Remarkable Tolerance of Tumor Cells to Nutrient Deprivation: Possible New Biochemical Target for Cancer Therapy

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ABSTRACT
We hypothesized that the tolerance for nutrient deprivation as well as angiogenesis might be an important factor for tumor progression under hypovascular conditions. When normal human fibroblasts were subjected to extreme nutrient starvation by culturing in a medium without serum, glucose, and amino acids, cells died within 24 h. When substituted with liver cancer cell lines HepG2, Hep3B, HLE, and HuH-7, cell death occurred within 36 h. In contrast, four of six pancreas cancer cell lines, PANC-1, AsPC-1, BxPC-1, and KP-3, survived for remarkably longer periods: >50% of the cells survived, even after starvation for 48 h. Among three gastric cancer cell lines, MKN28, MKN45, and MKN74, only the most poorly differentiated MKN45 cells survived >36 h. More than 50% of the cells in colon cancer cell lines SW480, WiDr, and DLD-1 survived after 36 h, and the most undifferentiated SW480 cell line survived longest. We examined the possible involvement of PKB/Akt expression in the survival of various cell lines under nutrient starvation conditions. High expression of PKB/Akt was found to be associated with tolerance for nutrient starvation. When Akt antisense RNA expression vectors were introduced into PANC-1 cells, the tolerance was partially but significantly diminished by vectors for Akt1 and Akt2 but not Akt3. Because elimination of the tolerance might serve as a new strategy for cancer therapy, several compounds were tested for this purpose, and troglitazone, an insulin sensitizer, as well as LY294002, a phosphatidylinositol 3-kinase inhibitor, were found to kill PANC-1 cells only under nutrient starvation conditions.

INTRODUCTION
Tumor cells in general are well known to have high glycolytic activity (1). This is partly because tumor cells progress through multiple steps of carcinogenesis exposed to insufficient oxygen supply because of excessive oxygen demand and thereby insufficient vascularization. Even after the tumor increases in size, the immediate environment of cancer cells often becomes heterogeneous. In addition, some regions of large tumors often have microenvironmental niches, displaying a significant gradient of critical metabolites including oxygen, glucose, other nutrients, and growth factors (1–3). Therefore, angiogenesis is regarded as the key step in tumor progression, and antiangiogenesis is the most promising cancer therapy, with extensive studies conducted to prevent tumor angiogenesis (4–6).

Tissue hypoxic response is critical for maintaining the microenvironmental tissue of the issue as well as its homeostasis and is sophisticatedly regulated by complex mechanisms. Hypoxia inducible factor-1 is a central transcriptional regulator of these processes of hypoxic response. Once it is activated, hypoxia inducible factor-1 stimulates transcriptions of a series of genes for hypoxic response including vascular endothelial growth factor, erythropoietin, and anaerobic glycolysis (1, 7, 8). These reactions explain very well how tissue survives under oxygen deficiency; however, these reactions do not satisfactorily explain how cancer tissues survive under hypoxic conditions, which are always associated with an insufficient supply of nutrients. Therefore, we hypothesize that some cancer cells might have acquired a tolerance for nutrient deficiency through their progression, in addition to the ability to stimulate angiogenesis.

To test the above hypothesis, we selected pancreatic cancer, well known to be a hypovascular tumor from clinical angiography. In this study, we found that four of six pancreatic cell lines display extremely long survival, even under complete nutrient deficiency conditions. Moreover, some poorly differentiated colon and gastric cancer cell lines were also found to be tolerant. These observations raise the possibility that the tolerance for nutrient deficiency might be a new biological character of cancer that is critical for cancer progression as well as angiogenesis. In addition, because normal organs are seldom faced with extreme deficiency of oxygen and nutrients, tolerance might serve as a new target for anticancer drugs. In the present study, we identified two possible candidate drugs for this purpose.

