1999 JOE DOUPE YOUNG INVESTIGATORS' AWARD

Bcl-2 intersects the NFκB signalling pathway and suppresses apoptosis in ventricular myocytes

Lorrie A. Kirshenbaum, PhD

Dr. Kirshenbaum is with the Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, and the Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Man.

MeSH headings: apoptosis; genes, bcl-2; NF-kappa B; ventricular remodeling

Clin Invest Med 2000;23(5):322-30.

Abstract

As a first step toward identifying putative regulators of apoptosis in the heart, the impact of the anti-apoptosis protein Bcl-2 (B-cell lymphoma gene) on the NFκB (nuclear factor kappa beta) signalling pathway in suppressing apoptosis in ventricular myocytes was studied. The data indicate that adenovirus-mediated delivery of Bcl-2 resulted in a significant increase in NFκB-dependent DNA binding and NFκB-directed gene transcription. No change in NFκB protein content was observed in myocytes expressing Bcl-2. Moreover, the Bcl-2-mediated NFκB activation was found to be related to changes in the activity of the NFκB regulatory protein IκBα (inhibitor of kappa beta). In this regard, a marked reduction in IκBα protein content was observed in ventricular myocytes expressing Bcl-2. The mode by which Bcl-2 regulates IκBα was related to the N-terminal phosphorylation and degradation of IκBα by the proteasome since an N-terminal deletion mutant of IκBα or the proteasome inhibitor lactacystin abrogated Bcl-2's inhibitory effects on IκBα and prevented NFκB activation. Furthermore, adenovirus-mediated delivery of a phosphorylation defective form of IκBα rendered ventricular myocytes incapable of NFκB activation and susceptible to tumour necrosis factor alpha-mediated apoptosis. Moreover, Bcl-2's anti-apoptotic function was lost in cells defective for NFκB activation. The data provide evidence for a link between Bcl-2 and the NFκB signalling pathway for the suppression of apoptosis in ventricular myocytes.

Résumé

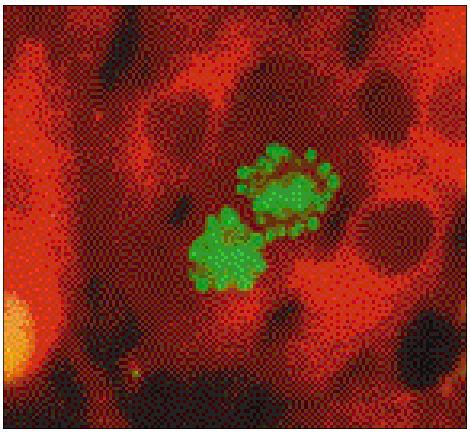
Pour identifier les régulateurs présumés de l'apoptose dans le cœur, on a étudié dans un premier temps l'effet de la protéine anti-apoptose Bcl-2 (gène du lymphome B) sur la voie de signalisation du facteur NFκB (facteur nucléaire kappa beta) dans la suppression de l'apoptose dans les myocytes ventriculaires. Les données indiquent que la distribution de la protéine Bcl-2 provoquée par l'adénovirus a entraîné une augmentation importante de la fixation de l'ADN tributaire du facteur NFκB et de la transcription génique dirigée par le facteur NFκB. On n'a observé aucun changement de la teneur en protéines du facteur NFκB dans les myocytes qui expriment la protéine Bcl-2. On a constaté de plus que l'activation du facteur NFκB provoquée par la protéine Bcl-2 était liée au changement de l'activité de la protéine IκBα (inhibiteur du facteur kappa beta) régulatrice du facteur NFκB. À cet égard, on a observé une réduction marquée de la teneur en protéine IκBα dans les myocytes ventriculaires qui expriment la protéine Bcl-2. On a établi un lien entre le mode de régulation de l'IκBα par la protéine Bcl-2 et la phosphorylation du N-terminal et la dégradation de l'IκBα par le protéasome puisqu'une forme mutante de l'IκBα par délétion du N-terminal ou la lactacystine inhibitrice du protéasome a annulé les effets inhibiteurs de la protéine Bcl-2 sur l'IκBα et a empêché l'activation du facteur NFκB. De plus, la distribution provoquée par l'adénovirus d'une forme à phosphorylation défectueuse de l'IkBa a rendu les myocytes ventriculaires incapables d'activer le facteur NFκB et vulnérables à l'apoptose à médiation alpha du facteur de nécrose des tumeurs. De plus, la fonction anti-apoptose de la protéine Bcl-2 a été perdue dans les cellules défectueuses en ce qui concerne l'activation du facteur NFκB. Les données démontrent l'existence d'un lien entre la protéine Bcl-2 et la voie de signalisation du facteur NFκB pour la suppression de l'apoptose dans les myocytes ventriculaires.

