Switching of mouse spermatogonial proliferation from the *c-kit* receptor-independent type to the receptor-dependent type during differentiation

Y. Tajima¹, K. Sawada², T. Morimoto¹ and Y. Nishimune¹

¹Research Institute for Microbial Diseases, Osaka University, Yamadaoka, Suita, Osaka 565, Japan; and

²Department of Pathology, Osaka Medical College, Takatsuki, Osaka 569, Japan

Testicular cells composed mostly of germ cells and immature Sertoli cells from neonatal mice 2 and 5 days old were cultured to investigate germ-cell proliferation mediated by the *c-kit* receptor. The addition of antibody to block the interaction of the *c-kit* receptor with its ligand inhibited the proliferation of cultured spermatogonia from 5-day-old mice in a dose-dependent manner, but not from that of 2-day-old mice. The addition of anti-*c-kit* ACK2 monoclonal antibody also inhibited the proliferation of spermatogonia from 5-day-old mutant *Sl^{fl}/Sl^{fl}* mice but not of 5-day-old mutant *W^e/W^e* mice. The results indicate that *c-kit*-positive type A spermatogonia in the testes of 5-day-old mice require steel factor (kit ligand) for their proliferation, whereas self-renewal and differentiation of *c-kit*-negative primitive type A spermatogonia in the testes of 2-day-old mice do not.

Introduction

Mutations at the murine *dominant white-spotting* (*W*) and the *steel* (*Sl*) loci lead to defects in haematopoiesis, melanogenesis and gametogenesis (Russell, 1979). Recent progress in the studies of these loci has demonstrated that the *c-kit* gene that encodes a transmembrane tyrosine kinase receptor is allelic with the *W* locus (Chabot *et al.*, 1988). Characterization of the growth factor binding to the *c-kit* receptor led to the identification of the gene encoding the steel factor (SF; the kit ligand), which is allelic with the *Sl* locus (Anderson *et al.*, 1990; Huang *et al.*, 1990; Williams *et al.*, 1990; Zsebo *et al.*, 1990).

Several severe mutations of *Sl* (*Sl^{sl.gb,8H,10H,12H,18H}*) have been shown by Southern blot analysis to be deletions of the gene encoding SF, which lead to loss of function (Copeland *et al.*, 1990). Although the *Steel–Dickie* allele (*Sl^{fl}*) is viable and less severe, in the homozygous condition it displays all of the pleiotropic effects associated with *Sl* mutations, suggesting some residual functional activity of SF. Molecular analysis has indicated that the *Sl^{fl}* allele arose as a result of an intragenic deletion, including the transmembrane domain and the carboxy terminus, which generated a secreted, biologically active SF protein product (Brannan *et al.*, 1991; Flanagan *et al.*, 1991). The *Sl^{pan}* (*Sl^f*) mutations have normal SF coding sequences but the expression of such SF transcripts is consistently reduced in several tissues including the testis and ovary of homozygous mutant mice, suggesting that *Sl^{pan}* affects SF expression (Huang *et al.*, 1993). In homozygous *Sl^{pan}/Sl^{pan}* and *Sl^f/Sl^f* mice, males are fertile while females are sterile (Beechey *et al.*, 1986). The *Sl^{17H}* mutation results from a defect that causes the SF cytoplasmic domain to be read from an alternative reading

frame. *Sl^{17H}* produces male sterility in the homozygous state. In *Sl^{17H}/Sl^{17H}* mice, the first wave of spermatogenesis is completed, but later on sperm development is almost completely stopped leading to a depletion of differentiating germ cells by 8 weeks of age (Brannan *et al.*, 1992).

In the testis, expression of the *c-kit* receptor can be observed by *in situ* hybridization and immunohistochemical analysis in type A spermatogonia, and remains during further spermatogonial proliferation up to the early preleptotene spermatocyte stage; it is also present on Leydig cells (Manova *et al.*, 1990; Yoshinaga *et al.*, 1991). We have reported that the *W* and *Sl* genes play some important roles in spermatogenesis (Nishimune *et al.*, 1980; Koshimizu *et al.*, 1991; Sawada *et al.*, 1991; Tajima *et al.*, 1991a) and have demonstrated that Sertoli cells are a main producer of biologically active SF in a membrane-bound form (Tajima *et al.*, 1991b). Furthermore, cAMP analogues stimulate an increase in the amount of mRNA (Rossi *et al.*, 1993) and biologically active SF (Tajima *et al.*, 1993) produced in cultured primary mouse Sertoli cells. Injection of an antagonistic anti-*c-kit* antibody (ACK2 mAb) *in vivo* results in a loss of differentiating type A spermatogonia, but has no effect on the transition of intermediate spermatogonia to type B spermatogonia and on spermatocytes (Yoshinaga *et al.*, 1991).

