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Rapid and Simple Method for Quantitative Evaluation of Neurocytotoxic Effects of Radiation on Developing Medaka Brain

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Developmental neurotoxicity/Brain/X-rays/Apoptosis/Medaka.

We describe a novel method for rapid and quantitative evaluation of the degree of radiation-induced apoptosis in the developing brain of medaka (*Oryzias latipes*). Embryos at stage 28 were irradiated with 1, 2, 3.5, and 5 Gy x-ray. Living embryos were stained with a vital dye, acridine orange (AO), for 1–2 h, and whole-mount brains were examined under an epifluorescence microscope. From 7 to 10 h after irradiation with 5 Gy x-ray, we found two morphologically different types of AO-stained structures, namely, small single nuclei and rosette-shaped nuclear clusters. Electron microscopy revealed that these two distinct types of structures were single apoptotic cells with condensed nuclei and aggregates of apoptotic cells, respectively. From 10 to 30 h after irradiation, a similar AO-staining pattern was observed. The numbers of AO-stained rosette-shaped nuclear clusters and AO-stained single nuclei increased in a dose-dependent manner in the optic tectum. We used the number of AO-stained rosette-shaped nuclear clusters/optic tectum as an index of the degree of radiation-induced brain cell death at 20–24 h after irradiation. The results showed that the number of rosette-shaped nuclear clusters/optic tectum in irradiated embryos exposed to 2 Gy or higher doses was highly significant compared to the number in nonirradiated control embryos, whereas no difference was detected at 1 Gy. Thus, the threshold dose for brain cell death in medaka embryos was taken as being between 1–2 Gy, which may not be so extraordinarily large compared to those for rodents and humans. The results show that medaka embryos are useful for quantitative evaluation of developmental neurocytotoxic effects of radiation.

INTRODUCTION

It is important to examine the hazardous effects of chemical substances and ionizing radiations on our central nervous system (CNS) for protection of public health. The developing CNS is particularly sensitive to ionizing radiation because of its structural complexity, its long developmental period, the vulnerability of undifferentiated neural cells, the need for neurons to migrate to functional positions, and the inability to replace lost neurons.[1,2)]

For humans, data from the fetuses of atomic bomb survivors in Hiroshima and Nagasaki provide the primary basis for risk estimation of radiation damage to a developing CNS.[3)] However, these data are limited and incomplete, making studies on other species essential. Mammalian embryos develop in the mothers' uteri; hence, their developmental process is hard to be examined directly. In contrast, fish embryos, such as those of zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), develop outside the mothers' bodies, and their chorions are transparent. Consequently, all gross abnormalities in these living embryos, including those in their CNS, can be detected throughout the entire period of their development under a conventional stereomicroscope.[4–6)] Moreover, the CNS of medaka embryos can be examined easily in whole-mount preparations, because it is much smaller than that of mammalian embryos.[7)] Furthermore, gene expression and histogenetic patterns during brain morphogenesis in medaka are essentially similar to those reported in mammals,[8–11)] suggesting that medaka and mammals share many common mechanisms underlying brain formation.

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In a previous study, late medaka embryos (stages 28–30) were irradiated with 10 Gy x-ray, a lower dose than LD50.[6] Transient radiation-induced apoptosis in the CNS was observed in all living irradiated embryos under a stereomicroscope, and the dead cells were distributed mainly in the marginal proliferating regions of the optic tectum in the dorsal part of the midbrain.

To assess whether this finding could provide a method for quantitative evaluation of radiation-induced effects, we studied the effects of radiation-induced apoptosis in the optic tectum at lower doses in late medaka embryos and tried to define the threshold above which discernible damage occurred. Also we developed a new method of staining using AO to visualize and count dead cells in whole-mount specimens. This AO-staining method proved to be rapid and convenient compared to histological examination by terminal deoxynucleotidyl trasferanse-mediated dUTP-biotin nick end labeling (TUNEL)[6] which requires the laborious preparation of histological sections and effort in counting TUNEL-positive cells in many sections. By using this method, we were able to evaluate quantitatively the neurocytotoxic effects of radiation on developing medaka brains.

MATERIALS AND METHODS

The protocol of this study was approved by the Committee of Experimental Animals of the National Institute of Radiological Sciences, Chiba, Japan.

Medaka strain

An inbred strain, HO4C,[12–14] was used. The fish of this strain were kept at room temperature (26–29°C) and under a controlled photoperiod (14 h light/10 h dark cycles). The fish were fed on powdered fish food (Tetra-min, Tetra Werke Co., Mells, Germany) once a day. Under these conditions, the fish laid clusters of eggs daily.

Medaka embryos

Egg clusters were rubbed between two small pieces of paper towel to remove filaments on chorions, and singly isolated eggs were placed in a petri dish containing 7 ml of distilled water supplemented with 10^{-5}% methylene blue. The eggs were incubated at 26–29°C to develop. Living medaka embryos were observed several times a day under a stereomicroscope (Leica Mz125, Nussloch, Germany) at 50–100x. The developmental stages of embryos were determined in accordance with the method described by Iwamatsu (2004).[15]

X-ray irradiation

X-ray irradiation was performed using a 200-kVp X-ray machine (PANTAK-HF320, Shimadzu, Kyoto, Japan) at 200 kV and 20 mA with 0.5 mm Cu and 0.5 mm Al filters. The target-to-object distance was 50.0 cm, and the average dose rate in air was 1.3 Gy min^{-1}.

