prolonged estrus phase [17]. Perinatal or prepubertal exposure of rats to RES causes abnormal estrous cycle [27], with an increase in the percentage of time spent in estrus phase [19]. Neonatal administration of ZEA disturbs the estrous cycle by prolonging estrus and/or diestrus [14,18,20]; irregular estrous cycle with prolonged estrus precedes persistent estrus [28]. In the present study, from 9 to 11 weeks of age, GEN, RES, BPA and DES increased the length of the estrous cycle by prolonging diestrus, whereas ZEA tended to increase the length of the estrous cycle by prolonging estrus phase. Neonatal exposure to DES causes squamous metaplasia of the uterine gland later in life [29]. In the present study, squamous metaplasia of the uterine gland was seen in ZEA-treated mice without corpora lutea (6/18). Treatment of CD-1 mice with DES (1 μg/kg/day) or GEN (50 mg/kg/day) at 1–5 days of age has been shown to cause considerable numbers of uterine adenocarcinomas at 18 months of age [30]. A carcinogenic response was not seen in reproductive organs in the present short-term study. Long-term observation may be necessary to accurately assess carcinogenecity of xenoestrogens in reproductive organs.

Mammary glands are more sensitive to neonatal DES exposure than the reproductive tract [23]. Maternal exposure to 0.25 mg/kg BPA alters the development of mammary gland of female mouse offspring [11]. ZEA stimulates developmental growth of the mammary gland in ovariectomized CD-1 mice [31]. In the present study, gestational exposure to BPA or ZEA accelerated mammary gland growth in female mice, with intact ovaries observed at 4 weeks of age, whereas GEN, RES and DES did not alter development of the mammary gland at 4 weeks of age; no adverse effects on mammary gland growth were observed at 8, 12 or 16 weeks of age. Maternal exposure to 0.1–10 μg/kg DES or 0.1–10 mg/kg GEN by gavage does not affect mammary gland morphology in pubertal female offspring [6]. However, in the present study, growth retardation of mammary glands was seen in ZEA-treated mice without corpora lutea. These mammary glands consisted only of the major duct system, with dilated ducts that exhibited a beaded appearance and were filled with secreted fluid. In BALB/c mice, neonatal estrogen exposure leads to mammary duct dilatation and filling of ducts with secretory product [32]. In GR mice, neonatal antiestrogen treatment causes persistent estrus and bulbous dilatation of mammary ducts [33]. Anovulatory ovaries characterized by persistent vaginal cornification show tonic high estrogen levels, low plasma progesterone levels, and the absence of a luteinizing hormone surge [34]. Neonatal gonadal hormone treatment of animals causes anovulatory estrus and mammary gland developmental arrest [33]. Consistent with these findings, in the present study, gestational exposure to ZEA produced anovulatory ovaries, leading to arrest of mammary gland growth. Induction of mammary carcinoma in rats has been shown to be suppressed by prepubertal ZEA treatment [20]. Although ZEA possesses tumor-suppressing activity, its dramatic effects on estrogen target organs suggest that ZEA should not be consumed during gestation. In an endogenous gene expression assay, the estrogenic potency of ZEA was found to be 2 orders of magnitude less than that of DES, but was 3 and 4 orders of magnitude greater than those of GEN and BPA, respectively [35].

In conclusion, high-dose GEN and RES, and low- and high-dose BPA and DES, had transient effects on the reproductive tract and mammary glands, whereas high-dose ZEA induced prolonged effects. Mammary gland growth was accelerated in ZEA- and BPA-treated mice with corpora lutea at 4 weeks of age, whereas mammary gland growth was suppressed in ZEA-treated mice lacking corpora lutea from 8 to 16 weeks of age.

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Effects of Prepubertal Zeranol Exposure on Estrogen Target Organs and *N*-Methyl-*N*-nitrosourea-induced Mammary Tumorigenesis in Female Sprague-Dawley Rats

TAKASHI YURI, YASUYOSHI NIKAIDO, NAOTO SHIMANO, NORIHISA UEHARA, NOBUAKI SHIKATA and AIRO TSUBURA

Department of Pathology II, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan

Abstract. *Background: There are no previous reports of the effects of prepubertal exposure to zeranol, an estrogenic substance, on estrogen-responsive reproductive organs and mammary glands in rats, or its effects on N-methyl-N-nitrosourea (MNU)-induced mammary tumorigenesis in rats. Materials and Methods: Prepubertal female Sprague-Dawley rats were treated daily with either 0, 0.1 or 10 mg/kg body weight of zeranol between 15 and 19 days of age. They were given 50 mg/kg body weight MNU at 28 days of age, and were monitored for occurrence of mammary tumors ≥1 cm in diameter. Body weight gain, structures and functions of estrogen target tissues, and mammary carcinogenesis were compared between dosage groups. Results: Zeranol did not affect body weight gain. At 28 days of age, zeranol-treated and -untreated rats showed similar development of reproductive organs and mammary glands. However, both low- and high-dose zeranol treatment caused significantly earlier vaginal opening, irregularity of estrous cycle (high frequency of prolonged estrous or prolonged diestrous) at 8 to 11 weeks of age, and anovulatory ovary (ovaries without newly formed corpora lutea). At 37 weeks of age, the high-dose zeranol-treated group exhibited increased relative uterine-ovarian weight, but mammary gland development was comparable to that of untreated rats. Mammary carcinogenesis was not affected by low- or high-dose zeranol treatment. Conclusion: Short-duration zeranol treatment in the prepubertal period severely damaged ovarian functions and structure, but mammary carcinogenesis was not affected. The present results suggest that ingestion of foods containing zeranol in the infantile period can cause dramatic endocrine disruption in later life.*

Correspondence to: Airo Tsubura, Department of Pathology II, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan. Tel: +81-6-6993-9431, Fax: +81-6-6992-5023, e-mail: tsubura@takii.kmu.ac.jp

Key Words: Zeranol, prepuberty, reproductive organ, mammary cancer.

The β-resorcylic lactones zeranol (α-zearalanol) [6-6,10-dihydroxyundecyl-β-resorcylic acid lactone] and zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcyclic acid-lactone] are naturally occurring substances that have estrogenic activity (1,2). Zearalenone is a mycotoxin synthesized by *Fusarium* molds, and is present as a natural contaminant in food as a result of infection of grain (3). Zeranol is a natural metabolic product of zearalenone (4). Zearalenone and zeranol are classified as mycoestrogens (5), and have been widely used to promote growth of livestock in the United States due to their potent anabolic effects (3). Zeranol has greater estrogenic potency than zearalenone (6), and has been commercially produced (7). Zeranol can enter the human food chain directly *via* ingestion of contaminated grain (8), or indirectly *via* consumption of meat products from animals fed a mold-infected grain or animals injected with zeranol for growth stimulation. Thus, it is difficult to avoid exposure to zeranol *via* consumption of human food products. Although no marked toxic, mutagenic or carcinogenic effects of zeranol have been observed (8), studies indicate that the timing and dosage of exposure to estrogenic chemicals can greatly influence their effects.

