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Contrasting effects of protein synthesis inhibition and of cyclic AMP on apoptosis in the developing retina

S. K. Rehen[1,†], M. H. Varella[1], F. G. Freitas[1], M. O. Moraes[1,2] and R. Linden[1,*]

[1]Instituto de Biofisica Carlos Chagas Filho, UFRJ, Centro de Ciencias da Saude, bloco G, Cidade Universitaria, 21949-900, Rio de Janeiro, Brasil
[2]Departamento de Biofisica e Biometria, UERJ, Rio de Janeiro, Brasil

*Author for correspondence (e-mail: rlinden@chagas.biof.ufrj.br)
†Present address: Department of Anatomy, ICB-UFRJ, Cidade Universitária, Rio de Janeiro, Brazil

SUMMARY

The role of protein synthesis in apoptosis was investigated in the retina of developing rats. In the neonatal retina, a ganglion cell layer, containing neurons with long, centrally projecting axons, is separated from an immature neuroblastic layer by a plexiform layer. This trilaminar pattern subsequently evolves to five alternating cell and plexiform layers that constitute the mature retina and a wave of programmed neuron death sweeps through the layers. Apoptosis due to axon damage was found in ganglion cells of retinal explants within 2 days in vitro and was prevented by inhibition of protein synthesis. Simultaneously, protein synthesis blockade induced apoptosis among the undamaged cells of the neuroblastic layer, which could be selectively prevented by an increase in intracellular cyclic AMP. Both the prevention and the induction of apoptosis among ganglion cells or neuroblastic cells, respectively, occurred after inhibition of protein synthesis in vivo. The results show the coexistence of two mechanisms of apoptosis within the organized retinal tissue. One mechanism is triggered in ganglion cells by direct damage and depends on the synthesis of proteins acting as positive modulators of apoptosis. A distinct, latent mechanism is found among immature neuroblasts and may be repressed by continuously synthesized negative modulators, or by an increase in intracellular cyclic AMP.

Key words: programmed cell death, killer genes, eye development, cyclic AMP, protein synthesis, apoptosis, retina

INTRODUCTION

Dependence on protein synthesis is generally considered a hallmark of programmed cell death (Lockshin and Zakeri, 1991). Apoptosis, originally defined on morphological grounds (Kerr et al., 1972) and later extended to biochemical criteria (Wyllie, 1980), consists of a series of controlled steps leading to cell demise (Kerr and Harmon, 1991) and is a major type of programmed cell death (Bowen, 1993). The defining criteria of apoptosis include the early condensation and fragmentation of nuclear chromatin, with preservation of cytoplasmic organelles and the cleavage of DNA into oligonucleosomes by an endogenous endonuclease (Kerr and Harmon, 1991). This mode of programmed cell death has been classically shown to depend on de novo protein synthesis (Wyllie et al., 1984).

Nonetheless, inhibition of protein synthesis can itself result in apoptosis in established cell lines, as well as in primary cultures (Martin, 1993; Martin et al., 1990, 1994). This indicates that apoptosis depends on counteracting signals relayed by proteins. Little is known of the control of programmed cell death in organized tissues, in which complex interactions of apoptosis-related genes with various extracellular signals and signal transduction pathways are likely to modulate sensitivity to cell death (Hoffman and Liebermann, 1994; McConkey and Orrenius, 1991, 1994; Kizaki and Tadakuma, 1993; Williams and Smith, 1993; Collins et al., 1994).

The formation of the highly structured and precisely interconnected nervous system of vertebrates includes a stage of naturally occurring neuron death (Oppenheim, 1991), which often takes the form of apoptosis (Clarke, 1990; Deckwerth and Johnson, 1993). In the rat retina, developmental cell death occurs mainly postnatally. At birth, the innermost ganglion cell layer is already formed, containing the cell bodies of the long-axon ganglion cells that convey information from the eye to the brain. The remaining retinal layers are then represented by a relatively undifferentiated neuroblastic layer, where cell proliferation still occurs. A wave of naturally occurring cell death sweeps through the depth of the retina, initially restricted to the ganglion cell layer and affecting the outer retinal tiers at progressively later stages. The identifiable dying cells show a morphology consistent with apoptosis, with condensation and fragmentation of chromatin (Perry et al., 1983; Beazley et al., 1987). Recent reports have shown that degeneration of ganglion cells after damage to their axons can be blocked by inhibitors of protein synthesis in rats (Harvey et al., 1994; Rabacchi et al., 1994) and that oligonucleosomal cleavage of DNA is found cotemporal with naturally occurring ganglion cell death in chicks (Ilschner and Waring, 1992).

Free-floating explants of newborn rat retinae can be maintained in vitro for several days while preserving the basic histological organization of the retina (Araujo and Linden, 1993). Explants allow both the detection of secreted products and experimental manipulation of the extracellular milieu of an organized piece of the central nervous system (Araujo and Linden, 1993; Rehen et al., 1993). These organotypic cultures are therefore particularly useful to study mechanisms dependent on the histotypical organization of nervous tissue, bridging the gap between the unstructured cultures of dissociated nerve cells and in vivo preparations. Here we report that inhibitors of protein synthesis have opposing effects on apoptosis in distinct layers of the developing retina, both in vitro and in vivo. Apoptosis induced by inhibition of protein synthesis can in turn be selectively prevented by an increase in intracellular levels of cAMP.

