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Mechanisms and Methods in Glucose Metabolism and Cell Death

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Abstract

Glucose metabolism represents a critical physiological program that not only provides energy to support cell proliferation, but also directly modulates signaling pathways of cell death. With the growing recognition of regulation of cell death by glucose metabolism, many techniques that can be applied in the study have been developed. This chapter discusses several protocols that aid in the analysis of glucose metabolism and cell death and the principles in practicing them under different conditions.

1. Introduction

Cell death is a critical mechanism in maintaining tissue homeostasis, and misregulation of cell death can lead to development of a variety of diseases. For example, too little cell death contributes to cancer development or onset of autoimmune responses, whereas excessive cell death can cause degenerative diseases, such as neuron degeneration. Cell death is regulated by intracellular machineries that respond to changes of environment both inside and outside cells. Accumulating data have shown that metabolism plays an important role in the regulation of cell death, and studies on relationship between metabolism and cell death are expanding quickly (Gottlob *et al.*, 2001; Nutt *et al.*, 2005; Rathmell *et al.*, 2003; Zhao *et al.*, 2007). Thus, there is a growing need for the development of methods and techniques to be used in the analysis of glucose metabolism so that researchers are able to study changes and roles of metabolism in cell death. This chapter summarizes general methods developed to monitor and manipulate glucose uptake and metabolism with the background of study on cell death.

Glucose metabolism is a primary source of energy and biomaterials for the maintenance of life. In the first rate-determining step of the metabolism, glucose is transported across the plasma membrane by the facilitative glucose transporter (Glut) down its concentration gradient. Hexokinase (HK) on the mitochondria then phosphorylates glucose to glucose-6-phosphate (G6P). The product generally enters the glycolytic pathway, generating NADH, ATP, and pyruvate, or the pentose phosphate pathway (PPP). In the presence of sufficient oxygen, pyruvate from glycolysis can be fed into mitochondria and fully oxidized to produce more ATP. When oxygen is limited, however, pyruvate is disposed in the form of lactate and glycolysis becomes the main source for ATP production (Gatenby and Gillies, 2004). PPP plays an important role in the synthesis of nucleic acids for DNA and RNA, as well as generation of NADPH for the synthesis of lipids and maintenance of intracellular redox homeostasis.

There are three major forms of cell death that may be influenced by glucose metabolism: necrosis, autophagy, and apoptosis (Edinger and Thompson, 2004). Necrosis can occur when ATP levels decrease too dramatically and cells lose the ability to maintain intracellular homeostasis of ions and water, leading to rupture of the plasma membrane and leakage of the cytoplasm into the extracellular environment. Autophagy is an energy-dependent process of self-digestion that can result in cell death (Lum *et al.*, 2005). It is initiated when the uptake of

extracellular nutrient sources such as amino acids, glucose, or lipids decreases and cells must use intracellular components to support mitochondrial oxidation and energy production. When autophagy occurs, double-membrane vesicles form and engulf cytoplasm and organelles (autophagosome). The autophagosome then fuses with lysosome and the compartment inside is degraded. Small molecules from this digestion can be fed into mitochondria to generate ATP. The onset of autophagy requires energy, but the outcome of autophagy can be beneficial as more ATP may be produced from mitochondria. Nutrient generation by autophagy is generally useful to cells and autophagy only becomes lethal when digestion becomes excessive and most of the cytoplasm is consumed (Shintani and Klionsky, 2004). Nevertheless, autophagy can lead to a clean form of cell death as cells are broken down from the inside and the extracellular environment is not affected. Apoptosis is also an energy-dependent program to eliminate cells without disturbing the extracellular environment (Danial and Korsmeyer, 2004). Apoptosis is initiated through two primary pathways: the mitochondrial, or intrinsic, and the death receptor, or extrinsic (Danial and Korsmeyer, 2004). The intrinsic death pathway is regulated by Bcl-2 family proteins, and commitment to death occurs when mitochondrial cytochrome *c* is released. The extrinsic pathway is initiated when death ligands such as tumor necrosis factor α and Fas ligand bind to the cell membrane to initiate death receptor signaling. Both pathways eventually activate a family of cysteine–aspartic acid proteases known as caspases to execute cells. The activation and function of caspases are ATP dependent, thus energy is required for the continuance of apoptosis (Budihardjo *et al.*, 1999). There is a specific form of cell death, eryptosis, in nonnucleate cells such as red blood cells that is apoptosis-like in morphology (Lang *et al.*, 2006). Eryptosis requires caspases as well as Ca^{2+} -dependent channels and formation of ceramide (Lang *et al.*, 2006), all of which can be affected by the status of glucose metabolism and levels of ATP (De Luca *et al.*, 2005; Henquin, 2000). Therefore, eryptosis is also an energy-dependent form of cell death.

The most studied connection between metabolism and cell death is the effect of glucose metabolism on the intrinsic pathway of apoptosis. Bcl-2 family members are key regulators of this mitochondrial death pathway and include antiapoptotic, proapoptotic, and BH3-only proteins. When cells are exposed to stresses such as growth factor deprivation or DNA damage, BH3-only proteins are induced and activated and then they translocate to mitochondria to antagonize the function of antiapoptotic proteins such as Bcl-2, Bcl-xL, and Mcl-1. Proapoptotic proteins, such as Bax and Bak, then undergo conformation change and oligomerize on mitochondria to release cytochrome *c*. As cellular stresses signal through Bcl-2 family proteins to induce apoptosis, the influence of metabolism on the mitochondria death pathway may be reflected by its impact on Bcl-2 family members. It has been reported that loss of glucose leads to a decreased level of Mcl-1 (Alves *et al.*, 2006; Zhao *et al.*, 2007), induction of BH3-only proteins Noxa and Bim (Alves *et al.*, 2006; Kuan *et al.*, 2003), and Bax activation (Chi *et al.*, 2000; Vander Heiden *et al.*, 2001). These findings strongly suggest that Bcl-2 family members are regulated by glucose metabolism.

