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Gamma irradiation leads to two waves of apoptosis in distinct cell populations of the retina of newborn rats

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SUMMARY

Gamma radiation induces apoptosis in the proliferative zone (neuroblastic layer) of the developing rat retina. We asked whether sensitivity to apoptosis might be related to distinct phases of the cell cycle. Explants of newborn rat retina or newborn pups were gamma-irradiated and apoptosis was detected by chromatin condensation, DNA fragmentation in situ and DNA electrophoresis. After 6 hours, early appearing apoptotic bodies were located mainly towards the outer tier of the neuroblastic layer. In contrast, after 24 hours, late-appearing apoptotic cells were located towards the inner margin of the neuroblastic layer, a region associated with the S phase of the cell cycle. Labeling of a cohort of cells with the nucleotide analog bromo-deoxyuridine (BrdU) at the time of irradiation, showed that these cells die in the late wave of apoptosis. BrdU given 3 hours before fixation labeled a large number of late apoptotic cells, but no early apoptotic cells. After labeling of all cycling cells with BrdU, 40% of the early apoptotic profiles were unlabeled, and thus post-mitotic. The same schedules of cell death were identified after gamma irradiation in vivo. The results show that irradiation leads to two waves of apoptosis in distinct cell populations. An early wave comprises both post-mitotic cells and proliferating cells out of the S phase. The late wave comprises cells in S phase, which pass through this phase again to die. The antioxidant pyrrolidinedithiocarbamate prevented the early but not the late wave of apoptosis following irradiation, and blocked lipid peroxidation at 6 hours after the insult, suggesting that the two waves of apoptosis are indeed mediated by distinct mechanisms.

Key words: Programmed cell death, Apoptosis, Radiation, Radiosensitivity, Cell cycle, Development, Central nervous system, Retina, Oxidative damage, Free radical, Antioxidant

INTRODUCTION

Cell death induced by ionizing radiation can occur either in the form of reproductive/mitotic cell death or, alternatively, by interphase cell death. Mitotic death was defined by the ability of the cell to divide before dying. In turn, interphase death was defined as an impairment of cellular metabolism and death of the cells before entering mitosis. Interphase cell death happens both in quiescent and in proliferating cells and usually occurs a few hours after irradiation (reviewed in Muschel et al., 1998; Szumiel, 1994).

Irradiation can induce both DNA and membrane damage (Haimovitz-Friedman, 1998; Szumiel, 1998, 1994). In many cases, irradiation-induced cell death has been identified as apoptosis (Yanagihara et al, 1995; Gobbel et al., 1998a, Haimovitz-Friedman, 1998; Barlow et al., 1997), which may depend on de novo protein synthesis (Ferrer et al., 1993b). It was suggested that the early interphase death is related to membrane lipid peroxidation (Ojeda et al., 1994), but recent reports suggest that lipid peroxidation induces necrosis rather than apoptosis (Gobbel et al., 1998b). In turn, mitotic death was associated with DNA damage that could lead to death after various cell cycles (Vidair et al., 1996).

The literature about apoptosis induced by ionizing radiation includes both animal models (D'Amato, 1982; Ferrer et al., 1993a) and cell lines (Kharbanda et al., 1997; Findley et al., 1997; Yanagihara et al., 1995). In most cell types studied, a higher sensitivity to apoptosis was reported for undifferentiated cells when compared with differentiated cells (Szumiel, 1994; Hicks et al., 1961).

Proliferating cells of various cell lines show fluctuating intrinsic levels of radiosensitivity throughout the phases of the cell cycle. In general, the highest radioresistance is found in middle to late S phase (Leonahrdt et al., 1997; Biade et al., 1997; Cheong et al., 1994). In contrast, developing cerebellar cells are most radiosensitive in the late G_1 and S phases (Ferrer, 1993a), while Myc-transfected fibroblasts were more sensitive to apoptosis induced by irradiation in S or G_2 than in G_1 phase (Guo et al., 1997).

In this study, we used the developing retina as a model of radiosensitivity in the organized central nervous system (CNS). The retina of newborn rats is composed of three strata: the

ganglion cell layer (GCL), the inner plexiform layer (IPL) and an outer cellular stratum (Fig. 1). The latter contains a layer of proliferating cells equivalent to the ventricular zone of other parts of the developing CNS, here referred to as the neuroblastic layer (NBL), and also contains a few rows of early differentiating amacrine cells that will compose the inner plexiform layer (INL) (reviewed in Linden et al., 1999).

The cell cycle takes place in the neuroblastic layer (NBL). The interkinetic nuclear migration (Fujita, 1962) allows the prediction of the phase of the cell cycle according to the position of the cell nucleus. The S phase occurs in the innermost region of the NBL (that is, towards the vitreal margin), while mitosis takes place at the outer edge of the NBL. The nuclei of proliferating cells migrate inwards in G_1 and outwards during G_2. The differential location of the nuclei of degenerating cells within the tissue may thus be ascribed to distinct phases of the cell cycle (Fig. 1).

We characterized apoptosis induced by gamma irradiation of the developing retinal tissue. Because most studies of radiosensitivity related to the cell cycle were done either with cell lines or with animal models, mechanistic studies are either uncertain to apply to organized tissues or difficult to perform in the intact animals. Therefore, an in vitro preparation of histotypical retinal explants was used, which allows the study of mechanisms of apoptosis induced by radiation in various cell types and at distinct stages of differentiation (Linden et al., 1999). The results show that both in vitro and in vivo, irradiation leads to two waves of apoptosis in the developing retina, and these are related with distinct phases of the cell cycle. Preliminary evidence also suggests that the two waves of cell death are mediated by distinct mechanisms.

MATERIALS AND METHODS

Tissue culture

Lister hooded rats were used throughout this study. Explants of rat retinae were prepared as previously described (Rehen et al., 1996; Araujo and Linden, 1993). Briefly, rat pups between postnatal day 0 (day of birth = P0) and day 7 (P7) were killed instantaneously by decapitation and the retinae were dissected out of the eyeballs with fine forceps. Fragments of about 1 mm side were cut and maintained in culture for the required incubation periods in Eagle's Basal Medium (Gibco BRL) with 5% fetal calf serum (Gibco BRL) in an atmosphere of 5% CO_2, 37°C. 1 μg/ml of anisomycin, a protein synthesis inhibitor, and gamma irradiation were both given at the beginning of the incubation.

Pyrrolidinedithiocarbamate (PDTC; 80 μM) was given either immediately after irradiation in explants fixed 6 hours after the insult, or during the last 6 hours of a 24-hour incubation period.

Irradiation

Rat pups were irradiated (2 Gy) using a Co^{60} unit from US Nuclear Corporation (Burbank, Ca, USA) at the laboratory of Radiation Biophysics, IBCCF, UFRJ, Rio de Janeiro, Brazil. After irradiation, the animals were kept warm in a plastic cup floating in a water bath at 37°C for either 6 or 24 hours. Then, the pups were deeply anaesthetized by ether inhalation, and were perfused through the heart with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The eyes were post-fixed in the same buffer and processed for histology.

Retinal explants were irradiated (2 Gy) immediately after preparation, except when used for DNA electrophoresis (see below). The incubation lasted for either 6 or 24 hours at 37°C, and explants were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. In some cases, we fixed explants after 1, 2, 3, 4 or 5 hours.