SF is produced from two alternatively spliced mRNAs as transmembrane proteins that may function as cell-associated proteins or may be proteolytically processed to produce the soluble form of SF (Flanagan *et al.*, 1991; Huang *et al.*, 1992). Both the soluble and membrane-bound forms of SF are biologically active. The membrane-bound form is more effective in promoting the survival and proliferation of primordial germ cells (Dolci *et al.*, 1991; Matsui *et al.*, 1991). The forms of SF mRNA present in the testes suggest that the

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membrane-bound form of SF predominates in 5-day-old mice (Manova *et al.*, 1993). In contrast, the soluble form of SF can promote DNA synthesis in type A spermatogonia (Rossi *et al.*, 1993). However, we previously noted that cell–cell contact is indispensable for the activity of SF produced by Sertoli cells to stimulate *c-kit* receptors on mast cells, that SF appears to be associated with the cell surface of Sertoli cells (Tajima *et al.*, 1991b), and that SF presumably also stimulates the growth and differentiation of germ cells.

In the present study, we investigated the role of the *c-kit* receptor and SF in spermatogonial proliferation and differentiation *in vitro*. Our results demonstrate that proliferation of *c-kit*-positive type A spermatogonia require SF produced by Sertoli cells but that self-renewal and differentiation of *c-kit*-negative primitive type A spermatogonia to *c-kit*-positive spermatogonia is independent of *c-kit* and SF.

Materials and Methods

Cell preparation

C57BL/6 mice (*Sf*^d/+, *W*^p/+ and ++) were raised in our animal centre. Sertoli cells and germ cells were isolated from 2-day-old and 5-day-old mice, as reported by Maekawa and Nishimune (1991). Approximately 5×10^4 cells in 250 μ l of minimum essential medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal calf serum were plated in each of the 16 chambers of a tissue culture slide (base area, 0.3 cm² per chamber; Nunc, Naperville, IL).

Antibodies

Germ cells were detected on the slides by immunoperoxidase staining using an anti-testicular germ cell TRA-98 monoclonal antibody (mAb) that recognizes exclusively germ cell nuclei from early spermatogonia to round spermatids (K. Sawada, unpublished). Anti-*c-kit* ACK2 mAb, which recognizes the extracellular domain of the murine *c-kit* receptor and antagonizes the interaction of the receptor with its ligand (Nishikawa *et al.*, 1991), was supplied by S-I. Nishikawa (Kyoto University Medical School, Kyoto).

Cell culture and [³H]thymidine labelling

Cultured testicular cells were maintained at 32.5°C in an incubator under an atmosphere of humidified 5% CO₂. Sixteen hours after plating, antibodies (ACK2 mAb and a rat antibody IgG2b, as control) were added to the culture medium. After 24 h the cultures were pulsed for 4 h with [³H]thymidine at 1 μ Ci ml⁻¹. Cells were fixed in cold acetone for 5 min and processed for immunostaining and autoradiography, as described below.

Immunostaining

The germ cells were immunostained by incubating the cultured slides with TRA-98 mAb at room temperature for 1 h. To stain for the *c-kit* receptor, the cultured slides were

incubated with purified 10 μ g ACK2 mAb antibody ml⁻¹ in phosphate buffer at 4°C overnight. Antibody binding to cells was detected using the avidin–biotin–peroxidase complex with the Vectastain elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's recommendation, and finally immersing in diaminobenzidine substrate solution.

Autoradiography

Autoradiograms were prepared by dipping the slides into NR-M2 emulsion. After exposure for 7 days, the slides were developed, fixed and mounted for examination under a microscope.

