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Brian V. Harmon
University of Queensland Medical School

David J. Allan
University of Queensland Medical School

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X-RAY-INDUCED CELL DEATH BY APOPTOSIS IN THE IMMATURE RAT CEREBELLUM

Brian V. Harmon[1]* and David J. Allan[1,2]

[1]Department of Pathology, University of Queensland Medical School, Herston, Queensland 4006, Australia.
[2]Department of Medical Laboratory Science, Queensland Institute of Technology, Brisbane, Queensland 4000, Australia.

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Abstract

The cells of the external granular layer (EGL) of the developing cerebellum are known to be particularly sensitive to radiation. In the past, changes induced in this layer by irradiation have been referred to by non-specific terms such as "pyknotic cells" and the mode of cell death has been assumed to be necrosis. However, in published light micrographs of these dying cells, the appearance is suggestive of apoptosis, a distinctive mode of cell death which occurs spontaneously in normal adult and embryonic tissues and can also be triggered by certain pathological stimuli.

This light and transmission electron microscopic study of control and irradiated (7 h post-irradiation) rat cerebellum from 18 day fetuses and 5 day-old neonates showed that the cell death was effected by apoptosis. The apoptosis was markedly enhanced by x-irradiation and quantification of the cell death in the EGL of 5 day-old rats exposed to 4, 8, 25, 100, and 400 cGy x-irradiation demonstrated that there was a positive dose response relationship. The extent of cell death by apoptosis which was 0.2% in control, ranged from 0.8% after 4 cGy to 62.3% after 400cGy x-irradiation.

The recognition that cell death by apoptosis can be a major component of x-irradiation damage has important implications for radiobiological studies.

Key Words: Cell death, cell survival, apoptosis, electron microscopy, cerebellum, immature, radiation.

*Address for correspondence:
Brian V. Harmon, Department of Pathology, University of Queensland Medical School, Herston, Queensland 4006, Australia.
Phone No. (07)-253-5331

Introduction

Cell death is a prominent feature of the developing nervous system of fetal and immature animals (Lewis, 1975; Hinchliffe, 1981; Cunningham, 1982; Clarke,1985) and is markedly enhanced by even low doses of ionizing radiation (Hicks and D'Amato,1963; Inouye and Kameyama, 1983). The cells of the external granular layer (EGL) of the developing cerebellum are known to be particularly sensitive to radiation (Altman et al.,1969; Das,1977; Inouye,1979; Inouye and Kameyama, 1983), and the pyknotic cells that are found in this layer following irradiation were considered by Altman and colleagues (1969) to be a manifestation of cell death by necrosis. However, it has since become apparent that there are at least two distinct types of cell death, apoptosis and necrosis which differ in their morphology, incidence, mechanism, and biological significance (Kerr et al., 1972, 1987; Wyllie et al., 1980; 1984). The light microscopic appearance of the pyknotic cells in the cerebellum (Altman et al.,1969) is very suggestive of cell death by apoptosis and not necrosis. In other rapidly proliferating cell populations, ionizing radiation is known to induce cell death by apoptosis (Kerr and Searle,1980; Allan et al.,1987a,b).

Necrosis, which is a process of passive cellular dissolution, is the result of severe injury to a cell by agents such as toxins or ischaemia. Cells undergoing necrosis are characterized by progressive swelling and degeneration of cellular components, with loss of ordered chemical activity (Trump and Ginn,1969). It is usual, with necrosis, for large numbers of contiguous cells within a damaged area to be affected. Apoptosis on the other hand appears to be an active, controlled process of selective and biologically meaningful cell deletion that plays an important kinetic role, opposite to that of mitosis, in the

maintenance of normal tissue homeostasis. Cells undergoing apoptosis are characterized by nuclear and cytoplasmic condensation and by their fragmentation into a number of membrane-bounded ultrastructurally well preserved apoptotic bodies. Apoptosis usually occurs in scattered single cells. Whilst it is often under physiological control, it can also be triggered by certain pathological stimuli (Kerr et al., 1972, 1987; Wyllie et al., 1980; 1984). The morphological distinction between these two modes of cell death is most readily made by transmission electron microscopy (Kerr et al.,1987).

In an attempt to establish the mode of cell death responsible for the production of the pyknotic cells in the EGL, normal and x-irradiated cerebella from 18 day rat fetuses and 5 day-old rats were examined by light and transmission electron microscopy. The extent of cell death in the granular layer was quantified in control and irradiated (4 - 400 cGy) cerebella from 5 day-old rats.

