はその電気抵抗性，熱安定性，低反応性などから，工業的に優秀な材料として熱交換や絶縁に汎用された．この物質の毒性知識が早期に浸透していれば食品を直接加熱する熱媒体にPCBを用いるという発想は回避されたのかもしれない．当時の生物学・臨床医学・病理学・毒性学では，PCBのような化学物質の生体影響は，肝臓などでの代謝酵素（P450など）の誘導現象として把握されていたが，その基礎となるリガンド依存的転写因子群（AhR, CAR, PXRをはじめとするorphan受容体群）とその関連シグナル伝達に関する事象が明らかとなってきたのは比較的最近のことである[1]．その結果を受けて胎児影響を含む毒性の分子機構が明らかになるに連れて，実際にどの程度の暴露が，どの発達時期の人体に，どのように有害であるかの判断がより正確にくだせるようになり

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つつある．さらには，個人を対象とした毒性評価から，集団（日本国民全体）を対象としたそれまでを，広く見渡すことが要求される．例として有名なのは，PCB暴露によるIQ低下論議である．ある集団のIQが5ポイント下がると，何らかの介護を必要とする人々の数が著増するというものである．すなわち，平均的なIQをもつ個人のIQが5ポイント下がっても実質上問題はないが，社会集団としての影響は無視できないという問題である[2)3)]．科学的には，高感受性亜集団，個体差，動物実験データからヒトへの外挿に際しての種差問題などが関連する．

近年の健康ブームは，いわゆる「サプリメント」など，健康に有益な効能を示唆あるいは謳う一連の食品関連製品を生み出している．他方の医薬品については「薬効」と引き換えに「副作用」が常に考慮されることから，使用者のcost-benefit（費用便益・費用対効果）の概念を基礎に，取り扱いの体系ができあがっており「処方箋」が必要であったり，注意書きが添付されていたりする．これに対してサプリメントなどは食品，および食品に由来する成分からなるとされることから，「食経験」にもとづいた安全性の概念が基本となっている．しかし，食品も医薬品も，生活の利便性のために毎日利用する化学物質も，体内で生体分子と相互作用を起こす．それらの毒性評価を生体側からみると，身体に入るまでの「物質の分類」や「能書き」はもはや重要ではなく，身体に入ったあとにどのような反応が如何に惹起されるかが問題となる．

人体に化学物質が何を引き起こすかを検討するためには，ヒトからの情報を得ることが一番正確なことはいうまでもない．薬の開発の過程では，ヒトによる「臨床試験」が可能である．この場合の毒性には，用量作用関係の概念が乏しい．すなわち，実際に薬として投与するときの薬用量において，どのような毒性（副作用）があらわれるかが，最大の焦点であり，わざわざ「自殺目的」の大量投与を行うことはなく，薬効が期待できない微量投与も行わないわけである．これに対して，いわゆる化学物質，例えば，家庭用品，工業製品，食品添加物などの現代生活の利便性に欠かせない物質に由来する化学成分の体内への侵入に対しては，一般的にcost-benefitの概念が弱く働き，可能ならばゼロにしたいという傾向がある．しかし，「完全ゼロ」は使用する限り基本的には不可能であるので，どの位の量までなら安全と見なせるかを検討することが行われてきている．これらについては，人体実験が倫理的にも現実的にもできないと考えるのが通常である．なお，薬でも「人体実験」が事実上できない対象がある．それは，胎児と子供である．いずれの場合も，現在のところ，人の身代わりとしてモデル動物を用いることになる．他方，食品そのもの，あるいは食品の主成分については「食経験有り」＝「安全」という考えの下に，毒性評価を行ってきていないのが現状である．しかし，成分などの濃縮や抽出により錠剤やエキスの形を取るサプリメントでは少なくとも「調理法」と「摂取量」のコントロールが「今までの経験の適用外」となる場合が多い．このようなものが「処方箋なし」に利用される場合の安全性を検討する際に，人体実験を行うか，動物実験を行うか，動物で得た情報はどのようにヒトに適用するのか，などが問題となる．

