The Fish Biology Journal MEDAKA, **11**: 23–30 (2007)

Apoptosis in neural tube during normal development of medaka

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Key words: developmental neuroscience, cell death, brain, embryo, vertebrate

Abstract Neural apoptosis in normal medaka (*Oryzias latipes*) embryos at stage 20 (the neural rod step in brain development), stage 27 (the neural tube step in brain development), and stage 29 (the late embryonic brain step in brain development) were examined, and the results were compared with those reported for other vertebrates, particularly zebrafish. Apoptotic cells were identified using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling), by which fragmented DNA, which is characteristic of apoptotic cells, is labeled. Whole-mount TUNEL showed the following gross pattern of apoptotic changes during development. At stage 20, numerous single apoptotic cells were distributed diffusely in the entire regions of the brain. At stage 27, in addition to the diffusely distributed apoptotic cells, several large aggregates of TUNEL-positive structures (apoptotic centers) appeared in the brain and eyes. Histological observations of sections of the stained embryos revealed that apoptotic centers are present in the zona limitans intrathalamica, the ventral region of the optic stalk, several sites of the floor plate, and the retina. At stage 29, both single apoptotic cells and apoptotic centers decreased in number. This apoptotic pattern in medaka embryos is generally similar to that in zebrafish embryos, but apoptosis in the nervous system continues for a longer period in medaka than in zebrafish. Apoptosis in the vertebrate nervous system seems to be ubiquitous, random and probabilistic in nature.

Introduction

Medaka (*Oryzias latipes*) embryos are suitable living materials for detecting radiation-induced brain cell death (Yasuda *et al.*, 2006). To quantitatively estimate the developmental neurocytotoxic effects of radiation, it is necessary to determine the extent and distribution of cell death during normal development of the medaka brain. However, there are few reports on neural degeneration during normal medaka development. We describe here cell death during normal development of medaka embryos at the neural tube step, a phylogenic stage of brain development in vertebrates (Kage *et al.*, 2004).

Normal development is a process in which construction and destruction occur simultaneously. Regressive developmental processes are considered to be the purposeful and determined removal of cells by the naturally occurring cell death or programmed cell death, which proceeds through a series of distinct morphological stages, known as apoptosis (Kerr *et al.*, 1972). The apoptosis results from an active developmental program often triggered by the withdrawal of trophic factors and local cell interactions (Kerr *et al.*, 1972; Oppenheim, 1985; Abrams *et al.*, 1993; Raff *et al.*, 1993).

Up to 50% or more of neurons normally die immediately after they differentiate during the development of the vertebrate nervous system (Oppenheim, 1985; Raff *et al.*, 1993). Apoptosis during the development of the nervous system has been examined in various vertebrate embryos (Källén, 1965; for a review, see Jacobson, 1991). Recently, genetic analyses of neural degeneration have been performed in zebrafish (Danio rerio), and apoptosis during normal zebrafish development has been described (Abdelilah *et al.*, 1996; Furutani-Seiki *et al.*, 1996).

Furutani-Seiki *et al.* (1996) used acridine orange, a vital dye, to stain apoptotic cells in living normal zebrafish embryos and observed them in whole mount. According to their results, the number of apoptotic cells in the neuroectoderm peaks at around the 10-somite stage (14 h after fertilization; which may correspond to Iwamatsu's stage 22 of medaka embryos; see Furutani-Seiki and Wittbrodt, 2004) and then decreases. Very few apoptotic cells are observed in the neural

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tube after the 20-somite stage (19 h after fetilization; which may correspond to Iwamatsu's stage 25 of medaka embryos; see Furutani-Seiki and Wittbrodt, 2004). They gave no precise description of the morphological locations of apoptotic cells in developing neural tubes, however, because of the low resolution in the whole-mount observations.

To determine the actual distribution of the apoptotic cells in the nervous system, we need to perform histological examinations of sections at a higher resolution. Hence, in this study, to detect apoptotic cells, we employed terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) (Gavrieli *et al.*, 1992), by which fragmented DNA, which is characteristic of apoptotic cells, is labeled, and then prepared serial sections of the labeled medaka embryos. Our results showed that a large number of apoptotic cells are distributed diffusely in the entire region of the medaka neural tube, and local centers of apoptosis are present in several regions.

