one tumor in the mammary gland region (a sarcoma) was observed, in an animal fed 5 ppm atrazine, at 20 weeks.

Final mammary tumor incidences and multiplicity data determined by histological examination are summarized in Table 2. In female Tg rats, the adenoma and adenocarcinoma incidences for rats fed 5 ppm atrazine were elevated as compared with controls ($P<0.01$ and $P<0.05$, respectively). The adenocarcinoma incidence of the rats fed 50 ppm atrazine was also increased ($P<0.05$) and a tendency for increase was evident at 500 ppm. Similar non-significant tendencies were observed for multiplicities of adenocarcinomas and total tumors. No significant differences were noted in male Tg rats.

Effects of atrazine on skin tumor induction. Skin tumors, squamous cell papillomas and carcinomas, were induced only in males. The first appearance was at week 10 in a control Tg rat, whereas it was at week 11 in Tg rats fed 5 or 50 ppm and at week 15 in Tg rats fed 500 ppm atrazine (Fig. 2). The incidence increased with time. Atrazine at the dose of 500 ppm significantly reduced skin tumor incidence at 15 and 16 weeks ($P<0.05$) (Fig. 2). Multiplicity was also significantly reduced ($P<0.05$) (Table 3).

General observations in experiment II. In experiment II, conducted to determine effects of nonylphenol, 6 rats died before the termination of the experiment, for reasons unrelated to the treatment or the Tg/non-Tg status. Data for final body weights and intakes of food are summarized in Table 4. Relative ovary weights in females Tg rats fed 25, 100, and 250 ppm nonylphenol were significantly decreased as compared to the control values ($P<0.05$, $P<0.01$, and $P<0.001$, respectively) (data not shown). Relative uterus weights in female Tg rats fed 250 ppm nonylphenol were also significantly decreased ($P<0.05$). No significant differences were noted in the body, liver, kidney, or testis weights. The daily food intake was significantly elevated in female Tg rats fed 10 ppm and in male Tg rats fed 100 or 250 ppm nonylphenol as compared to the relevant control groups ($P<0.005$). In non-Tg males, daily food intake with 10 and 25 ppm was decreased ($P<0.005$).

Effects of nonylphenol on mammary tumor induction. Cumulative incidence data for palpable mammary tumors are presented in Fig. 3. In female Tg rats, mammary tumors were first observed at 4 to 5 weeks after administration of DMBA. The incidence rapidly increased with time, especially in rats fed 10 ppm nonylphenol, with significant elevation of the tumor incidence at week 6 ($P<0.05$). In female non-Tg rats, the first appearance of mammary tumors was after 11 weeks. They were first observed after 10, 6, 8, and 6 weeks in female non-Tg rats fed 10, 25, 100, and 250 ppm nonylphenol, respectively. Incidence then increased with time, and finally was higher with 25 ppm than in the controls. Statistically significant earlier onset of mammary tumors relative to controls ($P<0.05$) was also apparent at 25 and 250 ppm, although the overall incidence of these neoplasms at the end of the study was similar in the treated and control groups. In male Tg rats, although mammary tumors were firstly observed at 11 weeks after administration of DMBA at all doses, incidences at this time point were higher in the rats fed 25 and 100 ppm nonylphenol than in the controls ($P<0.05$). No palpable tumors were encountered in male non-Tg rats.

Data for final mammary adenoma and adenocarcinoma and skin tumor incidences and multiplicity determined by histological examination are summarized in Tables 5 and 6. In female Tg rats, the adenocarcinoma and total tumor multiplicities in rats fed 10 ppm nonylphenol were increased as compared with control values, with a significant quadratic dose-response trend ($P<0.05$). In male Tg rats, sarcoma multiplicity with 25 ppm nonylphenol was also increased ($P<0.05$). Although not significant, a similar tendency was observed in female non-Tg rats, and also for tumor volume in female and male Tg and non-Tg rats (data not shown).

Effects of nonylphenol on skin tumor induction. Skin tumors were induced only in males, as in experiment I. The first appearance was at week 10 in Tg rats fed 0, 10, 100, and 250 ppm, and at week 12 in their counterparts receiving the 25 ppm dose. Nonylphenol at 25 ppm decreased skin tumor incidence at 10 and 11 weeks ($P<0.05$). Although final skin tumor incidence and multiplicity were not significantly decreased, a tendency for reduction with the treatment was observed with 100 ppm.

Effects of atrazine and nonylphenol on growth of human mammary cancer cells. Atrazine did not cause proliferation of MCF-7

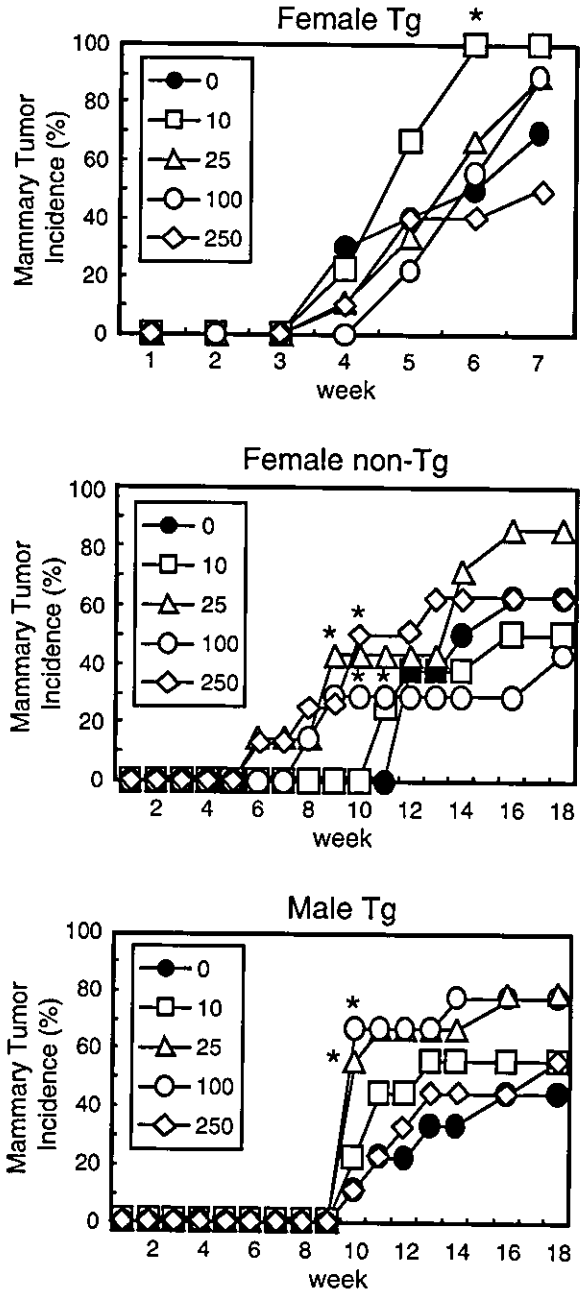

Fig. 3. Periodic observation of palpable mammary tumors in female Tg and non-Tg and male Tg rats fed nonylphenol (10, 25, 100, 250 ppm). * Significantly different from 0 ppm by the χ^2 test at $P<0.05$.

Table 5. Effects of nonylphenol on mammary tumor induction in female rats (experiment II)

Line	Nonylphenol (ppm)	Effective No. of rats	Adenoma		Adenocarcinoma	
			Incidence (%)	No./rat[1)]	Incidence (%)	No./rat[1)]
Tg	0	10	2 (20.0)	0.40±0.97	9 (90.0)	5.30±4.32*
	10	9	2 (22.2)	0.22±0.44	9 (100)	9.00±3.74#,*
	25	9	2 (22.2)	0.22±0.44	9 (100)	7.67±5.00*
	100	9	1 (11.1)	0.11±0.33	8 (88.9)	5.11±3.33*
	250	10	2 (20.0)	0.20±0.42	10 (100)	4.10±3.31*
Non-Tg	0	8	1 (12.5)	0.14±0.38	6 (75.0)	1.75±2.25
	10	8	1 (12.5)	0.13±0.35	5 (62.5)	1.25±1.28
	25	7	0	0	6 (85.7)	2.29±2.63
	100	7	1 (14.3)	0.14±0.38	4 (57.1)	1.29±2.14
	250	8	1 (12.5)	0.13±0.35	5 (62.5)	1.25±1.98

1) Mean±SD.
Significant quadratic trend by a test using the coefficients for the orthogonal polynomials ($P<0.05$).
* Significantly different from the control group by Student's t test ($P<0.05$).

Table 6. Effects of nonylphenol on mammary and skin tumor induction in male rats (experiment II)

Line	Nonylphenol (ppm)	Effective No. of rats	Adenoma		Adenocarcinoma		Skin tumors	
			Incidence (%)	No./rat[1)]	Incidence (%)	No./rat[1)]	Incidence (%)	No./rat[1)]
Tg	0	9	0	0	6 (66.7)	1.33±1.41	8 (88.9)	2.56±2.19
	10	8	1 (12.5)	0.13±0.35	5 (62.5)	1.75±1.75	8 (88.9)	2.00±1.73
	25	7	2 (28.6)	0.57±1.13	3 (42.9)	1.43±2.15	7 (77.8)	2.00±1.58
	100	7	0	0	5 (71.4)	2.57±1.99	6 (66.7)	1.67±1.74
	250	9	0	0	6 (66.7)	1.67±1.73	9 (100)	2.44±1.74
Non-Tg	0	10	0	0	1 (10)	0.10±0.32	0	0
	10	7	0	0	0	0	0	0
	25	7	0	0	0	0	0	0
	100	8	0	0	0	0	0	0
	250	7	0	0	0	0	0	0

1) Mean±SD.

cells at any dose. Nonylphenol, at high doses (10^{-6}–10^{-5} M) and 17β-estradiol even at low doses (10^{-11}–10^{-6} M) clearly caused cell growth (data not shown).

Serum nonylphenol and atrazine levels . At autopsy, serum levels of test chemicals were measured by LC/MS. Serum atrazine and nonylphenol were below the detectable levels (<50 ppb) in all groups (data not shown).