Histology

Following fixation for 1 hour at room temperature with 4% paraformaldehyde, the tissue fragments were infiltrated with 20% sucrose, and embedded in OCT tissue-embedding medium (Tissue-Tek). Transverse frozen sections were cut at 6 μm in a cryostat. The sections were either stained with Neutral Red or processed for immunocytochemistry (see below) or detection of DNA fragmentation in situ (ISNEL/TUNEL). For TUNEL reactions, the sections were incubated overnight at 37°C in a solution containing 0.5 U/μl of terminal-deoxyribonucleotidyl-transferase (TdT), 0.2 nmol/μl dATP and 0.013 nmol/μl biotin-14-dATP in TdT buffer (Gibco BRL). The reaction was stopped with 20 mM EDTA; the slides were washed with PBS and incubated with 1:100 streptavidin-Texas Red or streptavidin-FITC (Amersham) at 37°C for 1 hour, then washed with PBS and coverslips mounted with glycerol saturated with N-propyl-galate.

Labeling of proliferating cells

To label a cohort of cells in S phase of the cell cycle, an intraperitoneal injection (60 mg/kg body mass) of bromo-deoxyuridine (BrdU) was given 1 hour preceding retinal explantation. To label all proliferating

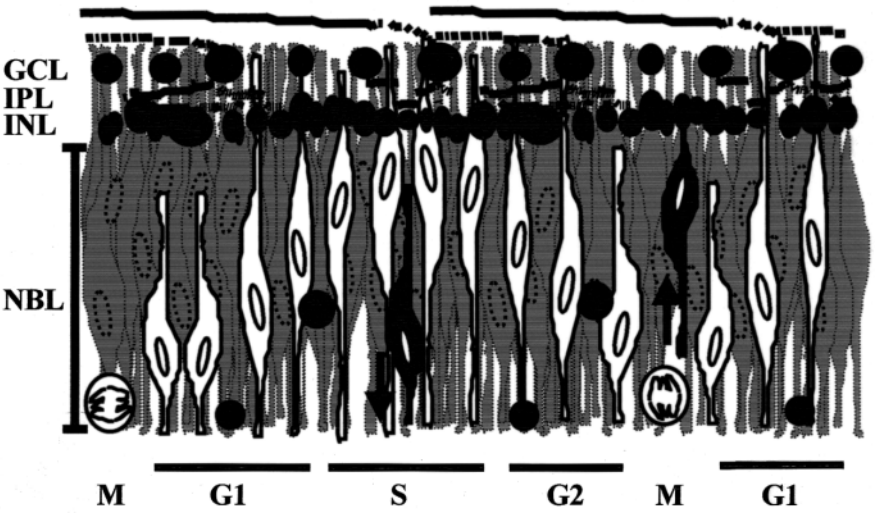


Fig. 1. Diagram of the retina in a newborn rat. The inner (or vitreal) margin is up. Proliferating neuroblasts are shown as white elongated profiles, and the interkinetic migration of their nuclei is shown as a single sequence, from left to right along the phases of the cell cycle. Post-mitotic cells are shown as dark elongated profiles. The ganglion cell layer (GCL) and inner nuclear layer (INL) at this age contain ganglion cells and early developing amacrine cells, respectively. The dark round profiles at about the middle of the neuroblastic layer (NBL) are horizontal cells and the smaller round profiles at the outer edge of the retina are photoreceptors. Abbreviations for this and the following figures: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, Inner nuclear layer; NBL, neuroblastic layer.

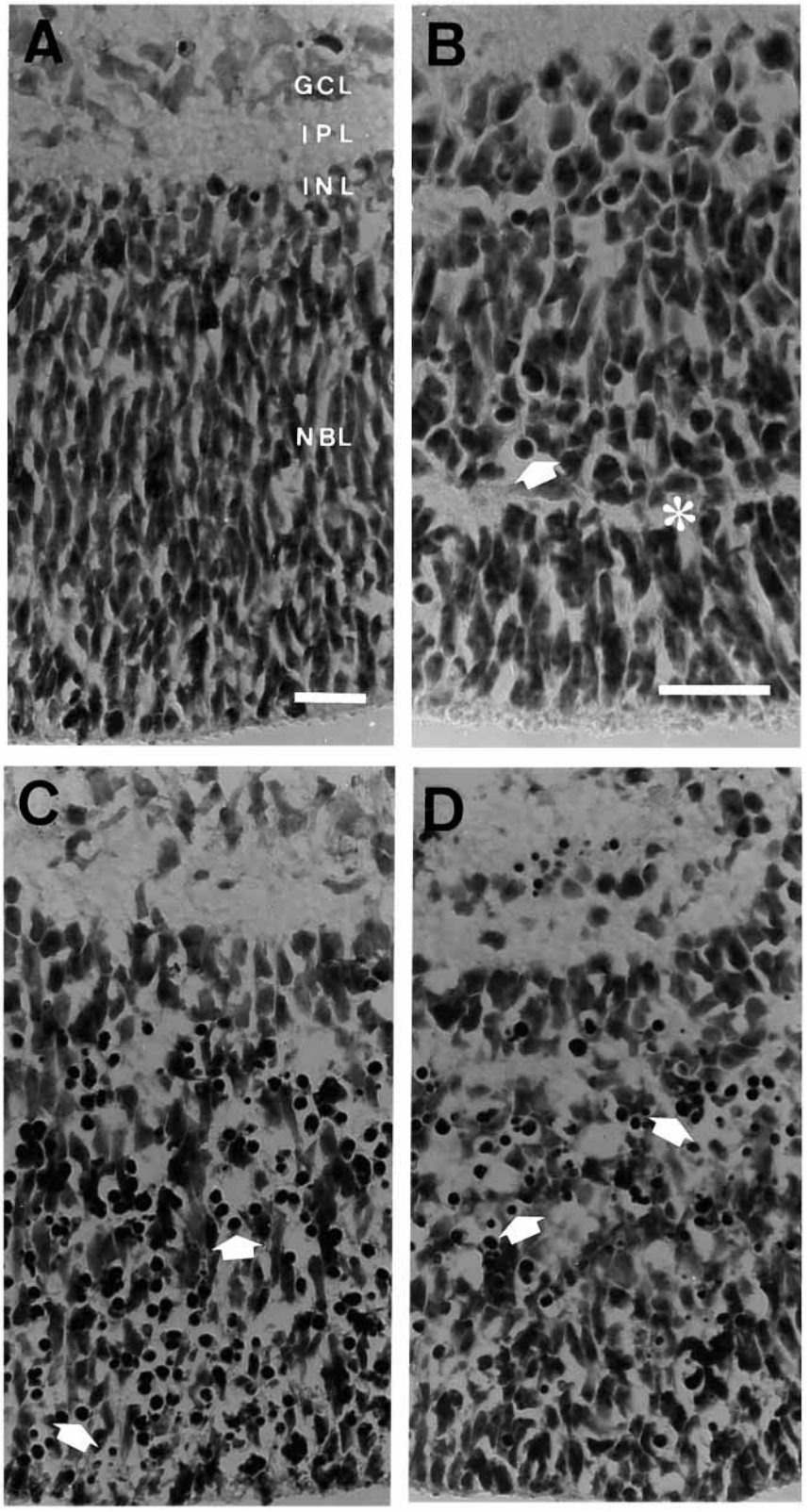


Fig. 2. Photomicrographs of transverse section from retinal explants stained with Neutral Red. (A) Explant from a newborn rat (P1) after 6 hours in control conditions. (B) Explant from a P6 animal 6 hours after gamma irradiation. (C,D) Explants from newborn rats (P1) either at 6 hours (C) or at 24 hours after irradiation (D). The arrows show apoptotic cells. The asterisk in B shows the outer plexiform layer (OPL). Bars, 20 μm (bar in A also applies to C and D).

cells in the developing retina, we used a schedule of intraperitoneal injections, of either BrdU (60 mg/kg body mass) as described elsewhere (Rehen et al., 1999), or [^{3}H]thymidine (5 μCi/g body mass). Briefly, we adopted an estimated cycle length of 36.1 hours and an S phase of approximately 18 hours at P1 (Alexiades and Cepko, 1996). The labeling was achieved with three intraperitoneal injections, at 0, 5 and 19 hours, and explants were prepared 1 hour after the last injection (see diagram in Fig. 10). The injection schedule guarantees that every cycling cell should be labeled at least by one injection, when passing through S phase.