MATERIALS AND METHODS

Retrograde labeling of retinal ganglion cells

Lister hooded rats were used throughout this study. In some experiments, ganglion cells were labeled by the retrograde transport of horseradish peroxidase (HRP). Rat pups were deeply anaesthetized either by hypothermia (P0) or by inhalation of ether (older pups) and a 20% solution of horseradish peroxidase (HRP) in 2% dimethyl sulphoxide was injected bilaterally into the upper layers of the superior colliculus. A total of 1 μl was infused through a series of small injections scattered across each colliculus. A minimum survival time of 16 hours was allowed before retinal tissue culture.

Tissue culture

Explants of rat retinae were prepared as described previously (Araujo and Linden, 1993). The animals 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 in culture medium and placed in 25 ml tight-lidded Erlenmeyer's with 5 ml of Basal Medium of Eagle (Gibco BRL) with 5% Fetal Calf Serum (WL Immunochemicals). CO_2 was adjusted to 5%. The flasks were kept in an orbital shaker at 80-90 revs/minute and 37°C for the required incubation periods. Drugs were added at the beginning of the incubation period.

In vivo experiments

To produce retrograde degeneration of ganglion cells, the rat pups were deeply anaesthetized by hypothermia and the left superior colliculus was removed under visual control by aspiration through a fine needle. The surgical wound was filled with gel foam and closed. Operated rats together with unoperated controls were either left untouched or injected immediately after the surgery with anisomycin to block protein synthesis in vivo.

The animals were kept warm in a plastic cup floating in a waterbath at 37°C. Two injections of 10 mg/kg b.w. were made at a 1 hour interval, under the slack posterior neck skin using an insulin syringe. A 24-hour survival was allowed after the first injection of anisomycin before killing the animals for histological processing.

Histology

The explants were fixed by immersion with a mixture of 1% paraformaldehyde and 2% glutaraldehyde in sodium phosphate buffer pH 7.2. The rat pups used for the in vivo experiments were painlessly killed by inhalation of chloroform and perfused through the heart with saline followed by the same fixative above at 4°C. The eyes were removed from the orbits for further tissue processing and the surgical lesions were confirmed by examination of the brains under a dissecting microscope.

The explants were oriented under a dissecting microscope, in an aluminum chamber filled with OCT embedding medium, and transverse sections were cut in a cryostat. The retina in situ was cut frozen after orienting the eye to allow transverse sectioning. When HRP was used a tracer, the material was processed for HRP histochemistry using tetramethyl benzidine as the chromogen. The sections were counterstained with neutral red.

Protein and DNA synthesis measurements

To measure protein synthesis in vitro, cultures either in control medium or with various concentrations of antibiotics were incubated with [^{35}S]methionine for either 1 hour (explants) or overnight (monolayers), washed twice with serum-free medium and homogenized following overnight incubation with 0.4 M NaOH. Aliquots of the homogenates were precipitated with 100 volumes of 10% trichloroacetic acid (TCA) and collected on GF/A Whatman filters. Following an additional wash with 10% TCA and three washes with ethanol, the filters were dried at 110°C in an oven and counted in a Packard model 1600TR liquid scintillation analyzer. DNA synthesis was similarly quantitated, by incubating either explants or monolayers with [^{3}H]thymidine.

The time course of inhibition of protein synthesis in vivo was evaluated following injections of anisomycin. Uninjected controls and injected animals at selected time points were instantaneously killed by decapitation and the retinae were quickly dissected out of the eyeballs in culture medium. The dissection procedure took less than 5 minutes in every case. In control experiments, we determined that intervals of up to 20 minutes between killing the animals and starting the incubation with radiolabeled aminoacid had no effect on the estimates of the rate of protein synthesis.

The retinae were collected in Eppendorfs with 500 μl culture medium and incubated with 10 μCi of [^{35}S]methionine for 30 minutes at room temperature. Then, the tissue was washed twice with serum-free medium and incubated overnight with 0.4 M NaOH. Aliquots of the homogenates were precipitated with TCA and the incorporated aminoacid was quantitated by scintillation counting.

In situ nick end labeling

Explants were fixed by immersion in 4% paraformaldehyde in phosphate buffer for 1 hour at room temperature, infiltrated with 20% sucrose, embedded in OCT and cut transversely at 10 μm in a cryostat. Sections mounted on glass slides were washed with PBS and preincubated in terminal deoxyribonucleotidyl transferase (TdT) buffer at room temperature for 20 minutes. This was followed by incubation with a solution containing 0.5 U/μl of TdT (Gibco BRL), 0.2 nmol/μl dATP and 0.013 nmol/μl biotin-14-dATP (Gibco BRL) in TdT buffer for 18 hours at 37°C. The reaction was stopped with 20 mM EDTA, the slides were washed with PBS and incubated with 1:100 Streptavidin-Texas Red (Amersham) at 37°C for 20 minutes, then washed with PBS and coverslipped with glycerol-N-propyl-galate.