化学物質の毒性の量と質の問題

多量に摂取すれば毒性は強く，少量になれば毒性は弱まるという大原則（毒性は用量に関して単調増加する）の下では，「毒性に閾値がある」と考えられる場合と，「閾値が存在しない」と考えられる場合とで扱いを分けている．前者の場合は無毒性量あるいは無作用量を実験動物で求め，種差や個体差を勘案した係数（不確実係数あるいは安全係数と呼び，通常100を用いる）で除して，安全の目安となる基準値とする．後者の場合は，無毒性量の代わりに，俗に「運悪く雷に打たれて死ぬ確率」を目安とする実質安全量（virtually safe dose，通常10^{-5}ないし10^{-6}の危険率を適用）を採用し，同様の手続きを経てヒトへの外挿を行っている．これらの判断が正しいか否かを検討する材料としては人での中毒事例，自殺事例，事故事例やそれらに関する疫学調査が活用され，それにもとづく基準設定法の修正が折にふれて加えられてきた歴史がある．ところで，食品あるいは食品関連製品（サプリメントなど）の場合，安全性評価に不確実係数100を使用するとどういうことになるであろうか．例えば，ニンニクや玉ねぎを毎日1個食べても安全であるという結果を引き出そうとすると，実験動物に毎日100個相当を食べても何も起こらないことを示す必要がある．

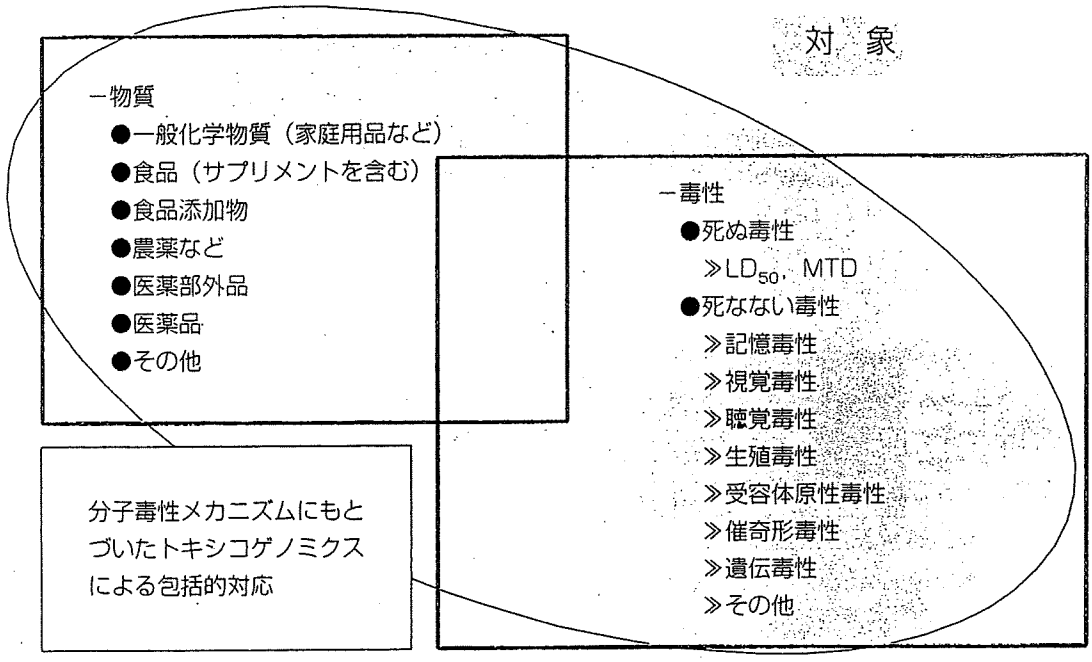

図1　分子毒性メカニズムにもとづいたトキシコゲノミクスが目指す包括的毒性
毒性を分類する際に，物質のカテゴリーを用いたり，毒性の症状を用いたりする．しかし，生体側からみれば，体内に入った物質がどのような生体反応を誘導するかが問題である．分子毒性メカニズムにもとづいたトキシコゲノミクスでは，生体反応を遺伝子発現カスケードとして把握することにより，このような従来の分類を包括した対応を目指す

ニンニクや玉ねぎ中のアリシンが動物に溶血を引き起こすが，100倍量を摂取すれば影響がみられる可能性が高い．すなわち，食品に関して動物実験を行った場合，一般論として不確実係数は利用できず，問題とする成分に対する生体反応のヒト・動物間の種差そのものを検討することが必要となる．

