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SHORT COMMUNICATION

An improved technique for repeated gavage administration to rat neonates

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ABSTRACT **The technique for gavage administration to rat nurslings was improved to allow determination of the direct effects of chemical substances in the nurslings. Rat neonates were treated with distilled water from postnatal day 1 through 20 using this technique. The viability of neonates during the administration period was comparable to that of untreated neonates. No adverse effects of this technique on the development of neonates were found, and no histological alterations of the esophagus or pharynx. Therefore, we conclude that use of our improved gavage administration method will contribute to ensuring successful neonatal development and thus allowing accurate assessment of the toxicological effects of test compounds on rat nurslings.**

Key words: gavage administration, rat neonates, histological changes, development

INTRODUCTION

In toxicological studies, a test substance is commonly administered to mature rats or mice to evaluate its adverse effects or to determine the no-observed adverse effect dose level (NOAEL). In particular, gavage administration is used in mature animals or immature animals after weaning for the above purpose. Hitherto, the toxicological effects of test substance on rat neonates have been evaluated via the milk of dams treated with the test substance during nursing. However, it is important to estimate the direct effects of test substances on nurslings in toxicological studies, using rodents in order to draw inferences about the effects on human infants. We reported the direct effects in male and female rats from neonatal exposure to environmental chemicals (Nagao *et al.*, 1999, 2000, 2001a; Kuwagata *et al.*, 2001). In addition, we demonstrated that direct neonatal exposure to phytoestrogen, genistein caused dysfunction of postpubertal reproductive performance as well as abnormal development of gonads in female but not in male rats (Nagao *et al.*, 2001b).

Problems encountered with daily gavage administration to neonatal rats include mortality due to cannibalism or neglect by the dam, resultant retardation of development, and injury of the esophagus or pharynx by the gastric tube. We aimed to develop a method of gavage administration which would enable us to test the effects of chemical compounds administered repeatedly to rat neonates. In this report, improved techniques for repeated gavage administration in rat neonates are described.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (Crj:CD, IGS) were purchased from Charles River, Atsugi, Japan, at 12 weeks of age. The animals were acclimated to the laboratory for 1 week prior to the start of the experiments. Animals were housed individually in metal cages in a room with controlled temperature (24 ± 1°C) and humidity (50 ± 5%), with lights on from 07:00 to 19:00 daily. Rats were given access to food (CE-2, Clea Japan) and tap water ad libitum.

Estrous female rats at 13 weeks of age were placed together overnight with a single male. The next morning, females with sperm in their vaginal smears were regarded as pregnant, and this day was designated as day 0 of gestation. Once insemination was confirmed, the females were weighed. The dams were allowed to deliver naturally and nurse their pups until postnatal day (PND) 21 (PND 0: the day of birth). On PND 1, all pups were weighed and sexed and the litters were culled randomly to 12 (6 pups/sex/litter where possible). Litters of twelve pups or less were not reduced. The remaining pups were discarded and the nurslings were weighed daily.

Administration

Three male and female pups per litter were given daily gavage administration of distilled water from PND 1 through 20, and the remaining littermates (3 males and 3 females) were not given any treatment. The number of males and females that were treated

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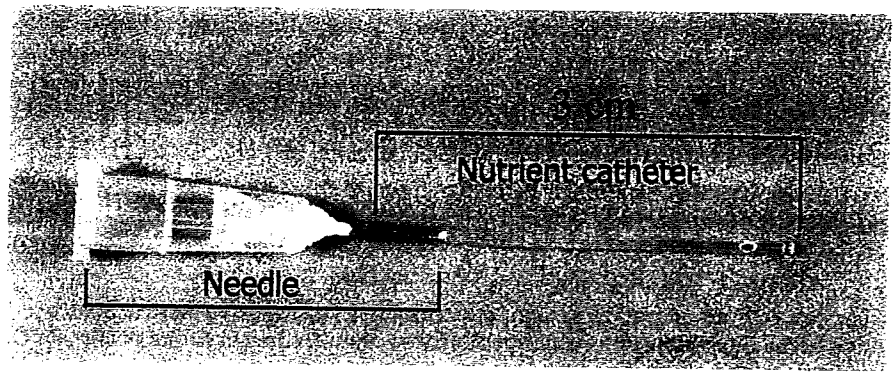

Fig. 1 Gastric tube with a needle for gavage administration to rat nurslings from PND 3 through 10.

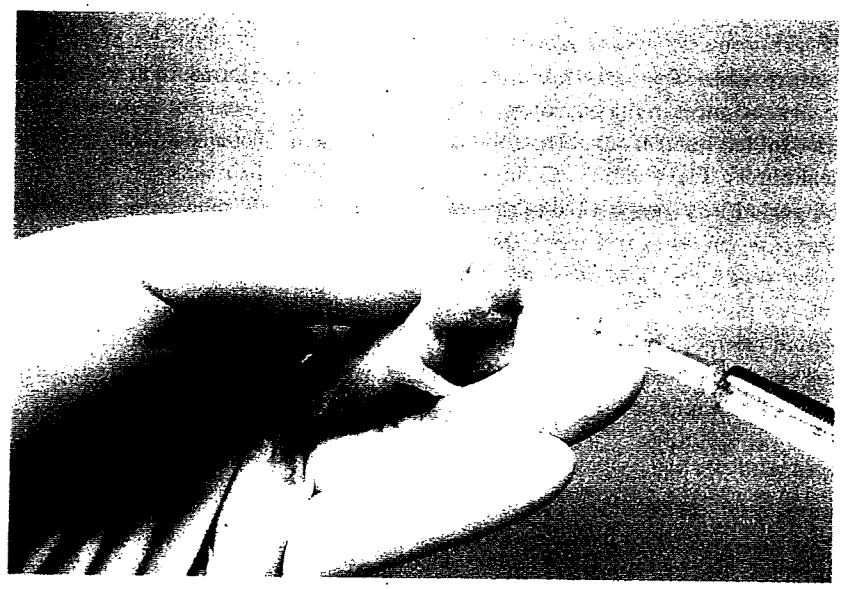

Fig. 2 Gavage administration to rat nursling on PND 1. The pup was held between the thumb and the 3rd finger.

with distilled water was 41 and 42, and the number of males and females that were not treated was 42 and 41, respectively (the total number of litters was 14).

We developed the following method for direct gavage administration to rat newborn pups on PND 1 through 20 in our laboratory. A needle (23G × 1", 0.65 × 25 mm, TERUMO Co., Tokyo) was cut off to a 10-mm length and the basal part of an indwelling feeding tube for infants (Nutrient catheter, Type 3Fr, Atom Medical Co., Tokyo) was attached to the needle (**Fig. 1**). This tube with the needle mounted to a microsyringe (Hamilton Gastight #1750, 500 μL, Hamilton Co., U.S.A.) or disposable syringe (1 mL, TERUMO Co., Tokyo) was used as a gastric tube. The length of the catheter attached to the needle was 2 cm for administration on PND 1 through 4, 3 cm for administration on PND 5 through 10, and 6 cm for administration on PND 11 and thereafter. The volume administered (10 μL/g body weight) was calculated based on the body weight measured daily. The rate of administration was approximately 10 μL/sec or less for administration on PND 1 through 7, 100 μL/sec or less for administration on PND 8 through 14, and 500 μL/sec or less for administration on PND 15 and thereafter. The pup was held between the thumb and the 3rd finger (**Fig. 2**) during the administration.

Observation

The numbers of live and dead pups were recorded for each litter on PND 1 through 21, and the viability from PND 1 to 21 was determined. Pups were observed daily for clinical signs and weighed daily. Developmental landmarks in the offspring were monitored on a daily basis for individual rats. All pups were examined for the development of neural reflexes (negative geotaxis and cliff-drop aversion) from PND 1 until the day of completion, for upper tooth eruption from PND 5 until the day of completion, and for eyelid opening from PND 10 until the day of completion as the endpoint of the physical milestones. Pups from each litter on PND 7 and 21 were weighed, anesthetized by ether and subjected to autopsy. The stomach was weighed. Subsequently,

the esophagus and pharynx were fixed in 0.1 M phosphate buffered 10% formalin solution and embedded in paraffin, sectioned at 4 μm, stained with hematoxylin and eosin (H&E), and examined histologically.

Animal care and use conformed to published guidelines (NIH, 1985).