Medaka embryos at stage 28 (30-somite stage, 64 h after fertilization) were irradiated with single acute doses of 1, 2, 3.5, and 5 Gy x-ray, all of which are lower than the LD50 for the embryos.[6] This developmental stage corresponds approximately to the early fetal stages in human development.[10]

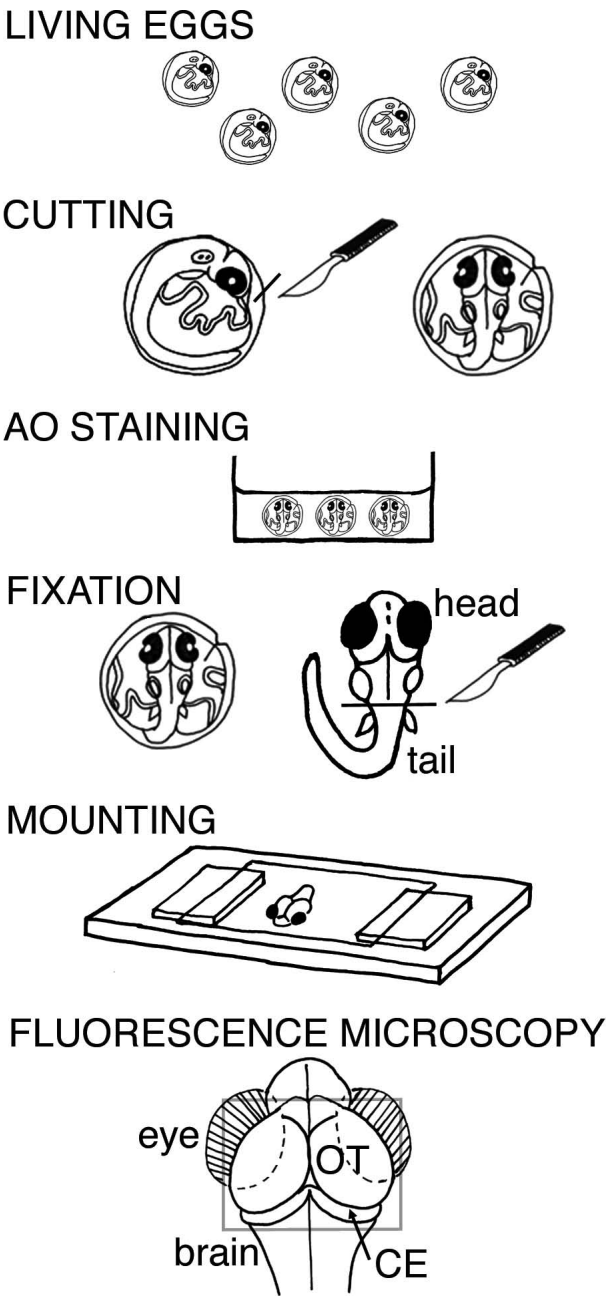

Fig. 1. Overall scheme of AO staining method used in this study. Medaka living embryos were stained with AO before fixation. The brain of the fixed embryo was mounted on a glass slide and observed under a fluorescence microscope. The boxed areas in the brains are shown in Fig. 2. CE = cerebellum; OT = optic tectum.

Staining with AO and counting number of AO-stained structures

The vital dye of AO (acridinium chloride hemi-[zinc chloride], Sigma), a DNA intercalating dye, stains selectively the nuclei of apoptotic cells and does not label significantly those of necrotic cells.[16] Living whole embryos were stained with AO as follows (Fig. 1): Embryos with their chorions were placed in 17 μg/ml AO in Iwamatsu's medium (110 mM NaCl, 5 mM KCl, 0.9 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1.2 mM $NaHCO_3$),[17] and the chorions were partly cut using a scalpel to enhance AO penetration into the embryos. After 1–2 h of incubation, the embryos with their chorions were rinsed with Dulbecco's PBS(–) (Nissui Pharmaceutical Co., Ltd.) for 10 min and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After 30 min of fixation, embryos were isolated from their chorions, and the heads of the embryos were cut off using a scalpel in PBS(–). Each head was placed in a drop of PBS(–) on a glass slide, on which a pair of spacers (0.3 mm in thickness) had been glued on both sides, so that the dorsal aspect of the brain could be observed, and covered with a cover glass. The brain was observed under a fluorescence microscope (Nikon, Eclipse E600, Tokyo, Japan) with a green filter at 200x.[18] Images were taken using a Fujichrome SensiaIII 100