Discussion

The present study showed that atrazine may enhance mammary tumor development in female Tg rats, but without any dose dependence, while inhibiting skin tumorigenesis in their male counterparts. The findings for nonylphenol point to significant promotion of mammary tumor development in female Tg rats and acceleration of induction in non-Tg females, but only at the 10 and 25 ppm doses, with no effects being apparent at 100 or 250 ppm. To our knowledge, this is the first concrete evidence that nonylphenol has enhancing effects on tumorigenesis *in vivo*, although it is reported that it can act as a promoter *in vitro*.[40] Since both Tg and non-Tg rats were affected, the role of the transgene itself is unclear. However, from the incidence data, it would appear that overexpression of the human ras protooncogene increases the susceptibility of the mammary tissue.

Atrazine does not have intrinsic estrogenic activity *in vivo* or *in vitro*.[41–43] As evidenced by our observations in MCF-7 assay, it does not bind to the estrogen receptor. The estrogenic effects associated with the triazine herbicides are not estrogen receptor-mediated, but may be explained partly by the ability of the compounds to induce aromatase.[44] Most of the work has been directed towards the effects of atrazine on the hypothalamus-pituitary-gonadal axis,[12,45–47] and the related hormonal imbalance appears to be significant for interpretation of possible carcinogenic effects on the mammary gland.

When Fischer F344/LATI rats of both sexes were given atrazine in the diet at concentrations of 0, 375, and 750 ppm for 126 weeks, a significantly increased number of combined leukemias/lymphomas and elevated incidence of mammary tumors in the high-dose male group was found, together with dose-dependent formation of uterine carcinomas.[48] However, in other investigations, mammary tumor development was only noted in SD and not F344 rats, and only at or above a threshold dose (the MTD) that interferes with normal estrous cycling, causing prolonged exposure to endogenous estrogen.[16,17] SD rats exhibiting persistent estrus also have a prolonged elevation of estrogen secretion, so it is proposed that the triazine-associated mammary tumor response is promoted by the test animal's own estrogen from ovarian follicles that fail to ovulate.[10] Chloro-*S*-triazine herbicides, such as cyanazine (CZ), atrazine (AZ), simazine (SZ), increased mammary tumors in Crl:CD BR rats, but not in F344 rats or in mice, but this is mediated through a prolactin mechanism not relevant to humans.[49] While one study suggested a link between agricultural exposure to atrazine and non-Hodgkins lymphomas,[50] the epidemiological evidence regarding triazines points to a null association for breast cancer across all exposure indices and an inverse association with ovarian cancer.[51] Individual population groups may show associations with specific cancers and atrazine contami-

nation levels (range 50–649 ng/liter, maximum acceptable concentration [MAC]=60,000 ng/liter) were positively associated ($P<0.05$) with stomach cancer incidence and negatively associated with colon cancer incidence in one study,[52] but an earlier review of the literature, with a pooled analysis of three of the case-control studies and the combined analysis of two retrospective follow-up studies, did not demonstrate the types of dose-response or induction time patterns that would be expected if triazines were causal factors.[53] The doses applied in the present study were in the range of 10,000 times the highest level of pollution conceivable in the human situation.[54]

Nonylphenol is estrogenic in rat, fish, avian, and mammary cells.[24, 25, 55] However, it may exert a promoting activity on cell transformation without binding to estrogen receptors.[40] Presently, we consider mammary tumors induced in the Tg rats to be estrogen-independent, because ovariectomy does not affect their development.[56] With regard to the literature, in contrast to the increase observed here in our transgenic animals, we earlier reported inhibition in the same model with bisphenol A.[37] This antagonist of PPARγ induces apoptosis of tumor cells, although the mechanisms are unclear.[57] In another study after DMBA initiation in SD rats, ovarian tumor development was significantly suppressed by 4-nonylphenol.[58] No effects of bisphenol A or methoxychlor were found on DHPN-initiated thyroid carcinogenesis,[59] or of the phenol in the prostate after DMAB-initiation.[60] However, in adult Donryu rats, ENNG-initiated endometrial carcinogenesis was promoted by high-dose exposure, possible due to uterotrophic effects.[61]

In the present study, atrazine and nonylphenol inhibited development of DMBA-induced skin tumors in male Tg rats, in line with earlier data for estradiol.[36] In Tg rats, feminization by endocrine disruptors may thus play a role in reducing the skin tumors, which do not develop in females.

The present findings on multiplicity of mammary tumors in female Tg rats given nonylphenol, and specifically the significant quadratic dose-response pattern, require comment. While the lack of a simple positive dose dependence might cast doubt on the validity of the results, a similar tendency has also been observed in an *in vitro* study. Thus nonylphenol-induced cell proliferation was observed only at 10^{-6} to 10^{-5} *M* in MCF-7 cells (reference 24 and our unpublished observations). Therefore, the predictive value of hazard risk assessment at exceedingly high doses may be questioned. Atrazine is one of the most frequently detected pesticides in surface water and sediment samples collected in the United States, the highest concentrations found being 18 ppb and 50 ppb, respectively.[62] WHO has established an international guideline for atrazine in drinking-water of 2 ppb.[63] Concentrations of alkylphenol ethoxylate metabolites in treated wastewater effluents in the US, Spain, and the UK ranged from <0.1 to 369 ppb, with a much larger range for sediments. Fish in the UK were found to contain up to 0.8 ppb nonylphenol in muscle tissue.[64] In fact, few published studies have addressed the effects of atrazine and nonylphenol at these very low concentrations. No animal models have been developed in which tumors are induced by very low doses of estradiol, presumably because of the cost of maintenance of a large number of animals for such a model and the difficulty of dosing in view of the varying levels of endogenous estrogen in cycling females. In a recent study[37] and the present investigation, we showed that Tg rats can be used in medium-term (8 to 12 weeks) bioassay models to test for endocrine disruptors on mammary tumor development. Further experiments are now required to elucidate what influence endocrine disrupters might exert at low-dose levels (ppb) in our c-Ha-*ras* transgenic rats. In conclusion, atrazine and nonylphenol, and especially the latter, may cause enhancement of mammary carcinogenesis within a certain restricted low-dose range, though all the doses employed in the present study were very much larger than any conceivable human intake under normal conditions.

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Mouse anococcygeus muscle: sexual dimorphism and responsiveness to sex hormones

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Abstract

The anococcygeus muscle (AcM) is one of a pair of thin sheets of smooth muscle inserting on the rectum, having a tendinous origin largely on sacral vertebrae. The cross-sectional area of AcM in the juxtarectal region in 90-day-old male mice was significantly larger than that in females of three strains: BALB/cCrgl, ICR/Jcl and C57BL/Tw. The AcM area in female mice showed strain differences: BALB/c>ICR>C57BL. Five daily injections of testosterone into newborn ICR mice from the day of birth significantly increased the areas of AcM in both sexes at 30 days of age, but five daily injections of oestradiol-17β (OE) decreased them. The AcM area in 60-day-old ICR male mice castrated at 30 days of age was significantly smaller than in intact males, and that in ovariectomized females was significantly larger than in intact females. In both sexes, implantation of a testosterone pellet (12 mg) into gonadectomized mice on the day of gonadectomy stimulated the growth of AcM, and implantation of an OE pellet (12 mg) inhibited the growth of AcM. The AcM in both ICR and C57BL strains showed positive androgen receptor and oestrogen receptor immunostaining at 15 days. Female ICR mice exposed neonatally to diethylstilboestrol (DES) had significantly larger AcM than controls; ovariectomy at 30 days of age did not change the AcM area in 60-day-old DES-exposed mice. However, male mice exposed neonatally to DES had significantly smaller AcM than controls; castration at 30 days of age nullified this inhibition. These results suggest that both androgen and oestrogen play an important role in sexual dimorphism of the mouse AcM. Neonatal exposure to DES (but not to oestradiol) had an irreversible stimulatory effect on the AcM area in female mice.

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Introduction

The anococcygeus muscle (AcM) of rats has been used to examine the pharmacology of neurotransmitters (Gillespie 1971, 1972). This muscle is one of a pair of thin sheets of smooth muscle inserting on the rectum, having a tendinous origin largely from sacral vertebrae; it might better be named 'the sacrorectal muscle' (Larson *et al.* 1985). A dense adrenergic innervation is distributed throughout the AcM; there is also a peptidergic innervation but apparently no cholinergic innervation (Gillespie & Lüllmann-Rauch 1974, Gillespie 1980, Brave *et al.* 1993). Nevertheless, acetylcholine as well as noradrenaline causes contraction of the muscle (Gillespie 1972, Gillespie & McGrath 1973, Gibson & James 1977). Dail *et al.* (1990) found evidence for acetylcholinesterase-positive fibres and for vasoactive intestinal polypeptide in rat AcM. Gibson *et al.* (1984) have demonstrated that the muscle responds both to fish urotensin II and to somatostatin (Larson *et al.* 1985). Stimulatory effects of testosterone on rat AcM contractility have been reported by Gibson (1977). The physiological role of the muscle, however, has not yet been elucidated.

