Promotion, but not progression, effects of tamoxifen on uterine carcinogenesis in mice initiated with N-ethyl-N'-nitro-N-nitrosoguanidine. Carcinogenesis 2002; **23**: 1549–1555.

57. Pinter A, Torok G, Borzsonyi M, Surjan A, Csik M, Kelecsenyi Z, and Kocsis Z. Long-term carcinogenicity bioassay of the herbicide atrazine in F344 rats. Neoplasia 1990; **37**: 533–544.
58. Watanabe T, Kashida Y, Ueda M, Onodera H, Hirose M, and Mitsumori K. Promoting effects of ethinylestradiol but not atrazine on N-ethyl-N-nitrosourea-induced uterine carcinogenesis in ICR mice. J Toxicol Pathol 2003; **16**: 139–145.
59. IPCS (International Programme on Chemical Safety). Vinclozolin. In: Pesticide Residues in Food-1995 (Joint FAO/WHO Meeting of Pesticide Residues), Geneva: WHO, 375–404, 1996.
60. Rier SE, Martin DC, Bowman RE, Dmowski WP, and Becker JL. Endometriosis in rhesus monkeys (Macaca mulatta) following chronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Fund Appl Toxicol 1993; **21**: 433–441.
61. Moriya M, Mitsumori K, Kato K, Miyazawa T, and Shirasu Y. Carcinogenicity of N-nitroso-ethylenethiourea in female mice. Cancer Lett 1979; **7**: 339–342.
62. Yoshida A, Harada T, Hayashi S, Mori I, Miyajima H, and Maita K. Endometrial carcinogenesis induced by concurrent oral administration of ethylenethiourea and sodium nitrite in mice. Carcinogenesis 1994; **15**: 2311–2318.
63. Nishiyama K, Ando-Lu J, Nishimura S, Takahashi M, Yoshida M, Sasahara K, Miyajima K, and Maekawa A. Initiating and promoting effects of concurrent oral administration of ethylenethiourea and sodium nitrite on uterine endometrial adenocarcinoma development in Donryu rats. In Vivo 1998; **12**: 363–368.
64. Katsuda S, Yoshida M, Kuroda H, Ando J, Takahashi M, Kurokawa Y, Watanabe G, Taya K, and Maekawa A. Uterine adenocarcinoma in N-ethyl-N'-nitro-N-nitrosoguanidine-treated rats with high-dose exposure to p-tert-octylphenol during adulthood. Jpn J Cancer Res 2002; **93**: 117–124.
65. Zhu BT and Conney AH. Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis 1998; **19**: 1–27.
66. Takahashi M, Shimomoto T, Miyajima K, Yoshida M, Katashima S, Uematsu F, Maekawa A, and Nakae D. Effects of estrogens and metabolites on endometrial carcinogenesis in young adult mice initiated with N-ethyl-N'-nitro-N-nitrosoguanidine. Cancer Lett 2004; in press.
67. Kojima T, Tanaka T, and Mori H. Chemoprevention of spontaneous endometrial cancer in female Donryu rats by dietary indole-3-carbinol. Cancer Res 1994; **54**: 1446–1449.
68. Certa H, Fedtke N, Wiegand HJ, Muller AMF, and Bolt HM. Toxicokinetics of p-tert-octylphenol in male Wistar rats. Arch Toxicol 1996; **71**: 112–122.
69. Upmeier A, Degan GH, Schuhmacher US, Certa H, and Bolt HM. Toxicokinetics of p-tert-octylphenol in female DA/Han rats after single i.v. and oral application. Arch Toxicol 1999; **73**: 217–222.
70. NTP. NTP final report on the reproductive toxicity of nonylphenol (CAS# 84852-15-3) administered by gavage to Sprague-Dawley rats. NTP Report # RACB 94021, 1997.
71. Chapin RE, Delaney J, Wang Y, Lanning L, Davis B, Collins B, Minz N, and Wolfe G. The effects of 4-nonylphenol in rats: a multi-generation reproduction study. Toxicol Sci 1999; **52**: 80–91.
72. Nagano T, Wada K, Marumo H, Yoshimura S, and Ono H. Reproductive effects of nonylphenol in rats after gavage administration: a two-generation study. Reprod Toxicol 2001; **15**: 293–315.
73. Yoshida M, Shimomoto T, Katashima S, Shirai T, Nakae D, Watanabe G, Taya K, and Maekawa A. Effects of maternal exposure to nonylphenol on growth and development of the female reproductive system and uterine carcinogenesis in rats. J Toxicol Pathol 2003; **16**: 259–266.
74. Ashby J and Tinwell H. Uterotropic activity of bisphenol A in the immature rat. Environ Health Perspect 1998; **106**: 719–720.
75. Morrissey RE, Lamb JC. IV, Morris RW, Chapin RE, Gulati DK, and Heindel JJ. Results and evaluations of 48 continuous breeding reproduction studies conducted in mice. Fund Appl Toxicol 1989; **13**: 747–777.
76. Morrissey RE, George JJ, Price CJ, Tyl RW, Marr MC, and Kimmel CA. The developmental toxicity of bisphenol A in rats and mice. Fund Appl Toxicol 1987; **8**: 1294–1302.
77. Nagao R, Saito Y, Usumi K, Kuwagata M, and Imai K. Reproductive function in rats exposed neonatally to bisphenol A and estradiol benzoate. Reprod Toxicol 1999; **13**: 303–311.
78. Kwon S, Stedman DB, Ilswick RC, Cattley RC, and Welsch F. Pubertal development and reproductive functions of Crl:CD BR Sprague-Dawley rats exposed to bisphenol A during prenatal and postnatal development. Toxicol Sci 2000; **55**: 399–406.
79. Sharpe RM, Fisher J, Milar MR, Jobling S, and Sumpter JS. Gestational and/or neonatal exposure of rats to environmental estrogenic chemicals results in reduced testis size and daily sperm production in adulthood. Environ Health Perspect 1995; **103**: 1136–1143.
80. vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, Parmigiani S, and Welshons WV. 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 1998; **14**: 239–260.
81. Fisher JS, Turner KJ, Brown D, and Sharpe RM. Effects of neonatal exposure to estrogenic compounds on development of the excurrent ducts of the rat testis through puberty to adulthood. Environ Health Perspect 1999; **107**: 397–405.
82. Howdeshell KL, Hotchkiss AK, Thayer KA, Vanderbergh JG, and vom Saal FS. Exposure to bisphenol A advances puberty. Nature 1999; **401**: 763–764.
83. Ashby J, Tinwell H, and Haseman J. Lack of effects for low dose levels of bisphenol A and diethylstilbestrol on the prostate gland of CF1 mice exposed in utero. Regul Toxicol Pharmacol 1999; **30**: 156–166.
84. Cagen SZ, Waechter JM Jr, Dimond SS, Breslin WJ, Butala JH, Jekat FW, Joiner RL, Shiotsuka RN, Veenstra GE, and Harris LR. Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A. Toxicol Sci 1999; **50**: 36–44.
85. Cagen SZ, Waechter JM Jr, Dimond SS, Breslin WJ, Butala JH, Jekat FW, Joiner RL, Shiotsuka RN, Veenstra GE, and Harris LR. Normal reproductive organ development in

Wistar rats exposed to bisphenol A in the drinking water. Regul Toxicol Pharmacol 1999; **30**: 130–139.
86. Tyl RW, Myers CB, Marr MC, Thomas BF, Keimowitz AR, Brine DR, Veselica MM, Fail PA, Chang TY, Seely JC, Joiner RL, Butala JH, Dimond SS, Cagen SZ, Shiotsuka RN, Stropp GD, and Waechter JM. Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. Toxicol Sci 2002; **68**: 121–146.
87. Kawamura Y, Inoue K, Nakazawa H, Yamada T, and Maitani T. Cause of bisphenol A migration from cans for drinks and assessment of inproved cans. Syokuhin Eiseigaku Zassi 2001; **42**: 13–17.
88. Yoshida M, Shimomoto T, Katashima S, Watanabe G, Taya K, and Maekawa A. Maternal exposure to low doses of bisphenol A has no effects on development of female reproductive tract and uterine carcinogenesis in Donryu rats. J Reprod Dev 2004; **50**: 349–360.
89. Takahashi O and Oishi S. Disposition of orally administered 2,2-bis(4-hydroxyphenol)propane (bisphenol A) in pregnant rats and the placental transfer to fetuses. Environ Health Perspect 2000; **108**: 931–935.
90. Johnson MD, Kenney N, Stoica A, Hilakivi-Clarke L, Singh B, Chepko G, Clarke R, Sholler P, Lirio AA, Foss C, Reiter R, Trock B, Paik S, and Martin MB. Cadmium mimics the in vivo effects of estrogen in the uterus and mammary gland. Nature Med 2003; **9**: 1081–1084.
91. Takasugi N. Cytological basis for permanent vaginal changes in mice treated neonatally with steroid hormones. Int Rev Cytol 1976; **44**: 193–224.
92. Newbold RR and Liehr JG. Induction of uterine adenocarcinoma in CD-1 mice by catechol estrogens. Cancer Res 2000; **60**: 235–237.
93. Okada A, Ohta Y, Buchanan DL, Sato T, Inoue S, Hiroi H, Muramatsu M, and Iguchi T. Changes in ontogenic expression of estrogen receptor alpha and not of estrogen receptor beta in the female rat reproductive tract. J Molecular Endocrinol 2002; **28**: 87–97.
94. Watanabe H, Suzuki A, Mizutani T, Khono S, Lubahn DB, Handa H, and Iguchi T. Genome-wide analysis of changes in early gene expression induced by estrogen. Genes to Cells 2002; **7**: 497–507.
95. Watanabe H and Iguchi T. Evaluation of endocrine disruptors based on gene espression using a DNA microarray. Environ Sci 2003; **10**: 061–067.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Toxicology Letters 163 (2006) 121–129
Toxicology Letters
www.elsevier.com/locate/toxlet

OECD validation of the Hershberger assay in Japan: Phase 3. Blind study using coded chemicals

Kanji Yamasaki [a,*], Ryo Ohta [b], Hirokazu Okuda [c]

[a] *Chemicals Evaluation and Research Institute, 3-822 Ishii, Hita, Oita 877-0061, Japan*
[b] *Food Drug Safety Center, Kanagawa, Japan*
[c] *Japan Bioassay Research Center, Kanagawa, Japan*

Received 3 August 2005; received in revised form 6 October 2005; accepted 6 October 2005
Available online 10 November 2005

Abstract

The Organization for Economic Co-operation and Development (OECD) has initiated the development of new guidelines for the screening and testing of potential endocrine disrupters. The Hershberger assay is one of the assays selected for validation based on the need for in vivo screening to detect androgen agonists or antagonists by measuring the response of five sex accessory organs and tissues of castrated juvenile male rats: the ventral prostate, the seminal vesicles with coagulating glands, the levator ani and bulbocavernosus muscle complex (LABC), Cowper's glands, and the glans penis. The Phase 1 feasibility demonstration stage of the Hershberger validation program has been successfully completed with a single androgen agonist and a single antagonist as reference substances. The Phase 2 validation study was performed, employing a range of additional androgen agonists and antagonists. Recently, the Phase 3 validation study was conducted and performed in several International laboratories. Three Japanese laboratories have contributed to the blind study using coded materials of Phase 3 validation. Four coded test substances in the agonistic version and seven substances in the antagonistic version were orally administered by gavage for 10 consecutive days, respectively. In the antagonist version of the assay, 0.2 mg/kg/day of testosterone propionate (TP) was coadministered by subcutaneous injection. All five accessory sex reproductive organs and tissues consistently responded with statistically significant changes in weight within a narrow window in both versions. Therefore, the Japanese studies support the Hershberger assay as a reliable and reproducible screening assay for the detection of androgen agonistic and antagonistic effects.

