Jpn. J. Cancer Res. **93**, 752–759, July 2002

Enhancing Effects of β-Estradiol 3-Benzoate but Not Methoxychlor on the Promotion/Progression Stage of Chemically-induced Mammary Carcinogenesis in Ovariectomized Rats

Makoto Ueda,[1, 2] Hisayoshi Takagi,[1] Hiroshi Onodera,[1] Kazuo Yasuhara,[1] Tamotsu Takizawa,[1] Toshio Imai,[1] Kunitoshi Mitsumori,[1, 2] Takane Matsui[2] and Masao Hirose[1, 3]

[1]Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501 and [2]United Graduate School of Veterinary Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193

Modifying effects of β-estradiol 3-benzoate (EB) and methoxychlor (MXC), a pesticide which possesses weak estrogenic activity, on 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary carcinogenesis were investigated in ovariectomized or intact female Sprague-Dawley rats. Twenty-eight weeks after a single DMBA (100 mg/kg body weight) initiation, when the incidence of mammary tumor-bearing rats had reached 75%, a number of the animals were subjected to ovariectomy in order to obtain 3 groups: i) tumor-bearing, ovariectomized group; ii) tumor-bearing, intact group; iii) no-tumor, ovariectomized group. Subsequently animals of each group were subjected to subcutaneous implantation of 0.5 mg EB or given diet containing 1000 ppm MXC for 13 weeks. Although the incidences, multiplicities and volumes of the palpable tumors gradually decreased after ovariectomy, EB treatment stimulated tumor growth in the tumor-bearing, ovariectomized group thereafter. A similar effect of EB treatment was also observed in the no-tumor, ovariectomized group. However, MXC did not show any effect in the tumor-bearing, or no-tumor ovariectomized groups, except that the multiplicity of tumors was significantly decreased by MXC treatment in the tumor-bearing, intact group. The results of our study suggest that MXC has no promotion/progression effect, but rather possesses a weak inhibitory effect, whereas the strongly estrogenic substance EB clearly enhanced DMBA-induced mammary tumorigenesis.

Key words: Mammary carcinogenesis — β-Estradiol 3-benzoate — Methoxychlor — Rat

Endocrine-disrupting effects of environmental xenoestrogens in humans and animals have become an important issue. In the mammary glands, xenoestrogens have been reported to alter glandular differentiation and epithelial proliferation.[1, 2] Since natural and synthetic estrogens have been shown to be potent modifiers of breast cancer in humans, related risk assessment of endocrine disrupting chemicals is clearly a high priority.[1, 3]

Methoxychlor (MXC) is a pesticide derived as the methoxylated isomer of dichlorodiphenyltrichloroethane (DDT), and is thought to be less toxic.[4, 5] MXC is approved for use on agricultural crops and livestock, and humans could be exposed to it.[4] Estrogenic effects of MXC have been shown in numerous *in vivo* rodent studies, impacting on the development of the female reproductive organs in rats[6, 7] and increasing prolactin release in male rats,[8] uterine epithelial height in ovariectomized mice,[9] uterine weight in immature mice[10] and number of epidermal growth factor receptors in the uteri of immature female rats.[11] MXC itself has weak affinity for estrogen receptor (ER) α and β,[12, 13] and its metabolite 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) is considered to be the principal active metabolite, because it shows agonistic activity to ERα with an affinity about 100-fold higher than that of MXC, and 15-fold lower than that of 17β-estradiol.[12, 14] Therefore, *in vivo* estrogenic activity of MXC is generally considered to be derived from HPTE. Stresser and Kupfer showed the presence in human hepatic microsomes of enzymes that convert MXC to estrogenic metabolites, and they suggested the compound to have estrogenic potency in humans as it does in animals.[15] With regard to its carcinogenic potential, MXC has been classified as a Group 3 agent by the International Agency for Research on Cancer, while DDT could be associated with a breast cancer risk in humans.[16, 17] There is some evidence that MXC may induce mammary tumors in female rats,[18] but further studies are needed to clarify whether MXC is indeed carcinogenic for the mammary glands in rodents and human beings.

Ovariectomy (OVX) removes the promoting effects of endogenous estrogens on mammary gland carcinogenesis.[19, 20] For accessing the impact of estrogenicity of endocrine-disrupting chemicals, ovariectomized female rats are necessary, since they are more sensitive to estrogenic effects than their intact counterparts. It has been shown

[3] To whom correspondence should be addressed.
E-mail: m-hirose@nihs.go.jp

that β-estradiol 3-benzoate (EB) is a potent synthetic estrogen which enhances the growth of breast tumors induced by chemicals or γ-rays in rodents.[21, 22]

In the present study, we examined the modifying effects of EB and MXC on development of 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary tumors in the promotion or progression stage using both intact and ovariectomized female rats.