A direct interaction between glucose metabolism and apoptosis can also be found in cancer, as both metabolic and apoptotic pathways are altered in cancer cells (Gatenby and Gillies, 2004; Hanahan and Weinberg, 2000; Warburg, 1956). Glucose metabolism is increased greatly in many cancer cells and glycolysis becomes the main source for ATP production (Gatenby and Gillies, 2004). This phenotype has been appreciated for decades as the Warburg effect and is often associated with overexpression of glycolytic genes such as Glut1 and HKs (Smith, 2000; Warburg, 1956; Younes *et al.*, 1997). A similar metabolic phenotype can also be found in growth factor-stimulated cells. Upon stimulation, cells undergo hypertrophy and their glucose metabolism increases dramatically to meet the energy demand for proliferation. In addition, in both cancer cells and growth factor-stimulated cells, cell death programs are suppressed to ensure cell survival. There are also reports that the status of glucose metabolism changes prior to cell death when cells are stressed (Byfield *et al.*, 2005; Campbell *et al.*,

2004; Haberkorn *et al.*, 2001; Jones *et al.*, 2005; Morissette *et al.*, 2003; Rathmell *et al.*, 2003a,b; Ward *et al.*, 2007; Wolin *et al.*, 2007; Zhao *et al.*, 2007; Zhou *et al.*, 2002). All these correlations have led to a hypothesis that mutual regulation may exist between these two physiological machineries (Rathmell *et al.*, 2003a; Zhao *et al.*, 2007; Zhou *et al.*, 2002).

Recent findings have supported the notion that glucose metabolism regulates cell death pathways (Fig. 22.1). We have demonstrated that maintenance of glucose metabolism by overexpression of Glut1 and/or HK1 in both cell lines and primary hematopoietic cells stabilizes Mcl-1 and attenuates cell death induced by growth factor withdrawal (Zhao *et al.*, 2007). Increased glucose metabolism protects cells by activating protein kinase C and subsequent inhibition of GSK-3, which, upon activation, promotes the degradation of Mcl-1. It has also been reported that NADPH, which is generated primarily from the PPP, mediates antiapoptotic function in a variety of conditions. For example, during DNA damage or reactive oxygen species (ROS) burst, p53 is activated to induce protein TIGAR, which lowers the flux of glucose metabolism through glycolysis and increases the relative flux through PPP (Bensaad *et al.*, 2006). As additional NADPH and glutathione are generated, ROS damages are reduced and cells are protected from cell death. Another novel antiapoptotic signal by NADPH was identified in *Xenopus* egg extracts (Nutt *et al.*, 2005). A sufficient level of NADPH is required to maintain CamKII phosphorylation of caspase-2 to inhibit its cleavage and activation. Inhibition of PPP and loss of NADPH lead to increased caspase-2 activity and cytochrome *c* release from mitochondria. In addition, loss of glucose-6-phosphate dehydrogenase (G6PD) has been associated with increased eryptosis, suggesting that PPP is important in maintaining the survival of red blood cells (Lang *et al.*, 2006).

Although the direct contribution from glucose metabolism to evasion of apoptosis in cancer cells is still not clear, it has been found that oncogenes such as Akt utilize signals from glucose metabolism to protect cells from apoptosis (Gottlob *et al.*, 2001; Rathmell *et al.*, 2003b). Akt is a master regulator of glucose metabolism. It maintains surface localization of Glut1 and mitochondrial localization of HKs and increases glucose uptake in cells (Majewski *et al.*, 2004; Wieman *et al.*, 2007). Akt also has a potent antiapoptotic function as it can inhibit Bax conformation change and cytochrome *c* release upon growth factor withdrawal (Rathmell *et al.*, 2003b). Interestingly, when glucose is limited, Akt fails to prevent cell death (Rathmell *et al.*, 2003b). This suggests that Akt needs glucose metabolism to mediate its antiapoptotic function. Other oncogenes, such as Ras and BCR-abl, also have the ability to both upregulate glucose metabolism and prevent cell death (Bentley *et al.*, 2001; Valverde *et al.*, 1998). It will be interesting to determine whether their antiapoptotic function also requires metabolic signals.

Given the importance of glucose metabolism in the regulation of cell death, it is necessary to combine the techniques from both fields and to develop novel methods to monitor the changes of glucose metabolism in stressed cells. Models and approaches to study the effects of glucose metabolism on cell death are needed to establish the role of glucose metabolism in a variety of experimental systems. This chapter describes some approaches to directly test the effect of glucose metabolism on apoptosis.

2. Methods

2.1. Metabolism and forms of cell death

Apoptosis, necrosis, and autophagy each have different metabolic requirements that can influence the cell death processes. At its extreme, necrotic cells simply break down and do not require any ATP for death. In contrast, apoptosis requires ATP-dependent caspase activation via the apoptosome to digest cells from the inside and autophagy depends on lysosomes and ATP-dependent regulation and trafficking of intracellular membranes to degrade cells. Changes in cellular nutrients or metabolic status can, therefore, change cell death pathways.

Decreased glucose availability, for example, can switch apoptosis to necrosis when ATP becomes depleted (Leist *et al.*, 1997; Lieberthal *et al.*, 1998).