Materials and Methods

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 (Hyodo-Taguchi, 1980; Hyodo-Taguchi and Egami, 1985; Hyodo-Taguchi, 1996), was used. Fish of this strain were kept in a room under controlled water temperature (26–29°C) and photoperiod (14-hr light/10-hr dark cycle). The fish were given 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

The clusters of eggs were rubbed between two small pieces of paper towel to remove filaments on the chorions, and singly isolated eggs were placed in a petri dish containing 7 ml of distilled water supplemented with 0.00001% methylene blue. The eggs were incubated at 26°C and allowed to develop. Living medaka embryos were observed under a stereomicroscope (Leica Mz125, Nussloch, Germany) at magnifications of 50-100x. The developmental stages were determined according to the method described by Iwamatsu (2004).

It has been shown that the development of the medaka brain can be subdivided into six sequential steps of development: 1) the gastrula, 2) neurula, 3) neural rod, 4) neural tube, 5) late embryonic brain, and 6) fry brain steps (Ishikawa, 1997; Kage *et al.*, 2004; Ishikawa *et al.*, 2007). We examined apoptosis in developing medaka brains at three developmental phases, namely, at the neural rod (stage 20, 4-somite stage, 31.5 h after fertilization at 26°C), neural tube (stage 27, 24-somite stage, 58 h after fertilization at 26°C), and late embryonic brain (stage 29, 34-somite stage, 74 h after fertilization at 26°C) steps. The earliest axons start to project at stage 22 (9-somite stage, 38 h after fertilization at 26°C) (Ishikawa *et al.*, 2004). Hence, all the brain walls are still composed of neuroepithelial cells (matrix layer) only, and all neurons are undifferentiated at the neural rod step (stage 20).

Whole-mount TUNEL

Embryos were anesthetized by cooling on ice and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). TUNEL-positive cells in the embryos were detected by whole-mount TUNEL (Yamamoto and Henderson, 1999) using an ApopTag Peroxidase *In Situ* Apoptosis Detection kit (S7100, Chemicon International Inc., Temecula, CA, USA). Stained embryos were mounted in a viewing chamber, in which an agar bed (2 mm in depth) with several holes (1 mm in diameter) had been prepared. The embryos were placed in the holes, so that a correct dorsal aspect of the brain could be observed under a compound microscope (Nikon, Eclipse E600, Tokyo, Japan). Images of the brains were taken at a magnification of 100x.

Histological examination

To examine histological features in more detail, some TUNEL-stained embryos were fixed again in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), dehydrated, embedded in Technovit 8100 (Kulzer, Wehrheim, Germany) and cut into a complete series of serial sections (10 μm). The sections were Nissl-stained with cresyl violet. Images of the sections were taken at a magnification of 400x.

Photomicrography production

Photomicrographs were scanned using a scanner and a slide mount folder (Dimage Scan Multi II, Minolta Co., Ltd., Tokyo). Adobe Photoshop, Macintosh version 7.0 (Adobe Systems Incorporated, Mountain View, CA) was used to prepare the figures and enhance contrast.

