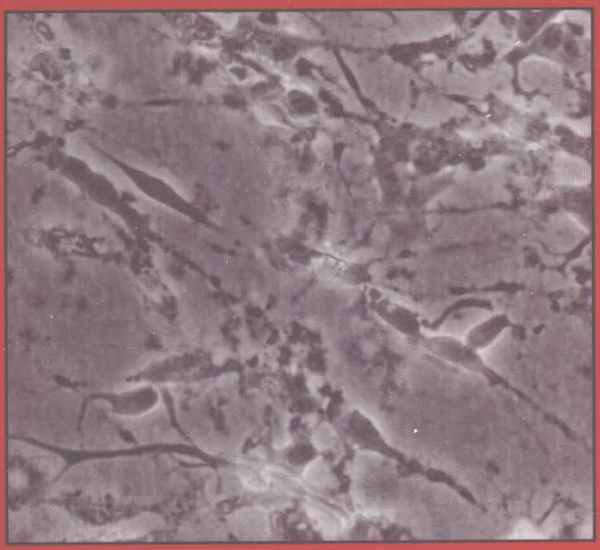
Apoptosis Techniques and Protocols

Edited by

Judes Poirier



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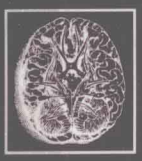

NEUROMETHODS ▪ 29

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Judes Poirier

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Preface

After many years of neglect, the study of normal cell death and the so-called programmed cell death has finally gained some needed momentum. Evidence obtained over the last five years indicates that programmed cell death (also referred to as apoptosis) occurs normally in most animal tissues at some well-defined stages of their development and/or maturation. The molecular mechanisms by which it is executed remains unknown. However, key regulatory and metabolic steps have been identified in the apoptotic cascade and a central concept has progressively emerged: Apoptosis appears to occur by default in many different cell types unless suppressed by signals originating from neighboring cells. As Martin Raff noted a few years ago, this molecular cascade probably imposes a form of social control on cell survival and cell death.

Since our understanding of the apoptosis phenomena has grown significantly over the last few years, we have been empowered by recent technological advances to biochemically dissect the key metabolic components of apoptotic and necrotic cascades in normal and injured brains. Moreover, the development of improved histochemical tools has allowed us to better define the fundamental criteria of cell death, and transgenic technology has allowed scientists to test working hypotheses pertaining to the mechanism of action of the so-called "cell death gene." Sophisticated molecular biological tools have also enabled us to determine the key elements of normal and abnormal cell cycles, indicating novel functions for the so-called cell-cycle regulated markers that appear to do much more than just regulate cell division.

The present volume of *Apoptosis Techniques and Protocols* now offers neuroscientists at all levels of experience a combination of theoretical and practical information for understanding and studying apoptosis. Topics include the practical

analysis of the concepts of necrosis and apoptosis in the CNS as well as histological, biological, and molecular criteria for investigating apoptosis and programmed cell death. Topics range from cellular and invertebrate to animal and human models of apoptosis in various pathological situations in the brain, including Alzheimer's disease, AIDS, and stroke. Numerous techniques are described for examining the critical steps involved in the apoptotic process. These methods range from PCR analysis of cell-cycle regulated proteins, histochemical analysis of DNA degradation, and DNA laddering analysis, to cytochemical alterations of living cells.

It is our hope that *Apoptosis Techniques and Protocols* will prove useful not only as a technical reference, but also as an introduction to the key notions associated with the phenomena of apoptosis and necrosis.