RESULTS

Neonatal viability, body weight changes and developmental landmarks

No overt signs were apparent in any pups during the postnatal period including the administration period from PND 1 through 20. There were no significant differences in the number or viability of pups on PND 7 or 21 in the groups treated orally with distilled water as compared with those in the control (untreated) group. No significant differences were detected in the body weight of male pups between the treated and control groups throughout the study. In female pups, the body weights on PND 10, 11 and 14 in the treated group were significantly higher than those in the controls.

No significant differences in the PND on which the cliff-drop aversion response or righting reflex was completed were found between the treated and control groups in either sex. In addition, there were no significant differences in the PND on which teeth eruption, ear opening or eye opening was completed between the treated and control groups in either sex.

Organ weight and histological findings

There were no significant differences in body weight on PND 7 and 21 between the treated and control groups in either sex. In addition, no significant differences were detected in the absolute and relative weights of the stomachs of male and female pups treated with distilled water as compared with those of the controls.

There were no inflammatory changes such as hemorrhage and neutrophilia that were considered to be related to physical stimulation by the gavage administration in the esophagus and pharynx of neonates on PND 7 or 21.

DISCUSSION

In this report, an improved technique for repeated gavage administration from PND 1 in rat neonates was described. No adverse effects were observed on the growth or developmental landmarks of pups treated orally with distilled water. Significantly higher body weights of female pups were found in the treated group on PND 10, 11 and 14. However, this was a slight and transitory change, and not common to both sexes. In addition, the body weight gains from PND 1 through 21 were 44.6 g in male pups and 43.0 g in female pups of both groups. Thus, it is reasonable to consider that the higher body weights of female pups on PND 10, 11 and 14 were not related to the repeated gavage administration of distilled water. From these data, it would appear that our improved technique for repeated gavage administration does not affect the development of rat neonates, and will enable us to evaluate the toxicological effects of test substances administered to rat neonates.

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—Original—

Reduction of Primordial Follicles Caused by Maternal Treatment with Busulfan Promotes Endometrial Adenocarcinoma Development in Donryu Rats

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Abstract. Ovarian dysfunction leading to hormonal imbalance plays a crucial role in uterine carcinogenesis in rats as well as women. However, the effects of a reduction in primordial follicles at birth on uterine adenocarcinoma development have hitherto not been determined. The present study was therefore conducted using female Donryu rats, a high incidence rat strain of uterine adenocarcinoma. The animals were maternally exposed to 2.5 or 5.0 mg/kg of busulfan on gestation day 14 to reduce primordial follicles, and were then initiated by intrauterine treatment with *N-ethyl-N'-nitro-N- nitrosoguanidine* at 11 weeks of age. Both busulfan treatment doses caused earlier occurrence of persistent estrus, with dose-dependence as compared to controls. At 15 months of age, the rats were euthanized. The incidence of uterine adenocarcinomas and multiplicity of uterine neoplastic lesions were significantly increased by the 5.0 mg/kg, but not the 2.5 mg/kg busulfan treatment. Morphologically, the ovaries exposed to busulfan treatment exhibited severe atrophy, with few or no follicles and corpus lutea. Serum 17β-estradiol (E2), progesterone, and inhibin levels were significantly decreased in the busulfan treatment groups, with a clear dose-relation. Interestingly, only the 5.0 mg/kg busulfan treatment elevated the E2/progesterone ratio. These results provide evidence that the reduction of primordial follicles promotes uterine adenocarcinoma development in rats in association with an earlier occurrence of the persistent estrus status.

Key words: Busulfan, Rat, Reduction of follicles, Uterine cancer

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Ovarian hormones are crucial for uterine carcinogenesis in humans and rats, and atrophic ovaries characterized by atretic and/or cystic follicles and a lack of corpus lutea are known to be associated with an increased blood 17β-estradiol (E2)/progesterone ratio and a high risk for uterine as well as mammary cancer [1, 2]. Many aspects of the aging process in the ovary remain unclear, but changes in follicles presumably play a central role [1, 3, 4]. Studies have provided morphological evidence that a reduction in the number of primordial follicles accelerates follicle growth in intact mice and mice treated with ovotoxic agents [5], or rats that were unilaterally ovariectomized [6].

Treatment with busulfan, an alkylating agent, during the period of germ cell proliferation reduces the number of oogonia in rats [7], and consequently reduces the number of primordial follicles formed in the ovary [8]. Hirshfield found an inverse correlation between the number of primordial

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follicles in the ovary and the rate at which they moved into the growing pool [8]. In addition, Shirota *et al.* [9] have demonstrated that a reduction in the number of primordial follicles resulting in decrease in the number of follicles entering the growing phase, a major source of circulating inhibin in the neonatal and infantile ovary. A consequent elevation in circulating FSH may accelerate follicular development and cause early puberty in rats treated with busulfan maternally. However, it is not known how this impacts on aging in the uterus and uterine adenocarcinoma development.

Uterine cancers in the corpus, the majority being endometrial adenocarcinomas, are relatively prevalent in developed countries and constitute a leading cause of cancer deaths [1]. In rats, naturally occurring endometrial adenocarcinomas are rare, but Maekawa *et al.* found a high incidence of such lesions with morphological and biological similarities to human tumors in aged animals of the Donryu strain [10]. They further demonstrated that endometrial adenocarcinoma development was remarkably linked to an age related ovarian hormonal imbalance that resulted in an increase in the serum estrogen/progesterone ratio [11, 12]. Using this rat strain, they have also established a 2-stage uterine carcinogenesis model to detect promoting or preventive effects of test-chemicals [13, 14].

The present study was conducted to clarify the effects of a reduction of primordial follicles on uterine carcinogenesis in rats maternally treated with busulfan. Busulfan was used as an agent since this chemical has been reported to reduce the number of oogonia during the period of germ cell proliferation and to consequently reduce the number of primordial follicles formed in the ovary in rats [8, 9]. In addition, the endocrinological status of the treated rats was analyzed.

Materials and Methods

Animals and housing conditions

Thirty pregnant female Crj:Donryu rats were purchased from Charles River Japan (Kanagawa, Japan) on gestation day 2. The animals were maintained in an air-conditioned animal room under constant conditions of 24 ± 2 C and 55 ± 10% humidity with a 12-h light/dark cycle (light, 8:00–20:00; dark, 20:00–8:00), and housed individually in cages until weaning. Offspring were also maintained in the same conditions and housed 3 or 4 to a cage. A commercial pellet diet (CRF-1, Oriental Yeast, Kanagawa, Japan) and drinking tap water were available *ad libitum* for dams and offspring. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

Chemicals and selection of a busulfan dosage

Busulfan (Sigma, St Lous, MO) was weighed to give doses of 2.5 and 5.0 mg/kg body weight of dams, suspended in a small amount of corn oil (Wako Pure Chemical, Osaka, Japan) and the concentration was adjusted for use at a constant volume of 5 ml/kg body weight of the dams.

Treatment and maintenance of animals

2.5 and 5.0 mg/kg of busulfan were administered intraperitoneally (ip) to 10 pregnant females per group on day 14 of gestation, and the females were then allowed to deliver spontaneously. The size of each litter was standardized to ten on day 4 after birth, and offspring were weaned on day 21 after birth. Control offspring were maternally treated with the corn oil vehicle on day 14 of gestation in the same manner. The numbers of offspring obtained were 27, 24, and 24 females for the controls, 2.5, and 5 mg/kg busulfan treated groups, respectively.

Uterine carcinogenesis and histopathological examination

For initiation, the female offspring were treated with a single dose of 20 mg/kg *N-ethyl-N'-nitro-N-nitrosoguanidine* (ENNG, Nacalai Tesque, Kyoto, Japan) into one of the uterine horns via the vagina using a stainless steel catheter at 11 weeks of age. This initiation is known to exert no carcinogenic effects in other organs [13]. At 15 months of age, all surviving animals were decapitated and necropsied, and organs and tissues of reproductive and endocrine systems, including the uterus, ovaries, adrenals, liver, kidneys, brain, and spleen were weighed and fixed in 10% neutral buffered formaldehyde solution. These tissues and the pituitary, thymus, mammary gland, brain, vagina, bone with bone marrow and macroscopic abnormalities were fixed and routinely processed for histopathological examination. The upper, middle and lower parts of each uterine horn and

the uterine cervix were cross-sectionally cut into 3 pieces to evaluate uterine neoplastic lesions, and classified into three degrees of atypical hyperplasia (slight, moderate, or severe) and adenocarcinomas, according to criteria described previously [11–13]. In addition, adenocarcinomas were subdivided into well, moderately and poorly differentiated types, and also classified as to the degree of invasion: limited to the uterus, invading into the serosa and/or surrounding adnexae, and with distant metastasis, in accordance with the simplified FIGO histopathological grades for human uterine cancers [15]. Animals found dead or euthanized when moribund were also examined in the same manner. The tissues and/or organs fixed were routinely processed, paraffin embedded and stained with hematoxylin and eosin for histopathological examination. Throughout the experiment, all animals were checked for growth, clinical signs, and their estrous cyclicity by vaginal cytology. Estrus lasting for continuous for 4 days or more was defined as persistent estrus (PE).