Materials and Methods

Twenty Sprague-Dawley rats (12 treated, 8 control) aged 5 days were selected at random from 4 litters, and eight fetuses (18 th day of development) were obtained from two pregnant rats just prior to use. All animals were housed in a laboratory in which the ambient temperature was 26 ± 2°C. Treatments were performed between 0900 and 1100 hours.

X-irradiation

The five day-old rats were not anesthetized for irradiation, but were restrained by placing them in a small Petri dish and surrounding them with cotton wool. The dish was positioned so that the animals would be in the center of the x-ray beam. Treated animals were exposed to 4 (2 animals), 8 (2 animals), 25 (2 animals), 100 (2 animals), or 400 cGy (4 animals) x-irradiation using a Toshiba Therapy X-Ray Unit operated at 200 kV and 15 mA, with 2.0 mm Al filtration, 5.4 Gy/ minute, and target-tissue distance of 8 cm. Dosages were measured using a Farmer dosimeter. Control animals were sham irradiated.

The two pregnant rats were anesthetized with pentobarbitone sodium, 40 mg/kg i.p. One of the animals was then positioned so that the fetuses would be in the center of the x-ray beam and the animal exposed to 400cGy x-irradiation. The control animal was sham irradiated.

Light and Electron microscopy

All animals were fixed by vascular perfusion 7 h after the completion of irradiation. To accomplish this, the 18 day fetuses were first surgically removed from the uteri of the anaesthetized pregnant rats. The fetal and 5 day-old rats were then deeply anaesthetized with pentobarbitone sodium, 80 mg/kg i.p. The blood was flushed out by injecting heparinized saline into the left ventricle and allowing it to drain out of the jugular veins which were cut. Animals were perfused with a mixture of 5% glutaraldehyde and 4% paraformaldehyde in 0.067 M sodium cacodylate buffer, pH 7.2 (Karnovsky,1965).

The cerebellum was removed from each animal 1h after perfusion-fixation and bissected with a sagittal cut through the vermis. For light microscopy, one half of the cerebellum was post-fixed in phosphate-buffered formalin for a further 24-48 h. The blocks were then embedded, cut surface down, in paraplast wax and 3µm sections through the vermis were cut, and stained with haematoxylin and eosin (H & E). For electron microscopy, a 1 mm thick slice was taken from the cut surface of the other half of each cerebellum and diced into strips approximately 2x1x1 mm in size. These were placed in fresh glutaraldehyde/paraformaldehyde fixative overnight before being washed for 24 h in cacodylate buffer, post-fixed in 1% osmium tetroxide for 2 h, washed in de-ionized water, stained en bloc in uranyl acetate, dehydrated through a series of graded ethanol solutions, cleared in propylene oxide and embedded in an Epon/Araldite mixture.

Sections 1 µm in thickness were cut on an LKB Ultratome V and stained with toluidine blue for use in light microscopy and in selecting areas for electron microscopy. Ultrathin sections were picked up on uncoated copper grids, stained in lead citrate for 1-2 minutes and examined in a Hitachi 300 electron microscope.

Quantification

The extent of cell death by apoptosis in the EGL of the cerebellum was quantified in H & E-stained sections from 5 day-old rats. Two animals were examined from the 4, 8, 25, and 100cGy group, and four animals from the control and 400cGy groups. In each H & E section, forty high power fields (oil immersion) were selected at random during a systematic scan of the EGL. The area occupied by EGL was measured using a MOP Videoplan, and the total number of cells and bodies showing the morphological features of apoptosis were counted. The apoptotic count was expressed as a number per mm^2 of EGL and the mean and standard error calculated for the control group and each irradiated group of animals. The number of cells undergoing apoptosis in the EGL was also expressed as a percentage of the mean number of cells

present in forty high power fields of control animals.

Results

Light microscopy

The light-microscopic appearance of the pyknotic cells, which were present throughout the cerebellum of control and irradiated animals (18 day fetuses and 5 day-old rats), conformed to previous descriptions of cell death by apoptosis (Wyllie et al.,1980; Kerr et al.,1987).