There has been increasing concern about the impact of exposure to dietary compounds with hormone-like action during critical periods on human development and reproductive health. Estrogens play important roles in the maintenance and function of the reproductive tract and mammary glands. Zearalenone disrupts reproductive function when given to prenatal mice (9) or prepubertal rats (10). Risk assessment of endocrine disruption by xenoestrogens and their metabolites requires accurate evaluations of their estrogenic potency. However, the effects of zeranol exposure during development on estrogen target tissues have not previously been precisely analyzed.

In addition, estrogens have long been recognized as important mitogens in the breast, and are associated with increased risk of breast cancer. Estrogens and estrogenic compounds can have considerable effects on breast carcinogenesis in humans and animals during critical

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periods of development. Epidemiological evidence suggests that high intake of soy (rich in phytoestrogens) during adolescence affects the risk of breast cancer later in life (11), and experimental results indicate that prepubertal exposure to estrogenic chemicals such as genistein (12), resveratrol (13) and zearalenone (10) modifies mammary cancer risk in female rats. There have been no studies of the effects of consumption of zeranol in the early period of development on mammary cancer risk. Thus, the object of the present study was to examine the effects of prepubertal administration of zeranol on estrogen target tissues and the occurrence of *N*-methyl-*N*-nitrosourea (MNU)-induced mammary tumors in female Sprague-Dawley rats.

Materials and Methods

Animals. Ninety 14-day-old female Sprague-Dawley rats (10 pups per 1 nursing mother) were obtained from Charles River Japan (Hino). To avoid exposure to endocrine-disrupting chemicals, rats were housed in standard rat polyisopentene cages (TPX, Charles River Japan) with sterilized white pine chips (White Flake, Charles River Japan, Yokohama) as bedding. To avoid phytoestrogens in the diet, rats were fed the low-phytoestrogen diet NIH-07 PLD (Oriental Yeast, Chiba, Japan), which effectively reduces adverse endocrine-disrupting activity (14), and water was supplied in polycarbonate bottles with rubber stoppers, throughout the experiment. Thus, known endocrine-disrupting agents were eliminated from the environment of the rats. The animal facility was maintained at 22±2°C with 60±10% humidity and a 12-h dark/light cycle.

Chemicals. Zeranol was purchased from Wako Pure Chemical Industries (Osaka, Japan). The purity was 99%, as analyzed by high-performance liquid chromatography. It arrived in powder form, and was kept at 0°C in the dark. Immediately before use, zeranol was dissolved in 100% dimethylsulfoxide (DMSO) (purity ≥ 99%, Nacalai Tesque, Kyoto, Japan), and stored at -80°C.

MNU was obtained from Nacalai Tesque. Upon arrival, it was kept at -20°C in the dark. MNU was dissolved in physiological saline containing 0.05% acetic acid immediately before use.

Experimental procedures. Animals were randomly divided into 3 groups of 30 animals each. From 15 to 19 days of age, 2 groups received a daily subcutaneous injection of zeranol at 0.1 (low-dose group) or 10 (high-dose group) mg/kg body weight, and the third group (untreated controls) received an equal volume of the vehicle (100% DMSO). There have been no reports of measurements of serum concentrations of zeranol in humans, but human exposure to zearalenone in the United State is 1 to 5 mg/day (0.02-0.1 mg/kg body weight per day) (15). Thus, zeranol doses of 0.1 and 10 mg/kg per day were selected for the present study. Rats were weaned at 21 days of age, and animals were then observed daily for vaginal opening (puberty onset). At 28 days of age, 6 randomly selected rats per group were autopsied to assess the effects of zeranol. Rats were killed by an overdose of ether followed by cervical dislocation. On the same day, the remaining rats (24 rats per group) received an intraperitoneal injection of freshly prepared MNU at 50 mg/kg body weight. They were then housed in groups of 3 to 4 animals and palpated weekly for mammary tumors. Rats were autopsied when their largest tumor was ≥ 1 cm in diameter. At 33 weeks after MNU administration, all remaining animals were killed and the experiment was terminated. Body weight was recorded weekly from 2 weeks of age to the end of the experiment (37 weeks of age), and growth rate was compared among groups. The Animal Experimentation Committee at Kansai Medical University, Japan approved all procedures involving animals.

Evaluation of estrogen target organs. At 28 days of age (the time of MNU exposure; 6 animals in each group) and at 37 weeks of age (all remaining rats in each group), animals were weighed and killed and the acute and chronic effects of prepubertal zeranol exposure, respectively, were assessed. Uterus and ovaries were excised and weighed, and formalin-fixed samples of uterus, vagina, ovaries and mammary glands were cut into sections (thickness, 4 μm), which were stained with hematoxylin and eosin (HE). Mammary gland growth and differentiation were also evaluated on whole-mount sections. Vaginal opening was checked, and estrous cyclicity was monitored by examination of vaginal smears (16), performed at the same time daily from 8 weeks of age for 4 consecutive weeks. Estrous cycles were classified as follows: i) normal length, 4 or 5 days; ii) prolonged estrous, irregular cycle in which estrous phase constitutes more than 50% of the observation period; iii) prolonged diestrous, irregular cycle in which the diestrous period constitutes more than 30% of the observation period. When rats were sacrificed during the experimental period, reproductive organs were routinely fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned and stained with HE.

Evaluation of mammary tumorigenesis. To determine the effect of prepubertal zeranol exposure on mammary tumorigenesis, all visible mammary tumors were dissected, fixed in 10% neutral buffered formalin, and cut into sections (thickness, 4 μm) that were stained with HE. In addition, normal-appearing mammary glands were dissected and processed to produce routine histological preparations, to detect microscopic tumors. Histopathology of mammary tumors of all sizes was evaluated from HE-stained sections. The histological criteria for identification of mammary tumors were based on those of Russo *et al.* (17). Data analysis included the number of animals with gross mammary carcinomas (≥1 cm) and latency (from MNU administration until largest mammary tumor reached ≥1 cm in diameter). Also, numbers of histologically detected mammary carcinomas (total number of carcinomas of all sizes) and the number of carcinomas per animal (multiplicity) were analyzed.

Statistical analysis. All data were expressed as mean±SE. Vaginal opening and cumulative incidence of gross mammary tumors (≥1 cm) was analyzed by Mantel-Cox Log-rank test. For all other data, after assurance of homogeneity of variance, analysis was performed using non-repeated measure ANOVA parametric test or Kruskal-Wallis non-parametric test. If the p value of these pre-tests was < 0.05, *post-hoc* analysis was performed using Fisher's protected least significant difference test. Differences between groups were considered significant if the p value was < 0.05.