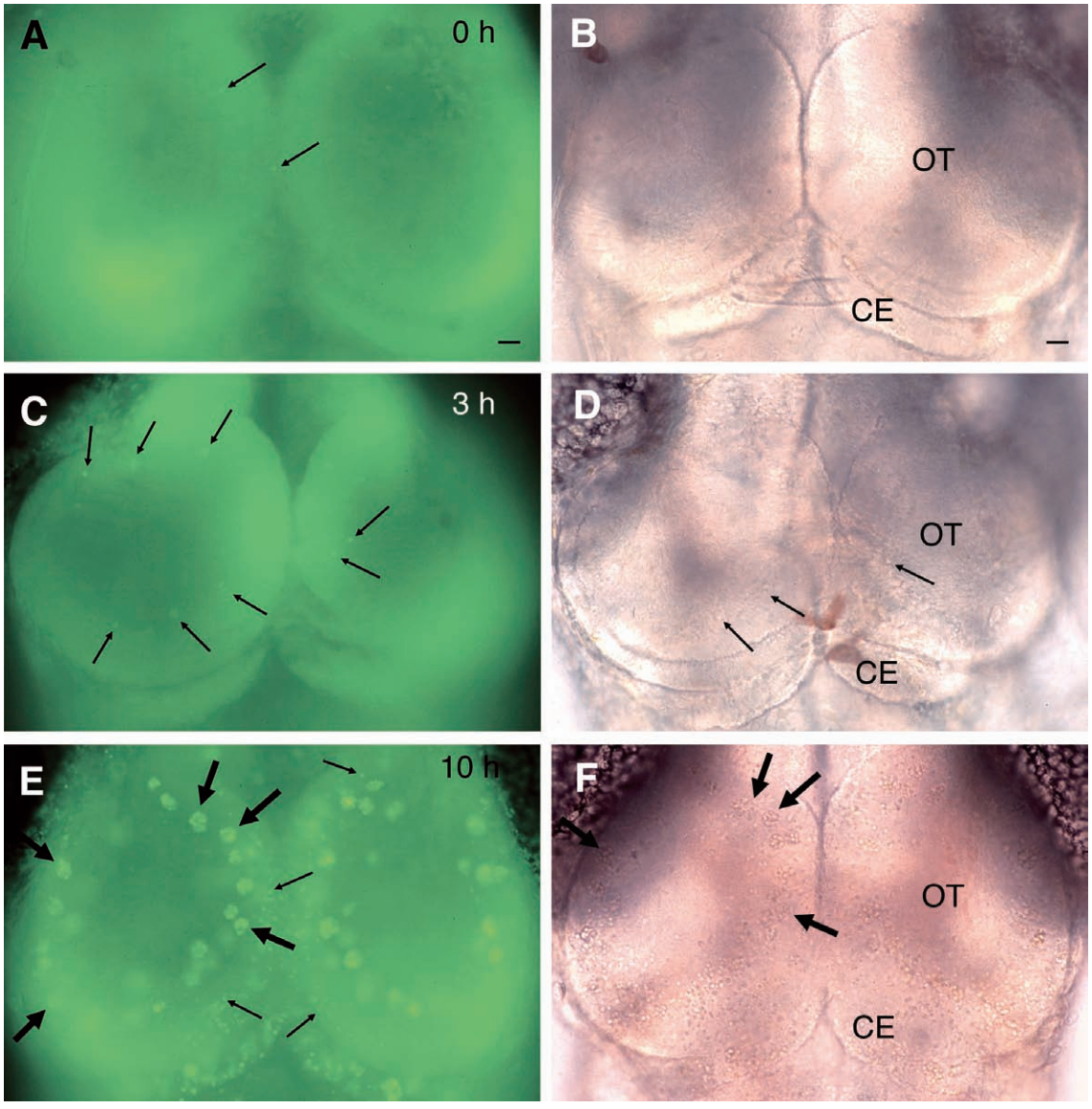

Fig. 2. Whole-mount AO staining of embryonic brains. The optic tectum (OT) of a nonirradiated normal embryo at stage 30 (A, B), and those of irradiated embryos stained 3 h (C, D) and 10 h (E, F) after 5 Gy x-ray irradiation. Fluorescence microscopy images (A, C, E) and their bright field counterpart images (B, D, F, respectively). Dorsal views, rostral to top. Thin arrows indicate small single nuclei and thick arrows indicate rosette-shaped clusters. CE = cerebellum. Scale bar = 20 μm.

daylight film.

Two types of AO-stained structures were found in the brain: small single nuclei and rosette-shaped clusters of apoptotic cells (see Results). For the quantitative evaluation of the neurocytotoxic effects of radiation, we counted the number of rosette-shaped clusters/optic tectum.

Sections for light microscopy and electron microscopy

Embryos were separated from their chorions in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) within 5 min, and fixed by immersion in this mixture of fixatives for 24 h at 0–4°C. After washing with the same buffer for 2 h, the embryos were postfixed with 2% osmium tetroxide in the same buffer for 2 h at room temperature. After dehydration in graded concentrations of ethanol, they were incubated with propylene oxide and embedded in Epok812 epoxy resin. Serial sections (1 μm) were cut and stained with toluidine blue for light microscopy. Ultrathin sections were also cut and stained with lead citrate for observation under an electron microscope. The sections were observed under a Hitachi H-7500 electron microscope operated at 80 kV.

Photomicrographs

Photomicrographs were scanned using a scanner and a slide mount folder (Dimage Scan Multi II, Minolta Co., Ltd.,

