Brazilian Journal of Medical and Biological Research (1999) 32: 813-820
ISSN 0100-879X

Nuclear exclusion of transcription factors associated with apoptosis in developing nervous tissue

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Abstract

Programmed cell death in the form of apoptosis involves a network of metabolic events and may be triggered by a variety of stimuli in distinct cells. The nervous system contains several neuron and glial cell types, and developmental events are strongly dependent on selective cell interactions. Retinal explants have been used as a model to investigate apoptosis in nervous tissue. This preparation maintains the structural complexity and cell interactions similar to the retina *in situ*, and contains cells in all stages of development. We review the finding of nuclear exclusion of several transcription factors during apoptosis in retinal cells. The data reviewed in this paper suggest a link between apoptosis and a failure in the nucleo-cytoplasmic partition of transcription factors. It is argued that the nuclear exclusion of transcription factors may be an integral component of apoptosis both in the nervous system and in other types of cells and tissues.

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Presented at the I International Symposium on "Signal Transduction and Gene Expression in Cell Proliferation and Differentiation", São Paulo, SP, Brasil, August 31-September 2, 1998.

Received November 26, 1998
Accepted January 27, 1999

Key words
- Programmed cell death
- Retina
- Neurogenesis
- Development
- Nuclear membrane

Introduction: transcription factors and apoptosis

Mechanisms of programmed cell death by apoptosis (1) involve a network of metabolic events and may be triggered by a variety of stimuli leading to the activation of caspases and the proteolysis of specific substrates (2). While in several instances the course of programmed cell death has been clearly shown to depend on *de novo* protein synthesis (3), in other cases the apoptotic machinery is available to use without the transcription or the translation of otherwise silent genes (4).

Nevertheless, a number of studies have suggested that the expression of specific transcription factors may be either associated with or necessary for the induction of programmed cell death. The simplest explanation would be that these transcription factors control the expression of genes associated with the commitment of a cell to the death pathway and the execution of the cell death program. For example, the enhanced expression of Fos and Jun family genes might affect the expression of either pro-apoptotic or anti-apoptotic genes driven by AP1-responsive promoters.

Indeed, early studies have shown that the expression of a LacZ construct under the c-Fos promoter is associated with programmed cell death in many organs and tissues (5). This work has suggested that the continuous

expression of c-Fos precedes programmed cell death, and may be an important step in cell demise. Later studies showed that the overexpression of c-Myc leads to apoptosis in fibroblasts (6). Both c-Myc and c-Fos are associated with the control of the cell cycle, a feature that reinforces the idea that both cell proliferation and cell death share common elements within their pathways (7).

The c-Jun protooncogene has often been associated with apoptosis in the nervous system. Thus, expression of c-Jun was detected in neurons undergoing apoptosis following either deprivation of neurotrophic factors (8) or exposure to the neurotoxic ß-amyloid peptide (9). The need for c-Jun in neuronal apoptosis was supported by the finding that the expression of a c-Jun dominant-negative mutant protein protected neurons from the withdrawal of their specific neurotrophic factor (10). More recently, it was found that phosphorylation of c-Jun in its transactivation domain is strongly associated with neuronal apoptosis induced by withdrawal of neurotrophic support, although the upstream pathways leading to phosphorylation of c-Jun remain somewhat elusive (11,12). In contrast with those findings, studies of the timing of both the activation of JNK and of c-Jun phosphorylation suggested that neither is rate-limiting to apoptosis following withdrawal of neurotrophic support (13).

While the expression of c-Jun has been associated with apoptosis *in vivo*, its subcellular localization is not entirely consistent with a canonical role as a transcription factor. For example, Jun immunoreactivity found in apoptotic cells following irradiation of the brain accumulates not only in the nucleus but also in the cytoplasm of the degenerating cells (14). On the other hand, evidence has been reported of an association of c-Jun expression with regeneration of retinal axons (15), thus raising the possibility that the expression of c-Jun after a lethal insult may be actually linked to an abortive regenerative response of the cell, rather than leading to the expression of death genes.

