TIMING OF PROLIFERATION OF SPERMATOGONIA AND SPERMATOGONIUM-SUPPORTING SERTOLI CELLS IN THE NEWT *CYNOPS PYRRHOASTER*

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ABSTRACT

The proliferative activities of spermatogonia and spermatogonium-supporting Sertoli cells in the newt *Cynops pyrrhogaster* were examined by means of repeated labeling with bromodeoxyuridine (BrdU). The animals were given from one to six injections of BrdU at 6-h intervals for 30 h, and some were killed 2 h after each injection. The labeling index of the spermatogonia surrounded by labeled Sertoli cells increased remarkably with the advance of time, while the labeling index of the Sertoli cells did not change significantly. These results indicate that during spermatogonial proliferation in the newt spermatogonium-supporting Sertoli cells enter the S-phase first, followed by the spermatogonia.

Spermatogenesis is one of the most attractive systems for the study of cell differentiation and morphogenesis, but this mechanism has not yet been clearly defined (3, 8, 16, 18, 19). Concerning the proliferation of spermatogonia, the cell cycle in each class of spermatogonia has been examined in mammals (12, 13). In amphibians, on the other hand, the deduction of the normal timing of this process from cyto-morphological observations is difficult (14), and there is little information about spermatogonial proliferation. In amphibians, however, clonally derived clusters of germ cells are surrounded by Sertoli cells to form spermatogenic cysts, which are the basic morphological unit within the seminiferous tubules; and the progression of spermatogenesis occurs synchronously within the cysts (3, 16, 20).

Recently, we reported that not only spermatogonia but also spermatogonium-supporting Sertoli cells proliferate in the testes of the toad, *Xenopus laevis* (2). This phenomenon was also observed in other amphibian species, e.g., *Rana nigromaculata*, *R. japonica*, *Hyla japonica*, and *Cynops pyrrhogaster* (Kobayashi, unpublished). However, the relation-

ship between the proliferation of spermatogonia and that of Sertoli cells is not defined.

In the present study, to clarify the details of the proliferation of spermatogonia and spermatogonium-supporting Sertoli cells, we used a thymidine analog, bromodeoxyuridine (BrdU) (2, 5, 6, 10, 11, 15, 22) to repeatedly label spermatogonia and Sertoli cells in S-phase, and investigated the proliferative activities of spermatogonia and Sertoli cells by means of this technique in the newt *Cynops pyrrhogaster*.

MATERIALS AND METHODS

Animals and Chemicals

Adult male newts, *Cynops pyrrhogaster*, 8-10 g in body weight, were collected in a suburb of Murakami, Niigata Prefecture in November 1990. The animals were kept thereafter in dechlorinated tap water at $22 \pm 1^\circ\text{C}$ and fed a commercial diet. A 12L:12D cycle with the light phase from 6 00 to 18 00 was employed. The experiment was performed one month after transfer of the animals to warm tap water, because the proliferation of spermatogonia in this species is activated by transfer to warm temperature in winter (Kobayashi,

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Fig. 1 Detection of proliferating cells in newt testis. Section was stained using an anti-BrdU antibody and counter-stained by hematoxylin. a: Primary spermatogonia and primary spermatogonium-supporting Sertoli cells. Primary spermatogonia incorporated BrdU surrounded by Sertoli cells incorporated BrdU (large arrow), non-immunoreactive primary spermatogonia surrounded by immunoreactive Sertoli cells (black asterisk), and immunoreactive primary spermatogonia surrounded by non-immunoreactive Sertoli cells (arrowhead) were observed. In some case, immunoreactive Sertoli cells (white asterisk) and non-immunoreactive Sertoli cells (small arrow) were seen within a single cyst. b: Secondary spermatogonia and secondary spermatogonia-supporting Sertoli cells. Immunoreactive secondary spermatogonia surrounded by immunoreactive Sertoli cells (large arrow), immunoreactive secondary spermatogonia surrounded by non-immunoreactive Sertoli cells (G) and non-immunoreactive secondary spermatogonia surrounded by immunoreactive Sertoli cells (large asterisk) were observed. Within a cyst, immunoreactive Sertoli cells (small asterisk) and non-immunoreactive Sertoli cells (arrowhead) were seen in some case. Interstitial cells incorporated BrdU were also seen (small arrow). a, b: $\times 300$

unpublished) as in other lower vertebrates (9, 17).

5-Bromo-2'-deoxyuridine, BrdU (Sigma, U.S.A.), and a 'Cell Proliferation Kit' (Amersham, U.K.) were purchased from commercial sources.