毒性の質的な問題はどのように取り扱われてきたか．生物学が現象の記述学に基礎を置いていた段階での毒性学は，対象が医薬品であれ，一般的な化学物質であれ，その要求される役割を果たすために，投与された化学物質と症状との連関性にもとづいた化学物質の体系化を基盤として発達してきた（図1）．その過程でのさまざまな経験を取り入れる形で，前述の「不確実係数」や「LD_{50}」の概念が利用され，現在まで，非常に有効に機能してきている．ここまでの毒性学は，化学物質の投与とそれによる症状（毒性）発現の関連性を分類し体系化するものであり，実験動物と人とをつなぐために，回帰モデル（regression model）の概念に根差した後向きの検討が行われることが多かった．しかし，サリドマイド禍（奇形発生）に象徴されるように，げっ歯類の実験動物では毒性が確認されず，人に使用して初めて催奇形性が明らかになった事例の存在は，この方法の限界を示している．

火事場の現場検証？

近年，科学の進歩により，毒性学は生体内で引き起こされる反応の分子レベルから形態レベルまでのメカニズム記述を基礎とするものへと変貌しつつある．ここで，活躍するのがハイスループット性の高いマイクロアレイ技術である．しかし，マイクロアレイから得られた遺伝子発現プロファイルによる検討も，そのときに観測される毒性形質と関連付け，いわゆる化学物質のフィンガープリント（指紋）として毒性反応の類型化を行うことが多い．このような関連付けを「phenotypic anchoring」と呼ぶことがある[4]．

分子毒性学の立場からは，化学物質が生体内で引き起こしている一連の事象を理解することが直近の目標である（図2）．毒性所見が明瞭に現れた段階では，化学物質による遺伝子発現はすでに十分にタンパク発現を引き起こしており，その結果としての組織改変までも完了してしまっている．この段階での遺伝子発現プロファイルは，所見と直結したものであることに間違いはないが，そこに至る過程を端的に示すものでは

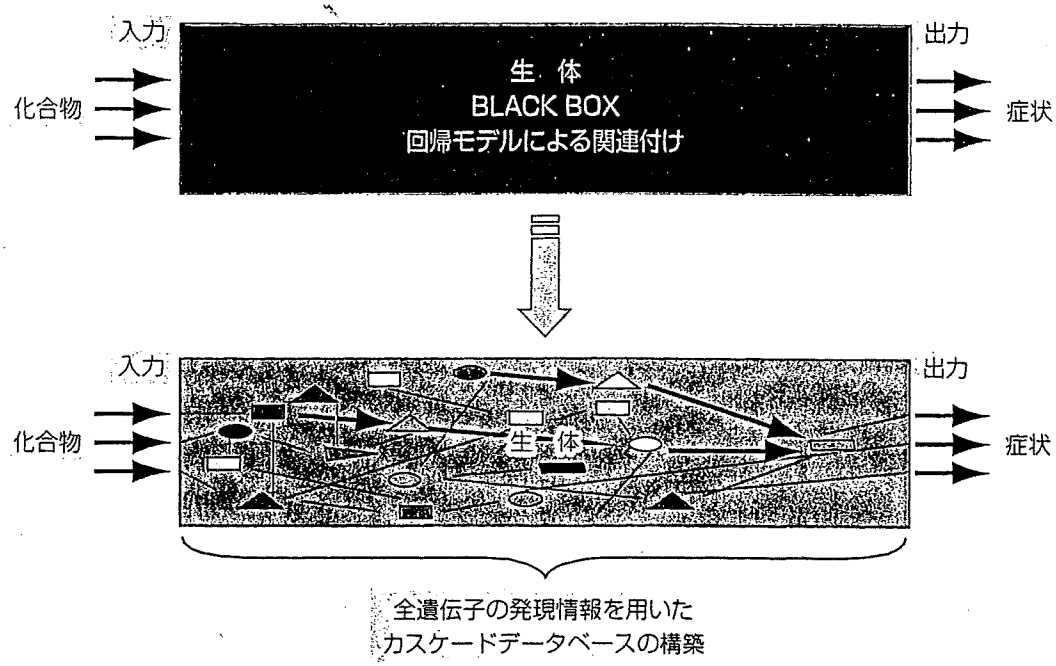

図2　経験則からメカニズムによる予測へ

生物学が現象の記述学に基礎を置いていた段階での毒性学は，投与された化学物質と症状との間を回帰モデルにより関連付けることで体系化が行われてきた．しかし，分子毒性学の立場から一番知りたいことは，生体内で実際に起こっている一連の事象であり，遺伝子発現解析の場合にはすべての遺伝子の情報をもとにした遺伝子カスケードの全容解明である．毒性学的に重要なマーカー遺伝子（数十～数百のことが多い）についてのこのようなデータベースは存在するが，ここではすべての遺伝子を対象としたものを指向する