The slides with mounted sections were heated in citrate buffer, pH 6, for 8 minutes in a microwave oven to expose hidden epitopes (Dover and Patel, 1994), then the incorporated BrdU was detected by immunohistochemistry using a monoclonal antibody (RPN 202, Amersham) according to the manufacturer's instructions, and developed with an anti-mouse ABC kit (Vector) using diaminobenzidine as chromogen.

To label a cohort of cells in S phase after irradiation we used a pulse of 3 μg/ml BrdU in vitro for 30 minutes, at 3 hours before fixation (see diagram in Fig. 7).

Immunocytochemistry

The slides were incubated with 0.5% Triton X-100 in phosphate-buffered saline (PBS), pH 7.4, for 15 minutes and washed with PBS followed by incubation with a blocking albumin solution (1% BSA in PBS) for 30 minutes. Then, the sections were reacted overnight at 37°C with monoclonal antibodies against rhodopsin (rho 4D2; kindly provided by Dr R. Molday, University of British Columbia, Vancouver, Canada) to stain rod photoreceptors, or PCNA (Calbiochem, La Jolla, CA) to label proliferating cells, or with a polyclonal antibody against calretinin (Chemicon, Temecula, CA), to stain amacrine and ganglion cells. The sections were washed in PBS, incubated with appropriate secondary antibodies, and stained with an HRP-ABC kit (Vector) with DAB as chromogen.

In one experiment, the sections were incubated with a monoclonal antibody to calbindin (Sigma), which stains horizontal cells, followed by a TUNEL reaction as described above. Calbindin staining was developed with an FITC-conjugated anti-mouse secondary antibody (Jackson) and TUNEL labeling was developed with Texas Red-conjugated streptavidin.

DNA electrophoresis

In control conditions, ganglion cells show internucleosomal DNA fragmentation after 24 hours in culture, as a result of axotomy during the production of retinal explants. For DNA electrophoresis, retinal explants were therefore irradiated after 2 days in culture, at the time when most ganglion cells have already died (Rehen et al., 1996). Explants from 5 retinas per group, in control conditions or at either 6 hours or 24 hours after irradiation, were frozen in liquid nitrogen. The tissue was resuspended in 0.5 ml of lysis buffer (0.2% Triton X-100, 10 mM Tris, pH 8.0, 1 mM EDTA), left at room temperature for 30

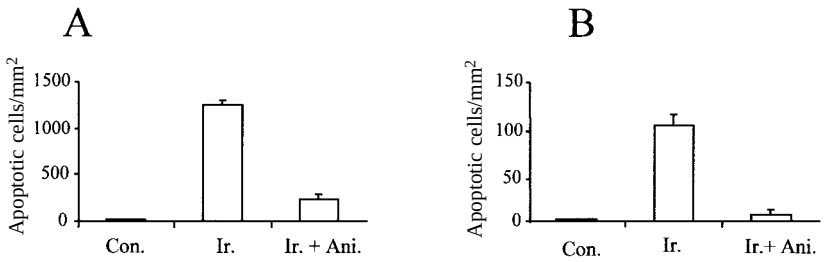


Fig. 3. Histograms showing the numbers of apoptotic cells in the outer cellular stratum (INL + NBL) of the retina of P1 rats (A), or within the INL, NBL and ONL of P6 animals (B). Data refer to groups in control conditions (Con.), 6 hours after irradiation in control medium (Ir.), or under inhibition of protein synthesis with anisomycin 1 μg/ml (Ir. + Ani.) throughout the whole interval of 6 hours after irradiation.

minutes and the lysates were centrifuged at 10000 rpm for 20 minutes at 4°C. The pellet containing high molecular mass DNA was discarded. The supernatant containing fragmented DNA was extracted twice with an equal volume of phenol-chloroform and once with chloroform and centrifuged at 10000 rpm for 20 minutes at 4°C. The supernatant was precipitated with an equal volume of isopropanol and 0.5 M NaCl. The samples were stored at −20°C overnight and centrifuged at 10000 rpm for 20 minutes at 4°C. The pellet was washed with 70% ethanol, air dried and resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8.0) followed by incubation with 100 μg/ml RNAse A (Gibco BRL) for 1 hour at 37°C. The optical densities of samples were read at 260 nm. Approximately the same amounts from each sample were electrophoresed in a 1.5% agarose gel, which was then stained with 0.5 μg/ml ethidium bromide. The gel was intended for qualitative comparisons only.

Methods of counting

Apoptotic cells were recognized either by their condensed profiles under differential interference contrast microscopy, or by their pyknotic nuclei following staining with Neutral Red, and counted at 1000× magnification. Work from our laboratory has shown that condensed, globular profiles recognized by differential interference contrast microscopy can be stained by the TUNEL technique (Rehen et al., 1999; Linden et al., 1999), and with an antibody to activated caspase-3 (C. A. Guimarães and R. Linden, unpublished results; see Namura et al., 1998). For the ganglion cell layer, apoptotic cells are reported as a percentage of the total number of cells; for the outer cellular stratum, the apoptotic cells were counted between the outer limiting membrane and the IPL (inner plexiform layer) in fields of 0.12 mm^2 using a Zeiss Standard microscope, or in fields of 0.14 mm^2 using a Zeiss Axiophot microscope. Counts were made in three fields from each of three explants in each group, and were normalized as apoptotic cells/mm^2. The same procedure was used to count BrdU-labeled cells. To locate precisely either the apoptotic or BrdU-labeled cells cells in the outer cellular stratum, we drew three explant sections per group using a camera lucida, and divided the outer stratum into 10 equal strips parallel to the IPL. The average number of apoptotic cells in each strip was expressed as a percentage of the total number of degenerating profiles in the outer cellular stratum.

Measurements of lipid peroxidation

Retinal explants were either maintained in control conditions, or irradiated, either in the presence or absence of 80 μM of PDTC. Then, the concentration of thiobarbituric acid reactive substances (TBARS) in homogenates of the explants were measured following a procedure slightly modified from North and Tappel (1997).

Briefly, four retinas per group were homogenized with 50 μl of PBS, pH 7.5, on ice. We took 1 μl of the tissue homogenate for protein measurements. The homogenate was then mixed with 150 μl of trichloroacetic acid (TCA, 15% w/v) and centrifuged for 10 minutes at 3000 ***g*** at 4°C. The supernatant was mixed with a half volume of a 0.7% thiobarbituric acid (TBA) solution previous prepared in 1:1 (v/v) water:acetic acid solution. The samples were then incubated at 95°C for 1 hour, and absorbance was read at 532 nm. Values were expressed as nanomoles of TBARS (malondialdehyde equivalents) per gram of tissue and plotted as fold-increase with respect to the values at 6 hours in control conditions.