Keywords: Blind study; Endocrine; Hershberger assay; OECD validation

1. Introduction

Certain reproductive and developmental toxicants may have the potential to interfere with normal sexual differentiation and development in animals and humans by modulating or interfering with the endocrine system (McLachlan, 1993; McLachlan and Korach, 1995). The Organization for Economic Co-operation and Development (OECD) has initiated an activity to revise existing guidelines and develop new screening and testing guidelines to aid in the identification and assessment of such toxicants (OECD, 1998, 2000, 2002).

One proposed assay, referred to as the Hershberger assay, uses the androgen sensitivity of several accessory sex organs and tissues of the male reproductive tract. The assay was originally developed in the 1930s by Korenchevsky and coworkers, and a number of accessory sex organs and tissues were shown to be use-

* Corresponding author. Tel.: +81 973 24 7211; fax: +81 973 23 9800.
E-mail address: yamasaki-kanji@ceri.jp (K. Yamasaki).

0378-4274/$ – see front matter © 2005 Published by Elsevier Ireland Ltd.
doi:10.1016/j.toxlet.2005.10.001

ful by these and other investigators including the ventral prostate (Deanesly and Parkes, 1936; Dingemanse et al., 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b), the seminal vesicles and coagulating glands (Deanesly and Parkes, 1936; Dingemanse et al., 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b), the preputial glands (Bülbring and Burn, 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b), Cowper's glands (Wainman and Shipounoff, 1941), and the glans penis (Bülbring and Burn, 1935; Dingemanse et al., 1935; Korenchevsky, 1932; Korenchevsky et al., 1932, 1933a,b). In the 1940s, it was discovered that the levator ani and bulbocavernosus muscles also responded to androgens, but in a differential way from the other tissues (Wainman and Shipounoff, 1941; Eisenberg et al., 1949; Eisenberg and Gordan, 1950). The basis for this differential sensitivity is the presence of 5α-reductase in most accessory tissues of the male reproductive tract, but its absence in the muscle complex (Di Salle et al., 1994). The capabilities of the assay were demonstrated in 1953 by Hershberger et al. when they analyzed the response of the ventral prostate, seminal vesicles and coagulating glands, and the levator ani without the bulbocavernosus muscle to a number of active chemicals, including estrogens and progesterones (Hershberger et al., 1953).

In the 1970s and 1980s, with the discovery of the androgen receptor and the first compounds such as cyprotone acetate that were antagonists of the receptor, the assay was modified to address antagonistic activity. Briefly, a set dose of a reference agonist was coadministered to several groups of animals to whom a set of doses of the purported antagonist was also administered. This modified system was successfully used by several investigators for assaying androgen antagonists (Peets et al., 1973; Raynaud et al., 1980, 1984; Wakeling et al., 1981).

Therefore, based upon the recommendation of scientific workshops, both the US Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) (USEPA, 1998) and the OECD Endocrine Disrupter Testing and Assessment Group (EDTA) of the OECD (OECD, 2000) have proposed this assay as a Tier-1 screen to identify possible reproductive and developmental toxicants acting through androgen agonist and antagonist mechanisms.

The OECD Phase 1 validation program for the Hershberger assay was completed in 2001. In this phase, a standardized protocol using the ventral prostate, the seminal vesicles with coagulating glands, the levator ani and bulbocavernosus muscle complex (LABC), Cowper's glands, and the glans penis was successfully tested against a reference androgen compound, testosterone propionate (TP), and a reference antagonist, flutamide (OECD, 2002). The OECD proposed a Phase 2 validation program using additional androgen agonistic and antagonists as the next step to validate the assay, but the final results of Phase 2 studies were not opened by the OECD.

Recently, the OECD conducted a Phase 3 validation program as a final blind study using coded agonistic and antagonistic chemicals (OECD, 2003). In Phase 3, the coded test substances were to be used to investigate the reliability of the assay, including a demonstration of the protocol's transferability among laboratories and the reproducibility of the protocol's results. Three Japanese laboratories participated in the Phase 3 validation study using four coded agonistic test substances and seven antagonistic substances. The participation of the laboratories in the OECD Phase 3 validation study was performed as part of a national validation program in Japan.

2. Materials and methods

2.1. Laboratories

The three participating Japanese laboratories were: the Chemicals Evaluation and Research Institute (CERI); the Food Drug Safety Center; and the Japan Bioassay Research Center. Each laboratory performed the study in compliance with the principles of Good Laboratory Practice guidelines.

2.2. Test substance

All coded test substances except for TP were sent to each laboratory from a centralized chemical repository at TNO, Zeist, the Netherlands. TP and corn oil as vehicles were prepared in each laboratory. The coded substances A, B, L and E were used in the agonistic version, and F, G, I, C, K, D and H were used in the antagonistic version. We did not receive any information regarding the coded substances before all tests were started.

2.3. Animals

Laboratory details regarding rat strain, age of castration, age at start of dosing, day of autopsy, animal diet, and the number of animals housed per cage are summarized in Table 1. Two laboratories used Crj:CD (SD) rats castrated at 6-weeks old, and the test substances were administered 1 week after castration. One laboratory used Brl Han: WIST Jcl (GALAS) rats castrated at 6-weeks old, and the test substances were administered 2 weeks after castration. In all the laboratories, the rats were weighed, weight-ranked, and assigned randomly to each of the experimental and control groups after they had recovered from their operation. Body weight and clinical signs were recorded daily throughout the study. Rats were provided with water and

Table 1
Laboratory detail for rat strain, age of castration, age at start of dosing, day of autopsy, animal diet, and the number of animals housed per cage

Lab	Rat strain	Age of castration	Age at start of dosing	Day of autopsy	Diet	Number of rats per cage
1	Brl Han: WIST Jcl (GALAS)[a]	6-weeks old	8-weeks old	10-weeks old	MF[b]	3
2	Crj:CD (SD)[c]	6-weeks old	7-weeks old	9-weeks old	CE-2[d]	1
3	Crj:CD (SD)[c]	6-weeks old	7-weeks old	9-weeks old	CRF-1[d]	1

[a] Clear Japan Inc., Tokyo, Japan.
[b] Oriental Yeast Co., Ltd., Tokyo, Japan.
[c] Charles River Japan, Kanagawa, Japan.
[d] Clear Japan Inc.

a commercial diet ad libitum. The animals were kept under SPF conditions. All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by The Japanese Association for Laboratory Animal Science.

2.4. Administration

We performed each test according to the protocol proposed by the OECD (OECD, 2000, 2002, 2003). Each test substance was orally administered via a stomach tube for 10 consecutive days at approximately the same time each day. A vehicle control group receiving only corn oil was used in both versions. For the antagonistic version, 0.2 mg/kg/day of TP was coadministered each day by subcutaneous injection in the dorsal region after the oral administration of each chemical. The volume of the corn oil solution containing the TP was 0.5 ml/kg. In the agonistic version, a positive control group of animals received TP injections alone. The group size in all cases was six rats. The volume of the corn oil solutions containing each of the test chemicals was 5 ml/kg. The animals were killed by bleeding from the abdominal vein under deep ether anesthesia approximately 24 h after receiving their final dosage. The five mandatory tissues, the ventral prostate and fluid, seminal vesicle and fluid, LABC, glans penis, and Cowper's gland, were carefully dissected free of adhering fat and weighed to the nearest 0.1 mg. We also weighed the liver in three laboratories, and paired kidney and adrenal weights were measured in one laboratory.

2.5. Statistical analysis

We received the information from the coordinator of this Phase 3 validation after all tests were finished that the participating laboratories received pairs of the test chemicals (i.e. L and E, F and G, I and C, or K and D), so we analyzed the data using the following analytical methods between the vehicle control group and the same chemical groups in the agonistic version, and the TP group and the same chemical groups in the antagonistic version. In addition, coded A and F were nonylphenol, B and G were dinitrophenol, E and L were trenbolone, C and I were *p,p'*-DDE, and D and K were linurone. Body weight and organ weight data were analyzed by Bartlett's test for homogeneity of variance. When the variance was homogeneous at a significance level of 5%, one-way analysis of variance was performed. If a significant difference was found, the difference between the control group/TP group and each of the dosage groups was analyzed with Dunnett's test. If the variance was not homogeneous, the Kruskal–Wallis test was used. If a significant difference was found, the difference between the control group/TP group and each of the dosage groups was analyzed by the non-parametric Dunnett's test. On the other hand, differences in body weight and organ weight between the control group and the TP group, coded A or B in the agonistic version and between TP group and the group using coded H, G or F in the antagonistic version were assessed for statistical significance by the two-tailed Student's *t*-test. For graphical presentation, the sex accessory organ data were normalized to visually compare the shapes of the responses produced by each laboratory. For this normalization, the control value was set to 100% in the agonistic study, and 100% in the TP without coded compound in the antagonistic study. Analyses of variance were performed on the data from each laboratory and for the pooled laboratory data; these normalized values were not analyzed statistically.

3. Results

3.1. Agonistic version

3.1.1. Body weights, clinical observations, and optional organ weights

The body weights and the optional organ weight changes are shown in Table 2. Terminal body weights in rats given L were significantly lower than in rats given vehicle alone in Labs 2 and 3, and tendency towards lowering of the terminal body weights was observed in Lab 1. No abnormal clinical signs were observed in any of the rats that were treated with each substance. The paired kidney weights in rats given substance A and TP were significantly higher than in rats given only the vehicle in Lab 3, and the liver weights in rats given A and TP were also higher than in rats given the vehicle only in Lab 2.

Table 2
Optional organ weights including the liver, adrenal, and kidney in agonistic version

Lab	Body weights/organ weights	Substances					
		V.C.	A	B	L	E	TP
1	Starting body wt. (g)	214.8 ± 10.6	214.4 ± 11.0	213.1 ± 10.1	219.1 ± 10.5	215.0 ± 8.0	214.9 ± 9.3
	Terminal body wt. (g)	262.9 ± 17.0	250.6 ± 12.0	252.4 ± 12.8	243.7 ± 9.2	249.4 ± 12.9	266.0 ± 12.7
	Liver (g)	10.0 ± 1.1	10.2 ± 1.3	9.6 ± 0.5	11.1 ± 0.7	9.7 ± 0.7	10.1 ± 0.8
2	Starting body wt. (g)	231.0 ± 5.3	227.5 ± 5.0	229.6 ± 6.9	229.1 ± 3.9	226.8 ± 7.9	230.9 ± 6.9
	Terminal body wt. (g)	280.8 ± 7.9	275.9 ± 6.3	285.2 ± 12.4	261.5 ± 7.1*	281.8 ± 15.0	305.6 ± 15.0*
	Liver (g)	11.1 ± 0.6	12.1 ± 0.8*	10.7 ± 1.1	11.5 ± 0.6	11.5 ± 0.9	12.8 ± 1.2*
3	Starting body wt. (g)	257.1 ± 8.9	256.7 ± 8.9	256.0 ± 8.3	257.4 ± 7.9	256.8 ± 10.1	255.0 ± 12.2
	Terminal body wt. (g)	303.2 ± 15.5	297.3 ± 17.6	308.3 ± 14.9	264.6 ± 26.4*	300.8 ± 14.9	320.0 ± 22.7
	Liver (g)	12.9 ± 0.9	14.0 ± 1.9	13.0 ± 1.3	12.2 ± 1.6	13.0 ± 1.3	13.6 ± 2.0
	Adrenals (mg)	58.8 ± 9.3	57.0 ± 9.2	61.5 ± 10.6	49.9 ± 7.8	50.2 ± 5.1	51.0 ± 11.7
	Kidneys (mg)	2110 ± 72	2344 ± 138*	2290 ± 162	2229 ± 226	2189 ± 192	2435 ± 244*

V.C., vehicle control; TP, testosterone propionate. $n = 6$ rats/group/Lab.
* Significantly different from control group at $P < 0.05$.

