Molecular Mechanisms of Programmed Cell Death

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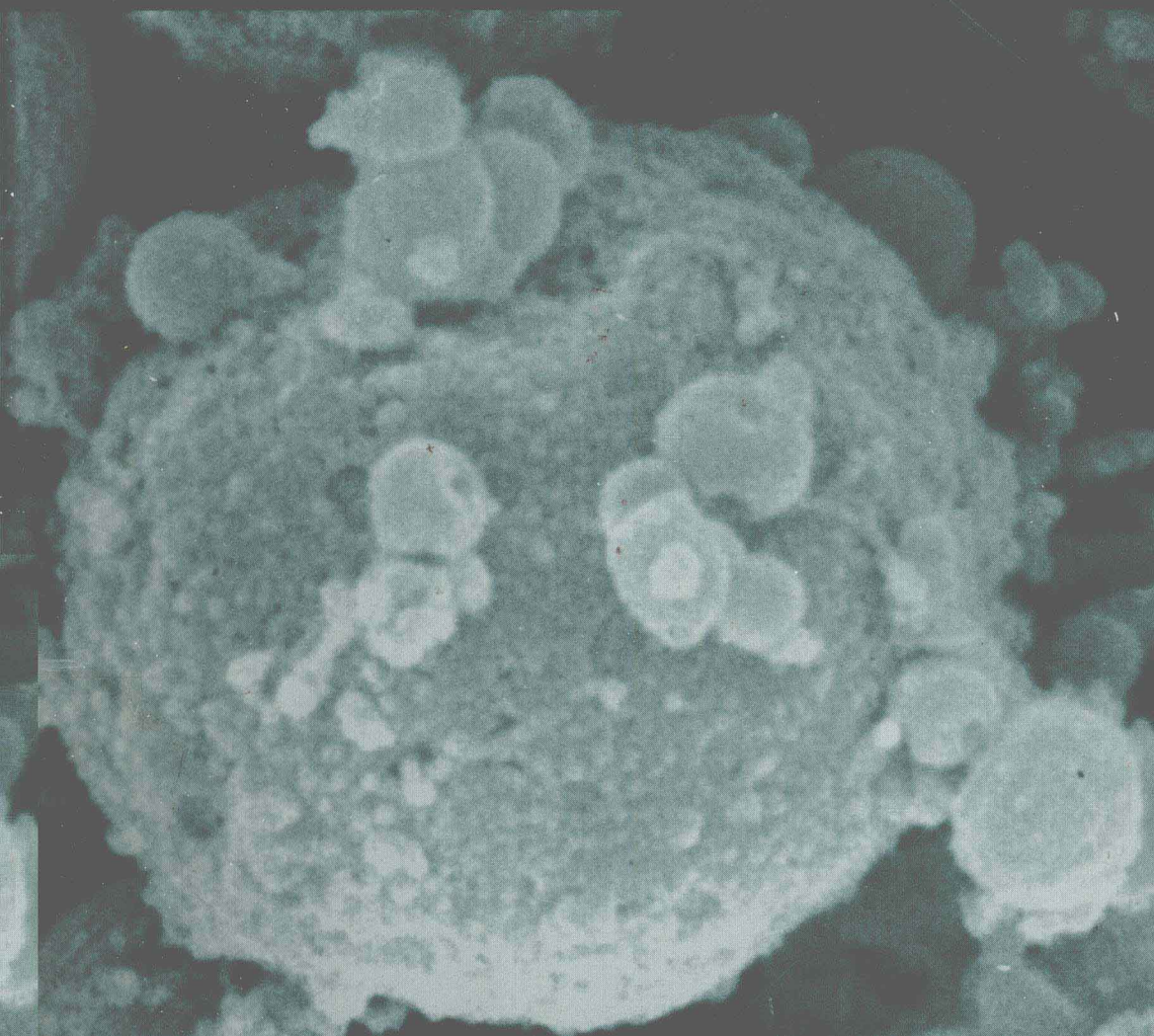

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Preface

The 2002 Nobel Prize in Physiology or Medicine was awarded to Sydney Brenner, H. Robert Horvitz, and John E. Sulston for their seminal discoveries concerning "genetic regulation of organ development and programmed cell death." This clearly marked the prime importance of understanding the molecular mechanisms controlling cell death.

