The Role of Spermatogonia in the Recovery Process from Temporary Sterility Induced by Gamma-Ray Irradiation in the Teleost Oryzias latipes

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INTRODUCTION

It has been demonstrated by several workers\textsuperscript{1-7} that, in fishes as in mammals, some spermatogonia and spermatocytes are particularly sensitive to ionizing radiation. In fish given 0.5-2 kR of X-rays, spermatogenesis was temporarily inhibited and recovered from the radiation damage, showing a number of spermatocytes and spermatids newly formed from the spermatogonia which had been dormant at the time of the irradiation.\textsuperscript{3,4} However, the nature of the dormant spermatogonia has not been investigated.

In a previous paper\textsuperscript{8} the spermatogonia in the testis of Oryzias latipes were classified into three types on the basis of cell morphology and the grade of differentiation. The first type of spermatogonia, named “spermatogonia A-stem” (As), found in the most peripheral part of the testis, has an ovoid or spherical, deeply staining nucleus. The second type, named “spermatogonia A-differentiated” (Ad), found in the periphery of the testis, has a large spherical, pale-staining nucleus with one nucleolus. The third type, named “spermatogonia B”, found near the cyst of the spermatogonia Ad, has a small, spherical, rather deeply staining nucleus with one or
two nucleoli. The spermatogonia Ad and the spermatogonia B were previously called the primary spermatogonia and the secondary spermatogonia respectively by many investigators. However, no special attention has been paid to the spermatogonia As in the response to ionizing radiation.

The present experiment was designed to observe the role of the spermatogonia As and the spermatogonia Ad in the recovery process from radiation injury in the fish testis.

MATERIALS AND METHODS

Experiments were carried out with adult males of the orange-red variety of the fish *Oryzias latipes* during the non-breeding season. Until the commencement of the experiments, thirty-three groups of eight males had been kept in 2 liters of tap water under low-temperature conditions (below 10°C). During gamma-ray irradiation, the males were kept at 7°C in a small plastic vessel containing water. 137Cs-gamma-rays were exposed at an exposure rate of 100 R per minute. Immediately after the irradiation with 0, 0.5, or 1 kR of gamma-rays, the fish were transferred from the cold water to warm water at 25±1°C with a long photoperiod (14 hrs light, 10 hrs dark) and fed on tetramin (Tetra Werke, West Germany). They were killed 0, 2, 5, 10, 15, 20, 25, 30, 40, 50, and 60 days after the irradiation. Two hours before sacrifice each animal was injected intraperitoneally with a single dose (1 pCi per fish) of tritiated thymidine (22.3 Ci/mM). After fixation in Bouin’s fluid, the body and testicular weights were measured. For the autoradiographic preparation of testicular sections, the testes were embedded in paraffin and cut at a thickness of 5 μm. The dewaxed preparations were air-dried and then covered with a liquid emulsion (Sakura NR-M2) diluted 1:1 for dipping application. The autoradiographs were stored for one week at 4°C prior to development and then stained with Mayer's acid-haemalum and eosin. The numbers of the spermatogonia As and the spermatogonia Ad per cross section were counted in three randomly selected cross sections near the middle part of the testis.

RESULTS

*Histological changes in the testis following gamma-ray irradiation*

The changes in the weight of the testis after gamma-ray irradiation are shown in Figure 1 as the gonadosomatic indices (testicular weight/body weight x 100). In non-irradiated controls, a rapid increase in the gonadosomatic index was found within 10 days following the transfer to warm water. No significant increase in the gonadosomatic index of the fish irradiated with 0.5 kR was found for 15 days after the irradiation. Thereafter, however, the index progressively increased in the group of 0.5 kR. On the other hand, the gonadosomatic index of the fish irradiated with 1 kR profoundly decreased to only 0.31 (on an average) 30 days after the irradiation, thereafter, it slowly increased.
Fig. 1. Changes in gonadosomatic index after irradiation. Fish were irradiated with 0 kR (●), 0.5 kR (■) and 1 kR (▲). Vertical bars indicate the standard error of means.