3.1.2. *Accessory sex organ weights*

Five accessory sex organ and total five organ weight changes are shown in Table 3, and normalized organ weight changes are shown in Fig. 1. The accessory sex organ weights of rats given TP only in all laboratories were higher than these of rats given the vehicle alone, confirming the reliability of this study. Almost all accessory sex organ weights and total five organs in rats given L were higher than in rats given the vehicle in all laboratories. The LABC weights in rats given E was significantly higher than in rats given the vehicle in Lab 2, but the normalized change in this organ was

Table 3
Mean body weights and mean organ weights in agonistic version

Lab	Body weights/organ weights	Substances					
		V.C.	A	B	L	E	TP
1	Terminal body wt. (g)	262.9 ± 17.0	250.6 ± 12.0	252.4 ± 12.8	243.7 ± 9.2	249.4 ± 12.9	266.0 ± 12.7
	Ventral prostate (mg)	16.8 ± 1.0	17.0 ± 3.2	15.6 ± 3.4	34.1 ± 8.0	14.9 ± 1.2	93.6 ± 11.0*
	Seminal vesicles (mg)	27.9 ± 5.6	26.5 ± 1.9	25.7 ± 4.9	61.2 ± 9.6*	29.0 ± 3.5	190.4 ± 19.1*
	LABC (mg)	136.8 ± 22.2	128.2 ± 19.2	128.3 ± 11.0	298.5 ± 28.1*	141.5 ± 15.2	312.3 ± 26.9*
	Glans penis (mg)	29.5 ± 5.6	29.9 ± 2.8	28.3 ± 7.2	49.8 ± 6.5*	32.6 ± 5.1	64.4 ± 6.0*
	Cowper's glands (mg)	4.1 ± 1.3	4.4 ± 1.4	3.9 ± 1.5	10.3 ± 2.5*	4.5 ± 1.1	20.4 ± 3.5*
	Total of five organs (mg)	215.0 ± 21.3	206.0 ± 23.5	201.9 ± 21.3	453.8 ± 48.3*	222.4 ± 19.0	681.0 ± 42.3*
2	Terminal body wt. (g)	280.8 ± 7.9	275.9 ± 6.3	285.2 ± 12.4	261.5 ± 7.1*	281.8 ± 15.0	305.6 ± 15.0*
	Ventral prostate (mg)	16.0 ± 5.2	19.8 ± 5.1	15.8 ± 6.5	33.4 ± 6.3*	18.4 ± 3.2	121.8 ± 25.6*
	Seminal vesicles (mg)	42.0 ± 14.5	40.9 ± 11.2	38.8 ± 12.2	178.7 ± 60.4*	41.6 ± 11.5	420.4 ± 32.1*
	LABC (mg)	163.6 ± 38.3	178.6 ± 23.5	189.9 ± 30.4	426.8 ± 46.2*	216.3 ± 17.3*	527.5 ± 23.5*
	Glans penis (mg)	44.1 ± 4.3	42.9 ± 2.2	41.8 ± 2.3	58.5 ± 3.7*	45.5 ± 2.5	73.9 ± 3.9*
	Cowper's glands (mg)	5.8 ± 1.3	5.6 ± 1.1	4.4 ± 1.7	10.1 ± 2.6*	5.8 ± 1.8	34.4 ± 8.1*
	Total of five organs (mg)	271.6 ± 51.3	287.8 ± 29.7	290.7 ± 35.3	707.6 ± 104.7*	327.7 ± 14.6	1177.9 ± 35.1*
3	Terminal body wt. (g)	303.2 ± 15.5	297.3 ± 17.6	308.3 ± 14.9	264.6 ± 26.4*	300.8 ± 14.9	320.0 ± 22.7
	Ventral prostate (mg)	22.0 ± 3.1	20.6 ± 1.4	24.0 ± 1.7	43.7 ± 11.5*	26.2 ± 3.8	186.5 ± 48.4*
	Seminal vesicles (mg)	61.2 ± 5.9	58.1 ± 7.0	58.4 ± 8.2	165.5 ± 37.1*	61.2 ± 10.9	431.3 ± 55.1*
	LABC (mg)	191.3 ± 16.0	178.6 ± 25.2	190.7 ± 6.6	452.3 ± 34.5*	221.1 ± 35.8	543.5 ± 83.5*
	Glans penis (mg)	53.0 ± 8.0	54.6 ± 5.4	54.8 ± 5.6	72.9 ± 3.2*	52.0 ± 2.6	95.1 ± 8.0*
	Cowper's glands (mg)	8.5 ± 2.2	7.4 ± 1.8	8.0 ± 1.3	18.2 ± 5.2*	8.8 ± 2.4	37.1 ± 6.6*
	Total of five organs (mg)	336.0 ± 19.9	319.3 ± 29.6	336.0 ± 13.7	752.5 ± 66.4*	369.4 ± 45.8	1293.6 ± 112.7*

V.C., vehicle control; TP, testosterone propionate. $n = 6$ rats/group/Lab.
* Significantly different from control group at $P < 0.05$.

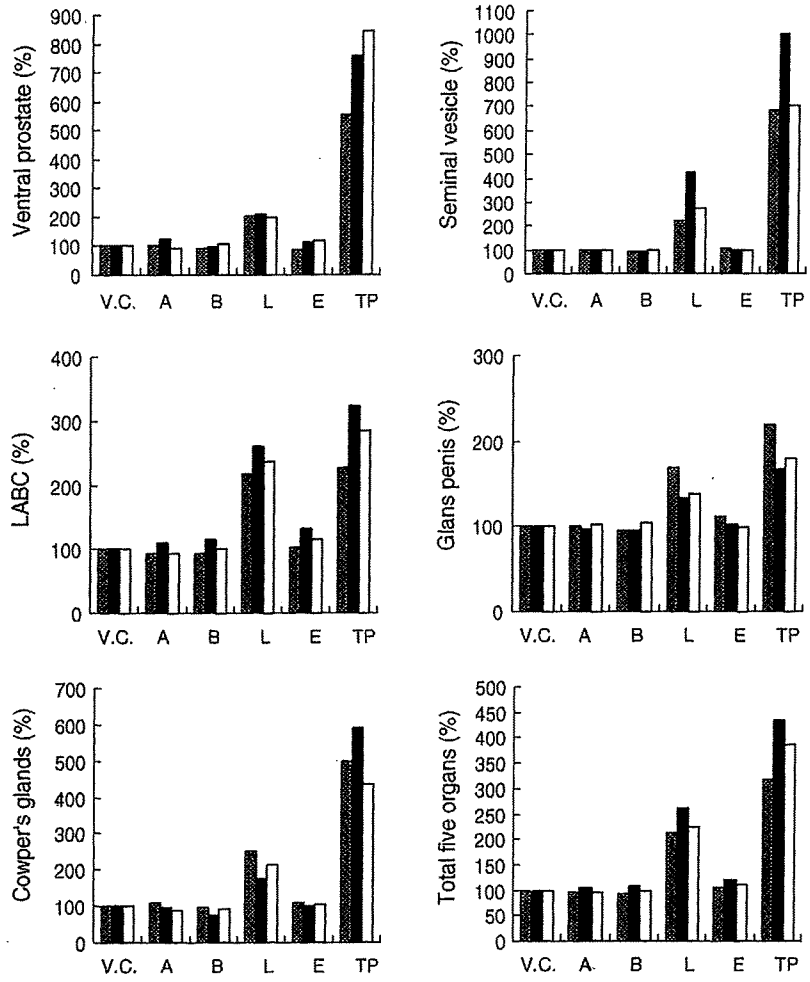

Fig. 1. Organ weights of accessory sex organs in agonistic version. Values from each laboratory were normalized to the control value set equal to 100%. LABC: levator ani and bulbocavernosus muscle; V.C.: vehicle control; A, B, L, and E: coded chemicals; TP: testosterone propionate. $n=6$ rats/group/Lab (⊠, Lab 1; ■, Lab 2; □, Lab 3).

not apparent. Normalized weight changes of the glans penis in rats given coded L showed the weakest response among five organs (Fig. 1).

3.2. Antagonistic version

3.2.1. Body weights, clinical general observations, and optional organ weights

The body weight changes and the optional organ weight changes are shown in Table 4. Two rats given I plus TP died with toxic signs such as decreasing body weight, soft feces, reddish urine, and weakness at 7–10 days after the administration in Labs 2 and 3, respectively. The terminal body weights in rats given I plus TP or K plus TP were significantly lower than in rats given TP only in two laboratories. The paired adrenals in rats given K plus TP were significantly higher than in rats given TP in Lab 3. The liver weights in rats given I plus TP were higher than in rats given TP in all laboratories, and increased liver weights were also observed in rats given C in Lab 1.

3.2.2. Accessory sex organ weights

Five accessory sex organ and total organ weight changes are shown in Table 5, and normalized organ weight changes are shown in Fig. 2. All accessory sex organ weights of rats given H, which is a positive compound, flutamide, plus TP were lower than those of rats given TP, confirming the reliability of this version. Almost all the accessory sex organ weights in rats given I plus TP and K plus TP were significantly lower than in rats given TP in all laboratories. Some accessory sex organ weights in rats given C plus TP and D plus TP were also lower than in the rats given TP. Although the

Table 4
Optional organ weights including the liver, adrenal, and kidney in antagonistic version

Lab	Body weights/organ weights	Substances								
		V.C.	TP	F	G	I	C	K	D	H
1	Starting body wt. (g)	216.2 ± 10.9	216.1 ± 8.8	214.9 ± 10.6	216.6 ± 10.8	218.1 ± 12.5	216.5 ± 8.1	216.0 ± 8.2	219.4 ± 11.3	215.2 ± 9.7
	Terminal body wt. (g)	260.0 ± 17.4	267.4 ± 16.5	263.3 ± 17.8	272.2 ± 9.5	248.7 ± 17.1	266.3 ± 11.7	245.7 ± 11.1*	263.6 ± 11.8	261.5 ± 12.5
	Liver (g)	10.0 ± 1.0	10.4 ± 0.9	11.3 ± 1.3	10.9 ± 0.9	17.3 ± 1.2*	12.5 ± 0.8*	9.6 ± 0.8	10.4 ± 0.5	10.0 ± 0.8
2	Starting body wt. (g)	256.8 ± 11.0	258.9 ± 9.2	259.3 ± 11.6	257.6 ± 10.9	256.8 ± 9.6	258.9 ± 10.8	258.0 ± 10.6	258.9 ± 10.7	255.4 ± 12.7
	Terminal body wt. (g)	313.2 ± 14.7*	331.9 ± 14.3	326.1 ± 16.4	332.2 ± 20.7	270.8 ± 44.6*	329.8 ± 18.7	309.5 ± 13.9*	335.3 ± 15.6	327.4 ± 23.1
	Liver (g)	13.8 ± 0.7	15.6 ± 2.1	15.8 ± 2.3	15.1 ± 0.9	22.8 ± 3.6*	17.7 ± 1.7	14.1 ± 0.8	15.6 ± 1.4	15.1 ± 2.2
3	Starting body wt. (g)	254.1 ± 13.8	254.4 ± 13.6	255.1 ± 12.3	255.1 ± 12.4	255.6 ± 13.1	255.3 ± 14.7	255.2 ± 14.9	254.3 ± 15.9	254.6 ± 16.6
	Terminal body wt. (g)	303.3 ± 16.4	314.7 ± 20.9	307.0 ± 24.0	317.5 ± 10.9	248.1 ± 62.4*	322.7 ± 23.5	292.7 ± 18.1	310.6 ± 23.1	313.2 ± 24.2
	Liver (g)	12.9 ± 1.2	13.7 ± 1.7	14.2 ± 1.7	13.7 ± 0.5	18.6 ± 2.5*	16.0 ± 1.3	13.0 ± 0.9	12.8 ± 1.4	13.5 ± 2.5
	Adrenals (mg)	57.0 ± 10.8	49.5 ± 8.3	54.3 ± 12.5	58.0 ± 5.9	55.5 ± 3.6	56.6 ± 3.5	63.2 ± 7.0*	56.5 ± 7.8	55.5 ± 7.7
	Kidneys (mg)	2106 ± 212	2207 ± 228	2316 ± 273	2342 ± 34	2152 ± 216	2329 ± 197	2236 ± 132	2184 ± 198	2195 ± 201

V.C., vehicle control; TP, testosterone propionate. Each substance was coadministered with 0.2 mg/kg TP. $n = 6$ rats/group/Lab.
* Significantly different from TP group at $P < 0.05$.