5 day-old rats. The cells in the EGL of the control animals (Figs.1-3) were actively proliferating and many mitotic figures were observed (Figs.2 and 3). The amount of cell death by apoptosis in control and irradiated animals is shown in Table 1. Cell death by apoptosis, which was only occasionally found in control animals (Fig.3), was markedly enhanced by x-irradiation (Table 1, Figs.4-6). Following 400 cGy x-irradiation (Figs.5 and 6), approximately 62% of the cells in the EGL were apoptotic, and in some areas the figure was as high as 80%.

18 day fetal rats. The EGL of the cerebellum in control fetal rats was characterized by the presence of many mitotic figures (Fig.7). Apoptotic cells

Table 1. Incidence of cell death by apoptosis in EGL of immature cerebellum following irradiation. (Mean ± S.E.)

Treatment	Apoptotic count per mm^2	% Apoptosis
Control	31 ± 3	0.2 ± 0.02
4 cGy	124 ± 49	0.8 ± 0.3
8 cGy	276 ± 31	1.7 ± 0.2
25 cGy	1815 ± 166	11.4 ± 1.0
100 cGy	8288 ± 670	51.8 ± 4.2
400 cGy	9885 ± 715	62.3 ± 4.5

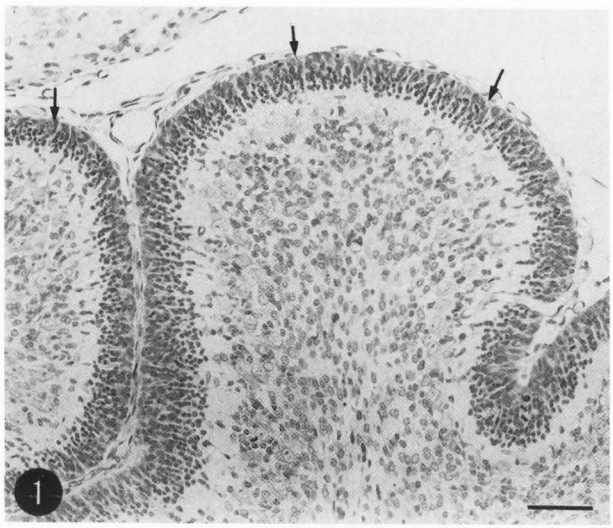


Figure 1. Control 5 day-old cerebellum showing the EGL (arrows) and underlying white matter. 3 μm paraffin section. Haematoxylin and eosin stain. Bar = 200 μm

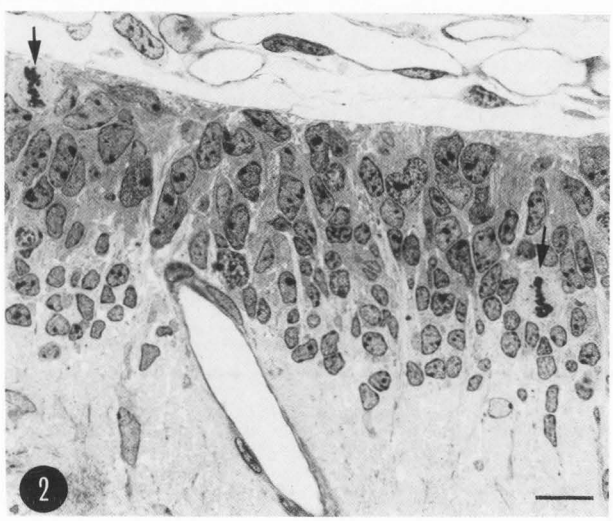


Figure 2. Control 5 day-old cerebellum. Cells in mitosis (arrows) are a prominent feature of the EGL. 1 μm toluidine blue-stained section. Bar = 10 μm.