必ずしもない．丁度，火事場の現場検証で出火元を特定する作業に似ている．これに対する別のアプローチとして，出火直後の変化から逐次検索することが考えられる．すなわち，化学物質に暴露され始めた初期からの遺伝子カスケードの全容解明である（図2）．全ゲノムが明らかになった現在，形質発現の有無にかかわらずすべての遺伝子の発現をモニターするこのようなアプローチ，すなわち「形質非依存型トキシコゲノミクス（phenotype-independent toxicogenomics）」を考慮せざるを得ない．

動物実験を主な手段として駆使しうる立場からは，この目的のための実験プロジェクトを企画することが可能である．タンパクのリン酸化や発現の変化も同時に観測できれば理想的であるが，それらに関する網羅的観測手法が整っていない現段階では，マイクロアレイ技術による遺伝子発現が頼りである．十分に精密かつ実態的に生体反応が記載されれば，従来の膨大な時間と費用のかかる長期毒性試験（ラットなどを用いる）の代替として，より早く，安く，正確な評価，さらに，種差や個人差を勘案した正確なヒト毒性予測が可能となることが強く期待される．特に胎児，新生児，小児，成人，老人の各発達段階における生体側の反応様式・感受性の変化や，複数の物質の進入による複合作用なども包括的に扱えるようになると考えられる．マウスにおいては遺伝子ノックアウト手法により遺伝子ごとの機能解析が可能であり，ヒトではSNPs解析が同様に利用できるであろう．これらについても，形質発現が伴わないために解析が行き詰まった場合には，形質発現に依存しない手段を選ばざるを得ない．恒常性維持機構に深くかかわる内分泌かく乱化学物質の問題など，外界からの影響が効率よく中和されてしまい，形質変化がモニターしにくい対象を扱う場合にも，形質発現の有無にかかわらずmRNAやタンパクの発現修飾を観測することが有効な影響解析手段となることが考えられる．

今後の毒性学における遺伝子発現解析（transcriptome），すなわちトキシコゲノミクス（toxicogenomics）は，従来の「形質依存型」のものに「形質非依存型（phenotype-independent）」のものを加える時期にきているといえよう．

形質非依存型トキシコゲノミクス（phenotype-independent toxicogenomics）の条件

形質依存型では，ある特定の毒性所見に連関した遺伝子をマーカーとして選択し，それが毒性発現に重要であると認定することから始まる．これに対して，形質非依存型トキシコゲノミクスは，まずは形質発現情報などの情報を用いずに，自らの遺伝子発現プロファイル情報のみを頼りに遺伝子発現変化の解析を開始しようとする点に特徴がある．このためには，測定するすべての遺伝子はどれも平等に重要であると仮定する必要がある．そして，そのすべてがどれだけ変動したかを正確に観測する必要がある．さらに，幾多の実験の結果を統合してはじめて全体像が明らかになるため，複数の実験の結果を長期にわたり集積し，それらのデータを縦横に解析する必要がある．

この条件を満たすためには，今までのマイクロアレイ手法には問題があった．まず，マイクロアレイの性能として，mRNAの測定可能な範囲が比較的狭いためにチップ1枚当たりに用いる総mRNA量を一定量に揃える必要があった点である．この場合，サンプル中の細胞1個当たりのmRNAの絶対的な多寡に関する情報は消失してしまう．これを補う種々の標準化手法が編み出されている[5)～12)]が，それらは原則的には統計学的な有意差検定をもとにした変動遺伝子の抽出を行う．このような計算では，一般に大半の遺伝子はサンプル間で不変であるとの前提から，多数の遺伝子が「変動したとはいえない」と位置付けられることが多い．また，変動を表現するために対照サンプルに対する比率表示をすることが多い．この場合，異なる時期に実施した複数の実験から得られたデータを比較する際に，対照群の実験間変動を吟味することが難しいという問題が加わる．