The spinal nucleus of the bulbocavernosus (SNB) and its target muscles of the perineal muscle complex (ischiocavernosus, bulbocavernosus and levator ani) are reduced or absent in normal female rats (Cihak *et al.* 1970, Breedlove & Arnold 1980, McKenna & Nadelhaft 1986, Tobin & Joubert 1988). Organizing and activational effects of perinatally administered testosterone on the tissues mediating mating behaviour have been reported (Phoenix *et al.* 1959, Mills *et al.* 1992). Perinatal antiandrogen treatment of male rats completely demasculinizes SNB (Breedlove & Arnold 1983*a*) and perinatal androgen treatment of females increases the number of SNB neurones found in adulthood (Breedlove & Arnold 1983*b*). Forger *et al.* (1992) raised the possibility that androgen rescues motoneurone cells in SNB of postnatal female rats from death through a ciliary neurotrophic factor, and consequent establishment of the neuromuscular system causes calcitonin gene-related peptide expression in SNB, which may act as an anterograde muscle trophic factor (Forger *et al.* 1993). The rat levator ani muscle exhibiting sexual dimorphism (Cihak *et al.* 1970) has been reported as a suitable model for studying the effects of testosterone on

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the development of mammalian muscle by Tobin & Joubert (1988); its growth and/or weight gain is stimulated by testosterone (Tobin & Joubert 1991, Rand & Breedlove 1992, Joubert *et al.* 1994). On the other hand, the female rat costo-uterine muscle which provides a skeletal attachment for the longitudinal myometrial layer of the uterus is also responsive to sex steroids (Guglielmone & Vercelli 1991). Muscle cell length increases during pregnancy and after oestrogen treatment. In rat AcM, sexual dimorphism in size (Gibson & Gillespie 1973) and the stimulation by testosterone of the contractile response to both noradrenaline and acetylcholine (Gibson 1977) suggest an effect of sex hormones. However, the effect of sex steroids on growth of the muscle and on the development of sexual dimorphism has not yet been delineated. In the present study, therefore, we studied sexual dimorphism, responsiveness to sex hormones administered postnatally, and androgen receptor (AR) and oestrogen receptor (OER) expression in this muscle. Disorders of uterine smooth muscle in rodents exposed neonatally to the synthetic oestrogen diethylstilboestrol (DES) have been reported (Ostrander *et al.* 1985, Iguchi & Takasugi 1987, Brody & Cunha 1989); therefore, we chose to study the effects of the neonatal exposure to DES on the AcM. In addition, DES was the primary oestrogen responsible for reproductive effects in female and male humans ('DES syndrome').

Materials and Methods

In the present study, ICR/Jcl, C57BL/Tw and BALB/cCrgl mice were used. All animals were provided with pine shavings for bedding, fresh water, and sterilizable rodent block diet available *ad libitum* and kept at 24 ± 1·0 °C under 12-h artificial illumination (from 0800 to 2000 h). All chemicals used in the present study were from Wako Pure Chemical Co. (Tokyo, Japan) except sex hormones (Sigma, St Louis, MO, USA). All procedures were carried out according to the NIH Guide for Care and Use of Laboratory Animals.

Both male and female mice of the three strains were killed at 90 days of age by cervical dislocation. In order to study the development of sexual dimorphism, ICR mice were killed at 30, 60, 90 and 120 days of age. ICR mice were gonadectomized (GX) at 30 days of age. GX male and GX female mice were separated into three groups: untreated, receiving a 12 mg testosterone implant, or receiving a 12 mg oestradiol-17β (OE) implant under the abdominal skin at the time of operation. These mice were killed at 60 days of age, and reproductive organs and gubernacula testis were weighed to confirm the effects of steroid implants. The steroid implants remained after the 30-day implantation period, and 3–4 mg of the implant were absorbed. The AcM was fixed in Bouin's solution. Seven to eleven mice were used for each time-point.

In order to study the role of neonatal sex hormones on the development of sexual dimorphism in the AcM, newborn ICR mice of both sexes were given five daily injections of 50 μg testosterone or 30 μg OE dissolved in 0·02 ml sesame oil or the vehicle alone from the day of birth. These doses of hormones induce sexual dimorphism in the mouse pubis as well as irreversible changes in the vagina (see Iguchi 1992). Mice were killed at 30 days of age. In addition, newborn ICR mice were given five daily injections of 3 μg DES (Sigma) from the day of birth, and half of these mice were gonadectomized at 30 days of age. All mice treated neonatally with DES and their controls were killed at 60 days of age, and AcM were dissected and fixed in Bouin's solution.

AcM was embedded in paraffin and serially cross-sectioned at 8 μm thickness. The sections were stained with Delafield's haematoxylin and eosin. The cross-sectional areas of AcM were measured by Color Image Analyzer CIA-102 (Olympus, Tokyo, Japan). The areas are expressed as actual area (μm^2) and as relative area (μm^2/20 g body weight). Data were analyzed by ANOVA.

For immunohistochemistry of AR (Maeda *et al.* 1994) and OER (Yamashita & Korach 1989, Iguchi *et al.* 1991, Sato *et al.* 1992) in AcM, ICR and C57BL strains, mice were killed at 15, 30, 60, 90 and 120 days of age. Dissected AcM embedded in OCT compound (Miles Laboratories, Elkhardt, IN, USA) were immediately frozen in liquid nitrogen and sectioned with a cryostat microtome at 6 μm; sections were then fixed in 4% paraformaldehyde solution at pH 7·2 for 10 min at room temperature (Maeda *et al.* 1994). Sections were incubated with 1% BSA fraction V (Sigma) in PBS at pH 7·4 for 30 min at room temperature and then incubated with primary anti-AR monoclonal antibody (10 μg/ml; Affinity Bioreagents, Neshanic Station, NJ, USA) or anti-OER monoclonal antibody (1/2 concentration of supplied solution; Abbott Laboratories, Chicago, IL, USA) for 2 h at room temperature. For negative controls, 1% non-immunized rat serum was used as a primary antibody solution. After washing with PBS, the sections were incubated with the second antibody (horseradish peroxidase-labelled anti-rat $F(ab')_2$ fragment; Amersham, Chicago, IL, USA) for 45 min at room temperature. Diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan) reaction with imidazole (Sigma) was carried out for 15 min for AR demonstration and 10 min for OER demonstration. Since the density of immunochemical reaction products *in situ* is influenced by several factors (e.g. fixation, section thickness, reagent concentrations, staining times), the procedure described above was strictly followed. The staining intensity was automatically quantified by the Color Image Analyzer in arbitrary densitometric units (ADU) for each specimen, and normalized by deducting ADUs of negative controls from each value. Densitometric data were statistically analyzed by ANOVA.

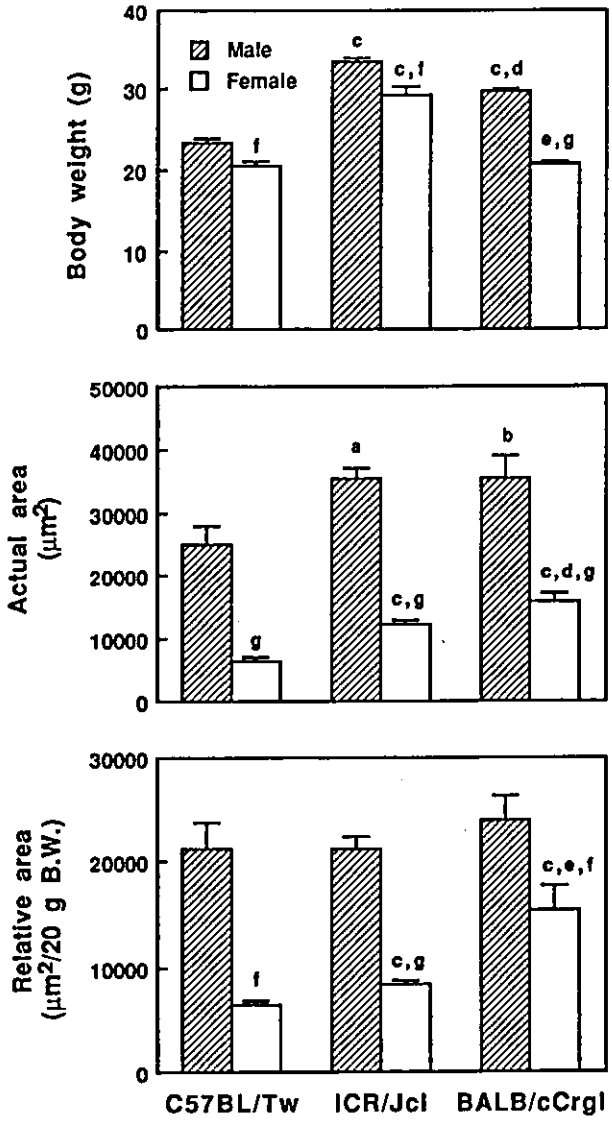

Figure 1 Body weight, actual cross-sectional area and relative area of AcM in 90-day-old mice of three strains. a, $P<0.05$; b, $P<0.01$; c, $P<0.001$ vs C57BL: d, $P<0.05$; e, $P<0.001$ vs ICR: f, $P<0.01$; g, $P<0.001$ vs male.

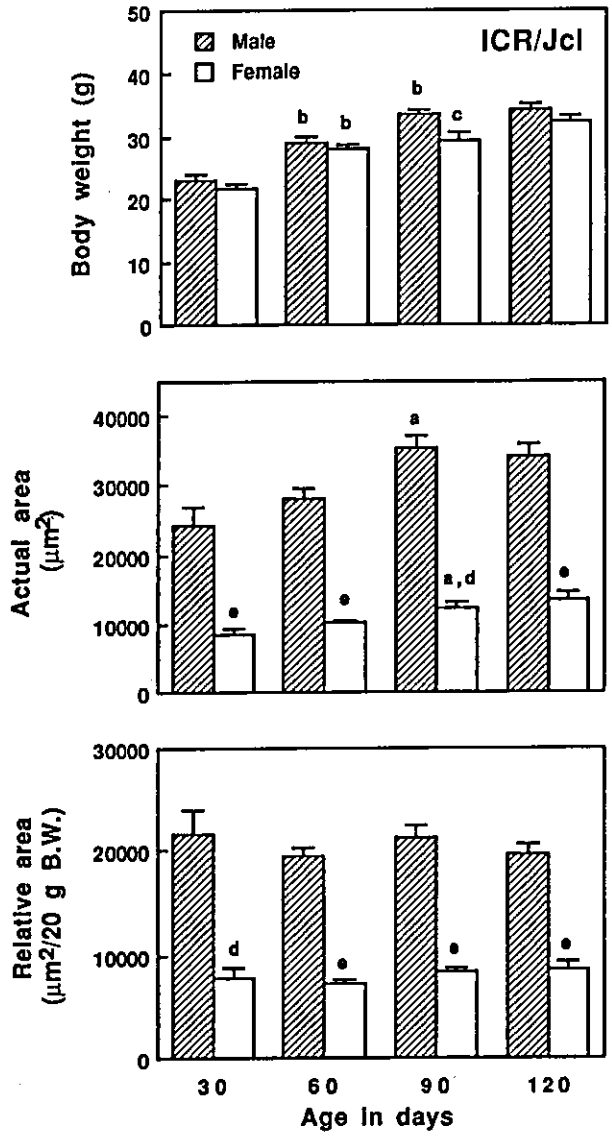

Figure 2 Developmental changes in body weight, actual cross-sectional area and relative area of AcM in ICR/Jcl mice. a, $P<0.05$; b, $P<0.001$ vs the prior age: c, $P<0.05$; d, $P<0.01$; e, $P<0.001$ vs male.