LABC weight in rats given G plus TP was significantly lower than that in the TP group in Lab 2, the normalized change of this organ was not so apparent. The total of the five accessory sex organ weights in rats given I plus TP and K plus TP was lower than in rats given TP in all laboratories. The seminal vesicle weight changes in rats given I plus TP and K plus TP were most sensitive among the five organs (Fig. 2).

4. Discussion

Japanese laboratories performed the validation studies of Phase 2 using methyltestosterone, vinclozolin, and *p,p'*-DDE as a part of the national validation program with the result that the Hershberger assay proposed by the OECD was suggested to be a good screening assay to detect androgen agonistic and antagonistic effects (Yamasaki et al., 2003a).

We also performed the Hershberger assay using coded chemicals as part of a national validation Phase 3 as the next step for the OECD guideline process of this assay. The weights of all the accessory sex organs from the experimental animals in all the laboratories exhibited significantly the same changes in the agonistic version; almost all organ weights increased in the rats given coded substance L, and no organ showed any response in rats given coded substances A and B. We received the information from the coordinator of this validation study after all tests were finished that a group of L and E was the same compound and a dose of L was higher than that of E, and that A and B were reported to have no agonistic properties and L and E were a weak agonistic compound. In addition, the normalized weights of all the tissues treated with coded substances in each assay fell within narrow ranges. Therefore, we think that the Hershberger assay is a good screening assay for detecting the androgen agonistic effects of chemicals. The findings that the terminal body weights in rats given coded L were depressed in all laboratories and no body weight changes were detected in rats given coded substance E in all laboratories means that a dose of L was a toxic level and a dose of E had no observed effect. The androgen agonistic effects were detected by the administration of toxic level in this study, but weak agonistic and antagonistic properties of some weak chemicals were detected when non-toxic level doses were administered (Yamasaki et al., 2003a,b).

In the antagonistic version, almost all the sex accessory organs decreased in rats given coded substances I plus TP and K plus TP in all laboratories compared with each organ weight in the rats given TP only, and some organ weights also decreased in the coded substance C

Table 5
Mean body weights and mean organ weights in antagonistic version

Lab	Body weights/organ weights	Chemicals								
		V.C.	TP	F	G	I	C	K	D	H
1	Terminal body wt. (g)	260.0 ± 17.4	267.4 ± 16.5	263.3 ± 17.8	272.2 ± 9.5	248.7 ± 17.1	266.3 ± 11.7	245.7 ± 11.1*	263.6 ± 11.8	261.5 ± 12.5
	Ventral prostate (mg)	14.4 ± 1.5*	102.4 ± 17.6	82.0 ± 7.7	85.9 ± 19.3	28.3 ± 5.9*	78.9 ± 14.6*	41.4 ± 5.9*	80.6 ± 10.7*	24.9 ± 3.6*
	Seminal vesicles (mg)	23.8 ± 3.1*	194.8 ± 38.9	191.4 ± 40.0	185.7 ± 33.5	35.5 ± 6.2*	144.5 ± 29.1	60.6 ± 8.2*	154.7 ± 32.5	28.2 ± 3.8*
	LABC (mg)	119.0 ± 7.0*	306.2 ± 39.1	307.8 ± 40.0	302.3 ± 29.4	125.7 ± 13.4*	280.7 ± 29.7	155.8 ± 21.8*	302.9 ± 40.4	148.3 ± 18.6*
	Glans penis (mg)	29.4 ± 2.9*	60.9 ± 5.3	61.9 ± 5.5	61.2 ± 2.1	32.0 ± 3.9*	53.6 ± 3.3*	41.7 ± 6.4*	64.5 ± 6.1	30.3 ± 4.2*
	Cowper's glands (mg)	3.2 ± 1.3*	18.4 ± 4.6	20.1 ± 1.7	16.2 ± 3.5	4.9 ± 0.8*	16.4 ± 1.9	8.3 ± 1.8*	18.1 ± 3.3	4.7 ± 1.3*
	Total of five organs (mg)	189.9 ± 10.6*	682.7 ± 85.8	663.2 ± 81.3	651.2 ± 65.6	226.5 ± 20.6*	573.9 ± 62.2	307.7 ± 25.4*	620.8 ± 74.6	236.3 ± 24.2*
2	Terminal body wt. (g)	313.2 ± 14.7*	331.9 ± 14.3	326.1 ± 16.4	332.2 ± 20.7	270.8 ± 44.6*	329.8 ± 18.7	309.5 ± 13.9*	335.3 ± 15.6	327.4 ± 23.1
	Ventral prostate (mg)	17.7 ± 2.7*	144.9 ± 19.4	135.0 ± 12.9	129.0 ± 31.5	28.0 ± 8.9*	126.5 ± 40.0	61.6 ± 25.7*	115.5 ± 26.3	28.2 ± 8.9*
	Seminal vesicles (mg)	47.5 ± 8.4*	463.2 ± 70.1	405.9 ± 69.8	439.7 ± 68.7	77.8 ± 26.4*	352.5 ± 80.3*	177.7 ± 43.8*	357.1 ± 40.3*	65.9 ± 11.2*
	LABC (mg)	217.7 ± 25.2*	575.3 ± 31.8	539.6 ± 53.0	529.9 ± 32.2*	218.1 ± 39.6*	496.4 ± 71.6*	336.0 ± 46.3*	532.4 ± 56.5	235.9 ± 29.0*
	Glans penis (mg)	49.9 ± 2.9*	73.0 ± 3.7	75.6 ± 5.9	75.2 ± 2.1	49.8 ± 1.7*	71.7 ± 2.5	63.9 ± 6.2*	73.0 ± 3.3	49.7 ± 2.0*
	Cowper's glands (mg)	5.7 ± 0.9*	33.0 ± 5.4	28.5 ± 5.6	31.0 ± 2.6	10.5 ± 3.8*	30.9 ± 6.5	22.9 ± 9.1	25.7 ± 6.2	10.4 ± 3.4*
	Total of five organs (mg)	338.4 ± 29.0*	1290.2 ± 111.4	1184.6 ± 97.1	1204.6 ± 115.7	384.2 ± 71.6*	1078.0 ± 174.3*	662.0 ± 111.1*	1103.7 ± 89.4*	390.0 ± 29.6*
3	Terminal body wt. (g)	303.3 ± 16.4	314.7 ± 20.9	307.0 ± 24.0	317.5 ± 10.9	248.1 ± 62.4	322.7 ± 23.5	292.7 ± 18.1	310.6 ± 23.1	313.2 ± 24.2
	Ventral prostate (mg)	20.7 ± 3.5*	159.1 ± 31.2	142.0 ± 34.5	132.8 ± 20.1	36.2 ± 9.4*	107.2 ± 17.8*	67.8 ± 24.5*	133.6 ± 24.2	39.8 ± 5.2*
	Seminal vesicles (mg)	53.9 ± 5.2*	459.7 ± 97.2	437.1 ± 120.5	411.6 ± 59.7	73.9 ± 7.9*	323.7 ± 59.0	159.6 ± 45.0*	317.1 ± 62.3*	76.3 ± 10.8*
	LABC (mg)	193.1 ± 11.3*	518.1 ± 74.2	460.2 ± 84.7	494.4 ± 50.1	165.4 ± 35.5*	396.4 ± 50.9*	262.6 ± 47.8*	452.4 ± 64.2	221.8 ± 51.5*
	Glans penis (mg)	50.9 ± 3.8*	93.5 ± 4.6	86.3 ± 7.9	89.6 ± 7.6	60.2 ± 7.3*	83.9 ± 4.3	73.2 ± 10.0*	90.2 ± 6.1	60.8 ± 3.4*
	Cowper's glands (mg)	8.3 ± 2.8*	35.6 ± 6.5	37.5 ± 11.1	33.4 ± 5.3	9.3 ± 2.5*	23.5 ± 5.9*	20.9 ± 4.6*	34.8 ± 6.7	12.0 ± 3.8*
	Total of five organs (mg)	326.9 ± 21.3*	1266.0 ± 151.6	1163.1 ± 217.1	1161.6 ± 84.1	345.0 ± 57.2*	934.7 ± 130.1*	584.2 ± 90.7*	1028.0 ± 110.7*	410.7 ± 61.4*

V.C., vehicle control; TP, testosterone propionate. Each substance was coadministered with 0.2 mg/kg TP. $n = 6$ rats/group/Lab.
* Significantly different from TP group at $P < 0.05$.

