How Do Cancer Cells Acquire the Fuel Needed to Support Cell Growth?

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In this paper we consider whether the dependency of metazoan cells on extracellular signals to maintain cell survival results in an important barrier that must be overcome during carcinogenesis. It is now generally accepted that a major barrier to cancer comes from the inability of cells to enter and progress through the cell cycle in a cell-autonomous fashion. Most of the oncogenes studied over the last two decades contribute to the ability of the cancer cell to enter and progress through the cell cycle in the absence of the instructional signals normally imparted by extracellular growth factors. Over the last two decades, it has begun to be appreciated that there is a second potential barrier to transformation. It appears that all cells in multicellular organisms need extracellular signals not only to initiate proliferation, but also to maintain cell survival. Every cell in our body expresses the proteins necessary to execute its own death by apoptosis. A cell will activate this apoptotic program by default unless it receives signals from the extracellular environment that allow the cell to suppress the apoptotic machinery it expresses. It now appears that the molecular basis of this suppression lies in the signaling pathways that regulate cellular nutrient uptake and direct the metabolic fate of those nutrients.

METAZOAN CELLS LACK THE CELL AUTONOMOUS ABILITY TO TAKE UP NUTRIENTS

Over the last decade, we have been studying the dependency of cells on lineage-specific growth factors. Although this paper focuses on hematopoietic cells, additional data suggest that the findings are equally true of neuronal cells, epithelial cells, and fibroblasts. In all of these cells, the lineage-specific receptors that promote cell survival regulate the ability of the cell to express the transporters needed to take up nutrients from the extracellular environment. Every cell in the human body has an adequate supply of extracellular nutrients to maintain its survival. However, it appears that the cells in multicellular organisms lack the cell-autonomous ability to take up nutrients from the extracellular space. For example, glucose uptake depends on signal transduction that directs the cell to produce glucose transporters, hexokinase, and phosphofructokinase (Vander Heiden et al. 2001). These proteins are required to maintain the supply of glucose utilized by the glycolytic pathway to produce substrates for mitochondrial oxidative phosphorylation. When a cell is deprived of survival signals, the ability of the cell to take up, capture, and metabolize sufficient nutrients to maintain ATP production disappears. In every cell lineage in which this experiment has been done, after 48–96 hours in the absence of the signal transduction that maintains nutrient uptake and metabolism, the cell initiates apoptosis. This apoptotic response depends on the activity of the proapoptotic Bcl-2-related proteins, Bax and Bak (see, e.g., Lindsten et al. 2000).

Bcl-2 PROTEINS REGULATE THE DURATION OF CELL SURVIVAL FOLLOWING GROWTH FACTOR WITHDRAWAL

The first question we have addressed concerns the role of antiapoptotic Bcl-2 proteins in maintaining cell survival following growth factor withdrawal. Surprisingly, we found that Bcl-2 proteins play no role in regulating nutrient uptake. Cells overexpressing antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-x_L still need survival signal transduction to maintain nutrient transporters. For example, interleukin-3 (IL-3)-dependent cells deprived of IL-3 lose the ability to express both glucose and amino acid transporters. Despite this, antiapoptotic Bcl-2 proteins can keep the cell alive several weeks in the absence of nutrient transporter expression (Rathmell et al. 2000).

Alternative explanations for the decline of nutrient transporters following growth factor withdrawal have been suggested. For example, it could be that the decline in transporters results simply from the cell withdrawing from the cell cycle and no longer requiring a high level of nutrients to support growth. This concept would suggest that changes in nutrient transporter expression are a compensatory response to withdrawal from the cell cycle. In this view, survival receptors regulate cell survival through altering the expression/function of Bcl-2 family members. If Bax and Bak are in excess, apoptosis is initiated by cytochrome *c* release into the cytosol and activation of caspase 9. If Bcl-2 or other antiapoptotic family members dominate, the cell can survive for a long period of time.