The 1st International Symposium on Programmed Cell Death was held in the Shanghai Science Center of the Chinese Academy of Sciences on September 8–12, 1996. A number of key issues in apoptosis were discussed at the meeting, and progress in major areas of apoptosis research was summarized by expert participants at the meeting and published by Plenum Publishing Corporation as a book entitled *Programmed Cell Death.* In the last six years, we have witnessed a real explosion in our knowledge on how cells undergo apoptosis, thereby participating in various developmental and pathophysiological processes. At this ever-exciting time, we organized the 2nd International Symposium on Programmed Cell Death.

The chapters in the present volume include contributions from invited speakers. Given the explosive growth and progress in the apoptosis field, it is clear that no single meeting will be able to cover all important areas. This volume emphasizes key areas such as cell volume changes, the role of Bcl-2 family proteins, signaling of the TNF family molecules, extracellular matrix, and the role of apoptosis in the regulation of the immune system and cancer. In each case, the contributors have emphasized the areas that are still open for further exploration. In addition, potential applications for understanding and treatment of diseases are discussed. Some diagrammatic representations are provided, which will be invaluable for summarizing the wealth of information. We expect that this volume will help those in basic research in this fascinating area, as well as those actively involved in drug discovery.

We are fortunate to have had financial support from National Institute of Child Health and Development of the National Institutes of Health, National Institute of Environmental Health Sciences of the National Institutes of Health, the American Red Cross, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, and Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences. We are also deeply indebted to Mr. Richard Wernoski, Ms. Bo Zhou, and Ailan Chang for their support in putting the meeting together. Their efforts not only ensured the success of the meeting but also made it exciting and memorable.

YUFANG SHI
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Contents

Chapter 1

Akt and Bcl-x_L Are Independent Regulators of the Mitochondrial Cell Death Pathways

DAVID R. PLAS, JEFFREY C. RATHMELL, JAMES E. THOMPSON, AND CRAIG B. THOMPSON*

ABSTRACT: In vivo, hematopoietic cells require continuous signals from their microenvironment to prevent activation of the endogenous programmed cell death machinery. Cell survival is therefore limited by the availability of ligands for the receptors that can influence cell survival. Following loss of receptor engagement, IL-3-dependent hematopoietic cells undergo a rapid decline in cellular metabolism, characterized by reductions in surface expression of the glucose transporter GLUT-1, mitochondrial potential, and cellular ATP. Two distinct classes of oncogenes can prevent cell death in response to declines in glucose uptake and metabolism following growth factor withdrawal: pro-survival Bcl-2 proteins, such as Bcl-x_L, or an activated form of Akt. However, Bcl-x_L and Akt appear to promote survival by distinct mechanisms. Expression of activated Akt leads to maintenance of glucose transporter expression, glycolytic activity, mitochondrial potential, and cell size, while Bcl-x_L-expressing cells deprived of growth factor survive in a more vegetative state characterized by small cells with reduced mitochondrial potential and glycolytic activity. Akt-mediated survival is dependent on promoting glycolysis and maintaining a physiologic mitochondrial potential. In contrast, Bcl-x_L maintains mitochondrial integrity in the face of a reduced mitochondrial membrane potential in growth factor-deprived cells. Thus, Akt and Bcl-x_L suppress mitochondrial-initiated apoptosis by distinct mechanisms.