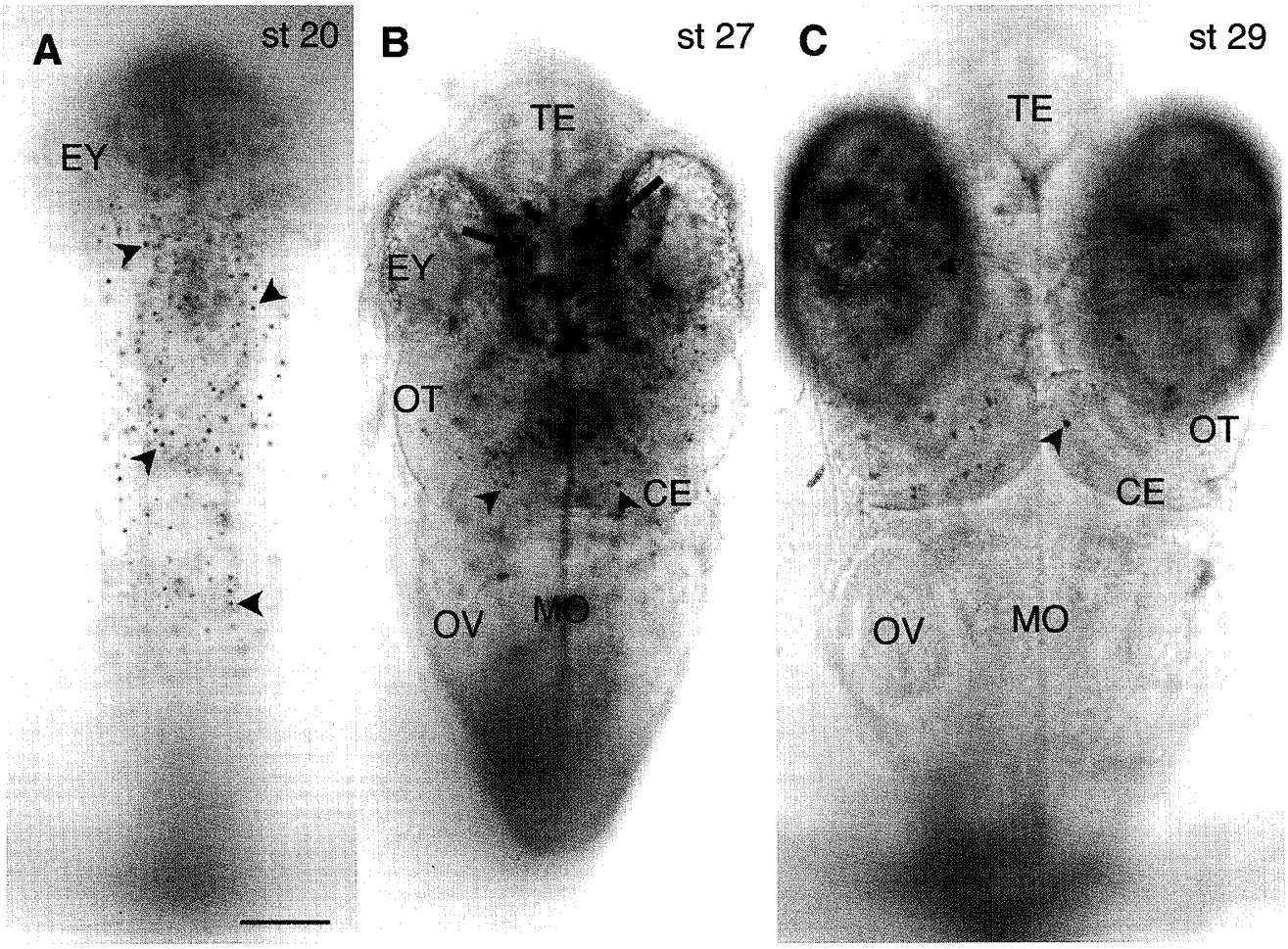

Fig. 1 Whole-mount TUNEL of normal embryos at stages 20 (**A**), 27 (**B**), and 29 (**C**). Dorsal views, rostral to top. Arrowheads indicate small single TUNEL-positive dots, and arrows indicate large patches of TUNEL-positive structures. CE = cerebellum; EY = optic vesicle; MO = medulla oblongata; OT = optic tectum; OV = otic vesicle; TE = telencephalon. Scale bar = 100 μm.

Results

Apoptotic cells detected in whole-mount specimens

Normal embryos at stage 27 (neural tube), at a younger stage (stage 20, neural rod), and at an older stage (stage 29, late embryonic brain) were examined.

Whole-mount TUNEL showed that the gross pattern of apoptosis can be observed easily by this method (Fig. 1). Apoptosis occurred in all regions of the body, including brain and eye regions, of medaka embryos at stages 20, 27 and 29 (Fig. 1). We focused on the features in the brain and eye regions and did not further examine in detail those in the other body regions.

At stage 20, numerous single TUNEL-positive cells, which are observed as small single brown dots, were distributed diffusely in the entire regions of the brain and eyes (Fig. 1A, arrowheads). At stage 27, several large aggregates of TUNEL-positive structures, which are observed as large brown patches, started to appear in the brain and eyes (Fig. 1B, arrows). At stage 29, both small single dots and large patches decreased in number (Fig. 1C).

Apoptotic cells observed in sections

We focused on the features of apoptosis in embryos at the neural tube step (Figs. 2 and 3), a phylogenic stage of vertebrate brain development (Kage *et al.*, 2004).

Closer observations of Nissl-stained sections of the whole-mount specimens revealed that there are two morphologically distinct TUNEL-positive structures: small single nuclei (arrowheads in Figs. 2 and 3) and their large aggregates (arrows in Figs. 2 and 3).