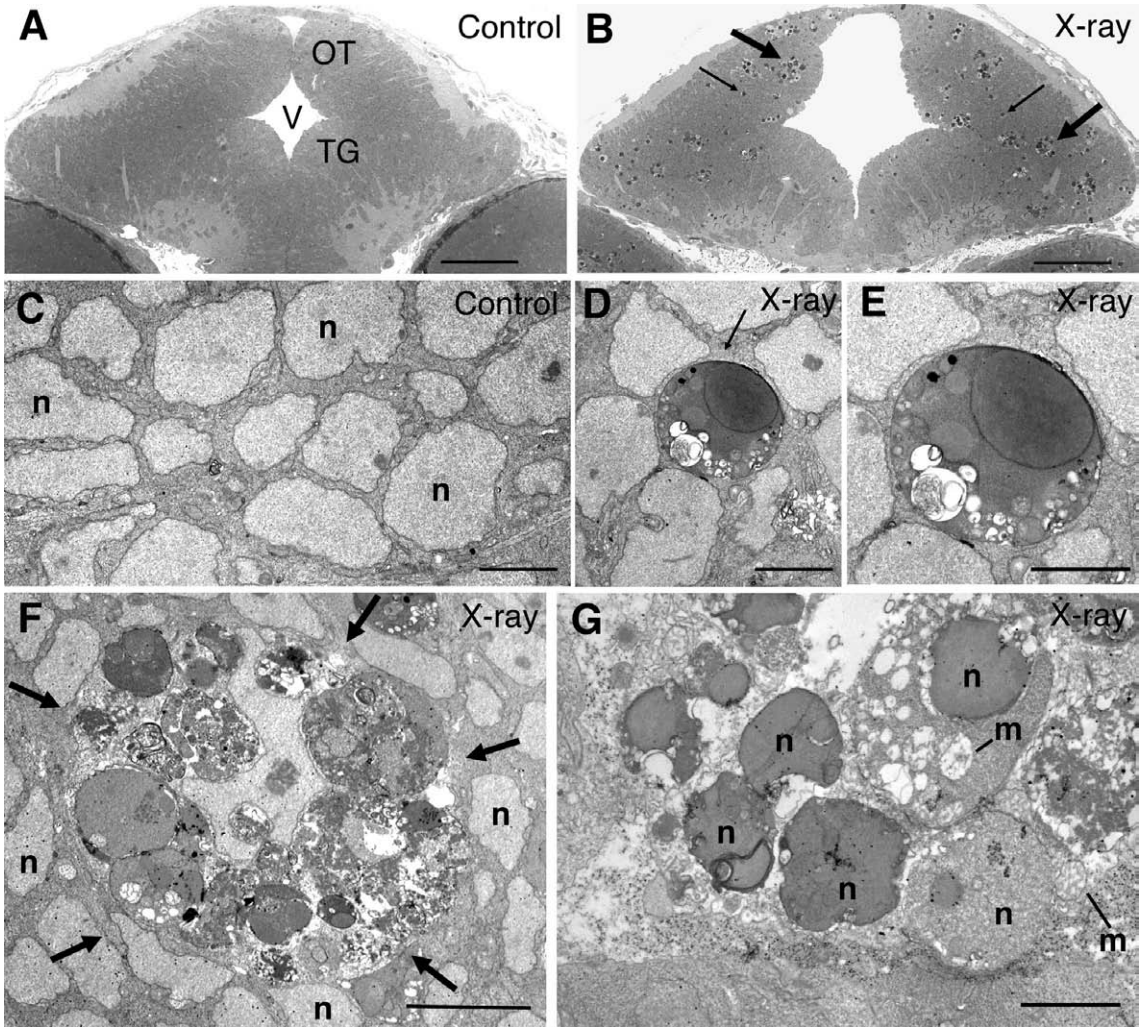

Fig. 3. Histological features of optic tectum of nonirradiated control embryos (A, C) and those of irradiated embryos 24 h after irradiation with 5 Gy x-ray (B, D–G). **(A, B)** Frontal semithin sections (1 μm) at the level of the midmesencephalon (toluidine blue staining) are observed by light microscopy. Dorsal to top. Thin arrows indicate small single nuclei and thick arrows indicate rosette-shaped clusters. Scale bar = 50 μm. **(C–G)** Electron micrographs of optic tectum of nonirradiated normal embryo (C) and irradiated embryos (D–G). A single apoptotic cell with condensed nucleus (thin arrow in D) and its magnified image (E). An aggregation of apoptotic cells (thick arrows in F) and the magnified image of another aggregation of apoptotic cells (G). n = nucleus; m = mitochondria; OT = optic tectum; TG = mesencephalic tegmentum; V = ventricle. C, D: Scale bar = 3 μm. E, G: Scale bar = 2 μm. F: Scale bar = 6 μm.

Tokyo). Adobe Photoshop, Macintosh version 7.0 (Adobe Systems Incorporated, Mountain View, CA) was used to prepare the figures and enhance contrast.

Statistical analysis

Differences in the numbers of rosette-shaped clusters/optic tectum at the different x-ray doses used were analyzed using analysis of variance (ANOVA) followed by Bonferroni post-hoc test for multiple comparisons. A p value less than 0.05 was considered to be statistically significant, while a value less than 0.01 was considered to be highly statistically significant.

RESULTS

Brain cell death detected by AO staining in non-irradiated embryos at stage 30

Normal nonirradiated embryos (n = 5) at stage 30 (24 h after stage 28) were stained with AO, and the optic tectum was examined under a fluorescence microscope (Fig. 2A). We observed only 4–10 AO-positive single nuclei appeared to be associated with the programmed cell death during normal development. They were distributed diffusely in the optic tectum (Fig. 2A, thin arrows). This was almost consistent with previous results obtained by TUNEL.[19)]

Time-lapse changes in AO staining pattern after irradiation

Embryos (n = 30) at stage 28 were irradiated with 5 Gy x-ray. The irradiated embryos were stained with AO 3, 7, 10, 20, 30, and 34 h after irradiation (Figs. 2C, E). Three hours after irradiation, many small single nuclei less than 5 μm in diameter started to appear predominantly in the marginal tectal regions (Fig. 2C, thin arrows). It is reported that the marginal tectal regions are the proliferating zones of the optic tectum.[20)] From 7 to 10 h after irradiation, 100–300 small single nuclei were observed in the entire region of the optic tectum (Fig. 2E, thin arrows). In addition to these single nuclei, 25–60 rosette-shaped clusters having diameters in the range of 15–30 μm and composed of 4–12 nuclei each, were observed in the marginal regions of the tectum (Fig. 2E, thick arrows). From 10 to 30 h after irradiation, a similar staining pattern was observed (Fig. 2E): about 60 rosette-shaped nuclear clusters and a few hundreds of small single nuclei were present in the optic tectum. Thirty-four hours after irradiation, the numbers of rosette-shaped nuclear clusters and small single nuclei started to decrease (data not shown).