DNA electrophoresis

Retinal explants were frozen under liquid nitrogen. The tissue was resuspended in 0.5 ml of lysis buffer (0.2% Triton X-100, 10 mM Tris buffer, 1 mM EDTA), left to stand at room temperature for 30 minutes and the lysates were centrifuged at 13,000 revs/minute for 15 minutes at 4°C. The pellet containing high molecular weight DNA was discarded. The supernatant containing fragmented DNA was precipitated with an equal volume of isopropyl alcohol and 0.5 M NaCl. The samples were stored at −20°C overnight and centrifuged at 13,000 revs/minute for 15 minutes at 4°C. The pellet was washed with 70% ethanol, air dried and resuspended in 100 μl TE buffer (10 mM Tris, 1 mM EDTA). Following incubation with RNAse A (20 μg/ml) and Proteinase K (100 μg/ml) for 3 hours at 37°C, the DNA was reprecipitated with 0.2 M NaCl and 2.5 volumes of absolute ethanol, the

pellet was washed with 70% ethanol and resuspended in 50 μl TE. The optical densities of aliquot samples were read at 260 nm and approximately 2-3 μg DNA from each sample were loaded in buffer containing 30% glycerol, 0.025% bromophenol blue and 0.025% xylene cyanol. The samples were electrophoresed in a 1% agarose gel, the gel was stained with ethidium bromide at 0.5 μg/ml and photographed with a Polaroid camera. A negative control was prepared with DNA from the freshly dissected retina of a 3-week-old rat, that is well after the end of the period of naturally occurring cell death (Perry et al., 1983; Beazley et al., 1987). This sample had small amounts of low molecular weight nuclei acid detected by OD_{260}. Therefore, an amount equivalent to 12× as many cells as compared with control explants was loaded in the gel.

Table 1. Inhibition of protein synthesis in retinal explants incubated with antibiotics

Concentration (μg/ml)	Cycloheximide	Anisomycin	Deacetyl-anisomycin
0	100*	100	100
0.01	65.5±6.5**	94.5±11.2	
0.1	57.0±4.2	31.6±7.6	
0.32	35.8±6.5	11.7±1.9	
1	11.1±3.2	3.5±0.5	83.6±10.0
3.2	10.1±2.8	0.4±1.0	
10	6.8±4.4	2.1±2.4	83.8±7.2

*Incorporation of [^{35}S]methionine is expressed as percentages of untreated control explants.

**The figures are the means±s.e.m. of three independent experiments.

RESULTS

Cell death in retinal explants in vitro

The ganglion cells in retinal explants degenerate due to the section of the optic axons when the eye is removed from the orbit. Retrograde labeling with horseradish peroxidase previously injected in vivo allows the time course of ganglion cell death to be followed (Fig. 1A,B) together with the detection of condensation and fragmentation of chromatin typical of apoptosis (Fig. 4A). We have previously shown that axotomized ganglion cells in retinal explants degenerate within 2-3 days in vitro (DIV) and the maximum frequency of cells with condensed chromatin was found at 1 DIV (Rehen et al., 1993). During the first 3-4 DIV, there was almost no cell death in the underlying neuroblastic layer. An outer plexiform layer started to appear at about 4 DIV (see Fig. 11 in Araujo and Linden, 1993), similar to the time course in vivo (Craft et al., 1982), indicating that major events of retinal differentiation are preserved in the explants.

Contrasting effects of protein synthesis inhibition in vitro

Incubation with the inhibitor of protein synthesis cycloheximide prevented the degeneration of retinal ganglion cells (Fig. 1C). The effect was dose-dependent and concentrations of 1-10 μg/ml of cycloheximide in the culture medium, which inhibited over 90% of the incorporation of aminoacid into protein (Table 1), led to the preservation of most ganglion cells at 2 DIV (Rehen and Linden, 1994). Consistent with this, the frequency of cells with condensed chromatin was reduced at 1 DIV (Figs 2A, 4B).

Surprisingly, however, cycloheximide also resulted in a dose-dependent increase in the frequency of pyknotic nuclei in the neuroblastic layer (Figs 1C, 4B; see also Rehen and Linden, 1994). There was no effect in this layer within the first 8 hours of incubation, but the rate of cell death in the NBL was severely increased at both 1 and 2 DIV (Fig. 2B). However, the degenerating cells were always found among healthy-looking neighbouring cells.

The condensation of chromatin in either the ganglion cell layer or the neuroblastic layer was accompanied by typical internucleosomal cleavage of DNA (Fig. 3). Thus, both in the presence and in the absence of protein synthesis, the degeneration induced in the respective layers conformed to the biochemical criterion for apoptosis.