Many small single nuclei less than 5 μm in diameter, which are considered to be condensed nuclei of single apoptotic cells, were distributed diffusely in the entire regions of the neural tube and retina (Figs. 2 and 3, arrowheads). These apoptotic cells were distributed in the entire walls of the neural tube, including the matrix layer (ven-

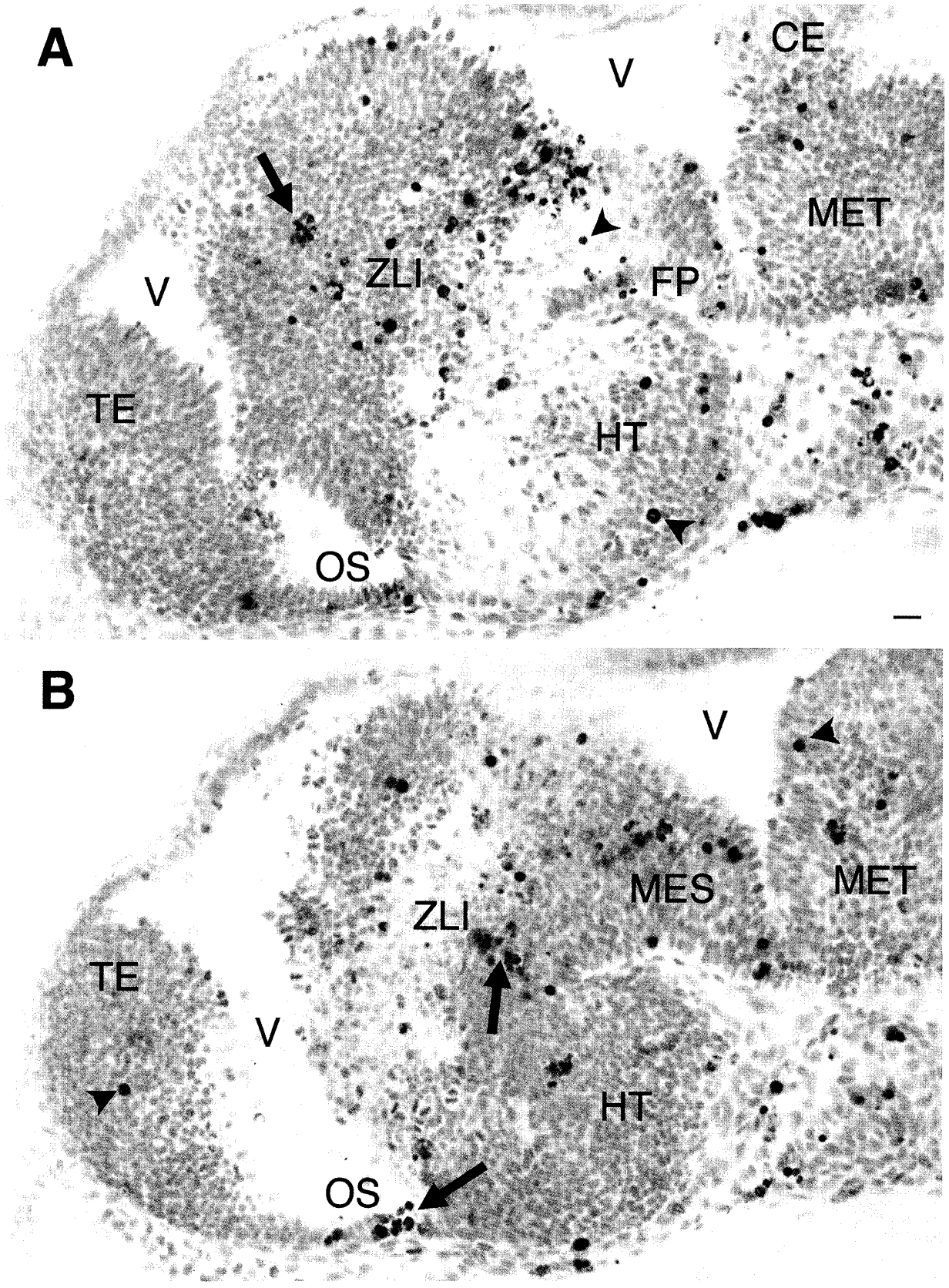

Fig. 2 Histological features of neural tube of embryo at stage 27 stained using whole-mount TUNEL (**A, B**). Parasagittal sections are shown. Rostral to left. The sections are stained with cresyl violet. Small single nuclei (arrowheads) and their large aggregates (arrows) are indicated. CE = cerebellum; FP = floor plate; HT = hypothalamus; MES = mesencephalon; MET = metencephalon; OS = optic stalk; TE = telencephalon; V = ventricle; ZLI = zona limitans intrathalamica. Scale bar = 10 μm.