Results

Body weight gain and reproductive organ function and structure. Prepubertal exposure to zeranol did not affect

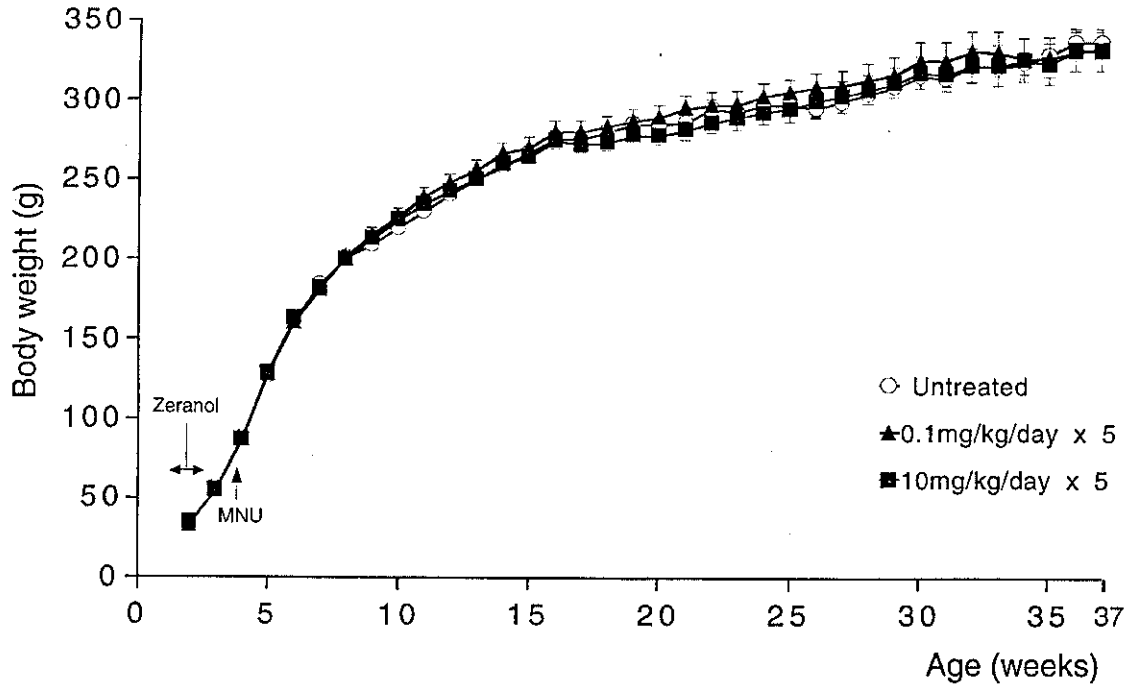

Figure 1. *Body weight gain in female Sprague-Dawley rats treated daily with 0.1 or 10 mg/kg zeranol or vehicle from 15 to 19 days of age and administered 50 mg/kg MNU at 28 days of age. Differences between zeranol-untreated and zeranol-treated groups were not significant.*

body weight gain (Figure 1). The doses of zeranol used were not toxic. In the untreated and 0.1 and 10 mg/kg zeranol-treated groups, there were 2, 2 and 1 moribund animals, respectively. These rats were excluded from the calculation. At 28 days of age (time at carcinogen administration), differences in body weight among groups were not significant, and relative uterine-ovarian weights were comparable among groups (Table I); histological sections of uterus, vagina and ovaries showed no detectable difference. At 28 days of age, whole-mount preparations of mammary gland showed similar differentiation; mammary ducts ended in club-shaped terminal end buds, and alveolar differentiation was seen proximal to the nipple.

Vaginal opening (puberty onset) occurred from 27 to 42 days of age. Compared with zeranol-untreated controls, 0.1 and 10 mg/kg zeranol-treated rats had significantly earlier vaginal opening (Figure 2): untreated controls, 36.4±0.6 days of age; 0.1 mg/kg zeranol, 31.2±0.6 days; 10 mg/kg zeranol, 32.2±0.7 days. Daily vaginal smears taken from 8 weeks of age for 4 weeks indicated that, whereas zeranol-untreated animals had an average cycle length of 4.6 days, both low- and high-dose zeranol-treated groups had a significant increase in cycle length due to prolonged estrous (Table II). In zeranol-untreated and 0.1% and 10% zeranol-treated groups, prolonged estrous was seen in 0% (0/22), 59% (13/22) and 78% (18/23) of animals, respectively, and prolonged diestrous was seen in 5% (1/22), 9% (2/22) and 9% (2/23) of animals, respectively. In rats sacrificed at the termination of the experiment (Table III), body weight was comparable among groups, but 10 mg/kg zeranol significantly increased the relative uterine-ovarian weight. In addition, the frequency of rats with no newly formed corpora lutea in the ovaries (indicating anovulation) increased in the zeranol-treated groups. Prepubertal zeranol treatment (both high- and low-dose) disrupted endocrine function and ovarian structure in adulthood. However, at 28

Table I. *Effect of prepubertal zeranol treatment on body weight and relative uterine-ovarian weight in female Sprague-Dawley rats at 28 days of age.*

Zeranol treatment	No. of rats	Body weight (g)	Relative uterine-ovarian weight (mg/100g B.W.)
Untreated	6	88.1±1.9	267.5±29.9
0.1 mg/kg/day x5	6	84.2±2.0	258.2±23.7
10 mg/kg/day x5	6	84.9±2.1	232.6±26.1

Values are mean±S.E; **p* value <0.05 compared with untreated group.