Radioimmunoassays

Using serum obtained after decapitation, concentrations of follicle-stiumulating hormone (FSH), lutenizing hormone (LH), inhibin, E2, and progesterone were determined using double-antibody radioimmunoassays and ^{125}I-labelled radio-ligand. National Institute of Diabetesand Digestive and Kidney Disease (NIDDK) radioimmunoassay kits were employed for rat FSH and LH (NIAMDD, NIH, Bethesda, MD), as described by Taya *et al.* [16] and Watanabe *et al.* [17]. Immunoreactive inhibin in the serum was analyzed using rabbit anti-serum, TNDH-1[18]. Serum concentrations of E2 and progesterone were also measured, as described by Taya *et al.* [19].

Statistical analysis

Values for incidences were statistically analyzed using the Fisher's exact probability test. Other data were analyzed using ANOVA, and post hoc comparisons between the treated and control groups were made using the Dunnett's *t*-test. P values of less than 0.05 were considered to be statistically significant.

Results

Growth and estrous cyclicity

The busulfan treatment did not affect the body weights and clinical status of the animals. Regarding estrous cyclicity, all animals showed regular 4-day cycling during first 3 months after commencement of the experiment. Thereafter some animals treated with 5.0 mg/kg busulfan showed an estrus stage that lasted 2 or 3 days. Consequently, the incidence of persistent estrus (PE) in this group began to increase at 4 months of age, and most of the animals showed PE at 6 months of age, 4 months earlier than in the controls (Fig. 1). In the 2.5 mg/kg group, PE was observed 2 months earlier than in controls.

Effects of busulfan on uterine carcinogenesis

Incidences of uterine neoplastic lesions, including atypical hyperplasias and adenocarcinomas, and data concerning their multiplicity, which was indicated as total number of the neoplastic lesions per rat, are shown in Table 1. The incidence of adenocarcinomas was significantly increased in the 5.0 mg/kg busulfan-treated animals compared with the controls, but not in the 2.5 mg/kg busulfan group. Similarly, the multiplicity was significantly increased at the high dose, but not at the low dose. Histologically, almost all uterine adenocarcinomas were of a well-differentiated type limited to the uteri, without invasion or metastasis to other organs, and morphological or biological malignancy was not influenced by either dose of busulfan.

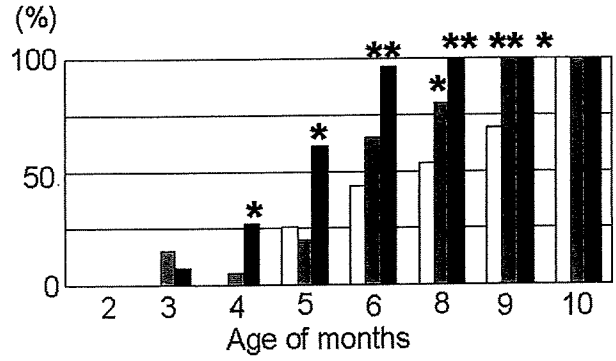

Fig. 1. Sequential changes in incidences of persistent estrus in the controls (□), 2.5 mg/kg busulfan (■), and 5.0 mg/kg busulfan treated (■) groups. *, ** Significantly different from the controls at P<0.05, and P<0.01, respectively.

Table 1. Incidence (%) and multiplicity data for uterine endometrial lesions

Group	No. of rats	Atypical hyperplasias				Adc	Multiplicity[a]
		–	+	++	+++		
Control	16	6.3	6.3	43.8	18.8	25.0	1.41 ± 0.80
Busulfan 2.5 mg/kg	18	33.3	5.6	16.7	11.1	33.3	1.21 ± 1.11
Busulfan 5.0 mg/kg	26	11.5	0	23.1	11.5	53.8*	3.03 ± 5.79*

[a] Total number of neoplastic lesions, including atypical hyperplasias and adenocarcinomas, per rat.
–: No lesion; +: Slight; ++: Moderate; +++: Severe
Adc: Adenocarcinoma. * Significant difference from the control group at 5%.

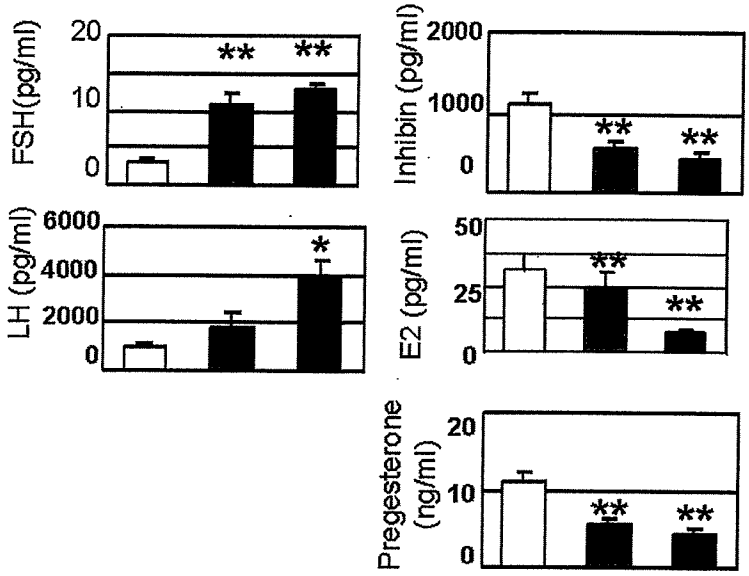

Fig. 2. Hormonal profiles of serum FSH, LH, E2, progesterone and inhibin at the termination of the experiment at 15 months of age in the controls (□), 2.5 mg/kg busulfan (■), and 5.0 mg/kg busulfan treated groups (■) groups. Results presented are mean ± SE values. *, ** Significantly different from the controls at $P<0.05$, and $P<0.01$, respectively.

Hormone profiles

Data concerning the serum concentrations of E2, progesterone, FSH, LH, and inhibin at 15 months of age are shown in Fig. 2. Inhibin, E2, and progesterone were significantly decreased by the busulfan treatment with dose-dependence. Conversely, serum FSH and LH were increased, although this was not significant for the LH level at 2.5 mg/kg. A remarkable increase in the serum E2/progesterone ratio was evident after the 5.0 mg/kg busulfan treatment, but significance was not achieved due to a high standard deviation (Fig. 3).

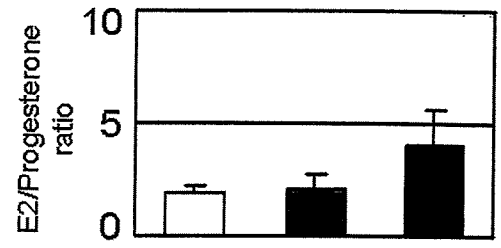

Fig. 3. E2/progesterone ratios at 15 months of age, and at termination of the experiment for the controls (□), 2.5 mg/kg busulfan (■), and 5.0 mg/kg busulfan treated (■) groups. Results presented are the mean ± SE values.

Organs weights and histopathology

In the uteri, macroscopic lesions such as nodules, hemorrhages, and bead-like horns were found in most animals, including the controls. Relative uterine weights per body weight were decreased in the 5.0 mg/kg busulfan group compared with the control values (Table 2).

