Steel–Dickie (Sl^d) mutation affects both maintenance and differentiation of testicular germ cells in mice*

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Summary. The effects of Steel–Dickie (Sl^d) mutations on testicular germ cell differentiation were investigated using experimental cryptorchidism and its surgical reversal in mutant, C57BL/6-Sl^d/+ and wild-type C57BL/6-+/+ mice. In Sl^d/+ cryptorchid testes the maintenance of undifferentiated type-A spermatogonia was impaired and their numbers decreased. In contrast, the proliferative activity of type-A spermatogonia in the cryptorchid testis of mutant mice appeared normal as judged by their progression through the cell cycle. Surgical reversal of cryptorchidism resulted in regenerative differentiation of mature germ cells in +/+ testes. However, the regenerative differentiation of type-A spermatogonia which remained in Sl^d/+ cryptorchid testes was strongly impaired, particularly at two steps of cellular differentiation, from type-A spermatogonia to intermediate or type-B spermatogonia and at meiotic division. Furthermore, in mutant mice, no significant recovery of testicular weight was observed after surgical reversal compared with +/+ mice.

Keywords: Sl^d mutation; testis; germ cell; Sertoli cell; cryptorchidism; mouse

Introduction

A double gene dose of mutant alleles at the Steel (Sl) locus produces the pleiotrophic effects of sterility, macrocytic anaemia (Russell, 1979), and a deficiency of melanocytes (Russell & Bernstein, 1966) and mast cells (Kitamura & Go, 1979). The two best-studied mutations at this locus, Sl and Sl^d (Steel–Dickie), exhibit effects that are typical of the majority of Steel mutations. Sl/+ and Sl^d/+ mice are only mildly affected. They are viable, and mildly anaemic, having smaller gonads and a lighter coat colour with white belly spot, white feet and white tail tip (Sarvella & Russell, 1956). Sl homozygotes die of severe anaemia before birth. On the other hand, Sl^d homozygotes and Sl/Sl^d heterozygotes display all the pleiotrophic effects of Sl homozygotes, but can survive for a few days to a few months after birth. They have a white coat and black eyes. Although the phenotypic expression of each mutation is slightly different from one mutant to another, they showed similar pleiotrophic effects on the differentiation of bone marrow cells, melanocytes and germ cells.

The sterility of Sl/Sl mice of both sexes results from the absence of germ cells in the gonads which has been attributed to an interference with the migration of primordial germ cells from the epiblast to the genital ridges (Bennett, 1956; Snow & Monk, 1983). However, McCoshen & McCallion (1975) were unable to find such interference and thought that the reduced number of primordial germ cells in Sl/Sl^d embryos was caused either by failure in proliferation or by an excessive rate of cell death.

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Experimentally induced cryptorchid testes are used to assess the process of spermatogenesis, since seminiferous tubules of such testes contain only type-A spermatogonia as a germ cell and supporting cells remain unaffected and fulfil their functions effectively. After surgical reversal of experimental cryptorchidism (orchidopexy), newly differentiated germ cells can be easily detected (Nishimune *et al.*, 1978). The ability of type-A spermatogonia to resume spermatogenesis and kinetic analysis of germ cell differentiation *in vivo* were demonstrated by using experimental cryptorchidism and its surgical reversal in a previous study (Nishimune *et al.*, 1978). Using these techniques, we have already demonstrated that there were no differences in the functions of the stem cells of testicular germ cells between $Sl^d/+$ mutant and wild-type cryptorchid testes, whereas the processes of regenerative differentiation of germ cells in $Sl^d/+$ mutant testes were retarded in surgically reversed testes (Nishimune *et al.*, 1980). In the present study the effects of the Sl^d mutation on the maintenance of undifferentiated type-A spermatogonia and regenerative differentiation from spermatogonia were studied in $Sl^d/+$ testes, since no germ cells are actually observed in the testes of Sl^d/Sl^d mice.

Materials and Methods

Animals. C57BL/6 mice ($Sl^d/+$ and $+/+$) were raised in our animal facilities using parental stocks originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The $Sl^d/+$ genotype of animals was inferred from the presence of white spots and dilution of coat colour. Normal littermates were used as control wild-type ($+/+$) animals.