RESULTS

Gamma irradiation induces cell death in the neuroblastic layer of the retinal tissue

Gamma irradiation led to cell death only in the neuroblastic layer (NBL) of explants from the retina of rats within the first postnatal week (Fig. 2). In the retina of newborn rats, the earliest degenerating cells were seen at 3 hours after irradiation in the outermost region of the NBL, near the sites where mitosis takes place (data not shown). In pups older than P4-P5, which is the age when the outer plexiform layer (OPL) appears in the retina of the rat (Craft et al., 1982), the apoptotic cells were found adjacent to the inner border of the emerging OPL, where there are residual proliferating cells (Fig. 2B) (Fujieda et al., 1994). The number of apoptotic cells in P6 animals was roughly 1/10 of that in P1. Inhibition of de novo protein synthesis rescued the NBL from cell death at 6 hours after irradiation both in newborn and in older pups (Fig. 3). In addition, inhibition of protein synthesis only during the first 2

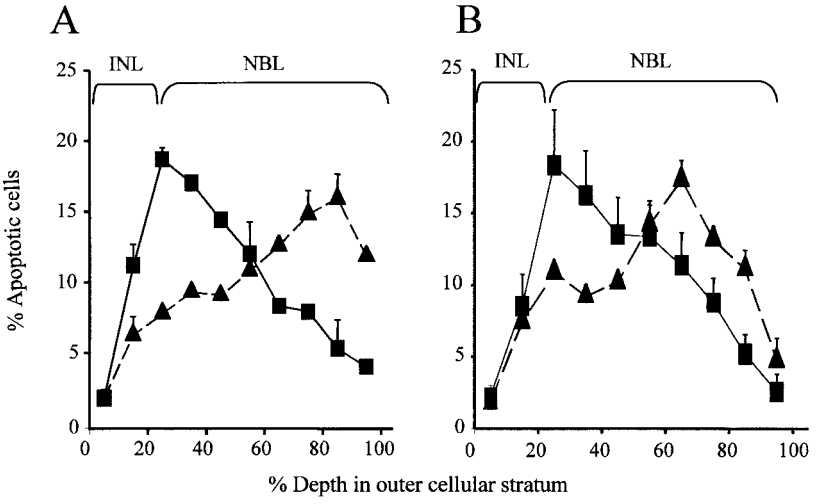
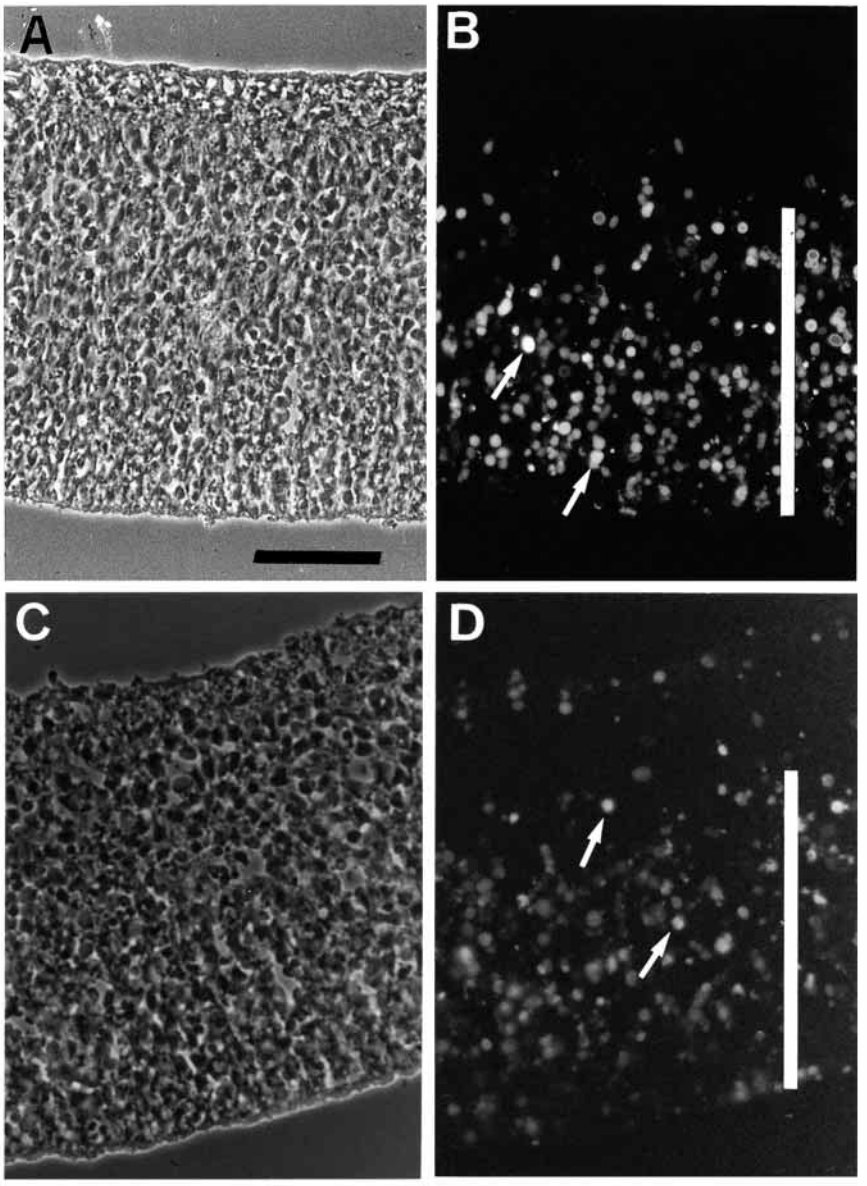


Fig. 4. Distribution of apoptotic cells as a function of depth in the outer cellular stratum at 6 hours (triangles) and 24 hours (squares), following irradiation in vitro (A) or in vivo (B). The vertical axis shows the percentage of apoptotic profiles found in each segment along the depth of the retina. In the horizontal axis 100% represents the outermost region and zero is the innermost margin (see Fig. 1).



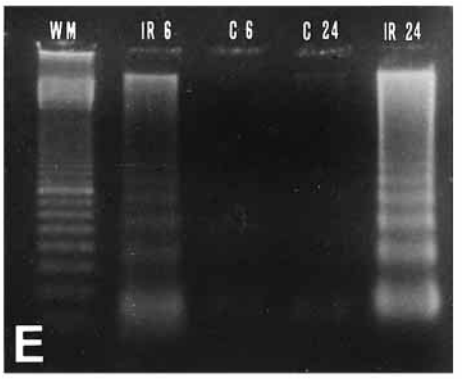


Fig. 5. Phase-contrast photomicrographs (A,C) and in situ detection of DNA fragmentation at 6 hours (A,B) and 24 hours (C,D) after irradiation. The white bar represents the extent of NBL; arrows, TUNEL-positive profiles.
(E) Oligonucleosomal fragments of DNA resolved by 1.5% agarose gel electrophoresis. Lanes: WM, 100 pb-interval molecular mass markers; IR 6, irradiated 6 hours; C 6, control 6 hours; C 24, control 24 hours; IR 24, irradiated 24 hours. Bar, 50 μm.

hours after irradiation was sufficient to block cell death examined at 6 hours after the insult (data not shown).