Judes Poirier

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Contents

Gene Tools to Study Neuronal Apoptosis

Isabelle Martinou, Harald Frankowski, Marc Missotten, Remy Sadoul, and Jean-Claude Martinou

1. Introduction

Naturally occurring cell death (NOCD) has been recognized for many years as a critical phase in the development of the nervous system (Hamburger and Levi-Montalcini, 1949; Oppenheim, 1991). During this process, immature neurons undergo an active process of cell death, probably because they receive insufficient quantities of trophic factors from their target cells. Active cell death implies a molecular program of cellular-self-destruction and is therefore often called programmed cell death (PCD) (Schwartz and Osborne, 1993). Diverse morphological types of PCD have been described, with apoptosis being the most commonly encountered (Kerr et al., 1972; Clarke, 1990). After the NOCD period, neuronal apoptosis is considered to be pathological and is found to be implicated in clinical outcomes, such as Alzheimer's disease, Parkinson's disease, or Huntington's disease. It is also observed in acute stresses, such as ischemia. The goal of our laboratory is to identify the molecular mechanisms that underlie PCD in neurons. In this article, we describe the technical approaches undertaken by our laboratory over the last few years to reach this goal.

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2. Culture of Sympathetic Neurons as an In Vitro Model System of Neuronal Apoptosis

One of the best documented examples of developmental neuronal death in vertebrates is the demise of 30–50% of the sensory and sympathetic neurons, that depend on nerve growth factor (NGF) for survival (Levi-Montalcini and Brooker, 1960; Thoenen et al., 1987). The type of death triggered by NGF deprivation in vivo can be reproduced in culture. We use sympathetic neurons from rat cervical ganglia that we can maintain in culture for several weeks in the presence of NGF. If NGF is withdrawn from the culture medium, neurons undergo an active death that requires RNA and protein synthesis (Martin et al., 1988; Deckwerth and Johnson, 1993). During PCD, first the cell bodies shrink, then the neurites disintegrate. One of the first morphological signs that can be observed after Hoechst staining is the condensation of the nucleus.

2.1. Microinjection of cDNAs Encoding Diverse Proteins in Sympathetic Neurons

To overexpress different proteins in sympathetic neurons, we microinject DNAs encoding proteins of interest directly into the nucleus. We have found that cytoplasmic injections lead to unefficient transfection. The cDNAs are inserted in an expression vector under the control of a viral promoter. We noticed that cytomegalovirus (CMV) and respiratory syncyalvirus (RSV) promoters are the most potent whereas SV40 promoters give lower expression. For reasons that are unclear, identical promoters confer different levels of expression according to the plasmid vector in which they have been inserted. Microinjection presents several advantages compared to DNA transfer by viruses, the other alternative to transfect efficiently neurons: DNA constructs for microinjection experiments are easy to prepare; and efficacy of transfection can be 100% as all injections can be successful if monitored under fluorescent microscope when a fluores-

cent dye (Dextran-FITC, for example) has been added to the DNA solution. The disadvantage of this technique is the limited number of neurons that can be microinjected, which impairs biochemical studies of microinjected neurons. Another problem encountered with this technique, as well as with the use of viruses, is associated to the cell system itself. As already mentioned, sympathetic neurons can be protected from NGF deprivation by protein synthesis inhibitors. Therefore, anything that causes a decrease in the overall protein synthesis will protect cells. This may explain why injection of high quantities of DNA that lead to very robust expression of any protein (β-galactosidase, for example) has a rescuing effect. To avoid this problem, we microinject solution at low DNA concentration (0.1–0.05 μg/μL). Moreover, in all experiments, a neutral protein (β-galactosidase) is used as a control. Injection *per se* has no significant effect on neuronal survival.

2.1.1. Proteins that Rescue Sympathetic Neurons from NGF Deprivation

The first protein that was identified as an antidote to neuronal death following NGF deprivation is the bcl-2 protooncogene. Bcl-2 was identified at the chromosomal breakpoint of t(14;18), which constitutes the most common translocation in human lymphoid malignancy (Tsujimoto, 1984). Bcl-2 favors malignancy not by acting on proliferation *per se,* but by blocking programmed cell death. Indeed bcl-2 was first shown to block death of hematopoietic cells induced by withdrawal of IL-3 and IL-4 (Hockenberry et al., 1990). We have microinjected sympathetic neurons with an expression vector containing the cDNA encoding bcl-2. Neurons overexpressing bcl-2 were able to survive for up to 7 d of culture in the absence of NGF, whereas all of the mock-injected neurons died whithin 2–3 d (Garcia et al., 1992). Since then Allsopp et al. (1993) and Mah et al. (1993) have also shown that other neurons that depend on brain-derived neurotrophic factor or neurotrophin 3 for survival can also be protected by bcl-2. Surprisingly, bcl-2 had no effect on cili-