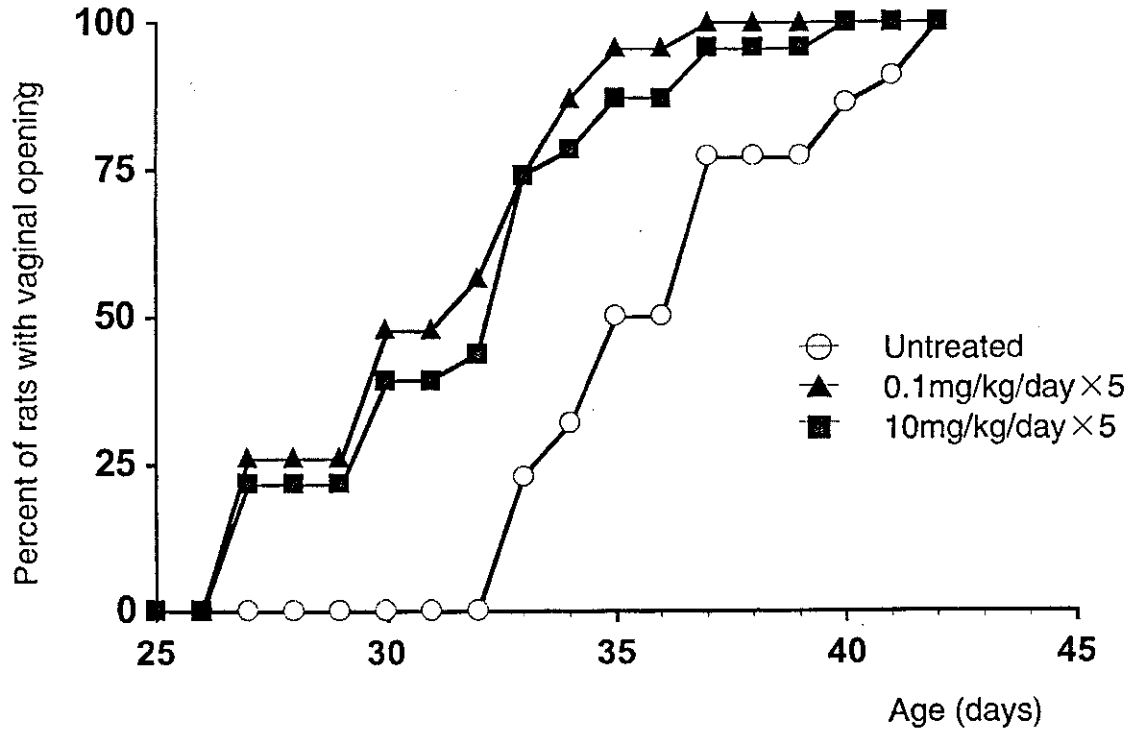

Figure 2. *Vaginal opening in female Sprague-Dawley rats after prepubertal zeranol treatment. Zeranol-treated groups showed earlier vaginal opening than untreated controls ($p<0.01$, respectively).*

Table II. *Estrous cycle in prepubertal zeranol-treated and -untreated female Sprague-Dawley rats administered MNU at 28 days of age.*

Zeranol treatment	One cycle length (days)	% of time spent in each phase of cycle			
		Proestrus	Estrous	Metestrous	Diestrous
Untreated	4.6±0.2	25.6±1.6	31.2±1.3	25.6±2.4	17.0±1.9
0.1 mg/kg/day x5	5.3±0.2	10.6±1.5*	56.6±2.5**	18.4±2.2*	14.0±2.0
10 mg/kg/day x5	5.9±0.4**	11.4±1.5**	56.2±2.4**	18.1±2.1*	13.8±2.1

Examined from 8 weeks of age for 4 weeks. Values are mean±S.E. *p value<0.05 and **<0.01 compared with untreated group.

days of age, zeranol treatment caused no apparent histological changes in the vagina or uterus, and mammary gland development was comparable among groups.

Mammary tumorigenesis. Gross mammary tumors (≥1 cm) were all histologically confirmed to be carcinomas. Development of gross mammary carcinomas (≥1 cm) tended to be delayed in zeranol-treated groups, especially in the 0.1 mg/kg group, compared with the untreated group (Figure 3). However, log-rank test analysis indicated that differences in cumulative incidence of gross mammary carcinoma among groups were not significant. Also, incidence of gross mammary

Table III. *Effect of prepubertal zeranol treatment on body weight and relative uterine-ovarian weight in female Sprague-Dawley rats at 37 weeks of age.*

Zeranol treatment	No. of rats	Body weight (g)	Relative uterine-ovarian weight (mg/100 g B.W.)
Untreated	8	336.0±8.2	291.7±27.7
0.1 mg/kg/day x5	12	330.3±13.6	294.9±24.4
10 mg/kg/day x5	10	328.5±14.5	340.7±27.8*

Values are mean±S.E; *p value <0.05 compared with untreated group.

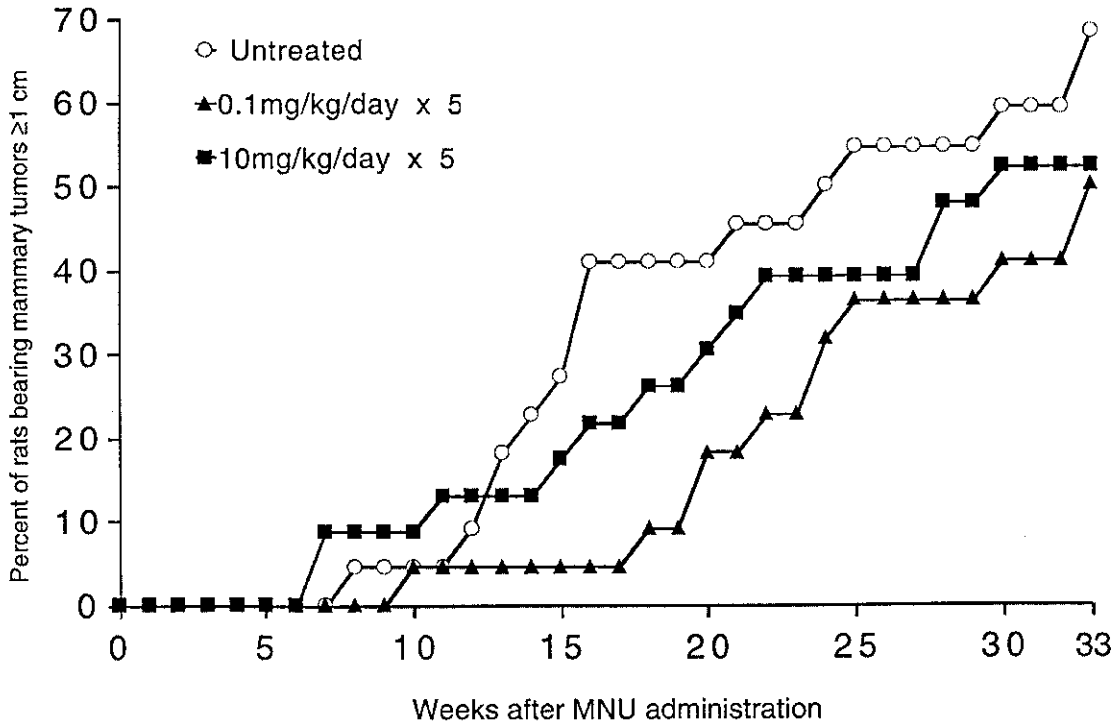

Figure 3. *Effects of prepubertal zeranol exposure on cumulative incidence of MNU-induced mammary tumors ≥1 cm in female Sprague-Dawley rats.*

carcinoma at the final time point did not significantly differ among groups (Table IV), and latency was also not significantly affected by zeranol treatment. When all sizes of histologically detected mammary carcinomas were analyzed, the number of mammary carcinomas per rat (multiplicity) did not significantly differ among groups, although the 0.1 mg/kg group tended to have a higher number of carcinomas per rat. Zeranol did not significantly affect mammary carcinoma yield, as indicated by cumulative incidence of gross mammary carcinomas, number of carcinomas per rat and latency. Zeranol tended to increase the incidence of fibroadenomas dose-dependently, and the reasons for this are unclear. Ovarian thecoma and adenoma, squamous cell carcinoma of the skin and renal mesenchymal tumors were observed after MNU administration, but the incidence of these tumors did not correlate with zeranol treatment.