Macroscopically, most of the ovaries were small at termination (15 months of age), and weights did not significantly differ across groups. Morphologically, marked atrophy was evident after the busulfan treatment, characterized by very few atretic and/or cystic follicles and a lack of

Table 2. Ovarian and uterine weights

Groups	No. of rats Examined	Final body weight	Uterus	Ovaries
Control	17	357.06 ± 32.26	1297.31 ± 342.05[a]	63.11 ± 44.11
			(361.62 ± 85.44)[b]	(17.59 ± 12.30)
Busulfan 2.5 mg/kg	18	399.81 ± 72.67	1156.62 ± 516.84	58.86 ± 43.28
			(259.54 ± 160.91)	(15.73 ± 12.14)
Busulfan 5.0 mg/kg	23	386.61 ± 46.56	1008.60 ± 405.35*	54.60 ± 47.71
			(268.50 ± 123.84)	(14.60 ± 13.12)

[a] Absolute weight, [b] Relative weights to body weight (mg/kg body weight × 100).
* Significant difference from the control group at 5%.

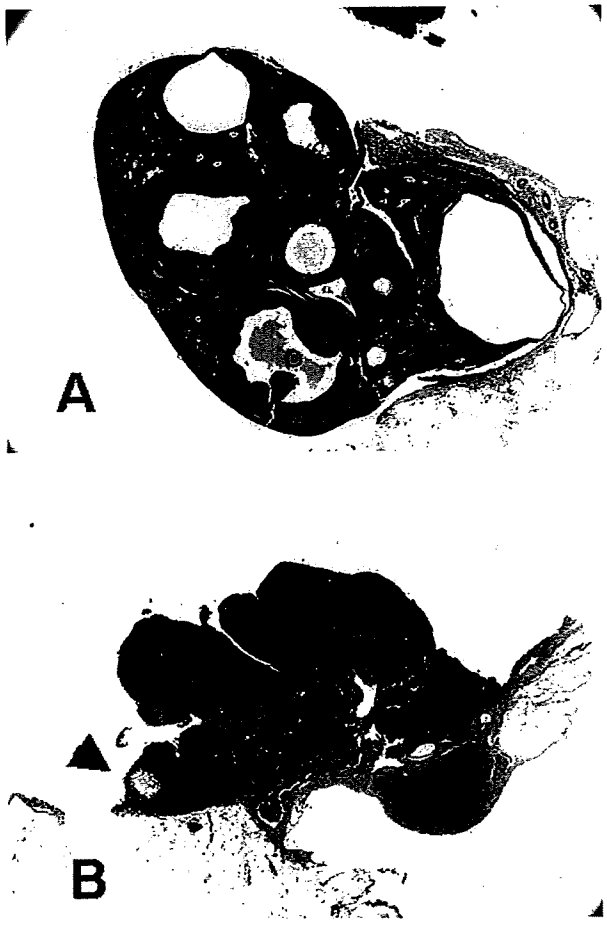

Fig. 4. Microphotographs of ovaries from controls (A) and 5.0 mg/kg busulfan treated animals (B) at 15 months of age. Note several atretic and/or cystic follicles with a lack of corpus and increased interstitial glands in the stroma in the control group (A). Only a small cystic follicle is apparent (arrowhead) in the stroma with 5.0 mg/kg busulfan (B). Hematoxylin and eosin staining. × 20.

corpus lutea, whereas the atrophic ovaries in controls demonstrated appreciable numbers of follicles (Fig. 4). In addition, collagen fibers in the ovarian stroma were more evident in busulfan treated rats, although aggregation of lipid containing stromal cells, or so-called interstitial glands, was not prominent. Busulfan, an alkylating agent, is well known to damage bone marrow and lungs [20, 21], but the results of the present study showed no effects of treatment in these body sites. A number of histopathological changes, including age-related ones, were observed in all animals, however there were no treatment-related changes in other organs or tissues.

Discussion

The present study clearly demonstrated that maternal treatment with a high dose of busulfan to enhance uterine carcinogenesis in Donryu rats is associated with an early occurrence of PE and severe ovarian atrophy, with lack of both follicles and corpus lutea. In this rat model, imbalance in ovarian hormones leads to elevation of the serum E2/progesterone ratio, and it was recognized that ovarian atrophy plays an essential role for the endometrial adenocarcinoma development, similar to the case in humans [1, 2, 11]. Using this model, many studies have proven that early and delayed occurrence of PE induced by chemicals promotes and prevents the endometrial adenocarcinoma development, respectively [22, 23]. Therefore, the earlier occurrence of PE in the present study is considered to be crucial for the promoting effect on uterine cancer.

Busulfan is known to accelerate the rate of follicular recruitment, in spite of the smaller

number of growing follicles [8]. Shirota *et al.* have demonstrated that the number of preantral follicles in the ovaries of Sprague-Dawley rats prenatally exposed to 2.5 mg/kg busulfan was comparable to the age-matched control value by day 13 after birth, and that the number of oocytes shed at the first ovulation with 5.0 mg/kg busulfan was also comparable to that in the controls [9]. In general, PE corresponds to an anovulation status, and appears in various situations such as neonatal exposure to high doses of estrogens and/or androgens and with aging in rodents, although the latter greatly varies depending on the strain [3, 4, 24, 25]. Ovaries with PE in rats and in postmenopausal women exhibit a gradual increase in the severity of atrophy, with final appearance as fibrous tissue in the end stage [1, 11, 26]. This is morphologically similar to the atrophy of ovaries treated with busulfan. Our present results suggest that a reduction in follicle resources due to maternal treatment with busulfan leads to earlier occurrence of PE and might accelerate ovarian changes with aging, although no sequential observations of the ovaries could be conducted in the present study.

The hormonal profiles exactly reflected the atrophic ovary status, with marked decreases in E2, progesterone, and inhibin levels, and increases in LH and FSH. Inhibin is a regulatory peptide that inhibits FSH synthesis and release from the pituitary, resulting in regulation of ovulation in mammals [17, 27–30]. Previous studies have indicated that the concentration of inhibin reflects the number of primary and preantral follicles until antral follicle formation in the ovary [9, 31]. Our results provide evidence that the hypothalmo-pituitary-gonadal control system still responds at 15 months of age after busulfan treatment. The observed decrease in uterine weights might be related to lower levels of E2, although a number of uterine lesions were detected in all animals examined at 15 months of age. Interestingly, the 5.0 mg/kg busulfan treatment elevated the serum E2/progesterone ratio, although both E2 and progesterone were markedly decreased. Thus, the results might support the previous finding that elevation of this ratio plays a crucial role in uterine carcinogenesis in our rat model.

In the present study, the possibility remains busulfan, an alkylating agent, might exert direct cytotoxicity damage to the uterus, as well as bone marrow and the lungs [20, 21, 32], but no necrotic changes were observed that suggested cytotoxiciy was increased in the uterus of the busulfan treated groups, nor were they reported in the previous study by Shirota *et al.* [9]. In addition, estradiol receptor mediated responsiveness plays an important role for the uterine adenocarcinoma development in rats [1] and women [2], although estrogen receptors were expressed only in a few areas of the epithelial cells in the fetal uterus on day 15 of gestation, and the receptor mediated responsiveness was absent in the development of the prenatal female reproductive tract in mice and rats [33–35]. Busulfan exerts any estrogenic activity. Therefore, these results suggest that a direct action of busulfan on the fetal uterus might be excluded.

In conclusion, maternal exposure to busulfan at dose of 5.0 mg/kg on day 14 gestation promoted the uterine adenocarcinoma development in Donryu rats that were subsequently initiated with ENNG, and this was associated with an earlier occurrence of PE, severe atrophy of the ovaries, and marked decreases in both serum E2 and progesterone levels. The E2/progesterone ratio, however, revealed an increased trend in the high dose group.