Thus, we found two morphologically distinct AO-stained structures in the optic tectum 7 h after irradiation, namely, small single nuclei and rosette-shaped clusters.

Histological observations

To examine the histological features of the two morphologically distinct AO-stained structures, we prepared histological sections of the optic tectum 24 h after irradiation with 5 Gy x-ray (Fig. 3). Light microscopy showed that there were two morphologically different types of condensed nuclei, namely, single condensed nuclei (Fig. 3B, thin arrows) and aggregates of those nuclei (Fig. 3B, thick arrows), similar to the results of AO staining. Electron microscopy revealed in more details these two distinct types of structures (Fig. 3D&F, thin arrows showing single apoptotic cells with condensed nuclei and thick arrows showing aggregates of apoptotic cells, respectively). The nuclei of the singly distributed apoptotic cells were less than 5 μm in diameter, indicating that the AO-stained single nuclei are the nuclei of single apoptotic cells (Figs. 3D, E). Apoptotic cell aggregates, which were composed of about 10 apoptotic cells, were generally round or ovoid and more than 15 μm in diameter, indicating that the AO-stained rosette-shaped clusters are the groups of nuclei of aggregated apoptotic cells (Figs. 3F, G).

Degree of brain cell death in embryos irradiated at various X-ray doses

Embryos (n = 45) at stage 28 were irradiated with x-ray at various doses, and the irradiated embryos were stained with AO 20–24 h after irradiation. We found that the number of AO-stained rosette-shaped clusters/optic tectum as well as that of AO-stained single nuclei/optic tectum increased with increasing x-ray dose (Fig. 4 and data not shown). To evaluate the degree of radiation-induced effects quantitatively,

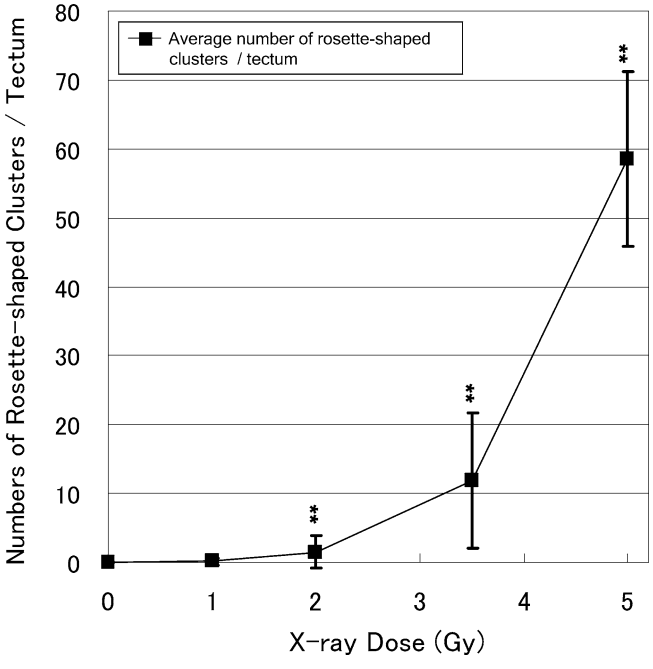

Fig. 4. Effects of x-rays on the number of rosette-shaped nuclear clusters in optic tectum of medaka embryos. The error bars show the standard deviations of the means. **p < 0.01 vs control (0 Gy).

we counted only the number of AO-stained rosette-shaped nuclear clusters/optic tectum for the following reasons. First, AO-stained rosette-shaped nuclear clusters were not observed in the nonirradiated embryos (Fig. 2), indicating that they are radiation-specific structures. Second, AO-stained small single nuclei were too numerous to count precisely and their images sometimes overlapped. Third, the number of AO-stained rosette-shaped nuclear clusters was small and the clusters can be easily detected because of their characteristic morphology and their positions in the optic tectum (Fig. 2E). Fourth, the number of AO-stained rosette-shaped nuclear clusters was almost unchanged from 10 to 30 h after irradiation (see above).

Because the thickness of the optic tectum of medaka embryos at stage 30 is only less than 80 μm (Figs. 3A, B), we were able to count all of the rosette-shaped clusters by changing the focus during fluorescence microscopy. Figure 4 shows the dose dependence of the number of AO-stained rosette-shaped nuclear clusters/optic tectum. The numbers of clusters/optic tectum in embryos exposed to 2 Gy (1.5 ± 2.3, $n = 10$), 3.5 Gy (12 ± 9.8, $n = 12$), and 5 Gy (59 ± 13, $n = 10$) x-ray, were highly significant compared to control embryos (0 ± 0, $n = 20$) (Fig. 4). The number of clusters/optic tectum in the embryos irradiated with 1 Gy (0.15 ± 0.55, $n = 13$) x-ray was not significantly different from that in nonirradiated control embryos. Thus, it was suggested that the threshold dose for the developmental neurocyotoxic effects of radiation was taken as between 1–2 Gy x-ray.

