—Brief Note—

Xenografting of Bovine Secondary Follicles into Male and Female SCID Mice

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Abstract: *Xenografting of ovarian tissue into immuno-deficient mice is useful in studying the dynamics of follicular development. We have demonstrated that xenografted bovine secondary follicles develop to the antral stage in female severe combined immunodeficient (SCID) mice. We did this by examining the development of bovine secondary follicles (140–190 μm in diameter) that had been grafted into male and female SCID mice for 4 and 6 weeks. We then compared the results for the two groups. The rate of surviving follicles in the grafts was similar in male and female mice, but the survival rate of oocytes was lower in male mice in these follicles, especially the antral follicles. In addition, the basement membranes of relatively large follicles were thinner and torn in the male mice, and erythrocytes had invaded the follicular cavity. The mean diameters of surviving follicles and oocytes were significantly larger in both male and female mice than before grafting. In female mice, the diameter of antral follicles increased gradually as the grafting was prolonged, although the difference was not in significant. Surviving oocytes in the follicles increased in diameter. In contrast, development of antral follicles in male mice seemed to be accelerated, but, in contrast to female mice, the mean diameters of antral follicles and surviving oocytes showed no further increase after 4 weeks of grafting. These results suggest that bovine follicles can develop in male SCID mice, but oocyte degeneration together with the follicular degeneration occurs in large antral follicles at a higher rate in males than in the females.*

Key words: *Cow, Follicular development, Male SCID mouse, Xenografting*

Received: September 9, 2004
Accepted: September 16, 2004
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Introduction

Artificial growth of small immature oocytes in the ovaries can provide a viable new source of mature oocytes to be used in livestock production as well as human reproduction and propagating endangered species. Several culture systems have been developed for domestic animals, but it is difficult to efficiently grow oocytes *in vitro* to the final size and to provide them with the necessary maturational competence. Severe combined immune deficient (SCID) mice which lack functional B and T lymphocytes have been found to be good recipients for xenografting of ovarian follicles, including small oocytes. Such xenografting of ovarian tissue into immuno-deficient mice has been used as a model to verify follicular viability as well as to study the dynamics of follicular development [1–4]. We demonstrated that bovine oocytes in secondary follicles grafted into female SCID mice grew to the final size, and that the grown oocytes matured to the second metaphase [5]. When bovine secondary follicles were grafted into ovariectomized SCID mice, development of the follicles was accelerated [6]. In the present study, bovine secondary follicles were grafted into male SCID mice. At 4 and 6 weeks after grafting, development of the grafted follicles was examined in comparison with those grafted to intact female mice.

Materials and Methods

Ovaries were obtained from pure-bred Japanese Black cows slaughtered at a local slaughterhouse, and were washed in Dulbecco's phosphate buffered saline three times. Approximately 0.5 mm × 0.5 mm × 0.5 mm

slices of ovarian cortex containing only one secondary follicle 140–190 μm in diameter were dissected with fine blades while immersed in TCM199 (pH 7.4, Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 0.1% polyvinylalcohol, 0.85 mg/ml $NaHCO_3$, 0.08 mg/ml kanamycin, and 25 mM HEPES. Secondary follicles were randomly separated into two groups, one for measuring diameters of follicles and oocytes, and the other for xenografting. Groups of 5 to 8 secondary follicles enclosed in connective tissue were inserted under the kidney capsule of 7 male and 10 female SCID mice (8 to 13 weeks old, C. B-17/Icr-*scid* Jcl, Clea Japan, Inc., Tokyo, Japan) with a pipette.

The mice were killed 4 and 6 weeks after xenografting by cervical dislocation. The kidneys containing secondary follicles were carefully torn free with fine forceps. After washing in Dulbecco's phosphate buffered saline, the kidneys were fixed in 3% formaldehyde in phosphate buffered saline. They were dehydrated, embedded in JB-4 (Polyscience, Inc., Niles, IL, USA), serially sectioned at 5 μm, stained with hematoxylin and eosin, and then examined. Surviving follicles were counted and classified into two groups according to the morphology of granulosa cell layers and antrum formation: secondary follicles containing an oocyte encapsulated by two or more layers of granulosa cells but no antrum, and antral follicles having an antral cavity with multiple layers of granulosa cells. The follicles were examined for structural normality in terms of oocyte degeneration, integrity of basement membrane, and the invasion of erythrocytes into the follicular cavity. The serial sections were examined to find the largest cross-section of follicles and oocytes. The diameters of the follicles and oocytes (excluding the zona pellucida) were measured with an ocular micrometer (Nikon, Tokyo, Japan) attached to a microscope, taking two perpendicular measurements to the nearest 1 μm, and recording the average.