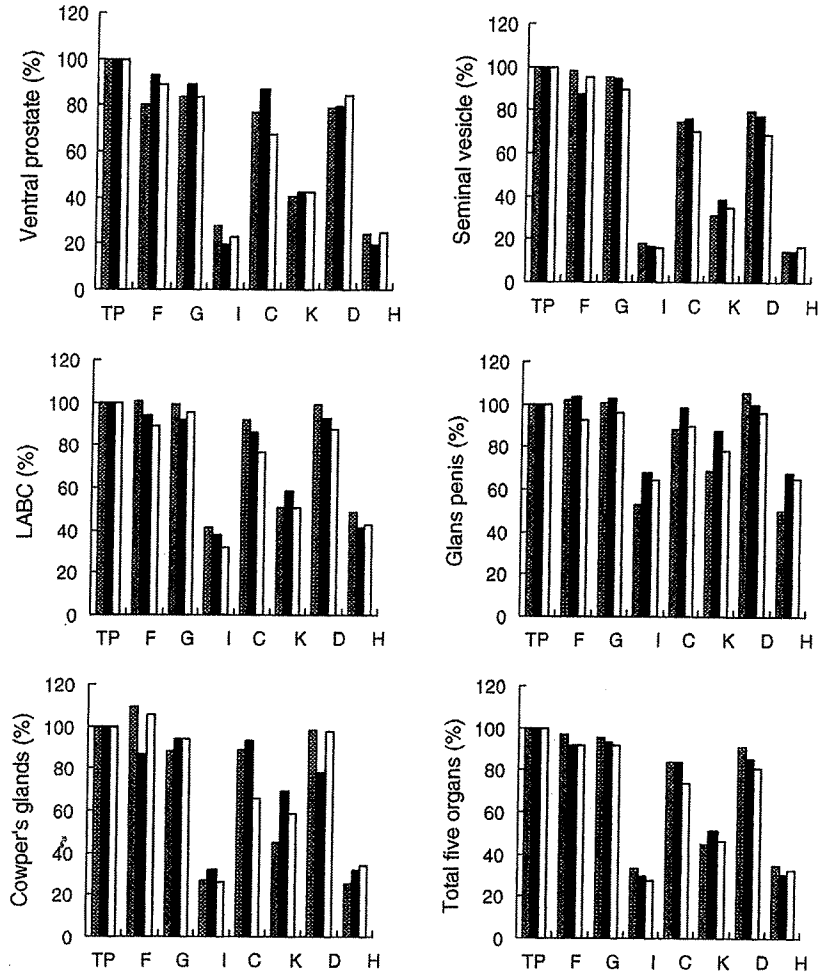

Fig. 2. Organ weights in antagonistic version. Values from each laboratory were normalized to the value of TP group set equal to 100%. LABC: levator ani and bulbocavernosus muscle; F, G, I, C, K, D, and H: coded chemicals; TP: testosterone propionate. $n = 6$ rats/group/Lab (⊠, Lab 1; ■, Lab 2; □, Lab 3).

plus TP and D plus TP groups. No changes were detected in rats given coded substances F and G. These findings demonstrate that coded substances I, C, K, and D had antagonistic properties and coded substances F and G had no antagonistic properties. We accepted the information; substances F and G were negative compounds, I, C, K, and D were weak antagonistic compounds, and H was a positive control compound, flutamide; the groups of substances I and C, or K and D were the same compound, and dose levels of I and K were higher than those of C and D. We also received the information that C and I were *p,p'*-DDE and D and K were linurone. The ventral prostate and glans penis in Lab 1, the seminal vesicle and LABC in Lab 2, and the ventral prostate, LABC and Cowper's glans in Lab 3 were significantly affected in the rats given coded substance C plus TP. In addition, the ventral prostate in Lab 1 and seminal vesicle in Labs 2 and 3 were significantly affected in rats given coded substance D plus TP. The differential effects across laboratories were observed in rats given coded C plus TP and D plus TP. We found that the most sensitive organ among the five accessory sex organs was the prostate and/or seminal vesicle in our previous validation Phase 2 study, and in the Hershberger assays using various chemicals (Yamasaki et al., 2003a,b). The ventral prostate and/or seminal vesicle were responded with or without significant differences in rats given coded substances C plus TP and D plus TP, so we determined that coded C and D have androgen antagonistic properties. On the other hand, the LABC weight in rats given coded substance G plus TP was significantly lower than that in the TP group in Lab 2, but the normalized change of this organ was not so apparent. Therefore, the Japanese data in this study demonstrated that the Hershberger assay

is considered to be a good screening assay for detecting the androgen antagonistic effects of chemicals. The findings that some animals died in rats given coded substance I and decreased body weights were detected in rats given K and I, and the liver weights increased in rats given I means the coded substances I and K were at a toxic dose level. In addition, the liver weights increased in rats given C in one laboratory, so a dose of C may be at a toxic level. The general toxicity is considered to be important for this assay, because a 10% change in terminal weight is suggested to affect some Hershberger assay endpoints (Marty et al., 2003).

In conclusion, we performed the OECD validation study Phase 3 using coded chemicals. All five accessory sex organs responded with statistically significant changes in weight within a narrow window in the agonistic and antagonistic versions, and no false positive or false negative results were observed in this study. Therefore, the Japanese studies support the Hershberger assay as a reliable and reproducible screening assay for the detection of androgen agonistic and antagonistic effects.

Acknowledgments

This study was supported by the grant by the Ministry of Economy, Trade and Industry, the Ministry of Health, Labour and Welfare, and Ministry of the Environment in Japan.

References

Bülbring, E., Burn, J.H., 1935. The estimation of oestrin and of male hormone in oily solution. J. Physiol. 85, 320–333.

Deanesly, R., Parkes, A.S., 1936. Comparative activities of compounds of the androsterone-testosterone series. Biochem. J. 30, 291–303.

Di Salle, E., Briatico, G., Giudici, D., Ornati, G., Panzeri, A., 1994. Endocrine properties of the testosterone 5α-reductase inhibitor turosteride (FCE 26073). J. Steroid Biochem. Mol. Biol. 48, 241–248.

Dingemanse, E., Frued, J., Laquer, E., 1935. Differences between male hormone extracts from urine and from testes. Nature 135, 184.

Eisenberg, E., Gordan, G.S., 1950. The levator ani muscle of the rat as an index of mytrophic activity of steroidal hormones. J. Pharmacol. Exp. Therap. 99, 38–44.

Eisenberg, E., Gordan, G.S., Elliott, H.W., 1949. Testosterone and tissue respiration of the castrate male rat with a possible test for mytrophic activity. Endocrinology 45, 113–119.

Hershberger, L.G., Shipley, E.G., Meyer, R.K., 1953. Myotrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. Proc. Soc. Exp. Biol. Med. 83, 175–180.

Korenchevsky, V., 1932. The assay of testicular hormone preparations. Biochem. J. 26, 413–422.

Korenchevsky, V., Dennison, M., Schalit, R., 1932. The response of castrated male rats to the injection of the testicular hormone. Biochem. J. 26, 1306–1314.

Korenchevsky, V., Dennison, M., Kohn-Speyer, A., 1933a. Changes produced by testicular hormone in normal and in castrated rats. Biochem. J. 27, 557–579.

Korenchevsky, V., Dennison, M., Kohn-Speyer, A., 1933b. On the assay and the absorption of testicular hormone dissolved in oil. Biochem. J. 27, 778–782.

Marty, M.S., Johnson, K.A., Carney, E.W., 2003. Effect of feed restriction on Hershberger and pubertal male assay endpoints. Birth Defects Res. (Part B) 68, 363–374.

McLachlan, J.A., 1993. Functional toxicology: a new approach to detect functionally active xenobiotics. Environ. Health Perspect. 101, 386–387.

McLachlan, J.A., Korach, K.S., 1995. Estrogens in the environment global health implications. Environ. Health Perspect. 103 (Suppl. 7), 3–4.

Organisation for Economic Cooperation and Development (OECD), 1998. Report of the first meeting of the OECD endocrine disrupter testing and assessment (EDTA) working group.

Organisation for Economic Cooperation and Development (OECD), 2000. The second meeting of the OECD validation management group (VMG) for the screening and testing of endocrine disrupters.

Organisation for Economic Cooperation and Development (OECD), 2002. Final OECD report of the work towards the validation of the rat Hershberger assay: Phase 1, androgenic response of testosterone propionate, and anti-androgenic effects of flutamide. Task force on endocrine disrupters testing and assessment (EDTA) of the test guidelines program.

Organisation for Economic Cooperation and Development (OECD), 2003. The fourth meeting of the OECD validation management group (VMG) for the screening and testing of endocrine disrupters.

Peets, E.A., Henson, M.F., Neri, R., 1973. On the mechanism of the anti-androgenic action of flutamide (α-α-α-trifluoro-2-methyl-4′-nitro-m-propionotoluidide) in the rat. Endocrinology 94, 532–540.

Raynaud, J.P., Bouton, M.M., Moguilewsky, M., Ojasoo, T., Philibert, D., Beck, G., Labrie, F., Mornon, J.P., 1980. Steroid hormone receptors and pharmacology. J. Steroid Biochem. 12, 143–157.

Raynaud, J.P., Bonne, C., Moguilewsky, M., Lefebvre, F.A., Bélanger, A., Labrie, F., 1984. The pure anti-androgen RU 23908 (Anandron®), a candidate of choice for the combined anti-hormonal treatment of prostatic cancer: a review. Prostate 5, 299–311.

USEPA, 1998. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) final report. EPA/743/R-98/003.

Wainman, P., Shipounoff, G.C., 1941. The effects of castration and testosterone propionate on the striated perineal musculature in the rat. Endocrinology 29, 975–978.

Wakeling, A., Furr, B.J.A., Glen, A.T., Hughes, L.R., 1981. Receptor binding and biological activity of steroidal and non-steroidal anti-androgens. J. Steroid Biochem. 15, 355–359.

Yamasaki, K., Sawaki, M., Ohta, R., Okuda, H., Katayama, S., Yamada, T., Ohta, T., Kosaka, T., Owens, W., 2003a. OECD validation of the Hershberger assay in Japan: Phase 2-Dose response of methyltestosterone, vinclozolin and *p,p′*-DDE. Environ. Health Perspect. 111, 1912–1919.

Yamasaki, K., Takeyoshi, M., Yakabe, Y., Sawaki, M., Imatanaka, M., Shinoda, K., Takatsuki, M., 2003b. Immature rat uterotrophic assay of 18 chemicals and Hershberger assay of 30 chemicals. Toxicology 183, 95–115.

[秦野研究所年報
27, 28-35 (2004)]

マウスを用いる子宮肥大試験

太田　亮[1]，田面喜之[2]，宮原　敬[2]，丸茂秀樹[1]

The Mouse Uterotrophic Assay

Ryo OHTA[1], Yoshiyuki TAZURA[2], Takashi MIYAHARA[2], Hideki MARUMO[1]

The mouse uterotrophic assay was attempted in ovariectomized mice using ethinyl estradiol as a reference compound and the result was comparable with the rat uterotrophic assay. Ovariectomized mice were exposed to bisphenol A and twenty other selected chemicals by gavage or subcutaneous injection according to the protocol drafted by the OECD Validation Management Group for the Screening. The results demonstrated that bisphenol A had potency not only of estrogenicity but also of anti-estrogenicity, and that ten of the twenty chemicals revealed either estrogenic or anti-estrogenic activity on the uterus of mice. These results support the mouse uterotrophic assay as a useful screening method for detection of estrogenic and anti-estrogenic compounds.