The coexistence of both decreased and increased rates of

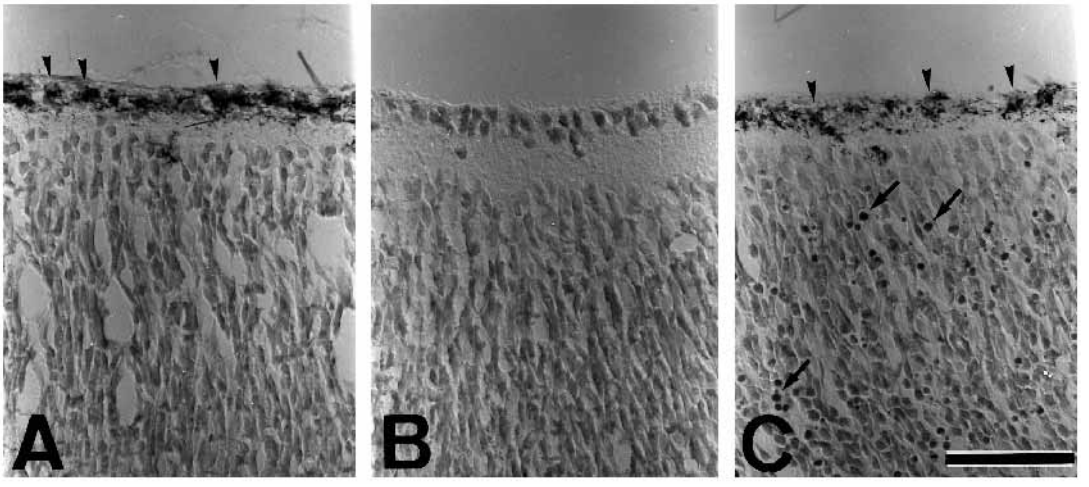

Fig. 1. Photomicrographs of transverse sections through explants of the rat retina. Ganglion cells were retrogradely labeled with HRP. (A) Control explant at 2 hours in vitro, showing extensive labeling of ganglion cells (arrowheads). (B) Explant kept in control medium for 2 days, showing the disappearance of the ganglion cells and no signs of degeneration in the neuroblastic layer. (C) Explant maintained for 2 days in medium containing 1 μg/ml cycloheximide, showing the rescue of the axotomized ganglion cells and simultaneous induction of degeneration in the neuroblastic layer (arrows). Bar, 50 μm.

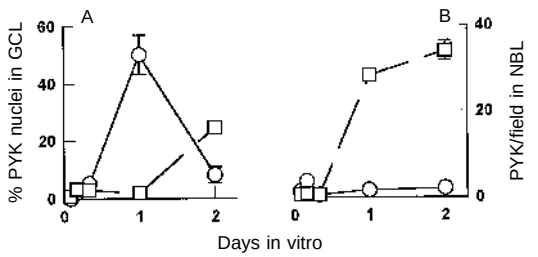

Fig. 2. Quantitation of cell death and the effects of cycloheximide in retinal explants in vitro. (A) Time course of cell death in the ganglion cell layer in control explants (circles) and in the presence of 1 μg/ml cycloheximide (squares). (B) Time course of cell death in the neuroblastic layer in control explants (circles) and in the presence of 1 μg/ml cycloheximide (squares).

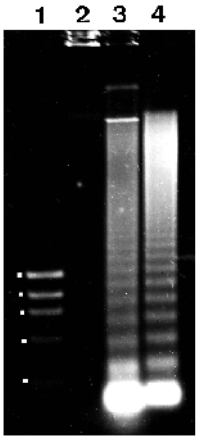

Fig. 3. Gel electrophoresis of DNA from retinal tissue. Lane 1: molecular weight markers from a *Hae*III digest of ϕX174 phage DNA (0.3; 0.6; 0.8; 1.0; 1.3 kbp); lane 2: DNA from the retina of a 3 week-old rat; lane 3: DNA from explants of P1 retina kept for 1 DIV in control medium; lane 4: DNA from explants of P1 retina kept for 1 DIV in medium supplemented with 1 μg/ml cycloheximide. Notice the oligonucleosomal cleavage of DNA both in the absence and in the presence of the inhibitor of protein synthesis.

apoptosis in distinct layers of the same explants argues strongly against non-specific toxic effects of cycloheximide. In addition, similar results were obtained with anisomycin, a less toxic protein synthesis inhibitor. Incubation of retinal explants with anisomycin prevented the degeneration of the axotomized ganglion cells, while inducing apoptosis in the neuroblastic layer (Fig. 4C). The inactive analog deacetyl anisomycin had no effect (Fig. 4D). Concentrations of cycloheximide and anisomycin that produced similar levels of inhibition of protein synthesis (ca. 90%, see Table 1) had similar effects on the rates of cell death in both layers of the developing retinal explants (Table 2).

Cell death in either layer was further examined by in situ labeling of DNA fragmentation (Gavrieli et al., 1992). Degenerating cells both in the ganglion cell layer in control explants and in the neuroblastic layer of explants treated with anisomycin, were labeled with polynucleotides synthesized in situ upon DNA strand breaks (Fig. 5). Again, the labeled cells were always found amongst unlabeled neighbours and most labeled nuclei were round and pyknotic, in contrast with the usual elongated morphology of the nuclei of retinal neuroblasts.

Cyclic AMP prevents apoptosis induced by protein synthesis inhibition

The degeneration induced in the neuroblastic layer by inhibition of protein synthesis was blocked by increasing the intracellular concentration of cyclic AMP (Table 3). The activator of adenylyl-cyclase forskolin at a concentration of 10 μM, prevented the induction of apoptosis by either cycloheximide or anisomycin in the neuroblastic layer. This concentration raises 15-fold the level of intracellular cAMP in the retina when tested in the presence of phosphodiesterase inhibitors (M. H. Varella, F. G. de Mello and R. Linden, unpublished results). The effect of forskolin was mimicked by incubation of the explants with the cell-permeant compound 8-bromo-cAMP at 1 mM. Neither forskolin nor 8-Br-cAMP at those concentrations prevented apoptosis in the ganglion cell layer at 1 DIV. Forskolin had no significant effect on either the incorporation of labeled methionine into protein or of thymidine into nucleic acid (data not shown), indicating that it did not interfere with either protein synthesis or cell proliferation in our conditions.

Table 2. Effects of various antibiotics on the rates of apoptosis in retinal explants

Treatment	GCL*	NBL**
Control	32.1±0.4***	0.2±0.1
Cycloheximide (1μg/ml)	6.8±3.2	15.3±2.2
Anisomycin (0.32 μg/ml)	5.6±2.9	14.9±0.5
Deacetyl-anisomycin (1 μg/ml)	40.8±4.1	0.4±0.1

*% pyknotic nuclei in the ganglion cell layer.
**Number of pyknotic nuclei per field in the neuroblastic layer.
***The figures are means±s.e.m. of three separate explants.

