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
follicles in the ovary and the rate at which they moved into the growing pool [8]. In addition, Shirot
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 C57: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 Louis, 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-stimulating hormone (FSH), luteinizing hormone (LH), inhibin, E2, and progesterone were determined using double-antibody radioimmunoassays and ¹²⁵I-labelled radio-ligand. National Institute of Diabetes and 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.
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).

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).

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

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>–</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>Adc</th>
<th>Multiplicity a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16</td>
<td>6.3</td>
<td>6.3</td>
<td>43.8</td>
<td>18.8</td>
<td>25.0</td>
<td>1.41 ± 0.80</td>
</tr>
<tr>
<td>Busulfan 2.5 mg/kg</td>
<td>18</td>
<td>33.3</td>
<td>5.6</td>
<td>16.7</td>
<td>11.1</td>
<td>33.3</td>
<td>1.21 ± 1.11</td>
</tr>
<tr>
<td>Busulfan 5.0 mg/kg</td>
<td>26</td>
<td>11.5</td>
<td>0</td>
<td>23.1</td>
<td>11.5</td>
<td>53.8*</td>
<td>3.03 ± 5.79*</td>
</tr>
</tbody>
</table>

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%.

Macrophosphically, 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...
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 hypotalmo-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 cytotoxicity 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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References


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