DISCUSSION

In this study, living irradiated embryos were stained with AO and whole-mount brains were examined under an epifluorescence microscope. We found two morphologically distinct AO-stained structures in the optic tectum 7 h after irradiation with 5 Gy x-ray, namely, small single nuclei and rosette-shaped clusters (Fig. 2E). Electron microscopy revealed that these two distinct structures are single apoptotic cells with condensed nuclei and aggregates of apoptotic cells, respectively (Fig. 3 D–G). The numbers of the single apoptotic cells and aggregates of apoptotic cells started to decrease 34 h after irradiation (data not shown).

Radiation-induced apoptosis has been classified into several types on the basis of mechanisms related to the cell cycle.[21–23] Shinomiya proposed two types of radiation-induced cell death: "premitotic apoptosis" and "postmitotic apoptosis".[22] The former is a rapid (within 4–6 h) apoptosis that occurs before cell division and results in single dead cells. In contrast, the latter is a delayed apoptosis that occurs after several cycles of cell division and results in clonal clusters of dead cells. Each rosette-shaped nuclear cluster in the present study is likely the clonal cluster of dead cells caused by postmitotic apoptosis, because it is first observable 7 h after irradiation and appears to be the descendant cells of a single cell that was damaged by radiation. This possibility is supported by our observation that one-cell medaka embryos did not die immediately after irradiation with 5 Gy x-ray, but cell division proceeded for more than 10 h before embryonic death (data not shown). Similar clonal clusters of dead cells have also been reported in irradiated yeast cells.[24,25] Radiation-induced apoptosis also exhibits a series of distinct morphological changes such as nuclear condensation, fragmentation and engulfment by macrophages.[26,27] The decrease of AO-stained structures 34 h after irradiation in this study seems to be due to loss of apoptotic cells by phagocytosis.

The continual formation of apoptotic cells and their continual elimination by macrophage degradation after irradiation are very dynamic processes. Consequently, an image of apoptotic cells at a time point after irradiation provides only a static snapshot of the entire radiation-induced cytologic changes. For this reason, the counting of apoptotic cells at a fixed time after irradiation may lead to a significant underestimation of the total number of apoptotic cells. Nevertheless, counting the number of apoptotic cells at a time point after irradiation is a useful method for the quantitative estimation of the neurocytotoxic effects of radiation, because radiation-induced apoptosis is one of the important events that can be used for evaluating the biological effects of radiation.[28,29] Many investigators employ the apoptotic index by counting the numbers of apoptotic cells and normal cells at a fixed time after irradiation in certain areas of sections of irradiated organs to estimate radiation-induced cytological effects.[30,31]

In the present study, we employed the number of AO-stained rosette-shaped nuclear clusters/optic tectum 20–24 h after irradiation as an index of the neurocytotoxic effects of radiation, for the reasons described in the Results section, and found that the number increased in a dose-dependent manner (Fig. 4). This method enabled us to estimate the lowest irradiation dose that can cause cytotoxic effects on developing medaka brains and the highest dose that causes no cytotoxic effects, as 2 Gy and 1 Gy, respectively. The threshold dose for developmental neurocyototoxic effects on medaka embryos was therefore taken as between 1–2 Gy (Fig. 4).

Many previous studies of acute cell death in the prenatal brain of rodents showed that the threshold range for radiation-induced neurocytototoxic effects is 0.05–0.25 Gy during the most sensitive developmental period,[32] namely, the beginning of corticogenesis (embryonic day 13 in the mouse corresponding to embryonic day 15 in the rat). In humans, during the most sensitive period of development (approximately 8–15 weeks postovulation), a dose-response relationship including a threshold with a lower boundary range of 0.15–0.25 Gy or 0.06–0.31 Gy is more likely than a linear nonthreshold relationship.[33–35] The period of 8–15 weeks postovulation in humans, when neural cells proliferate rap-

idly and neurons migrate to their final functional sites in the cerebral cortex,[36] and the corticogenesis periods in rodents correspond approximately to developmental stages 28–30 in medaka embryos.[10] Moreover, gene expression and histogenetic patterns during brain morphogenesis in medaka are essentially similar to those reported in mammals,[8–11] suggesting that many mechanisms underlying brain development are common in all vertebrates.[37] The radiosensitivity of medaka embryos markedly changes during their development, and that of late-stage embryos is much lower than that of mammalian embryos.[6,38–43] Although extrapolations in systems that are different in terms of radiosensitivity, genome size,[44] and radiation quality (x-rays, γ-rays, and neutrons)[45] must be carried out with care, our finding that the threshold dose for developmental neurocytotoxic effects was taken between 1–2 Gy, may not be so extraordinarily different from those reported in mammals.

Although radiation-induced brain cell death can be visualized by TUNEL in irradiated embryos,[6] TUNEL requires the laborious preparation of histological sections and efforts in counting TUNEL-positive cells in many sections. In contrast to TUNEL, this whole-mount method of staining using AO provides a more rapid and easy method to evaluate quantitatively the neurocytotoxic effects of radiation as shown in the present study (Figs. 2 and 4). In this study, however, the standard deviations of the counting numbers were large, probably because of the developmental differences in individual embryos and the errors in counting rosette-shaped clusters. In future studies, we should improve this method to obtain better reproducibility. For example, use of a confocal fluorescent microscope may be more suitable to detect precisely and easily all the rosette-shaped clusters at different depths in the irradiated embryos.