Discussion

Zeranol does not exhibit toxicity when properly used as an anabolic in animal production (18). However, because zeranol has strong estrogenicity, as indicated by *in vivo* bioassays (19), detailed analysis of the effects of zeranol exposure during development on estrogen-responsive reproductive system functions and structure is of clinical importance. In rodents, neonatal and prepubertal zearalenone treatment has been shown to cause accelerated vaginal opening, persistent estrous and structural changes in ovaries indicating sterility (9,10,20-22). In the present study, although we did not see structural changes in female genital organs at 28 days of age, even low (0.1 mg/kg)-dose prepubertal zeranol exposure induced early puberty onset and profound disruption of estrous cyclicity in a considerable number of animals examined at 8 to 11 weeks of age. The ovaries of some zeranol-treated rats sacrificed during the experiment lacked newly formed corpora lutea, indicating anovulation and sterility. Taken together, the present data indicate that zeranol causes profound endocrine disruption, leading to anovulation (sterility) in female rats. Neonatal exposure to DES or genistein (a phytoestrogen) induces development of uterine adenocarcinoma in mice (23). In the present study, zeranol did not induce abnormalities in the uterus or vagina. However, longer observation is necessary to exclude the possibility that zeranol can induce tumorigenesis in female reproductive organs. Prenatal zeranol treatment (0.15 mg/kg x 2) causes abnormal testicular differentiation in male mice (24). There is a need for further studies of the effects of zeranol on the male reproductive tract, including its tumor-producing effects.

Table IV. *Effects of prepubertal zeranol exposure on mammary tumorigenesis in female Sprague-Dawley rats administered 50 mg/kg MNU at 28 days of age.*

Zeranol treatment	No. of rats	No. of rats with carcinoma >1 cm (%)	Total no. of carcinoma	No. of carcinoma per rat	Latency (weeks)	Total no. of fibroadenoma	Other tumors
Untreated	22	18 (82)	33	1.5±0.3	19.3±2.1	1	2 ovarian thecomas, 1 skin squamous cell carcinoma
0.1 mg/kg/day x5	22	14 (64)	50	2.3±0.4	23.7±2.0	6	1 ovarian adenoma, 2 renal mesenchymal tumors
10 mg/kg/day x5	23	16 (70)	34	1.5±0.3	18.6±2.3	13	1 ovarian thecoma

Values are mean±S.E

In addition to its effects on genital organs, zeranol exhibits acute hepatotoxicity and induces hepatic carcinogenesis in American hamsters, a rodent that is especially sensitive to the hepatotoxic effects of exogeneous estrogens (25). Mammary glands are also sensitive to estrogenic chemicals, and timing and dosage of exposure of immature animals to xenoestrogens influence the development of mammary tumors. The phytoestrogens genistein (12) and resveratrol (13) and the mycoestrogen zearalenone (10) affect the occurrence of MNU-induced mammary tumors (carcinomas) in female Sprague-Dawley rats when administered during the prepubertal period. Exposure of prepubertal children to zeranol may pose a risk of increased mammary tumorigenic potential when the endogenenous estrogen level is low (26). Zeranol, which is a more potent estrogen than zearalenone, stimulates developmental growth of mammary glands in ovariectomized mice (27). The degree of differentiation of mammary glandular structure at the time of carcinogen exposure appears to play a critical role in mammary cancer risk (28). Human exposure to carcinogenic stimuli can occur at any time of a woman's life. MNU can induce mammary tumors in sexually immature rats (29). In the present study, MNU was administered at 28 days of age and, at this time point, results of qualitative analysis of mammary gland differentiation did not significantly differ between females not exposed to zeranol and those exposed daily to zeranol from 15 to 19 days of age. Zeranol administered in the prepubertal period did not affect mammary tumorigenic potential, but had irreversible effects on the female reproductive system.

In conclusion, prepubertal exposure to zeranol did not affect occurrence of mammary carcinoma in female rats later in life, but it had profound effects on ovarian function and structure, indicating that consumption of zeranol-containing foods by humans in the prepubertal period should be avoided.

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Establishment of an apoptosis-sensitive rat mammary carcinoma cell line with a mutation in the DNA-binding region of p53

Tetsuya Hamaguchi[1], Yoichiro Matsuoka[2], John Bechberger[3], Takamasa Ohnishi[4], Ken-ichi Fujita[5], Christian C. Naus[3], Masato Kusunoki[1], Airo Tsubura[2] and Hiroyuki Tsuda[6]

[1]Second Department of Surgery, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514-0001; [2]Second Department of Pathology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Japan; [3]Department of Anatomy, University of British Columbia, Vancouver, BC V6T 1Z3, Canada; [4]Department of Molecular and Environmental Pathology, The University of Tokushima Graduate School of Medical Sciences, 3-18-15 Kuramoto-cho, Tokushima 770-8503; [5]Experimental Pathology and Chemotherapy Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045; [6]Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

Corresponding author: Dr. Yoichiro Matsuoka, Second Department of Pathology, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi, Osaka 570-8506, Tel.: 81-6-6993-9432; Fax: 81-6- 6992-5023; E-mail: matsuoyo@takii.kmu.ac.jp or Dr. Hiroyuki Tsuda, Department of Molecular Toxicology, Nagoya City University Graduate School of Medical Sciences, 1 Kawazumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan, Tel.: 81-52-853-8991; Fax: 81-52-853-8996; E-mail: htsuda@med.nagoya-cu.ac.jp

Abstract

Seven mammary carcinoma cell lines were established from 7,12-dimethylbenz[a]anthracene-induced tumors developed in a human *c-Ha-ras* transgenic rat. Without apoptotic stimuli, a large amount of p53 protein was detected in the C11 cell line (C11), whereas all cell lines expressed variable levels of the assayed death receptor/ligand, *bcl-2* family and p53 cascade-related genes. The *p53* gene in C11 had a mutation at codon 246, in the DNA-binding region of p53. Transcriptional activity of the mutant protein appeared to be lower than that of the wild-type p53. Despite the presence of p53 mutation, C11 was more sensitive to apoptosis triggered by etoposide, paclitaxel and staurosporine than the cell lines expressing wild-type p53. These data suggest that the apoptosis induced by intracellular injury occurs via the transcriptionally impaired mutant p53 in C11.

Keywords: apoptosis, Bcl-XL, mammary carcinoma, p53, ras, transgenic rat

1. Introduction

Apoptosis can be induced by 2 major pathways. The extrinsic pathway is triggered by extracellular death ligands such as the tumor necrosis factor (TNF) relatives Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL), which bind Fas and DR4 or DR5, respectively [1]. The intrinsic pathway is initiated by intracellular injury such as DNA damage, which induces caspase activation via the Bcl-2 gene family [2, 3]. Many physical and chemical DNA-damaging agents used in cancer therapy are potent activators of the tumor suppressor p53. Activation of p53 results in the expression of a number of genes that are involved in both major apoptotic pathways, including genes encoding pro-apoptotic members of the Bcl-2 family [4, 5] and death receptors [6, 7]. Thus, p53 plays roles in both the intrinsic and extrinsic apoptotic pathways.