細胞1個当たりのmRNA絶対量を得る方法

このような問題を解決し，形質非依存型トキシコゲノミクスに適用するため，われわれは，細胞1個当たりのmRNA絶対量を得る方法（"Percellome"）を，当時それに必要な条件を満たしていたアフィメトリクス社のGeneChip®を対象に開発した（特許出願中，投稿中）．このシステムは大きく4つの要素からなっている．

①RNA用に準備したサンプル破砕液の一部からそのDNA濃度を簡便に測定する方法：細胞1個当たりのmRNA情報を得るために，サンプルを構成する総細胞数を測定する．実際に細胞数を計測することは特に実質臓器の場合には困難であるため，その代替指標として細胞核内のゲノムDNA量を用いる．サンプルをDNA測定専用に消費することを避けるため，RNA調整用の組織破砕液の極一部（通常，10 μl）からDNAを測定するプロトコールを確立した．

②用量関係を考慮し工夫された多段階濃度スパイクカクテル（GSC：dose-graded spike cocktail）の調整と，それの破砕液への添加法：細胞1個当たりのmRNAの標準として，組織破砕液に添加するスパイクRNAには，GeneChip®が使用者のために用意していた5種類の枯草菌由来遺伝子のRNAを用いた．5種類の枯草菌RNAをおのおの約2,000塩基の長さに合成し，5段階の用量に配合したカクテルを作製した．これにより，広い濃度範囲をカバーする標準用量作用曲線をすべてのサンプルに導入することが可能となった．

③Hill式にもとづいた絶対化アルゴリズム：GeneChip®では，蛍光シグナルとmRNA量との間にHill式に従う関係が成立することを後述のLBM標準サンプルなどにより確認した．その結果からHill式の直線化式によりGSCを直線化して絶対量化を行う変換アルゴリズムを開発し，それを自動実行するプログラムを独自に開発した．

④マイクロアレイの用量相関性確認およびバージョン間・プラットフォーム間データ変換対応のためのLBM（liver-brain mix）標準サンプルおよびデータ変換アルゴリズム：遺伝子発現プロファイルが大きく異なる一対の組織を一定の比率で相互に希釈しあったサンプルセットを表記の目的のために用意した．具体的には，肝と脳を用い，100：0，75：25，50：50，25：75，および0：100の混合比の5サンプルからな