There are both morphological and biochemical methods to distinguish these cell death pathways. As these models of death were initially described morphologically, the most accurate approach to determine the form of cell death is to determine the morphology of dying cells by electron microscopy (EM). Rupture of cells and organelles, loss of membrane integrity, and no sign of chromatin condensation are characteristic of necrosis. If the morphology of cells appears to be membrane blebbing, nuclear condensation, fragmentation of cells into small membrane-bound vesicles, it indicates that cell death is through apoptosis. When autophagy is initiated, many double membrane vesicles will be observed in cells and organelles are often engulfed in these vesicles. The cell membrane still maintains integrity and no leakage of cytosol into the extracellular environment will be observed. Molecular approaches are also available to help determine the form of cell death. ATP content in dying cells can be measured using a luciferase-based ATP assay kit. Dramatic loss of ATP may suggest that cells do not have sufficient energy to engage ATP-dependent death pathways and may only die by necrosis. An assay of caspase activity will provide information on the executioners of cell death. Increased caspase activity in dying cells suggests that cells die of apoptosis, whereas necrotic cells do not show activity of caspases. Modification of LC3 will indicate autophagy, which can be monitored by altered mobility on SDS-PAGE or a punctuated distribution of LC3–GFP by confocal microscopy. Results from individual approaches, however, may not be conclusive and are most useful when combined or with EM analysis of cell death.

2.2. How to monitor glucose metabolism

When the effect of glucose metabolism on cell death is studied, it is important to know what changes happen to glucose metabolism after cell stresses. After uptake, glucose is phosphorylated by HK1 to become G6P, which can enter either the glycolysis pathway or the PPP. The following protocols can be used to measure the uptake of glucose and flux of glucose to glycolysis. Since only live cells should be used to measure glucose metabolism, these experiments should be done before cells commit to die or live cells need to be sorted before the performance of these experiments.

2.2.1. Glucose uptake—Glucose is essential for the survival of most cells, and glucose homeostasis can be monitored by measuring glucose uptake levels. Levels of glucose uptake are dynamic and regulated, which can be seen as cells display increased glucose uptake during growth and proliferation and decreased glucose uptake when cells atrophy and die. Glucose uptake can be measured by exposing cells to a radiolabeled glucose analog 2-deoxyglucose (2-DOG), which can be taken into cells via facilitative glucose transporters and phosphorylated by hexokinases to glucose-6-phosphate but is not metabolized further.

2.2.1.1. Reagents: Kreb's Ringer HEPES (KRH) buffer: 136 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl₂, 1.25 mM MgSO₄, 10 mM HEPES, pH 7.4

2-Deoxy-D-[H³]glucose (2 μ Ci/rxn)

Phloretin 200 μ M

Dow Corning 550 silicon fluid

Dinonyl phthalate

NaOH 1 M

Phosphate-buffered saline (PBS)

2.2.1.2. Protocol: To measure glucose uptake in nonadherent cells, cells are washed once in PBS and resuspended in KRH. 2-Deoxy-D-[H³]glucose (2 μ Ci/rxn) is then added for a period of either 1 or 5 min at 37°. The reactions are quenched by the addition of ice-cold 200 μ M phloretin and centrifugation through an oil layer, which consists of a 1:1 ratio of Dow Corning 550 silicon fluid and dinonyl phthalate. After centrifugation, the cell pellets are washed with KRH and solubilized in 1 M NaOH, and radioactivity is measured with a scintillation counter. Figure 22.2 demonstrates that levels of glucose uptake can be measured with endogenous levels of glucose transporters in both the presence and the absence of growth factor. The assay also indicates a threefold increase in glucose uptake when FLAG-tagged Glut1 is stably overexpressed. For adherent cells, simply wash cells in PBS, culture in KRH with radiolabeled glucose for 1 or 5 min, rinse three times in PBS, and lyse cells in the dish to determine the amount of radiolabeled glucose internalized. The measurement of glucose uptake, however, does not differentiate between surface and total glucose transporter levels and glucose transporter activity, which are all key factors in the amount of glucose uptake. In order to decipher between these factors, total and surface levels of glucose transporters can be measured.

2.2.2. Glut1 protein levels—In addition to measuring glucose uptake, it is important to quantify the total amount of Glut1 in cells. Glut1 is highly hydrophobic, with 12 transmembrane segments, and can form large aggregates upon cell lysis. In addition, Glut1 is glycosylated on an asparagine residue on the first exofacial loop (Asano *et al.*, 1991) that causes even nonaggregated Glut1 to run as a smear on immunoblots. Using a standard cell lysis, 10 min on ice in a RIPA lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA, protease inhibitors) followed by centrifugation for 10 min at 4° to preclear the lysates, and boiling for 10 min in 5 $\times$ sample buffer (50% glycerol, 250 mM Tris, pH 6.8, 10% SDS, 0.05% bromophenol blue) led to a Glut1 smear at a high molecular weight by immunoblot (Fig. 22.3, lane 1). Glut1 aggregation can be minimized by using an alternative cell lysis protocol.

2.2.2.1. Reagents: Glut1 lysis buffer: 1% Triton X-100, 0.1% SDS, protease inhibitors

Glut1 5 $\times$ sample buffer: 1.56 ml 2 M Tris-HCl, pH 6.8, 1 g SDS, 5 ml glycerol, 2.5 ml 2-mercaptoethanol, 5 mg bromophenol blue

NP-40

PNGaseF kit (New England Biolabs)

2.2.2.2. Protocol: Cells are lysed in Glut1 lysis buffer for 1 h on ice and then precleared by centrifugation for 10 min at 4°. Glut1 sample buffer is then added at 1 $\times$ and samples are incubated at room temperature for 30 min (Fig. 22.3, lane 2). To fully collapse the Glut1 band, it is also necessary to remove Glut1 glycosylation by treating the cells with PNGaseF before loading the samples on a gel as seen in Fig. 22.3 (lane 3). Cell lysates that have been prepared via the Glut1 lysis method can be used for PNGaseF treatment. For the treatment as indicated by the manufacturer, dilute 10 μ g protein in 9 μ l of H₂O, add 1 μ l of denaturing buffer, and incubate for 30 min at room temperature. After incubation, add 4 μ l of H₂O, 2 μ l NP-40, 2 μ l G7 reaction buffer, and 2 μ l PNGaseF for 1 h at 37° followed by the addition of 1 $\times$ Glut1 sample buffer and an additional 30-min incubation at room temperature before loading the gel. The ability to collapse the Glut1 band allows precise quantitation of total Glut1 protein.