The p53 tumor suppressor is mutated in over 50% of human cancers. The majority of these gene alterations are missense mutations, which often cause single-residue changes in the conserved DNA-binding core domain (residues 102-292 in human p53) of the protein [8]. Some common mutations in the DNA-binding domain lead to loss of the transcription- and apoptosis-inducing activity of p53. On the other hand, some transcriptionally inactive forms of p53 have been shown to mediate apoptosis via a transcription-independent mechanism [9-12]. Most recently, p53 has been shown to activate the pro-apoptotic protein Bax via binding to the anti-apoptotic proteins Bcl-XL and Bcl-2, resulting in mitochondrial membrane permeabilization and induction of apoptosis without target gene expression [13, 14].

We have generated a rat strain that carries the human *c-Ha-ras* protooncogene and exhibits increased susceptibility to chemical carcinogens that target the mammary gland, urinary bladder and skin [15-17]. All of these transgenic (Tg) rats develop pre-neoplastic mammary lesions within 20 days after injection of N-methyl-N-nitrosourea (MNU) [18], and mammary carcinomas appear within 8 weeks after treatment with a variety of chemical carcinogens including MNU and 7,12-dimethylbenz[a]anthracene (DMBA) [15, 19]. Interestingly, these carcinomas contain activating mutations preferentially in the human transgene [15]. These Tg rats also spontaneously develop alveolar hyperplasia and adenocarcinomas. Elevated expression of the *c-Ha-ras* protooncogene, rather than mutations in this gene, appears to be sufficient to cause a highly proliferative phenotype of mammary alveoli [20]. On the other hand, mammary tumorigenesis in these Tg rats is suppressed by pregnancy (T. Hamaguchi, unpublished data) and soy isoflavones [18].

In the present study, we established and characterized 7 cell lines isolated from mammary tumors developed in a Tg rat. The *p53* tumor suppressor gene was mutated in only 1 cell line (C11), whereas the human (but not rat) *Ha-ras* genes were mutated in all 7 cell lines. C11 cells, which

overexpress the mutant p53 with a single amino acid substitution in the DNA-binding domain, were more sensitive to apoptosis triggered by DNA-damage than the other cell lines, which had wild-type p53. The mutant p53 may induce apoptosis in a transcription-independent manner in C11 cells.

2. Materials and methods

2.1. Establishment of carcinoma cell lines from DMBA-induced mammary tumors in a Tg rat and culture

Mammary carcinoma cells were isolated and cultured according to the method described by Hallowes et al. [21] with minor modifications. Briefly, 1.5 g of mammary tumor tissue was minced and incubated in 20 ml of 199 medium (Gibco/BRL, Grand Island, NY) containing 0.2% (300 u/ml) collagenase type I (Gibco/BRL, Grand Island, NY), 0.1% (460 u/ml) hyaluronidase (Gibco/BRL, Grand Island, NY) and 5% fetal calf serum (FCS) for 2 hr at 37 °C. Dissociated cells were pelleted at 200 rpm for 5 min, resuspended in 20 ml of 199 medium, and sequentially passed through 500 μm and 70 μm filters (Becton Dickinson, Franklin Lakes, NJ) to remove large clumps. The filtrate was then poured over a 40 μm filter to collect the cell pellet by trapping it on the filter surface. Cells were incubated to induce attachment to culture dishes for 2 hr in DMEM/F12 containing 5% FCS, 5 μg/ml insulin (Gibco/BRL, Grand Island, NY), 0.5 μg/ml hydrocortisone (Sigma, St. Louis, MO) and 0.1 μg/ml progesterone (Sigma, St. Louis, MO). The unattached epithelial cells were re-plated onto new dishes and incubated in the same medium. After establishment of cell lines derived from the single colonies by limiting dilution, another round of limiting dilution was performed to obtain the individual cell lines (C1, C2, C3, C6, C11, C15 and C17) using DMEM containing 10% FCS as a selection medium. RCN-9 rat colon carcinoma cells were purchased from RIKEN Bio Resource Center (Tsukuba, Japan). All cell lines were maintained in DMEM containing 10% FCS.

2.2. Cell treatment and measurement of cell viability

Cells were plated at 10^3 cells/well onto 96-well plates and incubated for 24 hr. Then, the cells were treated with etoposide (10 or 100 μM), paclitaxel (1 or 10 nM), staurosporine (0.1 or 1 μM), or TNFα (1 or 10 ng/ml) and cycloheximide (CHX) (1 μg/ml) for 24 hr. Viable cells were counted using a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) reduction assay kit (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer's protocol. In some experiments, RCN-9, C3 and C11 cells were incubated with 0.5 mg/ml of cisplatin (Wako Pure Chemicals, Osaka, Japan) for the indicated time.

2.3. Assessment of apoptotic cells

Apoptotic cells were assessed by nuclear morphology after staining with 4',6-diamidino-2-phenylindole (DAPI; Wako Pure Chemicals, Osaka, Japan). Briefly, cells were stained with 10 μg/ml of DAPI for 30 min at room temperature, and fluorescent images of 3 fields of view per sample were

collected using an LSM510-META confocal microscope (Carl Zeiss, Oberkochen, Germany). Cells with condensed or fragmented DNA were counted as apoptotic cells in a minimum number of 100 cells per field.

2.4. RT-PCR

First-strand cDNA synthesis from total RNA was performed as described elsewhere [22]. PCR amplifications was performed with 30 cycles of denaturing for 15 sec at 95 °C, annealing for 30 sec at 60 °C and extension for 2 min (*fas* and *tnfα* reactions) or 1 min (all others) at 68 °C, using KOD plus (Toyobo, Osaka, Japan) and the following primers: *fas* forward, 5'-GCGGATCCGATATGCTGTGGATCATGGC-3', reverse, 5'-GCGAATTCTTTCACTCCAGACTTTGTCC-3'; *fasl* forward, 5'-GCGGATCCGGGTGCCATGCAGCAGCCCG-3', reverse, 5'-GCGAATTCCTTTAAAGCTTATATAAGCC-3'; *tnfr1* forward, 5'-GCGAATTCATGGGTCTCCCCATCGTGCC-3', reverse, 5'-GCAAGCTTTTATCGCGGGAGGTGGGTCG-3'; *tnfα* forward, 5'-GCGAATTCATGAGCACGGAAAGCATGATCC-3', reverse, 5'-GCAAGCTTCGCTTCACAGAGCAATGACTCC-3'; *bcl-2* forward, 5'-GCGAATCCATGGCGCAAGCCGGGAGAAC-3', reverse, 5'-GCAAGCTTTCACTTGTGGCCCAGGTATGC-3'; *bcl-xl* forward, 5'-GCGAATCCATGTCTCAGAGCAACCGGGAG-3', reverse, 5'-GCCTGCAGTCACTTCCGACTGAAGAGTG-3'; *bax* forward, 5'-GCGAATCCATGGACGGGTCCGGGGAGCAGC-3', reverse, 5'-GCAAGCTTTCAGCCCATCTTCTTCCAGATG-3'; *bid* forward, 5'-GCGAATCCATGGACTCTGAGGTCAGCAA-3', reverse, 5'-GCGGATCCTCAGTCCATCTCATTTCT-3'; *bad* forward, 5'-GCGAATCCATGTTCCAGATCCCAGAGTTTG-3', reverse, 5'-GCAAGCTTTCACTGGGAGGGAGTGGAGC-3'; *bak* forward, 5'-GCGAATCCATGGCATCCGGACAAGGACCAG-3', reverse, 5'-GCAAGCTTTCATGATCTGAAGAATCT-3'; *p53* forward, 5'-ATGGAGGATTCACAGTCGGA-3', reverse, 5'-CCTTCCACCCGGATAAGATG-3'; $p21^{Waf1}$ forward, 5'-ATGTCCGATCCTGGTGATGTC-3', reverse, 5'-TCAGGGCTTTCTCTTGCAGA-3'; *mdm-2* forward, 5'-GTCTATCAGGCAGGAGAAAGCGATG-3', reverse, 5'-ACGGGGCAGGGCTTATTCCTCTTCT-3'; *arf*, forward, 5'-ATGGGTCGCAGGTTCGTGGT-3', reverse, 5'- TTATGCCTGTCGGTGACCCG-3'; *GAPDH* forward, 5'-TTCAACGGCACAGTCAAGG-3', reverse, 5'-CATGGACTGTGGTCATGAG-3'.