Results

Expression of the *c-kit* receptor on testicular germ cells cultured in vitro

Germ cells and supporting cells from 2-day-old and 5-day-old mice were cultured for 24 h, and immunostained with TRA-98 mAb and ACK2 mAb. As shown in Fig. 1, round cells from both 2-day-old and 5-day-old testes were stained with TRA-98 mAb; frequently, two cells were connected. In contrast, flat adherent cells that spread out on the slides were not immunostained. These results identified the round cells as germ cells or spermatogonia and the flat cells as immature Sertoli cells. Furthermore, many germ cells from 5-day-old testes were *c-kit*-positive, whereas few positive cells were detected in the culture from 2-day-old testes (Fig. 1), indicating that the *c-kit*-positive spermatogonia in 5-day-old testes derived from *c-kit*-negative germ cells in 2-day-old testes.

Effects of the antagonistic anti-*c-kit* ACK2 mAb on the proliferation of spermatogonia in vitro

The function of the *c-kit* receptor in the proliferation of spermatogonia *in vitro* was investigated with ACK2 mAb. After plating the testicular cells from 2-day-old and 5-day-old mice and treating them with various concentrations of ACK2 mAb (2.5–50 μ g ml⁻¹) for 24 h, they were labelled with [³H]thymidine. The number of labelled germ cells stained with TRA-98 mAb was counted. The proportion of [³H]thymidine-labelled germ cells from 5-day-old mice decreased significantly ($P < 0.01$) with the addition of 5 μ g ACK2 mAb ml⁻¹ from 40% to 25%, but not from those of 2-day-old mice (Fig. 2). The class-matched control antibody at 10 μ g ml⁻¹ had no effect (Fig. 2). As germ cells from 5-day-old mice contain both *c-kit*-positive and *c-kit*-negative cells, 22% of germ cells from 5-day-old mice still proliferated even at the highest concentration of antibody used (50 μ g ml⁻¹) (Fig. 2).

The role of the *c-kit* receptor on the proliferation of spermatogonia was investigated further by studying the effect of the ACK2 mAb differentially on *c-kit*-positive or *c-kit*-negative germ cells. The proportion of [³H]thymidine-labelled *c-kit*-positive germ cells from 5-day-old mice decreased in

Fig. 1. Expression of the *c-kit* receptor in mouse testicular cell cultures. Testicular cell cultures from (a, b) 2-day-old and (c, d) 5-day-old mice were immunostained for germ cells with (a, c) TRA-98 monoclonal antibody (mAb) and for *c-kit* receptor with (b, d) ACK2 mAb. Scale bar represents 50 μm .

response to the ACK2 mAb ($5 \mu\text{g ml}^{-1}$) from 42% to 15% compared with no decrease in *c-kit*-negative germ cells. These results indicated that the majority of *c-kit*-positive germ cells in 5-day-old testes proliferated in a manner dependent on the *c-kit* receptor.

*Existence and proliferation of spermatogonia in mutant Sl^d/Sl^d and W^o/W^o mouse testes and the effects of anti-*c-kit* ACK2 mAb on their proliferation in vitro*

Although there are germ cells in the neonatal testes of mutant Sl^d/Sl^d and W^o/W^o mice, their number is small com-

pared with that of the testes of wild-type (+/+) mice. However, the spermatogonia derived from both Sl^d/Sl^d and W^o/W^o mutant mice can proliferate *in vitro* in a similar way to control +/+ spermatogonia (Table 1). Furthermore, some of the spermatogonia from 5-day-old Sl^d/Sl^d and W^o/W^o mice were immunofluorescence-stained with anti-*c-kit* ACK2 mAb, indicating that germ cells from the testes of 5-day-old Sl^d/Sl^d and W^o/W^o mice differentiated into *c-kit*-positive cells (data not shown). To investigate whether the *c-kit* receptor expressed on Sl^d/Sl^d and W^o/W^o spermatogonia affects proliferation *in vitro*, we studied the effect of the anti-*c-kit* ACK2 mAb on the uptake of [^3H]thymidine. As shown in Table 1, the number of labelled spermatogonia in +/+ and Sl^d/Sl^d mice decreased when ACK2