Results

Sexual dimorphism of AcM

Both actual and relative cross-sectional areas of the AcM of male mice were significantly larger than those of female mice in all strains at 90 days of age (Fig. 1). Strain differences in sexual dimorphism were observed; relative cross-sectional areas of male AcM were 3·2, 2·6 and 1·6 times larger than those of C57BL, ICR and BALB/c female mice respectively. In female mice, the relative muscle area of C57BL mice was significantly smaller than that of the other two strains. The relative muscle area of BALB/c female mice was significantly larger than that of ICR female mice. In male mice, strain differences in muscle area were not significant.

Development of AcM

Body weights of ICR mice increased linearly from 30 to 90 days and reached a plateau at 90 days (Fig. 2). Sexual dimorphism in AcM was observed from 30 days of age. Both actual and relative muscle areas of male mice were

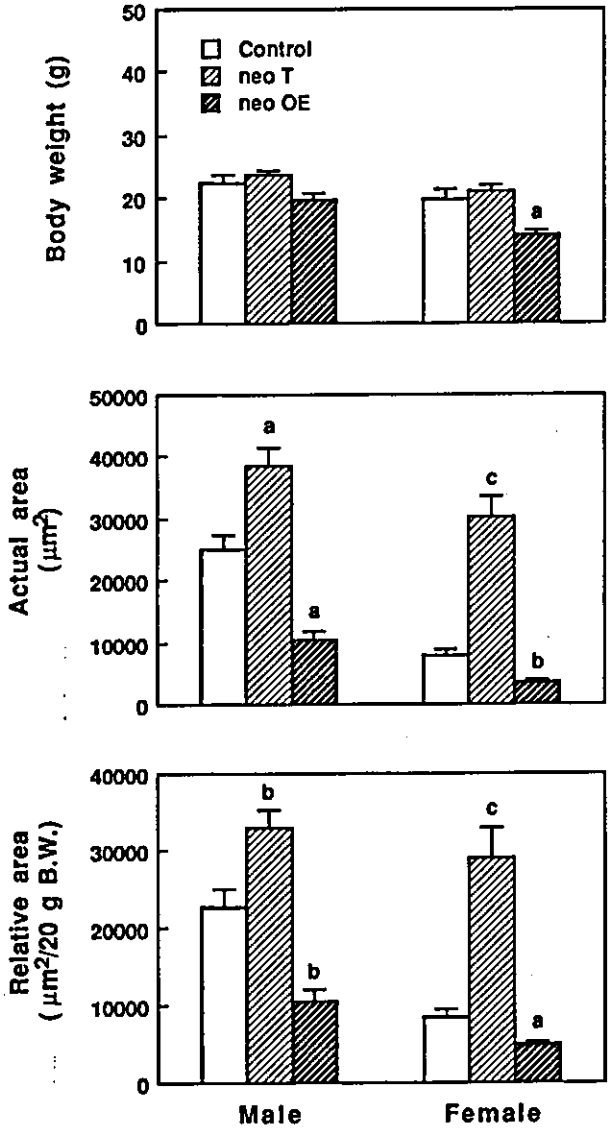

Figure 3 Effects of neonatal exposure to steroid hormones on the cross-sectional area of AcM in 30-day-old ICR/Jcl mice. Male and female neonatal mice were given a daily injection of testosterone (50 μg) or OE (30 μg) during the first 5 days after birth. Neo T=neonatally testosterone-exposed mice; neo OE=neonatally OE-exposed mice. a, $P<0.05$; b, $P<0.01$; c, $P<0.001$ vs control mice.

significantly larger than those of female mice at all ages examined. When expressed relative to 20 g body weight, there were no age differences in muscle area (Fig. 2).

Steroid hormone responsiveness of AcM during development

Neonatal exposure to OE significantly decreased the body weight of female mice ($P<0.05$ vs control) at 30 days of age (Fig. 3). Exposure of neonatal mice to testosterone

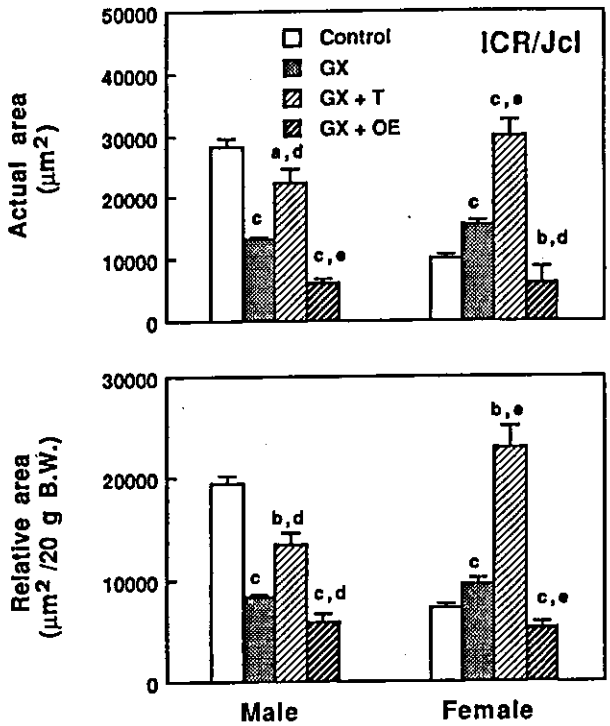

Figure 4 Effect of gonadectomy and steroid hormones on the cross-sectional area of AcM in 60-day-old ICR/Jcl mice. Male and female mice gonadectomized at 30 days of age received a subcutaneously implanted 12 mg pellet of testosterone (T) or OE for 30 days from the day of gonadectomy. a, $P<0.05$; b, $P<0.01$; c, $P<0.001$ vs control mice: d, $P<0.01$; e, $P<0.001$ vs gonadectomized mice.

caused increased actual and relative AcM areas at 30 days of age in both sexes, but OE significantly decreased them in comparison with controls.

The AcM areas of 60-day-old ICR mice gonadectomized at 30 days of age (GX), and gonadectomized and given an implantation of a 12 mg pellet of testosterone or OE (GX+T or GX+OE respectively) were calculated (Fig. 4). The actual and relative muscle area of GX male mice was significantly smaller than that of controls. The muscle area of GX+T male mice was significantly larger than that of GX males, but significantly smaller than that of control males. However, the muscle area of GX+OE mice was significantly smaller than that of control males and GX males. Actual and relative muscle areas of GX female mice were significantly larger than those of control females. The muscle area of GX+T female mice was significantly larger than that of controls and GX females. The muscle area of GX+OE female mice was significantly smaller than that of controls and GX females.

Expression of AR and OER in AcM

Positive staining for both AR and OER was found in seminal vesicle and uterus respectively, and staining was

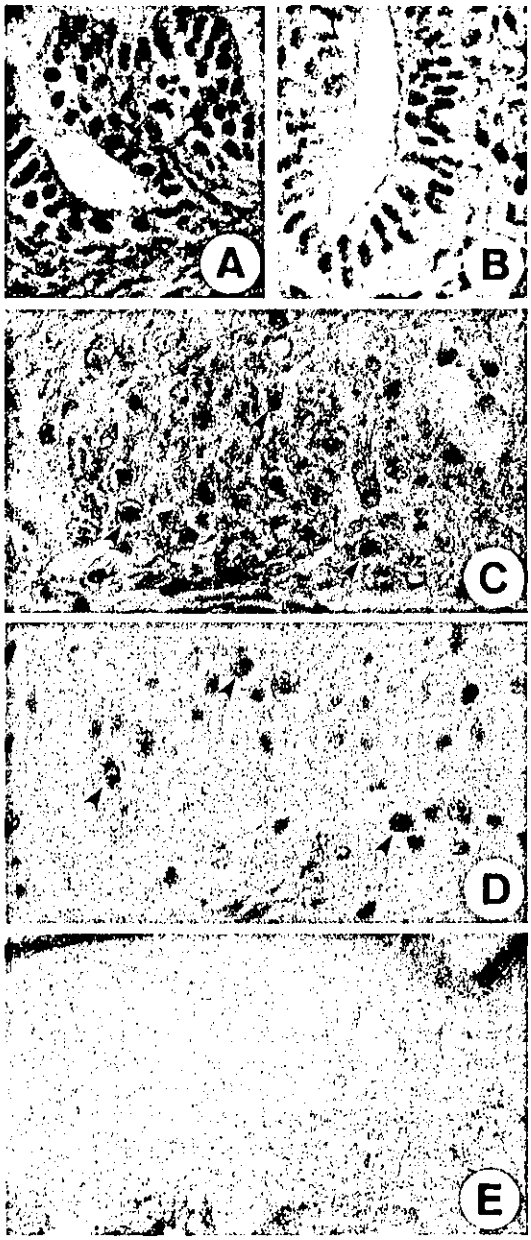

Figure 5 Immunohistochemical detection of (A and C) AR and (B and D) OER in the (A) seminal vesicle, (B) uterus and (C and D) AcM in 15-day-old ICR/Jcl mice. (E) For negative control, non-immunized rat serum was added to reaction mixture instead of the primary antibody. Positive AR and OER staining is found in the muscle nuclei (arrow heads), as occurs in epithelial nuclei of (A) seminal vesicle and (B) uterus respectively (× 500).

intense in the nucleus of both epithelial and stromal cells (Fig. 5). Relatively high background staining was observed in AR staining compared with that in OER staining. The staining intensity for AR immunostaining in the nucleus of epithelial cells of the seminal vesicle was 97·2 ± 2·7 ADU and the intensity for OER in the uterine epithelium was 92·3 ± 5·6 ADU in 15-day-old ICR mice.