Introduction

It is poorly understood how multicellular organisms maintain relatively constant numbers of cells throughout adult life. It has long been hypothesized that one critical mechanism underlying the control of cell numbers is the observation that the majority, if not all, cells in multicellular organisms lack the autonomous ability to replicate. Thus, metazoan cells have become dependent on extracellular signals for both initiating and progressing through the cell cycle. As such, cells are incapable of accumulating in a cell autonomous way, establishing a mechanism whereby the accumulation of excess cells during adult life is prevented by limiting the availability of necessary growth factors (Hanahan and Weinberg, 2000). Recently, we and others have hypothesized that a simple extension of this model could

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also serve to explain the regulation of cell death in multicellular organisms. This proposal suggests that all cells within a multicellular organism are also dependent on extracellular survival signals to prevent the induction of cell death. When cells are deprived of necessary survival factors for a sustained period of time, an endogenous cell suicide pathway commonly referred to as apoptosis, or programmed cell death, is activated (Rathmell and Thompson, 2002).

A number of laboratories have provided evidence for the dependency of cell survival on extracellular signals using lineage-specific survival factors (Marrack et al., 2000). Recent evidence suggests that apoptotic death has a number of common features, independent of the cell's lineage. A central event in the induction of apoptosis in response to numerous apoptotic stimuli is the formation of a caspase-9-activating complex comprised of cytochrome c and Apaf-1 and ATP or dATP (Zou et al., 1997). In intact cells, cytochrome c is sequestered in the intermembrane space of mitochondria, where it functions as a component of the electron transport chain. Apoptotic stimuli induce events which eventually trigger the translocation across the outer mitochondrial membrane into the cytosol where it oligomerizes with Apaf-1. As a result of the cleavage of the proenzyme caspase-9 to its active form, a caspase cascade is activated that is responsible for the morphologic features associated with apoptosis (Shi, 2002). Studies to characterize how cytochrome c translocation is accomplished suggest a disruption in the integrity of the outer mitochondrial membrane that releases not only cytochrome c but also a number of other proapoptotic molecules normally resident in the inter-membrane space, such as AIF or Smac (Du et al., 2000; Susin et al., 1999; Verhagen et al., 2000).

Much attention has now been focused on the molecular basis for the loss in the mitochondrial outer membrane integrity that results in redistribution of cytochrome c and other proapoptotic factors. This step in many cell types appears to be a point of irreversible commitment to cell death. Two hypotheses concerning the role of mitochondria in programmed cell death have been developed. In the prevailing model, mitochondria are viewed primarily as a storage site for various proapoptotic proteins. In this view, mitochondrial permeability is triggered as a result of events in the cytosol, which stimulate apoptotic control proteins in the cytosol to directly induce mitochondria to release cytochrome c (Huang and Strasser, 2000). In the extreme, this model proposes that mitochondrial physiology plays no central role in the regulation of apoptosis. An alternative model suggests that an impairment in mitochondrial function causes the loss in the integrity of the outer mitochondrial membrane (Vander Heiden and Thompson, 1999). In this model, loss of the integrity of the outer mitochondrial membrane is viewed as an irreversible loss of the ability of mitochondria to maintain organelle physiology.

A family of proteins localized to the outer mitochondrial membrane can regulate the ability of mitochondria to release cytochrome c and other pro-apoptotic molecules. The prototype of this family, Bcl-2, was first demonstrated to regulate the induction of apoptosis in leukemia cells overexpressing Bcl-2 as a result of a chromosomal translocation (Tsujimoto et al., 1985). Following the identification of cytochrome c as a critical mitochondrial constituent that is required for the activation of caspase 9, it was shown that the ability of Bcl-2 and related proteins to block programmed cell death is explained by an ability to prevent cytochrome c exit from mitochondria (Kluck et al., 1997; Yang et al., 1997). This finding has implications for models explaining the mechanism of cytochrome c release from mitochondria. If mitochondria act to simply release cytochrome c when acted upon by events in the cytosol, then Bcl-2 family proteins must regulate these cytosolic events. Alternatively,

if a failure in mitochondrial function is tied to the release of cytochrome c, then Bcl-2 family proteins would be expected to actively support mitochondrial physiology.