The results for all replicates were pooled and analyzed. Statistical differences in the mean diameters of follicles and oocytes were analyzed by Student's *t*-test. Other values were analyzed by chi-square analysis with Yates' correction for continuity. A probability of less than 0.05 was considered significant.

This study was conducted with the approval of the Committee on Animal Experimentation of Kobe University, Rokkodai Campus, Japan.

Results and Discussion

After xenografting, all mice contained developed antral follicles (Fig. 1a). In male mice, there were some hemorrhaged antral follicles that were larger than the others (Fig. 1b). Histological examination revealed that 60 to 73% of follicles survived the grafting for 4 and 6 weeks (Table 1). About one half of the surviving follicles developed to the antral stage irrespective of the sex of the host animals and duration of grafting (Fig. 1c). In the secondary follicles, almost all oocytes in both male and female mice had degenerated (Fig. 1d). Alternatively, those follicles might have stopped growing at the secondary stage after the death of the oocyte. Antral follicles which had a normal structure were observed at a higher rate in female mice (4w: 87%, 6w: 73%) than in male mice (4w: 50%, 6w: 27%) both 4 and 6 weeks after grafting. Furthermore, the antral follicles that developed in female mice contained a morphologically normal oocyte at a significantly higher rate than in male mice. Only in the male mice, basement membranes in some large antral follicles become partially torn with erythrocytes invading the follicular cavity (Fig. 1e).

Table 2 shows follicular development and oocyte growth after xenografting. The mean diameters of follicles and oocytes after grafting increased significantly, compared with those before grafting. Of follicles which developed to the antral stage, mean diameters of structurally abnormal follicles were larger than those of normal follicles both in male and female mice. In female mice, the diameter of total antral follicles had a tendency to increase as grafting was prolonged, and morphologically normal antral follicles also tended to increase. The surviving oocytes in the follicles increased in diameter. In contrast, in male mice, there was no significant difference in the mean diameter of total antral follicles at 4 to 6 weeks after grafting. Nevertheless, the mean diameter of morphologically normal antral follicles in male SCID mice was significantly smaller at 6 weeks than that at 4 weeks. Also, the mean diameter of surviving oocytes in the follicles showed no significant increase after 4 weeks. We know that, in female SCID mice, bovine secondary follicles develop to the antral stage, and the oocytes grow and acquire maturational competence [5]. Results in our study demonstrated that bovine secondary follicles developed in male SCID mice as well. In fact, in both mice and humans, ovarian follicles xenografted into heterogeneous male animals survived and developed in the hosts [7, 8]. Moreover, mouse oocytes grown in male host animals acquired fertilizable competence [8].