2.5. Immunoblot

Western blot analysis was performed as described previously [22, 23]. The following antibodies were used: rabbit polyclonal anti-p53 antibody (1:1000 dilution; Novocastra Laboratories Ltd., Newcastle, UK; reacts with both wild-type and mutant p53), anti-p42/44-MAPK (16 ng/ml; Upstate Biotechnology, Lake Placid, NY), anti-Rb (1 μg/ml; Oncogene Research

Products, Boston, MA), anti-phospho-p42/44-MAPK (1 μg/ml), anti-phospho-Rb (Ser 780, 1:10000 dilution; Cell Signaling Technology, Inc., Beverly, MA) and anti-β-actin (1:10000 dilution; Sigma, St. Louis, MO). The bound first antibodies were detected using HRP-conjugated anti-rabbit and anti-mouse IgG antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL) and ECL plus western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). For assays of phospho-p42/44-MAPK and phospho-Rb in cells, mammary carcinoma cells were pre-incubated in DMEM without serum and phenol red for 48 hr, and then were lysed in a minimal volume of lysis buffer (50 mM Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 1mM Na_3VO_4, 10 mM NaF, pH7.4).

2.6. Mutation analysis

Mutations in the rat p53 genes of the mammary carcinoma cells were initially screened by directly sequencing PCR products of the full-length cDNAs (1176 bp) using the forward (5'-ATGGAGGATTCACAGTCGGA-3') and reverse (5'-TCAGTCTGAGTCAGGCCCCA-3') primers. Only C11 cells (codon 246) were found to have a mutation in the full-length rat *p53* cDNA (excluding the first and last 20 nucleotides) in the initial sequencing analyses. This was confirmed by restriction fragment length polymorphism (RFLP) analysis with Aci I digestion (New England Biolabs, Beverly, MA) after PCR amplification of exons 5 and 6 of rat *p53* genes using the forward (5'-ACTTTGACCCTTGATCCTTAGTTGG-3') and reverse (5'-TAGATAGGGTAGGGTAAAGAGGGCT-3') primers, and direct sequencing of the PCR products. Mutations of codons 12 and 61 of the rat *Ha-ras* genes were analyzed by direct sequencing of PCR products. Briefly, 735-bp fragments flanking exons 1 and 2 were first amplified using KOD Dash (Toyobo, Osaka, Japan) and specific primers for the rat *Ha-ras* gene (forward, 5'-CCTTGGGTTAGGCATCTATTAGCAGTCTCA-3'; reverse, 5'-TGGGGTGTATATGAGCCAGCTAGCA-3'), with 25 cycles of annealing for 2 sec at 60 °C and extension for 30 sec at 74 °C. Second-round PCR was performed using KOD plus (Toyobo, Osaka, Japan), the same primer set, and a 1:50 diluent of the first PCR products as the template, with 35 cycles of annealing for 30 sec at 60 °C and extension for 60 sec at 72 °C. Mutation analyses of codons 12 and 61 of the human *Ha-ras* genes were performed using PCR-RFLP analysis as described previously [15]. The primers used in PCR of codon 12 were as follows: forward, 5'-GCAGGCCCCTGAGGAGCGAT-3'; reverse, 5'-AGCAGCTGCTGGCACCTGGA-3'. The primers used in PCR of codon 61 were as follows: forward, 5'-AGCCCTGTCCTCCTGCAGGAT-3'; reverse, 5'-GGCCAGCCTCACGGGGTTCA-3' and 5'-CGCATGGCGCTGTACAGCTC-3'. Direct sequencing was performed using Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA) and a ABI PRIZM3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). To confirm the presence of 2 mutations at codon 12 of the transgenes in C1, C2, C6 and C15 cells, a 3rd round of limiting dilution

was performed (see Text).

3. Results

Seven individual mammary carcinoma cell lines (C1, C2, C3, C6, C11, C15 and C17) were established and characterized in terms of expression of apoptosis-related genes and molecules. Without apoptotic stimuli, all cell lines other than C1 expressed varying levels of the assayed death receptor/ligand, anti-apoptotic, pro-apoptotic and p53 cascade-related genes; C1 but not other cells expressed *tnfα* (Fig. 1). No expression of *fasl*, *bcl-2* or *bak* was detected in any of the cells (data not shown). The expression level of p53 protein was then assessed by immunoblot analysis. While various amounts of both un-ubiquitinated and ubiquitinated forms of p53 were detected in all cell lines, C11 cells contained at least a 4-fold higher level of un-ubiquitinated p53 than the other cell lines (Fig. 2A).

The extraordinarily high level of p53 in C11 may be due to a point mutation in the *p53* gene; some common mutations of this gene are known to cause accumulation of mutant p53 proteins [8]. Therefore, sequence analysis of the *p53* gene of C11 cells was performed. As suspected, a point mutation at codon 246 (CGC to GGC) of the *p53* gene was identified (Table 1). Both of the alleles were found to be mutated since two bands (477 bp and 114 bp) corresponding to the wild allele were not observed with PCR-RFLP analysis (Fig. 2B). This mutation causes an Arg-to-Gly amino acid substitution in the DNA-binding domain of p53 (p53RG246). No mutations were found in the *p53* gene of the other cell lines (Table 1 and Fig. 2B). Also, PCR amplification and sequencing of full-length cDNAs showed no deletions or truncations in the *p53* genes, as described in 'Materials and methods'.