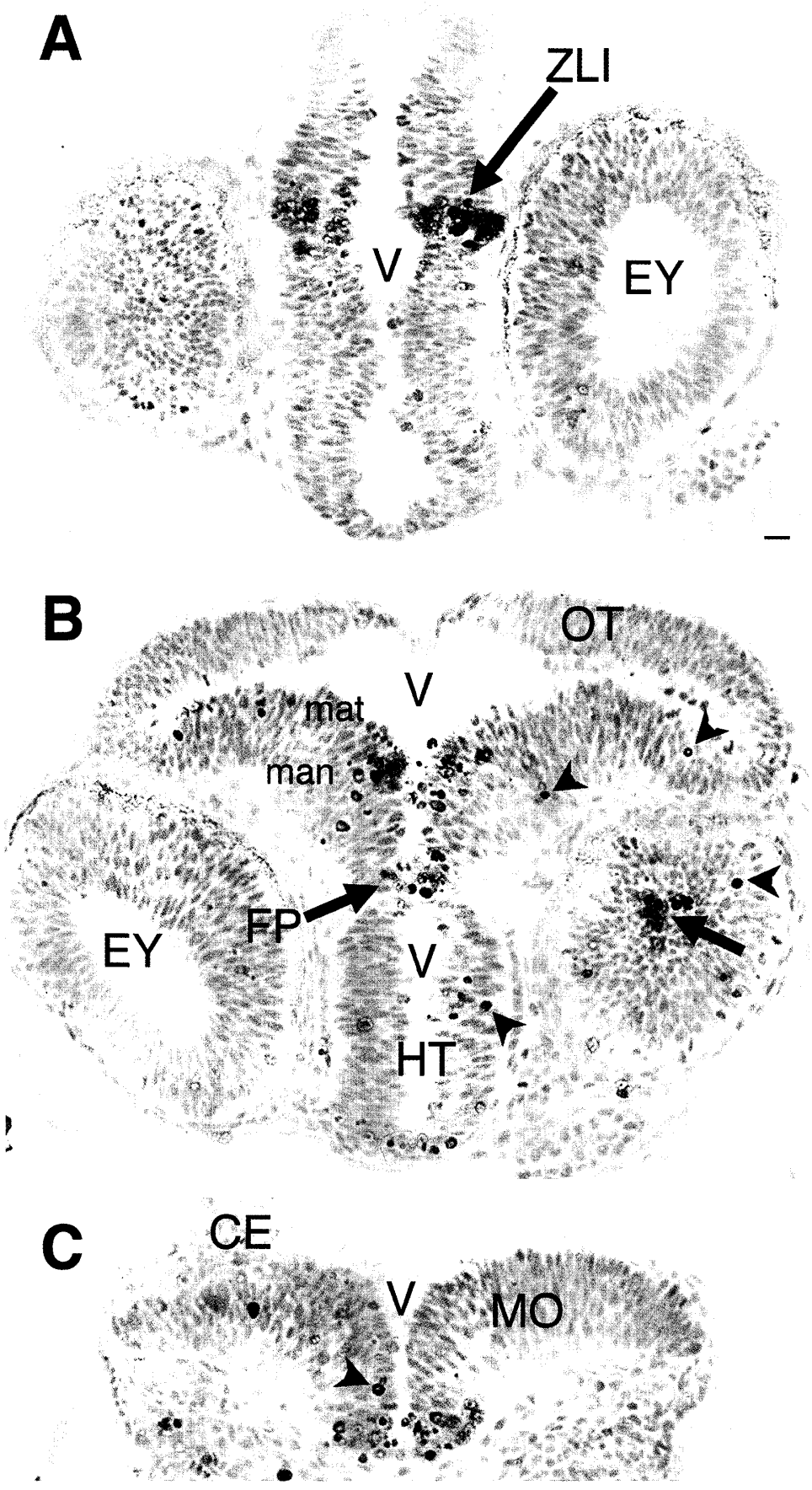

Fig. 3 Histological features of neural tube of embryo at stage 27 stained using whole-mount TUNEL (**A, B, C**). Frontal sections at the levels of the diencephalon (A), mesencephalon (B), and rhombencephalon (C) are shown. Dorsal to top. The sections are stained with cresyl violet. Small single nuclei (arrowheads) and their large aggregates (arrows) are indicated. CE = cerebellum; EY = optic vesicle; FP = floor plate; HT = hypothalamus; man = mantle layer; mat = matrix layer; MO = medulla oblongata; OT = optic tectum; V = ventricle; ZLI = zona limitans intrathalamica. Scale bar = 10 μm.

tricular neuroepithelium) and mantle layer (Fig. 3B).

Large nuclear aggregates, each of which had a diameter larger than 10 μm and was generally composed of 6-15 small single nuclei, were also found (Figs. 2 and 3, arrows). These large nuclear aggregates are probably clusters of nuclei of many apoptotic cells, and are considered to be local centers of apoptosis. They were observed mainly in the zona limitans intrathalamica, the ventral region of the optic stalk, several sites of the floor plate, and the retina (Figs. 2 and 3, arrows). These clusters of many apoptotic cells were distributed in the entire walls of the neural tube, including the matrix layer (ventricular neuroepithelium) (Fig. 3A).

Discussion

In this study, the degree and distribution of apoptosis were described in the medaka neural tube step, a phylogenic stage of brain development in vertebrates (Kage *et al.*, 2004). Our results showed that many apoptotic cells are distributed diffusely in the entire region of the neural tube, as well as in the neural rod at earlier stages of normal brain development (Fig. 1A, B).

Our observations that the number of apoptotic cells is large in the neural rod step (stage 20) are consistent with those reported in early zebrafish embryos (Furutani-Seiki *et al.