In the AcM, although positive staining for both AR and OER was found in the muscle cell nuclei in 15-day-old ICR male mice (Fig. 5), the intensities for both AR and OER throughout the present study were lower than in seminal vesicle and uterus respectively (Fig. 6). The staining intensity for both AR and OER in AcM was stronger in young animals in both sexes of ICR and C57BL. In ICR mice, however, positive staining for AR and OER could not be detected at 60–120 and at 90–120 days of age in either sex respectively. In male C57BL mice, the AcM showed positive staining for both AR and OER throughout the experimental periods (15–120 days). In females, OER expression was observed throughout the experimental period; however, AR expression at 90 and 120 days of age in the muscle was not detected in one-third and two-thirds of mice tested respectively (Fig. 6).

Effects of neonatal exposure of AcM to DES

In 60-day-old male mice, both actual and relative areas of AcM were significantly smaller in neonatally DES-exposed males than those of controls (Fig. 7). However, the muscle area of GX DES-exposed male mice was significantly larger than that of intact DES-exposed males. In contrast, muscle area of DES-exposed female mice was significantly larger than that of the controls, and the muscle area of GX DES-exposed females was not different from that of intact DES-exposed females.

Discussion

Sexual dimorphism in four perineal neuromuscular systems has been reported (Breedlove & Arnold 1981, 1983*c*, Jordan *et al.* 1982, Leslie *et al.* 1991, Maeda *et al.* 1993). The development of the SNB innervating the levator ani and bulbocavernosus muscles in the rat, which exhibits sexual dimorphism in the number of motoneurones, was reported to be controlled by perinatal testosterone; testosterone reduces SNB motoneurone death through a ciliary neurotrophic factor (Forger *et al.* 1992, Breedlove & Arnold 1983*a*,*b*). The rat levator ani muscle, a striated muscle of the perineal muscle complex, shows sexual dimorphism prenatally (22-day-old embryo), and testosterone increased its weight and cross-sectional area by increasing the number of muscle units (Tobin & Joubert 1991, Rand & Breedlove 1992). Jordan *et al.* (1982) found sexual dimorphism in the dorsolateral nucleus in the ventral horn of the rat lumbar cord; perinatal testosterone was involved in its development. Not only in neuromuscular systems which relate to sexual function mentioned above, but also in systems which are not involved in sexual function, sexual dimorphism and androgen

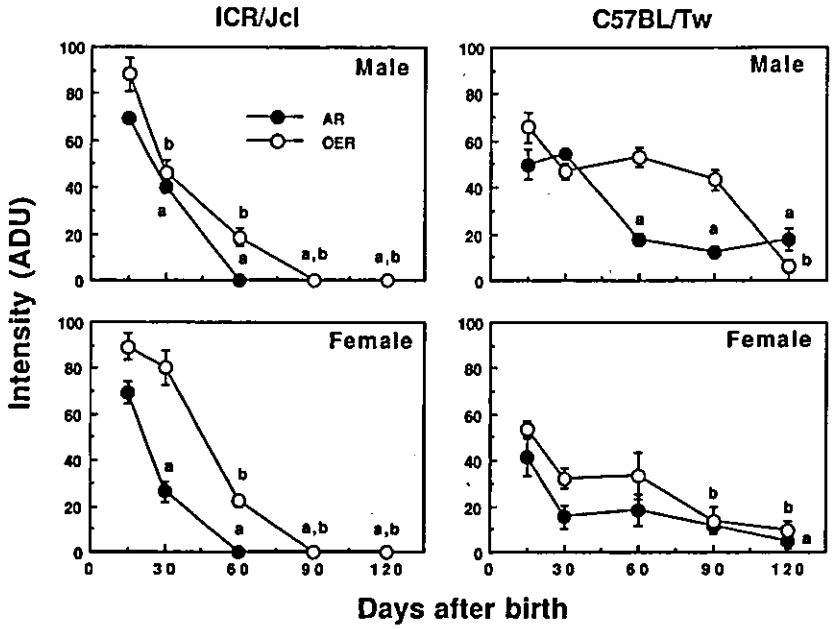

Figure 6 Developmental changes in the immunostaining intensity in mouse AcM. The stain intensity was measured with a Color Image Analyzer as ADU for each specimen and normalized by deducting ADU of negative control from each value. a and b, $P<0{\cdot}05$ compared with immunostaining intensity in 15-day-old mice for AR and OER respectively.

participation in development occur, as in the flexor digitorum brevis-retrodorsolateral nucleus (Leslie *et al.* 1991) and in the masseter muscle (Maeda *et al.* 1993).

Studies on the effects of androgenic hormones on striated muscles were initiated many years ago (Papanicolaou & Falk 1938, Wainman & Shipounoff 1941). These early studies indicated that androgens had pronounced effects on the growth of the striated perineal musculature in the rat, resulting in sexual dimorphism. In the present study, sexual dimorphism of the smooth AcM was demonstrated in C57BL, ICR and BALB/c mice at 90 days of age. Male AcM is larger than that of females in the three strains, as it is in the rat (Gibson & Gillespie 1973). The AcM of ICR mice showed an increase in cross-sectional area during development until 90 days after birth, but sexual dimorphism was already evident by 30 days of age; thus, sexual dimorphism of this muscle develops in the perinatal period.

Takeda *et al.* (1990), using mono- and polyclonal antibodies raised against AR, localized immunohistochemically AR in rat skeletal, cardiac and smooth muscles, as well as in all male sexual organs. In the present study, the AcM of both male and female ICR mice expressed AR at least until 30 days of age, and neonatal testosterone treatment significantly increased the AcM areas in both sexes. Androgens are secreted from both prenatal and postnatal testes (Motelica-Heino *et al.* 1988), suggesting that testicular androgen (mainly testosterone) during perinatal life may play an important role in the development of sexual dimorphism of the AcM as well as of other muscles. Implantation of testosterone on the day of gonadectomy resulted in an increase in the weight of the gubernaculum testis (control castrated mice, $5 \pm 0{\cdot}5$ vs testosterone pellet-implanted castrated mice, $14 \pm 0{\cdot}5$ mg/20 g body weight (B.W.), $P<0{\cdot}001$; for comparison, intact male mice $19 \pm 0{\cdot}7$ mg/20 g B.W.) and in the AcM cross-sectional area in both male and female mice, suggesting that testicular androgens act through AR to promote growth and thus result in sexual dimorphism of AcM in male mice.

On the other hand, neonatal exposure to OE caused significantly reduced AcM area in both sexes at 30 days of age. Ovariectomy at 30 days of age induced increased AcM area in females at 60 days of age, and resulted in the expected decrease in uterine and vaginal weights from $77 \pm 2{\cdot}4$ and $61 \pm 1{\cdot}8$ mg/20 g B.W. to $9{\cdot}0 \pm 0{\cdot}6$ and $14 \pm 0{\cdot}6$ mg respectively. Implantation of an OE pellet on the day of ovariectomy prevented the decrease in uterine and vaginal weights and stimulated their growth to $106 \pm 1{\cdot}3$ and $114 \pm 1{\cdot}9$ mg, but significantly reduced mean AcM area compared with both control and OVX mice. In male mice, OE treatment also decreased AcM area. OER was expressed until 60 days of age in both sexes, suggesting that OE acts as a suppressing factor during AcM development. Even though the doses used in the present study were large and the results obtained might be in part pharmacological (see Iguchi 1992), the findings suggest that both androgen and oestrogen play important

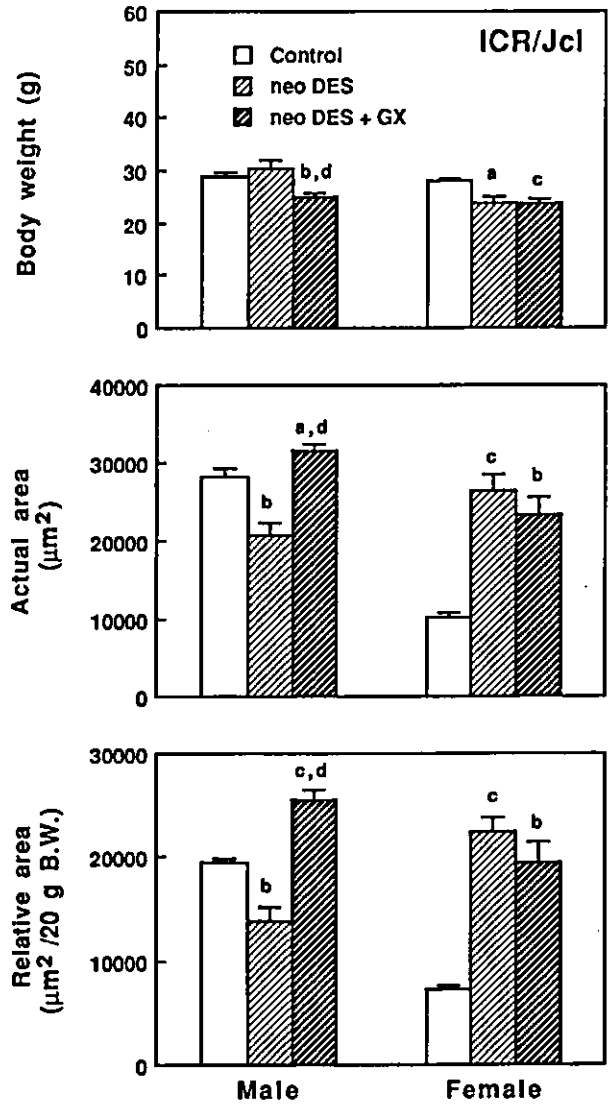

Figure 7 Effects of neonatal exposure to DES on body weight, actual cross-sectional area and relative area of AcM in 60-day-old ICR/Jcl mice. Neo DES=neonatally DES-exposed mice. a, $P<0.05$; b, $P<0.01$; c, $P<0.001$ vs control mice: d, $P<0.001$ vs neonatally DES-exposed mice.

roles in inducing the sexual dimorphism of the AcM, and that the muscle is under the control of both androgen and oestrogen at least until puberty.