2.2.3. Glut1 trafficking—Glut1 must be on the cell surface to transport glucose into the cell, and approaches to measure cell surface Glut1 levels are critical in understanding how cell stresses and death pathways affect glucose uptake. Surface staining of endogenous Glut1 may be accomplished using labeled HTLV-env proteins, which bind Glut1 to serve as a coreceptor for the virus, or anti-Glut1 extracellular domain-specific antibodies. Binding of HTLV-env does not depend solely on Glut1, however, and we have found the alternative approach of using antibodies directed against the extracellular domain of Glut1 to provide poor sensitivity. In addition, while these techniques can be successful for human cells, it is less clear how well such approaches work with murine cells. To bypass these concerns and allow highly specific tracking of Glut1 surface levels and trafficking, we have generated an epitope-tagged version of Glut1. A tandem 2X-FLAG tag is inserted in the first exofacial loop of Glut1, allowing surface levels of Glut1 to be detected via an anti-Flag antibody and flow cytometry analysis. While this approach does not measure changes to endogenous Glut1 trafficking, we have found that FLAG–Glut1 trafficking effectively represents the trafficking pathway of endogenous Glut1 and, therefore, provides an effective tool in determining how Glut1 trafficking is affected by various cell stresses. To measure surface FLAG–Glut1 levels, stably transfected or retrovirally infected cells are washed once in PBS/2% FBS and blocked with anti-Fc γ III/II (to block background staining from Fc receptors in hematopoietic cells) and 5% rat serum for 10 min on ice. Primary rabbit anti-FLAG is added at 1:100 for an additional 20 min. Cells are then washed twice with PBS/2% FBS to wash off the unbound FLAG antibody. Cells are then incubated in PBS/2% FBS, 5% rat serum, and R-PE donkey anti-rabbit at 1:100 for 20 min on ice. The remaining secondary antibody is washed off with PBS/2% FBS and cells are fixed in 1% paraformaldehyde in PBS to allow subsequent analysis of surface levels of FLAG–Glut1 via flow cytometry. Figure 22.4 shows a histogram of the mean surface FLAG–Glut1 levels of FL5.12 cells in the presence or absence of the cytokine interleukin-3 (IL3). The histogram plot indicates a loss (shift to the left) of surface FLAG–Glut1 when the cytokine is withdrawn from the cells. It is important to note that since the total levels of Glut1 can also be a factor in glucose metabolism, it is necessary to normalize the surface levels of FLAG–Glut1 to total levels of FLAG–Glut1. This can be done by intracellular flow cytometry of parallel samples or by measurement of the pixel density on an immunoblot. Measuring glucose uptake, total Glut1 protein levels, and surface levels of Glut1 together provides a detailed picture of glucose metabolism and glucose transporters in cell stress and death.

2.2.4. Measurement of glycolysis—After glucose uptake, glucose flux through glycolysis can be highly regulated and impact cell fate by the control of cellular ATP levels. When cells undergo death upon stresses such as DNA damage, an increase in glycolysis and ATP levels may favor the activation of caspases and lead to apoptotic cell death (Zamaraeva *et al.*, 2005). Conversely, decreased glycolysis is likely to induce autophagy to supplement cells with ATP from self-digestion, and as a consequence, cells may die of apoptosis and/or autophagy, depending on the extent of autophagy and ATP availability (Lum *et al.*, 2005). Glycolytic flux can be determined by analyzing the conversion of [5-³H]glucose to ³H₂O (Vander Heiden *et al.*, 2001). ³H on C5 of glucose is released in the form of H₂O at the second to last step of glycolysis, when 2-phosphoglycerate is converted to phosphoenolpyruvate by enolase.

2.2.4.1. Reagents: Krebs buffer: 115 mM NaCl, 2 mM KCl, 25 mM NaHCO₃, 1 mM MgCl₂, 2 mM CaCl₂, 0.25% FBS, pH 7.4

[5-³H]Glucose

HCl 0.2 M

2.2.4.2. Protocol: To measure the rate of glycolytic flux and to determine if cell stresses affect this pathway, cells are washed in PBS once and resuspended in Krebs buffer. After a 30-min

incubation, Krebs buffer containing glucose and [5-³H]glucose is added to cells to make final concentration of 10 mM glucose containing [5-³H]glucose (10 μ Ci/ml) in 0.5 ml and incubated for 1 h at 37°. After 1 h, 0.1 ml of 0.2 M HCl is then added to the mixture to stop the reaction. Then 0.2 ml of the reaction mixture is transferred to a small open tube that is placed in a scintillation vial that contains 0.5 ml water. The scintillation vial is then sealed to allow ³H-H₂O to evaporate from the tube and condense in the 0.5 ml water in the bottom of the scintillation vial. In time, ³H₂O will establish an equilibrium between the cell lysate and the water in the scintillation vial. In contrast, ³H-glucose will not evaporate and will remain in the cell lysate. After at least 24 h, ³H in water and the cell lysate are counted to determine the rate of glycolytic flux. The fraction of conversion of glucose to H₂O is calculated as the following:

$$F = (\text{activity}_{\text{vial}} / V_{\text{H}_2\text{O in vial}} * V_{\text{tube}} + \text{activity}_{\text{vial}}) / (\text{activity}_{\text{tube}} + \text{activity}_{\text{vial}})$$

2.2.5. Mitochondrial potential—Mitochondria are key organelles of ATP production. Products from glycolysis such as pyruvate are fed into mitochondria to generate ATP through oxidative phosphorylation, which is driven by transmembrane electrical potential, known as mitochondrial potential. When glucose metabolism is altered, the availability of substrates changes and mitochondrial potential will change accordingly. Cytochrome *c* release from mitochondria, such as occurs in apoptosis, also decreases mitochondrial potential because cytochrome *c* is a key component of the electron transportation chain. Thus, mitochondrial potential can be used as an indicator of the status of glucose metabolism, and a decrease in mitochondrial potential suggests a loss of glucose metabolism and a possible onset of apoptosis.