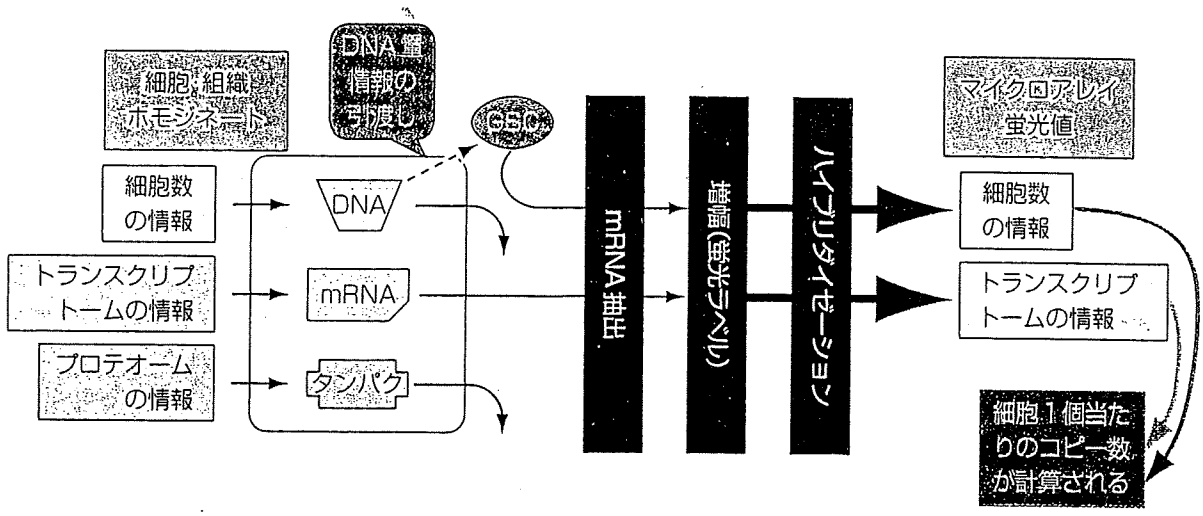

図3　絶対量化の原理
マイクロアレイなどから絶対量を得る方策は，まず，サンプル・ホモジネートのもととなった検体の細胞数の情報をDNA量として捉える．mRNA抽出段階でこのDNA情報が失われてしまうことを回避するために，DNA量の代わりに相当量の多段階濃度スパイクRNAカクテル（GSC）を添加する．mRNA抽出以降，GSCとサンプルのmRNAがともに増幅・蛍光ラベリング，マイクロアレイ表面へのハイブリダイゼーションなどの段階をほぼ平等に経験する．その結果，マイクロアレイの蛍光値を適切に比較・補正することにより，mRNAの細胞1個当たりのコピー数が計算される

るセットを用意した．

絶対量化の基本的原理は，サンプルの細胞数（ゲノムDNA濃度で代替）に比例した分子数のスパイクRNAを添加することで，サンプルの細胞1個当たりのmRNA絶対量（コピー数）の指標をサンプル中に導入するものである（図3）．ただし，スパイクRNAは1点を規定するものではなく，5種類の枯草菌遺伝子に対するRNA（哺乳類の配列と交叉しない）を適切な公比をもたせて5段階の濃度に割り振ったカクテルとして用いることが特長である．これにより，絶対コピー数の指標になると同時に，広い用量範囲について検量線を各サンプルに導入したことになり，mRNA抽出からGeneChip®の蛍光測光までの過程で生じるデータ全体の歪みを補正する際に威力を発揮する

他方，チップ内での異なる遺伝子の発現量の正確さに関しては，GeneChip®のプローブセットの設計に依存する．アフィメトリクス社はプローブの設計に際してそれらのTm値を一定に保つアルゴリズムを用いている．これについては，利用者として個々に定量的PCRなどにより検証する必要がある．本手法の特徴の1つとして，真の値が明らかになった時点で，すべての既測定値を一括修正することが可能であることがあげられる．

LBM（liver-brain mix）標準サンプルとの組合せ

LBMは実験動物サンプルに対しては肝と脳の組合せを用いたが，遺伝子発現プロファイルの異なるペアであればどのような組合せでも利用可能である（ヒトサンプルに対しては2種類のヒト培養細胞株も可）．複数のペアを併用すればさらに精度のよい検定が可能となる．GSCをDNA濃度に応じて添加したLBMセットを測定し，絶対量化した結果は，グラフ化すると直線を描くはずであり，さらに50：50のサンプルで除した場合，理想的にはすべての遺伝子が50：50のところで1の値を取り，100：0あるいは0：100では0から2の間の値を取るところの直線を描くはずである．この結果から，マイクロアレイの定量性が確認される．

さらに，LBMをバージョンアップ前後の新旧GeneChip®で測定しておくことにより（図4），LBMに含まれるすべての遺伝子（プローブセット）について，5点からなる新旧のチップ間の換算関数を求めることができる．LBMに他の臓器の組合せを用いることで取り扱える遺伝子数を増やすことが可能である．

本システムのGSCを添加したサンプルはスパイクRNAを検出するプライマーセットを用意することでPCRにおいても容易に絶対量化データを得ることができる．詳細は他に譲るが，プライマーペアの増幅効率