To examine the role of mitochondrial physiology in the induction of programmed cell death, we have for several years been studying growth factor-dependent cell lines derived from hematopoietic lineages, characterizing changes in mitochondrial bioenergetics following removal of growth factors from cultures. It was originally hypothesized that decreases in signal transduction through growth factor receptors might result in an increased level of cellular ATP and a decline in ADP, since growth factor signal transduction depends on phosphorylation reactions to engage kinase cascades, activate new transcription, and stimulate translation. All three of these processes are energy-dependent. Despite this expectation, we have found in multiple cell lines that withdrawal of growth factor or serum leads to a reproducible and continuous decline in the ATP/ADP ratio (Vander Heiden et al., 1999). Following withdrawal of growth factors, the fall in the ATP/ADP ratio can be accounted for by a decline in mitochondrial substrates with which to maintain electron transport chain activity and the mitochondrial membrane potential (Vander Heiden et al., 2001). Careful analysis of NADH compartments in cells suggests that the majority of the decline in NADH available to maintain electron transport comes from loss of the NADH produced through glycolysis, and demonstrated that a common feature of growth factor signal transduction is to maintain glucose uptake and glycolytic metabolism (Harris et al., manuscript submitted). Anti-apoptotic Bcl-2 proteins such as Bcl-2 or Bcl-x_L act prior to apoptosis to dampen the decline in the ATP/ADP ratio, suggesting that they act to sustain the ability of mitochondria to maintain ATP production in the face of growth factor withdrawal (Vander Heiden et al., 1999).

Experiments on a number of cellular systems suggest that one common feature of cytokines involved in cell survival is their ability to maintain cellular glucose uptake. In studies characterizing IL-3 withdrawal-induced death in the IL-3-dependent cell line, FL5.12, we find that following IL-3 withdrawal, there is a rapid loss of the expression of three enzymes involved in the proximal steps of glucose uptake and glycolytic commitment (Rathmell et al., 2000). There is a rapid decline in both the mRNA and protein levels of GLUT-1, the major glucose transporter of hematopoietic cells, hexokinase, and phosphofructokinase-2 (Vander Heiden et al., 2001). As a result, within 12 hours of growth factor withdrawal, the cell experiences a 10-fold decline in its ability to take up glucose from its extracellular environment and cannot generate a sufficient supply of NADH to maintain electron transport at levels that would sustain cellular ATP levels. This has led us to a relatively simple model that suggests a common feature of growth factor survival-mediated signal transduction is the stimulation of nutrient uptake necessary for the production of NADH required to maintain mitochondrial bioenergetics (Figure 1). When growth factors are withdrawn, the loss of the ability of cells to autonomously take up sufficient nutrients to maintain the NADH supply leads to a progressive loss of remaining intracellular stores of metabolites that can be utilized to produce NADH. When NADH levels fall beyond a given threshold, mitochondria are no longer able to generate a sufficient mitochondrial membrane potential to maintain ion homeostasis. This results in a disruption in their physiology, the non-specific rupture of the outer mitochondrial membrane, and the release of cytochrome c into the cytosol, where it can initiate formation of an apoptosome.

Surprisingly, we have found that Bcl-2 or Bcl-x_L overexpression, while preventing cytochrome c release, has no effect on the loss of glucose uptake and glycolysis in response to growth factor withdrawal (Rathmell et al., 2000). As a result, Bcl-x_L can promote cell