Fig. 2. Effects of antagonistic anti-*c-kit* ACK2 monoclonal antibody (mAb) on the proliferation of mouse spermatogonia *in vitro*. The dose-dependent effect of ACK2 mAb on the proliferation of spermatogonia was tested by adding various concentrations of ACK2 mAb and the cultures were incubated for 24 h followed by the addition of [³H]thymidine for 4 h. The uptake of [³H]thymidine in germ cells from 2-day-old testes (●) and 5-day-old testes (○) stained with TRA-98 mAb were then counted. The reported values are the percentages of labelled germ cell nuclei observed out of more than 600 nuclei. Data represent means $\pm$ SEM of four independent cultures. C: control rat, 10 μ g IgG2b antibody ml⁻¹.

mAb (25 μ g ml⁻¹) was added, although the total number of spermatogonia was small in the mutant mice. In contrast, the proliferation of spermatogonia from 5-day-old *W^o/W^o* mutant mice, which have a defective *c-kit* receptor owing to a missense mutation but a normal size and cell surface expression (Nocka *et al.*, 1990), was not inhibited by ACK2 mAb (Table 1). These results support the notion that (i) some of the *+/+* and *S^d/S^d* spermatogonia differentiated into *c-kit*-positive spermatogonia in 5-day-old testes, which then proliferated in a *c-kit*-SF-dependent manner (Table 1), and (ii) the differentiation step from *c-kit*-negative to *c-kit*-positive spermatogonia is independent of *c-kit* and SF, as the testes of both *S^d/S^d* and *W^o/W^o* 5-day-old mutant mice contain *c-kit*-positive germ cells.

Discussion

In mice and rats there are four generations of type A spermatogonia (A₁–A₄) and one generation each of intermediate (In) and type B spermatogonia (Monesi, 1962; Clermont and Bustos-Obregon, 1968). Kluin and de Rooij (1981) reported that at day 3 after birth the mouse testes contain solely type A spermatogonia, at day 4 the first intermediate spermatogonia appear, and at days 5 and 6 the first type B spermatogonia and primary spermatocytes are observed. Although we cannot analyse the type of spermatogonia of cultured germ cells of 5-day-old testes, the culture we used was designed to contain both differentiated *c-kit*-positive and undifferentiated *c-kit*-negative spermatogonia. The inhibitory effect of anti-*c-kit* antibody on proliferation was observed in the population of *c-kit*-positive germ cells but not in *c-kit*-negative cells. However, some of the *c-kit*-positive cells take up [³H]thymidine even in the presence of an excess amount of blocking antibody. This may be due to the action of other paracrine growth factors; alternatively, Sertoli cells expressing membrane-bound SF may form a tight association with germ cells that blocking antibodies cannot inhibit.

Our data also demonstrate that differentiation of spermatogonia proceeds from 2-day-old *c-kit*-negative germ cells to 5-day-old *c-kit*-positive germ cells. Approximately half of the germ cells in 5-day-old mice are *c-kit* positive. However, this differentiation step does not seem to require *c-kit* receptor stimulation, as the testes of both *S^d/S^d* and *W^o/W^o* 5-day-old mice contain *c-kit*-positive germ cells (the *S^d/S^d* mutant is known to produce soluble SF, and the kinase activity of *W^o/W^o* *c-kit* receptor is only partially impaired *in vitro*; Nocka *et al.*, 1990). From some experiments it might be hypothesized that the *S^d* SF protein product sustains cell migration but not cell proliferation; consequently, the cell membrane form of SF may play a critical role in the proliferative response of cells to *c-kit* (McCoshen and McCallion, 1975; Silvers, 1979).

S^d/S^d fibroblasts and *S^d/S^d* Sertoli cells do not support the proliferation and maintenance of bone marrow mast cells in the absence of interleukin 3 (IL-3), in contrast to wild-type cells (Fujita *et al.*, 1989; Tajima *et al.*, 1991b). Although the testes of *W^o/W^o* mice contain *c-kit*-receptor-positive germ cells (data not shown), and the size and cell surface expression of the *c-kit* receptor kinase and the antigenicity detected by ACK2 mAb

Table 1. Effects of ACK2 monoclonal antibody (mAb) on the proliferation of germ cells in the testes of 5-day-old wild-type, *S^d/S^d*, and *W^o/W^o* mice