In the present study, histological examination

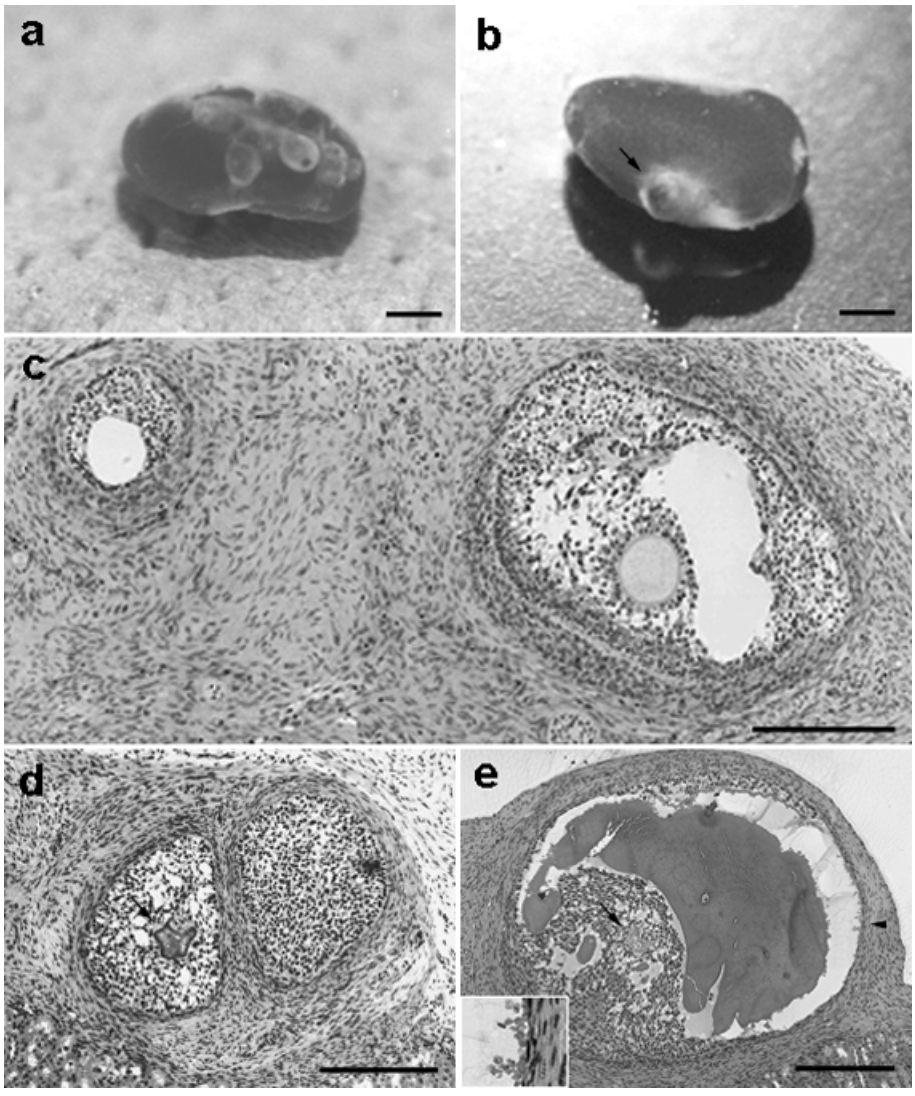

Fig. 1. Xenografted bovine secondary follicles developed under the kidney capsules of female (a) or male (b) SCID mice at six weeks after grafting. A hemorrhaged large follicle is observed in a male mouse (b, arrow). Three antral follicles in a histological section of a xenograft in a female mouse (c), and a secondary follicle containing a degenerated oocyte in a male mouse (d, arrow) after 6 weeks. In male mice, some large antral follicles allowed invasion of erythrocytes from a tear in the basement membrane (e, arrowhead and inset). In this follicle, the oocyte degenerated (e, arrow). Bars represent 2 mm in (a) and (b), 0.4 mm in (c), and 0.2 mm in (d) and (e).

revealed that 27 to 40% of bovine secondary follicles had been lost in xenografts at 4 to 6 weeks after grafting, which suggests that some follicles degenerated and disappeared during the grafting period. The rate of surviving follicles in the grafts was similar in male and female mice, but the survival rates of oocytes and the rates of morphologically normal follicles were lower in male mice, especially in the antral follicles. We observed that the basement membrane of relatively large follicles became thinner and torn, and erythrocytes invaded the follicles only in male mice. It is interesting that such abnormalities have also been observed when bovine secondary follicles are xenografted into ovariectomized female SCID mice [6]. Furthermore, after grafting, the survival rate of antral follicles was similar in male and female mice, but the mean diameter of the follicles in male mice was larger than that in female mice. Therefore, the differences

Table 1. Abnormal structures of bovine follicles in male and female SCID mice after xenografting

Duration of grafting[1] (weeks)	Sex of mice	No. of mice	No. of grafted follicles	No. (%) of surviving follicles	Follicular stage	No. of follicles examined	No. (%) of follicles with abnormal structure	Abnormal structures of follicles[2]		
								Oocyte	Loss of basement membrane integrity	Erythrocyte invasion
4	♂	3	19	12 (63)	Antral	6	3 (50)^{ab}	3 (50)^{ab}	3 (50)	1 (17)
					Secondary	6	5 (83)^{bc}	5 (83)^{ab}	0	—
4	♀	4	26	19 (73)	Antral	8	1 (13)^{a}	0	1 (13)	0
					Secondary	11	8 (73)^{bc}	8 (73)^{ab}	1 (9)	—
6	♂	4	32	20 (63)	Antral	11	8 (73)^{bc}	8 (73)^{ab}	5 (45)	3 (27)
					Secondary	9	4 (44)^{ab}	4 (44)^{bc}	2 (22)	—
6	♀	6	42	25 (60)	Antral	11	3 (27)^{a}	1 (9)^{c}	3 (27)	0
					Secondary	14	13 (93)^{c}	13 (93)^{a}	3 (21)	—