MATERIALS AND METHODS

Animals and housing One hundred and sixty female Sprague Dawley rats were purchased at 5 weeks of age from Charles River Japan Co., Ltd. (Yokohama). Through the acclimatization and experimental periods, animals were housed at a maximum of 5 to a plastic cage with absorbent hardwood bedding (White Flakes, Charles River, Inc., Tokyo) in an air-conditioned animal room (room temperature, 24±2°C; relative humidity, 60±10%; lighting cycle, 12-h light/12-h dark). All animals were transferred to clean cages with fresh bedding twice a week. The rats were quarantined for 2 weeks and only those without any abnormal findings at the end of this acclimatization period were selected for the experiment. Powdered basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo) and tap water via automatic stainless steel nozzles were freely available throughout the study. This study was carried out in accordance with the Guide for Animal Experimentation, National Institute of Health Sciences, Japan.

Chemicals DMBA (purity: ≥95.0%) and MXC (purity: ≥95.0%) were purchased from Sigma Chemical Co. (St. Louis, MO), and EB (purity: 97.0−103%) and cholesterol from Wako Pure Chemical Industries, Ltd. (Osaka). EB-containing pellets were prepared by mixing 1000 mg of cholesterol and 1.0 ml of olive oil with 33 mg of EB and introducing the mixture into medical-grade silicone tubes which were then cut into segments. The EB content in each pellet was approximately 0.5 mg.

Experimental design The experimental design is outlined in Fig. 1. All rats received an intragastric intubation of 100 mg/kg body weight of DMBA (10 mg/ml sesame oil) at

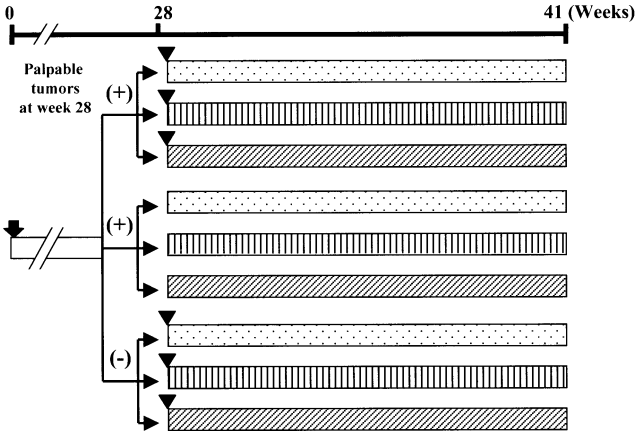

Fig. 1. Study protocol. All female rats received an intragastric intubation of 100 mg/kg body weight of DMBA (10 mg/ml sesame oil) at the age of 7 weeks. Twenty eight weeks thereafter, they were divided into 3 groups: tumor-bearing ovariectomized; tumor-bearing intact; and no tumor ovariectomized. Each group consisted of 3 subgroups; receiving no (basal diet+vehicle pellet), EB (basal diet+EB pellet), or MXC treatment (1000 ppm MXC in diet+vehicle pellet) for 13 weeks. ⬇ DMBA (100 mg/kg, i.g.), ▼ ovariectomy, ▭ basal diet, ▭ basal diet+vehicle pellet, ▭ basal diet+EB (0.5 mg) pellet, ▨ MXC (1000 ppm)+vehicle pellet.

Table I. Survival Rate, Body Weight and Number of Regressing Palpable Tumors in DMBA-initiated Female Rats after 13-Week Dosing with β-Estradiol 3-Benzoate (EB) or Methoxychlor (MXC)

Tumors at the start of dosing	Ovariectomy	Treatment	Survival rate (%)	Body weight	No. of regressed tumors (%)
Bearing	Subjected	Control	8/10 (80)	395.8±57.1	15/24 (63)
		EB	17/20 (85)	322.0±55.6**	21/48 (44)
		MXC	15/20 (75)	288.6±17.8**	24/40 (60)
Bearing	Intact	Control	5/8 (63)	348.1±35.1	0/16 (0)
		EB	4/9 (44)	323.6±33.9	4/19 (21)
		MXC	5/8 (63)	295.8±41.8	8/16 (50)**
None	Subjected	Control	12/13 (92)	422.4±73.0	— —
		EB	15/15 (100)	314.8±29.3**	— —
		MXC	13/14 (93)	291.0±21.7**	— —

* $P<0.05$, ** $P<0.01$.

the age of 7 weeks. Animals were checked every day and individual body weights were determined every week. When the incidence of palpable tumor-bearing animals reached 75% (28 weeks after the administration of DMBA), 50 tumor-bearing and 42 no-tumor rats were ovariectomized under ether anesthesia (32 rats died of mammary tumors before OVX). The tumor-bearing, ovariectomized rats [Tumor(+)-OVX group] were divided into 3 subgroups of 10, 20 and 20 animals for no, EB and MXC treatment, respectively. No-tumor, ovariectomized animals [Tumor(−)-OVX group] were also divided into 3 subgroups of 13, 15 and 14 animals, respectively. The remaining 26 tumor-bearing rats [Tumor(+)-intact group] were similarly divided into subgroups of 8, 9 and 9 animals. EB and MXC were given for 13 weeks. The control and EB subgroups were allowed free access to powdered