Mitochondrial potential can be measured with fluorescent dyes such as tetramethylrhodamine ethylester (TMRE). There are a number of other dyes that may fulfill this purpose, but TMRE is known to be highly sensitive to small changes in potential that may occur prior to apoptosis. TMRE is a cell-permeable lipophilic cation that can accumulate in mitochondria, and its fluorescence increases in proportion to mitochondrial potential. To stain cells with TMRE, cells are treated by death stimuli over a certain time course and 150 nM of TMRE is added to the culture for 30 min at 37°. For negative staining control, 50 μ M of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is added together with TMRE. CCCP is a protonophore and can diminish mitochondrial potential to show the background staining of TMRE that is not due to mitochondrial potential. After incubation, cells are washed with PBS once and resuspended in PBS containing 2% FBS with 15 nM of TMRE to maintain TMRE equilibrium. Cells are then subject to flow cytometry to analyze the relative intensity of red fluorescence.

2.3. Overexpression of glycolytic genes to increase glucose metabolism

Loss of glucose metabolism may contribute to cell death or simply occur concurrently with cell death. Methods to modify glucose metabolism are needed, therefore, to study the effect of glucose metabolism on cell death. One approach is to promote elevated glucose uptake by expression of a glucose transporter and to determine the effects of this alteration on cell metabolism on cell death. In the absence of this or a similar gain-of-function experiment, it is impossible to fully ascertain the role of loss of metabolism on cell death. In order to increase glucose metabolism without disturbing other signal pathways, we overexpressed glycolytic genes in nontransformed, growth factor-dependent cell lines to boost metabolic pathways (Rathmell *et al.*, 2003b). In particular, Glut1 and HK1 were chosen because they regulate the first rate-limiting steps of the whole glucose metabolism. Glut1 transports glucose down its concentration gradient into cells and HK1 phosphorylates glucose to glucose-6-phosphate, which is the starting material for both glycolysis and the PPP. Glut1 is highly regulated at localization, trafficking, and protein half-life, and the activity of HK1 is sensitive to localization and glucose concentration (Edinger *et al.*, 2003; Rathmell *et al.*, 2000; Wieman *et al.*, 2007; Wilson, 2003). Upon growth factor deprivation, Glut1 is internalized and degraded in

lysosomes, leading to decreased glucose uptake (Edinger *et al.*, 2003; Rathmell *et al.*, 2000; Wieman *et al.*, 2007). Overexpression of Glut1 can overwhelm this regulation and, thus, maintain the inflow of glucose under conditions when Glut1 is normally internalized and glucose uptake is reduced (Rathmell *et al.*, 2003b; Zhao *et al.*, 2007). Overexpression of HK1 can “trap” glucose inside cells because phosphorylated glucose cannot be transported out of cells, but can only enter downstream metabolic pathways.

There are several advantages of overexpressing glycolytic genes to increase glucose metabolism compared to simply increasing the concentration of glucose in media, which also can promote glucose uptake. First, glucose uptake rates are often more limited by surface Glut1 levels than the availability of glucose based on the low K_m of Glut1 and the relatively high levels of glucose normally available *in vivo*. Second, because Glut1/HK1 overexpression does not require a change of glucose concentration to increase glucose uptake, cells will not be affected by increased osmotic pressure. High extracellular glucose can also increase ROS levels in cells, which can affect cell death by inducing proapoptotic BH3-only proteins (Callaghan *et al.*, 2005; Sade and Sarin, 2004). We have observed Glut1/HK1 cells, however, to have lower levels of ROS than control cells (unpublished results). Third, overexpression of Glut1/HK1 reflects a physiological model of high cell intrinsic glucose metabolism, such as occurs in cancer or activated lymphocytes (Frauworth and Thompson, 2004; Semenza *et al.*, 2001). In contrast, increased extracellular glucose for sustained periods models another pathological condition, diabetes, in which loss of systemic metabolic control may have an important impact on individual cell physiology and fate. Selecting which approach, therefore, may be dictated by the disease process in question, with hyperglycemia leading itself to increased glucose levels and altered signaling of cancer leading itself to overexpression of glucose transporters.

When overexpressing glycolytic genes to promote glucose metabolism, several criteria should be kept in mind. Glucose transporters and HKs are preferred candidates because they determine the first rate-limiting steps in glucose metabolism. Increased G6P from the phosphorylation of glucose by HKs is often sufficient to drive the progress of the pathways of glucose metabolism for a certain period of time. Increased levels of downstream genes, however, may not have a similar effect if the need for increased glucose uptake cannot be met. It is also important to determine which Gluts and HKs to overexpress, as they have different properties in different cells. For example, Glut1 is expressed ubiquitously, has a low K_m and high transport rate, and often localizes to the cell surface. Glut3, however, may localize differently, such as to intracellular compartments, thus making it not suitable for maintaining glucose uptake. HK1 and HK2 have a similar K_m for glucose at 0.05 mM, whereas glucokinase (GK), which is also known as hexokinase 4 and is expressed mainly in the liver, has a much higher K_m for glucose at 5 mM (Wilson, 2003). Because intracellular glucose rarely reaches a concentration of 5 mM, the physiologic concentration of glucose in blood, GK will have very low activity in normal culture conditions and is not ideal for catalyzing glucose phosphorylation in cell culture. The role that specific glucose transporters may play in specific pathological conditions is unclear, but the different cell surface trafficking patterns of different transporters may profoundly influence the overall cellular glucose uptake and cell death pattern.