Table 1

Cell types in the testis at various times following gamma-irradiation

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Plus and minus signs give a semi-quantitative estimate of the numbers of germ cells observed in the testis.
At the beginning of the experiments, the testis contained a small number of the spermatogonia As and large numbers of the spermatogonia Ad, the spermatogonia B, primary spermatocytes, and spermatozoa. However, the secondary spermatocytes and spermatids could not be observed (Fig. 2). In the non-irradiated and warmed fish, the spermatogenesis was actively initiated. Within 2 days after the transfer to warm conditions, the number of the spermatogonia B increased and newly differentiated primary spermatocytes were observable. After 5 days the secondary spermatocytes and spermatids appeared (Table 1 and Fig. 3).

In the irradiated fish, the spermatogonia Ad, the spermatogonia B, and the primary spermatocytes were severely damaged and disappeared within 2 days after the irradiation, but the mature spermatozoa apparently remained intact (Table 1 and Fig. 4). The histological damage in the testis of the fish irradiated at a dose of 1 kR was more marked than in those irradiated with 0.5 kR (Figs. 5 and 6). In the testis of the fish irradiated with 0.5 kR, small numbers of the newly differentiated primary spermatocytes were observable 15 days after the irradiation, and the testis became approximately normal in histological character 30 days after the irradiation. In the testis of the fish irradiated with 1 kR, the primary spermatocytes differentiated 40 days after the irradiation and the recovery of the testis took place 50 days after the irradiation (Table 1 and Fig. 7).

Changes in the number of spermatogonia following irradiation

The changes in the number of the spermatogonia As are shown in Figure 8. In the non-irradiated fish, a temporary increase in the number of the spermatogonia As was found 2 days after the transfer to warm water. At 5 days, the number of the spermatogonia As decreased with a rapid differentiation of the cells into the spermatogonia Ad. After the early response, a distinct increase in the spermatogonia As population was induced. Fifteen days after the transfer to warm water, the number of the spermatogonia As markedly increased to about twice as many as those in the initial control.

In the irradiated fish the increase in the spermatogonia As population was not observed within 2 days after the irradiation. However, 15 days after the irradiation the number of the spermatogonia As significantly increased at a dose of 0.5 kR. The number of the spermatogonia As showed the highest value 15 days after the irradiation with 1 kR. Thereafter, in the irradiated fish, the number of the spermatogonia As persisted at a higher level than that in the controls up to 50 days after. In all cases, no reduction in the cell number of the spermatogonia As in comparison with the cell number of the initial control was observed.

The changes in the number of the spermatogonia Ad are shown in Figure 9. The changes were more marked than those in the spermatogonia As. In the non-irradiated fish, the number of the spermatogonia Ad temporarily increased 2 days after the transfer to warm water. At 5 days, the number of the spermatogonia Ad decreased to the initial level due to the rapid differentiation of the cells into the spermatogonia
Fig. 2. Spermatogenetic cells in the testis during the non-breeding season. A few spermatogonia A-stem (As), numerous spermatogonia A-differentiated (Ad), spermatogonia B, primary spermatocytes, and spermatozoa can be seen, but no secondary spermatocytes and spermatids are observed. Bottom bar, 20 μm.

Fig. 3. Spermatogenetic cells in the testis of the non-irradiated control fish at 5 days. The spermatogenesis was actively initiated. An increased number of primary spermatocytes and newly differentiated secondary spermatocytes and spermatids can be seen.

Fig. 4. Two days after irradiation with 0.5 kR. The spermatogonia Ad, spermatogonia B, and primary spermatocytes are severely damaged, but the mature spermatozoa apparently remain intact.

Fig. 5. Ten days after irradiation with 0.5 kR. Damaged spermatogenetic cells have disappeared.

Fig. 6. Ten days after irradiation with 1 kR. The testis is profoundly damaged. Few spermatogenetic cells can be seen.

Fig. 7. Fifty days after irradiation with 1 kR. Recovery of the testis has taken place. Spermatogenetic cells at various developmental stages are newly differentiated.