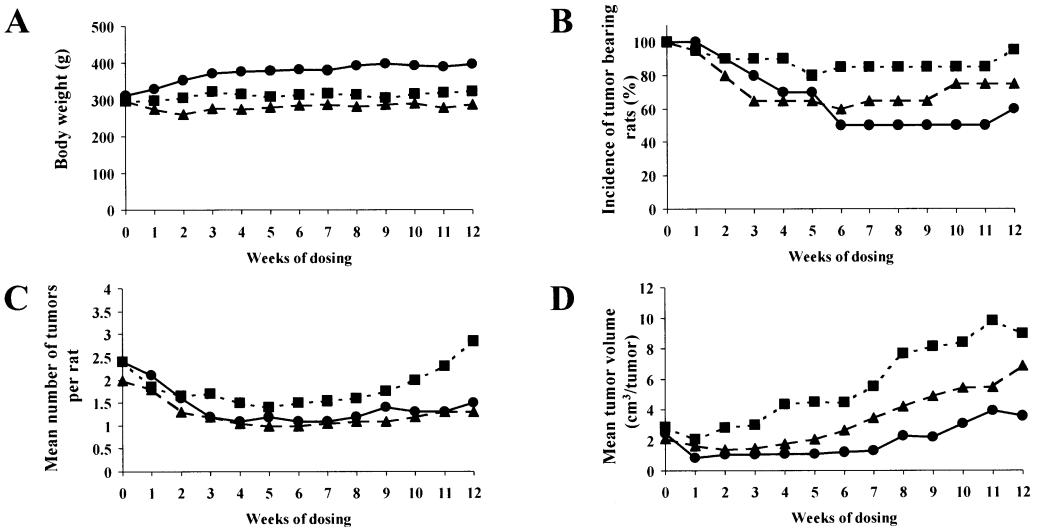

Fig. 2. Sequential changes in body weight (A), incidence of palpable mammary tumor-bearing rats (B), mean number of tumors per rat (C), and mean tumor volume (D) for the tumor-bearing ovariectomized rats. ● control, ■ β-estradiol 3-benzoate, ▲ methoxychlor.

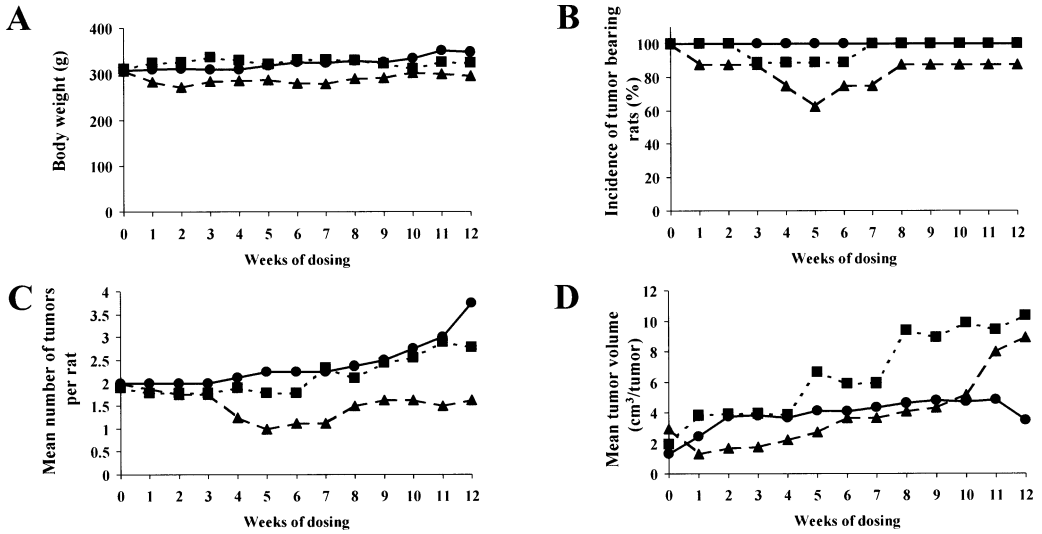

Fig. 3. Sequential changes in body weight (A), incidence of palpable mammary tumor-bearing rats (B), mean number of tumors per rat (C), and mean tumor volume (D) for the tumor-bearing intact rats. ● control, ■ β-estradiol 3-benzoate, ▲ methoxychlor.

basal diet throughout the administration period. EB pellets were implanted in the subcutis of the interscapular area under light anesthesia with ether and replaced every 4 weeks. For the control and MXC subgroups, pellets containing only cholesterol and olive oil were implanted. MXC was mixed into powdered basal diet at a concentration of 1000 ppm for *ad libitum* consumption. The location, number and size (long and short diameters) of subcutaneous thoracic and abdominal tumors of all animals were examined by palpation and recorded weekly throughout the EB or MXC-treated period. The tumor volumes were calculated as follows:

$$\text{Tumor volume} = (\text{long diameter})^2 \times (\text{short diameter})/2.$$

After the end of the 13-week administration period, the surviving animals were killed by exsanguination from the posterior vena cava under ether anesthesia and subjected to autopsy.