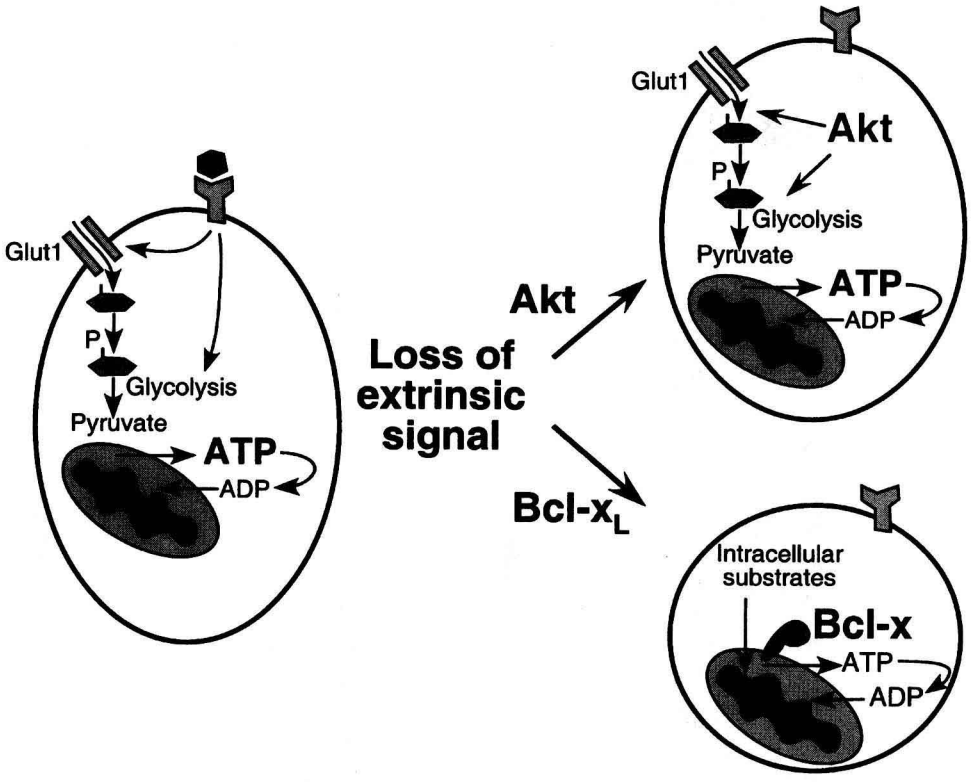

Figure 1. Bcl-x_L and Akt mediate cell survival via distinct mechanisms. Signals emanating from growth factor receptors support both glucose uptake and glycolysis, providing substrates required for maintaining mitochondrial homeostasis. When growth factors are removed from the medium, activated Akt can promote cell survival by supporting continued glucose uptake and metabolism (top), while Bcl-x_L can prevent the disruption of mitochondrial homeostasis, allowing cells to adapt to a diminished supply of energy driven by the consumption of intracellular nutrients (bottom).

survival under conditions of not only growth factor withdrawal but also glucose withdrawal (Figure 2). Nevertheless, the changes in mitochondrial physiology that immediately precede cytochrome c release are not detected when Bcl-x_L is expressed, suggesting that in the presence of Bcl-x_L, the decline in glycolysis is not causing a disruption in mitochondrial physiology. Thus, when antiapoptotic Bcl-2 proteins are overexpressed, cells may have sufficient time to alter their physiology to adapt to long-term maintenance of their mitochondrial integrity through the induction of autophagy, in which efficient utilization of intracellular substrates through lysosomal degradation is utilized to maintain a supply of NADH and thus mitochondrial integrity over a prolonged period of time (Vander Heiden et al., 1997). Under this model, the expression of the proapoptotic Bcl-2 family members, such as Bax and Bak, prevents cells from undergoing such an adaptation and leads to the induction of apoptosis (Figure 2A). Based on these models, it appears that the relative balance of pro- and anti-apoptotic Bcl-2 family members determines the sensitivity of cells to undergo cell death in response to growth factor withdrawal. Sensitivity of cells to apoptosis can be further modulated by transcriptionally-activated or post-translationally-modified BH3-containing proteins, which act as regulators of the functions of Bcl-2 proteins (Huang

