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Neonatal administration of diethylstilbestrol has adverse effects on somatic cells rather than germ cells

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Abstract

Neonatal administration of diethylstilbestrol (DES) to rodents has adverse effects on spermatogenesis. However, not many studies have been conducted to determine which type of cell – germ or somatic – is the major target of DES. In order to clarify this, we tried reciprocal germ cell transplantation—transplantation of germ cells from DES-treated mice into intact mice and germ cells from normal mice into DES-treated mice. The donor germ cells were tagged with the green fluorescent protein (GFP) gene in order to distinguish the exogenous germ cells from the endogenous cells. Moreover, to obtain a large number of spermatogonia from the testes of adult mice, we performed fractionation by centrifugation with Percoll. Consequently, we found that the germ cells collected from DES-treated mice have differentiated into normal sperms in normal seminiferous tubules. However, in the case of the transplantation of normal germ cells into the seminiferous tubules of DES-treated mice, defective spermatogenesis was observed. In conclusion, DES has adverse effects on the somatic cells that are involved in spermatogenesis rather than the germ cells.

Keywords: Diethylstilbestrol; Busulfan; Germ cell transplantation; Green fluorescence; Germ cell; Somatic cell

1. Introduction

Diethylstilbestrol (DES) is an estrogenic compound that was administered to prevent miscarriage and other pregnancy complications in the 1940s–1970s [1,2]; however, this gestational exposure resulted in children at high risk for congenital anomalies of their reproductive tracts [1,3]. DES induced a high frequency of cryptorchidism and hypospadias in boys at birth and in small testis and low sperm counts in adulthood [1,4]. Neonatal treatment with DES also has adverse effects on spermatogenesis in male rodents [5,6].

Several studies showed that neonatal administration of DES caused a variety of abnormalities including low serum testosterone, small testis and abnormal spermatogenesis [3,4,7–10]. Other studies showed that neonatal exposure to DES retards the development of the blood–testis barrier [11,12]. However, it is not clear which type of cell – germ or somatic – is the major target of DES.

Brinster and co-workers showed that germ cells undergo spermatogenesis when they are transplanted into the seminiferous tubules in which the endogenous germ cells had been destroyed by busulfan [13,14]. After 1 week of transplantation, the exogenous germ cells reached and remained on the basal membrane. Furthermore, the colonies derived from the exogenous germ cells randomly spread as foci in the recipient seminiferous tubules. After transplantation, spermatogenesis occurred by fourth week and sperm were recognized by the second or third month [15,16]. Thus, exogenous germ cells have the ability to undergo spermatogenesis in the recipient seminiferous tubules after transplantation. Studies using germ cell transplantation have reported that abnormal spermatogenesis due to inherited defects is caused by either the germ cells or the somatic cells [17,18]. The use of

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this transplantation method might help in defining the interaction between the donor germ cells and the recipient somatic cells by observing the transplanted germ cells, and specifically to address whether a chemical has a negative influence on either the germ cells or the somatic cells. Suitable *in vitro* methods for spermatogenesis has not yet been established.

The abnormalities in spermatogenesis that are caused by neonatal treatment with DES are clearly evident in adulthood. In our previous study, we performed germ cell transplantation by using the stem cells collected from the testes of adult donor mice [19]. In the present study, we focused on the interaction between germ cells and the somatic cells supporting spermatogenesis. Furthermore, by using a transplantation method, we clarified the type of cell – germ or somatic – that gives rise to DES-induced abnormal spermatogenesis. We performed the reciprocal germ cell transplantation between DES-treated and DES-untreated mice [18] and evaluated the proliferation and differentiation of the transplanted germ cells. The results of the present study suggest that neonatal administration of DES has adverse effects on the somatic cells involved in spermatogenesis rather than the germ cells.

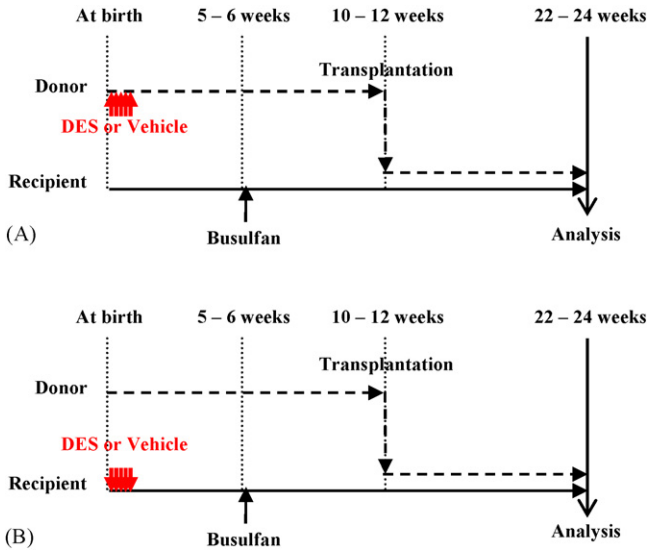

Fig. 1. Experimental design. (A) Transplantation of vehicle- or DES-treated germ cells to normal recipients. (B) Transplantation of normal germ cells to vehicle- or DES-treated recipients.