Histopathology Mammary tumors, other grossly abnormal lesions and major organs including the uterus, vagina,

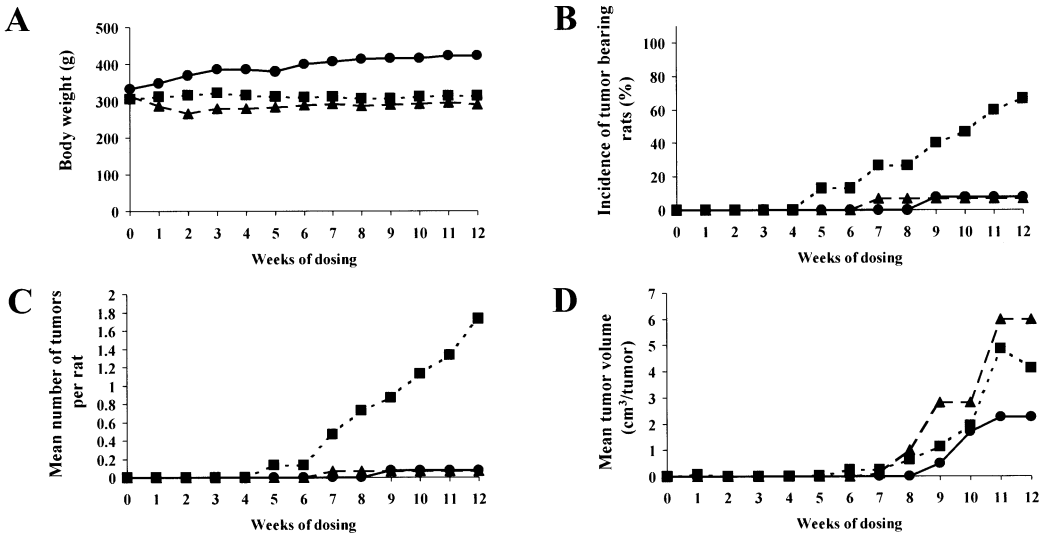

Fig. 4. Sequential changes in body weight (A), incidence of palpable mammary tumor-bearing rats (B), mean number of tumors per rat (C), and mean tumor volume (D) for no-tumor ovariectomized rats. ● control, ■ β-estradiol 3-benzoate, ▲ methoxychlor.

Table II. Incidence, Volume, Multiplicity and Histological Classification of Mammary Tumors in DMBA-initiated Female Rats after 13-Week Dosing with β-Estradiol 3-Benzoate (EB) or Methoxychlor (MXC)

Tumors at the start of dosing	Ovariectomy	Treatment	Incidence of tumor-bearing rats (%)	Multiplicity (Number of tumors/rat)	Tumor volume (cm^3/tumor)	Tumor type (%)	
						Benign[a)]	Carcinoma
Bearing	Subjected	Control	4/8 (50)	1.00±2.05	12.28±13.52	2/8 (25)	6/8 (75)
		EB	17/17 (100)**	4.77±8.28**	14.26±31.76	7/90 (8)	83/90 (92)
		MXC	13/15 (87)	1.80±1.65	6.80±16.26*	13/27 (48)	14/27 (52)
Bearing	Intact	Control	5/5 (100)	5.80±4.90	6.70±9.22	2/28 (7)	26/28 (93)
		EB	4/4 (100)	6.50±8.43	13.18±15.41	3/26 (11)	23/26 (89)
		MXC	5/5 (100)	2.00±1.22*	13.70±15.77	1/10 (10)	9/10 (90)
None	Subjected	Control	1/12 (8)	0.17±0.39	1.38±1.24	0/2 (0)	2/2 (100)
		EB	14/15 (93)**	3.80±2.40**	5.61±13.97	10/57 (17)	47/57 (83)
		MXC	5/13 (39)	0.38±0.51	3.00±5.32	2/5 (40)	3/5 (60)

* $P<0.05$, ** $P<0.01$.
a) Adenoma, fibroadenoma, fibroma.

pituitary gland and ovaries (except for ovariectomized animals) were fixed in 10% neutral buffered formalin. The mammary tumors and these organs were processed routinely, embedded in paraffin, sectioned at 4–5 μm and stained with hematoxylin and eosin (H-E) for microscopic examination.