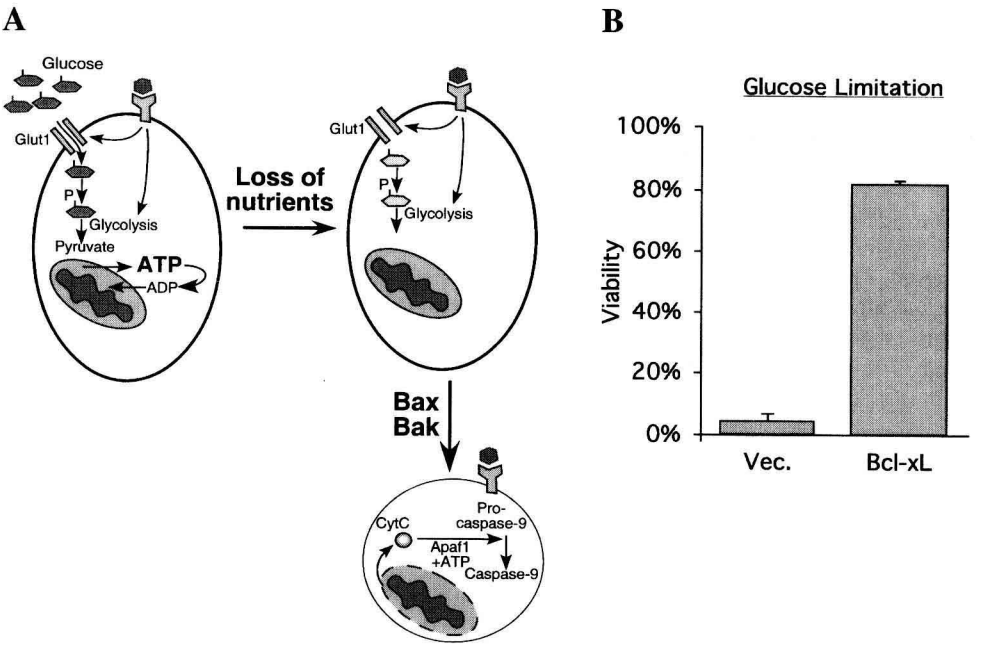

Figure 2. Control of apoptosis under nutrient-limited conditions. A. When nutrients such as glucose become limiting, cells fail to provide mitochondria with the nutrients required to maintain mitochondrial homeostasis, even though growth factor may still be present. Cytochrome c is released in a Bax- and Bak-dependent manner, resulting in the activation of Caspase 9 and downstream caspases. B. Bcl-x_L prevents cell death when glucose is limiting. Vector control or Bcl-x_L-expressing FL5.12 cells were cultured in medium containing a limiting concentration of glucose (0.02 mM). Cell viability was measured by propidium iodide exclusion after 48 hours of culture.

and Strasser, 2000). Consistent with this, we find that cells deficient in both Bax and Bak are profoundly resistant to apoptosis following growth factor withdrawal (Figure 2A) (Cheng et al., 2001; Lindsten et al., 2000; Zong et al., 2001). This model has important implications for how long cells can survive in the absence of growth factor withdrawal.

Based on the model presented in Figure 1, either a loss of the proapoptotic functions of Bax and Bak, or an overexpression of Bcl-2 or Bcl-x_L, will allow cells to acclimate to a more efficient, highly coupled form of mitochondrial maintenance. However, this occurs as a result of the progressive consumption of cellular constituents, since intracellular organelles and contents must be oxidized to provide a continuous supply of NADH. As a result, cells progressively atrophy in the absence of extracellular signal transduction, and although they remain alive, they exist in a state which requires prolonged recovery before they can reinitiate cell proliferation following growth factor readdition (Casey J. Fox and C.B.T, manuscript submitted). This is best seen when cells overexpressing Bcl-x_L are followed over a long period of time of growth factor withdrawal. Under these conditions, Bcl-x_L-expressing cells maintain nearly uniform survival for approximately one week. Thereafter there is a continual loss of cell viability, with few, if any, recoverable cells three weeks after growth factor withdrawal. Throughout this entire time course, cells undergo progressive atrophy, which causes a progressively longer delay before cells can reenter S phase following readdition of growth factors. Thus, Bcl-x_L-dependent cell survival is

accompanied by a cost: the progressive atrophy of the cell and loss of its capabilities to carry out effector functions or cell proliferation.