Topographical distribution of apoptosis induced by protein synthesis inhibition in the retinal tissue

In explants from newborn rats, the degeneration induced by protein synthesis blockade was found throughout the depth of the neuroblastic layer (Figs 1C, 4B,C). We therefore examined the effects of cycloheximide upon explants from older rat pups, following the emergence of the outer retinal layers. In explants from 6-day-old rats (P6), retrogradely labeled ganglion cells

Table 3. Effects of raising the intracellular concentration of cyclic AMP on the rates of apoptosis in retinal explants

Treatment	GCL*	NBL**
Control	27.2±1.1***	0.7±0.2
Ethanol (0.1%)	24.8±3.3	1.5±0.2
Forskolin (10 μM)	29.0±2.4	1.6±0.5
Cycloheximide (1 μg/ml)	3.2±1.1	24.1±2.5
Cycloheximide + Forskolin	1.1±0.3	5.9±1.6
Control	33.8±4.2	0.9±0.2
8-Br-cAMP (1 mM)	28.6±5.6	0.2±0.1
Cycloheximide (1 μg/ml)	2.7±1.3	48.0±8.2
Cycloheximide + 8-Br-cAMP	2.3±0.6	17.0±2.0

*% pyknotic nuclei in the ganglion cell layer.
**Number of pyknotic nuclei per field in the neuroblastic layer.
***The figures are means±s.e.m. of three separate explants and each set of data belongs to an independent experiment.

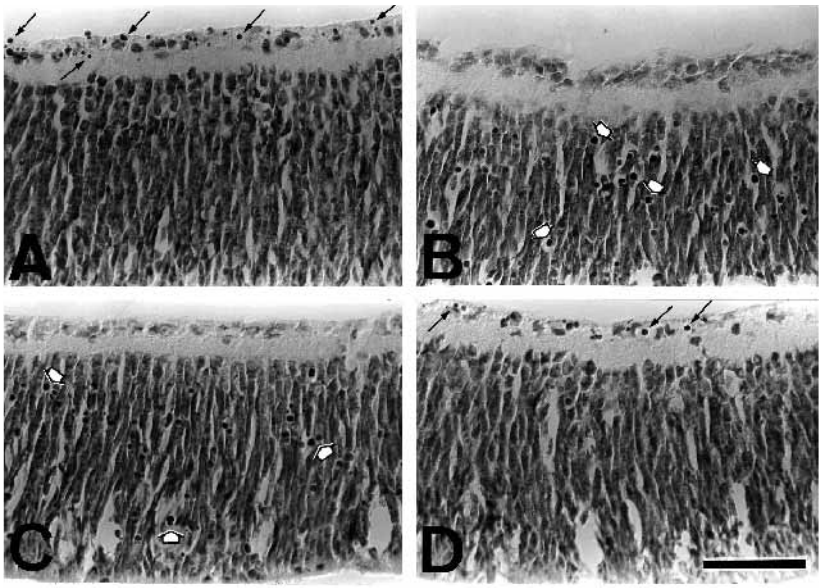

Fig. 4. Effects of distinct inhibitors of protein synthesis on retinal apoptosis. Photomicrographs of transverse sections of explants kept for 1 DIV and counterstained with neutral red, viewed under differential interference contrast microscopy. (A) Control medium; (B) 1 μg/ml cycloheximide; (C) 0.32 μg/ml anisomycin; (D) 1 μg/ml deacetyl-anisomycin. Notice the similar effects of cycloheximide and anisomycin. Arrows, apoptotic cells in the ganglion cell layer; arrowheads, apoptotic cells in the neuroblastic layer. Bar, 50 μm.

were rescued from degeneration at 2 DIV by the antibiotic. The inhibition of protein synthesis also produced apoptosis outside the GCL at 1 DIV. The dying cells were, however, concentrated in the midportion of the outer retinal layers, straddling the limits of the emerging outer plexiform layer. Rare degenerating profiles were found scattered either in the inner half of the inner nuclear layer or in the outermost portion of the retina. In explants from P10 or P13 rats, the cell death induced by cycloheximide was restricted to the immediate vicinity of the outer plexiform layer. At these stages, there was little degeneration in the ganglion cell layer of explants kept in control medium, probably due to increased resistance of maturing ganglion cells to axotomy-induced cell death (e.g. Perry and Cowey, 1982; Linden and Perry, 1983). In explants from P33 rats, there were virtually no pyknotic nuclei in the GCL at 1 DIV and inhibition of protein synthesis had no effect. Thus, the older retina became more resistant both to the axotomy-induced death of ganglion cells and to apoptosis induced by protein synthesis blockade.

Contrasting effects of protein synthesis inhibition in the retina in vivo

The effect of protein synthesis inhibition was further tested in vivo. In order to avoid inflicting direct damage to the eye with intraocular injections, we administered the drugs subcutaneously. Initial attempts at using cycloheximide in vivo failed due to the high mortality rate following systemic injections of this drug in neonatal rats. The effects of systemic injections of anisomycin were thus tested both upon naturally occurring cell death in the ganglion cell and neuroblastic layers, and upon the retrograde degeneration of ganglion cells produced by damage to their axons after tectal lesions (Fig. 6A). A series of two subcutaneous injections of 10 mg/kg b.w. of anisomycin produced approximately 60% inhibition of protein synthesis in the retina over a 24-hour period (Fig. 6B), during which the animals appeared healthy.