Experimental Procedure

The newts were given BrdU, 50 $\mu\text{g/g}$ body weight, intraperitoneally every 6 h for 30 h, i.e., one to six injection at 12 00, 18 00, 24 00, 6 00, 12 00, and

20 00. A group of four newts was killed by decapitation 2 h after the injection at each time. The left testis was excised and fixed in Bouin's solution for 12 h at room temperature, and then embedded in Paraplast (Sherwood Medical, St. Louis, U.S.A.), and serial sagittal sections were cut at 4 μm . The sections were stained immunohistochemically with the reagent included in the 'Cell Proliferation Kit'. Details of this procedure were described previously (2).

Estimation of Proliferative Activities in Spermatogonia and Spermatogonium-Supporting Sertoli Cells

To elucidate the labeling index (L.I.) for spermatogonia and Sertoli cells, a mid-sagittal plane was chosen among sections. In amphibians, spermatogonia are classified into primary spermatogonia and secondary spermatogonia; the former are identified as one spermatogonium surrounded by Sertoli cells (single cyst), and the latter, as two or more spermatogonia surrounded by Sertoli cells (14, 16, 18). In amphibians, spermatogonia proliferate synchronously within a cyst (2, 16). But the number of Sertoli cells surrounding the primary spermatogonium or secondary spermatogonia was one or more, respectively, and these Sertoli cells did not always proliferate synchronously within a cyst (see Results). To clarify the relationship between the proliferation of spermatogonia and Sertoli cells, in this study, the number of the cysts was counted instead of the number of spermatogonia and spermatogonium-supporting Sertoli cells. About 800 and 1,000 cysts bearing primary and secondary spermatogonia, respectively, were counted per newt. As to the number of labeled Sertoli cells within a cyst, similarly, the number of cysts containing labeled Sertoli cells was counted as the number of labeled Sertoli cells, irrespective of the actual number of labeled Sertoli cells within a given cyst.

According to the above-mentioned counting method, the L.I. was calculated by the following formula: $\text{L.I. (\%)} = (\text{number of labeled cysts} / \text{total cyst number}) \times 100$. The results were expressed as the mean $\pm$ SE.

Statistics

The results were assessed statistically by one-way analysis of variance, followed by Duncan's multiple range test, Student's *t*-test, or the Cochran-Cox test.

RESULTS

In the animals used in this study, the spermatogenic stages other than those of spermatogonia and spermatozoa were not seen. Not only spermatogonia but also spermatogonium-supporting Sertoli cells were observed to have incorporated BrdU irrespective of the time of the injection. Immunoreactive interstitial cells were also frequently seen in the spermatogonial zone. As for all the secondary sper-

matogonia in any given cyst, all were either labeled or unlabeled. Concerning the primary and secondary spermatogonium-supporting Sertoli cells in any given cyst, however, this was not the case; i.e., within a cyst one Sertoli cell was immunoreactive, but the others were not so (Fig. 1).

Proliferative Activity of Spermatogonia

The results of the repeated BrdU labeling are shown in Fig. 2. Fig. 2a shows that the L.I. of primary spermatogonia increased with time and reached 37.8% in 26 h (0.8% in 2 h). Similarly, the L.I. of secondary spermatogonia increased and reached 48.4% in 32 h (7.3% in 2 h).

Proliferative Activity of Sertoli Cells

The frequency of Sertoli cells that incorporated BrdU is shown in Fig. 2b. The L.I. of primary spermatogonium-supporting Sertoli cells (pg-Sertoli cells) was always about 30% with significant variation recognized in the L.I. over the entire labeling period ($P < 0.05$). The L.I. of secondary spermatogonium-supporting Sertoli cells (sg-Sertoli cells) did not increase significantly until 20 h after the first injection, reaching 29.1% after the last injection (17.1% in 2 h).

Proliferative Activity of Spermatogonia Surrounded by BrdU-Labeled Sertoli Cells

The proliferative activity of primary and secondary spermatogonia surrounded by labeled Sertoli cells was expressed as the L.I. per the number of the cysts containing labeled Sertoli cells (Fig. 2c). The L.I. increased remarkably with time and reached the maximum value in 32 h. That is, the L.I. of primary and secondary spermatogonia surrounded by the labeled Sertoli cells was 1.6% at 2 h and 28.7% at 32 h, and 0% at 2 h and 54.8% at 32 h, respectively.

DISCUSSION

In the present study, the proliferative activities of spermatogonia and spermatogonium-supporting Sertoli cells during spermatogonial proliferation were examined in the newt *Cynops pyrrhogaster*.