MATERIALS AND METHODS

Cell Lines and Nutrient Starvation. Stomach cancer cell lines MKN28, MKN45, and MKN74; colon cancer cell lines DLD-1, WiDr, and SW-480; pancreas cancer cell lines PANC-1, AsPC-1, PSN-1, KP-3, BxPC-3, and MiaPaCa-2; liver cancer cell lines HepG2, Hep3B, HLE, and HuH-7; and normal human colonic fibroblasts, HF, which were established in our laboratory from human colon, were used in this study. Cell lines were cultured in a suitable medium, DMEM or RPMI 1640. All cells were seeded at a concentration of 1.5 × 10^4 cells/well in six-well plates and incubated in complete fresh medium for 20 h. The cells were then washed with PBS, and nutrient deprivation was initiated by replacement with nutrient-deprived medium. Basal medium was prepared to contain only electrolytes and vitamins, according to the composition of DMEM as follows: CaCl_2 (2H_2O), 265 mg/l; Fe (NO_3)_3 (9H_2O), 0.1 mg/l; KCl, 400 mg/l; MgSO_4 (7H_2O), 200 mg/l; NaCl, 6400 mg/l; NaHCO_3, 700 mg/l; NaH_2PO_4, 125 mg/l; phenol red, 15 mg/l; 25 mM HEPES buffer (pH 7.4); and MEM vitamin solution (Life Technologies, Inc., Rockville, MD). When glucose was supplemented, 10% glucose was added to 1000 mg/l. Two hundred mm t-glutamine, MEM amino acids solution, and MEM nonessential amino acids solution (Life Technologies, Inc.) were used as stock solution and were supplemented at 100 dilution. The FCS (Sigma Chemical Co., St. Louis, MO) was dialyzed three times against large excesses of 0.9% NaCl before use. Cell viability after nutrient deprivation was estimated by the trypan blue dye-exclusion method.

Detection of Protein and Phosphorylation of PKB/Akt. Cell lysates were prepared at 0, 2, 12, and 24 h after the nutrient deprivation. Proteins were separated by gel electrophoresis on a polyacrylamide gel containing SDS, and specific bands were visualized by Western blot using Akt antibody and the phospho-specific (Ser-473) Akt antibody (New England BioLabs, Beverly, MA). Horseradish peroxidase-conjugated goat antirabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as a secondary antibody for enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Construction of Akt Antisense RNA Expression Vectors. cDNAs for human Akt1, Akt2, and Akt3, which contain the entire coding sequences, were generated by reverse transcription–PCR amplification of each human brain cDNA. Each cDNA contained nucleotides –104 to 1514, –10 to 1459, and –9 to 1520 of Akt1, Akt2, and Akt3, respectively. Each cDNA was cloned in

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its antisense orientation into an expression vector, PCR3, and verified by sequencing. cDNAs were transfected into PANC-1 cells with Lipofectamine-Plus reagent (Life Technologies, Inc.). G418 selection was started 48 h after transfection; G418-resistant colonies were pooled after 2–3 weeks of selection and grown as a mixture.

**Effect of LY294002 and Troglitazone on Cell Survival in a Low-Nutrient Medium.** LY294002, wortmannin (Carbiochem, La Jolla, CA), and troglitazone (a generous gift from Sankyo Pharmaceutical Company, Tokyo, Japan) were dissolved in DMSO. LY294002, wortmannin, human insulin (Wako Chemicals, Tokyo, Japan), and troglitazone were added to a final concentration of 50 μM, 100 nM, 5 μg/ml, and 20 μM, respectively. The final concentration of DMSO was 0.1%, which did not affect cell survival or Akt phosphorylation. DMEM containing 10% FCS was diluted 10 times with basal medium to produce low-nutrient medium. After 24 h in low-nutrient medium, cell survival was estimated by the trypan blue dye-exclusion method.