Every area or nucleus within the nervous system is composed of several neuron and glial types, and developmental events are strongly dependent on selective cell interactions, which cultures of either dissociated cells or cell lines fail to reproduce. Structural complexity, as well as the changing requirements of developing neurons to survive during embryogenesis (16) warrant the investigation of cell death *in vitro* using histotypical preparations. We use retinal explants as a model to investigate the control of apoptosis in the developing central nervous system. This preparation maintains the structure and cell interactions of the retinal tissue similar to the retina *in situ*. In addition, the retina of newborn rats contains cells in all stages of development, from proliferating neuroblasts to relatively differentiated neurons presenting advanced phenotypic traits. The time course of neurogenesis is represented spatially by the segregation and arrangement of distinctive, albeit overlapping strata of cells in various stages of differentiation (17).

In this article, we review the use of the retinal explant model to investigate apoptosis induced by various agents, and the finding that several transcription factors are excluded from the nucleus of apoptotic cells within the retinal tissue. It will be argued that the nuclear exclusion of transcription factors may be a widespread event in neural apoptosis.

The retinal explant model: apoptosis in developing nervous tissue

A transverse section through the neural retina of a newborn rat is schematically represented in Figure 1A. Proliferating neuroblasts within the ventricular zone of the retinal tissue (here termed neuroblastic layer - NBL) undergo interkinetic nuclear migration, and therefore the nucleus of neuroblasts may be found at any level across the depth of the NBL. DNA synthesis is restricted to this layer's innermost stratum,

while mitosis occurs at the outermost margin of the NBL. In this outer tier a few photoreceptors, mostly the early developing cones, can also be identified with appropriate antibodies. Within the NBL, a row of precocious horizontal cells can be recognized by their relatively large size and immunoreactivity to calbindin. Towards the center of the eye, the NBL abuts a few rows of early developing amacrine cells that show immunoreactivity to calretinin and define the inner portion of the emerging inner nuclear layer (INLi). The NBL and the INLi together comprise the outer stratum of the retina in the newborn rat. The inner stratum is the ganglion cell layer (GCL), which in turn is separated from the outer stratum by the inner plexiform layer (IPL) containing dendrites of retinal ganglion cells and processes of the early developing amacrines.

This arrangement of the neural retina evolves due to progressive neuron migration and differentiation (Figure 1B). By the end of the first postnatal week the outer nuclear layer (ONL) of photoreceptor cell bodies is now separated from the remainder of the NBL by the outer plexiform layer (OPL). The OPL contains processes of both photoreceptors and horizontal cells and will eventually house the processes of the late developing bipolar cells. The INLi has grown to contain many more rows of amacrine cells adjacent to the NBL. Since most mitotic figures are still found at the outer rim of the retina, the actual NBL traverses the ONL, but the concentration of photoreceptor cell bodies leads to the appearance under light microscopy of a compact NBL only between the INLi and the OPL.

Thus, the major stages of neurogenesis in the central nervous system can be traced in single transverse sections of the rat retina within the first postnatal week. Proliferating neuroblasts, early undifferentiated neurons and several cell types at variable stages of phenotypic differentiation are found spatially segregated in a precise arrangement within the structure of the retinal tissue.

Explants of retinal tissue can be maintained *in vitro* for several days (18). At least for periods of up to one week, the explants maintain the histotypical organization and undergo progressive development with a time course similar to the retina *in situ*. The OPL appears at 4-5 days in culture, there is progressive development of amacrine cell rows in the INLi, and the death of the axotomized ganglion cells occurs within 2-3 days, similar to the fate of these cells following axotomy *in vivo* (17). This preparation serves therefore as a useful tool for the study *in vitro* of both differentiation and degeneration within the retinal tissue, and as a generalized model for developmental events, including apoptosis, within the immature nervous tissue.