緒言

子宮肥大試験（Uterotrophic assay）は，エストロゲン活性を調べるための*in vivo*試験系として，1930年代から用いられてきた古い手法であるが，現在は，内分泌かく乱化学物質のスクリーニング試験法としての応用が期待され，OECDなどが中心となって，その有用性を確認する作業（バリデーション作業）が国際レベルで進められている[1-3]．OECDが提唱している子宮肥大試験のガイドライン案では，「嚙歯類を用いる子宮肥大試験」となっているが，これまで実施されてきたOECDバリデーション作業は，ラットのみである[1-3]．一方，同じ嚙歯類でもマウスはこれまで子宮肥大試験に使用されてこなかったが，マウスはラットに比べて体が小さく，子宮も著しく軽量なため，重量測定の前処理などに熟練を要することや，測定値に正確性を欠くことなどが敬遠される主な理由と考えられる．しかしながら，マウスには，被験物質量がラットの約1/10で済むことや，飼育スペースが節約できること，遺伝子発現変化に関する情報が多いことなど利点も多く，将来，マウスが子宮肥大試験に使用される可能性は高いと考えられる．そこで，本稿では，マウスを用いる子宮肥大試験の検証を目的として，エチニルエストラジオール（EE）投与による子宮重量増加をマウス－ラット間で比較した結果と，ビスフェノールA（BPA），さらには，エストロゲン様作用が疑われている種々の化学物質について実施したマウス子宮肥大試験の結果を紹介する．

材料および方法

実験1．EE投与による子宮重量増加の用量反応性

実験は，OECDバリデーション作業のプロトコール案[1]に準拠して，幼若マウスを用いる3日間経口投与法，幼若マウスを用いる3日間皮下投与法，卵巣摘出マウスを用いる7日間経口投与法および卵巣摘出マウスを用いる7日間皮下投与法の4種類を実施した．動物は，ICR系雌マウス（日本チャールス・リバー，横浜）を用い，本館飼育室内で，ペパークリーン（日本エスエルシー，浜松）を入れたTPX樹脂製ケージに収容し，固型飼料（CE-2，日本クレア，東京）および水道水を自由摂取させて飼育した．EE（純度99.0％，和光純薬工業，大阪）は，少量のエタノールに溶解後，コーン油で所定の濃度に調製して，投与した．EEの投与量は，幼若マウスの経

1 Safety Testing Laboratory
2 Animal Husbandry Unit

口投与法では0.3～150 μg/kg/day，皮下投与法では0.1～50 μg/kg/day，卵巣摘出マウスの経口投与法では0.3～100 μg/kg/day，皮下投与法では0.03～10 μg/kg/dayの範囲に設定し，対照群の動物にはコーン油のみを投与した．投与は，幼若マウスの場合は哺育19日から，卵巣摘出マウスの場合は8週齢から開始し，1群当たりの例数は5～6とした．投与した動物は，最終投与の約24時間後に頚椎脱臼し，速やかに放血致死させた．その際，子宮は膣とともに摘出し，実体顕微鏡下で脂肪を除去した後，膣を子宮頚の外子宮口の位置で切り離した．子宮重量は，子宮内液を含んだ状態でまず測定し（wet weight），次に子宮壁の一部を切開し，子宮内液をガーゼで吸い取った後，再度，重量を測定した（blotted weight）．得られたデータは一元配置の分散分析で解析し，有意性が認められた場合には，対照群を基準群としてDunnettの多重比較検定を行った．なお，本稿ではblotted weightのみを指標とした．

実験2．BPAの卵巣摘出マウスを用いた子宮肥大試験

実験は，OECDバリデーションプロトコール案[1]の卵巣摘出動物を用いる7日間皮下投与法に準拠して行い，エストロゲン作用と抗エストロゲン作用を調べた．動物は，6週齢で卵巣を摘出したC57BL/6J系マウス（日本エスエルシー）を動物繁殖研究所（茨城）から入手し，膣スメアで性周期が回帰していないことを確認した後，8週齢で試験に供した．動物の飼育条件は，実験1と同様にした．BPA（純度99.0％，和光純薬工業）は，メノウ乳鉢で破砕後，少量のエタノールに溶解し，コーン油で調製した．EEは，実験1と同様に調製した．群構成は，表1に従い，BPAの投与量は10～300 mg/kg/dayの範囲に設定した．陰性対照群の動物には，コーン油を皮下投与した．陽性対照群の動物には，実験1の結果のED_{10}～ED_{20}に相当する0.2 μg/kg/dayのEEを皮下投与し，抗エストロゲン作用を調べる試験系では，ED_{60}～ED_{70}に相当する0.6 μg/kg/dayのEEを併用皮下投与した．1群当たりの匹数は6とした．子宮重量の測定およびデータの解析は，実験1と同様にした．ただし，陽性対照群と陰性対照群の比較については，Studentのt検定を用いた．

実験3．化学物質の卵巣摘出マウスを用いた子宮肥大試験

実験は，OECDバリデーションプロトコール案[1]に準拠して，卵巣摘出マウスを用いる7日間経口投与法と卵巣摘出マウスを用いる7日間皮下投与法で行った．動物の系統，飼育条件等は，実験2と同様にした．実験に供した化学物質は，以

表1　群構成

Evaluation / Group	Estrogenic action	Anti-estrogenic action
Negative control	Vehicle	Vehicle plus EE [b)]
Low dose	Test substance	Test substance plus EE [b)]
Medium low dose	Test substance	Test substance plus EE [b)]
Medium high dose	Test substance	Test substance plus EE [b)]
High dose	Test substance	Test substance plus EE [b)]
Positive control	EE [a)]	Not applicable

EE: エチニルエストラジオール
a): 皮下投与法では0.2 μg/kg皮下投与，経口投与法では6 μg/kg経口投与
b): 0.6 μg/kg皮下投与

下の通りであり，いずれも構造活性相関や，*in vitro*試験系の結果などからホルモン活性を有すると疑われた20物質を選択した：2-〔ビス(4-ヒドロキシフェニル)メチル〕ベンジルアルコール(CAS No. 81-92-5，東京化成工業，東京)，6-ジンゲロール（CAS No. 23513-14-6，和光純薬工業)，ロスマリン酸（CAS No. 20283-92-5，Aldrich Chemical, Milwaukee, WI)，フェノールフタレイン（CAS No. 77-09-8，シグマアルドリッチジャパン，東京)，2,2',4,4'-テトラヒドロキシベンゾフェノン（CAS No. 131-55-5，THBP，和光純薬工業)，*N*-ヘプチル4-ヒドロキシベンゾエイト（CAS No. 1085-12-7，東京化成工業)，マラカイトグリーンベース（CAS No. 510-13-4，Sigma Chemical, St. Louis, MO)，ニューフクシン（CAS No. 3248-91-7，ICN Biomedicals, Irvine, CA)，テトラゾリウムバイオレット（CAS No. 1719-71-7, Sigma Chemical, St. Louis, MO)，アルファナフトールベンゼイン（CAS No. 6948-88-5，和光純薬工業)，プラバスタチン（CAS No. 81093-37-0，和光純薬工業)，フィゾスチグミン（CAS No. 57-64-7，シグマアルドリッチジャパン)，コルヒチン（CAS No. 64-86-8，和光純薬工業)，ノルジヒドログアイアレティック酸（CAS No. 500-38-9，ICN Biomedicals, Irvine, CA)，レセルピン（CAS No. 50-55-5，和光純薬工業)，フェンブコナゾール（CAS No. 114369-43-6，ジーエルサイエンス，東京)，*o*-クレゾールフタレイン(CAS No. 596-27-0，和光純薬工業)，*N,N'*-ジフェニル-*p*-フェニレンジアミン（CAS No. 74-31-7，和光純薬工業)，1,3-ジニトロベンゼン(CAS No. 99-65-0，和光純薬工業)，ピグメントオレンジ（CAS No. 12236-62-3，国立医薬品食品衛生研究所，東京)．

本試験に先立ち，まず，投与量を設定するための毒性予備試験を実施した．すなわち，C57BL/6J系雌マウスを用いて100，300および1000 mg/kg/dayの投与群を設け，各群3匹に3日間反復投与し，死亡が認められなかった最高用量を子宮肥大試験の最高用量とした．100 mg/kg/day投与群でも死亡が認められた場合は，さらに低い用量を設定して，死亡が認められない用量を求めた．化学物質は，コーン油または注射用水で所定の濃度に調製した．群構成は，表1に従い，各物質とも公比約3とし，4用量を設定した．陰性対照群の動物には，溶媒のみを投与した．陽性対照群には，経口投与法では6 μg/kg/dayのEEを経口投与し，皮下投与法では0.2 μg/kg/dayのEEを皮下投与した．抗エストロゲン作用を調べる試験系では，経口投与法，皮下投与法のいずれの場合も，0.6 μg/kg/dayのEEを併用皮下投与した．1群当たりの匹数は6とした．子宮重量の測定およびデータの解析は，実験2と同様にした．子宮および膣は，必要に応じて病理組織学的検査を実施した．

結果

実験1：幼若マウスを用いた場合，EE投与により，経口投与法（図1A）では30 μg/kg/day以上，皮下投与法（図1B）では1 μg/kg/day以上の投与群において，子宮重量が対照群より有意に増加した．一方，卵巣摘出マウスを用いた場合，経口投与法（図2A）では10 μg/kg/day以上，皮下投与法（図2B）では0.3 μg/kg/day以上のEE投与群において，子宮重量が対照群より有意に増加した．

実験2：BPA単独投与群では，BPAの用量が増加するに従って子宮重量が増加し，100および300 mg/kg投与群で有意差が認められた（図3A)．一方，BPAとEEの併用投与群では，BPAの用量が増加するに従って子宮重量が低下し，100および300 mg/kg投与群において，有意差が認められた（図3B)．

実験3：マウスを用いて化学物質の子宮肥大試験を実施した結果，エストロゲン作用あるいは抗エストロゲン作用のいずれかを示した物質は，20物質中10物質（50%）であった（図4A)．そのうち，抗エストロゲン作用のみを示した物質は6物質（60%)，エストロゲン作用と抗エストロゲン作用の両方を示した物質は4物質（40%)，エストロゲン作用のみを示した物質はなかった(図4B)．投与経路を比較すると，経口と皮下の両方で作用がみられた物質は5物質（50%)，皮下のみが3物質（30%)，経口のみが2物質(20%）であった（図4C)．子宮重量を変化させ

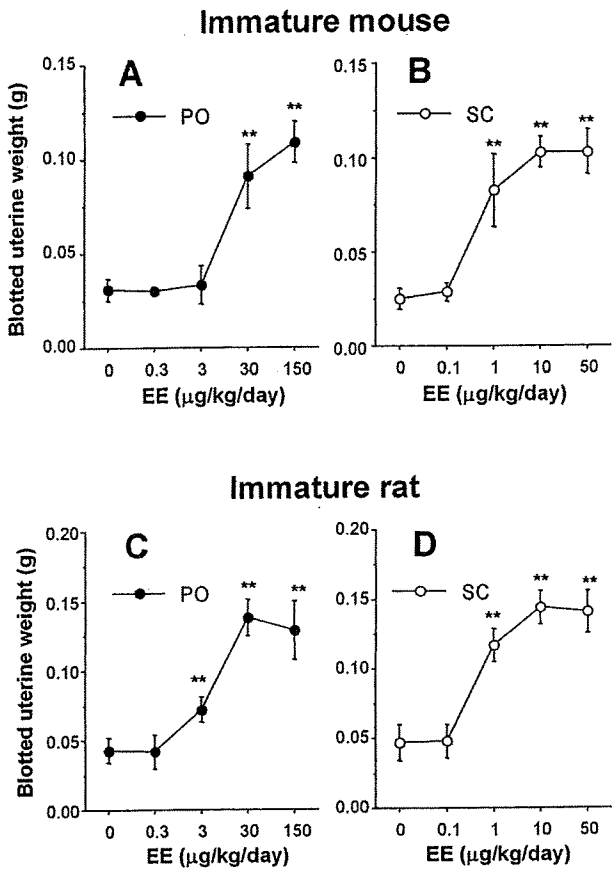

図1　幼若動物のエチニルエストラジオール（EE）投与による子宮重量増加の用量反応性

幼若動物にEEを投与し，最終投与の24時間後に測定した子宮重量（平均±標準偏差）を示す．
A：幼若マウスを用いる3日間経口投与法（PO）
B：幼若マウスを用いる3日間皮下投与法（SC）
C：幼若ラットを用いる3日間経口投与法（PO）
D：幼若ラットを用いる3日間皮下投与法（SC）
マウスの系統はICR，ラットの系統はSprague-Dawley．1群当たりの例数は5～6．
*，** は0 μg/kg群と比較して有意差（$p<0.05$, $p<0.01$）があることを示す．