**FACS Analysis.** Cells were incubated without or with 50 μM LY294002 in medium deprived of amino acids for 12 h and without or with 20 μM troglitazone in medium deprived of glucose and serum for 4 h. The cells were then collected and double-stained with FITC-conjugated Annexin V and propidium iodide by an apoptosis detection kit (Bender MedSystems Diagnostics, Vienna, Austria) according to the manufacturer’s instructions, followed by analysis using a FACS (FACS Caliber; Becton Dickinson, Franklin Lakes, NJ).

**RESULTS**

**Survival of PANC-1 under Various Starvation Conditions.** Because we hypothesized that some cancer cells might have acquired tolerance for nutrient starvation, the effect of withdrawal of each nutrient component on cell survival was examined. For this purpose, we used a pancreatic cell line, PANC-1. As we expected, PANC-1 showed extreme resistance against glucose starvation (Fig. 1A). Amino acid starvation was more critical for survival than glucose starvation, regardless of the presence of serum. In addition, >50% of PANC-1 cells survived after 48 h, even under complete nutrient deficiency (Fig. 1B). Under insufficient blood supply to the tumor tissue, either the mobilization of glycogen storage or gluconeogenesis supplies glucose. Amino acids themselves also serve as substrates for oxidative metabolism in which they serve as an energy source. Amino acids are supplied not only via the bloodstream but also by the protein breakdown of either tumor cells themselves or surrounding cells or matrix. These findings, therefore, suggest that the tolerant cancer cells might alter the energy metabolism. As shown in Fig. 1A, PANC-1 cells survive for long periods of time in the absence of both glucose and amino acids when serum is present. This observation is consistent with the hypothesis that cancer cells might use proteins as a source of amino acids and thereby as an energy source.

**Survival under the Nutrient Starvation of Various Cell Lines.** To examine the tolerance for nutrient starvation, cell survival under extreme nutrient deprivation was examined by culturing cells in the medium containing only electrolytes and vitamins. When normal human fibroblasts were cultured under this condition, cells died within 24 h (Fig. 2A). Similarly, most of cells in the four human liver cancer cell lines, Hep3B, HepG2, HLE, and HuH-7, died within 36 h. By contrast, four of the six pancreas cancer cell lines, PANC-1, AsPC-1, BxPC-3, and KP-3, survived remarkably for long periods after nutrient deprivation (Fig. 2B). Even for relatively sensitive cell lines, PSN-1 and MiaPaCa-2, >30% of cells survived after 36 h. These observations well supported our hypothesis. Gastric and colon cancer cell lines were also examined for tolerance (Fig. 2, C and D). Well-differentiated gastric cancer cell lines, MKN28 and MKN74, died similar to the normal fibroblasts, but poorly differentiated gastric cancer cell line MKN45 was found to be as tolerant as the pancreatic cancer cell lines. Three colon cancer cell lines, SW-480, WiDr, and DLD-1, survived relatively long periods with the least differentiated, SW-480, most tolerant.

**Akt Might Be Involved in Tolerance.** What is the molecular basis of the resistance? In a previous study, it has been reported that Akt is overexpressed in some pancreatic cancers (9, 10). To examine the relationship between cell survival and Akt expression, Akt expression was examined by Western blot analysis. Higher expression of Akt was observed in three of four tolerant pancreas cell lines, whereas only a low level of expression was observed in all of the liver cancer cell lines (Fig. 3). In addition, in six gastric and colon cancer cell lines, high expression was found in two tolerant cell lines, MKN45 and SW-480. In seven cancer cell lines with a high level of Akt expression, five were tolerant to extreme nutrient deprivation.

Akt activation was examined by immunoblotting with a phospho-specific Akt antibody in the PANC-1 cell line after nutrient deprivation. The deprivation of glucose, or amino acids, or both, caused phosphorylation of Akt within 2 h (Fig. 4, left). On the other hand, Akt was not phosphorylated after nutrient deprivation in the serum-deprived medium, although constitutively weak phosphorylation was observed. Therefore, phosphorylation of Akt does not necessarily correlate with the tolerance.