Strain difference in the expression time of AR and OER in AcM was observed. Both receptors disappeared by 60 days and 90 days respectively in ICR mice; however, they were present in the AcM of C57BL mice at 120 days. This longer expression of both receptors may be responsible for the greater sexual dimorphism in AcM cross-sectional area in C57BL than in ICR mice.

Neonatal exposure to DES can induce irreversible changes in reproductive tracts of male and female mice (see Arai *et al.* 1983, Iguchi 1992); in the present study, it reduced the AcM cross-sectional area of 60-day-old male mice, but increased the area significantly in female mice at the same age. The reason for the opposite effects of DES on male and female mice is unknown. In male mice, the weight of the gubernaculum testis (19 ± 0·7 mg/20 g B.W.) did not change as a result of neonatal exposure to DES (19 ± 0·9 mg/20 g B.W.), suggesting that serum androgen levels in neonatally DES-exposed mice may not be different from those in controls. Thus, decrease in the male AcM area may result directly from neonatal exposure to DES. The decreased AcM area in neonatally DES-exposed male mice at 60 days of age was not seen after castration at 30 days of age, indicating that the effect of neonatal DES exposure is reversible in male mice. Stromal and myometrial abnormalities occur in the uterus of neonatally DES-exposed mice (Ostrander *et al.* 1985, Iguchi & Takasugi 1987, Brody & Cunha 1989). The AcM area of neonatally DES-exposed females was significantly larger than that of controls, and ovariectomy at 30 days of age did not affect the cross-sectional area of the muscle, suggesting that the increase in AcM by neonatal DES exposure occurred before 30 days of age and that, once induced, it was not reversed by steroid hormone withdrawal.

The induction of an irreversible hypertrophy of the AcM by DES but not by OE is a unique and surprising finding for which we have no ready explanation. A neonatal dose of DES is generally 200 times more potent than that of OE (0·1 μg DES/day and 20 μg OE/day have equivalent effects) in induction of uterine stromal and myometrial abnormalities (Iguchi *et al.* 1986) and of polyovular follicles (Iguchi 1985), and in alteration of oestrogen and progesterone receptor levels in vagina and uterus (Shyamala *et al.* 1974, Bern *et al.* 1987). Cervicovaginal abnormalities and ovary-independent vaginal cornification are induced by 0·005 μg DES/day (Bern *et al.* 1987) and 0·01 μg DES/day (Iguchi *et al.* 1986, 1988) respectively, whereas both alterations are induced by 20 μg OE/day (see Takasugi 1976). In the present study, neonatal treatment with DES (3 μg/day) increased AcM area in females but OE (30 μg/day) did not, suggesting that as much as 600 μg OE/day might be needed to induce persistent enlargement of the AcM.

In general, our results indicate that both androgen and oestrogen play an important role in sexual dimorphism of the mouse AcM, a smooth muscle, and that neonatal exposure to DES has an irreversible stimulatory effect on the AcM of female mice, whereas similar exposure to OE does not.

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Existence of a Threshold for Induction of Aberrant Crypt Foci in the Rat Colon with Low Doses of 2-Amino-1-methyl-6-phenolimidazo[4,5-*b*]pyridine

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Until recently it has been generally considered that genotoxic carcinogens have no threshold in exerting their potential for cancer induction. However, the nonthreshold theory can be challenged with regard to assessment of cancer risk to humans. In the present study we show that a food derived, genotoxic hepatocarcinogen, 2-amino-1-methyl-6-phenolimidazo[4,5-*b*]pyridine (PhIP), does not induce aberrant crypt foci (ACF) as preneoplastic lesions at low dose (below 50 ppm) or 8-hydroxy-2′-deoxyguanosine (below 400 ppm) in the rat colon. Moreover PhIP-DNA adducts were not formed at the lowest dose (below 0.01 ppm). Thus, the dose required to initiate ACF is approximately 5000 times higher than that needed for adduct formation. The results imply a no-observed effect level (existence of a threshold) for colon carcinogenesis by a genotoxic carcinogen.

Key Words: **PhIP; risk assessment; carcinogenicity threshold; PhIP carcinogenicity.**

The possible existence of a dose threshold for chemical carcinogenicity is of great importance in the regulatory science field. It has been generally considered that genotoxic carcinogens have no threshold in exerting carcinogenic potential (Preussmann, 1980; Tomatis *et al.*, 1997), because classically carcinogens are mutagenic, interacting with DNA to produce irreversible genetic changes in target organ cells. This is based on acceptance of a linear curve down to zero at low doses for risk assessment of exposure to humans with chemicals found to be carcinogenic in animal studies. While there are only limited data available for estimation of cancer risk assessment in humans exposed to genotoxic carcinogens (Gaylor, 1979; Littlefield *et al.*, 1979; Peto *et al.*, 1991), it has been argued that the nonthreshold theory can be challenged. This is because life forms possess biological responses that can ameliorate genotoxic activity. It is very important to resolve this point from the viewpoint of cancer risk control and management, and recently we showed the existence of a threshold for hepatocarcinogenicity of both 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and diethylnitrosamine (DEN) in rats (Fukushima *et al.*, 2002, 2003). In addition, Williams *et al.* (1998, 2000) and Yoshino *et al.* (2002) also recently provided evidence of a threshold for 2-acetylaminofluorene and diethylnitrosamine hepatocarcinogenicity.

There are many genotoxic carcinogens occurring naturally in our environment, including the large group of heterocyclic amine mutagens (Sugimura *et al.*, 1995, 2000). The human daily intake of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), one of these food-derived agents, is estimated to be 0.1–13.8 μg/person (Wakabayashi *et al.*, 1993). PhIP can be detected in the urine of healthy volunteers after eating cooked meat (Donald *et al.*, 1995; Ushiyama *et al.*, 1991; Wakabayashi *et al.*, 1993) and in rats, it causes DNA adduct formation in the colon (Fretland *et al.*, 2001; Kaderlik *et al.*, 1994) and treatment at high doses induces carcinomas in the colon, breast, and prostate (Hasegawa *et al.*, 1993; Ito *et al.*, 1991; Shirai *et al.*, 1997).

Recently *in vivo* medium-term bioassays for carcinogens have been accepted as possible alternatives to long term carcinogenicity tests (Ito *et al.*, 1988) and appropriate for assessment of low dose effects because of their high sensitivity. Aberrant crypt foci (ACF) are established preneoplastic markers in the colon of rats (Bird, 1987; Tudek *et al.*, 1989) and their ready detectability underlies their acceptance as end-point lesions to assess carcinogenic responses in medium-term bioassays. PhIP has been shown to induce ACF in a dose-related fashion over a range of doses (Nakagawa *et al.*, 2002; Tudek *et al.*, 1989). In the present study, for clarification of human risk assessment of

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genotoxic carcinogens, we examined low dose carcinogenicity of PhIP in the rat colon in detail using a medium-term bioassay, with the primary aim of determining whether the response curve is indeed linear near zero.

DNA adduct formation is considered to be an important factor in carcinogenesis with heterocyclic amines and PhIP-DNA adducts are formed in rat colon (Ochiai *et al.*, 1996). Generation of oxygen free radicals is also a key step in carcinogenesis, again induced by various heterocyclic amines (Maeda *et al.*, 1995). 8-Hydroxy-2′-deoxyguanosine (8-OHdG) is the most abundant species of adduct associated with oxidative stress, resulting in DNA damage and specific types of mutation (Kasai *et al.*, 1987). Therefore, levels of PhIP-DNA adducts and 8-OHdG were also examined in the present study to cast further light on mechanistic aspects of PhIP carcinogenicity at low doses in the colon.

MATERIALS AND METHODS

Animals and chemicals. A total of 1835 male five-week-old F344 rats were obtained from Charles River Japan, Inc. (Atsugi, Kanagawa, Japan), and housed in rooms maintained on a 12-h light/dark cycle, at constant temperature and humidity, and observed daily. PhIP (purity, 99.8%) was purchased from the Nard Institute, Nishinomiya, Japan.

Experimental procedures. The experiment was started when the animals were six weeks of age. They received PhIP at doses of 0 (group 1, a control), 0.001 (group 2), 0.01 (group 3), 0.1 (group 4), 1 (group 5), 10 (group 6), 50 (group 7), 100 (group 8), and 400 ppm (group 9) in powdered basal diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) for 16 weeks, continuously. The lowest level, 0.001 ppm of PhIP was established as equivalent to the daily intake of this carcinogen in humans (Wakabayashi *et al.*, 1993). Numbers of rats were 240 in group 1, 242 in group 2, 241 in group 3, 243 in group 4, 244 in group 5, 212 in group 6, 214 in group 7, 62 in group 8, and 61 in group 9. The rats were killed at the end of week 16 under ether anesthesia for examination of ACF (61 to 244 rats) in the colon. Additional rats in groups 1 to 9 were given diets containing PhIP and killed at week 4 for examination of PhIP-DNA adducts (three or four rats) and 8-OHdG (five rats each) in the colon. The animals were carefully observed during the course of the experiment, body weights, water intake, and food consumption were measured every week. Calculations to achieve precise mole-per-rat of total PhIP ingested in every initiated group was estimated.

ACF counts. Colons were quickly excised, flushed with saline, and inflated by intraluminal injection of 10% phosphate-buffered formalin solution, slit open along the longitudinal median axis from the cecum to anus, and fixed flat between two pieces of filter paper in 10% phosphate-buffered formalin. After fixation for at least 24 h at 4°C, the colons were all stained with 0.2% methylene blue (in H_2O) for 3–5 min, and then examined for ACF by light microscopy at 40× and 100× magnification using the following criteria for identification: (1) increased size as compared to normal crypts, (2) enlarged pericryptal zone, (3) slight elevation above the surrounding mucosa, and (4) frequently more elongated shape of the luminal opening.