2. Materials and methods

2.1. Animals

C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). GFP mice – the transgenic C57BL/6 mice into which the green fluorescent protein (GFP) gene is inserted – were a gift from Mitsubishi Kagaku Institute of Life Science, Tokyo, Japan [20]. The mice were maintained under controlled light (12L:12D) and temperature (22–24 °C), and had free access to food and water. We designated the day of birth as Day 0. All animal protocols were reviewed and approved by the Laboratory Animal Care Committee and conducted in accordance with the Guidelines for Animal Experimentation of the Graduate School of Medicine, Chiba University.

2.2. Experimental design

Two experimental designs were used in this study. One involved the transplantation of the DES-exposed germ cells into intact mice (Fig. 1A). We reasoned that if the neonatal administration of DES has adverse effects on the germ cells, then normal spermatogenesis in the DES-exposed germ cells will not occur even in a normal spermatogenic environment. On the other hand, if DES does not affect the germ cells, then normal spermatogenesis in the DES-exposed germ cells can be expected in a normal spermatogenic environment.

The second experimental design involved the transplantation of the normal germ cells into DES-treated mice (Fig. 1B). We reasoned that if postnatal treatment with DES has adverse effects on the somatic cells that support spermatogenesis, then normal spermatogenesis in the normal germ cells will not occur in a DES-treated environment. On the other hand, if DES does not affect the spermatogenesis-supporting somatic cells, then normal spermatogenesis in the normal germ cells can be expected in a DES-treated environment.

The germ cells for transplantation were collected from the testes of GFP mice and conventional C57BL/6 mice were used as the recipient to distinguish the exogenous cells from the endogenous cells.

2.3. Chemical treatment and germ cell transplantation

DES (ICN Biomedicals Inc., Aurora, OH, USA) was dissolved in sesame oil (Sigma–Aldrich Co., Tokyo, Japan). Busulfan (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO; Dojindo Laboratories, Kumamoto, Japan). From Day 1–5, 25 μL of DES (0.5 μg/mouse/day) solution was subcutaneously injected into male mice. In the control group, 25 μL of sesame oil (vehicle) was injected.

Prior to the transplantation experiment, the adverse effect of the neonatal treatment with DES on spermatogenesis was evaluated using C57BL/6 mice. After 20 weeks of treatment, the body weight was measured and the testes were removed under deep anesthesia and weighed. The testes were fixed in the Bouin's fluid and embedded in paraffin: 5-μm-thick sections were cut and stained with hematoxylin and eosin (HE). Ten sections that were randomly selected from each section were observed. In this experimental group, eight male mice were vehicle-treated and nine male mice were DES-treated.

The endogenous germ cells were destroyed by single intraperitoneal administration of busulfan (50 mg/kg) in recipients of the transplantation, i.e., 5- or 6-week-old C57BL/6 mice. For the collection of the donor germ cells, the testes of GFP mice were excised and the tunica albuginea was removed and collected in the phosphate buffered saline (PBS). The peeled testes were treated with 1 mg/mL collagenase type IV (Sigma–Aldrich, St. Louis) in PBS for 20 min at 37 °C. The collagenase-treated testes were suspended in 0.25% trypsin in Hank's balanced salt solution (Invitrogen Co., Ltd., Tokyo, Japan) and incubated for 20 min at 37 °C. The cell suspension was mixed with a half the volume of fetal bovine serum (FBS; Invitrogen Co., Ltd., Tokyo, Japan) and then filtered through a 60-μm nylon mesh in order to remove the cell clumps. The cells were washed twice with Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich Japan, Tokyo, Japan) supplemented with 0.75% bovine serum albumin (DMEM-BSA). Next, the enzyme-treated cells were fractionated on a discontinuous density gradient that was prepared using the following concentrations of Percoll (Pharmacia Biotech, Tokyo, Japan): 65, 50, 40, 36, 33, 30, 25 and 20% in DMEM-BSA. After centrifugation (800 × *g*, 30 min, 18 °C), fractions of 33 and 36% of Percoll density were collected and washed twice with DMEM-BSA. The cells were then suspended in DMEM supplemented with 1% FBS at a concentration of 10^8 cells/mL.

For the transplantation, the cell suspension was mixed with equal volume of 0.4% trypan blue in PBS so that the injected cell suspension was visible. After anesthesia with 50 mg/kg pentobarbital sodium solution (Abbott Laboratories, North Chicago, IL, USA), both the testes of the recipient mice were exposed and the tunica albuginea near the rete testis was cut with a razor blade. The cell suspension was injected into the rete testis by using a glass pipette with an outer diameter of 50 μm (Narishige Co., Ltd., Tokyo, Japan) that had been coated with silicon (Sigma–Aldrich, St. Louis). The injected volume was approximately 7 μL for each testis. The success of the injections was confirmed by the spread of trypan blue in the seminiferous tubules.

In this experiment, the germ cells collected from the two testes were transplanted into a recipient testis. The total number of transplanted testes was 50 (vehicle donors: 16, DES donors: 16, vehicle recipients: 8 and DES recipients: 10).