2.4. Inhibition of glucose metabolism

Decreasing glucose metabolism can be an alternative approach to study effects of glucose metabolism on cell death. There are several ways to limit glucose metabolism, the most direct method being decreasing the concentration of glucose in culture or even depleting glucose from media. The concentration of glucose ranges from 3 to 5 mM physiologically in normal blood, but may go much lower in tissues or poorly vascularized areas. It has been suggested that cells can behave normally when the concentration of glucose goes down to 0.1 mM (Vander Heiden *et al.*, 2001). When the glucose concentration is lower than 0.1 mM, substrates for glucose

metabolism become limited and cell growth is slowed. Using a glucose analog to replace glucose in media is another method used to limit glucose metabolism. 2-DOG is often used as an analog of glucose and can be taken up by cells through glucose transporters. However, after phosphorylation by HK, 2-DOG cannot be metabolized further by glycolysis. Phospho-2-DOG then accumulates, resulting in feedback inhibition of HKs. Thus, 2-DOG limits overall glucose metabolism. Despite its inability to progress through glycolysis, it should be noted that 2-DOG may be metabolized through a few steps in the PPP (Mourrieras *et al.*, 1997), providing a limited amount of NADPH for cells. The few NADPH generated by metabolizing 2-DOG may provide a survival advantage for cells, as NADPH may inhibit caspase-2 activation and ROS accumulation. It should also be noted that low glucose or 2-DOG can also induce an unfolded protein response via hypoglycosylation of proteins in the ER. Apoptosis can be induced by ER stress through upregulation and activation of BH3-only proteins (Puthalakath *et al.*, 2007; Reimertz *et al.*, 2003). Thus, when studying effects of low glucose on cell death, contributions from ER stress should be considered.

Chemical inhibitors can also be used in cell culture to inhibit glucose metabolism. Trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP) is a protonophore and can uncouple mitochondrial oxidative phosphorylation. Use of FCCP will decrease mitochondrial potential and ATP production, and increase ROS generation in cells. Glycolysis and PPP may still go on as they do not proceed in mitochondria. Dehydroepiandrosterone (DHEA) and 6-aminonicotinamide (6-AN) are inhibitors of G6PD, which catalyzes the first step of the PPP. Inhibition of G6PD with DHEA or 6-AN stops the PPP, thus limiting the generation of NADPH and ribosugar for the synthesis of DNA and RNA. Iodoacetate can be added to the culture to stop glycolytic flux, as it inhibits the function of glyceraldehyde-3-phosphate dehydrogenase, a critical enzyme in glycolysis. The advantages of using chemical inhibitors are easy access, low cost, and relatively fast screening. The general concern, however, is their specificity, and the results from chemical inhibitors can be misleading. Thus, data from inhibitors are not conclusive and should be used only as supportive evidence. Ultimately, it will be necessary to confirm the result of chemical inhibitors by downregulation of target genes by RNAi.

2.5. Bcl-2 markers for glucose metabolism

Ultimately, cell death occurs via the Bcl-2 family of proteins when glucose metabolism is prevented. Several Bcl-2 family members have been reported to regulate cell death in response to changes in metabolic status and their levels can be analyzed when cells are exposed to stresses that may affect metabolism. Bax changes its conformation and becomes activated when cells are deprived of glucose (Chi *et al.*, 2000; Vander Heiden *et al.*, 2001). To monitor the conformation change of Bax, a conformation-specific antibody (6A7) that only recognizes active Bax can be used to immunoprecipitate Bax and the precipitates can be subjected to immunoblot to determine the levels of active Bax. Alternatively, stressed cells can be fixed and immunostained with the antibody, and active Bax can be analyzed by flow cytometry. Levels of Mcl-1 decrease when glucose becomes limited, whereas increased glucose metabolism protects Mcl-1 from degradation (Alves *et al.*, 2006; Zhao *et al.*, 2007). In BH3-only proteins, Noxa has been reported to be induced and initiate cell death in T cells upon loss of glucose (Alves *et al.*, 2006). Thus, Mcl-1 and Noxa can be analyzed by immunoblot to determine the effect of glucose metabolism on cell death.

3. Conclusion

This chapter described general approaches that can be applied to the study of glucose metabolism and cell death. With these methods, glucose metabolism in cells can be increased or decreased to analyze its effect on cell death programs. Glucose metabolism can also be monitored to provide information on metabolic changes in response to cell stresses. These

protocols should be subject to modifications to fit different experimental settings and data from them should be interpreted in combination with information on forms of cell death. In particular, there is a need for single cell assays and biochemical approaches that utilize minimal material to allow more accurate analysis of small sample size. As more and more researchers begin to focus on metabolic regulation of cell death, this field is expanding quickly and new techniques and methods are expected to emerge to meet the growing need for accurate and easy analysis on both glucose metabolism and cell death.