Recent studies using TUNEL show that exposure of endocrine disrupting compounds at nonlethal level results in apoptosis in medaka brains.[46] We consider that our AO-staining method would be applicable to examining not only radiation-induced brain cell death, but also to screening the developmental neurocytotoxic effects of various hazardous factors such as chemical pollutants that may potentially cause brain cell death.

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REFERENCES

1. ICRP (1986) Developmental effects of irradiation on the brain of the embryo and fetus. Publication 49, Annals of the ICRP **16**(4): 1–43, Pergamon Press, Oxford.
2. ICRP (1991b) Risks associated with ionizing radiations. Ionizing radiation and the developing human brain. Annals of the ICRP **22**(1): 95–118, Pergamon Press, Oxford.
3. UNSCEAR (1993) Sources and Effects of Ionizing Radiation, United Nations Scientific Committee on the Effects of Atomic Radiation, 1993 UNSCEAR Report to the General Assembly, with Scientific Annexes, Radiation effects on the developing human brain. United Nations Publication, New York.
4. Ishikawa, Y. (2000) Medaka fish as a model system for vertebrate developmental genetics. BioEssays **22**: 487–495.
5. Furutani-Seiki, M. and Wittbrodt, J. (2004) Medaka and zebrafish, an evolutionary twin study. Mech. Dev. **121**: 629–637.
6. Yasuda, T., Aoki, K., Matsumoto, A., Maruyama, K., Hyodo-Taguchi, Y., Fushiki, S. and Ishikawa, Y. (2006) Radiation-induced brain cell death can be observed in living medaka embryos. J. Radiat. Res. **47**: 295–303.
7. Ishikawa, Y. and Hyodo-Taguchi, Y. (1994) Cranial nerves and brain fiber systems of the medaka fry as observed by a whole-mount staining method. Neurosci. Res. **19**: 379–386.
8. Ishikawa, Y., Kage, T., Yamamoto, N., Yoshimoto, M., Yasuda, T., Matsumoto, A., Maruyama, K. and Ito, H. (2004) Axonogenesis in the medaka embryonic brain. J. Comp. Neurol. **476**: 240–253.
9. Kage, T., Takeda, H., Yasuda, T., Maruyama, K., Yamamoto, N., Yoshimoto, M., Araki, K., Inohaya, K., Okamoto, H., Yasumasu, S., Watanabe, K., Ito, H. and Ishikawa, Y. (2004) Morphogenesis and regionalization of the medaka embryonic brain. J. Comp. Neurol. **476**: 219–239.
10. Ishikawa, Y., Yamamoto, N., Yoshimoto, M., Yasuda, T., Maruyama, K., Kage, T., Takeda, H. and Ito, H. (2007) Developmental origin of diencephalic sensory relay nuclei in teleost. Brain. Behav. Evol. **69**: 87–95.
11. Ishikawa, Y., Yasuda, T., Kage, T., Takashima, S., Yoshimoto, M., Yamamoto, N., Maruyama, K., Takeda, H. and Ito, H. (2008) Early development of the cerebellum in teleost fishes: a study based on gene expression patterns and histology in medaka embryo. Zool. Sci. **25**: 407–418.
12. Hyodo-Taguchi, Y. (1980) Establishment of inbred strains of the teleost, *Oryzias latipes* (in Japanese with English abstract). Zool. Mag. (Tokyo) **89**: 283–301.
13. Hyodo-Taguchi, Y. and Egami, N. (1985) Establishment of inbred strains of the medaka, *Oryzias latipes*, and the usefulness of the strains for biomedical research. Zool. Sci. **2**: 305–316.
14. Hyodo-Taguchi, Y. (1996) Inbred strains of the medaka, *Oryzias latipes*. Fish Biol. J. Medaka **8**: 11–14.
15. Iwamatsu, T. (2004) Stages of normal development in the medaka *Oryzias latipes*. Mech. Dev. **121**: 605–618.
16. Abrams, J. M., White, K., Fessler, L. I. and Steller, H. (1993) Programmed cell death during *Drosophila* embryogenesis. Development **117**: 29–43.
17. Iwamatsu, T. (1983) A new technique for dechorination and observations on the development of the naked egg in *Orizias latipes*. J. Exp. Zool. **228**: 83–89.
18. Furutani-Seiki, M., Jiang, Y. J., Brand, M., Heisenberg, C.-P.,