Acknowledgements

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Original

α-Smooth Muscle Actin-positive Stromal Cells Reactive to Estrogens Surround Endometrial Glands in Rats but not Mice

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Abstract: In human endometrium, α-smooth muscle actin (α-SMA)-positive stromal cells (SMA-SCs) surround endometrial glands, and the α-SMA expression is regulated by estrogen. The biological significance of these cells remains to be elucidated, and no information is available with regard to their animal counterparts. The present study, therefore, investigated SMA-SCs in the uteri of female Donryu rats and CD-1 mice. SMA-SCs with morphological similarities to those in the human were detected around the endometrial glands in normal cycling rats, but not in mice. Furthermore, the rat SMA-SCs disappeared after ovariectomy but returned with estrogen replacement in a duration-dependent manner, suggesting the regulatory role of estrogens similar to the human situation. Thus, SMA-SCs are present in rats, but not in mice, with characteristics close to their human counterparts. Their biological significance now needs to be elucidated by comparative studies. (J Toxicol Pathol 2005; **18**: 47–52)

Key words: endometrial stromal cells, α-smooth muscle actin, 17β-estradiol, octylphenol, Donryu rat

Introduction

Actin, a cytoskeletal protein involved in cell contraction, cell movement and cell-to-substrate adhesion[1–6], has been divided into six isoforms: two non-muscle actins (β and γ) known to be cytoplasmic, two smooth muscle actins (α and γ), and two sarcomeric actins (α-cardiac and α-skeletal)[7–9]. The α-smooth muscle actin (α-SMA) is found in smooth muscle cells, pericytes and myoepithelial cells[10–12], and in humans normal endometrial stromal cells around endometrial glands have also been shown to immunostain for α-SMA[13,14]. This α-SMA expression in stromal cells changes during the estrous cycle, greater numbers of positive cells being present in the proliferative than in the secretory phase[13]. Furthermore, in the proliferative phase α-SMA-positive stromal cells (SMA-SCs) can be detected occasionally in the more superficial mucosa and around non-dilated glands as well as in the lower, basal layer of the endometrial mucosa and around dilated or cystic glands, whereas SMA-SCs in the secretory phase are mostly evident in the basal, inactive layer and around single non-secretory glands[14]. This suggests that estrogen influences α-SMA expression in the endometrial stromal cells, although their significance remains to be elucidated. Furthermore, no information is available in the literature about their existence in experimental animals, such as rodents. The present study was thus conducted to determine whether SMA-SCs are a feature of the uteri of rats and mice. Finding them present in rats, we then assessed the reactivity of SMA-SCs to 17β-estradiol and *p-tert*-octylphenol, an endocrine disrupting chemical with estrogenic activity, using ovariectomized rats.

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Materials and Methods

Ethical considerations for animal experiments

The animal experiments conducted in this study were approved by the Animal Experimentation Committee of the Sasaki Institute prior to their execution and were conducted under monitoring by the committee in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law Number 105 and Japanese

Government Notification on Feeding and Safekeeping of Animals Number 6.

Animals

A total of 30 virgin female Donryu rats (Crj:Donryu, 6 or 10 weeks of age) and 12 virgin CD-1 mice (Crj:CD-1, 7 weeks of age) were purchased from Charles River Japan Inc. (Kanagawa, Japan). They were housed in plastic cages, kept in an air-conditioned animal room (under constant conditions of 24 ± 2°C, 55 ± 10% humidity, and a 12-hour light/dark cycle), and maintained on a basal diet, CRF-1 (Oriental Yeast Inc., Tokyo, Japan) with tap water *ad libitum*.

Experimental design

Experiment I: Twelve animals each of the two species showing normal estrous cyclicity were selected, and vaginal smears were checked every morning. At ages of 12 or 15 weeks (rats) and 9 weeks (mice), 3 animals each were euthanized in the 4 stages of the estrous cycle (proestrus, estrus, metestrus and diestrus), and the uteri were excised for histological and immunohistochemical examination. The uterine horns were fixed in 10% neutrally buffered formalin solution and embedded in paraffin. Appropriate numbers of serial sections at a thickness of 4 μm were then prepared from each specimen, one being routinely stained with hematoxylin and eosin for histological examination. The other sections were processed for immunohistochemical analyses using mouse monoclonal antibodies against α-SMA (clone 1A4, Dakocytomation Japan, Kyoto, Japan, 100-fold diluted) and cytokeratin 14 (CK14; clone LL002, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK, 20-fold diluted), the latter for the rat uteri only, at 4°C overnight, then processed for the labeled polymer method using an Envision Plus kit (Dakocytomation) according to the manufacturer's instructions, and counterstained with hematoxylin. Negative controls were included with primary antibodies omitted.

Experiment II: At 8 weeks of age, 18 rats underwent ovariectomy via the dorsal route under light ether anesthesia. Three weeks after the operation, they were equally divided into 3 groups, given subcutaneous injections of vehicle (dimethylsulfoxide), 5 μg/kg/day of 17β-estradiol (E_2; Wako Pure Chemical Industries Ltd., Osaka, Japan) or 100 mg/kg/day of *p-tert*-octylphenol (OP; Wako), respectively, for 2 or 14 successive days (3 animals each). The animals were euthanized 24 hours after the last administration, and the uteri were excised. The uterine horns were processed in the same manner as for experiment I except that CK14 immunostaining was not performed.

Results

Experiment I

In rats, the endometrial stroma was cellular, especially in the subluminal layer surrounding the glandular epithelium. The stromal cells surrounding the glands were spindly in shape with oval or spindle nuclei, resembling fibroblasts rather than basket-shaped myoepithelial cells. They were arranged in one or several layers around the glands (Fig. 1A) in all stages of the estrous cycle. In mice, the endometrial stroma was also cellular, but stromal cells surrounding the endometrial glands were not very conspicuous (Fig. 2A).

Immunohistochemically, the stromal cells surrounding the endometrial glands in one or several layers were positive for α-SMA in rats (Figs. 1B and 1C). Positive cells were observed through all stages of the estrous cycle, but the cell layers were thickest in proestrus (Figs. 1B and 1C). The stromal cells were negative for CK14 (Fig. 1D). In mice, SMA-SCs were not detected at any stage of the estrous cycle (Fig. 2B). In the uteri of both rats and mice, smooth muscle fibers in the blood vessels and the myometrium were positive for α-SMA (Figs. 1B, 1C and 2B).

Experiment II

In the ovariectomized rats, the uteri were severely atrophic, and the luminal epithelial cells in the endometrium were cuboidal rather than columnar in shape. The endometrial stromal cells and smooth muscle in the myometrium were reduced in size (Fig. 3A). In rats treated with E_2 for 2 days, the size of the uteri recovered remarkably, and the luminal epithelial cells in the endometrium were again columnar in shape. The endometrial stromal cells also recovered, and the myometrium was multi-layered (Figs. 3A and 3B). In rats treated with E_2 for 14 days, the uteri were as large as those of 2-day-treated animals while the endometrial stromal cells were larger (Figs. 3B and 3C) and the myometrium was thicker (Figs. 3B and 3C). In rats receiving OP, the uteri generally demonstrated similar histological findings to those in the rats treated with E_2 for the same term (Figs. 3C and 3D).

Table 1 summarizes the data of the α-SMA immunohistochemistry of stromal cells surrounding the endometrial glands in experiment II. In ovariectomized rats treated with vehicle stromal cells were negative for α-SMA (Fig. 4A), but 2 out of 3 rats treated with E_2 for 2 days exhibited weakly-positive spindle cells around the glands (Fig. 4B). Furthermore, α-SMA-positive cells were observed in all rats injected with E_2 for 14 days (Fig. 4C). Similarly, one out of 3 rats treated with OP for 2 days and all rats receiving OP for 14 days had SMA-SCs (Fig. 4D). Smooth muscle fibers in the blood vessels and the myometrium were positive for α-SMA in rats of all groups (Figs. 4A-D).

Discussion

The present study unequivocally demonstrated the presence of SMA-SCs surrounding endometrial glands in untreated rats but not mice. The SMA-SCs clearly differed from myoepithelial cells, characterized as basket-shaped with a positive CK14 phenotype[15,16], and were similar to SMA-SCs in the human endometrium, negative for