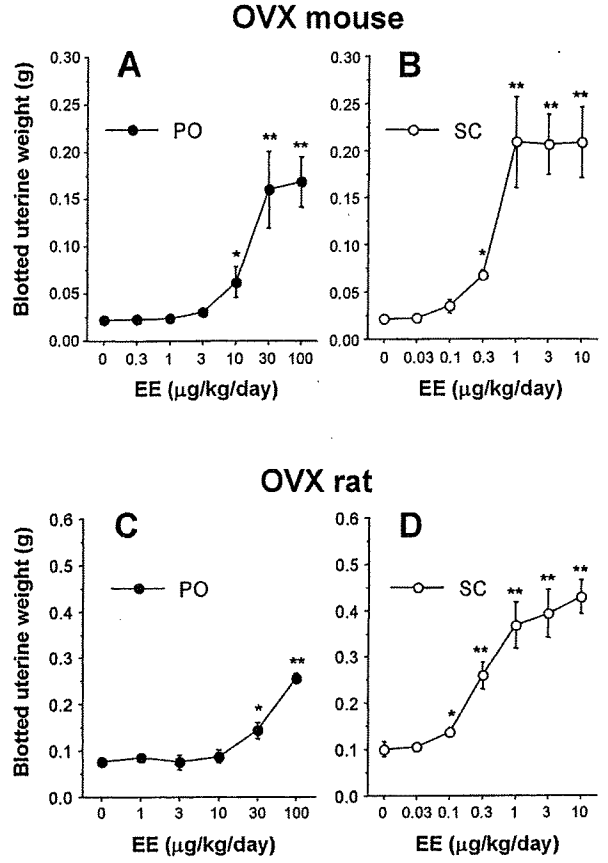

図2　卵巣摘出（OVX）動物のエチニルエストラジオール（EE）投与による子宮重量増加の用量反応性

卵巣摘出動物にEEを投与し，最終投与の24時間後に測定した子宮重量（平均±標準偏差）を示す．
A：卵巣摘出マウスを用いる7日間経口投与法（PO）
B：卵巣摘出マウスを用いる7日間皮下投与法（SC）
C：卵巣摘出ラットを用いる7日間経口投与法（PO）
D：卵巣摘出ラットを用いる7日間皮下投与法（SC）
マウスの系統はICR，ラットの系統はSprague-Dawley．1群当たりの例数は6．
*，** は0 μg/kg群と比較して有意差（$p<0.05$, $p<0.01$）があることを示す．

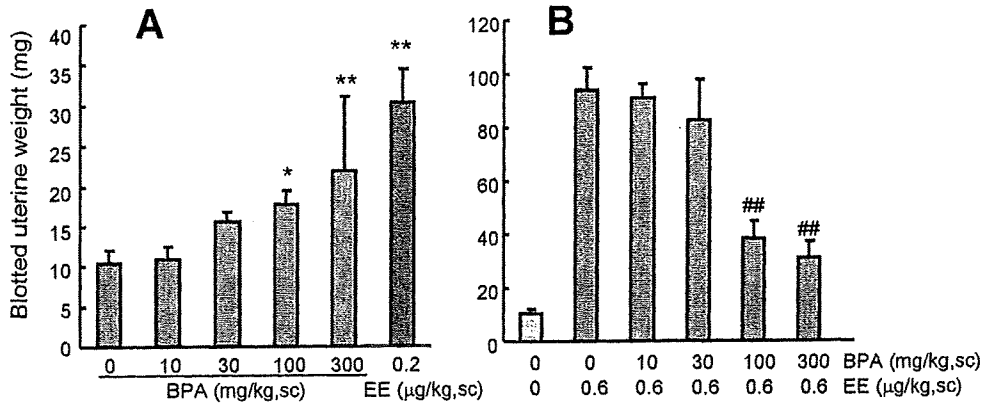

図3　ビスフェノールA（BPA）の卵巣摘出マウスを用いた子宮肥大試験

卵巣摘出マウスにBPAを7日間皮下投与し，最終投与の24時間後に測定した子宮重量（平均±標準偏差）を示す．A：エストロゲン作用を確認する試験，B：抗エストロゲン作用を確認する試験．

マウスの系統はC57BL/6J．1群当たりの例数は6．

*，** は0 mg/kg群と比較して有意差（$p<0.05$, $p<0.01$）があることを示す．

#，## は0 mg/kg BPAと0.6 μg/kg エチニルエストラジオール（EE）の併用投与群と比較して有意差（$p<0.05$, $p<0.01$）があることを示す．

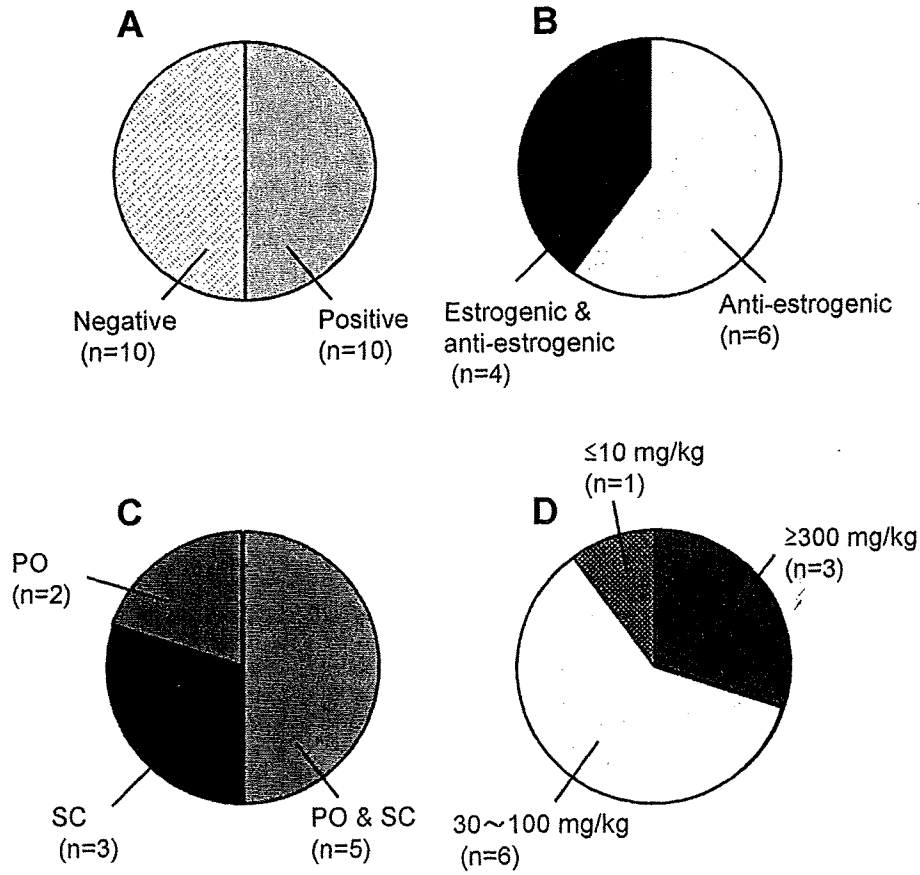

図4　化学物質の卵巣摘出マウスを用いた子宮肥大試験

卵巣摘出マウスを用いて20の化学物質について，7日間経口投与法および7日間皮下投与法で子宮肥大試験を実施し，結果を集計した．A：子宮重量に変化がみられた物質の割合，B：子宮重量に変化がみられた物質を作用別に分類，C：子宮重量に変化がみられた物質を投与別に分類，D：子宮重量に変化がみられた物質を最小有効用量で分類．

マウスの系統はC57BL/6J．POは経口投与，SCは皮下投与．

る最小有効用量は，300～1000 mg/kgが3物質（30％），30～100 mg/kgが6物質（60％），10 mg/kg以下が1物質（10％）であった（図4D）．図5には，THBPの経口投与法による子宮肥大試験の結果を示し，表2にはその時採取した子宮および膣の病理組織学的検査の結果を示した．THBP単独投与群では，1000 mg/kg投与群で子宮重量が有意に増加し（図5A），THBPとEEの併用投与群では，1000 mg/kg投与群で子宮重量が有意に低下した．病理組織像では，300 mg/kg以上の投与群で子宮内膜，腺上皮および筋層，さらには膣の粘膜上皮に陽性対照群と同様の変化がみられた．

考察

実験1のEE投与によるマウス子宮肥大試験の結果を，以前実施したSprague-Dawley系ラットを用いた子宮肥大試験[4]の結果と比較したところ，幼若動物の場合，EEに対する感度は，経口投与法ではラットの方がやや高いが（図1C），皮下投与法では差のないことがわかった（図1D）．一方，卵巣摘出動物の場合，EEに対する感度は，経口投与法ではラットの方が低く（図2C），皮下投与法ではラットの方が高いことがわかった（図2D）．エストロゲンに対する子宮の反応には，系統差がラット[5]およびマウス[6]で報告されていることから断定はできないが，今回の試験結果からは子宮肥大試験の感度にラット-マウス間の本質的な差はないと推察される．

マウス子宮肥大試験の問題点として，子宮が著しく小さいことから，重量を測定する前の脂肪除去や膣切断などに熟練を要すると考えられたが，今回の試験結果から，実体顕微鏡を用いることで，この問題点への対応は可能であると判断した．

実験2のBPA単独投与群では，用量に依存して子宮重量が増加したことから，BPAのエストロゲン作用がマウスにおいて確認された．また，今回の子宮重量を増加させる最小有効用量（100 mg/kg）は，BPAを卵巣摘出ラットに皮下投与した際の最小有効用量[4]と一致したことから，BPAに対する子宮肥大試験の感度にもラット-マウス間の差はないと推定される．一方，BPAとEEの併用投与群では，子宮重量が低下したことから，BPAの抗エストロゲン作用も確認された．これは，エストロゲン活性の弱いBPAがエストロゲン活性の強いEEの作用を阻害したものと推察されるが，実験3の化学物質の中にもエストロゲン作用と抗エストロゲン作用の両方を示す物質がみられており，山崎ら[7]も幼若ラットを用いた子宮肥大試験において，18物質中10物質にみられることを報告している．

実験3において，20の化学物質についてマウス子宮肥大試験を実施した結果，化学物質の半数に子宮重量の変化がみられたが，そのうち，エストロゲン作用のみを示した物質はなかった．このことから，化学物質の子宮肥大試験では，エストロゲン作用と抗エストロゲン作用の両方が確認できるように群構成を設定することが必要と考えられる．また，投与経路についても，約半数の物質は皮下のみ，あるいは経口のみで子宮重量に変化がみられたことから，経口と皮下の両方を実施することが望ましいと考えられる．なお，子宮重量を変化させる最小有効用量は，ほとんどが30 mg/kg以上であり，毒性が強い化学物質では，低用量から他の毒性が発現し，子宮肥大試験では変化が見えにくい傾向にあった．

マウスの子宮および膣の病理組織学的検査は，ラットに比べて標本作製が難しいと予想されたが，ホルマリン固定時に子宮を適度に伸展させたことで，標本が作製し易くなった．また，子宮重量の変化との相関も高く，ラットでみられた所見とほぼ同じ像を観察することができた．子宮および膣の組織像には明らかに系統差がみられることから，子宮肥大試験での組織観察には，種や系統間でグレード付けが異なる事態は避けられないと考えられる．しかしながら，病理組織検査は，子宮重量の変化がエストロゲン活性によるものであることを確認する重要な手段であることに変わりはないと考えられる．