Death of axotomized ganglion cells is not affected by gamma irradiation

We have previously shown that axotomized ganglion cells degenerate with a peak rate at about 1 day after axotomy in retinal explants from newborn rats (Rehen et al., 1996). At 6 hours after irradiation, the percentage of apoptotic profiles in the ganglion cell layer (GCL) was 3.7±1.4 (mean ± s.e.m.), similar to the rate of 3.4±0.4 found in unirradiated control explants. At 24 hours after irradiation, the rate of cell death was of 24.4±2.6 in irradiated explants and 18.6±1.3 in control explants.

Thus, irradiation had no clear effect upon the rate of apoptosis induced by axotomy of ganglion cells.

Distribution of cell death in the NBL

Within the NBL of retinal explants, there were distinct topographical distributions of degenerating cells at either 6 hours or 24 hours after gamma irradiation in vitro. At 6 hours after the insult, degenerating cells were located mainly in the outer half of the neuroblastic layer (Fig. 2C). At 24 hours after the insult, however, dead cells were found mainly within the inner half of the NBL (Fig. 2D), in a region that coincides with the area where the S phase of the cell cycle takes place (Fig. 1).

We mapped the distribution of cell death following irradiation of the explants as a function of depth within the outer cellular stratum (Fig. 4A). The graphs confirm that the highest incidence of cell death at 6 hours after irradiation occurs near the outer border of the retina, where M phase takes place. In turn, 24 hours after irradiation the highest incidence of apoptosis is seen in the S phase region of NBL, while very few degenerating cells were found in the inner nuclear layer (INL). The distributions of cell death that occur at either 6 hours or 24 hours after irradiation will be referred to as the early and late waves of cell death, respectively (Fig. 4A).

Irradiation-induced retinal cell death is apoptotic

Both the morphology of degenerating cells and the detection of DNA fragmentation in situ (Fig. 5A-D) were similar in the early and late waves of cell death in the NBL of retinal explants. Internucleosomal DNA fragmentation was detected by eletrophoresis of the DNA extracted at either 6 hours or 24 hours after irradiation (Fig. 5E), confirming that cell death following the insult was apoptotic. It should be noted that in this particular experiment, control explants show almost no DNA laddering because nearly all ganglion cells were allowed to degenerate during the 2 days of incubation prior to irradiation. Sections of explants fixed immediately after

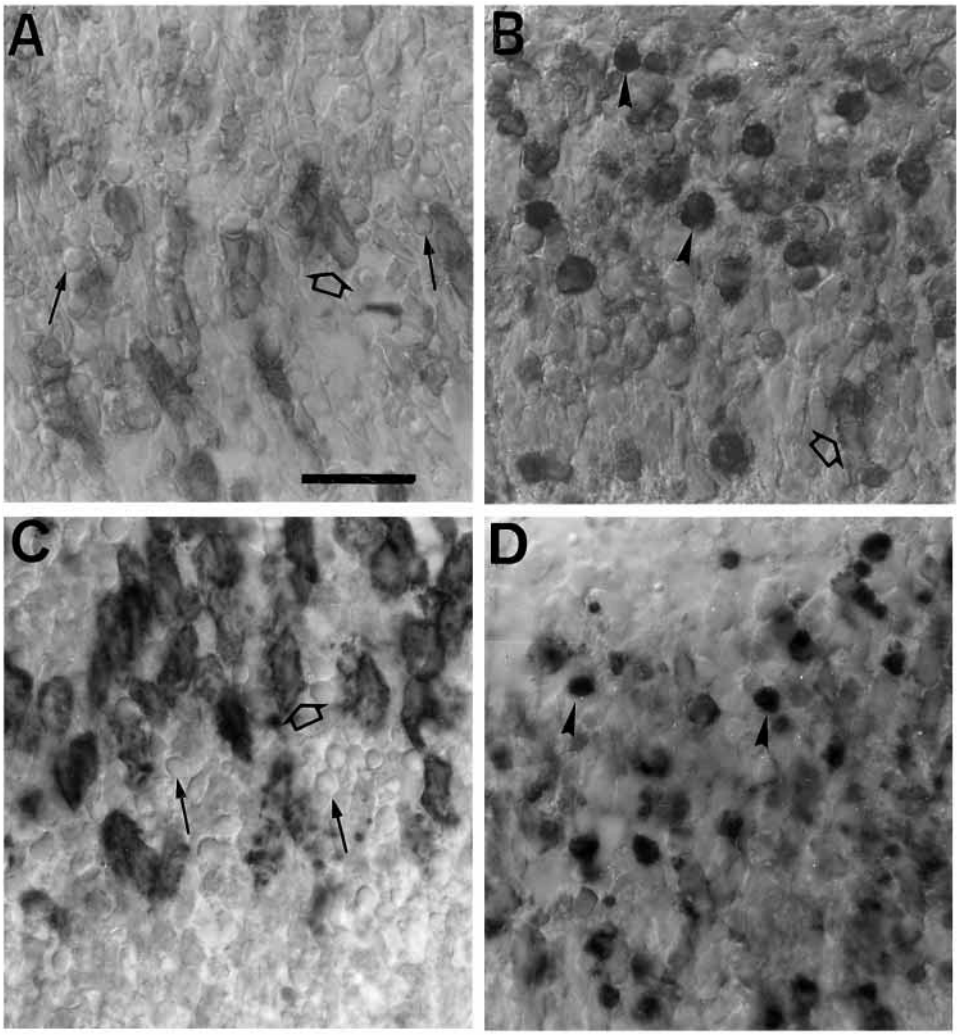


Fig. 6. Photomicrographs of explants at 6 hours (A,C) and 24 hours (B,D) after irradiation. (A,B) Explants from the retinae of rats that received a pulse of BrdU immediately before the irradiation. (C,D) Explants that received an in vitro pulse of BrdU 3 hours before fixation. The thin arrows show BrdU-negative apoptotic cells identified by differential interference contrast microscopy. Arrowheads show apoptotic cells labeled with BrdU. The thick arrows show healthy BrdU-positive cells. Bar, 20 μm.

irradiation contained almost no TUNEL staining profiles, similar to unirradiated control explants (not shown).

The late wave of apoptosis affects cells in the S phase of the cell cycle

To test whether distinct phases of the cell cycle are associated with either the early or the late waves of apoptosis, we injected BrdU in vivo to label a cohort of cells in S phase, 1 hour before explants were irradiated. The majority of these BrdU-labeled cells died at 24 hours after the irradiation (Figs 6A,B, 7). However, the highest incidence of apoptosis in this late wave of apoptosis occurred in the S phase region of the retina. To verify if dying cells were indeed in S phase, we gave a pulse of BrdU in vitro 3 hours before fixation of the explants at either 6 hours or 24 hours after irradiation. Half of the apoptotic cells at 24 hours after irradiation (Fig. 6D), but almost none of the early degenerating profiles (Fig. 6C), had synthesized DNA just prior to cell death (Fig. 7).