Apoptosis of developing retinal cells

Using retinal explants, we have been able to selectively induce apoptosis in cell popu-

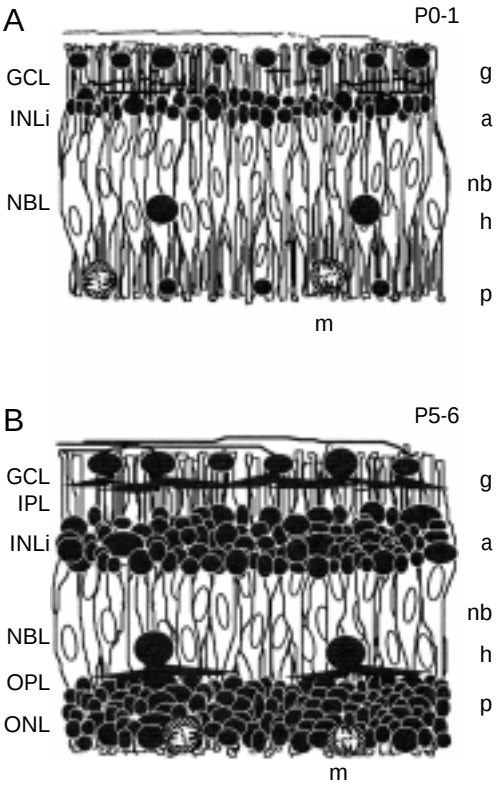

Figure 1 - Schematic representation of the developing retina of rats at birth (A, postnatal days 0-1 = P0-1) or towards the end of the first postnatal week (B, P5-6). Abbreviations on the left refer to the retinal layers, while characters on the right side and below each drawing refer to cell types: GCL = ganglion cell layer; IPL = inner plexiform layer; INLi = inner portion of the inner nuclear layer; NBL = neuroblastic layer; OPL = outer plexiform layer; ONL = outer nuclear layer; g = ganglion cells; a = amacrine cells; nb = proliferating neuroblasts; h = horizontal cells; p = photoreceptors; m = cell in mitosis.

lations at distinct stages of development within the retinal tissue (Figure 2). Retinal ganglion cells, the axons of which are damaged by the explantation procedure, die by apoptosis with a peak rate at about one day *in vitro* in explants from newborn rats and are rescued by inhibition of protein synthesis (19). In contrast, cells within the neuroblastic layer are killed by apoptosis following inhibition of protein synthesis (19), and the sensitive cells in this case have been identified essentially as postmitotic cells soon after withdrawal from the cell cycle (17).

Selective induction of apoptosis in either proliferating cells or differentiated photoreceptors can be achieved by incubation with various drugs in retinal tissue from newborn rats or towards the end of the first postnatal week (Figure 2). This selective induction of apoptosis allowed testing the association of transcription factors with apoptosis in various stages of retinal cell development.

Nuclear exclusion of c-Jun associated with apoptosis in retinal tissue

Immunoreactivity for c-Jun was found in apoptotic cells in all instances in retinal explants. Apoptotic bodies labeled for c-Jun are found in the ganglion cell layer following axotomy and the immunoreactivity is abolished together with the blockade of apoptosis produced by inhibition of protein synthesis. Cells killed within the neuroblastic layer under inhibition of protein synthesis are also immunoreactive for c-Jun. Proliferating cells killed by etoposide are labeled, as well as photoreceptors after treatment with either thapsigargin or okadaic acid (Chiarini LB, Freitas FG and Linden R, unpublished results).

The subcellular distribution of c-Jun, however, is not restricted to the nucleus of the apoptotic cells. In fact, only a few degenerating cells showed definite nuclear immunoreactivity to this protooncogene, and most of the protein is found in a perinuclear location. In several instances, particularly in the neuroblastic layer, unequivocal staining of the cytoplasm of degenerating cells was observed. In the ganglion cell layer, double labeling with both the antibody for c-Jun and nuclear markers showed that the previously found globular apoptotic bodies positive for c-Jun (20) are devoid of DNA, and therefore correspond to cytoplasmic fragments from the relatively large ganglion cells (Chiarini LB, Freitas FG and Linden R, unpublished results).