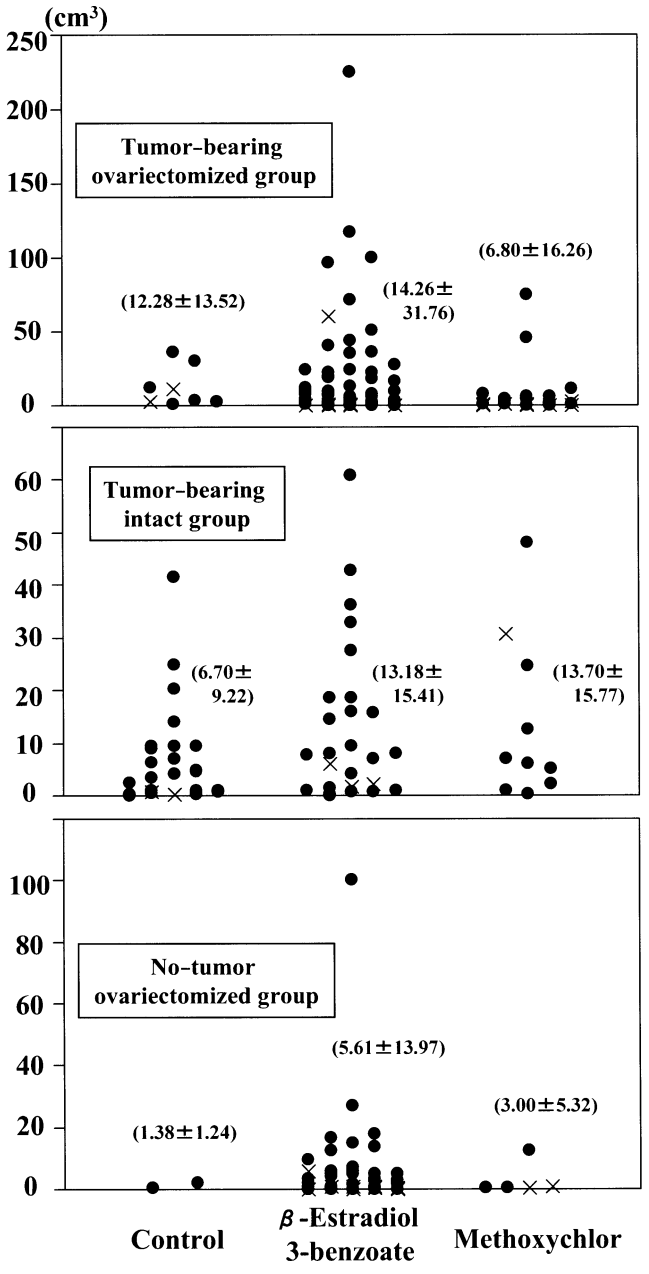

Fig. 5. Individual volume of mammary tumors in DMBA-initiated female rats after 13-week dosing with EB or MXC. × benign, ● carcinoma (mean±SD).

Statistical analysis The survival rates, number of regressing tumors after OVX, and incidences of mammary tumors and other histological lesions were analyzed for inter-group differences by using Fisher's exact test. Body weights were analyzed using Student's *t* test. Multiplicity and volume of mammary tumors were compared with the Mann-Whitney *U* test.

RESULTS

Mortality and body weight changes One MXC-treated animal in the Tumor(+)-intact group which died of a mammary tumor after 3 days of OVX was excluded from the evaluation. The survival rates at the termination were 75–85%, 44–63% and 92–100% in the Tumor(+)-OVX, Tumor(+)-intact and Tumor(−)-OVX groups, respectively, and there were no significant differences among control, EB and MXC subgroups within each group (Table I). The cause of death in almost all cases of mortality was mammary tumor-related. Pituitary tumors were also observed in 4 of 5 dead animals in the Tumor(+)-intact-EB group. In the Tumor(+)-OVX and Tumor(−)-OVX groups, body weights of EB or MXC-treated rats were significantly ($P<0.01$) lower than those of the controls (Figs. 2A, 4A, Table I). Also in the Tumor(+)-intact group, body weights showed a tendency for decrease in the MXC subgroups, but this did not reach statistical significance (Fig. 3A, Table I).

Sequential changes of palpable tumors Incidences, multiplicities and volumes of palpable tumors gradually regressed after OVX in the Tumor(+)-OVX groups in the initial few weeks (Fig. 2, B, C and D). EB treatment seemed to inhibit these OVX effects on incidence and mean volume. However, examination of individual tumors indicated that EB treatment did not significantly affect the disappearance of tumors (Table I). On the other hand, remaining tumors obviously grew under EB treatment, resulting in a continuous increase in mean volume (Fig. 2D). In the last half of the dosing period, the mean number of tumors also gradually increased in the EB treatment subgroup. In the Tumor(−)-OVX group, the incidence, multiplicity and volume of palpable tumors gradually increased in the EB treatment subgroup from the middle of the treatment period (Fig. 4, B, C and D). In the Tumor(+)-intact group, EB treatment caused no clear change (Fig. 3, B, C and D). In the MXC treatment subgroups, no clear changes were observed in the Tumor(+)-OVX group (Fig. 2, B, C and D, Table I). In the Tumor(+)-intact group, no tumors disappeared throughout the experimental period in the control subgroup, whereas MXC treatment induced statistically significant ($P<0.01$) regression (Fig. 3B, Table I). MXC treatment also inhibited the development of palpable tumors (Fig. 3C). In the Tumor(−)-OVX group, MXC treatment increased the vol-

