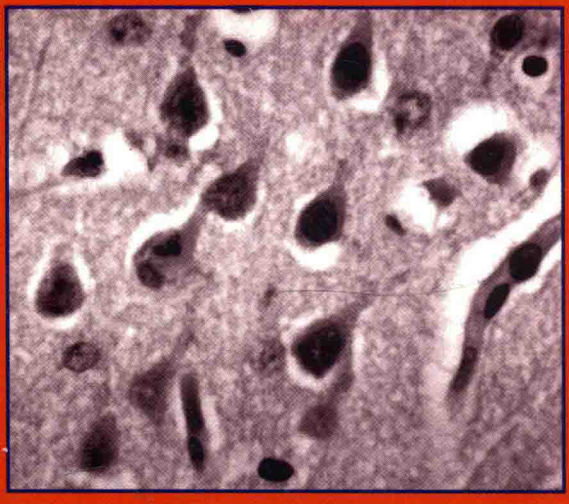
Apoptosis Techniques and Protocols

SECOND EDITION

Edited by

Andréa C. LeBlanc



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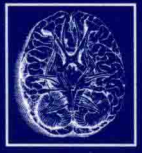

NEUROMETHODS ▪ 37

Series Editors: Alan A. Boulton and Glen B. Baker

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Cover artwork: Photomicrograph is courtesy of Dr. Steffen Albrecht, Department of Pathology, McGill University and Sir Mortimer B. Davis Jewish General Hospital. The cover illustrates a section of the hippocampus of a newborn human brain that suffered from hypoxia during birth. One apoptotic neuron with fragmented nucleus is surrounded by intact neurons. H&E, original magnification ×400.

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Preface to the Series

When the President of Humana Press first suggested that a series on methods in the neurosciences might be useful, one of us (AAB) was quite skeptical; only after discussions with GBB and some searching both of memory and library shelves did it seem that perhaps the publisher was right. Although some excellent methods books had recently appeared, notably in neuroanatomy, it was a fact that there was a dearth in this particular field, a fact attested to by the alacrity and enthusiasm with which most of the contributors to this series accepted our invitations and suggested additional topics and areas. After a somewhat hesitant start, essentially in the neurochemistry section, the series has grown and will encompass neurochemistry, neuropsychiatry, neurology, neuropathology, neurogenetics, neuroethology, molecular neurobiology, animal models of nervous disease, and no doubt many more "neuros." Although we have tried to include adequate methodological detail and in many cases detailed protocols, we have also tried to include wherever possible a short introductory review of the methods and/or related substances, comparisons with other methods, and the relationship of the substances being analyzed to neurological and psychiatric disorders. Recognizing our own limitations, we have invited a guest editor to join with us on most volumes in order to ensure complete coverage of the field. These editors will add their specialized knowledge and competencies. We anticipate that this series will fill a gap; we can only hope that it will be filled appropriately and with the right amount of expertise with respect to each method, substance or group of substances, and area treated.

Alan A. Boulton
Glen B. Baker

Preface

Neuronal loss that occurs in the central nervous system as a result of injury or neurodegenerative diseases is a devastating problem because of the lack of regenerative ability of the human body to functionally replace these neurons. Although there is promise of eventual replacement of these lost neurons through stem cell technology, inhibition of neuronal cell death in many instances would also provide a chance to reestablish their function. In many neurodegenerative diseases, however, there is no clear understanding of the cell death mechanism. Considerable debate focuses on whether these neurons undergo apoptosis, necrosis, or another yet undefined mode of cell death. The enigma is complicated by cell-type and by insult- and species-specific mechanisms of neuronal cell death. It is obvious that the identification and characterization of the mechanism of neuronal cell death can be resolved only through extensive research of the several types of neurons. Only when a fundamental understanding of the mechanisms of neuronal demise is achieved will we be in a position to identify the key pathways involved in human neurodegenerative diseases.