ary neurons that depend on ciliary neurotrophic factor (CNTF) for survival (Allsopp et al., 1993). Together, these observations suggest that bcl-2-sensitive and bcl-2-insensitive cell death pathways coexist during development of the nervous system. The apparent selectivity of bcl-2 action has its parallel in the hematopoietic system where, in contrast to observations with IL-3- or IL-4-dependent cells, bcl-2 is ineffective in preventing T-cell negative selection against super-antigen or apoptosis in hematopoietic cell lines following IL-6 or IL-2 deprivation (Nunez et al., 1991). However, these observations do not exclude the possibility that the CNTF removal in ciliary neurons or IL-6 and IL-2 deprivation in T-cells could initiate the death program downstream of bcl-2.

Bcl-2 is the prototype of a family whose members can be divided in two subgroups: antiapoptotic proteins bcl-2, bcl-x being the most important) and proapoptotic proteins (Bax, Bak, and Bad).

The bcl-x gene encodes two proteins, bcl-xl and bcl-xs, which are the products of alternative splicing. The longest protein, bclxl is similar in size and predicted structure to bcl-2. Like bcl-2, bcl-xl blocks cell death induced by IL-3 deprivation in lymphocytes (Boise et al., 1993). We found that Bcl-xl is expressed in the nervous system throughout development, whereas Bcl-2 is mainly expressed during early phases of development. Bcl-xl is as efficient as Bcl-2 in blocking PCD in sympathetic neurons (Frankowski et al., 1995; Gonzalez-Garcia et al., 1995).

Bcl-xs, which lacks domains BH1 and BH2 of bcl-2, was shown to counteract the death blocking effect of both bcl-2 and bcl-xl (Boise et al., 1993; Martinou et al., 1995). Bcl-xs was not detected in the rat nervous system (Frankowski et al., 1995).

Two viral proteins, E1B19kD from adenovirus and p35 from baculovirus, were also found to promote survival of sympathetic neurons in the absence of neurotrophic factors (Martinou et al., 1995). E1B19kD shows limited structural homology with bcl-2. p35 was recently found to be an inhibitor of proteases of the interleukin-1β converting enzyme (ICE) family.

Table 1
List of Proteins Overexpressed in Sympathetic Neurons Cultured in the Presence or Absence of NGF

	Prevents death	Triggers death
bcl-2	+	–
bcl-xL	+	–
bcl-xs	–	–
E1B19K	+	–
p 35	+	–
CrmA	+	–
Bak	–	+
Bax	–	+
Nedd-2	–	+
ICE	–	+/–
CPP 32	–	+/–
Cyclin D1	–	–
WAF1/p 21	–	–
cdc 2	–	–
p 53	–	–
E1A	–	–
E1B55K	–	–
Retinoblastoma gene	–	–
TIA 40	–	–
Ced 4	–	–

We have also tested other proteins whose functions suggested that they could be implicated in cell death. These proteins are listed in Table 1.

2.1.2. Proteins That Trigger Cell Death in Sympathetic Neurons Cultured in the Presence of NGF

We have shown that both Bax and Bak can not only accelerate the kinetics of death of sympathetic neurons after NGF deprivation, but also trigger cell death in neurons cultured in the presence of NGF, that is in the absence of any apparent cell death stimulus (Sadoul et al., submitted).

Studies with the nematode *Caenorhabditis elegans* have identified genes involved in programmed cell death. Two