Houart, C., Beuchle, D., Eeden, van F. J. M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., Kelsh, R. N., Mullins, M. C., Odenthal, J. and Nüsslein–Volhard, C. (1996) Neural degeneration mutants in the zebrafish, *Danio rerio*. Development **123**: 229–239.
19. Ishikawa, Y., Yasuda, T., Maeda, K., Matsumoto, A. and Maruyama, K. (2007) Apoptosis in neural tube during normal development of medaka. Fish Biol. J. Medaka. **11**: 23–30.
20. Nguyen, V., Deschet, K., Henrich, T., Godet, E., Joly, J.-S., Wittbrodt, J., Chourrout, D. and Bourrat, F. (1999) Morphogenesis of the optic tectum in the medaka (*Oryzias latipes*): A morphological and molecular study, with special emphasis on cell proliferation. J. Comp. Neurol. **413**: 385–404.
21. Halicka, H. D., Seiter, K., Feldman, E. J., Traganos, F., Mittelman, A., Ahmed, T. and Darzynkiewiez, Z. (1997) Cell cycle specificity of apoptosis during treatment of leukaemias. Apoptosis **2**: 25–39.
22. Shinomiya, N. (2001) New concepts in radiation-induced apoptosis: 'premiotic apoptosis' and 'postmitotic apoptosis'. J. Cell Mol. Med. **5**: 240–253.
23. Huang, X., Halicka, H. D., Traganos, F., Tanaka, T., Kurose, A. and Darzynkiewicz, Z. (2005) Cytometric assessment of DNA damage in relation to cell cycle phase and apoptosis. Cell Prolif. **38**: 223–243.
24. James, A. P. and Werner, M. M. (1966) Radiation-induced lethal sectoring in yeast. Rad. Res. **29**: 523–536.
25. James, A. P. and Werner, M. M. (1969) Multiple-step recovery from heritable lethal sectoring in yeast. Genetics **62**: 533–541.
26. Kerr, J. F. R., Wyllie, A. H. and Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br. J. Cancer **26**: 239–257.
27. Wyllie, A. H., Kerr, J. F. R. and Currie, A. R. (1980) Cell death: the significance of apoptosis. Int. Rev. Cytol. **68**: 251–306.
28. Warenius, H. M. and Down, J. D. (1995) RBE of fast neutrons for apoptosis in mouse thymocytes. Int. J. Radiat. Biol. **68**: 625–629.
29. Hendry, J. H., Potten, C. S. and Merritt, A. (1995) Apoptosis induced by high- and low-LET radiations. Radiat. Environ. Biophys. **34**: 59–62.
30. Hendry, J. H., Potten, C. S. and Chadwick, C. (1982) Cell death (apoptosis) in the mouse small intestine after low doses: effects of dose-rate, 14.7 MeV neutrons, and 600 MeV (maximum energy) neutrons. Int. J. Radiat. Biol. **42**: 611–620.
31. Ishida, Y., Ohmachi, Y., Nakata, Y., Hiraoka, T., Hamano, T., Fushiki, S. and Ogiu, T. (2006) Dose-response and large relative biological effectiveness of fast neutrons with regard to mouse fetal cerebral neuron apoptosis. J. Radiat. Res. **47**: 41–46.
32. ICRP (2003) Biological effects after prenatal irradiation (embryo and fetus). Publication 90, Annals of the ICRP 33 (1–2), Pergamon Press, Oxford.
33. Otake, M. and Schull, W. J. (1990) Radiation-related posterior lenticular opacities in Hiroshima and Nagasaki atomic bomb survivors based on the DS86 dosimetry system. Radiat. Res. **121**: 3–13.
34. ICRP (1991) 1990 Recommendation of the International Commission on Radiological Protection. Publication 60, Annals of the ICRP 21 (1–3), Pergamon Press, Oxford.
35. Otake, M., Schull, W. J. and Lee, S. (1996) Threshold for radiation-related severe mental retardation in prenatally exposed A-bomb survivors: a re-analysis. Int. J. Radiat. Biol. **70**: 755–763.
36. Williams, R. S. (1989) Cerebral malformations arising in the first half of gestation. Dev. Neurobiol. **12**: 11–20.
37. Nieuwenhuys, R. (1998) Morphogenesis and general structures. In: The central nervous system of vertebrates, Eds. Nieuwenhuys, R., Ten Donkelaar, H. J., Nicholson, C., pp. 158–228, vol. 1, Springer, Berlin.
38. Hyodo-Taguchi, Y. and Egami, N. (1969) Change in dose-survival time relationship after X-irradiation during embryonic development in the fish, *Oryzias latipes.* J. Radiat. Res. **10**: 121–125.
39. Hyodo-Taguchi, Y., Etoh, H. and Egami, N. (1973) RBE of fast neutrons for inhibition of hatchability in fish embryos irradiated at different developmental stages. Radiat. Res. **53**: 385–391.
40. Egami, N. and Hama, A. (1975) Dose-rate effects on the hatchability of irradiated embryos of the fish, *Oryzias latipes*. Int. J. Radiat. Biol. **28**: 273–278.
41. Suyama, I., Etoh, H., Maruyama, T., Kato, Y. and Ichikawa, R. (1981) Effects of ionizing radiation on the early development of *Oryzias* eggs. J. Radiat. Res. **22**: 125–133.
42. Etoh, H. and Hyodo-Taguchi, Y. (1983) Effects of tritiated water on germ cells in Medaka embryos. Radiat. Res. **93**: 332–339.
43. Ishikawa, Y. and Hyodo-Taguchi, Y. (1997) Heritable malformations in the progeny of the male medaka (*Oryzias latipes*) irradiated with X-rays. Mutation Res. **389**: 149–155.
44. Abrahamson, S., Bender, M. A., Conger, S. and Wolff, S. (1973) Uniformity of radiation-induced mutation rates among different species. Nature **245**: 460–462.
45. ICRP (2003) Relative biological effectiveness (RBE), quality factor (Q), and radiation weighting factor (W_R). Publication 92, Annals of the ICRP 33 (4), Pergamon Press, Oxford.
46. Stokes, E. A., Lonergan, W., Weber, L. P., Janz, D. M., Poznanski, A. A., Balch, G. C., Metcalfe, C. D. and Grober, M. S. (2004) Decreased apoptosis in the forebrain of adult male medaka (*oryzias latipes*) after aqueous exposure to ethinylestradiol. Comp. Biochem. Physiol. part C **138**: 163–167.

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