These observations suggest that growth factor signal transduction must have components that directly stimulate glucose uptake and glycolytic commitment. The insulin receptor signaling pathway represents a paradigm for growth factor signal transduction that directly modulates glucose metabolism (Hajduch et al., 2001). In both muscle and fat cells, the ligand-induced activation of the tyrosine kinase activity of the insulin receptor leads to the phosphorylation of IRS-1, which in turn acts as an adaptor in the activation of PI3 kinase. The products of PI3 kinase lead to the membrane recruitment and activation of the serine/threonine kinase Akt, which can activate the three proximal steps of glycolysis, including glucose transporter expression and surface translocation, hexokinase-2 expression, and PFK-1 activity through allosteric regulation of PFK-2 (J.C.R. and C.B.T. manuscript submitted).

Like many other survival cytokines involved in maintaining cell survival, IL-3 stimulates Akt activity, suggesting that growth factor receptors modulate cellular bioenergetics in hematopoietic cells, as has been described for the insulin receptor in fat and muscle cells (Songyang et al., 1997). Therefore, the bioenergetic effects of overexpression of a constitutively active form of Akt on the survival of FL5.12 cells in the absence of growth factor were examined (Plas et al., 2001). Cells overexpressing constitutively active myristoylated Akt were produced, and their ability to survive following growth factor withdrawal was examined over a three week time period. Akt provided potent ability of cells to survive over this period of time such that approximately 30% of cells were capable of surviving and undergoing rapid recovery following readdition of IL-3, even after three weeks of growth factor withdrawal. However, a decidedly different pattern of cell survival kinetics was observed. A large number of Akt cells underwent cell death in the first week following growth factor withdrawal. However, after the first week, cells apparently had undergone an accommodation that allowed them to survive for a prolonged period of time without significant further diminution in their survival. This occurred in the absence of proliferation and was observed in multiple clones. Furthermore, unlike Bcl-x_L-transfected cells, Akt-transfected cells (although they withdrew from the cell cycle) maintained greater cell size in G1 throughout the period of time of growth factor withdrawal. This correlated with the ability of Akt to maintain GLUT-1 expression, glycolytic activity, and mitochondrial membrane potential (Plas et al., 2001). Furthermore, although constitutive Akt activation provides prolonged protection from apoptosis induced by growth factor withdrawal, it fails to protect from glucose withdrawal (Figure 3). Thus, it appears that following growth factor withdrawal, the activity of Akt on the proximal steps of glycolysis allows cells to maintain a higher level of cell autonomous nutrient uptake, thereby preventing disruption of mitochondrial homeostasis and the release of cytochrome c.

The ability of Akt to maintain glucose transport expression on the cell surface was examined by confocal microscopy. In addition to maintaining the overall expression of GLUT-1 on cells, Akt selectively maintained its expression at the cell surface, accounting for its ability to sustain glucose uptake and glycolysis. This activity of Akt to maintain nutrient transporters was not confined only to the uptake of glucose. A number of amino acids can also contribute to the production of NADH under conditions of nutrient limitation, and cell culture media contains high levels of both essential and nonessential amino acids. In growth factor-dependent cells, removal of growth factor induces a rapid internalization of amino acid transporters as visualized by the antibody 4F2, which is directed against the light chain of the common amino acid transporter. This intracellular sequestration and

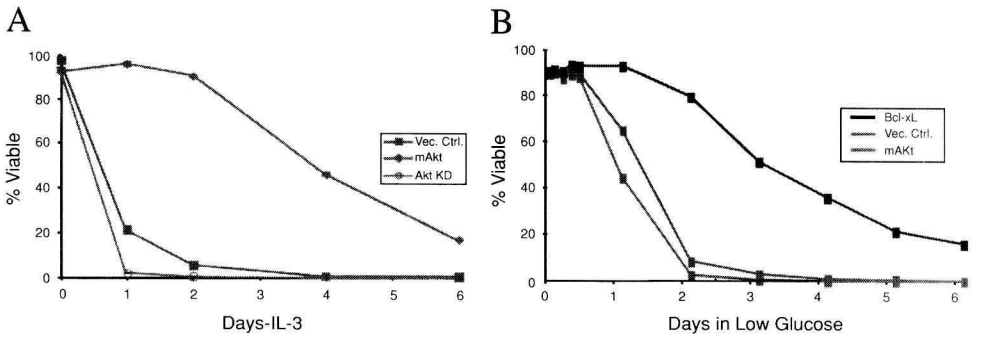

Figure 3. Akt prevents death in response to growth factor withdrawal, but not glucose withdrawal. A. Cells expressing activated Akt (mAkt) or kinase-deficient Akt (Akt KD) were cultured in the absence of growth factor for up to 6 days. Cell viability in mAkt, Akt KD, and vector control cells was measured by propidium iodide exclusion at the indicated time points. B. Vector control, mAkt-, or Bcl-x_L-expressing cells were cultured in limiting concentrations of glucose. Cell viability was measured as described in A.