PhIP-DNA adducts and 8-OHdG formation. The colons were excised and flushed in saline, and the mucosa was scraped off to obtain samples, which were frozen in liquid nitrogen and stored at −80°C until the levels of PhIP-DNA adducts in the colon were measured by the ^{32}p-postlabeling method as described previously (Uehara *et al.*, 1996). Measurement of 8-OHdG levels in colon DNA was performed with the method of Nakae *et al.* (1997).

Statistical analyses. Statistical analysis of our data was performed using the StatView-J 5.0 program (Abacus Concepts, Inc., Berkeley, CA). Differences from the control values were evaluated for significance by the Dunnet-test.

RESULTS

General Findings

All the rats survived in good condition until the scheduled sacrifices. No adverse effects on average body weight gain were observed in rats treated with PhIP except at 400 ppm. Final average body weights were significantly lower in group 9 (400 ppm PhIP-treated group) than those of group 1 (a control). There were no significant differences in liver and kidney weights among the group except group 9 (Table 1). Average total PhIP intake in each group was dose-dependent. No macroscopic lesions were apparent in any organs, including the colon.

Induction of ACF in the Colon

After 16 weeks treatment with PhIP at various doses in the diet, total numbers of ACF foci in the rat colon of groups

TABLE 1

Final Average Body Weights, Average Liver and Kidney Weights, and Average Total PhIP Intakes

Group	PhIP doses (ppm)	No. of rats	Final body weights	Relative organ weights (g)		Total PhIP intake (mg/rat)
				Liver	Kidney	
1	0	240	323.9 ± 21.9[a]	8.3 ± 1.5	1.9 ± 0.2	0
2	0.001	242	326.0 ± 24.0	8.4 ± 1.4	1.9 ± 0.2	0.0015
3	0.01	241	325.9 ± 19.2	8.4 ± 1.2	1.9 ± 0.2	0.0145
4	0.1	243	326.7 ± 21.3	8.5 ± 1.3	1.9 ± 0.2	0.1505
5	1	244	325.6 ± 20.5	8.2 ± 1.2	1.9 ± 0.2	1.5117
6	10	212	323.2 ± 20.1	8.4 ± 1.3	2.0 ± 0.2	15.0680
7	50	214	321.5 ± 28.3	8.2 ± 1.3	2.0 ± 0.5	77.6894
8	100	62	318.6 ± 18.1	8.5 ± 1.1	2.0 ± 0.2	153.725
9	400	61	235.8 ± 21.8*	6.5 ± 1.2*	1.5 ± 0.5*	581.872

[a]Values are mean ± SD.
*$p < 0.05$ (vs. Group 1).

TABLE 2

The Occurrence of Aberrant Crypt Foci (ACF) in the Colons of Rats Treated with PhIP at Various Doses for 16 Weeks

Group	PhIP dose (ppm)	No. of rats	No. of ACF comprising				
			1	2	3	4	Total
1	0	240	0.1 ± 0.4[a]	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.2	0.3 ± 0.7
2	0.001	242	0.1 ± 0.3	0.2 ± 0.5	0.1 ± 0.3	0.1 ± 0.2	0.4 ± 0.7
3	0.01	241	0.1 ± 0.4	0.1 ± 0.4	0.1 ± 0.3	0.1 ± 0.3	0.5 ± 0.8
4	0.1	243	0.1 ± 0.4	0.1 ± 0.3	0.1 ± 0.4	0.1 ± 0.4	0.4 ± 0.8
5	1	244	0.2 ± 0.4	0.2 ± 0.5	0.1 ± 0.3	0.1 ± 0.3	0.5 ± 0.9
6	10	212	0.1 ± 0.3	0.1 ± 0.4	0.1 ± 0.4	0.1 ± 0.3	0.4 ± 0.8
7	50	214	0.2 ± 0.4*	0.2 ± 0.4*	0.2 ± 0.4	0.1 ± 0.3	0.6 ± 1.0*
8	100	62	0.6 ± 0.9*	0.4 ± 0.7**	0.3 ± 0.6**	0.2 ± 0.5**	1.5 ± 1.4**
9	400	61	2.7 ± 2.1*	1.2 ± 1.3*	0.6 ± 0.8**	0.4 ± 0.8**	5.0 ± 2.8**

[a]Values are mean ± SD.
*$p < 0.05$; **$p < 0.01$ (vs. Group 1).

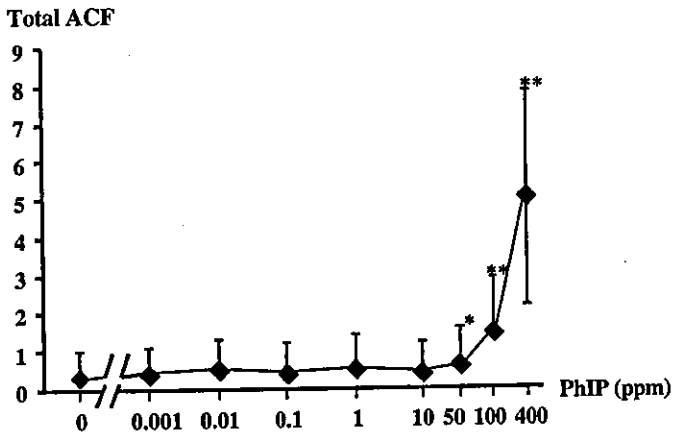

FIG. 1. Induction of aberrant crypt foci in the colons of rats treated with PhIP at various doses for 16 weeks. Significant differences from the 0 ppm group at *$p < 0.05$ or **$p < 0.01$. Bars, SD.

receiving 0.001–10 ppm of the carcinogen did not differ from the control value (Table 2 and Figure 1), in contrast to the dose-dependent significant increase observed with 50 ppm and above. Numbers of ACF comprising one and two crypts in the groups given 0.001–10 ppm PhIP were also not different from the control values, while those with 50 ppm PhIP and over were significantly increased (Table 2). Numbers of ACF with three crypts and > four crypts were significantly increased only with PhIP at doses of 100 and 400 ppm.

Formation of PhIP-DNA Adducts and 8-OHdG

At week 4, there was a linear relationship between the various doses (0.01–400 ppm) of PhIP and the levels of PhIP-DNA adducts at 0.01 ppm and above (Table 3 and Figure 2). However, no significant increase was evident at 0.001 ppm dose.

TABLE 3

Formation Values of PhIP-DNA Adducts and 8-OHdG in the Colons of Rats Treated with PhIP at Various Doses for 16 Weeks

Group	PhIP dose (ppm)	No. of Rats	PhIP-DNA adducts (adducts/10^8 ntd)	8-OHdG (8-OHdG/10^5 dG)
1	0	240	0.02 ± 0.008[a]	1.51 ± 0.85
2	0.001	242	0.02 ± 0.007	1.04 ± 0.30
3	0.01	241	0.08 ± 0.012*	1.56 ± 0.40
4	0.1	243	0.11 ± 0.054*	1.57 ± 0.77
5	1	244	0.16 ± 0.039*	1.46 ± 0.27
6	10	212	0.25 ± 0.056*	1.18 ± 0.57
7	50	214	1.63 ± 0.594*	1.17 ± 1.00
8	100	62	4.48 ± 0.805*	3.00 ± 1.58
9	400	61	18.7 ± 3.664*	3.26 ± 1.83**

Note. ntd, nucleotides; dG, deoxyguanosine.
[a]Values are means ± SD.
*$p < 0.05$; **$p < 0.01$ (vs. Group 1).

Concerning the 8-OHdG levels in the colon DNA at week 4, no significant differences were apparent among the groups receiving PhIP from 0.001–100 ppm and the control group and only 400 ppm PhIP caused a significant increase (Table 3 and Figure 3).

DISCUSSION

The present results clearly indicate that the curve for induction of ACF in the rat colon by PhIP is not linear down to zero. Similarly, no-response levels were evident for both PhIP-DNA adducts and 8-OHdG formation, indicating that there are threshold for carcinogenesis-related parameters with PhIP colon carcinogenicity.

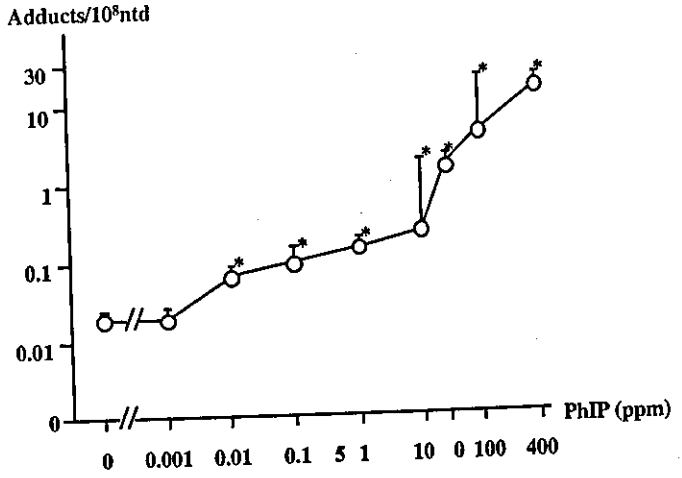

FIG. 2. PhIP-DNA adducts in the colons of rats fed diets containing PhIP for four weeks. Significant difference from the 0 ppm group at $*p < 0.01$. Bars, SD. Values in the figure are shown in logarithmic scale.

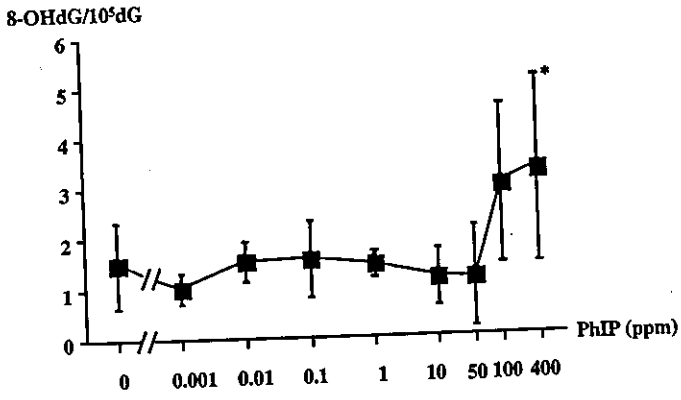

FIG. 3. The 8-OHdG formation levels in the colons of rats treated with PhIP for four weeks. Significant difference from 0 ppm group at $*p < 0.05$. Bars, SD.