[1] Groups of 5–8 bovine secondary follicles 140–190 μm in diameter were xenografted under the kidney capsule of each SCID mouse. [2] Oocyte degeneration, integrity of the basement membrane and the invasion of erythrocytes into the follicular cavity were examined. [a–c] Values with different superscripts in the same column differ significantly ($p<0.05$).

Table 2. Follicular development and oocyte growth in bovine secondary follicles xenografted into male and female SCID mice

Duration of grafting* (weeks)	Sex of mice	Follicular stage	No. of follicles examined	Mean diameter of follicles (μm ± SD)			No. (%) of surviving oocytes	Mean diameter of oocytes (μm ± SD)		
				Total	Normal follicles	Abnormal follicles		Total	Oocytes in normal follicles	Oocytes in abnormal follicles
Before	—	Secondary	119	174.8 ± 11.4^{a}	174.8 ± 11.4^{a}	—	119 (100)	55.2 ± 3.7^{a}	55.2 ± 3.7^{a}	—
4	♂	Antral	6	602.8 ± 235.8^{b}	547.7 ± 15.5^{b}	658.0 ± 294.0^{a}	3 (50)^{ab}	94.3 ± 4.0^{b}	94.3 ± 4.0^{bc}	—
		Secondary	6	334.0 ± 73.5^{c}	240.0	352.8 ± 57.3^{a}	1 (17)^{ac}	84.0	84.0	—
4	♀	Antral	8	402.5 ± 112.6^{bc}	385.7 ± 110.3^{cd}	520.0	8 (100)	98.4 ± 7.6^{c}	98.1 ± 8.2^{b}	—
		Secondary	11	258.2 ± 65.4^{d}	290.0 ± 103.9^{c}	246.3 ± 46.2^{b}	3 (27)^{ac}	82.3 ± 16.3^{b}	82.3 ± 16.3^{c}	—
6	♂	Antral	11	602.8 ± 382.0^{bc}	380.0 ± 53.9^{cd}	686.4 ± 395.1^{a}	3 (27)^{ac}	90.7 ± 7.6^{bc}	90.7 ± 7.6^{bc}	—
		Secondary	9	373.7 ± 56.4^{c}	369.2 ± 39.1^{cd}	379.3 ± 69.1^{a}	5 (56)^{ab}	94.0 ± 8.0^{bc}	94.0 ± 8.0^{bc}	—
6	♀	Antral	11	515.9 ± 191.7^{b}	474.4 ± 119.3^{bd}	626.7 ± 269.0^{a}	10 (91)^{b}	107.0 ± 5.6^{d}	106.4 ± 5.5^{d}	109.5 ± 5.5
		Secondary	14	212.2 ± 26.0^{e}	200.0	213.2 ± 25.8^{b}	1 (7)^{c}	98.0	98.0	—

*Groups of 5–8 bovine secondary follicles 140–190 μm in diameter were xenografted under the kidney capsule of each SCID mouse. [a–e] Values with different superscripts in the same column differ significantly ($p<0.05$).

between conditions of male and female mice, such as the balance and circulating concentration of hormones, for instance, may affect the development of follicles.

In female mice, the diameter of xenografted bovine oocytes increased with the increase in follicle size after grafting. In contrast, in male mice, the average size of oocytes did not continue to increase after 4 weeks. These results were possibly due to oocyte degeneration in large antral follicles, together with the follicular degeneration.

These results demonstrate that bovine follicles could also develop in male SCID mice. We conclude, however, that conditions are more suitable for growing bovine oocytes in female mice than in male mice.

Acknowledgments

The authors are grateful to the staff of the Kobe Meat Inspection Office and the Animal Biotechnology Center, Kobe (Mr. A. Hamawaki and Mr. M. Yoshikawa) for supplying bovine ovaries. This work was supported in part by a Grant-in-Aid for Creative Scientific Research (13GS0008) to T. M., and by a 21st Century COE Program to S. S. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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