Since publication of the first edition of the *Apoptosis Techniques and Protocols* in 1997, the study of apoptotic mechanisms has boomed, and a number of key proteins involved in neuronal apoptosis have been identified. It has become clear that Bax and the family of caspases that is regulated through mitochondrial cytochrome-*c* release or through an extrinsic receptor-mediated pathway are key pro-apoptotic regulators of neuronal cell death. The first three chapters of this book present comprehensive technical approaches to study Bax (Hsu and Smaili), cytochrome-*c* (Ethell and Green), and caspases (Bounhar, Tounekti, and LeBlanc) in neurons. The following two chapters describe two methods, viral infections (Maguire-Zeiss, Bowers, and Federoff) and microinjections (Zhang and LeBlanc), to assess the importance of apoptotic proteins in cultures of primary neurons and in brain. Though key regulators of apoptosis have been uncovered, undoubtedly there are additional factors involved in neuronal apoptosis. Therefore, we must continue to search for proteins that may be responsible for neuronal loss in neurodegenerative

diseases. DNA microarray assay is one of the current techniques used to identify differentially expressed genes in human disease (Eastman and Loring) and in transgenic mice models of neurodegeneration (Tucker and Estus) . These chapters provide helpful insight into the design of an appropriate experimental protocol and in the interpretation of data from these microarrays. It is evident that as pro-apoptotic proteins are discovered, inhibitors of their functions in neuronal apoptosis must be sought. Berry and Ashe describe the role of differentially expressed G3PDH as an early marker of apoptosis and how one can isolate drugs against such pro-apoptotic molecules.

Though extensive research on apoptosis is performed and key mechanisms provided for many cell types, these do not necessarily apply to neurons. Indeed, we must consider the very specialized architecture of the neuron. Neurons are compartmentalized and polarized cell types. It is possible that specific pathways of apoptosis may be restricted to a specialized compartment of these cells and that activation of certain apoptotic mechanisms could result in cellular dysfunction prior to complete cell death. It has recently been proposed that the apoptotic process may occur in synapses. The technical and theoretical aspects of neuronal compartmentalization and the study of synaptosis are explained in the next two chapters, by Campenot and colleagues and by Cole and Gylys.

As we define the mechanisms of apoptosis that regulate cell death of neurons in cultures, it is essential to extend the studies to the brain tissue of individuals who have suffered from neurodegenerative diseases. It is hoped that this will lead to the identification of key processes that may eventually be controlled to prevent neuronal demise. The chapters by Roth on *in situ* detection of apoptosis and Smith and colleagues on the role of oxidative stress and apoptosis in Alzheimer's disease describe technical approaches associated with *in situ* detection of apoptosis.

The field of apoptosis research has grown exponentially in the past few years, and it would be impossible to describe each aspect of apoptosis as it applies to neurons. The importance of signal transduction pathways, transgenic animal models to study apoptosis, the other Bcl-2 family members, and the role of cell cycle gene expression in neuronal apoptosis has not been addressed in this book. These are equally important aspects of neuronal apoptosis.

Apoptosis Techniques and Protocols, Second Edition is intended to provide a handbook for the laboratory as well as a description of the limitations and advantages of the techniques proposed. I hope that it will be useful to both new and seasoned investigators who have an interest in unraveling the molecular mechanisms of neuronal cell death.

Andréa C. LeBlanc

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Contents

Molecular Characterization of the Proapoptotic Protein Bax

Yi-Te Hsu and Soraya Smaili

1. Introduction

Bax is a proapoptotic member of the Bcl-2 family. Members of this family can promote either cell survival, as in the case of Bcl-2 and Bcl-X_L, or cell death, as in the case of Bax and Bak. Bax was first identified as a Bcl-2 binding partner by immunoprecipitation *(1)*. Subsequently it was shown that overexpression of Bax can accelerate cell death in response to various apoptosis stimuli *(2)*. Physiologically, Bax plays an important role in neuronal development and spermatogenesis. Animals that are deficient in Bax have increased number of neurons and the males are known to be sterile *(3,4)*. Under pathological conditions such as cerebral and cardiac ischemia, upregulation of Bax has been reported in the afflicted area of the tissues, implicating the participation of this protein in promoting neuronal and cardiomyocytic cell death *(5–7)*. In certain cases of human colorectal cancer, mutations were found in the gene encoding Bax, suggesting that inactivation of Bax promotes tumorigenesis by enabling the tumor cells to be less susceptible to cell death *(8)*.

Structurally, Bax, like other members of the Bcl-2 family, shares a common feature of having three conserved regions known as the BH (Bcl-2 homology) domains 1–3 *(9,10)* (*see* Fig. 1). These domains have been shown to be important for the apoptosis regulatory functions of these Bcl-2 family proteins. In addition, Bax and a number of Bcl-2 family members also possess a hydrophobic segment at their carboxyl terminal ends. For Bcl-2, this hydrophobic

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segment is required for anchoring the protein to various organelles, including endoplasmic reticulum, mitochondria, and nuclear outer membranes *(11,12)*. The three-dimensional structures of the Bax and its pro-survival antagonist Bcl-X_L have recently been deciphered *(13,14)*. These two proteins appear to share a significant structural homology with the translocation domain of diphtheria toxin, especially at a helical loop domain formed by α-helices 5 and 6. This particular domain of diphtheria toxin has been shown to insert into lipid bilayer to form pores *(15–17)*.