GROWTH FACTOR REGULATION OF CELL SURVIVAL DOES NOT DEPEND ON APOPTOSIS

Cells need a constant supply of energy to maintain themselves even in the absence of growth. Cells need ATP just to “maintain the pumps”—to exclude sodium or to sequester calcium. If a cell cannot take up effective amounts of nutrients from its environment, it will ultimately die a bioenergetic death independent of apoptosis. To test this prediction, we engineered mice that were deficient in Bax and Bak. Bax/Bak-deficient animals have a variety of developmental defects, but a few make it into adulthood, and they have been widely reported (Lindsten et al. 2003). From these mice we have derived cell lines that have allowed us to take our initial experiments one step farther. We have made a variety of cell lines: neural progenitor cell lines, IL-3-dependent hematopoietic cells, and 3T3 fibroblasts (Lindsten et al. 2003; Zong et al. 2004; Lum et al. 2005). All of these cell lines share the property that stress-inducing apoptotic initiators no longer lead to rapid cell death. Thus, Bax and Bak are essential for initiating the apoptotic fate of the cell in response to stress. For example, the apoptotic initiator staurosporine can induce both wild-type cells and p53-deficient cells to die. In contrast, Bax/Bak-deficient cells survive the drug treatment and recover after the drug is removed. If the cells are treated with a chemotherapeutic agent such as etoposide, a topoisomerase inhibitor, a similar result is observed. Normal cells die in a dose- and time-dependent fashion, whereas survival of Bax/Bak-deficient cells is unaffected. Next, we tested the growth factor dependence of these cells: the fibroblasts for serum dependency, the neural progenitor cells for FGF, and the hematopoietic progenitors for IL-3. The IL-3 results have recently been published (Lum et al. 2005). Just as in other

IL-3-dependent cell lines, IL-3 regulates the ability of Bax^{-/-}Bak^{-/-} cells to express glucose transporters and catabolize glucose to provide mitochondrial substrates for ATP production. When the cells are deprived of IL-3, the transporters are lost. Thus, cells must catabolize intracellular substrates to maintain ATP production. The IL-3-deprived cells essentially degrade themselves through the process of macroautophagy. As long as a cell can generate metabolites from its intracellular contents to maintain mitochondrial ATP production, the cell survives. Autophagy can keep a cell alive in complete medium for several weeks. After that there is nothing left to catabolize intracellularly, and the cell dies by necrosis. What this suggests is that antiapoptotic Bcl-2 proteins affect the time it takes from the loss of signaling information to the loss of cell viability, but they do not promote cell-autonomous survival. Although activation of proapoptotic family members initiates a more rapid death by apoptosis and the antiapoptotic proteins promote autophagy and longer survival, ultimately, growth-factor-deprived cells still die (Fig. 1).

AUTOPHAGY CAN MAINTAIN TRANSIENT GROWTH FACTOR-INDEPENDENT SURVIVAL

There has recently been a great deal of controversy concerning the role of autophagy in regulating cell survival. Autophagy is a process in which cells sense that they are bioenergetically compromised and initiate the formation of new double membrane vesicles that sequester off regions of the cytosol. These vesicles isolate a region of the cytosol along with its complement of organelles and deliver the contents to the lysosome. Following fusion, the lysosomal degradative enzymes break

Figure 1. Model for growth factor regulation of cell survival. As depicted, growth factor signal transduction is required to regulate the expression and function of nutrient transporters by which cells take up nutrients needed to maintain ATP production. Loss of growth factor signaling leads inexorably to cell death, unless growth factor-induced nutrient uptake is reestablished. Apoptosis provides a rapid and efficient elimination of unwanted or neglected cells following growth factor deprivation. In the absence of apoptosis, growth-factor-deprived cells persist for several weeks by maintaining their bioenergetics through autophagy. Ultimately, autophagy is a self-limited process and the cells die by necrosis.

the cytosolic and organellar contents into substrates that the mitochondria can utilize to maintain ATP production. Autophagy has been best characterized in yeast and plants, where this process is more easily characterized because there is no apoptosis to complicate the study of the process. The MAP protein, LC3, can be used to identify and study newly forming autophagic vacuoles. Using this marker, we have studied primary IL-3-dependent bone marrow cells out of a Bax/Bak-deficient animal that were subjected to IL-3 withdrawal. In the presence of IL-3, LC3 is diffusely expressed in the cytosol. However, 48 hours after IL-3 depletion, LC3 undergoes redistribution to newly formed autophagic vacuoles. The role of autophagy in maintaining cellular bioenergetics was demonstrated by using either 3-methyladenine or chloroquine to inhibit the degradative process that is initiated by autophagy. In the presence of IL-3, neither of these drugs has any effect on the viability of the bone marrow cells. Viability remains high in IL-3-deprived cells until they are treated with either of the autophagy inhibitors. In the presence of either drug, the IL-3-deprived cells undergo cell death, and only 30% are viable after 24 hours. Over the next 24 hours, the rest of these cells die. The suppression of autophagy kills IL-3-deprived Bax/Bak-deficient cells. This death results from the bioenergetic compromise of the cells, as cell death can be completely reversed by addition of a cell-permeant metabolite, methyl pyruvate, that mitochondria can degrade to produce ATP (Fig. 2).