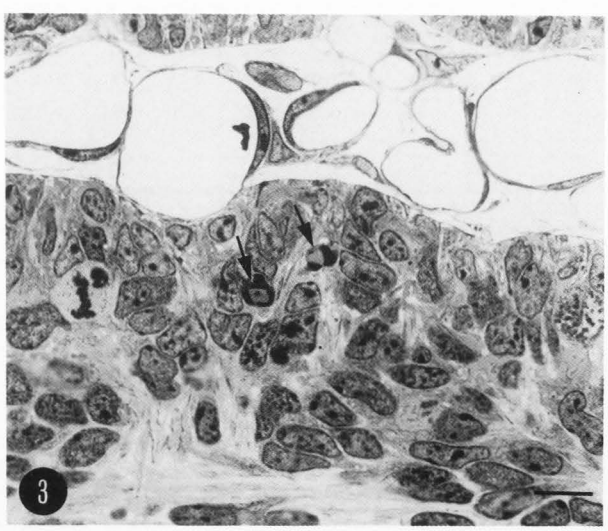


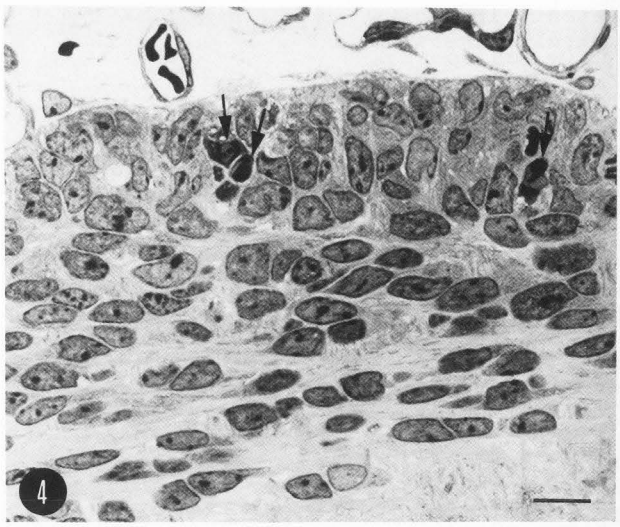
Figure 3. Control 5 day-old cerebellum showing spontaneous apoptosis (arrows). The condensed marginated nuclear chromatin displayed by these cells is characteristic of early apoptosis. Prolonged search is required to find apoptotic cells such as these in control tissue. 1 μm toluidine blue-stained section. Bar = 10μm.

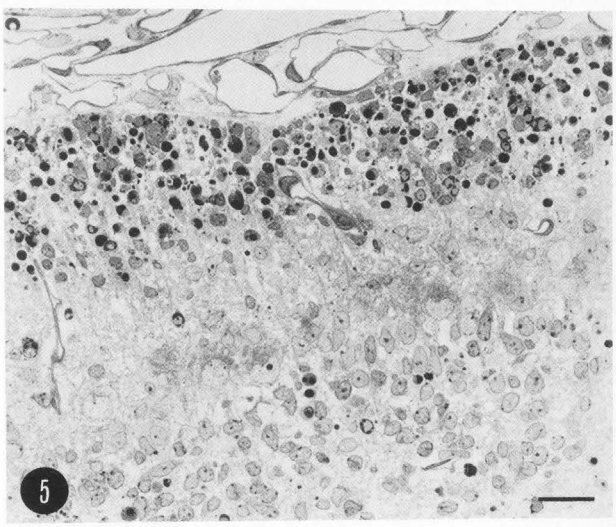
were only occasionally found. Following 400cGy x-irradiation, large numbers of cells with the characteristic morphological features of apoptosis were observed (Fig.8).

Morphology of cell death. In both groups of animals (18day fetuses and 5 day-old rats), the early stages of apoptosis could be recognized by the striking condensation and margination of the nuclear chromatin (Figs.3,4,6 and 8). Later stages were characterized by the presence of rounded apoptotic bodies, which typically contained one or more dense nuclear fragments (Figs.6 and 8).

Transmission electron microscopy

The identification of the cell death as apoptosis was confirmed by electron microscopy. Cells showing segregation of chromatin into sharply delineated masses that lay against the nuclear membrane, and condensation of the cytoplasm with maintenance of organelle integrity (Figs.9 and 10), were found in all control and irradiated cerebella. These early changes, which are diagnostic of apoptosis, were followed by nuclear and cytoplasmic fragmentation of the condensed cells into a number of membrane-bounded globules or apoptotic bodies that were still ultrastructurally well preserved (Fig.9). Apoptotic bodies consisting of one or more condensed masses of nuclear chromatin surrounded by variable amounts of cytoplasm (Figs.9 and 10) were the most common manifestation of the process.