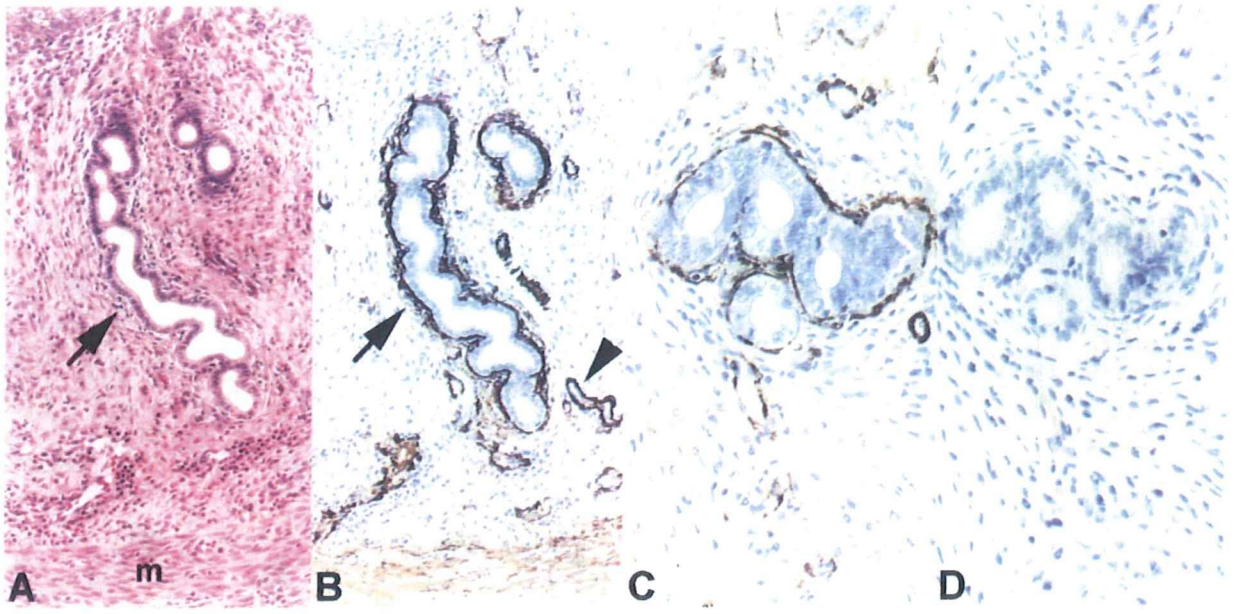

Fig. 1. Uteri of untreated rats (experiment I). (A) Representative histology in proestrus. Several layers of stromal cells surround endometrial glands (arrow). m: myometrium, × 90. (B) Representative α-SMA immunohistochemistry in proestrus. One or several layers of stromal cells surrounding endometrial glands are positive (arrow). Smooth muscle fibers in the blood vessels (arrowhead), as well as in the myometrium, are also positive, × 90. (C) Representative α-SMA immunohistochemistry in metestrus. One or two layers of stromal cells surrounding endometrial glands are positive, × 180. (D) Representative CK14 immunohistochemistry in metestrus. The endometrial stromal cells are negative, × 180.

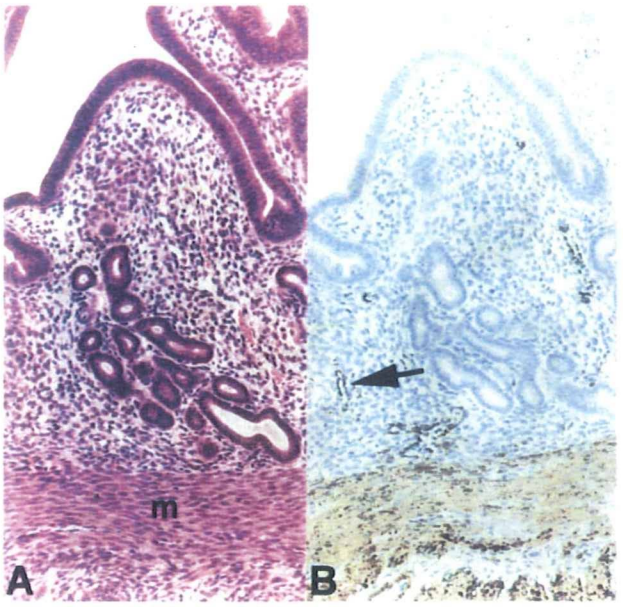

Fig. 2. Uteri of untreated mice (experiment I). (A) Representative histology in proestrus. Endometrial stromal cells are diffusely present throughout the stroma. m: myometrium, × 90. (B) Representative α-SMA immunohistochemistry in proestrus. The endometrial stromal cells are negative. Smooth muscle fibers in the blood vessels (arrow), as well as in the myometrium, are positive, × 90.

Table 1. Grades of α-SMA Immunohistochemistry for the Stromal Cells Surrounding Endometrial Glands in Ovariectomized Rats (Experiment II)

Group	Treatment		Grade		
	Compound	Period	–	±	+
1	Vehicle	2 days	3	0	0
		14 days	3	0	0
2	E_2	2 days	1	2	0
		14 days	0	0	3
3	OP	2 days	2	1	0
		14 days	0	0	3

Symbols used are: –, negative; ±, weakly positive; +, positive.

cytokeratins and distinguishable from myoepithelial cells[14]. Human SMA-SCs have been suggested to be a subset of myofibroblasts[13,14]. Myofibroblasts are characterized as having features of both smooth muscle cells and fibroblasts[17–19], and can be classified into 4 subtypes based on their differential immunoreactivity with antibodies against vimentin, desmin and α-SMA[20]. Results of the present study indicate that the SMA-SCs present in rats similarly have a myofibroblast origin, judging from the morphological findings.

In the rats, layers of SMA-SCs were apparent in proestrus, when serum E_2 levels are the highest of the estrous cycle. The fact that the endometrial stromal cells surrounding endometrial glands were small in size and negative for α-SMA in ovariectomized rats, with recovery

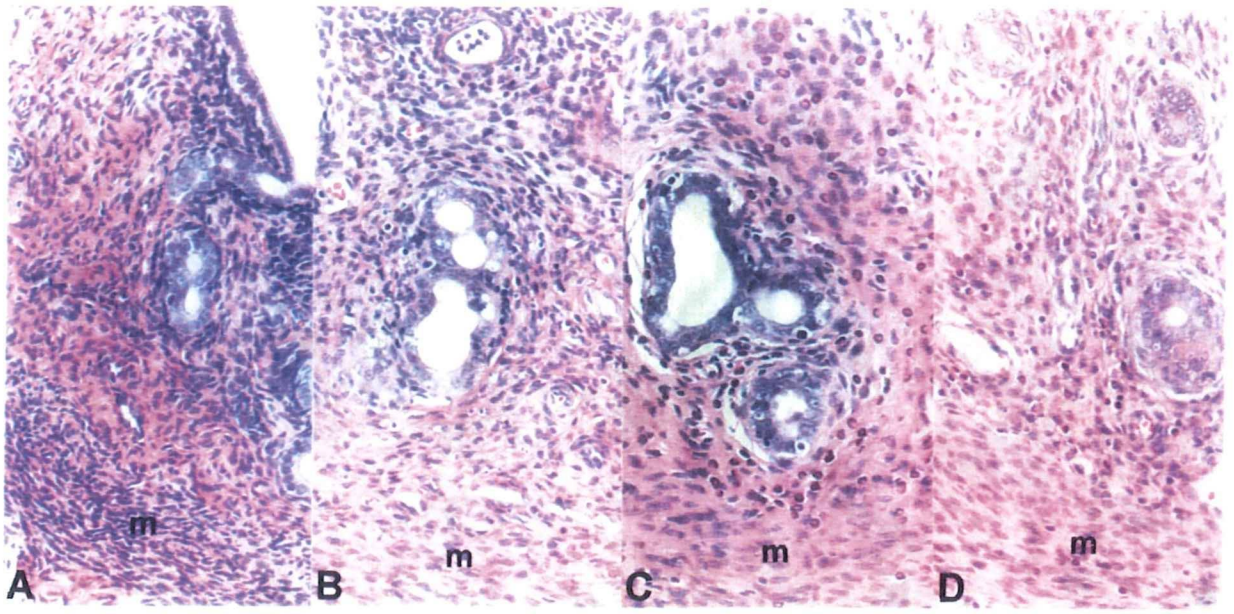

Fig. 3. Representative histology of uteri of ovariectomized rats (experiment II), × 180. (A) Treated with vehicle for 14 days. The endometrial stromal cells and smooth muscle in the myometrium (m) are small in size. (B) After treatment with E_2 for 2 days; the endometrial stromal cells are larger in size than those of the vehicle controls. Smooth muscle in the myometrium (m) is also thicker. (C) After treatment with E_2 for 14 days; the endometrial stromal cells are larger than those of the 2-day-treated rats and the myometrium (m) is thicker. (D) After treated with OP for 14 days; the endometrial stromal cells are large and the myometrium (m) is thick, like those of the rats receiving E_2 for 14 days.