Cell death was again evaluated by the frequency of profiles with condensed and fragmented chromatin. Following partial inhibition of protein synthesis for 24 hours in vivo, naturally occurring degeneration in the ganglion cell layer was reduced (Fig. 6C,D) and simultaneously the rate of cell death in the neuroblastic layer was increased (Fig. 6E,F). Protein synthesis blockade with anisomycin also prevented the retrograde degeneration of axotomized ganglion cells following the tectal lesion (Fig. 6C,D), while again causing an increased rate of apoptosis in the neuroblastic layer (Fig. 6E,F). The magnitude of the effects was consistent with the partial blockade in protein synthesis. We have previously shown that 60-70% inhibition of protein synthesis in retinal explants reduces approximately

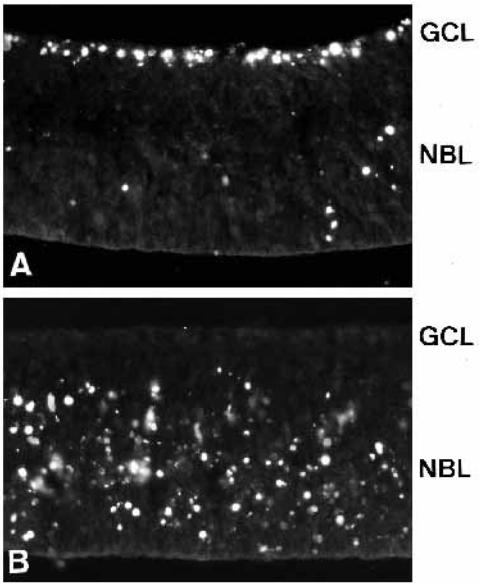

Fig. 5. In situ labeling of DNA strand breaks in apoptotic cells. Polynucleotides added to the 3′-OH nick ends were detected through the binding of streptavidin to biotinylated dATP. Apoptotic cells appear brightly labeled with Texas Red against the dark background of the retinal tissue section viewed under epifluorescence. (A) Control explant; (B) explant maintained for 1 DIV in the presence of 1 μg/ml anisomycin. GCL, ganglion cell layer; NBL, neuroblastic layer.

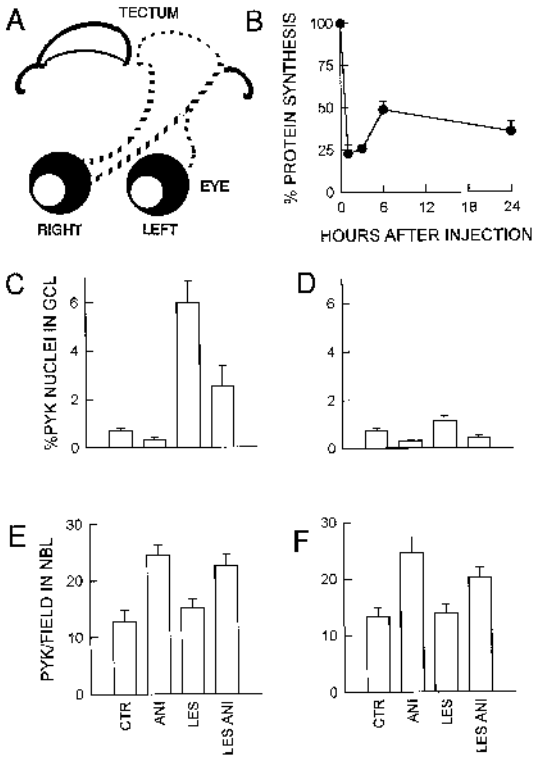

Fig. 6. Effects of inhibition of protein synthesis in vivo. (A) The diagram shows the design of the surgical procedure used to induce retrograde degeneration of the retinal ganglion cells. In neonatal rats, the massive contralateral retinotectal projection originates from 20 times as many cells as the ipsilateral projection. The latter cells are mostly located in a restricted part of the retina, in the ventrolateral quadrant. Axon damage following unilateral ablation of the left superior colliculus is therefore expected to produce massive retrograde degeneration in the right (contralateral) retina and minor localized effects in the left (ipsilateral) eye, when compared with the ongoing rate of naturally occurring ganglion cell death. (B) Blockade of protein synthesis with anisomycin following the two-injection schedule reached about 60% and persisted for 24 hours. (C,D) Frequency of pyknotic nuclei in the ganglion cell layer in the right retina (C) and in the left retina (D). Notice that the tectal lesion produced a massive increase in the rate of cell death in the contralateral retina and that both the naturally occurring cell death in unoperated rats and the induced retrograde degeneration were blocked by anisomycin. (E,F) Frequency of pyknotic nuclei in the neuroblastic layer in the right retina (E) and in the left retina (F). Notice that anisomycin induced increased degeneration in the NBL irrespective of the rate of cell death in the ganglion cell layer. ANI, anisomycin; CTR, control; LES, lesion.

by half the loss of axotomized ganglion cells and increases cell death in the neuroblastic layer to a similar extent as in the in vivo experiment shown here (Rehen and Linden, 1994). Within the neuroblastic layer, neither the rate of naturally occurring cell death nor the increased rate of cell death caused by anisomycin were affected by the enhanced degeneration of the axotomized ganglion cells. Thus, the results of protein synthesis inhibition in vivo were similar to the effects of the antibiotics in vitro.