The data show that apoptosis of retinal cells at any stage of development is accompanied by heavy cytoplasmic concentration of c-Jun immunoreactivity, indicating that c-Jun is excluded from the nucleus of the

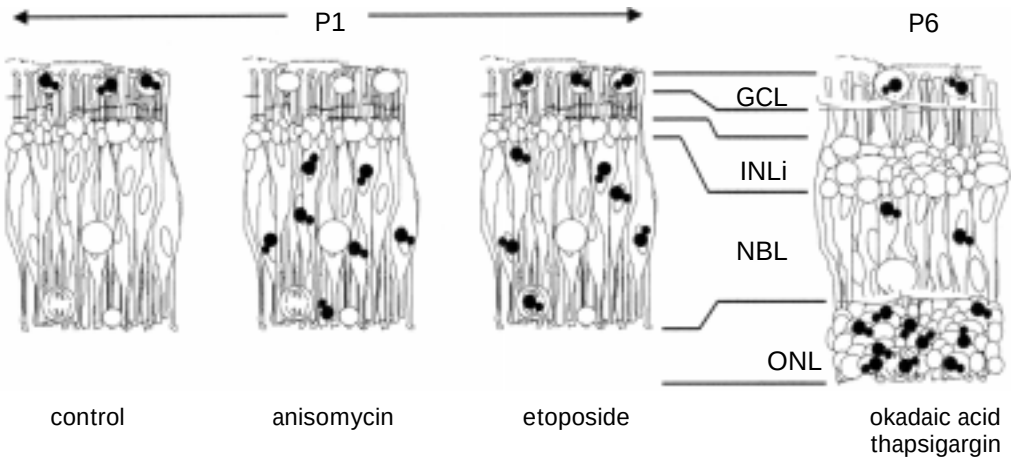

Figure 2 - Location of apoptotic bodies (filled twin circles) in retinal explants either at birth (P1) or at P6, following 24 h of treatment as indicated below each drawing. Apoptosis within the neuroblastic layer (NBL) following treatment with anisomycin occurs predominantly in recent postmitotic cells, while etoposide kills proliferating cells in the NBL of retinal explants from newborn rats. Abbreviations for retinal layers are as in Figure 1.

apoptotic cells. The accumulation of the c-Jun protein in the cytoplasm even under severe inhibition of protein synthesis suggests that a reduction in the rate of degradation of the c-Jun protein is also associated with apoptosis. An alternative explanation would, nonetheless, be that a residual rate of protein synthesis (19) is strongly biased towards this transcription factor.

Nuclear exclusion of other transcription factors associated with apoptosis in the retina

Expression of other transcription factors was also found associated with apoptosis in retinal cells. C-fos, c-Myc and JunB immunoreactivity were found in a fraction of axotomized ganglion, and at least c-Fos was also found in the neuroblastic layer following inhibition of protein synthesis. Again, the immunoreactivity for these proteins was found in an extranuclear location, and the pyknotic nuclei were devoid of labeling (Figure 3).

The transcription factor Max, the expression of which is necessary for the physiological functions of c-Myc in the cell cycle (21), is found within the nuclei of normal retinal ganglion cells. When explants from the retina of two-week-old rats are placed in culture, the ganglion cells degenerate with a slower time course than in explants from newborn rat retinae. This allows for better resolution of events occurring during the retrograde degeneration that follows axotomy, even though all ganglion cells eventually die in the cultured tissue. Immunoreactivity for Max gradually migrates towards the cytoplasm of the axotomized ganglion cells within the explants. At about 36 h after explantation, the dendrites of ganglion cells with apparently normal morphology were clearly delineated by cytoplasmic immunoreactivity for Max, while apoptotic bodies in advanced stages of degeneration were devoid of labeling for this protein (Chiarini LB, Petrs-Silva H and Linden R, unpublished results). This strongly indicates that Max is progressively excluded from the nuclei of the cells dying by apoptosis. We also detected a progressive loss of immunoreactivity for ATF2, which was also found excluded from the nucleus of a few degenerating cells in explants from newborn rat retinae.