To clarify the involvement of Akt in tolerance, antisense RNA expression vectors were transfected into PANC-1 cells. Transfectants of Akt1 and Akt3 grew normally in DMEM complete medium, but Akt2 transfectants grew slowly compared with the parental cells (data not shown). This might indicate that Akt2 is deeply involved in the mechanisms of malignant transformation of PANC-1 cells, as reported previously. Cell survival of the above transfectants was examined under nutrient starvation as described above. The results in Fig. 5a show clearly that the antisense RNA expression vector for Akt1 and Akt2 but not Akt3 significantly diminished tolerance, although the inhibition seemed partial. When Akt protein levels in parental

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The abbreviations used are: FACS, fluorescence-activated cell sorter; PI3, phosphatidylinositol 3-kinase.
PANC-1 and transfectants were examined, a clear decrease in Akt protein was seen in Akt1 antisense transfectants. However, a decrease in Akt protein was only marginal in Akt2 antisense transfectants (Fig. 5b). The reason for this is not clear, but it seems likely that a large decrease in Akt2 protein might result in strong growth suppression in PANC-1 cells.

Compounds That Prevent Tolerance. Angiogenesis is seldom activated in normal tissues of the adult, except in the female reproductive system (11), and this is one of the reasons why antiangiogenesis therapy is specific for cancer tissue. The same is true for oxygen and nutrient deprivation. If any compound prevents tolerance for nutrient deprivation, the compound might be applicable in sensitization of cancer cells to nutrient deprivation, resulting in the regression of cancer. Because we found that amino acids are efficiently used in cancer cells, we assumed that gluconeogenesis or oxidative metabolism of amino acids is the underlying biochemical mechanism. Insulin is known to suppress these metabolic events. Therefore, insulin and troglitazone, an insulin sensitizer (12), were used to inhibit possible changes in energy metabolism. After 24 h in a low-nutrient medium, 10% diluted DMEM, 5 mM insulin did not affect cell survival (Fig. 6). However, troglitazone significantly decreased cell survival. As mentioned previously, the tolerance and activation of Akt were found to be closely associated with each other; thus, the effects of wortmannin and LY294002, PI3 kinase inhibitors (13–15), were examined. Although 100 nM wortmannin did not affect cell survival under nutrient starvation conditions, 50 μM LY294002 remarkably decreased cell survival. To determine deprivation of which nutrient was responsible for these toxic activities, each nutrient was added to a base medium, and cell survival was examined.

Interestingly, the results show that different conditions were required for toxicity of the two compounds. Troglitazone inhibited survival under deprivation of both glucose and serum (Fig. 7A), whereas 50 μM LY294002 inhibited survival under amino acid deprivation, irrespective of the presence of glucose and/or serum, although the effect was more prominent in the presence of serum (Fig. 7B). Next, we asked whether troglitazone and LY294002 influence Akt activation in response to nutrient deprivation. Twenty μM trogli-
tazone inhibited Akt phosphorylation when both glucose and serum were absent under the same conditions used for prevention of survival (Fig. 4, middle). Phosphorylation of Akt in the medium without nutrient deprivation was weakened by LY294002 but not totally inhibited (Fig. 4, right). The effect of LY294002 on the phosphorylation of Akt was more prominent when amino acids were absent. On the other hand, it did not suppress phosphorylation of Akt by glucose deprivation. These observations suggest that block of Akt activation is necessary but not sufficient for cell death to occur.