Abbreviations used in this article

Bcl-2 — B-cell lymphoma gene
NFκB —Nuclear factor kappa beta
IκBα — Inhibitor of kappa beta
Lactacystin — A chemical inhibitor of the proteasome
TNFα — Tumour necrosis factor alpha
Rel homologue — The name of a gene that encodes stringent factor
p50Kd — 50 kilodalton subunit of NFκB
p65Kd/RelA — 65 kilodalton subunit of NFκB
ced-3, ced-4, ced-9 — Caenorhabditis elegans death genes
Bcl-2. x_L, W— B-cell lymphoma gene, long form Bfl-1 member of the Bcl-2 family
Mcl-1 member of the Bcl-2 family
Bax, Bak, Bad, Bid, Bin, Bik, Bok_S, Hrk (all members of the Bcl-2 family that promote cell death)
BH — Bcl homology
$\Delta\psi_m$ — Mitochondrial membrane potential
Ras/PI-3K/Akt — Ras-oncogene, phosphoinositol-3 kinase, protein kinase B
FLAG — a synthetic sequence tag to identify proteins

Background: apoptosis and the cardiac myocyte

One of the most intriguing and perplexing issues to impact on contemporary cardiology to date is the limited ability of heart muscle to repair itself after injury. In contrast to other cells of the body, which retain a regenerative potential and ability to multiply and divide, cardiac muscle cells inherently lose this property shortly after birth. This has significant consequence for patients who have suffered a heart attack or other forms of myocardial cell injury, since the heart cells once damaged do not actively repair themselves and are instead replaced by scar tissue. Undoubtedly, myocardial cell loss will immediately impair contractility and the ability of the heart to pump blood commensurate with the body's demands.



Apoptosis of neonatal ventricular myocytes. Nucleosomal DNA fragmentation of ventricular myocytes was detected by immunofluorescence microscopy using terminal deoxynucleotide nick-end labelling (TUNEL method) to detect fragmented DNA (green).

Recently, programmed cell death or "apoptosis" has been described as a pathophysiologic process that is essential for the removal of unwanted or damaged cells from the body. In this report, new, exciting information is provided regarding the molecular signalling pathways that underlie apoptosis in the heart. Using the tools of molecular genetics, the author identifies a new signalling pathway for the anti-apoptotic factor Bcl-2 in the heart. He shows that Bcl-2 turns on a transcription factor called nuclear factor kappa beta (NFκB) recently attributed with anti-apoptotic properties. Furthermore, he demonstrates that activation of this factor can play a crucial role in suppressing apoptosis in the heart. This may have direct therapeutic implications for preventing cell death in pathologic states such as cardiac hypertrophy and failure.

Introduction

Arguably, one of the most compelling issues to make an impact on contemporary cardiology to date is programmed cell death or "apoptosis." Apoptosis is an evolutionary conserved event that permits multicellular organisms to selectively discard cells through an intrinsic cell suicide program. This is characterized by distinct morphologic changes typified by DNA fragmentation and cell shrinkage without the loss of membrane integrity.[1] Importantly, apoptosis can be influenced by internal as well as external cues that promote the

removal of cells that were inappropriately produced during embryogenesis[2,3] or cells that have become genetically unstable.[4–6] Despite the beneficial effects, there is increasing awareness that defects in regulatory pathways that govern apoptosis may contribute to various disorders including cancer, Alzheimer's disease, Parkinson's disease and Huntington's disease.[5,7] Central to this issue is the recent suggestion that apoptosis may also play a role in cardiovascular disease. In this regard, apoptosis has been documented in the myocardium in a number of clinically important states including hypoxia,[8,9] ischemia followed by reperfusion, myocardial infarction,[10] and, more recently, in patients with end-stage heart failure.[11,12] Apoptosis has also been detected in atherosclerotic lesions.[13]

Although apoptosis has been documented in different cardiac conditions, its significance is undetermined. However, since ventricular myocytes are terminally differentiated and have exited from the cell cycle, the loss of potentially viable cardiac cells through an apoptotic process may profoundly influence cardiac structure and function, given the lack of *de novo* myocyte regeneration and the limited regenerative ability of heart muscle. This point becomes particularly important during conditions of increased hemodynamic loading such as that imposed by hypertension or cell loss due to infarction. Maladaptive or excessive cell loss may be critically linked to the compensatory remodelling process that occurs during heart hypertrophy and heart failure after injury. Though of considerable scientific and clinical interest, little is known of the molecular events that govern apoptosis in cardiac muscle cells. Abetter understanding of the cellular factors and signalling pathways that regulate apoptotic cell death in the heart would contribute significantly to the long-term goal of developing therapeutic interventions to suppress aberrant apoptotic cell death after myocardial injury. To this end, our laboratory has focused on studying putative regulators of apoptosis in the heart with the future objective of modulating these to abrogate aberrant myocardial cell death.