Autophagy-dependent survival is by its very nature a self-limited survival strategy. Autophagy-dependent cells begin to die several weeks after growth factor de-

privation. At the end of this time, the cells appear to be filled with residual autophagolysosomes. These vesicles are filled with degradative debris. Although morphologically the cells look horrible, they retain two properties that suggest they may represent a meaningful biological intermediate in the lineage homeostasis of adult animals. The first is that the cells are capable of moving in response to chemokine signals that normally recruit these progenitor cells to hematopoietic stem cell niches. Even though the cells have undergone profound self-catabolism, they are able to use their remaining energy to move to a chemokine gradient. Hematopoietic progenitors are recruited to hematopoietic niches in the bone marrow by chemokines such as SDF-1. Hematopoietic progenitor cells grown in the presence of IL-3 will respond in a transwell assay to 100 ng/ml of SDF, with essentially all of the cells migrating toward the chemokine gradient. However, the IL-3-deprived cells are even more sensitive and more active to chemokine gradients. It takes one log less chemokine to induce them to migrate. We interpret this to mean that the cells are using their residual energy to find a site with the trophic signals they need to sustain survival.

The one additional signal the cells are capable of responding to is IL-3. IL-3 readdition allows the cells to fully recover glycolysis within 20 hours. Despite the rapid recovery of glycolysis, the cells do not recover the ability to proliferate for a week. In the first 24 hours, the cells recover their ability to take up and metabolize nutrients; they then recover the ability to grow and increase in size over the next 6 days. When the cells reach a size threshold of 700 femtoliters, they are able to resume pro-

Figure 2. Autophagy maintains the survival of growth-factor-deprived cells. (A) Primary bone marrow cells derived from Bax^{-/-}Bak^{-/-} mice are placed into culture in the presence of IL-3. Under these conditions, the autophagy marker LC3 exhibits a diffuse cytosolic distribution (*upper panel*). When IL-3 is withdrawn for 48 hours (*lower panel*), LC3 redistributes to newly formed autophagic vacuoles. (B) Autophagy inhibitors 3-methyladenine (3MA) and chloroquine (CQ) inhibit the survival of growth-factor-deprived Bax^{-/-}Bak^{-/-} primary bone marrow cells. Growth-factor-deprived cells, in which autophagy is inhibited by 3MA or CQ, can be rescued from cell death by the addition of a cell-permeant metabolite, methyl pyruvate (MP).

Figure 3. Growth-factor-deprived cells undergo reversible atrophy. $Bax^{-/-}Bak^{-/-}$ IL-3-dependent cells were deprived of IL-3. The cells atrophy from 700 femtoliters to ~300 femtoliters in size over the next several weeks. Under these conditions, bioenergetics is maintained by autophagy (-IL-3). Despite this profound autophagy-induced atrophy, cells are able to fully recover following IL-3 readdition. Six days following IL-3 readdition (+IL-3), the cells have recovered their cell volume and reinitiated cell proliferation.

liferation. Upon IL-3 readdition, virtually every cell in the culture increases in size and recovers its ability to engage in exponential growth (Fig. 3).

GROWTH FACTOR SIGNALING PATHWAYS REGULATE NUTRIENT UPTAKE AND METABOLISM

The above results demonstrate that hematopoietic cells lack the ability to maintain sufficient nutrient uptake to maintain cell survival in the absence of instructional signals. This suggests that these cells have two barriers to overcome in order to proliferate in a cell-autonomous fashion. The first is the cell's dependence on growth factor signals to activate the genes needed to enter and progress through the cell cycle. The second is the cell's dependence on survival signals to take up and utilize the nutrients needed to fuel this growth process. A substantial increase in a cell's production of ATP and uptake of

macromolecular precursors is required to support net cell growth.