Proliferative Activity of Spermatogonia

The present study showed by means of repeated

Fig. 2 The labeling index (L.I.) of spermatogonia and spermatogonia-supporting Sertoli cells by repeated BrdU labeling experiment. The newts were given from one to six injections of BrdU at 6-h intervals for 30 h. a: The labeling index of primary (open circle) and secondary spermatogonia (closed circle). b: The labeling index of primary (open circle) and secondary spermatogonia (closed circle)-supporting Sertoli cells. c: The labeling index of primary (open circle) and secondary spermatogonia (closed circle) surrounded by labeled Sertoli cells. This value was expressed as the L.I. per the number of cysts containing labeled Sertoli cells. The vertical bars represent the mean $\pm$ SEM.

labeling treatment that in the newt L.I. of the primary and secondary spermatogonia increased with

time (Fig. 2a). In the anuran *Rana esculenta*, Rastogi *et al.* (21) classified primary spermatogonia into two types, i.e., pale and dark, and pointed out that the cells of the pale type were stem cells and that the cells of the dark type, which transdifferentiated from those of the pale type, differentiated into secondary spermatogonia. In *Xenopus laevis*, no significant variation in the L.I. of spermatogonia was recognized over a 24-h period, though each population of spermatogonia could not be classified as in *Rana esculenta* (21). Thus, a hypothesis was proposed that several populations are present among the spermatogonia in *Xenopus laevis* as in mammals (4, 12, 13); i.e., the length of the S-phase and/or the time-lag in timing of entrance into the S-phase would be different in each population (2). The present data (Fig. 2a) may also be interpreted according to the above-mentioned hypothesis, though the doubling time of spermatogonia could not be calculated in the present study.

Proliferative Activity of Spermatogonium-Supporting Sertoli Cells

In lower vertebrates, it is known that spermatogenesis progresses synchronously within the cyst (3, 16, 18), but information on the proliferation of Sertoli cells is scant. Recently we reported that the proliferative activity of spermatogonium-supporting Sertoli cells was detectable throughout a 24-h period in *Xenopus laevis* (2). The present study also indicates proliferative activity of spermatogonium-supporting Sertoli cells in the newt.

In this study, no significant change in the L.I. of pg-Sertoli cells was recognized over the labeling period (Fig. 2b), while the L.I. of primary spermatogonia increased (Fig. 2a). In the process of spermatogenesis, it is known that after cell division, one of the primary spermatogonia immediately transforms into a secondary spermatogonium, while the other remains as a primary spermatogonium (3, 16). In the method used in this study, we could not determine if the L.I. of sg-Sertoli cells was increased by the proliferation of sg-Sertoli cells within a cyst, because the L.I. of Sertoli cells was calculated from the number of cysts containing labeled Sertoli cells irrespective of the number of labeled Sertoli cells within a given cyst (see Materials and Methods). Thus, in the present study, the increase in the L.I. of sg-Sertoli cells could have been caused by the transfer of labeled pg-Sertoli cells into the cysts containing secondary spermatogonia.

gonia and/or by the labeling of Sertoli cells already present in the secondary spermatogonial cysts containing no labeled Sertoli cells. The present data (Fig. 2b), however, indicate that the L.I. of pg- and sg-Sertoli cells did not change significantly until 20 h after the first injection. From 0 to 20 h after the injection, therefore, it is suggested that the above-mentioned possibility is not accepted, and pg-Sertoli cells that had recently entered into the S-phase are not hardly present.

Relationship between the Proliferative Activity of Spermatogonia and Sertoli Cells

As mentioned above, the present data suggest that pg- and sg-Sertoli cells that entered into the S-phase newly were not present during the period of 0–20 h after the injection. However, the L.I. of primary and secondary spermatogonia surrounded by labeled Sertoli cells increased with time (Fig. 2c). Therefore, these findings indicate that the labeling of spermatogonia and Sertoli cells within a non-labeled spermatogonial cyst did not occur at the same time but that non-labeled spermatogonia surrounded by labeled Sertoli cells had incorporated BrdU. Furthermore, we also observed that spermatogonia supported by non-labeled Sertoli cells incorporated BrdU. Although the doubling time of spermatogonium-supporting Sertoli cells could not be calculated, this phenomenon may imply that spermatogonia surrounded by Sertoli cells that had already passed through the S-phase were labeled.

It is known that the Sertoli cells play an important role in spermatogonial proliferation (1, 7). As to the spatial relationship between germ cells and Sertoli cells during spermatogenesis, each seminiferous tubule contains numerous 'cysts', and the spermatogenic change in germ cells in each cyst progresses synchronously (3, 16, 18). The present results indicate that the proliferation of spermatogonia follows the proliferation of spermatogonium-supporting Sertoli cells. This phenomenon may be necessary to maintain spermatogonial proliferation spatially within a cyst.

In conclusion, the present study clearly defined the pattern of proliferation of spermatogonia and spermatogonium-supporting Sertoli cells in *Cynops pyrrhogaster*. That is, the entrance into the S-phase by spermatogonia is preceded by that by spermatogonium-supporting Sertoli cells.