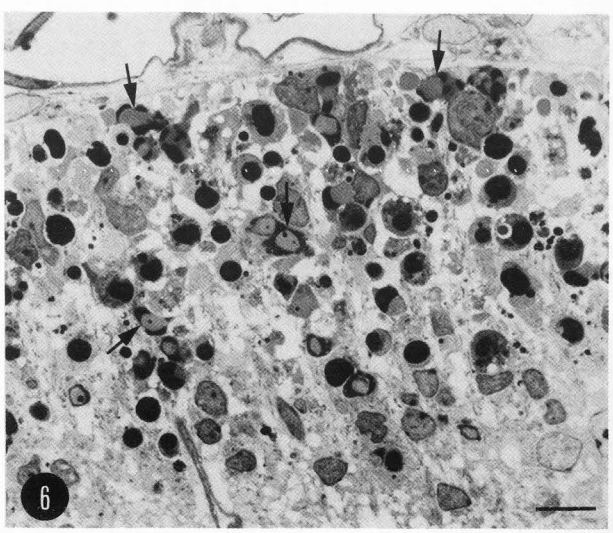
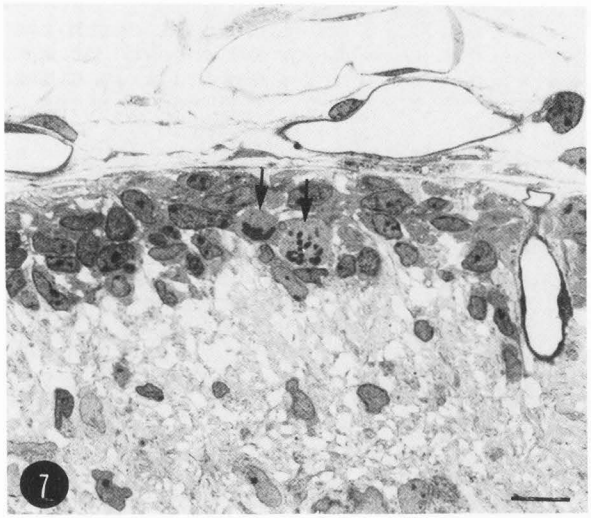
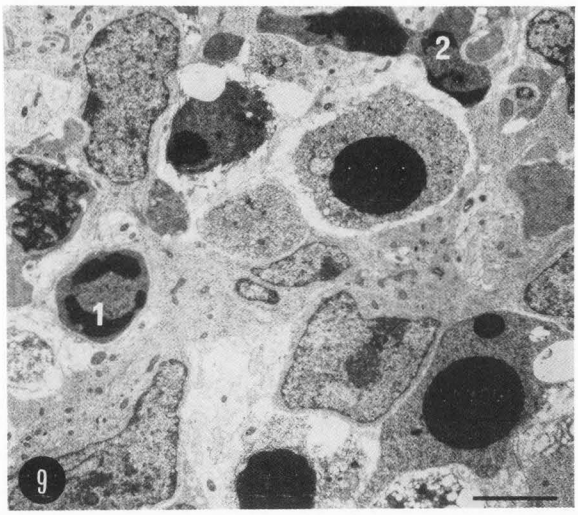


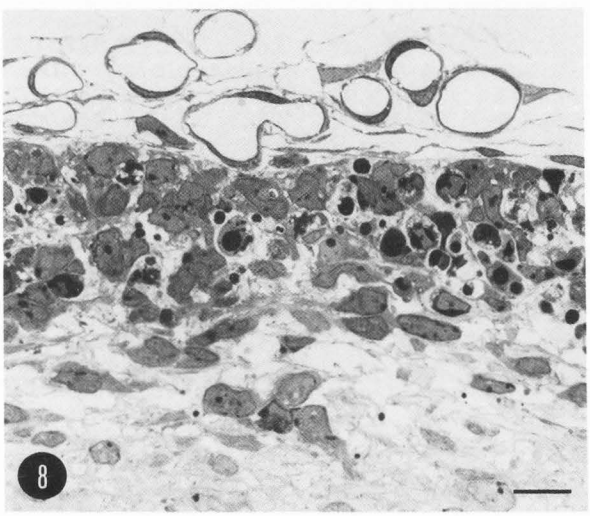
Figure 4. Seven hours after 8 cGy x-irradiation, 5 day-old rat. Several apoptotic bodies (arrows) show similar features to the spontaneous apoptosis in the previous figure. In irradiated tissue, apoptotic cells are widespread in the EGL. 1μm toluidine blue-stained section. Bar = 10 μm

Figure 5. Seven hours after 400 cGy x-irradiation, 5 day-old rat. Extensive death of cells in the EGL and scattered cell death in underlying areas. 1 μm toluidine blue-stained section. Bar = 40 μm