The major pathway that regulates nutrient uptake in all lineages tested so far is the PI3K/Akt/TOR pathway, which is negatively regulated by the tumor suppressors PTEN, Tsc-1, and Tsc-2 (Bauer et al. 2004). The primary effect of Akt in cellular transformation is to constitutively activate glucose uptake and metabolism. For example, expression of an activated Akt under a doxycyclin promoter in a cell line that lacks constitutive Akt activity leads the cell to increase glycolysis without any significant effect on cell proliferation. The enhanced glycolysis is a specific effect of Akt. There is no generalized increase in metabolism. If anything, the rate of oxidative phosphorylation goes down in response to Akt stimulation of glycolysis. Because Akt transformation does not stimulate increased proliferation, all of the excess carbon taken up as glucose under the direction of activated Akt is secreted into the supernatant as lactate (Fig. 4).

Figure 4. Constitutively active Akt stimulates glycolysis. (A) A continuously growing cell line was transfected with a doxycyclin-regulated form of activated Akt (myrAkt1-3). (B) Expression of activated Akt does not enhance the growth of the cell line in culture. (C) Akt stimulates glycolysis in a dose-dependent fashion, without a commensurate increase in the rate of oxidative phosphorylation. Because Akt stimulation of glycolysis does not result in enhanced cell growth, the excess carbon taken up is secreted into the medium as lactate.

Figure 5. ACL activity is required for cell growth. An Akt-dependent leukemia cell line is stably transfected with an shRNA that suppresses ATP citrate lyase (ACL) (*upper left panel*). Cells exhibiting ACL suppression demonstrated a loss of tumorigenicity when reintroduced in vivo (*middle panel*). Western blot of proteins from the tumors that arise in animals receiving tumors stably transfected with ACL shRNA show recovery of ACL expression (*upper right panel*).

GROWING CELLS RELY ON GLUCOSE-DEPENDENT METABOLISM TO MAINTAIN ATP PRODUCTION AND LIPID BIOSYNTHESIS

Converting to a glucose-dependent form of metabolism is adaptive during cell growth because this conversion allows cells to divert all the amino acids and lipid precursors that they are able to take up into membrane and protein synthesis. The conversion to a glucose-dependent metabolism provides a clear growth advantage in terms of lipid biology. Growing cells do not take up sufficient lipids from their environment to support membrane biosynthesis. Instead they synthesize many of the lipids they need for growth from cytosolic acetyl-CoA. Just as cells use amino acids to produce the new proteins they need, the cells build their own lipids from acetyl-CoA. The ability to engage in net lipid synthesis depends on a novel glucose-dependent metabolic cycle. This mitochondrial-cytosolic cycle turns pyruvate into mitochondrial NADH to maintain electron transport and cytosolic acetyl-CoA that is used for lipid synthesis. During growth, geranylgeranyl and farnesyl groups, membrane phospholipids, and sphingomyelin are all produced in the cytosol from acetyl-CoA. A key Akt-regulated enzyme in this pathway is ATP-citrate lyase (ACL), a direct phosphorylation target of Akt (Bauer et al. 2005). ACL activation appears to play a critical role in Akt-mediated tumorigenesis. We have generated Akt-dependent lymphoma cell lines. When we suppressed ACL in these cell lines by shRNA, the cell lines lost their tumorigenicity (Fig. 5). The few tumors that do arise in the animals no longer display ACL suppression. This suggests that key enzymes associated with metabolic pathways unique to

cell growth may represent potential targets for the treatment of cancer. It is an idea we are just beginning to explore.

ACKNOWLEDGMENTS

The authors thank members of the Thompson laboratory for critical insights and suggestions. We also acknowledge the excellent secretarial assistance of J. Joh and S. Kerns. J.J.L. is supported by a fellowship from the Leukemia and Lymphoma Society. G.H. is supported by a Fellowship Award from the Damon Runyon Foundation. W.X.Z. is supported by a National Cancer Institute Howard Temin Mentored Research Scientist Development Award (KO1). Funding for C.B.T. was provided in part by grants from the NCI and the National Institutes of Health.

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Cold Spring Harbor Symposia on Quantitative Biology

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Cold Spring Harb Symp Quant Biol 2005 70: 357-362

Access the most recent version at doi:[10.1101/sqb.2005.70.011](https://doi.org/10.1101/sqb.2005.70.011)

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