*, 1996). In zebrafish, few apoptotic cells are observed in the neural tube after the 20-somite stage (19 h after fetilization at 28.5°C), which may correspond to Iwamatsu's stage 25 (36 h after fetilization at 28°C) of medaka embryos (Furutani-Seiki and Wittbrodt, 2004). However, our results showed that the number of apoptotic cells is still large in the neural tube step (stage 27, 24-somite stage, 50 h after fertilization at 28°C) and starts to decrease as late as stage 29 (34-somite stage, 85 h after fertilization at 28°C) in medaka (Fig. 1). Thus, massive apoptosis continues to occur for a longer period in the medaka neural tube than in the zebrafish neural tube.

In the medaka neural tube, several local centers of apoptosis were also observed mainly in specific regions (Figs. 2 and 3, arrows). Another difference between medaka and zebrafish embryos may be the presence of these apoptotic centers in the medaka neural tube, because the presence of such centers has not yet been reported in normal zebrafish brain development. Our results are rather consistent with those of Källén (1965) obtained using rabbit and chick neural tubes, in which local apoptotic centers are found in several specific locations: the ventral regions of optic evaginations, the ganglionic anlargen and the roof plate. Although we observed no massive apoptosis in the ganglionic anlargen and roof plate, we found apoptotic centers in the ventral regions of optic evaginations (Fig. 2).

Our results showed that both single apoptotic cells and apoptotic centers decreased in number after stage 29 (Fig. 1C). Furutani-Seiki *et al.*, (1996) also reported that few apoptotic cells are observed in the nervous system of zebrafish from the 20-somite stage to the hatching stage (35-somite stage). Thus, our observations of later stages of medaka embryos are again consistent with those of late zebrafish embryos.

Apoptosis during normal development has been classified into three main types on the basis of its function, namely, phylogenetic, morphogenetic and histiogenetic degenerations (Glücksmann, 1951; for a critical review, see Jacobson, 1991).

Phylogenetic degeneration occurs during the regression of vestigial organs or larval structures; morphogenetic degeneration during morphogenesis in regions of cavitation, separation, or other morphogenetic changes; and histiogenetic degeneration during the differentiation of tissues or organs as an adjustment of the final number of differentiated cells after the initial period of excessive cell production.