Genotype	Fraction of germ cells in S phase (%) ^a			
	Without ACK2 mAb	With ACK2 mAb (25 μ g ml ⁻¹)	Without ACK2 mAb	With ACK2 mAb (5 μ g ml ⁻¹)
++	42.3 $\pm$ 0.8	27.5 $\pm$ 1.8 (35)*	40.7 $\pm$ 0.6	25.1 $\pm$ 1.0 (38)*
<i>S^d/S^d</i>	39.1 $\pm$ 2.2	23.4 $\pm$ 2.0 (40)*	44.2 $\pm$ 3.2	33.6 $\pm$ 3.8 (24)
<i>W^o/W^o</i>	NT	NT	37.8 $\pm$ 1.4	39.2 $\pm$ 1.4 (N)

Cells were immunostained with TRA-98 mAb. Values are means $\pm$ SEM of more than three independent cultures. More than 600 (++) and 100 nuclei (*S^d/S^d* and *W^o/W^o*) were observed. Percentage inhibition is shown in parentheses.

N: no inhibition; NT: not tested.

^aPercentages of labelled germ cells that incorporated [³H]thymidine after 24 h of culture with or without ACK2 mAb.

**P* < 0.01 (Student's *t* test) compared with the value when no antibody was added.

are not affected by the mutation (Nocka *et al.*, 1990), no effect of ACK2 on germ cell proliferation was observed in 5-day-old W^u/W^u mice despite the fact that no further proliferation and differentiation of germ cells could be observed in this mutant mouse. Furthermore, the proliferation and differentiation of spermatogonia into *c-kit*-positive cells in the testes of 5-day-old $+/+$ and Sl^d/Sl^d mice seems to require *c-kit* receptor stimulation. Taking the results from these two mutants together, the signal transduction of the *c-kit* receptor appears to regulate the proliferation and differentiation of germ cells in the testes of 5-day-old mice.

In the testes of mutant Sl^{pan}/Sl^{pan} mice the amount of SF expression is reduced, but this reduction does not have appreciable effects on spermatogenesis and male fertility. It may be that SF is not limiting in spermatogenesis and that the amounts expressed in Sl^{pan}/Sl^{pan} mice are sufficient to maintain spermatogonial proliferation and normal fertility, in contrast to oogenesis where the amount of SF is limiting. Thus, the demand for stimulation by SF in the testes does not seem to be so severe as in other cell systems such as melanocytes, oocytes or mast cells. In some cases, *c-kit* receptor stimulation seems to be more critical in the differentiation and proliferation of primordial germ cells than in spermatogenesis (Koshimizu *et al.*, 1992), whereas in other cases, such as in W/W^u or W^u/W^u mice where the gonocytes in testes cannot differentiate, primordial germ cells can differentiate into gonocytes or spermatogonia.

The SF cytoplasmic tail may have an important, as yet undefined, function that is impaired in Sl^{17H} and Sl^d mice. This could involve a feedback or signalling mechanism normally transmitted through the SF cytoplasmic tail upon interaction with *c-kit*-expressing cells. The germ cells could affect some biological function of Sertoli cells (Jegou, 1991), although the mechanism of germ-cell-mediated stimulation of Sertoli cells has not been identified as involving interactions between the *c-kit* receptor and SF. This notion is supported by the fact that nerve growth factor and tumour necrosis factor α are produced in germ cells while their receptors are synthesized on Sertoli cells, suggesting that germ cells have direct effects on Sertoli cells (Ayer-Le Lievre *et al.*, 1988; De *et al.*, 1993).

The factor that induces the differentiation step from *c-kit*-negative to *c-kit*-positive germ cells is unknown. Evidence from several observations has shown that the testis is an especially rich source of growth factors (Bellvé and Zheng, 1989). With respect to the paracrine action of some intratesticular factors, the roles of inhibin and activin as spermatogonial regulators have been investigated using different experimental approaches (van Dissel-Emiliani *et al.*, 1989; Mather *et al.*, 1990). It has also been proposed that IL-1 α may act as a spermatogonial mitogen on stage-specific DNA synthesis in segments of seminiferous tubules cultured *in vitro* (Parvinen *et al.*, 1991). These testicular growth factors may act as autocrine and paracrine modulators of male gonadal function, but their precise role in the regulation of germ cell proliferation has yet to be defined.

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