Recently we found that the hepatocarcinogens, MeIQx and DEN, do not induce the preneoplastic lesions, glutathione-S-tranceferase placented form (GST-P) positive foci, in rat liver at very low doses (Fukushima *et al.*, 2002, 2003). Morever, MeIQx-DNA adducts and particularly 8-OHdG levels demonstrated no-observed effect levels. Our findings thus indicated the existence of a threshold for carcinogenicity with genotoxic agents.

The present results for ACF, DNA-adducts, and 8-OHdG in the colons of rats treated with PhIP at various doses point to the same conclusion (Fig. 4). On the other hand, it is noteworthy to mention that the no-response level for adduct formation (below 0.01 ppm; about 21 adducts/cell), and for induction of ACF (below 50 ppm; about 60 adducts/cell), supports the notion that rather large threshold number of adducts must be exceeded in order to induce formation of ACF. i.e., here, the dose required

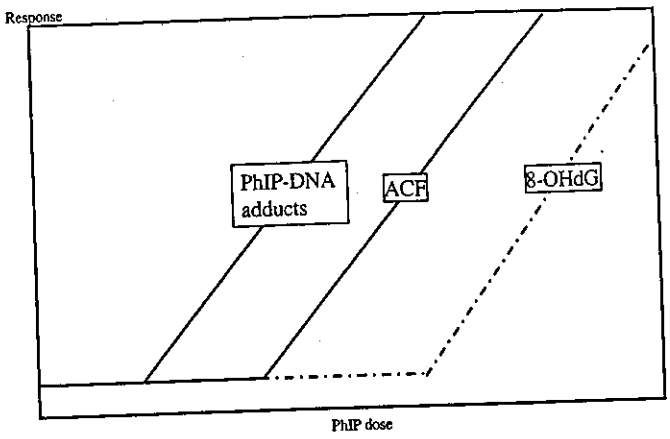

FIG. 4. Summarized relationships among carcinogenesis-related biomarkers in the colon of rats treated with PhIP.

to initiate ACF is approximately 5000 times higher than that for adduct formation. Maeda *et al.* (1995) reported that carcinogenic heterocyclic amines generate oxyradicals at differing levels, MeIQx giving the highest values as judged by electron-spin resonance (ESR) spin trapping and PhIP being much less active in this regard. Two different metabolic pathways have been indicated for activation of PhIP. One is the hepatic pathway, involving N-hydroxylation by CYP1A2 and O-acetylation by N-acetyltransferase-2 and the other is extrahepatic, rendering free-radical metabolites. On ESR examination, generation of free radicals was greater with 2-amino-3-methylimidazo[4,5-*f*] quinoline (IQ) than PhIP although DNA analysis showed adduct formation to be similar with the two carcinogens (Moonen *et al.*, 2002). From this evidence, participation of 8-OHdG to colon carcinogenesis due to PhIP may not be of direct importance, although this parameter also demonstrated a threshold in the present study.

Biological adaptive responses, resulting in physiological protection of cells against toxic agents, have recently become accepted for radiation carcinogenesis at low dose (Wollff *et al.*, 1998). This concept might also be useful for understanding low dose effects of chemical carcinogenesis, since adaptation might be expected to occur in response to low doses of all types of DNA damaging agents (Kleczkowska and Althaus, 1996; Olivieri *et al.*, 1984). It has been reported that extremely low doses of chemical carcinogens actually decrease the degree of DNA damage in treated animals, although the authors of the article in question hesitated to draw firm conclusions (Kitchin and Brown, 1994). In addition, the importance of toxicokinetics of chemicals for carcinogenicity has recently been stressed. Absorption of carcinogens into the body, distribution to target organs, metabolism to active ultimate forms which react with DNA, induction of detoxifying enzymes, formation of polar metabolites and excretion, all influence DNA damage. Moreover, various factors such as stimulation of the immune response, induction of different detoxification and repair enzymes, and upregulation of

tumor suppressor genes could result in beneficial effects with low dose exposure to carcinogens.

In conclusion, a threshold may exist for the colon carcinogenic potential of PhIP, and by analogy, probably also for other colon-genotoxic carcinogens. Recently Waddell (2003) stressed the existence of a threshold for DEN carcinogenicity in the lever and esophagus of rodent in his review article. Williams *et al.* (2000) postulated that mechanisms differ between low and high exposures, and reflect thresholds for hepatocellular initiating effects by low dose genotoxic carcinogens. Previously we provided evidence that genotoxic hepatocarcinogens may exhibit a threshold (Fukushima *et al.*, 2002). The present findings provide a new basis for extrapolation from animal carcinogenicity data to human risk assessment.

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WT1 and DAX-1 Inhibit Aromatase P450 Expression in Human Endometrial and Endometriotic Stromal Cells

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The orphan nuclear receptor steroidogenic factor-1 (SF-1) induces the expression of Müllerian inhibiting substance (*MIS*) and many steroidogenic genes, including aromatase P450 (P450arom). Dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome gene 1 (DAX-1) inhibits SF-1-mediated induction of *MIS* and other steroidogenic genes, whereas Wilms' tumor suppressor gene (WT1) augments SF-1-mediated *MIS* expression. The effects of WT1 on steroidogenesis or P450arom expression have not been explored to date. In human endometriotic stromal cells, extremely high levels of P450arom mRNA and enzyme activity are present. Prostaglandin E_2 stimulates cAMP formation, SF-1 binding activity, P450arom mRNA levels, and estrogen synthesis in endometriotic stromal cells. Stromal cells of eutopic endometrium from disease-free women, on other hand, do not contain readily detectable levels of P450arom mRNA. Thus, we evaluated herein the possible roles of WT1 and DAX-1 in cAMP/SF-1-mediated regulation of P450arom expression in endometriotic and endometrial stromal cells. We also determined the cellular distribution and levels of these transcription factors in pathological endometriotic *vs.* normal eutopic endometrial tissues by immunohistochemistry to understand their *in vivo* roles. *In vitro* transcriptional regulation studies showed that both WT1 and DAX-1 inhibited cAMP and/or SF-1-induced P450arom promoter activity in a dose-dependent fashion in cultured human endometriotic and endometrial stromal cells. Site-directed disruption of the SF-1 binding site (−136/−124 bp) in the P450arom promoter abolished basal or cAMP/SF-1-induced promoter activity in the presence or absence of WT1 or DAX-1. Immunohistochemistry and H-scoring showed that DAX-1 was ubiquitously present in epithelial and stromal cells of both tissues. WT1, on the other hand, was preferentially expressed in stromal (*vs.* epithelial) cells. Moreover, WT1 levels in endometriotic stromal cells are significantly down-regulated compared with normal endometrial stromal cells. In summary, WT1 or DAX-1 inhibits cAMP-SF-1 pathway-dependent P450arom expression in cultured human endometriotic and endometrial stromal cells. *In vivo* down-regulation of WT1 in endometriotic stromal cells (*vs.* normal endometrial stromal cells) may in part be responsible for aberrantly increased P450arom expression and estrogen formation in this pathological tissue. (*J Clin Endocrinol Metab* 87: 4369–4377, 2002)

ENDOMETRIOSIS IS A CHRONIC disease that is manifest by pelvic pain and infertility and defined as the presence of endometrial glands and stroma within the pelvic peritoneum and other extrauterine sites. It is estimated to affect 2–10% of women in the reproductive age group (1, 2). Endometriosis is viewed to be a polygenically inherited disease of complex multifactorial etiology (3). Implantation of eutopic (intrauterine) endometrium on peritoneal surfaces in the abdomen via retrograde menstruation through the uterine tubes has been proposed to be the most common mechanism responsible for this disease.

Both circumstantial and laboratory evidence indicate a proliferative role played by estrogen in the establishment and maintenance of endometriosis (4). Aromatase P450 (P450arom) is the rate-limiting enzyme for the synthesis of estrogen and catalyzes the conversion of C_{19} steroids to estrogens. This conversion takes place in a number of human tissues such as the ovary, placenta, adipose tissue, skin, and the brain (5). We previously demonstrated significantly higher levels of P450arom mRNA and activity in the stromal cell component of endometriotic tissues compared with the eutopic endometrium in which P450arom mRNA or enzyme activity was either undetectable or barely detectable (6, 7). We have also shown that aromatase activity and P450arom mRNA levels can be induced by cAMP analogs or prostaglandin (PG) E_2 in endometriotic stromal cells to levels comparable to those found in ovarian granulosa cells or the placental syncytiotrophoblast but not in eutopic endometrial stromal cells (7). The clinical significance of this local induction of aromatase activity in endometriotic tissue was exemplified recently by the successful use of an aromatase inhibitor to treat an unusually aggressive and resistant case of recurrent postmenopausal endometriosis (8). Therefore, aberrant P450arom expression in endometriotic tissue, in contrast to eutopic endometrium, accounts for local biosynthesis of estrogen that promotes the growth of these lesions and possibly mediates the resistance to conventional hormonal treatments observed in a number of women with endometriosis.

We reported previously that the high levels of baseline and cAMP-induced aromatase activity were partially mediated

Abbreviations: Bt_2cAMP, Dibutyryl cAMP; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; CRE, cAMP response element; DAX-1, dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome gene 1; FBS, fetal bovine serum; KTS, Lys-Thr-Ser; *MIS*, Müllerian inhibiting substance; NRHS, nuclear receptor half site; P450arom, aromatase P450; PG, prostaglandin; SF-1, steroidogenic factor-1; WT1, Wilms' tumor suppressor gene.

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