These data suggest that the cells sensitive to the late wave of apoptosis were in S phase at the time of irradiation and returned to S phase before dying. An alternative possibility, that the sensitive cells never actually left the S-phase stratum of the neuroblastic layer, is unlikely for the following reasons. First, labeling with BrdU did not interfere with either the rate or location of cell death, since the number of apoptotic cells in irradiated explants containing BrdU (1301±104/mm^2) did not differ from unlabeled irradiated explants (1500±129/mm^2) and their distribution along the NBL was similar in both cases (not shown). Second, the irradiation did not interfere with the interkinetic nuclear migration, as shown by similar dispersion of labeled nuclei from the inner (S-phase) margin towards the outer (M-phase) margin of the outer cellular stratum of either irradiated or control retinal explants at various time intervals after a pulse of BrdU (Fig. 8). These data are consistent with the hypothesis that the sensitive S-phase cells actually continue their cycle, divide and finally re-enter S phase before undergoing apoptosis in the late wave of cell death following gamma irradiation.

The early wave of apoptosis affects a mixture of both post-mitotic and proliferating cells

The early wave of apoptosis occurs in the proliferative zone (NBL) in the retina, which contains both proliferating cells and early post-mitotic cells. The dying cells at 6 hours after irradiation were not in the S phase of the cell cycle either at the time of irradiation (Fig. 6A) or afterwards at the time of their death (Fig. 6C). Following the multiple BrdU injections designed to label all proliferating cells, the majority of the early dying cells were labeled. This indicates that the radiation-sensitive cells are either neuroblasts or have just left their last round of cell division. Approximately 36.5% of the early apoptotic cells were devoid of BrdU and thus post-mitotic (Figs 9A,B, 10). As an alternative labeling method, that does not depend on immunocytochemistry, we used a series of injections of [^{3}H]-thymidine, and found a similar percentage (41%) of unlabeled cells (n=2).

Sections of irradiated explants were also stained with an antibody to PCNA. The percentage of apoptotic profiles stained for PCNA at 6 hours after irradiation was 59.5±5.1 (mean ± s.e.m.), which is consistent with the data obtained with either BrdU or ^{3}H-thymidine injections.

To test whether post-mitotic cells were already

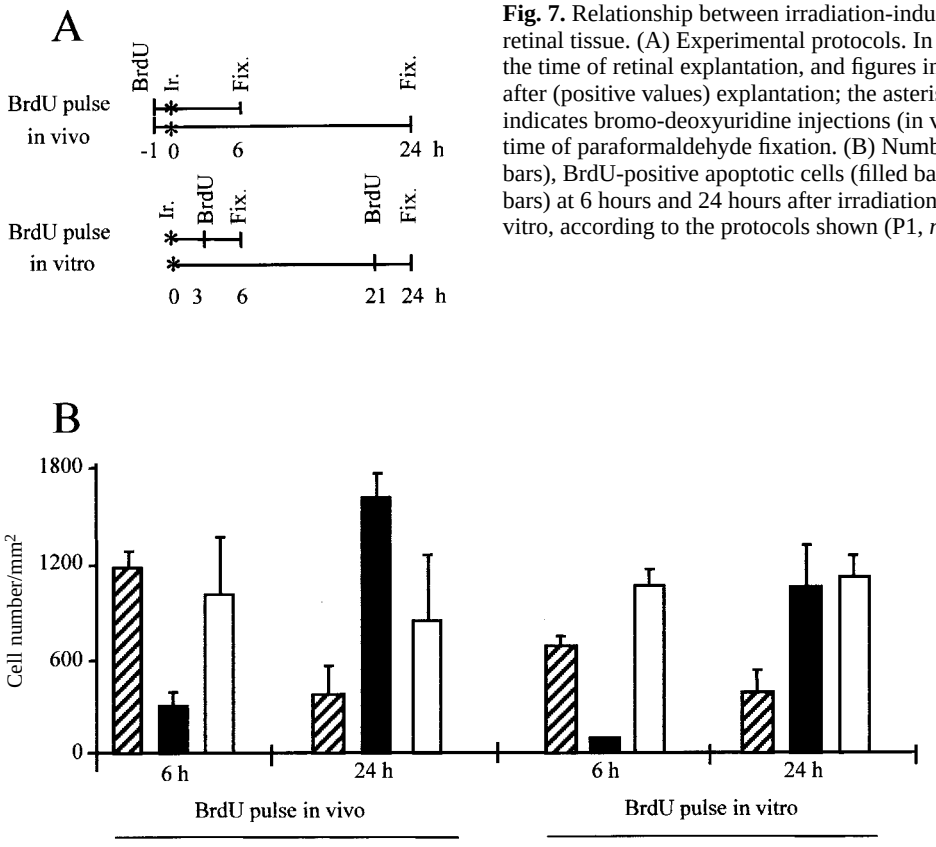


Fig. 7. Relationship between irradiation-induced cell death and S phase of the cell cycle in retinal tissue. (A) Experimental protocols. In this and in subsequent figures, zero indicates the time of retinal explantation, and figures indicate hours either before (negative values) or after (positive values) explantation; the asterisk indicates the time of irradiation; BrdU indicates bromo-deoxyuridine injections (in vivo) or additions (in vitro); Fix indicates the time of paraformaldehyde fixation. (B) Number of healthy BrdU-positive cells (hatched bars), BrdU-positive apoptotic cells (filled bars) and BrdU-negative apoptotic cells (open bars) at 6 hours and 24 hours after irradiation following BrdU pulses either in vivo or in vitro, according to the protocols shown (P1, *n*=3 in vivo, *n*=2 in vitro)

differentiated, sections were stained with various phenotypic markers of amacrine (calretinin), horizontal (calbindin) or rod photoreceptor (rhodopsin) cells. No immunolabeled cells were found among the apoptotic profiles at 6 hours after irradiation (data not shown).

Early and late waves of irradiation-induced apoptosis in vivo

We asked whether the same schedules of cell death would be induced by irradiation in vivo. Newborn animals were exposed to gamma irradiation and killed either at 6 hours or 24 hours later. The distributions of cell death that follow irradiation of the retina in vivo (Figs 4B, 9C,D) are nearly identical to those produced by the same insult upon retinal explants in vitro (compare Figs 2C,D, 4A).

The anti-oxidant pyrrolidinedithiocarbamate (PDTC) blocks the early, but not the late wave of apoptosis

PDTC is a metal chelator with antioxidant activity that has various biological effects (Marui et al., 1993; Verhaegen et al., 1995). We tested for protective effects upon irradiation-induced cell death. 80 μM of PDTC prevented the early wave of apoptosis at 6 hours after irradiation (Fig. 11A), but had no effect upon the late wave at 24 hours following the insult. The amount of TBARS produced at 6 hours after irradiation was higher than in control explants, but was similar in both control and irradiated explants at 24 hours of incubation. Treatment with 80 μM PDTC prevented the increase of TBARS at 6 h after irradiation (Fig. 11B).

DISCUSSION

The retina as a model of irradiation-induced apoptosis.