Experimental cryptorchidism and its surgical reversal (orchidopexy). Experimental cryptorchidism was carried out at the age of 2–3 months (Nishimune *et al.*, 1978). From 32 to 150 days after operation, some of the animals were killed by cervical dislocation and their testes were removed. Orchidopexy was carried out on the remaining animals after 2–2.5 months and the mice were killed at 100 days.

Diameter of seminiferous tubules and counting of cells. Testes were removed, weighed and fixed in Bouin's solution, and embedded in paraffin wax. Sections (5 μ m thick) were stained with haematoxylin and eosin and diameters of seminiferous tubules were measured with an eyepiece micrometer. The circumferences of the tubules were calculated by assuming the cross-section of the tubule to be a circle. Numbers of Sertoli cells and type-A spermatogonia in more than 30 cross-sections of seminiferous tubules of cryptorchid testes were randomly counted under the microscope and expressed as the number per 1-mm circumference of seminiferous tubule. Each tubule from a surgically reversed testes was classified into the following 6 categories: (1) degenerate in which only Sertoli cells were detectable; (2) no germ cells beyond type-A spermatogonia were present; (3) > 5 germ cells per tubule developed as far as the intermediate and/or type-B spermatogonia; (4) > 5 germ cells per tubule developed as far as the resting primary and/or leptotene spermatocytes; (5) > 5 germ cells per tubule developed as far as zygotene and/or pachytene spermatocytes; (6) > 10 spermatids per tubule. These abnormal tubules were calculated as a percentage of the total tubules observed. The numbers of spermatids in surgically reversed testes were counted from 30 cross-sections of tubules with spermatids.

Detection of type-A spermatogonia in S phase. The proportion of type-A spermatogonia which incorporated 5 bromodeoxyuridine (BrdU) (Sigma Chemical Co., St Louis, MO, USA) was used as an index for cell proliferation as described by Dolbeare *et al.* (1983). Five mice of each genotype that had been cryptorchid for 3 months were injected intraperitoneally with 1.2 mg BrdU per mouse in 0.5 ml PBS. At 1 h after injection 5 mice were killed. The testes were removed and fixed with 70% ethanol for 1 day at 4°C, embedded in paraffin wax, and sectioned at 5 μ m. The immunostaining to detect nuclei labelled with BrdU was performed using a monoclonal antibody against BrdU (Becton-Dickinson, Mountain View, CA, USA) (Grantzner, 1982). Bound monoclonal antibody was visualized using a Vectastain ABC®, anti-mouse IgG, biotin-avidin-peroxidase kit (Vector Labs, Burlingame, CA, USA). The incorporation of BrdU into the nucleus was recognized by the presence of dark brown granules of diaminobenzidine.

Results

Effects of duration of cryptorchidism on the cellular components of seminiferous tubules

Figure 1 shows histological features of testes from intact C57BL/6- $+/+$ and $Sl^d/+$ mice and cryptorchid mice and from mice 100 days after surgical reversal. All seminiferous tubules of $+/+$ and $Sl^d/+$ testes from intact mice contained normally developing, spermatids and spermatozoa (Figs 1a, b).

Fig. 1. Light micrographs of testicular cross-sections showing: intact testes of $+/+$ (a) and $Sl^d/+$ (b) mice, cryptorchid testes of $+/+$ (c) and $Sl^d/+$ (d) mice 3 months after the experimental cryptorchidism and 100 days after surgical reversal of $+/+$ (e) and $Sl^d/+$ (f) mouse testes which had been cryptorchid for 80 days. The bar indicates 100 μ m.