In healthy cells, Bax is predominantly a soluble monomeric protein *(18–20)* despite the fact that it posseses a C-terminal hydrophobic segment. This hydrophobic domain, unlike those of Bcl-2 and Bcl-X_L, is sequestered inside a hydrophobic cleft *(13)*. Upon the induction of apoptosis by a variety of agents, a significant fraction of Bax was observed to translocate from the cytosol to the membrane fractions, in particular, the mitochondrial membrane *(18,20–28)*. This translocation process appears to involve a conformational change in Bax leading to the exposure of its C-terminal hydrophobic domain *(21,29)*. Deletion of the Bax C-terminal hydrophobic domain abrogated the ability of the mutant protein to translocate to mitochondria and greatly attenuated its ability to promote cell death *(21)*. On the other hand, point mutations of Bax that target the expressed proteins to mitochondria greatly increased Bax toxicity *(29)*. The translocation of Bax to mitochondria is associated with the release of cytochrome-*c* and the loss of mitochondrial membrane potential *(30–32)*. These phenomena may be related to the recent observations that Bax can form ion channels or pores in mitochondrial membranes *(33,34)* and that it can also modulate the activity of the mitochondrial permeability transition pores through binding to the pore components VDAC channel *(35)* or adenine nucleotide translocase *(36)*. Cytochrome-c activates caspase-3, leading to the proteolysis of the cell *(37)*, while the loss of mitochondrial membrane potential results in a decrease in cellular energy production. The proapoptotic activity of Bax, however, can be counteracted by co-expression with pro-survival factors Bcl-2 and Bcl-X_L, which can block Bax translocation to mitochondria during apoptosis *(24,38)*.

In this chapter, we describe the methodology we employ to study the translocation of Bax during apoptosis. Specifically, we will detail the techniques involved in the tracking of intracellular Bax distribution and Bax immunoaffinity purification.

2. Methods

2.1. Determination of Bax Intracellular Localization

The intracellular distribution of Bax in healthy cells and in cells undergoing apoptosis can be determined by subcellular fractionation, green fluorescent protein tagging, and immunofluorescence microscopy using anti-Bax antibodies.

2.1.1. Localization of Bax by Subcellular Fractionation

During apoptosis, a significant fraction of Bax has been shown to translocate from the cytosol to membranes, in particular mitochondrial membranes. This can be shown by subjecting healthy or apoptotic cells to hypotonic lysis, Dounce homogenization, and differential centrifugation. The resulting protein samples are then analyzed by Western blotting with anti-Bax antibodies.

1. Treat the cells of choice with the desired apoptosis inducer. Typically, we treat thymocytes with 1 μ*M* dexamethasone and HL 60 promyelocytic leukemia cells with 1 μ*M* staurosporine.
2. Collect the cells, pellet them by centrifugation, and wash them with the culture medium minus fetal bovine serum.
3. Resuspend the cells in the lysis buffer (10 m*M* HEPES, pH 7.4, 38 m*M* NaCl, 25-μg/mL phenylmethylsulfonyl fluoride, 1-μg/mL leupeptin, and 1-μg/mL aprotinin) at a cell density of 1–5 × 10^7 cells/mL. Incubate the mixture on ice for 15 min.
4. Homogenize the cells in a Dounce homogenizer until greater than 95% of the cells are broken. Centrifuge the lysate at 900*g* to pellet the nuclei in a Sorval SA-600 rotor. Save the pellet.
5. Centrifuge the postnuclear supernatant at 130,000*g* in a Beckman Ti 80 rotor to pellet the membranes.
6. Resuspend both the nuclear and crude membrane pellets with lysis buffer equal to the volume of the supernatant.
7. Analyze the protein samples by a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel, followed by Western blotting analysis with an anti-Bax antibody. Typically, we transfer the protein samples from the SDS gel onto Immobilon-P membranes (Millipore) in Tris-glycine buffer containing 10% methanol. The membranes are blocked in the blocking buffer (phosphate-buffered saline [PBS] containing 5% fetal bovine serum and 0.05% Tween-20) for 90 min. The blots are then incubated in the primary antibody (1/10 dilution in the blocking buffer) for 45 min,