NFκB signalling pathway

Though the molecular events that govern apoptosis in cardiac muscle cells are poorly understood, there is increasing awareness that apoptosis is a highly regulated process involving the precise coordination of genes that promote or prevent the process. Recent evidence suggests that cellular factors, including NFκB, may play a fundamental role in apoptosis by directly modulating the activity of key anti-apoptotic genes to suppress apoptosis. NFκB was first identified as a key regulatory molecule necessary for the activation of B-lymphocyte gene transcription.[14] It is now widely appreciated that NFκB is a ubiquitously expressed transcription factor involved in activating genes associated with the immune system, inflammation, cell adhesion, viral gene transcription and recently anti-apoptosis.[15,16] NFκB belongs to a family of highly conserved transcription factors with Rel homology, which include Rel-A, c-Rel, v-Rel and *Drosophilia dorsal* proteins. The predominant form of NFκB exists in mammalian cells and binds to DNA as a heterodimeric complex of p50Kd and p65Kd/RelA protein subunits.[17–19] The p65 subunit is the transcriptionally active component of the p65/p50 NFκB complex necessary for gene transcription.[20] In contrast to other transcription factors that readily localize to the nucleus of the cell, NFκB is sequestered as an inactive complex in the cytoplasm by the IκBα protein.[21,22] The interaction of NFκB with IκBα[22–24] prevents NFκB from translocating to the nucleus under basal conditions. NFκB activity can be induced in ventricular myocytes by cytokines, including interleukin-1 and TNFα,[25–27] as well as other agents. Recently, an anti-apoptotic function for NFκB has been described.[16,28,29] This is supported by gene knockout studies in which loss of the p65 subunit of NFκB was embryonically lethal.[30] Presumably, the embryos die from excessive apoptosis, suggesting that NFκB is crucial for regulating apoptosis during embryonic development.[30]

The mechanism by which biologic signals activate NFκB in vivo remains poorly defined. Recent studies suggest that NFκB activation requires the phosphorylation and degradation of IκBα.[25,27] Inducible degradation of IκBα permits NFκB to translocate to the nucleus and affect gene transcription.[14,24,31] The N-terminal domain of IκBα represents an important site of regulation. In particular, studies of mutagenesis have revealed that the conserved serine residues at positions 32 and 36 of IκBα are necessary and sufficient

for signal-induced phosphorylation.[32] Ostensibly, the phosphorylation of these specific residues initiates the ubiquitination of IκBα followed by its degradation by the 26S proteasome.[33] Deletion of the N-terminus or substitution of the critical serine residues within the N-terminal domain render the IκBα molecule defective for phosphorylation and degradation. Therefore, regulation of IκBα through phosphorylation represents a crucial point in the activation of NFκB, important for regulating cellular activities including apoptosis.[34]