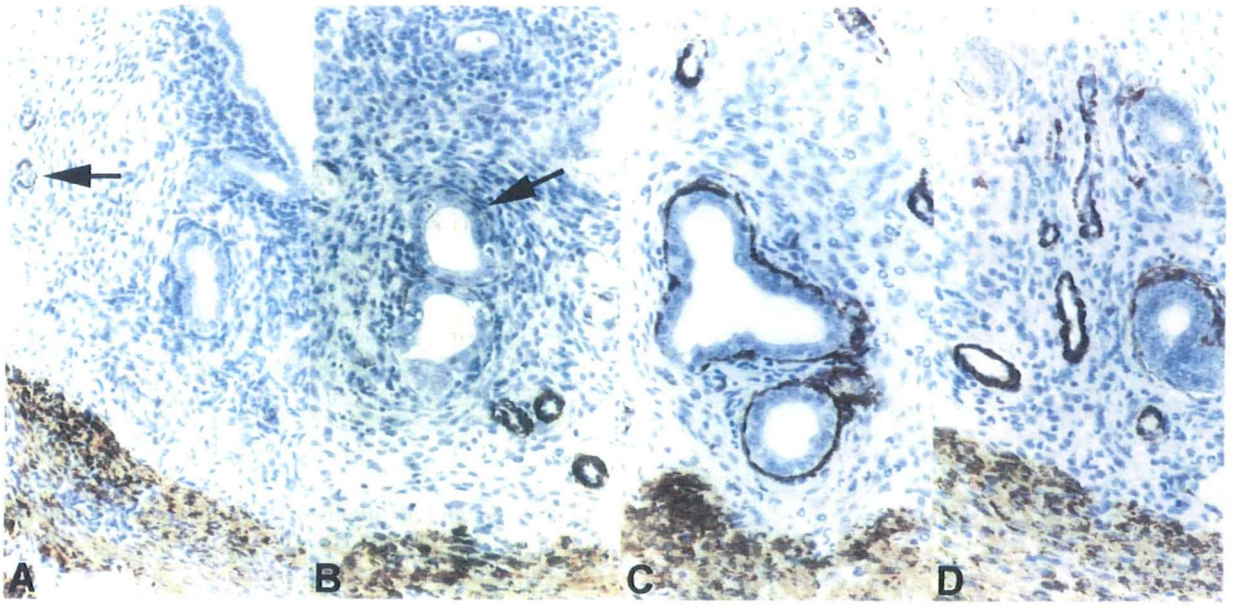

Fig. 4. Representative α-SMA immunohistochemistry of uteri of ovariectomized rats (experiment II), × 180. (A) Treated with vehicle for 14 days. Stromal cells surrounding endometrial glands are negative. Smooth muscle fibers in the blood vessels (arrow), as well as in the myometrium, are positive. (B) Treated with E_2 for 2 days. Some stromal cells surrounding endometrial glands are weakly positive (arrow). (C) Treated with E_2 for 14 days. One layer of the stromal cells surrounding endometrial glands is positive. (D) Treated with OP for 14 days. The stromal cells surrounding endometrial glands are positive.

on treatment with estrogen or OP, in a duration-dependent manner, clearly points to hormone dependence. OP is an endocrine disrupting chemical with estrogenic activity, from *in vitro* and *in vivo* evidence[21–28], and the dose of OP used in this study has been shown to be sufficient to exert estrogenic effects on the female reproductive tract in ovariectomized rats[27]. The results thus suggest that, similar to the human situation[13,14], estrogen modulates α-SMA expression in the

stromal cells surrounding endometrial glands in rats. Although the underlying mechanisms remain largely obscure, estrogen receptors can be immunohistochemically detected in endometrial stroma as well as in epithelium and in myometrium[29], and Hsu and Frankel[30] have demonstrated that mRNA expression of the *smooth muscle actin* gene is up-regulated by estrogens in immature rat uteri.

Myofibroblasts have been proposed as playing crucial roles in the contraction and relaxation of human granulation tissue on the basis of *in vitro* pharmacological reactivity similar to the smooth muscle[2]. α-SMA-positive myofibroblasts are also observed in rat granulation tissue[18] and may act similarly to their human counterparts. α-SMA is also expressed in passaged cultures of chick embryo fibroblasts[10], and stress fibers containing actin have been postulated as playing structural roles in the connection of the cytoplasmic matrix to the substrate rather than being contractile[4]. Thus, SMA-SCs surrounding endometrial glands might either participate in the contraction of glands or in the cell's adhesion to the surrounding substrate. Mechanistic studies are now needed to clarify, for example, the lack of SMA-SCs in mice.

In conclusion, SMA-SCs are present in rats, but not mice, and surround the endometrial glands, exhibiting morphological and endocrinological similarities to their human counterparts. Elucidation of their functional significance now needs to be performed by comparative studies in different species.

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Distinct patterns of gene expression in hepatocellular carcinomas and adjacent non-cancerous, cirrhotic liver tissues in rats fed a choline-deficient, L-amino acid-defined diet

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Gene expression profiles of HCC and surrounding non-cancerous tissues in rats fed a CDAA diet for 70 weeks, as well as normal liver tissues, were explored using an oligonucleotide microarray for 3757 genes. A total of 146 genes were identified as differentially expressed; the affected functions including metabolism, apoptosis, cell cycling, RNA splicing, Wnt signaling, reactive oxygen species-induced stress, and fibro/cirrhogenesis. The genes were found to fit into four distinct expression patterns after classification by hierarchical and *k*-means clustering procedures. Notably, genes within the same functional category tended to be found within the same cluster, thus gene functions appeared to be related to their expression patterns. For example, genes encoding receptors (Fisher's exact test, $P < 0.01$) and cytokines (Fisher's exact test, $P < 0.05$) were both enriched in a cluster characterized by low expression in HCC compared to their surrounding tissues. While some of the receptors in this cluster had cell-proliferative potential, others are known to be growth-suppressive. It was noted, however, that four of the 10 receptor genes encode G-protein-coupled receptors, for which growth-suppressive potential has been reported. The seven growth factors in the same cluster included two fibroblast growth factors. The current findings suggest the possibility that genes differentially expressed in this multistep carcinogenic model may be classified into relatively few clusters according to their expression patterns, and that these clusters may be associated with gene functional categories. (*Cancer Sci* 2005; 96: 414–424)

Hepatocellular carcinomas are common in Asia and Africa, and the incidence is increasing in Europe and North America. The prognosis for HCC is extremely poor. In human HCC, both genetic and epigenetic alterations have been detected with regard to particular genes such as *p53*, cyclin D, $p16^{Ink4}$, $p21^{Waf1/Cip1}$, *Rb*, β-catenin, mannose-6-phosphate/insulin-like growth factor II receptor (*M6P/IGF2R*), E-cadherin, cyclo-oxygenase (*COX*)-2, and telomerase reverse transcriptase (*hTERT*).[1–3] In addition to studies on individual genes, microarray technology has allowed the exploration of comprehensive changes in expression during HCC development. To gain insight into the overall picture, suitable animal models are necessary so that samples reflecting various stages of carcinogenesis can be collected. It should be noted, however, that in animal models featuring the use of chemical carcinogens, unavoidable carcinogen-specific molecular alterations may mask generic and essential changes. In addition, the major risk factor in human HCC has been established as being continuous chronic liver injury, which occurs in hepatitis virus infections.[1–3] Thus we have chosen to use an animal model that employs the administration of a CDAA diet, which induces HCC on a background of continuous hepatic injury and cirrhosis. It has been confirmed that this model resembles human carcinogenesis caused by chronic viral hepatitis, hemochromatosis, and Wilson's disease in many respects.[1–4]

The CDAA diet is hepatocarcinogenic in male rats of Fischer 344 and Wistar strains.[4] As with other diets deficient in choline and low in methionine (CMD diets), HCC are induced in the absence of chemical carcinogens. In the CDAA model, HCC occur at a high rate through the induction and growth of preneoplastic hepatocellular lesions, which are followed by progression to hepatocellular adenomas and subsequent conversion to malignancy.[4] Chronic feeding of CMD diets in rats has also been accepted as an animal model for nonalcoholic fatty liver disease/nonalcoholic steatohepatitis (NAFLD/NASH), a condition in which a substantial number of patients develop HCC.[5] Here we have performed microarray analyses to obtain gene-expression profiles for HCC and surrounding non-cancerous tissues in CDAA treated rats, as well as normal liver samples, so as to gain insight into the mechanisms of multistep hepatic carcinogenesis.

Materials and Methods

Ethical considerations. The experimental protocols were approved by the Animal Experimentation Committee of the Sasaki Institute prior to their execution. The experiment was conducted under monitoring by the committee in accordance with the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals, Japanese Government Animal Protection and Management Law Number 105, and Japanese Government Notification on Feeding and Safekeeping of Animals Number 6.