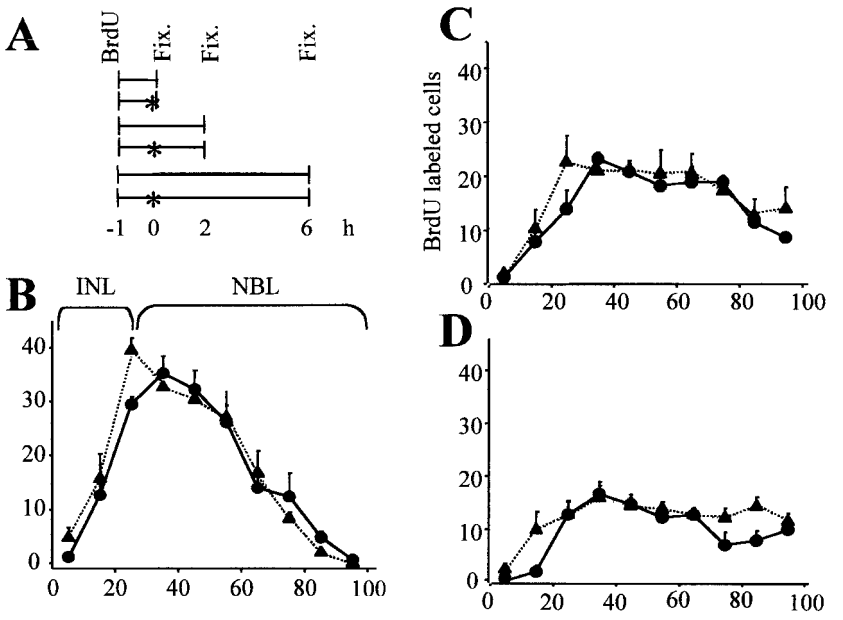
Present theories of radiation biology derive from a combination of work with intact animals and cell line assays. In many cases, cell lines were used because of their ease of operation, but they bear little resemblance to any tissue. Moreover, cell communication has only recently become an important topic in radiation biology. As reviewed by Mothersill (1998), primary tissue cultures are currently used in radiobiology, but most of them are derived from epithelial tissues.

Using an in vitro preparation of developing retina, we asked whether sensitivity to apoptosis is related to distinct phases of the cell cycle in the central nervous system. This model of radioinduced apoptosis was used because the major features of the developing nervous tissue are maintained, including the interkinetic nuclear migration, the recognition of distinct cell types by their location in the tissue, and the presence of cells at various stages of development. These features bridge the gap between cultures of dissociated cells or cell lines and animal models, because they allow experimental manipulation of the extracellular millieu, whilst maintaining the histotypical organization of the tissue.

In the present study, irradiation-induced apoptosis occurred only within the proliferative zone of the developing retina. Irradiation led to two waves of cell death affecting separate cell populations at distinct times after the insult. An early wave of apoptosis included both post-mitotic and proliferating cells out of the S phase. Cells that were in the S phase of the cell cycle at the time of irradiation were the main components of a late wave of apoptosis. These cells died after a single round of mitosis, again in the phase of DNA synthesis. The same two waves of apoptosis occurred after irradiation in vivo.

A single irradiation, two deaths

The first wave of apoptosis starts as early as 3 hours after irradiation. Approximately 60% of the apoptotic profiles found at 6 hours after irradiation could be labeled either with a series of BrdU injections designed to label all proliferating cells, or with an antibody against PCNA. These data indicate that most of the cells that die at 6 hours after irradiation are neuroblasts. The dying cells were preferentially located at the outermost region of the proliferative zone. This corresponds to the



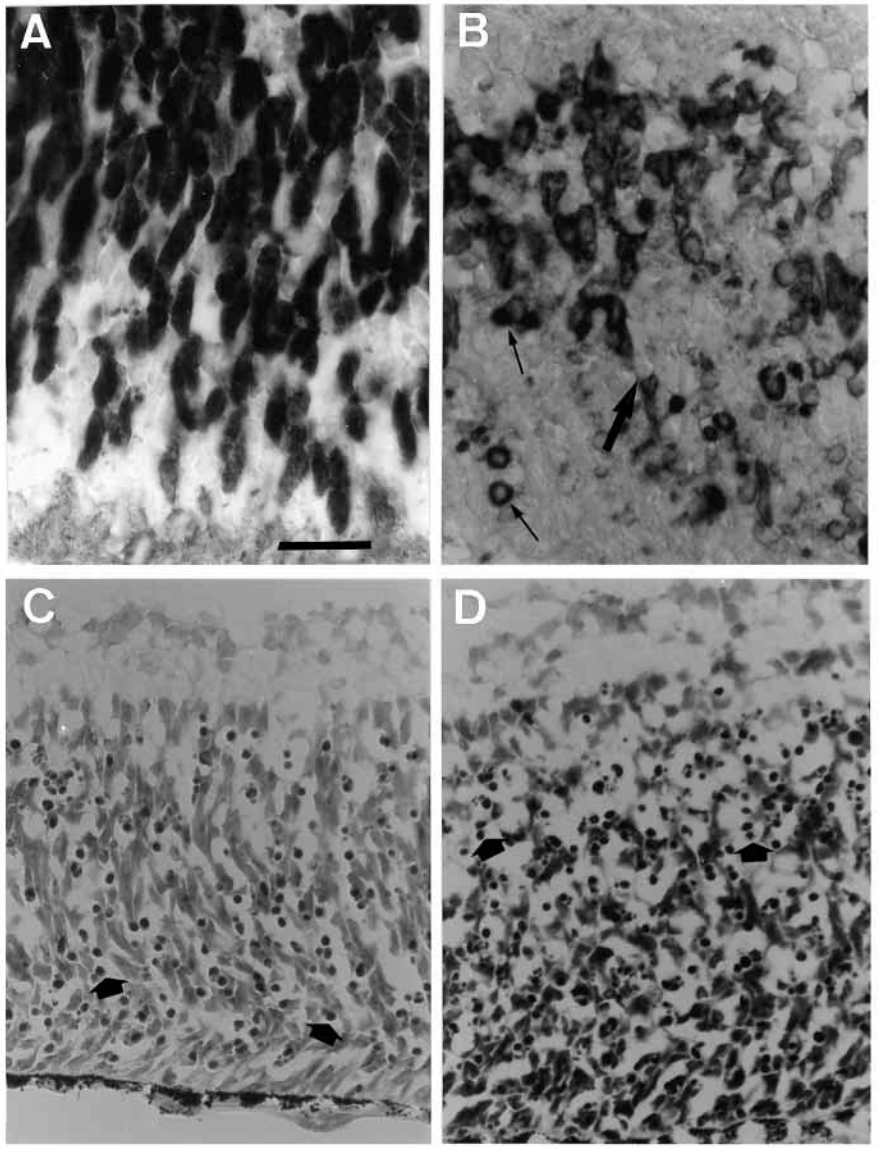


Fig. 8. Distribution of BrdU-labeled nuclei along the depth of the neuroblastic layer at various intervals following irradiation. (A) The protocol of the experiment. See Fig. 7 for abbreviations and symbols. (B-D) Distribution of labeled nuclei in control (triangles) and irradiated (circles) retinae at 10 minutes (B), 2 hours (C) or 6 hours (D) after irradiation. Notice the similar migration of the nuclei of the neuroblasts towards the outer margin of the retina in both groups.

expected location of checkpoint G_2/M (Fujita, 1962), which is usually one of the most radiosensitive phases of the cell cycle (Cheong et al., 1994). This G_2/M radiosensitive phase was previously noted in the developing spinal cord, cerebral cortex, and retina in vivo (Hicks et al., 1961).

However, after labeling of all cycling cells with BrdU, 40% of the early apoptotic cells were unlabeled and thus post-mitotic. A similar proportion of unlabeled cells was observed following immunocytochemistry for PCNA. These unlabeled cells must have recently left the cell cycle because none of the degenerating profiles could be stained with antibodies to any of various markers of differentiation. Cells labeled with antibodies for calbindin, calretinin or rhodopsin always showed a healthy morphology (see Peichl and Gonzales-Soriano, 1994; Papazafiri et al., 1995; Molday, 1998, for retinal phenotypic markers).