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References

- Alves NL, Derks IA, Berk E, Spijker R, van Lier RA, Eldering E. The Noxa/Mcl-1 axis regulates susceptibility to apoptosis under glucose limitation in dividing T cells. *Immunity* 2006;24:703–716. [PubMed: 16782027]
- Asano T, Katagiri H, Takata K, Lin JL, Ishihara H, Inukai K, Tsukuda K, Kikuchi M, Hirano H, Yazaki Y, Oka Y. The role of N-glycosylation of GLUT1 for glucose transport activity. *J Biol Chem* 1991;266:24632–24636. [PubMed: 1761560]
- Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, Gottlieb E, Vousden KH. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell* 2006;126:107–120. [PubMed: 16839880]
- Bentley J, Walker I, McIntosh E, Whetton AD, Owen-Lynch PJ, Baldwin SA. Glucose transport regulation by p210 Bcr-Abl in a chronic myeloid leukaemia model. *Br J Haematol* 2001;112:212–215. [PubMed: 11167806]
- Budihardjo I, Oliver H, Lutter M, Luo X, Wang X. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol* 1999;15:269–290. [PubMed: 10611963]
- Byfield MP, Murray JT, Backer JM. hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase. *J Biol Chem* 2005;280:33076–33082. [PubMed: 16049009]
- Callaghan MJ, Ceradini DJ, Gurtner GC. Hyperglycemia-induced reactive oxygen species and impaired endothelial progenitor cell function. *Antioxid Redox Signal* 2005;7:1476–1482. [PubMed: 16356110]
- Campbell M, Allen WE, Sawyer C, Vanhaesebroeck B, Trimble ER. Glucose-potentiated chemotaxis in human vascular smooth muscle is dependent on cross-talk between the PI3K and MAPK signaling pathways. *Circ Res* 2004;95:380–388. [PubMed: 15242975]
- Chi MM, Pingsterhaus J, Carayannopoulos M, Moley KH. Decreased glucose transporter expression triggers BAX-dependent apoptosis in the murine blastocyst. *J Biol Chem* 2000;275:40252–40257. [PubMed: 10995754]
- Danial NN, Korsmeyer SJ. Cell death: Critical control points. *Cell* 2004;116:205–219. [PubMed: 14744432]
- De Luca T, Morre DM, Zhao H, Morre DJ. NAD⁺/NADH and/or CoQ/CoQH₂ ratios from plasma membrane electron transport may determine ceramide and sphingosine-1-phosphate levels accompanying G1 arrest and apoptosis. *Biofactors* 2005;25:43–60. [PubMed: 16873929]
- Edinger AL, Thompson CB. Death by design: Apoptosis, necrosis and autophagy. *Curr Opin Cell Biol* 2004;16:663–669. [PubMed: 15530778]
- Edinger AL, Cinalli RM, Thompson CB. Rab7 prevents growth factor-independent survival by inhibiting cell-autonomous nutrient transporter expression. *Dev Cell* 2003;5:571–582. [PubMed: 14536059]
- Frauwirth KA, Thompson CB. Regulation of T lymphocyte metabolism. *J Immunol* 2004;172:4661–4665. [PubMed: 15067038]
- Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* 2004;4:891–899. [PubMed: 15516961]
- Gottlob K, Majewski N, Kennedy S, Kandel E, Robey RB, Hay N. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev* 2001;15:1406–1418. [PubMed: 11390360]

- Haberkorn U, Altmann A, Kamencic H, Morr I, Traut U, Henze M, Jiang S, Metz J, Kinscherf R. Glucose transport and apoptosis after gene therapy with HSV thymidine kinase. *Eur J Nucl Med* 2001;28:1690–1696. [PubMed: 11702112]
- Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70. [PubMed: 10647931]
- Henquin JC. Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* 2000;49:1751–1760. [PubMed: 11078440]
- Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, Birnbaum MJ, Thompson CB. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol Cell* 2005;18:283–293. [PubMed: 15866171]
- Kuan CY, Whitmarsh AJ, Yang DD, Liao G, Schloemer AJ, Dong C, Bao J, Banasiak KJ, Haddad GG, Flavell RA, Davis RJ, Rakic P. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Proc Natl Acad Sci USA* 2003;100:15184–15189. [PubMed: 14657393]
- Lang F, Lang KS, Lang PA, Huber SM, Wieder T. Mechanisms and significance of eryptosis. *Antioxid Redox Signal* 2006;8:1183–1192. [PubMed: 16910766]
- Leist M, Single B, Castoldi AF, Kühnle S, Nicotera P. Intracellular adenosine triphosphate (ATP) concentration: A switch in the decision between apoptosis and necrosis. *J Exp Med* 1997;185:1481–1486. [PubMed: 9126928]
- Lieberthal W, Menza SA, Levine JS. Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells. *Am J Physiol* 1998;274:F315–F327. [PubMed: 9486226]
- Lum JJ, Bauer DE, Kong M, Harris MH, Li C, Lindsten T, Thompson CB. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* 2005;120:237–248. [PubMed: 15680329]
- Lum JJ, DeBerardinis RJ, Thompson CB. Autophagy in metazoans: Cell survival in the land of plenty. *Nat Rev Mol Cell Biol* 2005;6:439–448. [PubMed: 15928708]
- Majewski N, Nogueira V, Bhaskar P, Coy PE, Skeen JE, Gottlob K, Chandel NS, Thompson CB, Robey RB, Hay N. Hexokinase-mitochondria interaction mediated by Akt is required to inhibit apoptosis in the presence or absence of Bax and Bak. *Mol Cell* 2004;16:819–830. [PubMed: 15574336]
- Morissette MR, Howes AL, Zhang T, Heller Brown J. Upregulation of GLUT1 expression is necessary for hypertrophy and survival of neonatal rat cardiomyocytes. *J Mol Cell Cardiol* 2003;35:1217–1227. [PubMed: 14519432]
- Mourrieras F, Foulfelle F, Foretz M, Morin J, Bouche S, Ferre P. Induction of fatty acid synthase and S14 gene expression by glucose, xylitol and dihydroxyacetone in cultured rat hepatocytes is closely correlated with glucose 6-phosphate concentrations. *Biochem J* 1997;326(Pt 2):345–349. [PubMed: 9291103]
- Nutt LK, Margolis SS, Jensen M, Herman CE, Dunphy WG, Rathmell JC, Kornbluth S. Metabolic regulation of oocyte cell death through the CaM-KII-mediated phosphorylation of caspase-2. *Cell* 2005;123:89–103. [PubMed: 16213215]
- Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, Hughes PD, Michalak EM, McKimm-Breschkin J, Motoyama N, Gotoh T, Akira S, et al. ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell* 2007;129:1337–1349. [PubMed: 17604722]
- Rathmell JC, Elstrom RL, Cinalli RM, Thompson CB. Activated Akt promotes increased resting T cell size, CD28-independent T cell growth, and development of autoimmunity and lymphoma. *Eur J Immunol* 2003a;33:2223–2232. [PubMed: 12884297]
- Rathmell JC, Fox CJ, Plas DR, Hammerman P, Cinalli RM, Thompson CB. Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival. *Mol Cell Biol* 2003b;23:7315–7328. [PubMed: 14517300]
- Rathmell JC, Vander Heiden MG, Harris MH, Frauwirth KA, Thompson CB. In the absence of extrinsic signals, nutrient utilization by lymphocytes is insufficient to maintain either cell size or viability. *Mol Cell* 2000;6:683–692. [PubMed: 11030347]
- Reimertz C, Kogel D, Rami A, Chittenden T, Prehn JH. Gene expression during ER stress-induced apoptosis in neurons: Induction of the BH3-only protein Bbc3/PUMA and activation of the mitochondrial apoptosis pathway. *J Cell Biol* 2003;162:587–597. [PubMed: 12913114]
- Sade H, Sarin A. Reactive oxygen species regulate quiescent T-cell apoptosis via the BH3-only proapoptotic protein BIM. *Cell Death Differ* 2004;11:416–423. [PubMed: 14713957]