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REFERENCES

1. ABÉ S.-I. (1987) Differentiation of spermatogenic cells from vertebrates *in vitro*. *Int. Rev. Cytol.* **109**, 159–209
2. ASAKAWA Y., KOBAYASHI T. and IWASAWA H. (1991) Immunohistochemical detection of the proliferative activity of spermatogonia in *Xenopus laevis* by application of bromodeoxyuridine. *Biomedical Res.* **12**, 269–272
3. CALLARD G. V. (1991) Spermatogenesis. In *Vertebrate Endocrinology: Fundamentals and Biochemical Implications* Vol. 4, Part A (ed. PANG K. T. and SCHREIBMAN M. P.) Academic Press, San Diego, pp. 303–341
4. CLERMONT Y. (1972) Kinetics of spermatogenesis in mammals: Seminiferous epithelium cycle and spermatogonial renewal. *Physiol. Rev.* **52**, 198–236
5. DEAN P. N., DOLBEARE F., GRATZNER H., RICE C. and GRAY J. W. (1984) Cell-cycle analysis using a monoclonal antibody to BrdUrd. *Cell Tissue Kinet.* **17**, 427–436
6. DEFazio A., LEARY J. A., HEDLEY D. W. and TATTERSALL M. H. N. (1987) Immunohistochemical detection of proliferating cells *in vivo*. *J. Histochem. Cytochem.* **35**, 571–577
7. DE KRETZER D. M. (1988) Local regulation of testicular function. *Int. Rev. Cytol.* **109**, 89–112
8. DE REVIERS M.-T. H., COURTENS J.-L. and DE REVIERS M. (1990) Spermatogenesis in mammals and birds. In *Marshall's Physiology of Reproduction* Fourth Ed., Vol. 2 (ed. LAMMING G. E.) Churchill Livingstone, Edinburgh, pp. 106–182
9. FRAILE B., PANIAGUA R., SÁEZ F. J. and RODRÍGUEZ M. C. (1989) Effects of moderately high temperature on the testis of the marbled newt, *Triturus marmoratus*. *Amphibia-Reptilia* **10**, 117–124
10. GRATZNER H. G. (1982) Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* **218**, 474–475
11. HAYASHI Y., KOIKE M., MATUTANI M. and HOSHINO T. (1988) Effects of fixation time and enzymatic digestion on immunohistochemical demonstration of bromodeoxyuridine in formaline-fixed, paraffin-embedded tissue. *J. Histochem. Cytochem.* **36**, 511–514
12. HUCKINS C. (1971) The spermatogonial stem cell population in adult rats. II. A radioautographic analysis of their cell cycle properties. *Cell Tissue Kinet.* **4**, 313–334
13. HUCKINS C. (1971) The spermatogonial stem cell population in adult rats. III. Evidence for a long-cycling population. *Cell Tissue Kinet.* **4**, 335–349
14. KALT M. R. (1976) Morphology and kinetics of spermatogenesis in *Xenopus laevis*. *J. Exp. Zool.* **195**, 393–408
15. LANGER E.-M., RÖTTGERS H. R., SCHLIERMANN M. G., MEIER E.-M., MILTENBURGER H. G., SCHUMANN J. and GÖHDE W. (1985) Cycling S-phase cells in animal and spontaneous tumours. I. Comparison of the BrdUrd and 3H-thymidine techniques and flow cytometry for the estimation of S-phase frequency. *Acta Radiol. Oncol.* **24**, 545–548
16. LOFTS B. (1974) Reproduction. In *Physiology of the Amphibia* Vol. II. (ed. LOFTS B.) Academic Press, New York, pp. 107–218
17. MICHIBATA H. (1975) Cell population kinetics of primary spermatogonia activated by warm temperatures in the teleost, *Orizias latipes* during the winter months. *J. Fac. Sci. Univ. Tokyo IV* **13**, 299–309
18. NAGAHAMA Y. (1986) Testis. In *Vertebrate Endocrinology: Fundamentals and Biochemical Implantations* Vol. 1. (ed. PANG P. K. T., SCHREIBMAN M. P. and GORBMAN A.) Aca-

- demic Press, Orlando, pp. 399-437
19. PARVINEN M., VIHKO K. M. and TOPPARI J. (1986) Cell interactions during the seminiferous epithelial cycle. *Int. Rev. Cytol.* **104**, 115-151
 20. PUDNEY J. and CALLARD G. V. (1984) Organization of interstitial tissue in the testis of the salamander *Necturus maculosus* (caudata: Proteidae). *J. Morphol.* **181**, 87-95
 21. RASTOGI R. K., DI MEGLIO M., DI MATTEO L., MINUCCI S. and IELA L. (1985) Morphology and cell population kinetics of primary spermatogonia in the frog (*Rana esculenta*) (Amphibia: Anura). *J. Zool.* **207**, 319-330
 22. WYNFORD-THOMAS D. and WILLIAMS E. D. (1986) Use of bromodeoxyuridine for cell kinetic studies in intact animals. *Cell Tissue Kinet.* **19**, 179-182