Table III. Histopathological Findings for Reproductive Organs in DMBA-initiated Female Rats after 13-Week Dosing with β-Estradiol 3-Benzoate (EB) or Methoxychlor (MXC)

Tumors at the start of dosing	Ovariectomy	Treatment	Ovary	Uterus		Vagina	
			Atrophy (%)	Atrophy (%)	Hypertrophy (%)	Atrophy (%)	Epithelial thickening/ mucinification (%)
Bearing	Subjected	Control	—	8/8 (100)	0/8 (0)	8/8 (100)	0/8 (0)
		EB	—	0/16 (0)**	16/16 (100)**	0/16 (0)**	1/16 (6)
		MXC	—	0/15 (0)**	15/15 (100)**	0/15 (0)**	13/15 (87)**
Bearing	Intact	Control	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
		EB	4/4 (100)**	0/4 (0)	4/4 (100)**	0/4 (0)	4/4 (100)**
		MXC	5/5 (100)**	0/5 (0)	5/5 (100)**	0/5 (0)	1/5 (20)
None	Subjected	Control	—	12/12 (100)	0/12 (0)	12/12 (100)	0/12 (0)
		EB	—	0/13 (0)**	13/13 (100)**	0/13 (0)**	2/13 (15)
		MXC	—	0/13 (0)**	13/13 (100)**	0/13 (0)**	7/13 (54)**

— not examined.
* $P<0.05$, ** $P<0.01$.

Table IV. Histopathological Findings for Non-reproductive Organs in DMBA-initiated Female Rats after 13-Week Dosing with β-Estradiol 3-Benzoate (EB) or Methoxychlor (MXC)

Tumors at the start of dosing	Ovariectomy	Treatment	Pituitary			Adrenal
			Castration cells (%)		Adenoma (%)	Necrosis (%)
			+	++		
Bearing	Subjected	Control	0/6 (0)	6/6 (100)	0/6 (0)	7/8 (87)
		EB	0/17 (0)	0/17 (0)**	12/16 (75)**	15/16 (94)
		MXC	12/15 (80)**	0/15 (0)**	0/15 (0)	14/15 (93)
Bearing	Intact	Control	0/5 (0)	0/5 (0)	0/5 (0)	5/5 (100)
		EB	0/4 (0)	0/4 (0)	4/4 (100)**	3/3 (100)
		MXC	0/4 (0)	0/4 (0)	0/4 (0)	5/5 (100)
None	Subjected	Control	0/13 (0)	12/13 (92)	0/13 (0)	10/12 (83)
		EB	0/15 (0)	0/15 (0)**	14/15 (93)**	15/15 (100)
		MXC	10/12 (83)**	0/12 (0)**	0/12 (0)	12/12 (100)

+ slight, ++ moderate.
* $P< 0.05$, ** $P< 0.01$.

ume of palpable tumors in the Tumor(−)-OVX group (Fig. 4D), which was considered not to be significant, since the small number of larger tumors affected the data.

Histopathological findings for mammary tumors At the terminal kill, histologically defined mammary tumors were classified as benign lesions including adenomas, fibroadenomas and fibromas and malignant adenocarcinomas (Table II, Fig. 5). The incidence and multiplicity of mammary tumors were significantly elevated and apparent volume increase in several adenocarcinomas was observed in the EB treatment subgroup of the Tumor(+)-OVX group. EB treatment also increased the incidence, volume and multiplicity of mammary tumors in the Tumor(−)-OVX group. In the MXC treatment subgroups, such enhancing effects were not observed. MXC treatment significantly decreased the number of mammary tumors compared to the corresponding control in the Tumor(+)-intact group. A similar change was observed in the Tumor(+)-OVX group, though the multiplicity was increased compared to the control subgroup, and this volume change was

considered not to be biologically significant. MXC had no influence in the tumor(−)-OVX group.

Histopathological findings for other organs As shown in Table III, hypertrophy of the uterine epithelium and thickening/mucinification of the vaginal epithelium were observed in the EB and MXC subgroups of each group. Furthermore, both treatments induced ovarian atrophic changes such as decrease or absence of corpora lutea in intact animals. In the pituitary, 'castration cells,' which are generally observed in the pituitary of castrated animals, were frequently observed in ovariectomized animals, and the frequency and severity were relatively low in MXC-treated subgroups compared to the corresponding controls. In the EB-treated subgroups, pituitary adenomas were observed in 75−100% of animals, though no pituitary tumors were observed in the MXC subgroups (Table IV).