In mice of the $+/+$ and $Sl^d/+$ genotypes, success of experimental cryptorchidism was confirmed histologically 3 months after operations. All differentiated germ cells (including intermediate and type-B spermatogonia) were absent, whereas type-A spermatogonia, Sertoli cells, Leydig cells, and other supporting elements of seminiferous tubules remained morphologically intact (Figs 1c, d). Furthermore, the degree of degeneration of $Sl^d/+$ mutant testes in the cryptorchid condition seems to be different from that in wild-type testes, i.e. a decrease in the number of type-A spermatogonia was observed in association with the length of time that testes were in the abdominal cavity (Fig. 2). The process of degeneration of type-A spermatogonia was studied by differential scoring of the seminiferous tubules having a different number of type-A spermatogonia (Fig. 3). In $Sl^d/+$ cryptorchid testes, reduction in the number of tubules having many type-A spermatogonia was predominant. Furthermore, there was a remarkable increase in the number of tubules having no type-A spermatogonia as the duration of cryptorchidism increased (Figs 1d & 3). No germ cells were present in 25% of the tubules after 3 months, whereas no significant degeneration of type-A

spermatogonia was observed in the testes of wild-type mice (Fig. 3). From the slope of the degeneration curve of type-A spermatogonia (Fig. 2), we calculated the apparent half-life of type-A spermatogonia to be approximately 60 days in the $Sl^d/+$ testis in the cryptorchid condition. Furthermore, the number of Sertoli cells, whose role seems to be important for the maintenance of type-A spermatogonia in cryptorchid testes (Nishimune *et al.*, 1986), also decreased more in $Sl^d/+$ testes than in $+/+$ testes during the period of cryptorchidism (Fig. 2). From the slope of the degeneration curve of Sertoli cells, the half-life of Sertoli cells was also estimated to be approximately 60 days in $Sl^d/+$ cryptorchid testes. In contrast, Sertoli cells in $+/+$ cryptorchid testes are rather stable though their numbers gradually decrease in the cryptorchid condition (Fig. 2) (Nishimune *et al.*, 1986).

Fig. 2. Changes in the number of type-A spermatogonia and Sertoli cells with duration of cryptorchidism. Each plot represents the average values of more than 4 testes. Error bar indicates s.e.

Proliferative ability of type-A spermatogonia in the cryptorchid condition

The reduced numbers of type-A spermatogonia may be due to a failure in proliferation and/or to an increased rate of cell death. To learn more about the proliferative ability of type-A spermatogonia, and to compare the functions of undifferentiated type-A spermatogonia of wild-type ($+/+$) mice with that of mutant ($Sl^d/+$) mice, we performed cytological analyses of germ cells by scoring the number of mitotic cells and cells in S phase among the population of BrdU-labelled type-A spermatogonia. Although the number of type-A spermatogonia decreased in mutant but not in wild-type cryptorchid testes staying in the abdominal cavity for 3 months (Figs 1 & 2), the labelling index (ratio of the number of labelled cells to total number of cells) was not significantly different between mutant and control wild-type testes. Mitotic index, which reflects the mitotic activity of type-A spermatogonia, was also the same in the testes of both genotypes (Table 1). These results indicate that type-A spermatogonia present in $Sl^d/+$ cryptorchid testes seem to progress through their cell cycle at a rate similar to that in $+/+$ testes, although they might degenerate sooner in mutant testes than in wild-type testes.

Fig. 3. Histograms showing the distribution of type-A spermatogonia during cryptorchidism for 1–5 months. The number of seminiferous tubules having 0–4 and more than 5 type-A spermatogonia per cross-section was differentially scored from a total of 30 tubular cross-sections of each testis and the average values of more than 4 testes are shown. Error bar indicates s.e.

Table 1. Labelling index and mitotic index of type-A spermatogonia in testes of mice made cryptorchid for 3 months

Genotype	No. of testes	Labelling index (%)*	Mitotic index (%)†
$Sl^d/+$	6	38.3 ± 2.2	6.4 ± 1.2
$+/+$	4	40.2 ± 3.1	6.0 ± 1.2

Values are mean $\pm$ s.e.m.

Approximately 300 type-A spermatogonia were randomly counted in microscopic fields in each testicular sample.

*% of type-A spermatogonia labelled with BrdU/total no. of type-A spermatogonia counted.

†% of the number of mitotic cells/total no. of type-A spermatogonia counted.