Mutations of the rat and human *Ha-ras* genes were also analyzed. In all cell lines, GGC-to-GAC mutations were identified in codon 12 of the human transgenes. In C1, C2, C6 and C15, GGC-to-GTC mutations were also detected. Because 3 copies of the transgene are integrated in chromosome 5 of the Tg rats [15, 20], each copy of the transgene in these cells may contain either one of the mutations. Three of the 7 cell lines (C2, C11 and C15) were found to also have mutations in codon 61 of the transgenes (Table 1). In clear contrast, no mutations were detected in the endogenous rat genes, as reported previously [15]. Because the mutant proteins produced by the transgenes are considered constitutively active Ras [24-26], we assessed the activity states of the down-stream effectors p42/44-mitogen-activated protein kinase (MAPK) and Rb (Fig. 2C). After serum starvation, active MAPK or phospho-MAPK was detected in all cell lines other than C11. Although no clear correlation was observed between the phosphorylation levels of MAPK and Rb, it is interesting that C17, which exhibit minimum phosphorylation of Rb, grow much slower than the other cell lines in the absence of serum (Y. Matsuoka, unpublished data).

Based on the above results, 3 mammary carcinoma cell lines (C3, C6 and C11) were chosen as representative lines for comparison of apoptotic responses triggered by different apoptotic stimuli (Fig. 3). In particular, C11 were initially predicted to be resistant to DNA-damaging reagents because of the mutation in their *p53* gene. Unexpectedly, C11 were the most sensitive to all of the reagents tested, and were more sensitive than RCN-9 colon carcinoma cells, in which comparable amounts of p53 mRNA were expressed and low levels of p53 were detected without apoptotic stimuli (Fig. 1 and 2A). Growth of all cell lines was strongly suppressed by TNFα and staurosporine, a protein kinase C inhibitor. C11 cells responded to etoposide (a DNA-damaging reagent) and TNFα to the same degree, whereas the other 3 representative cell lines responded less strongly to etoposide than to TNFα (Fig. 3). These results suggest that the p53-dependent apoptotic cascade initiated by DNA damage functions most efficiently in C11, and that the apoptotic pathways triggered by TNFα and a PKC inhibitor are equally functional in all 4 representative cell lines. Moreover, C11 responded relatively well to a clinically relevant dose of the mitosis blocker paclitaxel (Fig. 3). When cells were stained with DAPI after incubation with 10 μM etoposide for 48 hr, 6.0%, 5.7%, 9.1% and 18.5% of RCN-9, C3, C6 and C11 cells, respectively, were apoptotic (Fig. 4 and data not shown). Similar results were obtained at 24 hr of incubation: 3.9%, 4.1%, 4.9% and 8.2% of RCN-9, C3, C6 and C11 cells, respectively, were apoptotic. Therefore, we conclude that C11 mammary carcinoma cells, which have a single amino acid mutation in the DNA-binding domain of p53, are highly competent for apoptosis triggered by intracellular injury.

Mutations in the DNA-binding domain of p53 often impair transcriptional activity of the protein. Therefore, we investigated whether p53RG246, which is expressed at a high level in C11, would remain transcriptionally functional under various conditions. After challenge with cisplatin, expression levels of 2 known target genes of p53, *waf1/cip1* and *bax*, were significantly less in C11 cells than in RCN-9 and C3 cells, relative to p53 levels (Fig. 5 and Fig. 2A). In addition, confocal microscopy showed that a large amount of mutant p53 localized in the nuclei and cytoplasm of C11 cells (data not shown). Taken together, these results suggest that the mutation in p53RG246 impairs its transcriptional activity.

4. Discussion

Mihara et al. demonstrated that p53 can bind Bcl-XL, and that a region in the conserved DNA-binding domain (residues 239-248 and 237-246 in human and rat, respectively) is required for this interaction [13]. Moreover, another laboratory has recently reported that p53 binds Bcl-XL with a higher affinity than some Bcl-2 homology (BH)3-only proteins such as Bid, and that such binding can release the BH3-only proteins from Bcl-XL/BH3-only protein complexes, leading to Bax oligomerization [14]. Bax oligomerization is an

obligate step in activation of the protein to assist in mitochondrial outer membrane permeabilization [27-29]. It is also noteworthy that mutations in codon 248 of human p53 (codon 246 in the rat gene) disrupt the binding of the protein to DNA but affect weakly its apoptosis-inducing activity [8, 30]. We showed that C11 cells overexpress p53 (Fig. 2A) with a mutation at residue 246 (p53RG246) (Table 1) and are highly sensitive to DNA-damaging reagents (Figs. 3 and 4). Although a common p53 polymorphic variant at codon 72 (Pro to Arg) has been known to increase nuclear export, mitochondrial localization, and apoptosis [31], C11 is a novel apoptosis-sensitive cell line with a mutation at codon 246 (codon 248 in humans). It would be very interesting to compare the binding affinity of p53RG246 and wild-type p53 to Bcl-XL.

C11 cells also exhibit interesting changes in their Ras-MAPK pathway. We assumed that MAPK in C11 cells would be phosphorylated at an equal or greater level as in the other cell lines, because the human *Ha-ras* genes in all cell lines had the activating mutations at codon 12 (Table 1). However, a trace amount of MAPK phosphorylation was detected in C11 cells. Strangely, phosphorylation of Rb, the key molecule through which the Ras-MAPK pathway exerts its cell-proliferating effect, was highest in C11 cells (Fig. 2C). The PKB/Akt pathway is another important Ras-regulated pathway, and mediates stimulation of cell proliferation [32]. We are currently conducting biochemical studies of the molecule(s) responsible for the high phosphorylation level of Rb in C11 cells.

We have established 7 individual cell lines isolated from mammary tumors developed in a *c-Ha-ras* Tg rat. There were similarities and differences between these cell lines in characteristics related to apoptosis. This series of cell lines is useful for comparison of apoptotic responses to drugs and examination of the mechanisms underlying their differences in responsiveness. In addition, given the functional resemblance of rat mammary neoplasias to their human counterparts, further elucidation of their biological properties should facilitate development of treatments for the human form of this disease.

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Table 1 *p53* and *Ha-ras* gene mutations in rat mammary carcinoma cells

Cell line	Sequence of gene				
	p53	Rat *Ha-ras*[a]		Human *Ha-ras*[b]	
		exon 1	exon 2	codon 12	codon 61
C1	W[c]	W	W	GA/TC	W
C2	W	W	W	GA/TC	CGG
C3	W	W	W	GAC	W
C6	W	NA[e]	NA[e]	GA/TC	W
C11	GGC(codon 246[d])	W	W	GAC	CAT
C15	W	W	W	GA/TC	CAT
C17	W	W	W	GAC	W

[a]The entire region flanking exons 1 and 2 including codons 12 and 61, respectively, were sequenced.
[b]The wild-type sequences are as follows: codon 12 is GGC and codon 61 is CAG.
[c]W denotes the wild-type sequence.
[d]The wild-type sequence of codon 246 is CGC.
[e]The rat *Ha-ras* was not sequenced because of unsuccessful PCR amplification.