DISCUSSION

It is well known that chemically-induced mammary tumor development is dependent on the presence of estrogen, with regression occurring on ovariectomy in rats.[19, 20] Also in humans, estrogen exposure has been shown to be directly associated with the risk of breast cancer.[23, 24] As indirect effects of estrogen, weak mutagenicity[25] and potent stimulation of prolactin[26] have been documented. In the present study, differences in the modifying effects of EB and MXC in the promotion/progression stage of DMBA-induced mammary carcinogenesis were observed under intact and ovariectomized conditions. In line with expectation,[27] ovarietomy decreased the incidence, multiplicity and volume of palpable mammary tumors in the Tumor(+)-OVX group, while EB treatment countered this influence. EB also exerted similar promoting or progressing effects on mammary tumors in the other groups, furthermore, causing pituitary adenomas and changes in the ovaries (intact animals), uterus and vagina. The results thus confirmed that EB in the cholesterol pellets used in this study was effective as an estrogen treatment.

MXC also showed clear estrogenic effects on the ovaries (intact animals), uterus, vagina and pituitary. On the other hand, in contrast to EB, it did not exert any promoting/progressing effects on mammary tumors, with or without OVX, in fact significantly decreasing the number of mammary tumors in intact rats. Low body weight induced by 50% underfeeding is reported to cause a decrease in the size and number of DMBA-induced mammary tumors in intact rats.[21] In our study, non significant lowering of body weight ($P=0.06$) was observed in the Tumor(+)-intact, MXC-treated animals, but this was not considered sufficient to have had a major impact on the mammary tumor incidence. Recently, a number of estrogenic chemicals have been re-classified as selective estrogen receptor modulators (SERM), showing ER ligand activity and acting like estrogens in some tissues but blocking estrogenic action in others.[28] For example, both tamoxifen and raloxifen exhibit ER-antagonistic activity in the breast while acting as agonists in bone and the uterus in the case of tamoxifen.[28] Previously MXC was found to have antiestrogenic effects on the ovary, but an estrogenic influence on the uterus and oviduct in mice.[29] HPTE, the principal active metabolite of MXC, exhibits agonistic activity against ERα in contrast to antagonism of ERβ in estrogen-responsive promoter-transfected HepG2 and HeLa cells.[14] The available reports suggest that MXC could be a SERM and it may be antagonistic in mammary tissue, like tamoxifen or raloxifen. Furthermore, MXC can increase the concentrations of mRNAs for two estrogen-responsive proteins, lactoferrin and glucose-6-phosphate dehydrogenase, in the uterus of mice by an unknown mechanism that is not mediated through ERα or β.[30] Further investigations, including determination of the levels of such estrogen-responsive mRNAs in mammary tumors, are needed to elucidate the mechanisms of MXC-induced tumor suppression.

In conclusion, the present study indicated that subcutaneous application of 0.5 mg EB pellet results in promoting or progressing effects on DMBA-induced mammary tumors, under ovariectomized conditions. In contrast, 1000 ppm MXC in the feed was without influence after ovariectomy, while showing suppressive effects when ovarian hormones were present.

ACKNOWLEDGMENTS

This work was supported in part by a Health Sciences Research Grants (Research on Environmental Health) from the Ministry of Health, Labour and Welfare of Japan.

(Received January 31, 2002/Revised April 19, 2002/Accepted April 30, 2002)

REFERENCES

1) Safe, S. H. Endocrine disruptors and human health—is there a problem? An update. *Environ. Health Perspect.*, **108**, 487−493 (2000).
2) Brown, N. M. and Lamartiniere, C. A. Xenoestrogens alter mammary gland differentiation and cell proliferation in the rat. *Environ. Health Perspect.*, **103**, 708−713 (1995).
3) Henderson, B. E. and Feigelson, H. S. Hormonal carcinogenesis. *Carcinogenesis*, **21**, 427−433 (2000).
4) Cummings, A. M. Methoxychlor as a model for environmental estrogens. *Crit. Rev. Toxicol.*, **27**, 367−379 (1997).
5) U. S. Department of Health and Human Services. "Toxicological Profile for Methoxychlor TP-93/11," (1994).