DISCUSSION

This investigation showed that inhibition of protein synthesis in the developing retina has contrasting effects on apoptosis of distinct retinal cell types. In the present report, apoptosis was identified by multiple criteria. Although the chromatin condensation and the in situ nick end labeling of DNA strand breaks per se do not reliably discriminate between the apoptotic and necrotic modes of degeneration in individual cells (Grasl-Kraupp et al., 1995), their finding in scattered cells amidst a majority of healthy neighbours, together with their coincidence with the internucleosomal cleavage of DNA, argue strongly that classical apoptosis (Kerr et al., 1972; Wyllie 1980), and not necrosis, is indeed the mode of cell death in the retinal explants. The degeneration of the long-axon ganglion cells was prevented by inhibition of protein synthesis, either in normal rats or following direct damage to the cells' axons. In turn, apoptosis in the neuroblastic layer was induced by inhibition of protein synthesis both in vitro and in vivo.

An increase in the rate of retinal ganglion cell death was recently described after intraocular injections of cycloheximide in neonatal rats (Cui and Harvey, 1994). The effect, however, was detected only at 6-7 hours and not at 14 hours after the injections, and was not significantly different from intraocular injections of saline. It is therefore likely that those results are strongly affected by ocular injury (e.g. Mansour-Robaey et al., 1994) and they do not challenge the decrease in the rate of ganglion cell death described here, after 24 hours of inhibition of protein synthesis produced by systemic injections of anisomycin.

It is unlikely that the induction of apoptosis is due to a non-specific toxic effect of the antibiotics. Both the rescue of the axotomized ganglion cells and the degeneration in the neuroblastic layer of the explants were similarly dose-dependent and accompanied the degree of inhibition of protein synthesis (Rehen and Linden, 1994 and Tables 1, 2). Identical results were seen with two distinct antibiotics that block translation by different mechanisms and an inactive analogue had no effect. In explants from older retinae, the induced degeneration was restricted to specific sublayers, with little or no effect upon other portions of the retina. Finally, the degeneration induced by the antibiotics could be prevented by raising the intracellular concentration of cAMP. The data therefore indicate that the induction of apoptosis is directly related to the inhibition of protein synthesis and does not represent a catastrophic interference with the cells' metabolism.

Role of protein synthesis in apoptosis

Various studies have shown that apoptosis of both non-neuronal and neuronal cells can be blocked by inhibition of protein synthesis (Wyllie et al., 1984; Ferrer et al., 1993; Martin et al., 1988, 1992; Edwards et al., 1991; Deckwerth and Johnson, 1993; Comella et al., 1994; Harvey et al., 1994; Garciavalenzuela et al., 1994; Scott and Davies, 1990). In contrast, protein synthesis may not be necessary for the occurrence of apoptosis (Vaux and Weissman, 1993) and apoptosis may be triggered by protein synthesis blockade in other cells or in other contexts (Goldman et al., 1983; Griffiths et al., 1987; Keenan et al., 1986; Martin, 1993; Martin et al., 1990; Gong et al., 1993; Caronleslie and Cidlowski, 1994; Polunovsky et al., 1994).

The reasons for these discrepancies are not entirely clear. It is, however, possible that conflicting results obtained in apparently simple preparations such as primary cultures of thymo-

cytes (reviewed in Chow et al., 1995) may be due to the heterogeneity of cell content (Finkel et al., 1991; von Boehmer, 1992), with either distinct types or cells in various stages of maturation responding differentially to the drug treatment. In this respect, the analysis of apoptosis in organ cultures may offer an advantage over the monolayer cultures of dissociated cells.

Either corollary or collateral effects of inhibitors of macromolecular synthesis may be involved in the experimental modulation of apoptosis (Cano et al., 1994; Chow et al., 1995). For example, a recent report suggests that the protection conferred by inhibitors of macromolecular synthesis upon apoptosis may be due to increased antioxidant activity provided by shunting cysteine from global protein synthesis into the formation of glutathione (Ratan et al., 1994). In other circumstances, however, the protective effect of protein synthesis blockade was not reverted by inhibition of glutathione synthesis (Koh et al., 1995).

The most frequent interpretation of data showing that blockade of protein synthesis inhibits cell death is, nonetheless, that the signals that trigger apoptosis induce the expression of specific killer genes (Martin et al., 1988; Oppenheim et al., 1990; Scott and Davies, 1990). Genes related to the induction of apoptosis may, however, be continuously expressed and balanced by counteracting genes that block the action of the former by either heterodimerization of the respective protein products or other biochemical interactions (Hoffman and Liebermann, 1994; Oltvai and Korsmeyer, 1994; Reed, 1994).

Current data indicate that the sensitivity to death signals in each cell is controlled by a balance between positive and negative modulators of apoptosis. Viewed in this perspective, the effects of protein synthesis blockade suggest that distinct cells may be set either towards apoptosis by the dominance of positive modulators or away from cell death by the dominance of negative modulators. The present results suggest that, in the developing retina, ganglion cells are prone to apoptosis by dominance of positive modulators, while a latent mechanism of apoptosis in cells in the neuroblastic layer is continuously prevented by negative modulators. This hypothesis may be approached by mapping the expression of the known positive and negative modulators in various cell types within the organized retinal tissue and studying both their turnover rates and their responses to various signals that either induce or prevent apoptosis.