Animals, diets and animal treatment. A total of 10 male 5-week-old Fischer 344 rats were purchased from Charles River Japan (Atsugi, Kanagawa, Japan). They were divided into two groups of five animals, housed in plastic cages with white-flake

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Abbreviations: CDAA, choline-deficient, L-amino acid-defined; HCC, hepatocellular carcinoma; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; RT-PCR, reverse transcription polymerase chain reaction.

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bedding in an airconditioned room (25 ± 3°C temperature, 55 ± 8% relative humidity, 10–12 times/h ventilation and 12-h dark/light cycle). They were used after a 1-week acclimation on basal diet (CRF-1, Oriental Yeast Corporation, Itabashi, Tokyo, Japan) and allowed free access to food and tap water throughout the acclimation and experimental periods. Bodyweight, food consumption, and water intake were monitored weekly. The CDAA diet was obtained from Dyets (Bethlehem, PA, USA).

After acclimation, group 1 rats were administered the basal diet, while group 2 rats were placed on the CDAA diet for 70 weeks and then killed. The livers were then macroscopically examined. Portions of group 1 livers, and the macroscopic tumors and surrounding non-cancerous tissues of group 2 livers were fixed in 10% neutrally buffered-formalin for 24 h, embedded in paraffin, processed for the routine HE staining procedure, and histologically examined. Remaining portions of these three types of liver tissues were immediately frozen in liquid nitrogen and stored at −80°C. Group 1 liver tissues, group 2 liver tissues from non-cancerous areas, and group 2 liver tissues from tumors (after being histologically diagnosed as HCC) were employed as normal liver (CON), surrounding non-cancerous liver (NC), and HCC samples (CA). The microarray and RT-PCR experiments and the subsequent data analysis were performed using five CON samples from individual group 1 rats and five matched pairs of NC and CA samples from individual group 2 animals.

RNA isolation and probe labeling. Total RNA from liver tissues was isolated using an RNeasy Midi kit (QIAGEN, Hilden, Germany) and its integrity checked by electrophoresis on 1% agarose-formaldehyde gels. Five micrograms of total RNA from individual samples were then labeled with Cy3 (Amersham Biosciences, Uppsara, Sweden) using a BD Atlas PowerScript Fluorescent Labeling kit (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's protocols.

Hybridization, scanning and quantification. Cy3-labeled probes were hybridized to Atlas Rat 3.8 I microarrays (BD Biosciences Clontech) containing 3757 genes for 16 h at 50°C. After hybridization, microarrays were washed, dried and scanned using a GMS 418 confocal laser scanner (Genetic MicroSystems, Woburn, MA, USA). Fluorescence intensities of the Cy3 channels were quantified using ImaGene 4.0 software (BioDiscovery, El Segundo, CA, USA).

Microarray analysis and annotation of gene function. Data analysis was performed using GeneSpring software, version 5.1 (Silicon Genetics, Redwood City, CA, USA), including appropriate statistics. Dividing by the median calculated from all of the signal intensities for the Cy3 in a given sample, the fluorescence signal for each gene was normalized and the expression ratio was then calculated by dividing the normalized signal by the median for each gene to offset differences in expression levels between genes. Values were displayed as average of five animal samples for the three groups and were then logged (base 10) for further analyses. Standard deviations of the normalized values were calculated for the three groups, and genes in which standard deviation was more than two for any of the three groups were excluded. Genes differentially expressed among the three tissue types were detected by one-way ANOVA ($P = 0.02$).

The functions of the genes were assigned referring to a table attached to Atlas Rat 3.8 I microarrays (BD Biosciences Clontech), and classified into 21 categories: cell surface antigens, transcription factors, cell cycle-related factors, cell adhesion receptors/proteins, extracellular transport/carriers, stress response proteins, membrane channels/transporters, extracellular matrix proteins, trafficking/targeting proteins, metabolism-related factors, post-translational modification/protein folding-related factors, translation-related factors, apoptosis-related factors, RNA processing/turnover/transport-related factors, DNA binding and chromatin proteins, cell receptors, cell signaling/extracellular communicating factors, intracellular transducers/effectors/modulators, protein turnover-related factors, cytoskeleton/motility proteins, and others. Statistical significance for frequencies of genes of each functional category in each cluster was assessed as follows: values of the other categories in the relevant cluster, values of the other clusters in the relevant category, and values of the other clusters in the other categories were all combined, and the resultant combined values were compared with the relevant value by Fisher's exact test for the 2 × 2 table.

Semi-quantitative RT-PCR. Semi-quantitative RT-PCR was performed for seven genes, at least one gene from each cluster. They were endothelin receptor B (*EDNRB*), zinc finger protein 265 (*ZNF265*), neurofibromatosis type 1 (*NF1*), hepatic nuclear factor 1/transcription factor 1 (*HNF1/TCF1*), leukemia/lymphoma related factor (*LRF*), cyclin L (*CCNL*), and lamin A (*LMNA*). cDNA was synthesized from 3 μg of total RNA using a First-Strand cDNA Synthesis kit (Amersham Biosciences) and oligo$(dT)_{18}$ primer according to the manufacturer's instructions. RT-PCR was carried out using the GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) and cycling conditions were 2 min at 94°C, followed by 30 cycles (25 cycles for β-*actin*) of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Forward and reverse primer sequences were: *EDNRB*, 5′-TTGATGTGATTACGTCGGAC-3′ and 5′-GGACTGTTTTTCCTCAAACG-3′; *ZNF265*, 5′-AGAAGTACTACATCTGCTAG-3′ and 5′-TTCCCAGTTGTCAGCTTTGC-3′; *NF1*, 5′-GTACACCAAATACCATGAGC-3′ and 5′-ATGAAGAGGGTGTTGTTGGC-3′; *HNF1/TCF1*, 5′-TGCACTAGAAAGGCTGCTTC-3′ and 5′-GGTTTCTTGCAGTACCGAGG-3′; *LRF*, 5′-TGGGCCGCCTGAATGTAGCG-3′ and 5′-GTATGTCAGTGGTGGCCATG-3′; *CCNL*, 5′-TAATAGGCGAAGTCGATCTG-3′ and 5′-CATCGTCACACTGCATATGG-3′; *LMNA*, 5′-ATGAGAGCAGGTCTGAAGCC-3′ and 5′-AAGCATGGCAGATTTGCCTC-3′; β-*actin* (used as control), 5′-TTGAACACGGCATTGTAACC-3′ and 5′-ATCTCTTGCTCGAAGTCTAG-3′. PCR products were analyzed on 2% agarose gels with ethidium bromide and subsequently underwent densitometry. The obtained values were then normalized to those for β-*actin*.

Western blotting. Western blotting was conducted using the liver samples. Liver tissues were homogenized in 5 volumes of extraction buffer (10 mM Tris-HCl, pH 6.8, 1% SDS) using a polytron homogenizer at setting 7 for 90 s. The homogenates were heated on boiling water for 5 min and centrifuged at 13 000 × *g* for 30 min. The supernatants were used for western blotting. Protein concentrations of the lysates were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Samples containing 5 μg of protein were separated on 7.5% SDS polyacrylamide gel electrophoresis and transferred to Hybond-P PVDF membranes (Amersham Biosciences, Buckinghamshire, UK). The blots were blocked with 5% dried milk in PBS for 1 h, incubated with 1:200 dilution of rabbit anti-EDNRB polyclonal antibody (Chemicon International, Temecula, CA, USA), washed in 0.1% Tween 20 in PBS, and incubated with 20 000-fold diluted antirabbit donkey IgG conjugated with peroxidase (Amersham Biosciences). Both primary and secondary antibodies were diluted with 0.1% Tween 20 in PBS, and incubation was at room temperature for 1 h. The immunoreactive bands were detected using ECL Plus Western Blotting Detection Reagents (Amersham Biosciences).

Results

General findings. All rats survived until their scheduled killing in relatively healthy conditions, but the mean bodyweight of group 2 animals was lighter than that of group 1 animals (Table 1). Group 1 livers showed no particular pathological changes either macroscopically or histologically. All of group 2 livers were macroscopically yellowish-white and appeared cirrhotic with 1 or 2 large tumoral nodules with a dark color. The mean relative liver weight was greater in group 2 compared