degradation within the lysosome of amino acid transporters was prevented in Akt-expressing cells (Edinger and Thompson, 2002). Thus, Akt promotes not only the uptake of glucose as previously described in insulin responsive tissues, but also maintains amino acid uptake in hematopoietic cells. This increased nutrient uptake induced by Akt is potentially sufficient to account for its ability to maintain macromolecular synthesis and cell size in the face of growth factor withdrawal on a cell autonomous basis.

Based on these studies, it appears that the regulation of mitochondrial bioenergetics contributes to the susceptibility of cells to induction of programmed cell death, and that mitochondrial bioenergetics are directly regulated by growth factor receptor modulation of the ability of cells to take up nutrients. In the absence of extracellular signals to direct nutrient uptake, cells lack the autonomous ability to take up sufficient nutrients to maintain themselves. How long cells can survive in the absence of extracellular nutrients is regulated either directly or indirectly by the activities of the Bcl-2 family members. Alternatively, enzymes that control nutrient uptake downstream of growth factor receptors, such as Akt, can be directly activated in a cell-autonomous fashion and contribute to oncogenic transformation by supporting uptake of extrinsic nutrients. Thus, it appears that Akt and Bcl-x_L regulate metabolism by distinct mechanisms, and this regulation can directly contribute to the apoptotic sensitivity of cells (Figure 1). Investigation of other genes involved in cell survival is likely to reveal an intricate connection between the sustained cellular metabolism and the ability of cells to suppress the induction of programmed cell death.

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Chapter 2

Thyroid Hormone-Induced Apoptosis during Amphibian Metamorphosis

TOSIKAZU AMANO, LIEZHEN FU, ATSUKO ISHIZUYA-OKA, AND YUN-BO SHI*

ABSTRACT: Anuran metamorphosis involves thyroid hormone (TH)-induced, systematic transformations of individual organs. The vast majority of the larval tissues are removed during this process. Among them is the complete degeneration of the tail and gills and reduction of small intestine by about 90% (lengthwise). Various morphological and cellular studies have shown that the removal of larval organs/tissues is through programmed cell death or apoptosis. Recent cloning and characterization of TH-regulated genes revealed that a group of genes encoding matrix metalloproteinases (MMPs) are activated by TH during metamorphosis in various organs. The activation of MMPs, which are extracellular or membrane-associated enzymes capable of degrading extracellular matrix (ECM) proteins, are in agreement with the previously observed remodeling/degradation of the ECM during metamorphosis. In vivo and in vitro studies have provided evidence to support that ECM remodeling by MMPs plays an important role in regulating apoptosis and cell migration during tissue remodeling.

Key Words: *Xenopus laevis*; thyroid hormone receptor; extracellular matrix (ECM); matrix metalloproteinase (MMP).

Abbreviations: TH, thyroid hormone; TR, TH Receptor; RXR, retinoid X receptor or 9-cis-retinoic acid receptor; MMP, matrix metalloproteinase, ECM, extracellular matrix; ST1, stromelysin-1; ST3, stromelysin-3; Col3, collagenase-3; Col4, collagenase-4, GLA, gelatinase-A; GLB, gelatinase-B.

Introduction

Anuran development is a biphasic process. Their embyogenesis leads to the formation of free-living aquatic tadpoles. After a finite period of growth, the tadpoles then undergo metamorphosis. This postembryonic process involves systematic transformations of essentially every organ/tissue, leading to the formation of adult organs/tissues that have

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