Apoptosis of both neuroblasts and post-mitotic cells, similar to the early wave of apoptosis detected in the retina, was also found in other regions of the developing central nervous system, as noted by the location of sensitive populations 6 hours after ionizing irradiation. The developing cerebral cortex showed radioinduced cell death in both the ventricular zone and in the cortical plate of post-mitotic cells (Ferrer, 1992). In the developing cerebellum, most of the radiosensitive cells were found in the proliferative zone, the external granular (proliferative) layer, but degenerating cells were also found in the internal granular layer that contains post-mitotic, differentiating neurons (Ferrer et al., 1993a).

Fig. 9. Photomicrographs of control (A) or irradiated (B) explants cultured for 6 hours, following pre-labeling of all proliferating cells with three injections of BrdU (see Materials and Methods). Small arrows show labeled apoptotic cells, and the large arrow shows an unlabeled apoptotic cell. (C,D) Photomicrographs of retinal sections stained with Neutral Red, from rats killed at either 6 hours (C) or 24 hours (D) after irradiation in vivo. Arrows show apoptotic cells. Bar, 30 μm.

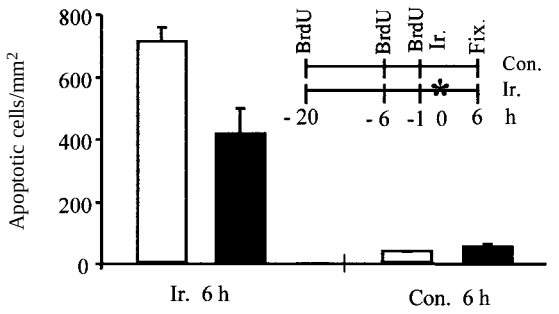


Fig. 10. Numbers of BrdU-labeled (open bars) and unlabeled (filled bars) apoptotic cells in control and irradiated explants at 6 hours of incubation, following pre-labeling of all proliferating cells with three injections of BrdU made in vivo before explantation, as shown in the diagram. See Fig. 7 for abbreviations and symbols.

In contrast, the late wave of apoptosis heavily affects neuroblasts in the S phase. Evidence for this includes the fact that most of the apoptotic cells found at 24 hours after the insult were labeled with bromodeoxyuridine given immediately before irradiation. In addition, half of the apoptotic cells were labeled with the nucleotide analog given a few hours before fixation. Since the BrdU pulse lasted for only 30 minutes of the 3 hours at the end of the incubation, some cells entering S phase and dying in the late wave of apoptosis may have escaped labeling, even though they are located mainly within the S-phase region. Thus, the percentage of cells that synthesize DNA just before dying may be higher than 50%. In addition, more than 90% of the apoptotic cells found at 24 hours after irradiation are stained with an antibody for PCNA. The irradiation did not affect the interkinetic nuclear migration, at least during the first 6 hours after the insult, which makes it unlikely that the irradiated cells may have been delayed for 24 hours within the S phase. It is therefore likely that the neuroblasts irradiated when in S phase continued along the G_2 phase and died after one round of mitosis when again in S phase, here detected at 24 hours after irradiation. Our results differ from those of Hicks and co-workers (1961), who reported that in the cerebral cortex, spinal cord and olfactory bulb of rat embryos irradiated 1 hour after an injection of [^{3}H]thymidine delayed cell death (24 hours after the insult) was composed mostly of unlabeled cells. In fact, in our study most of the retinal neuroblasts that were in S phase were killed at 24 hours after irradiation. It is not clear whether the distinct results reflect either the distinct areas of the central nervous system examined, or the use of different techniques.

The late wave of apoptosis is therefore similar to the mitotic/reproductive cell death induced by irradiation. In reproductive apoptosis, the cells lose viability after mitosis, as shown by the decline in colony-forming capacity (Yanagihara et al., 1995; Szumiel, 1994). In turn, the early wave of apoptosis is at least in part similar to interphasic cell death, because it occurs quickly and comprises post-mitotic cells. Thus, similar to leukemic cell lines, the organized retinal tissue both in vitro and in vivo is subject to a mixture of interphasic and mitotic cell death induced by irradiation (Szumiel, 1994).

The interphasic death has been attributed to membrane damage (lipid peroxidation) and/or increased production of ceramide by activation of sphingomyelinase (Bruno et al., 1998; Haimovitz-Friedman, 1998; Ojeda et al., 1994). In contrast, the reproductive apoptosis seems to be mostly induced by DNA or nuclear damage (Bruno et al., 1998; Haimovitz-Friedman, 1998; Godar and Lucas, 1995; Guo et al., 1997). Differential sensitivity to subcellular damage in distinct stages of development may explain the presence of the two waves of apoptosis in the developing retina.

Indeed, we found that pyrrolidinedithiocarbamate (PDTC) prevents the early apoptotic wave, but does not block the late wave of cell death. Blockade of TBARS production at 6 hours after irradiation suggests that the early, but not the late wave of irradiation-induced apoptosis depends on lipid peroxidation, while cells in the S phase would be relatively resistant to membrane damage. These preliminary results are consistent with the hypothesis that the two waves of apoptosis are triggered by distinct mechanisms. The model of apoptosis induced by gamma irradiation in explants of the rat retina may be therefore of particular value for the study of multiple mechanisms of apoptosis in the developing nervous tissue.

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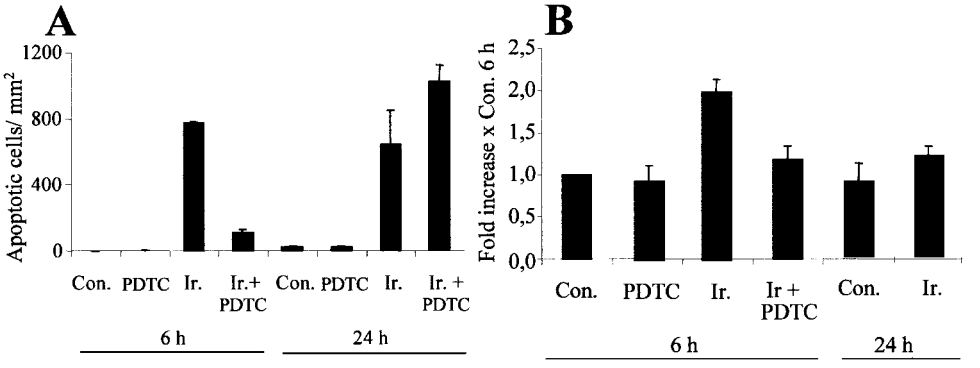

Fig. 11. Effects of the antioxidant PDTC on apoptotis and lipid peroxidation in retinal tissue. Explants were either fixed (A) or homogenized (B) either at 6 hours or at 24 hours of incubation, as shown below the lower horizontal lines. (A) Number of apoptotic cells in the outer cellular stratum after various treatments. Data are representative of three independent experiments. (B) Lipid peroxidation indicated by the amount of TBARS in retinal tissue following various treatments. Values are expressed as fold-increase with respect to control conditions at 6 hours of incubation. Treatments were: Con, control; Ir., irradiation; PDTC, 80 μM pyrrolidinedithiocarbamate, as described in Materials and Methods (n=2 for groups with PDTC, n=3 for the remaining groups).

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