Differential responses of retinal cells to inhibition of protein synthesis

Various features distinguish the ganglion cells from the cells in the neuroblastic layer that respond differentially to the inhibition of protein synthesis. First, the ganglion cells are subject to direct damage to their axons when preparing the explants and apoptosis dependent on protein synthesis might conceivably be restricted to degeneration induced by damage. This is unlikely because blockade of protein synthesis in undamaged retinae in vivo produced the same effects as those found in the explants. Second, the ganglion cells are severely depleted of target-derived neurotrophic support following axotomy, while cells in the neuroblastic layer make only local connections and may therefore be at equilibrium with respect to their neurotrophic requirements. The differential effects of the antibiotics may thus be related to the state of neurotrophic support, similar to the contrasting effects of protein synthesis blockade demonstrated in a GM-CSF-dependent myeloid leukaemic cell line either in the presence or in the absence of the growth factor (Han et al., 1995). This hypothesis may apply in vivo as well, because retinal ganglion cells in newborn rats are critically dependent on support from their central targets and are already passing through the period of naturally occurring cell death (Perry et al., 1983), while the period of natural neuron death in the outer layers starts later (Beazley et al., 1987).

A third possibility is that, in newborn rats, the ganglion cells are in a more advanced developmental stage than most cells in the neuroblastic layer and may have modified their dependence on key proteins, similar to the switch in the dependency of trophic factors observed during development of certain neurons (Davies, 1994; Buchman and Davies, 1993; Buj-Bello et al., 1994; Larmet et al., 1992). In this regard, a recent study of neuron-specific enolase activity in the developing rat retina revealed delayed maturation of enzyme activity in cells bordering the emerging outer plexiform layer at about 1 week after birth (Fujieda et al., 1994). This region contains most of the cells that degenerate following treatment with the antibiotics at P6, consistent with a selective sensitivity of immature cells to apoptosis triggered by protein synthesis blockade. Fourth, cell proliferation still occurs in the neuroblastic layer postnatally, and a link between the mechanisms of apoptosis and the cell cycle (Amati et al., 1993; Shi et al., 1994; Neiman et al., 1994; Demarcq et al., 1994; Shan and Lee, 1994; Zhu and Anasetti, 1995) may render the proliferating neuroblasts prone to apoptosis. Again, the region bordering the emerging outer plexiform layer at P6 contains proliferating cells, at a time when cell division has essentially ceased throughout the other retinal layers (S. K. Rehen, C. B. L. Campos and R. Linden, unpublished results). Finally, the mechanisms of apoptosis may depend on cell-type-specific events that are differentially regulated in distinct retinal cells. Experiments designed to distinguish among these alternatives are currently underway in our laboratory.

Cyclic AMP and apoptosis

Effects of cAMP on apoptosis have been tested in various cell types and the results are controversial. In many cells, increasing the intracellular concentration of cAMP prevents apoptosis (Rydel and Greene, 1988; Edwards et al., 1991; Lee et al., 1993; Dmello et al., 1993; Berridge et al., 1993; Hoshi et al., 1994; De et al., 1994) while, in other circumstances, the increase in intracellular cAMP may induce cell death (Azuma et al., 1993; Szondy, 1994; Lomo et al., 1995). The present results show that increasing intracellular cAMP overrides the induction of apoptosis by inhibition of protein synthesis. It is thus likely that phosphorylation of key apoptosis modulators by a cAMP-dependent protein kinase may be involved in the control of programmed cell death in the retina. It has, in fact, recently been suggested that the phosphorylation of Bcl-2, a major negative modulator of apoptosis, by protein kinase C may be involved in the transduction of the growth factor signals that prevent apoptosis in interleukin-3-dependent cell lines (May et al., 1994). On the other hand, the retinal neurotransmitters dopamine, adenosine and, to a lesser extent, glutamate have been shown to induce accumulation of cAMP in the embryonic retina (De Mello, 1978; Paes de Carvalho and

De Mello, 1982; Ventura and De Mello, 1990). These results raise the hypothesis that neurotransmitters may help control programmed cell death in the neuroblastic layer of the immature retina via cAMP-dependent mechanisms.

The current results also differ in an important way from previous reports on the effects of cAMP on retinal cell death. Raising the concentration of cAMP prevented the degeneration of ganglion cells produced by blockade of electrical activity in dissociated cell cultures (Lipton, 1986; Kaiser and Lipton, 1990) while, in the retinal explants, cAMP failed to block apoptosis in the ganglion cell layer. The discrepancy of the results may be due to distinct structural relationships of the ganglion cells in the explants when compared with the monolayers. Cyclic AMP has been shown to mediate the action of the vasoactive intestinal peptide in the retinal monolayers (Kaiser and Lipton, 1990), an effect that depends on glial cells in similar cultures from the spinal cord (Brenneman et al., 1985; Brenneman and Eiden, 1986; Brenneman et al., 1987). Since glial cells tend to proliferate abundantly in culture, the presence of altered ratios of glial to ganglion cells may lead to distinct effects in the two preparations. These data further emphasize the need to test for cellular mechanisms active in unstructured monolayer cultures in histotypic cultures more akin to the living tissue in situ, such as the currently described free-floating retinal explants.

Conclusion

The contrasting effects of protein synthesis blockade on distinct cell types within the organized retinal tissue are consistent with the simultaneous operation of two mechanisms of apoptosis. One mechanism depends on de novo protein synthesis and can be triggered by axon damage and/or withdrawal of trophic support in retinal ganglion cells. A complementary mechanism of apoptosis is latent in cells of the underlying neuroblastic layer and is continually repressed by negative modulators that include proteins.

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