Nuclear pore complex and apoptosis

It has been shown that apoptosis is consistently accompanied by early clustering of

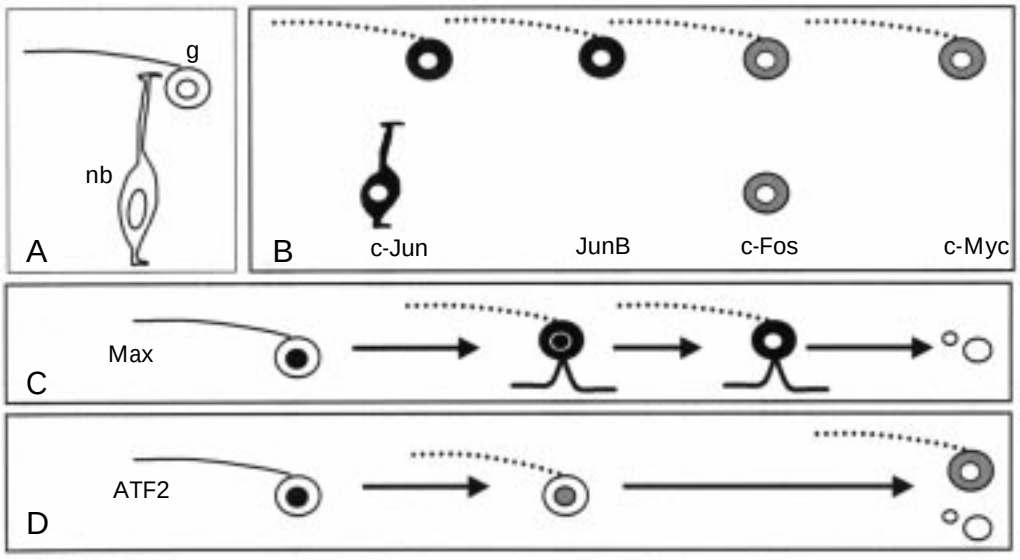

Figure 3 - Nuclear exclusion of transcription factors in retinal apoptosis. *A*, Symbolic depiction of retinal ganglion cells (g) and neuroblasts (nb). *B*, Representation of the location of immunoreactivity detected for 4 transcription factors in the cell types depicted in A. Immunoreactivity for c-Fos and c-Myc was less intense than obtained for c-Jun and JunB in our conditions. *C* and *D*, The course of nuclear exclusion of Max and ATF2, as detected by immunocytochemistry in retinal ganglion cells at progressively longer periods after axotomy due to explantation. Max was analyzed in explants from rats at P13, while all others were studied in explants from newborn rat retinae. The twin circles at the far right in C and D represent fragmented apoptotic bodies unlabeled for either transcription factor.

nuclear pores, detected by electron microscopy (22). The nuclear pore complex (NPC) is a major protein complex composed by several dozen distinct proteins known as nucleoporins, that mediate bidirectional nucleocytoplasmic transport (23). Transport is based on the binding of substrates such as transcription factors or RNA-protein complexes to shuttling proteins called importins (or karyopherins) and exportins (24-26), that bind to and carry the substrates through the NPCs in a translocation cycle driven by the activity of the GTPase Ran/TC4 (24).

The clustering of nuclear pores may either be a consequence of the disruption of the lamina network at late stages of apoptosis, or reflect early changes in nucleoporins (22). Indeed, it has been shown that nuclear pore clustering occurs in yeast mutants lacking the NUP133 nucleoporin (27). Recent reports have shown that the deletion of CAN/NUP214 nucleoporin in mice disturbs nucleocytoplasmic transport and leads to cell cycle arrest (28), and that overexpression of the same gene leads to apoptosis in addition to both effects above (29). Therefore, changes in nucleoporins may be associated both with impaired nucleocytoplasmic translocation and with apoptosis. It remains to be tested whether retinal apoptosis is accompanied by modifications in either the expression or the function of nucleoporins.