Regulation of apoptosis: the Bcl-2 family

Much of our understanding of the mechanisms that regulate apoptosis in mammalian cells stems from the discovery of *ced-3*, *ced-4* and *ced*-9 in the nematode *Caenorhabditis elegans*.[35,36] Genetic studies identified that the *ced-3* and *ced-4* gene products encode proteins that are crucial for initiating apoptosis, whereas the *ced-9* gene product encodes proteins that prevent cell death.[37] Using this approach, researchers postulated that coordinated and timed expression of these factors is essential for normal worm development.[35] Importantly, the mammalian homologue to the *C. elegans ced-9* gene includes the *Bcl-2* gene product.[35,38] Of historical note, the *Bcl-2* gene was first identified as a translocation break-point mutation (t14:18) in human B-cell lymphoma,[39–41] with cells derived from these tumours being resistant to pro-apoptotic signals. In gain-of-function studies, Bcl-2 protected cells from a variety of death-promoting signals, supporting the notion that Bcl-2 functions as an anti-apoptotic factor. It is now understood that Bcl-2 belongs to a large family of homologous proteins that can either promote or suppress apoptosis. Examples of Bcl-2 family members that prevent cell death include Bcl-2, $Bclx_L$, Bcl-W, Bfl-1, Mcl-1 and A1,[42,43] whereas those that promote cell death include Bax, Bak, Bad, Bid, Bim, Bik, Bok and Hrk.[44–47] Structural analysis studies have identified 4 key domains that are conserved among the Bcl-2 family proteins, referred to as BH domains.[48] Though the majority of the Bcl-2 family members share sequence conservation at the C-terminal transmembrane domain, variable homology exists between domains BH1 to BH4, suggesting that these differences may account for a given Bcl-2 family member to promote or prevent cell death. The BH1, BH2, BH3 domains are common to most Bcl-2 proteins including the death-promoting factors Bax, Bak and Bok.[49–51] A subgroup of death factors based on the presence of only the small BH3 domain has also been identified and includes Bid, Bad, BNIP3 and EGL-1. Since the conserved N-terminal amphipathic BH4 domain is restricted to those Bcl-2 family members with anti-apoptotic properties, it appears that this domain may be crucial for preventing apoptosis. This is supported by studies in which deletion of this region renders Bcl-2 defective for suppressing apoptosis.[49,50,52,53] Crystallographic analysis of Bcl-2 family proteins has revealed a hydrophobic cleft between the BH1, BH2 and BH3 domains that serves as a docking site for Bcl-2 family members.[48] An early model proposed that the physical interaction of death promoting factors such as Bax with Bcl-2 would provoke apoptosis by titrating out the cellular pool of available life-promoting Bcl-2 proteins.[42,54] Therefore, a disproportionate ratio of cell death to cell life factors could ultimately determine whether the cell would live or die. However, the limited ability to actively detect Bcl-2-Bax complexes in cells coupled with the Bcl-2's ability to prevent apoptosis independent of its presumed ability to dimerize with the pro-apoptotic factors Bax, Bak or Bad[50] has brought into question the relative importance of this model. This suggests that Bcl-2 may avert apoptosis by another mechanism.

Interestingly, the BH1 and BH2 domains of Bcl-2 proteins can form conductance ion channels in synthetic membranes, raising the possibility that these proteins may influence apoptosis by regulating ion fluxes in vital organelles such as mitochondria.[44,55] Furthermore, perturbations to mitochondria resulting in the collapse of $\Delta\Psi_m$ have been proposed as a crucial event in the mitochondrial-death pathway. Loss of $\Delta\Psi_m$ is thought to occur from the formation of the permeability transition pore, a large multiprotein complex comprising, in part, the adenine nucleotide translocator, porin/voltage dependent anion channel in the mitochondrial inner and outer membranes that can increase permeability of mitochondria in response to pro-apoptotic signals.[56,57] The fact that Bcl-2 can delay or prevent apoptosis by a diverse number of death-promoting signals suggests that it impinges on one or more signalling factors that lead to cell death. In this

regard, Bcl-2 has been reported to interact with cellular factors including Raf-1.[51,58–60] The fact that Raf-1 is activated as part of the Ras/PI-3K/Akt signalling pathway, recently ascribed to mediate life-promoting anti-apoptotic signals,[61,62] raises the interesting possibility that Bcl-2 may intersect this pathway and modulate key proteins in the apoptotic pathway.[63] Since it has been suggested that NFκB plays a beneficent role in preventing apoptosis provoked under certain conditions, my group investigated whether Bcl-2 modulates the activity of NFκB in neonatal ventricular myocytes.

Methods

Cell culture and transfection

Postnatal ventricular myocytes were isolated from 2-day old Sprague–Dawley rat hearts and submitted to primary culture as reported earlier.[64] Cells were maintained overnight in Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12, (DF) 1:1, containing 10% fetal bovine serum (FBS) and then transferred to serum-free DMEM. Myocyte cultures were infected with 20 plaque-forming units per cell of recombinant adenovirus encoding the human *Bcl-2* c-DNA for 4 hours. Myocytes were transfected immediately after removal of viral stocks with luciferase reporter plasmids. The myocytes were stimulated with 10 ng/mL of human recombinant TNFα for 24 hours (R & D systems). Human embryonic kidney 293 cells, (American Type Culture Collection, Rockville, Md.) were maintained in DMEM containing 10% fetal bovine serum (Gibco BRL).[65] For transfection experiments in 293 cells, cells were incubated in DMEM containing Superfect (Qiagen, Santa Clara, Calif.) and eukaryotic expression vectors for Bcl-2 (CMV Bcl-2)[66] and epitope-FLAG-tagged derivatives of wild type IκBα, N-terminal deletion mutant, or a serine to alanine substitution mutant at amino acids 32 and 36 respectively (kindly provided by D. Ballard).

Western blot analysis

Cell lysate derived from either cardiac myocytes or 293 cells, or both, were harvested in NP-40, containing, 150 mmol NaCl, 50 mmol TRIS-HCl (NP40 buffer). Cell lysates were subjected to Western blot analysis and probed with antibodies directed toward the p65 subunit of NFκB, IκBα and Bcl-2 proteins respectively.