Agency for Toxic Substance and Disease Registry, Atlanta.
6) Gray, L. E., Ostby, J., Ferrell, J., Rehnberg, G., Linder, R., Cooper, R., Goldman, J., Slott, V. and Laskey, J. A dose-response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. *Fundam. Appl. Toxicol.*, **12**, 92–108 (1989).
7) Laws, S. C., Carey, S. A., Ferrell, J. M., Bodman, G. J. and Cooper, R. L. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and Methoxychlor in rats. *Toxicol. Sci.*, **54**, 154–167 (2000).
8) Lafuente, A., Marquez, N., Pousada, Y., Pazo, D. and Esquifino, A. I. Possible estrogenic and/or antiandrogenic effects of methoxychlor on prolactin release in male rats. *Arch. Toxicol.*, **74**, 270–275 (2000).
9) Eroschenko, V. P., Johnson, T. J. and Rourke, A. W. Estradiol and pesticide methoxychlor do not exhibit additivity or synergism in the reproductive tract of adult ovariectomized mice. *J. Toxicol. Environ. Health A*, **60**, 407–421 (2000).
10) Shelby, M. D., Newbold, R. R., Tully, D. B., Chae, K. and Davis, V. L. Assessing environmental chemicals for estrogenicity using a combination of *in vivo* and *in vitro* assays. *Environ. Health Perspect.*, **104**, 1296–1300 (1996).
11) Metcalf, J. L., Laws, S. C. and Cummings, A. M. Methoxychlor mimics the action of 17β-estradiol on induction of uterine epidermal growth factor receptors in immature female rats. *Reprod. Toxicol.*, **10**, 393–399 (1996).
12) Gaido, K. W., Leonard, L. S., Maness, S. C., Hall, J. M., Mcdonnell, D. P., Saville, B. and Safe, S. Differential interaction of the methoxychlor metabolite 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane with estrogen receptors α and β. *Endocrinology*, **140**, 5746–5753 (1999).
13) Kuiper, G. G. J. M., Lemmen, J. G., Carlsson, B., Corton, J. C. and Safe, S. H. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. *Endocrinology*, **139**, 4252–4263 (1998).
14) Gaido, K. W., Maness, S. C., Mcdonnell, D. P., Dehal, S. S., Kupfer, D. and Safe, S. Interaction of methoxychlor and related compounds with estrogen receptor α and β, and androgen receptor: structure-activity studies. *Mol. Pharmacol.*, **58**, 852–858 (2000).
15) Stresser, D. M. and Kupfer, D. Human cytochrome p450-catalyzed conversion of the proestrogenic pesticide methoxychlor into an estrogen. Role of CYP2C19 and CYP1A2 in *o*-demethylation. *Drug Metab. Dispos.*, **25**, 617–622 (1998).
16) International Agency for Research on Cancer. Some halogenated hydrocarbons. *IARC Monogr. Eval. Carcinog. Risks Humans*, **20**, 259–282 (1979).
17) Wolff, M. S., Toniolo, P. G., Lee, E. W., Rivera, M. and Dubin, N. Blood levels of organochlorine residues and risk of breast cancer. *J. Natl. Cancer Inst.*, **85**, 648–652 (1993).
18) Reuber, M. D. Carcinogenicity and toxicity of methoxychlor. *Environ. Health Perspect.*, **36**, 205–219 (1980).
19) Russo, J., Gusterson, B. A., Rogers, A. E., Russo, I. H., Wellings, S. R. and Zwieten, M. Comparative study of human and rat mammary tumorigenesis. *Lab. Invest.*, **62**, 244–278 (1990).
20) Nandi, S., Guzman, R. C. and Yang, J. Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. *Proc. Natl. Acad. Sci. USA*, **92**, 3650–3657 (1995).
21) Thyagarajan, S., Meites, J. and Quadri, S. K. Underfeeding-induced suppression of mammary tumors: counteraction by estrogen and haloperidol. *Proc. Soc. Exp. Biol. Med.*, **203**, 236–242 (1993).
22) Yamanouchi, H., Ishii-Ohba, H., Suzuki, K., Onoda, M., Wakabayashi, K. and Inano, H. Relationship between stages of mammary development and sensitivity to gamma-ray irradiation in mammary tumorigenesis in rats. *Int. J. Cancer*, **60**, 230–234 (1995).
23) Martin, A. M. and Weber, B. L. Genetic and hormonal risk factors in breast cancer. *J. Natl. Cancer Inst.*, **92**, 1126–1135 (2000).
24) Dickson, R. B. and Stancel, G. M. Estrogen receptor-mediated processes in normal and cancer cells. *J. Natl. Cancer Inst. Monogr.*, **27**, 135–145 (2000).
25) Cavalieri, E., Frenkel, K., Liehr, J. G., Rogan, E. and Roy, D. Estrogens as endogenous genotoxic agents—DNA adducts and mutations. *J. Natl. Cancer Inst. Monogr.*, **27**, 75–93 (2000).
26) Fujimoto, N. Effects of endocrine disruptors on the pituitary gland. *J. Toxicol. Pathol.*, **14**, 65–69 (2001).
27) Talwalker, P. K., Meites, J. and Mizuno, H. Mammary tumor induction by estrogen or anterior pituitary hormones in ovariectomized rats given 7,12-dimethyl-1,2-benzanthracene. *Proc. Soc. Exp. Biol. Med.*, **116**, 531–534 (1964).
28) Dutertre, M. and Smith, C. L. Molecular mechanisms of selective estrogen receptor modulator (SERM) action. *J. Pharmacol. Exp. Ther.*, **295**, 431–437 (2000).
29) Hall, D. L., Payne, L. A., Putnam, J. M. and Huet-Hudson, Y. M. Effect of methoxychlor on implantation and embryo development in the mouse. *Reprod. Toxicol.*, **11**, 703–708 (1997).
30) Ghosh, D., Taylor, J. A., Green, J. A. and Lubahn, D. B. Methoxychlor stimulates estrogen-responsive messenger ribonucleic acids in mouse uterus through a non-estrogen receptor (Non-ER) α and Non-ERβ mechanism. *Endocrinology*, **140**, 3526–3533 (1999).