Fig. 8. Changes in the number of spermatogonia As after irradiation. After irradiation, males were transferred from cold water to warm water at 25±1°C. The number of spermatogonia As per cross section was counted. ●, 0 kR; ■, 0.5 kR; ▲, 1 kR.
B. Thereafter, the data revealed that the number of the spermatogonia Ad progressively approached a steady level.

In the irradiated fish, however, warming for 2 days had no influence upon the increase in the cell number of the spermatogonia Ad. The lowest number of the spermatogonia Ad was reached after 10 days in the fish irradiated with 0.5 kR. A smaller number of the spermatogonia Ad was counted in the testis irradiated with 1 kR than in the testis irradiated with 0.5 kR. Furthermore, in the testis irradiated with 1 kR the number of the spermatogonia Ad very slowly increased, reaching normal values within 50 days after the irradiation (Fig. 9).

![Graph showing changes in the number of spermatogonia Ad per cross-section over days after irradiation.](image)

**Fig. 9.** Changes in the number of spermatogonia Ad after irradiation. Cells were counted in the same way as has described in Fig. 8. •, 0 kR; ■, 0.5 kR; ▲, 1 kR.

*Changes in labeling index and mitotic index of spermatogonia following irradiation*

The labeling indices of the spermatogonia As and the spermatogonia Ad were examined in fish sacrificed at various intervals after the irradiation. The labeling indices and the mitotic indices obtained in irradiated animals were compared with
those obtained in non-irradiated animals. In the present experiment, no attempt was made to quantify the mitotic figures in the spermatogonia As because of their small number.

The labeling index of the spermatogonia As obtained in non-irradiated fish increased to a maximal value 15 days after the transfer to warm water. In the irradiated fish the labeling index of the spermatogonia As significantly increased; especially in the fish irradiated with 1 kR, the highest value was observed 10 days after the irradiation. The labeling index of the spermatogonia As in the fish irradiated with 0.5 kR was not different from the control level, but the labeling index of the spermatogonia As in the fish irradiated with 1 kR showed continuously higher values than the control value (Fig. 10).

![Fig. 10. Changes in the labeling index of spermatogonia As after irradiation. Males were given 1 μCi of 3H-TdR and killed 2 hours after each injection. •, 0 kR; ■, 0.5 kR; ▲, 1 kR.](image)

In the testis of the non-irradiated fish, the labeling index of the spermatogonia Ad rapidly increased 2 days after the warming, thereafter, it did not change very much. The mitotic index of the spermatogonia Ad showed a pattern similar to that of the labeling index. In the testis of the irradiated fish, the labeling index and the mitotic index of the spermatogonia Ad were depressed for 15 days after the irradiation. In the testis irradiated with 1 kR, the mitotic index was profoundly depressed. From 20 days after the irradiation, however, the labeling index and the mitotic index of the spermatogonia Ad recovered (Fig. 11).

Transformation of the spermatogonia As into the spermatogonia Ad

During the non-breeding season, most of the spermatogonia As, 3-4 μm in diameter, had an ovoid, deeply staining nucleus. The nucleolus was single and inconspicuous. They were morphologically distinguishable from the spermatogonia Ad, which meas-
ured 5-10 μm in diameter and which had a large, spherical, pale-staining nucleus (Fig. 12).

Fifteen days after the transfer to warm water, or 15 days after the irradiation, the nuclear morphological characters of some of the spermatogonia As underwent a dramatic change. The cells became more regularly spherical and progressively enlarged (reaching a size 5 μm in diameter). The nucleolus became darker and more conspicuous. Following these changes, the spermatogonia As resembled the typical spermatogonia Ad (Fig. 13). The rest of the spermatogonia As remained small and unchanged in appearance and were readily distinguished on morphological grounds from the typical spermatogonia Ad.

Fig. 11. Changes in the labeling index and mitotic index of spermatogonia Ad after irradiation. Males were injected and killed in the same way as has described in Fig. 10. ●, 0 kR; ■, 0.5 kR; ▲, 1 kR.
Fig. 12. Spermatogonia As (As) in the testis during the non-breeding season. An ovoid and deeply staining nucleus can be seen. The nucleolus was single and inconspicuous. They were morphologically distinguished from spermatogonia Ad. Bottom bar, 10 μm.