Electromobility gel shift assay

Nuclear extracts of cardiac myocytes were prepared and were analysed by electromobilityshift analysis for NFκB using a duplex oliogonucleotide probe labelled with phosphorus 32 containing NFκB consensus binding sites AGTTGAGGGGACTTTCGCAGGC. Nuclear-protein complexes were resolved on a native 5% polyacrylamide gel in TRIS-borate-EDTApH 8.0 and detected by autoradiography.

Assays of apoptosis

Cardiac nuclear morphologic features and DNA fragmentation were determined by counter staining myocytes with Hoechst 33258 dye (Sigma Chemical Co., St. Louis, Mo.) for nuclear DNA as previously described.[65] Replicate cultures using 200 or more cells for each condition were calculated. Genomic DNA was isolated from ventricular myocytes for nucleosomal DNA fragmentation by gel electrophoresis as described earlier.[65]

Results and discussion

Previously, my group reported that adenovirus-mediated delivery of Bcl-2 was sufficient to prevent apoptosis of neonatal and adult ventricular myocytes, establishing that Bcl-2 can operate as an anti-apoptotic factor in the heart.[64,67] To elucidate whether external signals result in the activation of NFκB in ventricular myocytes, myocytes were monitored for NFκB activity. In this regard, a greater than twofold induction of NFκB activity was observed in ventricular myocytes in the presence of TNFα, a known trigger of NFκB. Interestingly, a similar increase in NFκB activity was observed in ventricular myocytes expressing the *Bcl-2* gene. In addition, a significant increase in NFκB-dependent DNA binding was observed in cells treated with TNFα, verifying that ventricular myocytes are functionally coupled to biological signals that lead to NFκB activation.[68]

Interestingly, stimulation of myocytes with TNFα did not provoke apoptosis of ventricular myocytes as indicated by Hoescht 33258 staining. In contrast, the combination of TNFα and the protein synthesis inhibitor cycloheximide resulted in a significant increase in apoptosis as illustrated by increased chromatin condensation by Hoechst 33258 staining and nucleosomal DNA laddering. Expression of Bcl-2 in ventricular myocytes reduced the incidence of cell death triggered by TNFα and cycloheximide.[68]

The fact that TNFα only provoked apoptosis of ventricular myocytes in the presence of cycloheximide implies that certain life-promoting genes are required to abrogate the otherwise cytotoxic actions of TNFα. NFκB has been suggested as a key factor in suppressing apoptosis triggered by a variety of death-promoting signals including TNFα.[28,30] Because Bcl-2 is known to modulate the activity of certain cellular factors in the apoptotic pathway, we ascertained whether Bcl-2 might activate NFκB in ventricular myocytes. Adenovirus-mediated gene transfer of Bcl-2 to ventricular myocytes, resulted in a significant increase in nuclear DNAbinding activity of NFκB as indicated by electromobility shift analysis. Surprisingly, no change in NFκB protein content was detected in ventricular myocytes expressing Bcl-2 proteins, suggesting that Bcl-2 may lead to the activation of NFκB through an indirect pathway. Since NFκB is regulated by the inhibitor protein IκBα, we determined whether the observed increase in NFκB activity in the presence of Bcl-2 was related to an altered expression of IκBα. In contrast to control cells, Western blot analysis indicated that total IκBα protein content was markedly reduced in ventricular myocytes expressing Bcl-2, suggesting that the increased NFκB activity in ventricular myocytes expressing Bcl-2 may be linked to inactivation of IκBα.

Since the N-terminus of IκBα is necessary and sufficient for signal-induced phosphorylation and degradation by agents that activate NFκB,[65] we postulated that it may also serve as a target for Bcl-2. For these experiments we utilized IκBα DNA constructs containing mutations in the N-terminus of IκBα known to render IκBα resistant to signal-induced phosphorylation and degradation.

In contrast to wild type IκBα protein, no apparent reduction in the levels of either an N-terminal deletion mutant or serine to alanine point substitution mutant of IκBα was observed in the presence of Bcl-2. These findings suggest that the N-terminus of IκBα may be the site by which Bcl-2 targets the degradation of IκBα.[65]

Next we tested the functional significance of our observations by determining whether a block to NFκB activation would impair Bcl-2's ability to rescue TNFα-mediated apoptosis. For these experiments we generated a recombinant adenovirus encoding a phosphosphorylation mutant of IκBα that renders IκBα defective for phosphorylation and degradation. In the presence of the IκBα mutant protein, Bcl-2's ability to suppress apoptosis mediated by TNFα plus cycloheximide was lost, substantiating the need for a functional NFκB signalling pathway for the suppression of apoptosis in ventricular myocytes.