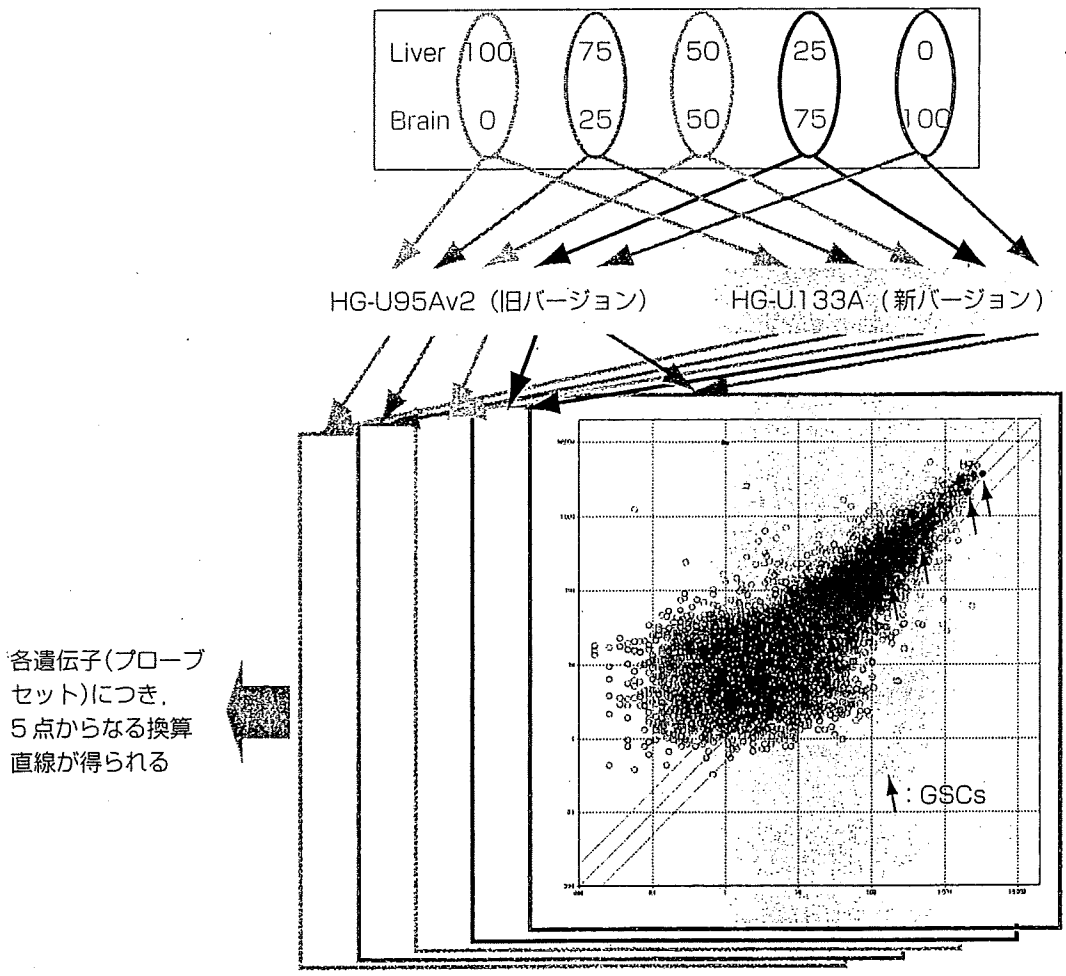

図4　GeneChipの新旧バージョン間のデータ互換
LBMサンプルセットを新旧のバージョンのGeneChip®において測定する．スキャッターグラフで示すような関係が5組得られる．矢印で示す黒丸がGSCである．ここにプロットされた遺伝子（両バージョンに同一または対応するアノテーションが得られ，かつ，LBMサンプルに発現されているもの）については個々について直接変換式が得られる．これは，定量的PCRやアフィメトリクス社以外のマイクロアレイプラットフォームにも拡張可能である

のばらつきを勘案した絶対化アルゴリズムとともにPercellome定量PCRシステムを構築中である．GeneChip®以外のプラットフォームとのデータ互換も可能である．本システムが適応可能なプラットフォームの条件としては，GSCを受け付けるプローブセットが用意されていること，および用量相関性が確保されてることの2点を満たしている必要がある（図5）（現在，2社の製品について検討開発中）．

遺伝子カスケード解析を目指した形質非依存型トキシコゲノミクスへの適用

厚生労働科学研究費のプロジェクトにこのPercellomeシステムが採用され進行中である〔厚生労働科学研究費補助金H14-トキシコ-001（創薬支援トキシコゲノミクス）およびH15-化学-002（化学物質トキシコゲノミクス）〕．4～5段階の用量（公比$\sqrt{10}$等）について，4時点（2，4，8，24時間等）での遺伝子発現を観測する16～20群（一群3匹）の構成からなるプロトコールにより，1つの化合物について48～60匹の動物のサンプルからPercellomeデータを生成している．化学物質トキシコゲノミクスプロジェクトでは，遺伝子の発現値を3次元表示することでその用量･時間依存性を視覚化し，データ解析を進めている．X軸に用量，Y軸に時間，Z軸に発現量（ゼロからの均等目盛り表示）をプロットすることにより，1つの遺伝子につき16～20格子点（48～60枚のGeneChipからのデータ）からなる1枚の局面を描くことができる（図6）．1つのGeneChipが45,000のプローブセットからなる場合，1つの化合物の用量･