Figure 6. Seven hours after 400 cGy x-irradiation, 5 day-old rat. Massive cell death in the EGL. Many cells show marginated, condensed nuclear chromatin characteristic of early apoptosis (arrows), while others have a single condensed mass of featureless chromatin. 1 μm toluidine blue-stained section. Bar = 10 μm







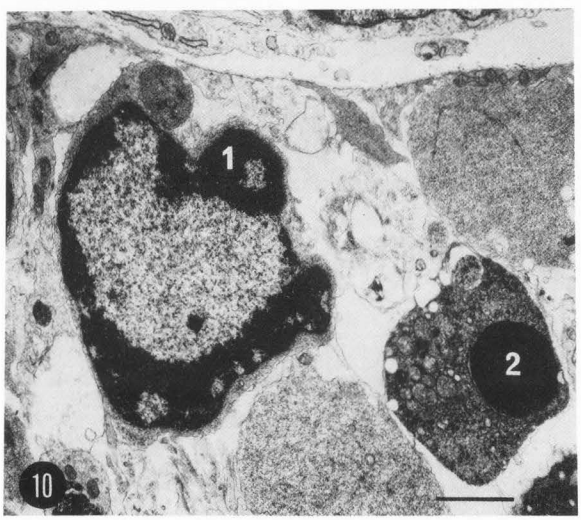


Figure 7. Control cerebellum, 18 day fetus. Note the mitotic figures (arrows) in the EGL. 1 μm toluidine blue-stained section. Bar = 10 μm

Figure 8. Seven hours after 400 cGy x-irradiation, 18 day fetus. Extensive cell death by apoptosis in the EGL. 1 μm toluidine blue-stained section. Bar = 10 μm

Figure 9. Electron micrograph of EGL 7h after 400 cGy x-irradiation, 5 day -old rat, showing extensive apoptosis. Apoptotic cells 1 and 2 show sharply delineated, compacted chromatin marginated against the nuclear membrane. Cell 2 is undergoing fragmentation into smaller apoptotic bodies. Bar = 5 μm.

Figure 10. Electron micrograph of EGL 7h after 400 cGy x-irradiation, 5 day-old rat. Cells (1 and 2) showing apoptotic changes. 1 is in the early stage and has marginated condensed nuclear chromatin. 2 is in a later stage and has a single mass of condensed chromatin and compacted cytoplasm with well-preserved organelles. Bar = 2 μm.

Discussion

This study confirms previous observations that cells in the EGL of developing rat cerebellum are extremely sensitive to x-irradiation (Hicks and D'Amato, 1963; Altman et al., 1969; Das, 1977; Inouye, 1979, 1983). Furthermore, the cell death occurring in both normal and x-irradiated cerebellum (fetal and neonatal) has now been categorized as apoptosis.Spontaneous apoptosis has been

observed in many other tissues during normal mammalian development. It plays an important and essential part in the deletion of redundant epithelium after fusion of the palatine processes, in the elimination of interdigital webs (Kerr et al.,1987), in the formation of intestinal villi (Harmon et al.,1984), and in the differentiation of the retina (Young, 1984).

Doses of ionizing radiation in the therapeutic range, which caused a massive enhancement of apoptosis in the present study, are known to trigger apoptosis in other rapidly proliferating cell populations such as the spermatogonia of the testes (Allan et al.,1987b), the epithelial cells of intestinal crypts (Potten,1977; Potten et al.,1978; Kerr and Searle, 1980), and in Sertoli cells of neonatal testes (Allan et al.,1987a). Radiation also causes apoptosis in lymphoid cells (Ohyama et al.,1985), and in acinar cells of the salivary gland (Pratt and Sodicoff,1972).

During guinea pig development, mild hyperthermia has been shown to produce apoptosis of the neuroepithelium (Wanner et al., 1976). In homozygous "staggerer" mice, signs of severe cerebellar dysfunction develop during the first few weeks after birth. The accompanying massive death of cells in the internal granular layer of the cerebellar cortex is by apoptosis (Kerr et al., 1987).

Although the ultimate fate of the apoptotic bodies was not followed in the present study, in other tissues they are phagocytosed by surrounding resident cells or macrophages and degraded in phagolysosomes (Kerr et al.,1987). The "pyknotic cells" induced in 18 day fetal rat cerebellum by x-irradiation were thought by Das (1977) to be eventually phagocytosed as they were no longer evident 24 h after treatment.