We believe the data presented, under the conditions tested, provides compelling evidence to link Bcl-2 to the NFκB signalling pathway for the suppression of apoptosis in ventricular myocytes (Fig. 1). Furthermore, our data indicate that Bcl-2 activates NFκB through a mechanism that involves the phosphorylation and subsequent degradation of IκBα through the proteasome. Our future investigations are directed toward understanding the involvement of Bcl-2 proteins and the NFκB signalling pathway for the suppression of apoptosis during load-induced hypertrophy and heart failure.

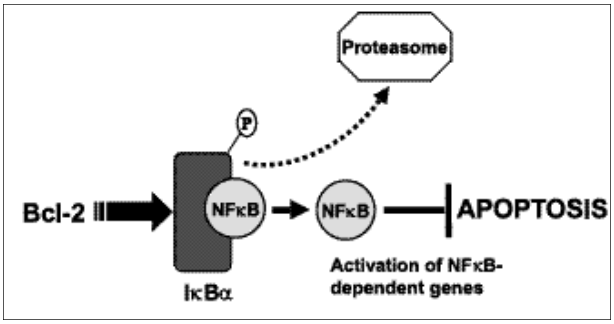


Fig. 1: The activation of NFkB (nuclear factor kappa beta) in ventricular myocytes by Bcl-2 (B-cell lymphoma). Bcl-2 activates NFkB through a mechanism that involves the N-terminal phosphorylation and degradation of IkBa (inhibitor of kappa beta) by the proteasome. This permits NFkB to translocate to the nucleus and activate downstream expression necessary to suppress apoptosis.

Acknowledgements

We thank D. Ballard, J. Reed, S. Koresmeyer and G. Chinnadurai for their generous gift of reagents cited; Dr. Arnold Greenberg and Kelly Regula for critical comments on the manuscript. Danielle de Moissac and Hui Zheng for expert technical assistance.

This work was supported by grants from The Canadian Institutes for Health Research.

Dr. Kirshenbaum is a Heart and Stroke Foundation of Canada Scholar.

References

1. Wyllie AH. Apoptosis (the 1992 Frank Rose Memorial Lecture). *Br J Cancer* 1993;67(2):205-8.
2. Wyllie AH. The genetic regulation of apoptosis. *Curr Opin Genet Dev* 1995;5(1):97-104.
3. Wyllie AH. Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. *Cancer Metastasis Rev* 1992;11(2):95-103.
4. Arends MJ, Wyllie AH. Apoptosis: mechanisms and roles in pathology. *Int Rev Exp Pathol* 1991;32:223-54.
5. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995;267(5203):1456-62.
6. Eastman A, Grant S, Lock R, Tritton T, Van Houten N, Yuan J. Cell death in cancer and development. AACR special conference in cancer research. *Cancer Res* 1994;54(10):2812-8.
7. Martin SJ, Green DR. Apoptosis and cancer: the failure of controls on cell death and cell survival. *Crit Rev Oncol Hematol* 1995;18(2):137-53.
8. Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, et al. Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. *Circ Res* 1994;75 (3):426-33.
9. Moissac D, Gurevich RM, Zheng H, Singal PK, Kirshenbaum LA. Caspase activation and mitochondrial cytochrome C release during hypoxia-mediated apoptosis of adult ventricular myocytes. *J Mol Cell Cardiol* 2000;32(1):53-63.
10. Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, et al. Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. *Lab Invest* 1996;74(1):86-107.
11. Narula J, Haider N, Virmani R, DiSalvo TG, Kolodgie FD, Hajjar RJ, et al. Apoptosis in myocytes in end-stage heart failure [see comments]. *N Engl J Med* 1996;335(16):1182-9. Comments in: *N Engl J Med* 1996;335(16):1224-6; *N Engl J Med* 1997;3;336(14): 1025-6.
12. Quaini F, Cigola E, Lagrasta C, Saccani G, Quaini E, Rossi C, et al. End-stage cardiac failure in humans is coupled with the induction of proliferating cell nuclear antigen and nuclear mitotic division in ventricular myocytes. *Circ Res* 1994;75:1050-63.
13. Isner JM, Kearney M, Bortman S, Passeri J. Apoptosis in human atherosclerosis and restenosis. *Circulation* 1995;91(11):2703-11.
14. Beg AA, Baldwin AS Jr. Activation of multiple NF-kappa B/Rel DNA-binding complexes by tumor necrosis factor. *Oncogene* 1994;9(5):1487-92.
15. Baeuerle PA, Baltimore D. NF-kappa B: ten years after. *Cell* 1996;87(1):13-20.
16. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death [see comments]. *Science* 1996;274(5288):782-4. Comments in: *Science* 1996;274(5288):724; *Science* 1997;11;276 (5310):185.
17. Baeuerle PA, Baltimore D. A 65-kappaD subunit of active NF-kappaB is required for inhibition of NF-kappaB by I kappaB. *Genes Dev* 1997;3(11):1689-98.
18. Hansen SK, Nerlov C, Zabel U, Verde P, Johnsen M, Baeuerle PA, et al. A novel complex between the p65 subunit of NF-kappa B and c-Rel binds to a DNA element involved in the phorbol ester induction of the human urokinase gene. *EMBO J* 1992;11(1):205-13.
19. Urban MB, Baeuerle PA. The role of the p50 and p65 subunits of NF-kappa B in the recognition of cognate sequences. *New Biol* 1991;3(3):279-88.
20. Schmitz ML, Baeuerle PA. The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. *EMBO J* 1991;10(12):3805-17.
21. Urban MB, Baeuerle PA. The 65-kD subunit of NF-kappa B is a receptor for I kappa B and a modulator of DNA-binding specificity. *Genes Dev* 1990;4(11):1975-84.
22. Baeuerle PA, Baltimore D. I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science* 1988;242(4878):540-6.
23. Nolan GP, Baltimore D. The inhibitory ankyrin and activator Rel proteins. *Curr Opin Genet Dev* 1992;2(2): 211-20.
24. Zabel U, Henkel T, Silva MS, Baeuerle PA. Nuclear uptake control of NF-kappa B by MAD-3, an I kappa B protein present in the nucleus. *EMBO J* 1993;12(1): 201-11.
25. Miyamoto S, Maki M, Schmitt MJ, Hatanaka M, Verma IM. Tumor necrosis factor alpha-induced phosphorylation of I kappa B alpha is a signal for its degradation but not dissociation from NF-kappa B. *Proc Natl Acad Sci U S A* 1997;91(26):12740-4.
26. Messer G, Weiss EH, Baeuerle PA. Tumor necrosis factor beta (TNF-beta) induces binding of the NF-kappa B transcription factor to a high-affinity kappa B element in the TNF-beta promoter. *Cytokine* 1990;2 (6):389-97.
27. Lenardo MJ, Baltimore D. NF-kappa B: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell* 1989;58(2):227-9.
28. Wang CY, Mayo MW, Baldwin AS Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science* 1996;274(5288):784-7.