本稿では，マウスを用いる子宮肥大試験の検証を目的として，EE投与によるマウス子宮重量増加の用量反応性，BPAやその他種々の化学物質について実施したマウス子宮肥大試験の結果を紹介し，マウス子宮肥大試験は，ラット子宮肥大試験と同様にエストロゲン作用や抗エストロゲン作

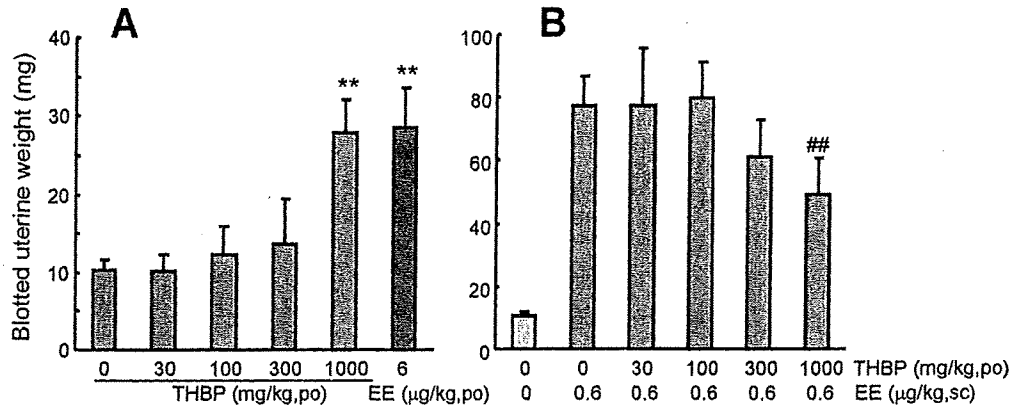

図5　2,2',4,4'-テトラヒドロキシベンゾフェノン（THBP）の経口投与法による卵巣摘出マウスを用いた子宮肥大試験

卵巣摘出マウスにTHBPを7日間経口投与し，最終投与の24時間後に測定した子宮重量（平均±標準偏差）を示す．A：エストロゲン作用を確認する試験，B：抗エストロゲン作用を確認する試験．
マウスの系統はC57BL/6J．1群当たりの例数は6．
*，** は0 mg/kg群と比較して有意差（$p<0.05$, $p<0.01$）があることを示す．
は0 mg/kg BPAと0.6 μg/kg エチニルエストラジオール（EE）の併用投与群と比較して有意差（$p<0.01$）があることを示す．
poは経口投与，scは皮下投与．

表2　2,2',4,4'-テトラヒドロキシベンゾフェノン（THBP）の子宮肥大試験における病理組織検査結果

		Estrogenic action						Antagonistic action				
	THBP (mg/kg)	0	30	100	300	1000	0	0	30	100	300	1000
Findings	EE (μg/kg)	0	0	0	0	0	6	0.6	0.6	0.6	0.6	0.6
Uterus												
Luminal epithelial cell												
Hypertrophy		-	-	-	±	±	±	++	++	++	++	+
Mitosis		-	-	-	-	+	+	±	±	±	±	±
Vacuolation		-	-	-	±	+	+	++	++	++	+	++
Glandular epithelial cell												
Hypertrophy		-	-	-	-	±	±	++	++	++	++	+
Vacuolation		-	-	-	-	+	±	±	±	±	±	±
Endometrial stroma												
Edema		-	-	-	-	-	-	±	±	±	±	-
Hypertrophy		-	-	-	±	±	±	++	++	++	++	+
Cellular infiltration, eosinophil		-	-	-	-	±	±	++	++	++	++	++
Myometrium												
Hypertrophy		-	-	-	-	±	±	++	++	++	++	++
Vagina												
Epithelium												
Cornification		-	-	-	-	±	±	+++++++++		++	+	
Thickening		-	-	-	±	+	+	+++++++++		++	+	

試験は，卵巣摘出マウスを用いる7日間経口投与法による．
マウスの系統はC57BL/6J．
エストロゲン作用のエチニルエストラジオール（EE）は経口投与，抗エストロゲン作用のEEは皮下投与．

用を有する化学物質を検出する試験系として利用できることを示した.

謝辞

最後に，本稿の連名者以外で，この研究に携わった者の氏名を掲載し，謝意を表する．野口聡，田子和美，代田眞理子，臼見憲司，加藤博康，横田俊二，関　剛幸，大澤徳子，千坂亜希子，今野和則，永田伴子，堀内伸二，稲田浩子，三枝克彦．この研究は，平成13年および14年度厚生労働省厚生労働科学研究費の補助により実施した.

文　献

1) Kanno, J., Onyon, L., Haseman, J., Fenner-Crisp, P., Ashby, J., Owens, W.: The OECD program to validate the rat uterotrophic bioassay to screen compounds for *in vivo* estrogenic responses: Phase 1. Environ. Health Perspect. **109**: 785–794 (2001)
2) Kanno, J., Onyon, L., Peddada, S., Ashby, J., Jacob, E., Owens, W.: The OECD program to validate the rat uterotrophic bioassay. Phase 2: Coded single-dose studies. Environ. Health Perspect. **111**: 1550–1558 (2003)
3) Kanno, J., Onyon, L., Peddada, S., Ashby, J., Jacob, E., Owens, W.: The OECD program to validate the rat uterotrophic bioassay. Phase 2: Dose-response studies. Environ. Health Perspect. **111**: 1530–1549 (2003)
4) 太田　亮，今井　清：ラットを用いる子宮重量試験とハーシュバーガー試験，アニテックス**13**: 24–32 (2001)
5) Diel, P., Schmidt, S., Vollmer, G., Janning, P., Upmeier, A., Michna, H., Bolt, H.M., Degen, G.H.: Comparative responses of three rat strains (DA/Han, Sprague-Dawley and Wistar) to treatment with environmental estrogens. Arch. Toxicol. **78**: 183–93 (2004)
6) Ashby, J., Owens, W., Odum, J., Tinwell, H.: The intact immature rodent uterotrophic bioassay: Possible effects on assay sensitivity of vomeronasal signals from male rodents and strain differences. Environ. Health Perspect. **111**: 1568–70 (2003)
7) Yamasaki, K., Takeyoshi, M., Sawaki, M., Imatanaka, N., Shinoda, K., Takatsuki, M.: Immature rat uterotrophic assay of 18 chemicals and Hershberger assay of 30 chemicals. Toxicology **183**: 93–115 (2003)

J. Biochem. **140**, 409–419 (2006)
doi:10.1093/jb/mvj167

AIB1 Promotes DNA Replication by JNK Repression and AKT Activation during Cellular Stress

Kikumi Horiguchi, Shigeki Arai*, Tsutomu Nishihara and Jun-ichi Nishikawa†
Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka, 565-0871

Received February 17, 2006; accepted July 19, 2006

Amplified in breast cancer 1 (AIB1) is a member of the p160 family of nuclear receptor coactivator protein. Recent studies have reported that high-level AIB1 production is involved in the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway for progression to malignant carcinoma in a steroid-independent manner. Here we demonstrate that, in AIB1-knockout DT40 chicken B-lymphocytes, loss of AIB1 results in induction of phosphorylation of c-Jun N-terminal kinase (JNK) and c-Jun, in addition to the inhibition of DNA replication. In contrast, high-level AIB1 production prevents proapoptotic activation of the JNK/c-Jun signal transduction pathway and induces DNA replication through phosphorylation of the Akt/p65 NF-κB subunit RelA under cellular stresses such as UV irradiation or serum deprivation. Moreover, we have found that AIB1 is essential for the phosphorylation of histone H3 at serine 10, which is associated with the signal transduction to chromatin, leading to the transient expression of immediate-early genes in response to UV stimulation. Our results therefore suggest that AIB1 directly links to cell cycle control mechanisms in concern with the balance between apoptosis and proliferation.

Key words: amplified in breast cancer 1, cellular stress, DNA replication, phosphorylation, signal transduction.

Abbreviations: AIB1, amplified in breast cancer 1; Akt, cellular homolog of v-akt oncogene; CARM1, coactivator-associated arginine methyltransferase 1; CBP, cyclic AMP response element binding protein; CDK, cyclin dependent kinase; ER, estrogen receptor; ERK, extracellular signal–regulated kinase; FCS, fetal cauf serum; FACS, fluorence-activated cell sorting; GSK3, glycogen synthase kinase 3; HAT, histone acetyltransferase; HER-2, human epidemal growth factor receptor-2; JNK, c-Jun amino-terminal kinase; MAPK, mitosis-activated protein kinase; NF-κB, nuclear factor-κB; PAS, Per/Arnt/Sim; PI3K, phosphatidylinositol 3 kinase; RSK2, ribosomal S6 kinase 2; SRC-1, steroid receptor coactivator-1; TIF2, transcriptional intermediary factor 2; TUNEL, terminal deoxynucleotidyl transferase–mediated nick end label.

The nuclear receptor coactivator known as AIB1 (also called p/CIP, ACTR, RAC3 and SRC-3) is a member of the p160 nuclear receptor coactivator family. This family contains SRC-1 (steroid receptor coactivator-1) and TIF2 (transcriptional intermediate factor-2) that interact with the general transcriptional coactivators CBP, p300 and p/CAF (*1–8*). These coactivator complexes possess intrinsic histone acetyl transferase activity and are responsible for the remodelling of chromatin and modification of components of the transcription machinery (*9*, *10*).

AIB1 increases estrogen-dependent transcriptional activation by interaction with estrogen receptor (ER) α in a ligand-dependent manner. Furthermore, AIB1 mRNA and protein have been shown to be amplified and overexpressed in primary human breast and ovarian cancer cell lines, in which transcription is upregulated and the AIB1 gene on chromosome 20q12 is amplified (*1*). Recent studies report that high levels of AIB1 production are relates to both a high DNA-synthesis phase fraction and HER-2/*neu* production with p53 mutations in breast cancer, which is a disease characterized by an imbalance between cell division and cell death (*11*, *12*). Her-2/*neu* protein activates the PI3K (phosphoinositide 3-kinase)/Akt (also known as protein kinase B, PKB) pathway, which, through NF-κB activation, plays an important role in preventing cells from undergoing apoptosis (*13*, *14*). More recently, it has been shown that overexpression of AIB1 in the mammary gland leads to activation of the PI3K/Akt pathway, with IGF-1 signaling (*15*). Because AIB1 (RAC-3) has also been shown to interact with NF-κB and enhance its transcriptional activity (*16*, *17*), it has been suggested that AIB1 is an altered regulator for the mechanism by which constitutive activity of an NF-κB–dependent promoter is involved in chemotherapeutic resistance in ER-negative cancer cells (*18*, *19*). However, the biological function of AIB1 in the signal transduction pathways influenced by complex cascades of phosphorylation events triggered by exposure to cellular stress is not completely understood. Therefore, we focused our attention on AIB1 activity, to determine whether this protein regulates the antiapoptotic process or perturbs signal integration in response to cellular stress.

Importantly, c-Jun N-terminal kinase (JNK) has also been shown to be a key regulator of programmed cell death and part of a subfamily of the mitogen-activated protein kinase (MAPK) superfamily (*20*). Recent studies indicate that JNK activation contributes to

*Present address: Research Center for Genomic Medicine, Saitama Medical School, 1397-1 Yamane, Hidaka, Saitama 350-1241.
†To whom correspondence should be addressed. Tel: +81-6-6879-8241, Fax: +81-6-6879-8244, E-mail: nisikawa@phs.osaka-u.ac.jp