Two apoptotic centers found in the medaka neural tube, namely, the zona limitans intrathalamica and floor plate (Figs. 2 and 3), both of which play developmental inductive roles only in the embryonic stages, may be characterized as phylogenetic degeneration. Apoptotic centers in the ventral regions of optic evaginations (Fig. 2) may be associated with morphogenesis of the chiasma ridge, and thus may be characterized as morphogenetic degeneration. The local centers of apoptosis in the retina and many single apoptotic cells in the mantle layer of the neural tube (Fig. 3B) may be characterized as histiogenetic degeneration.

However, the presence of numerous single apoptotic cells that are distributed diffusely in the entire regions of the neural rod (Fig. 1A) and in the matrix layer of the neural tube (Fig. 3B) is difficult to interpret with the conceptual framework of histiogenetic degeneration, because these cells have not yet differentiated (Kage *et al.*, 2004; Ishikawa *et al.*, 2004; Ishikawa *et al.*, 2007). In

the neural rod and the matrix layer of the neural tube, neuroepithelial cells (matrix cells) still continue to produce daughter cells before neuronal differentiation. Thus, apoptosis seems to occur numerously and randomly in the medaka neural rod and neural tube. Unlike that in some invertebrates, such as the nematode, in which specific and identifiable cells die in a highly ordered pattern, apoptosis in the vertebrate nervous system seems to be more ubiquitous, random and probabilistic in nature.

Acknowledgements

We thank Ms. Y. Matsumoto and Ms. T. Sato for technical assistance. The study was supported partly through a grant of Long-Range Research Initiative (LRI) by the Japan Chemical Industry Association (JCIA).

References

Abdelilah, S., Mountcastle-Shah, E., Harvey, M., Solnica-Krezel, L., Schier, A. F., Stemple, D.L., Malicki, J., Neuhauss, S.C.F., Zwartkruis, F., Stainier, D.Y.R., Rangini, Z. and Driever, W. (1996) Mutations affecting neural survival in the zebrafish *Danio rerio*. *Development* **123**, 217–227.

Abrams, J. M., White, K., Fessler, L.I. and Steller, H. (1993) Programmed cell death during *Drosophila* embryogenesis. *Development* **117**, 29–43.

Furutani-Seiki M, Wittbrodt J (2004) Medaka and zebrafish, an evolutionary twin study. *Mech. Dev.* **121**, 629–637.

Furutani-Seiki, M., Jiang, Y.-J., Brand, M., Heisenberg, C.-P., Houart, C., Beuchle, D., van Eeden, 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.

Gavrieli, Y. Sherman, Y. and Sasson, B.S. (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493–501.

Glücksmann, A. (1951) Cell deaths in normal vertebrate ontogeny. *Biol. Rev.* **26**, 59–86.

Hyodo-Taguchi, Y. (1980) Establishment of inbred strains of the teleost, *Oryzias latipes* (in Japanese with English abstract). *Zool. Mag. (Tokyo)* **89**, 283–301.

Hyodo-Taguchi, Y. (1996) Inbred strains of the medaka, *Oryzias latipes*. *Fish Biol. J. Medaka* **8**, 11–14.

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.

Ishikawa, Y. (1997) Embryonic development of the medaka brain. *Fish Biology Journal Medaka* **9**, 17–31.

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.

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.

Iwamatsu, T. (2004) Stages of normal development in the medaka *Oryzias latipes*. *Mech. Dev.* **121**, 605–618.

Jacobson M. (1991) Neuronal death and neurotrophic factors. In Developmental Neurobiology, Third Edition, Plenum Press, New York and London, pp. 311–358.

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.

Källén, B. (1965) Degeneration and regeneration in the vertebrate central nervous system during embryogenesis. *Progress Brain Res.* **14**, 77–96.

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.

Oppenheim, R.W. (1985) Naturally occurring cell death during neural development. *Trends Neurosci.* **8**, 487–493.

Raff, M.C., Barres, B.A., Burne, J.F., Coles, H.S., Ishizaki, Y. and Jacobson, M.D. (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* **262**, 695–700.

Yamamoto, Y. and Henderson, C.E. (1999) Patterns of programmed cell death in populations of developing spinal motoneurons in chicken, mouse, and rat. *Dev. Biol.* **214**, 60–71.

Yasuda T., Aoki K., Matsumoto A., Maruyama K., Taguchi Y., Fushiki S. and Ishikawa Y. (2006) Radiation-induced brain cell death can be observed in living medaka embryos. *J. Rad. Res.* **47,** 295–303.

Received 2 February 2007.