Recovery of testicular weight after surgical reversal

Surgical reversal was used to study the regenerative ability of germ cells in cryptorchid testes (Nishimune *et al.*, 1978). The process of germ cell differentiation in mutant testes was compared with that of wild-type mice. The effect of surgical reversal on the weight of cryptorchid testes is shown in Fig. 4. After surgical reversal in $+/+$ mice of cryptorchid testes that had been in the abdominal cavity for 2 months, a progressive recovery occurred up to 100 days. In the testes of mutant mice, however, the ability to recover testicular weight was almost completely lost; even by >100 days after surgical reversal, no significant increase in testicular weight was observed.

Fig. 4. Recovery of testicular weight after surgical reversal of cryptorchid testes. Each value represents the average value of more than 4 testes. Testicular weights of intact mice of the $+/+$ and $SI^d/+$ strains are also plotted, and each plot represents the average values of more than 8 testes. Error bar indicates s.e.

Regeneration of seminiferous epithelium by surgical reversal

Virtually all seminiferous tubules of $+/+$ mice contained normally developing, post-meiotic spermatids at 100 days after surgical reversal (Figs 1e & 5). Fertilizing ability is also recovered about 80–100 days after surgical reversal in wild-type mice (Nishimune *et al.*, 1978). In contrast, in the testes of mutant $SI^d/+$ mice, the regenerative differentiation of germ cells was extremely impaired (Fig. 1f); 35% of the tubules contained no germ cells, 10% of the tubules had no cells after type-A spermatogonia, and 15% of tubules had no cells beyond the zygotene–pachytene stages (Fig. 5, I, II and V, respectively). Very few tubules contained germ cells developed as far as the intermediate or type-B spermatogonia and resting primary stage-leptotene spermatocytes (Fig. 5, III and IV, respectively). These results indicate that 10% and 15% of the tubules showed impairment of differentiation at type-A spermatogonia to intermediate-B spermatogonia and at pachytene spermatocyte to meiotic division, respectively. The rate of increase of the differentiated tubules is therefore greatly impaired in $SI^d/+$ compared to $+/+$ testes. Although 35% of the tubules contained germ cells as far as the post-meiotic spermatid stage (Fig. 5, VI), quantitative evaluation of

Fig. 5. Differentiation of germ cells after surgical reversal of cryptorchid testes. Values are the average of 6 surgically reversed testes and recorded as a proportion of each tubule type per total tubular cross-sections counted. Open column; $Sl^d/+$ testes, hatched column; the $+/+$ testes. The vertical error bar indicates s.e. I = degenerative tubules in which only Sertoli cells were detectable; II = no germ cells beyond type-A spermatogonia were present; III = intermediate-B spermatogonia were the most advanced germ cells present; IV = resting primary and leptotene spermatocytes were the most advanced germ cells present; V = zygotene-pachytene spermatocytes were the most advanced germ cells present; VI = spermatids were the most advanced germ cells present.

Table 2. Number of spermatids and diameter of seminiferous tubules in testes of mice in which cryptorchidism lasting 2–2.5 months had been reversed for 100 days

Genotype	No. of tubules observed	Diameter (μ m)	No. of spermatids
$Sl^d/+$	180	103 ± 1.0	1243 ± 103
$+/+$	180	168 ± 1.7	5012 ± 152

Values are mean $\pm$ s.e.m.

Counted from 30 tubular cross-sections of VI tubules in Fig. 5 per testis.

differentiated germ cells indicates that substantial impairment of spermatogenesis exists in mutant testes. The number of spermatids in tubules of surgically reversed $Sl^d/+$ testes was approximately 25% that of $+/+$ control testes and their diameter was also less (Table 2). Germ cell differentiation is therefore greatly impaired in $Sl^d/+$ testes, both qualitatively and quantitatively.

Discussion

Little information exists on the effect of the single gene dose of Sl^d mutation on the differentiation of germ cells in adult testes. The present results show that the Sl^d mutation affects testicular germ cell differentiation in the $Sl^d/+$ condition in the same manner as does the $Sl/+$ mutation in the WC genetic background, i.e. germ cell differentiation is impaired at two stages, from type-A spermatogonia to intermediate or type-B spermatogonia and at meiotic division (Nishimune *et al.*, 1980).