Fig. 13. The transformation of spermatogonia As into spermatogonia Ad is observed in the testis of non-irradiated fish 15 days after the transfer into warm water. The nucleus became spherical and enlarged. The nucleolus became darker and more conspicuous. The spermatogonia As resembled the typical spermatogonia Ad.

DISCUSSION

Studies of histological changes in the testis following X-ray irradiation in the teleost Oryzias latipes have been made by several workers. The present histological results obtained from gamma-irradiation are concordant with the above findings.

During sexually inactive seasons, the testis contained a small number of the spermatogonia As and large numbers of the spermatogonia Ad, the spermatogonia B, the primary spermatocytes, and the spermatozoa. However, the secondary spermatocytes and the spermatids were not observed. The spermatogenesis was actively reinitiated following the transfer of the male fish to warm water (Table 1). In the irradiated fish, however, the reinitiation of the spermatogenesis induced by the warm condition was depressed. These histological changes following the irradiation suited the curve of the gonadosomatic indices (Fig. 1).

From the viewpoint of the cell population shift and cell differentiation, no attempt has been made to analyze the mechanism of the restoration of the spermatogenesis from radiation-induced damage in the teleostean fishes. In the present experiment, the number of the spermatogonia Ad was reduced 10 days after the irradiation. However, no reduction in the cell number of the spermatogonia As in comparison with the cell number of the initial control was observed in either the non-irradiated or irradiated fish (Figs. 8 and 9). The spermatogonia As, which were radioreistant with regard to the survival of the cells, began to proliferate after the spermatogonia Ad had been differentiated into the spermatogonia B in the non-irradiated fish, or after the spermatogonia Ad had degenerated and disappeared following the irradiation. The
extent of the proliferation of the spermatogonia As was strikingly related to the extent of cell loss of the spermatogonia Ad. From these findings, it is likely that the spermatogonia As are stem cells in a spermatogenetic system and functionally supply the spermatogonia Ad during the repopulation of the spermatogenetic cells following radiation-induced damage.

The overshoot of the cell number of the spermatogonia As observed 15 days after the irradiation may result from the extra division of the cells (Fig. 8). The degree of the overshoot depended on the increase in the dose of gamma-rays. An overshoot of the cell number of undifferentiated-type spermatogonia in the course of recovery has also been observed in the mouse seminiferous epithelium after a cell loss inflicted by the injection of an alkylating agent\textsuperscript{10,11}. It is possible that the cause of the overshoot involves the reduction in cell density after the depletion of the spermatogonia Ad. A mitotic inhibitory substance (chalone) suggested to exist in the rat seminiferous epithelium\textsuperscript{12} may give another explanation of these facts. On this, further research must be done.

The labeling index and the mitotic index of the spermatogonia Ad were depressed for 15 days after the irradiation, then they recovered to those in the control fish (Fig. 11). However, the labeling index of the spermatogonia As was not depressed for 15 days after the irradiation (Fig. 10). These results also suggest that the spermatogonia Ad do not play a role in the repopulation of the spermatogenetic cells following the irradiation, while the spermatogonia As contribute to the repopulation.

For 15 days after the irradiation, i.e., during the overshoot phase of the cell number of the spermatogonia As, the nuclear morphology of some of the spermatogonia As underwent a dramatic change, denoting their differentiation into the spermatogonia Ad (Fig. 13). From these morphological observations and the autoradiographic observations (unpublished data), it is evident that some of the spermatogonia As are transformed into the spermatogonia Ad, which contribute to the repopulation of the spermatogenetic cells in the testis following the cell loss induced by differentiation or radiation damages. Huckins\textsuperscript{13,14} and Oakberg\textsuperscript{15,16} have similarly suggested that undifferentiated spermatogonia are transformed into differentiated spermatogonia in the rat testis and in the mouse testis. The question arises as to whether the spermatogonia As must undergo a specific number of mitoses before their transformation into the spermatogonia Ad.

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REFERENCES