The quantification of cell death by apoptosis in the present study shows that there is a positive dose response relationship. The extent of cell death by apoptosis in the 5 day-old rat cerebellum was higher after 25 and 100cGy x-irradiation, than the corresponding incidence of "pyknotic cells" published by Inouye and Kameyama (1983) for 3 and 6 day-old rats. Their animals, however, were sampled 6 h after exposure to x-irradiation, one hour earlier than in the present study. The most likely reason for this apparent disparity between counts is that in cells showing the early changes of apoptosis, the nucleus retains its structure and contains only a thin rim of condensed, marginated chromatin and is not a dense, structureless mass (pyknotic). Therefore, cells in the early stage of apoptosis would most probably have been excluded from the "pyknotic cell" counts.

The recognition that cell death by apoptosis can be a major component of x-irradiation damage, particularly in rapidly proliferating cell populations, has important implications for radiobiological studies.

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Discussion with Reviewers

U.Heinzmann: What is the hypothetical role of x-ray induced apoptosis in the developing cerebellum in these experiments?

Authors: It is attractive to speculate that selective cell deletion constitutes one of a series of mechanisms for conserving the genetic integrity of a tissue, and that the active self-destruction (apoptosis) of these x-ray damaged cells would be in the interests of the animal as a whole.

U.Heinzmann: Does the incidence of cell death by apoptosis differ in 18 day postcoitus (fetal) rats from that of 5 day-old (neonatal) rats?

Authors: Yes, although our work has mainly concentrated on the morphological description and quantification of the cell death in the 5 day-old rats, preliminary counts of the radiation-induced cell death in 18 day fetal cerebellum suggests that the incidence of apoptosis is significantly lower.

U.Heinzmann: What are the first observable alterations (TEM and SEM) in the process of apoptosis?

Authors: The earliest recognised changes in the TEM occur in the nucleus and involve condensation and margination of the chromatin to form dense sharply delineated masses that abut on the nuclear membrane. In the SEM, the first observable alterations are the loss of surface microvilli and the development of pedunculated protuberances on the cell surface.

U.Heinzmann: When do the macrophages first emigrate into the injured cerebellum?

Authors: To our knowledge, it has not yet been established whether or not macrophages do emigrate into the injured cerebellum. As the observations in the present study were made at only a single (early) time interval post-irradiation, we are not able, at this stage to answer this question. Further studies, looking in more detail at the time course of

radiation-induced changes should provide the answer to this important question.

U.Heinzmann: Were any indicators of autophagocytosis observed in the injured tissue?

Authors: No, however the ultrastructural distinction between phagocytosed apoptotic bodies and autophagic vacuoles can be quite difficult. The contents of cytoplasmic vacuoles can only be confidently identified as apoptotic in origin if they include nuclear remnants with characteristically compacted chromatin. Most of the bodies in the present study contained such a nuclear remnant.

C.V.Mecklenburg: The irradiation of the specimens was performed between 9-11am. The fixation was performed 7 hours later. Why did you choose 7h and not 6h or 12h? Your result would have been directly comparable with those published by Inouye and Kameyama (1983).

Authors: The aim of the present study was primarily to establish the mode of cell death occurring in the cerebellum. The identification of cell death as apoptosis can be most readily made by examining the early stages of the process. After carrying out a pilot study which looked at the cerebellum 4h, 8h and 12 h post 8cGy x-irradiation, we considered that a time interval just short of 8 h would be the most suitable for this purpose.

C.V. Mecklenburg: In the introduction you state: "The morphological distinction between these two modes of cell death is most readily made by transmission electron microscopy (Kerr et al,.1987)." You have excellent micrographs showing apoptosis on light microscopical level but only two on ultrastructural level. Why don't you have more ultrastructural analysis of this very interesting phenomenon?

Authors: The morphological distinction between apoptosis and necrosis is most readily made by transmission electron microscopy. In this study, we have used electron microscopy precisely for this purpose. Having identified the pattern of cell death occurring in the cerebellum as apoptosis, we did not feel that including another two electron micrographs which would show essentially the same features as figures 9 and 10 could be justified. There are now a large number of published ultrastructural studies of apoptosis recording its distinctive morphological features. The paper referred to in the question provides a comprehensive bibliography as well as many illustrations of various stages of the process.