29. Van Antwerp DJ, Verma IM. Signal-induced degradation of I(kappa)B(alpha): association with NF-kappaB and the PEST sequence in I(kappa)B(alpha) are not required. *Mol Cell Biol* 1996;16(11):6037-45.
30. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature* 1995;376(6536):167-70.
31. Henkel T, Machleidt T, Alkalay I, Kronke M, Ben Neriah Y, Baeuerle PA. Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. *Nature* 1993;365(6442):182-5.
32. Sun SC, Ganchi PA, Ballard DW, Greene WC. NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. *Science* 1993;259(5103):1912-5.
33. Maniatis T. A ubiquitin ligase complex essential for the NF-kappaB, Wnt/Wingless, and Hedgehog signaling pathways. *Genes Dev* 1999;13(5):505-10.
34. Brockman JA, Scherer DC, McKinsey TA, Hall SM, Qi X, Lee WY, et al. Coupling of a signal response domain in I kappa B alpha to multiple pathways for NF-kappa B activation. *Mol Cell Biol* 1995;15(5):2809-18.
35. Hengartner MO, Horvitz HR. *C. elegans* cell survival gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. *Cell* 1994;76(4):665-76.
36. Hengartner MO, Horvitz HR. The ins and outs of programmed cell death during *C. elegans* development [review]. *Philos Trans R Soc Lond B Biol Sci* 1994;345 (1313):243-6.
37. Hengartner MO, Horvitz HR. Activation of *C. elegans* cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2. *Nature* 1994;369 (6478):318-20.
38. Hengartner MO, Horvitz HR. Programmed cell death in *Caenorhabditis elegans*. *Curr Opin Genet Dev* 1994;4 (4):581-6.
39. Lu QL, Hanby AM, Nasser Hajibagheri MA, Gschmeissner SE, Lu PJ, Taylor Papadimitriou J, et al. Bcl-2 protein localizes to the chromosomes of mitotic nuclei and is correlated with the cell cycle in cultured epithelial cell lines. *J Cell Sci* 1994;107(Pt 2):363-71.
40. Reed JC. Regulation of apoptosis by bcl-2 family proteins and its role in cancer and chemoresistance. *Curr Opin Oncol* 1995;7(6):541-6.
41. Korsmeyer SJ, Arnold A, Bakhshi A, Ravetch JV, Siebenlist U, Hieter PA, et al. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. *J Clin Invest* 1983;71(2):301-13.
42. Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB, et al. Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. *Proc Natl Acad Sci U S A* 1995;92(17):7834-8.
43. Korsmeyer SJ. Programmed cell death: Bcl-2 [review]. *Important Adv Oncol* 1993:19-28.
44. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998;281(5381):1309-12.
45. Reed JC. Bcl-2 and the regulation of programmed cell death. *J Cell Biol* 1994;124(1-2):1-6.
46. Cory S. Regulation of lymphocyte survival by the bcl-2 gene family. *Annu Rev Immunol* 1995;13:513-43.
47. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998;281(5381):1322-6.
48. Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, et al. X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. *Nature* 1996;381(6580):335-41.
49. Hunter JJ, Bond BL, Parslow TG. Functional dissection of the human Bc12 protein: sequence requirements for inhibition of apoptosis. *Mol Cell Biol* 1996;16(3):877-83.
50. Hanada M, Aime Sempe C, Sato T, Reed JC. Structure-function analysis of Bcl-2 protein. Identification of conserved domains important for homodimerization with Bcl-2 and heterodimerization with Bax. *J Biol Chem* 1995;270(20):11962-9.
51. Yin XM, Oltvai ZN, Korsmeyer SJ. BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax [see comment]. *Nature* 1994;369(6478):321-3. Comment in: *Nature* 1994;369(6478):272-3.
52. Huang DC, Adams JM, Cory S. The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. *EMBO J* 1998;17(4):1029-39.
53. Lee LC, Hunter JJ, Mujeeb A, Turck C, Parslow TG. Evidence for alpha-helical conformation of an essential N-terminal region in the human Bcl2 protein. *J Biol Chem* 1996;271(38):23284-8.
54. Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* 1995;80(2):285-91.
55. Minn AJ, Velez P, Schendel SL, Liang H, Muchmore SW, Fesik SW, et al. Bcl-x(L) forms an ion channel in synthetic lipid membranes. *Nature* 1997;385(6614): 353-7.
56. Bossy-Wetzel E, Newmeyer DD, Green DR. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. *EMBO J* 1998;17(1):37-49.
57. Bradham CA, Qian T, Streetz K, Trautwein C, Brenner DA, Lemasters JJ. The mitochondrial permeability transition is required for tumor necrosis factor alpha-mediated apoptosis and cytochrome c release. *Mol Cell Biol* 1998;18(11):6353-64.
58. Wang HG, Rapp UR, Reed JC. Bcl-2 targets the protein kinase Raf-1 to mitochondria [see comment]. *Cell* 1996;87(4):629-38. Comment in: *Cell* 1996;15;87(4): 589-92.
59. Hu Y, Benedict MA, Wu D, Inohara N, Nunez G. Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. *Proc Natl Acad Sci U S A* 1998;95(8):4386-91.
60. Pan G, O'Rourke K, Dixit VM. Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. *J Biol Chem* 1998; 273(10):5841-5.

61. Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling [see comment]. *Nature* 1999;401(6748):86-90. Comment in: *Nature* 1999;401(6748):33-4.
62. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* 1999;401(6748):82-5.
63. Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, et al. Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. *Science* 1999;284(5412):339-43.
64. Kirshenbaum LA, Schneider MD. Adenovirus E1A represses cardiac gene transcription and reactivates DNA synthesis in ventricular myocytes, via alternative pocket protein-and p300-binding domains. *J BiolChem* 1995;270(14):7791-4.
65. de Moissac D, Zheng H, Kirshenbaum LA. Linkage of the BH4 domain of Bcl-2 and the nuclear factor kappaB signaling pathway for suppression of apoptosis. *J Biol Chem* 1999;274(41):29505-9.
66. Subramanian T, Tarodi B, Chinnadurai G. p53-independent apoptotic and necrotic cell deaths induced by adenovirus infection: suppression by E1B 19K and Bcl-2 proteins. *Cell Growth Differ* 1995;6(2):131-7.
67. Kirshenbaum LA, de Moissac D. The bcl-2 gene product prevents programmed cell death of ventricular myocytes. *Circulation* 1997;96(5):1580-5.
68. de Moissac D, Mustapha S, Greenberg AH, Kirshenbaum LA. Bcl-2 activates the transcription factor NFkappaB through the degradation of the cytoplasmic inhibitor IkappaBalpha. *J Biol Chem* 1998;273(37):23946-51.

Reprint requests to: Dr. Lorrie A. Kirshenbaum, Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, Rm. 3042, 351 Taché Ave., Winnipeg MB R2H 2A6; fax 204 233-6723, Lorrie@sbrc.umanitoba.ca