Nuclear import of transcription factors

Nuclear translocation of proteins synthetized in the cytosol depends on specific nuclear localization signals (NLS). The prototypical NLS was originally described for the SV40 T-antigen (T-ag), and consists of a stretch of basic amino acids (a.a.) separated by a 10 a.a. spacer from another cluster of a.a. located towards the N-terminal of the T-ag. The latter cluster contains 2 sites of phosphorylation for casein kinase II and cdc2, both of which have been shown to regulate the kinetics of nuclear import (30). The structure of the T-ag NLS highlights two general properties of nuclear localization sequences, namely the need for recognition by importins of a specific domain in the subject protein, and the regulation of nuclear translocation by post-translational modifications of the subject proteins. Other sequences and arrangements of multiple relevant a.a. have been identified as the NLS of various proteins (30).

An additional and distinct mechanism of control of nuclear translocation is typified by the transcription factor NF-κB, which is held within the cytosol by combination with a retention factor I-κB. Certain stimuli lead to phosphorylation of I-κB and release of NF-κB with an unmasked NLS, thus allowing translocation of the latter to the nucleus (30). Evidence for analogous retention factors has been postulated for both c-Fos and c-Jun (31), and an AP1-inhibitory protein named IP1 and detected both in the nucleus and in the cytoplasm of several cell types may also be related to retention of both Fos and Jun in the cytoplasm (32).

It has also been reported that heat shock protein 70 (hsp 70) may have a role in the control of nucleo-cytoplasmic translocation (33,34). The mechanisms are still unclear, but hsp 70 may facilitate the interaction of the NLS with its receptor importin, modulate protein traffic by assisting the folding of the relevant polypeptides, or even function as an ancillary importin itself (33).

Recent data from our laboratory are likely to be relevant to the mechanisms of nuclear translocation affected in the course of apoptosis in developing retinal cells. We have shown that the bifunctional AP endonuclease/redox factor Ref-1 (also known as APE, APEX or HAP1) is lost in apoptotic cells following a variety of insults (Chiarini LB, Freitas FG and Linden R, unpublished results). Ref-1 was suggested as an anti-apoptotic protein with an increasing protective effect associated with its accumulation

during retinal differentiation, in line with data indicating its protective role in the context of DNA damage (35,36). Besides its role in DNA repair that depends on the C-terminal of the protein, the N-terminal of Ref-1 controls the activation of various transcription factors, such as c-Jun, c-Fos, NF-κB and p53 (37-40). A link between Ref-1 and translocation of c-Jun is predictable because Ref-1 reduces a cysteine residue in the DNA-binding site immediately adjacent to the NLS of c-Jun (41). This cysteine was not directly shown to control translocation. However, in v-Jun this residue is replaced by serine, the phosphorylation of which controls the translocation of this viral oncogene (42). If indeed reduction of the cysteine adjacent to the NLS should control the translocation of c-Jun, then the accumulation of c-Jun in the cytoplasm of apoptotic cells may be a consequence of the loss of Ref-1.

In addition, work in progress shows that hsp 70 family proteins, the expression of which also depends on retinal differentiation, are absent from apoptotic cells at the same time as they are induced in cells resistant to heat shock (Freitas FG, Chiarini LB and Linden R, unpublished results). Thus, a putative role of hsp 70 as a nuclear import factor (33) may also be related to the cytoplasmic retention of transcription factors observed in retinal apoptosis.

Finally, although no direct evidence has been gathered for inhibitory activities in the cytoplasm of developing retinal cells, it is perhaps significant that cyclic AMP, which was shown to relieve retention of c-Fos in the cytoplasm of fibroblasts (31), effectively counteracts the induction of retinal apoptosis by inhibition of protein synthesis (19,43). The apoptosis-related targets of cAMP-dependent protein kinase A are still unknown, but they may possibly include either inhibitory proteins or retention factors.

Conclusion

The data reviewed in this paper suggest an association of apoptosis with a failure in the nucleo-cytoplasmic partition of transcription factors. In addition to the examples cited above, closer examination of other circumstances of cell death should help establish whether the nuclear exclusion of transcription factors is indeed an integral component of apoptosis both in the nervous system and in other types of cells and tissues, and whether it occurs at the induction, modulation or execution phases in the program of cell demise.

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