- Semenza GL, Artemov D, Bedi A, Bhujwala Z, Chiles K, Feldser D, Laughner E, Ravi R, Simons J, Taghavi P, Zhong H. 'The metabolism of tumours': 70 years later. *Novartis Found Symp* 2001;240:251–260. [PubMed: 11727934]discussion 60–64
- Shintani T, Klionsky DJ. Autophagy in health and disease: A double-edged sword. *Science* 2004;306:990–995. [PubMed: 15528435]
- Smith TA. Mammalian hexokinases and their abnormal expression in cancer. *Br J Biomed Sci* 2000;57:170–178. [PubMed: 10912295]
- Valverde AM, Navarro P, Benito M, Lorenzo M. H-ras induces glucose uptake in brown adipocytes in an insulin- and phosphatidylinositol 3-kinase-independent manner. *Exp Cell Res* 1998;243:274–281. [PubMed: 9743587]
- Vander Heiden MG, Plas DR, Rathmell JC, Fox CJ, Harris MH, Thompson CB. Growth factors can influence cell growth and survival through effects on glucose metabolism. *Mol Cell Biol* 2001;21:5899–5912. [PubMed: 11486029]
- Warburg O. On the origin of cancer cells. *Science* 1956;123:309–314. [PubMed: 13298683]
- Ward MW, Huber HJ, Weisova P, Dussmann H, Nicholls DG, Prehn JH. Mitochondrial and plasma membrane potential of cultured cerebellar neurons during glutamate-induced necrosis, apoptosis, and tolerance. *J Neurosci* 2007;27:8238–8249. [PubMed: 17670970]
- Wieman HL, Wofford JA, Rathmell JC. Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking. *Mol Biol Cell* 2007;18:1437–1446. [PubMed: 17301289]
- Wilson JE. Isozymes of mammalian hexokinase: Structure, subcellular localization and metabolic function. *J Exp Biol* 2003;206:2049–2057. [PubMed: 12756287]
- Wolin MS, Ahmad M, Gao Q, Gupte SA. Cytosolic NAD(P)H regulation of redox signaling and vascular oxygen sensing. *Antioxid Redox Signal* 2007;9:671–678. [PubMed: 17511583]
- Younes M, Brown RW, Stephenson M, Gondo M, Cagle PT. Overexpression of Glut1 and Glut3 in stage I nonsmall cell lung carcinoma is associated with poor survival. *Cancer* 1997;80:1046–1051. [PubMed: 9305704]
- Zamaraeva MV, Sabirov RZ, Maeno E, Ando-Akatsuka Y, Bessonova SV, Okada Y. Cells die with increased cytosolic ATP during apoptosis: A bioluminescence study with intracellular luciferase. *Cell Death Differ* 2005;12:1390–1397. [PubMed: 15905877]
- Zhao Y, Altman BJ, Coloff JL, Herman CE, Jacobs SR, Wieman HL, Wofford JA, Dimascio LN, Ilkayeva O, Kelekar A, Reya T, Rathmell JC. Glycogen synthase kinase 3alpha and 3beta mediate a glucose-sensitive antiapoptotic signaling pathway to stabilize Mcl-1. *Mol Cell Biol* 2007;27:4328–4339. [PubMed: 17371841]
- Zhou R, Vander Heiden MG, Rudin CM. Genotoxic exposure is associated with alterations in glucose uptake and metabolism. *Cancer Res* 2002;62:3515–3520. [PubMed: 12067998]

Figure 22.1.

Glucose metabolism regulates cell death pathways through multiple mechanisms that culminate in the regulation of proapoptotic Bcl-2 family proteins, such as Bax, to control apoptosis.

Figure 22.2.

Glucose uptake can be raised artificially by the expression of glucose transporters. Glucose uptake was measured in control FL5.12 cells and FL5.12 cells stably expressing FLAG-Glut1 that were deprived growth factor (IL3) for 6 h.

Figure 22.3.

The Glut1 protein is sensitive to cell lysis conditions and runs as a smear on SDS-PAGE unless treated properly. Immunoblot of FLAG-Glut1 using cell lysates is treated as follows: lane 1, RIPA lysis buffer (smear around 150 kDa); lane 2, Glut1 lysis buffer (smear around 55 kDa); and lane 3, Glut1 lysis buffer followed by PNGaseF treatment (sharp band at 40 kDa).

Figure 22.4.

Surface Glut1 trafficking can be modeled by expression and analysis of tagged Glut1. Flow cytometric analysis of surface FLAG-Glut1 levels in FL5.12 cells in the presence of growth factor (IL3) or after 6 h withdrawal from growth factor.