Moreover, we have now demonstrated that the maintenance of type-A spermatogonia in $Sl^d/+$ testes is difficult in the cryptorchid condition (Figs 2 & 3), despite the fact that they showed no difference in proceeding through their cell cycles (Table 1). Detailed analyses of the degeneration processes of experimental cryptorchidism demonstrate that the number of type-A spermatogonia decreases with the duration of cryptorchidism, although the cell cycle progression of surviving type-A spermatogonia is not retarded. We estimate the apparent half-life of type-A spermatogonia in mutant testes in the cryptorchid condition to be approximately 60 days. The pattern of decrease in type-A spermatogonia begins with a decrease in the number of germ cells per tubule followed by an increase in the number of tubules having no germ cells at all (Fig. 3). This is quite different from the cryptorchid testes of littermate wild-type control mice and $Sl/+$ mutants in the WC genetic background (Nishimune *et al.*, 1980) which show no degeneration of type-A spermatogonia during the period of observation. Since spermatogenesis is so limited in the surgically reversed cryptorchid testes of the $Sl^d/+$ mutation (Fig. 5) compared with $Sl/+$ testes in the same condition (Nishimune *et al.*, 1980), the $Sl^d/+$ mutation and/or C57BL/6 genetic backgrounds may have more severe deleterious effects than the $Sl/+$ mutation in the WC genetic background. Alternatively, the Sl^d mutation may have a more severe effect on spermatogenesis specifically on the C57BL/6 genetic background.

The degeneration of type-A spermatogonia may be associated with the decrease in the number of Sertoli cells. The Sertoli cells in cryptorchids seem to be more sensitive in mutant than in wild-type mice. Although it is not yet known how the Steel mutation affects spermatogenesis, the cause of anaemia (McCulloch *et al.*, 1965; Bernstein, 1970) and lack of tissue mast cells (Kitamura & Go, 1979) and melanocytes (Mayer & Green, 1968) are known to result from a defect in microenvironments. The deficient spermatogenesis observed in present study may also, therefore, be attributed to a defect in supporting elements in the testes such as Sertoli cells in the seminiferous tubules and peritubular myoid cells in extratubular cells, whereas the activity of Leydig cells to produce androgens is not impaired in $Sl^d/+$ mice (data not shown). Furthermore, by transplanting seminiferous tubules from cryptorchid testes of $+/+$ mice into testes of Sl/Sl^d mice, the extratubular environment is demonstrated to be intact in these mutant mice (Kuroda *et al.*, 1989). By using aggregation chimaeras from Sl/Sl^d and $+/+$ embryos, Nakayama *et al.* (1988) demonstrated that some Sl/Sl^d germ cells had differentiated into functioning spermatozoa. These results suggested that Sertoli cells and/or tissue microenvironment of $+/+$ genotype may help mutant germ cells differentiate and that no intrinsic defects exist in mutant germ cells.

Although there are many differences between the testicular stem cells and the haematopoietic stem cells, the action of the Steel mutant gene seems to be similar in both types of cells. (1) Only slight reduction in spermatogenesis and erythropoiesis was observed in the intact $Sl/+$ or $Sl^d/+$ mice (Russell & Berstein, 1966). However, defects in the $Sl/+$ mutant mice became very evident in stressed conditions, i.e. cryptorchidism and surgical reversal for spermatogenesis (Nishimune *et al.*, 1980) and sublethal irradiation and erythropoietin injection for erythropoiesis (McCarthy *et al.*, 1977). (2) The Steel mutation affects various stages of differentiation. Spermatogenesis may be suppressed at the primordial germ cell level (Mintz & Russell, 1957), during the maintenance of type-A spermatogonia (Figs 2 & 3), or in the differentiation of type-A spermatogonia (Fig. 5) (Nishimune *et al.*, 1980). Similarly, erythropoiesis is suppressed both at the haematopoietic stem cell level (McCulloch *et al.*, 1965; Wiktor-Jedrzejczak *et al.*, 1979) and at the early erythroid progenitor cell level (Chui *et al.*, 1978). These impairments of cell differentiation caused by Steel mutations are common to spermatogenesis and haematopoietic cell differentiation. Therefore, it may be useful to study the factor(s) and mechanism(s) controlled by the Sl^d gene by using the regenerative differentiation of testicular germ cells.

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