**Troglitazone and LY294002 Sensitize Cells by Different Mechanisms.** Finally, we examined the mode of cell death caused by the above two compounds in PANC-1 cells by FACS using propidium iodide and annexin V double staining. The deprivation of glucose, amino acids, and serum, and their combinations, were found to induce apoptosis in 3 or 5 days of starvation (data not shown). Fig. 8 shows the results at 4 and 12 h after the addition of troglitazone (20 μM) and LY294002 (50 μM), respectively. The addition of troglitazone to glucose- and serum-deprived medium caused necrosis, whereas the addition of LY294002 to the amino acid-deprived medium caused apoptosis. These observations again suggest that the two drugs sensitize cells to nutrient deprivation by different mechanisms.
DISCUSSION

Tumors continuously grow because of their intrinsic unregulated growth program caused by genetic alterations. Therefore, tumors in general are always exposed to hypoxia and poor nutrition because of insufficient vascularization (1–3). Without angiogenesis or neovascularization, tumor size is generally limited to a few mm in diameter (3). Metabolic stress appears to trigger signal transduction and gene expression, causing the synthesis of angiogenic factors and vascular endothelial growth factors, resulting in neovascularization and tumor growth and invasion, as well as greater metastatic potential (16–18). This is the reason why angiogenesis could be one of the determinants of tumor progression. Is this always true?

Theoretically, there are two ways of adaptation to an insufficient supply of oxygen and nutrients that could be interlinked but distinct from each other. One way is by increasing the supply, an idea widely accepted. The other way is by tolerating insufficiency, austerity. A tumor grows beyond its ability to improve oxygen and nutrient supplies by angiogenesis, and this might be the reason why tumors are often associated with necrotic foci. Only the tumor cells that have acquired the ability to survive an unfavorable microenvironment might be malignant. The two ideas above are linked but distinct, like both sides of a coin. Pancreatic cancer is one of the most aggressive cancers known; however, it is mostly hypovascular (19). Why is liver metastasis of pancreatic cancer often hypovascular? Why is liver metastasis of colon cancer often hypovascular? Although most studies have suggested that neovascularization is pivotal for tumor progression, less attention has been paid to the tolerance of poor nutritional conditions. Our results showed that some cancer cells had acquired strong tolerance for nutrient deprivation. In particular, pancreatic cancer cell lines survived for considerably longer periods under extremely low nutrient conditions than cell lines of liver cancer, which are mostly hypervascular (20). These observations are in agreement with our hypothesis that the acquisition of tolerance by tumor cells might be another determinant of tumor progression. Similar observations were made of gastric and colon cancer cells. The tolerance for nutrient deficiency was well correlated with poor differentiation of tumor cells.

What is the molecular mechanism for tolerance? Our present observations clearly indicate that certain cancer cell lines have acquired an ability to tolerate extreme nutrient starvation and remind us of an interesting biological response in nematodes. The nematode Caenorhabditis elegans responds to overcrowding or nutrient deprivation by changing its metabolism and arresting the development at a long-living dauer diapause stage. By this response, they tolerate disadvantageous conditions and survive for longer periods (21–23). A genetic and molecular analysis study suggests that a PI3 kinase and Akt signaling pathways are involved in this response (24). Among cell lines used in the present study, tolerance was closely associated with the Akt expression. Recent investigations suggested that Akt and PI3 are highly expressed in ovarian and pancreatic cancers, and the high level of expression was found to be associated with poor prognosis and increased tumorogenicity (9, 10, 25–29). Once PI3 becomes activated, it generates phosphorylatedinositol 4,5-biphosphate or phosphatidylinositol 3,4,5-tris phosphate, which in turn activates various AMP-activated protein kinase is well recognized in mediating various stress signals including hypoxia, nutrient starvation, and physical stresses (41, 42). It is probable that pancreatic cancer and some poorly differentiated cancers have already acquired constitutive tolerance for nutrient and oxygen starvation through multiple carcinogenes steps. It is also plausible that the tolerance might be acquired by modulating the AMP-activated protein kinase pathway. Indeed, we found recently that the tolerance of PAN-1 cells was dramatically inhibited by introduction of antisense expression vector for α subunit of AMP-activated protein kinase. At present, nothing is known about interaction or cross-talk between the Akt pathway and the AMK-activated protein kinase pathway. This remains to be clarified.

In our study, wortmannin did not affect cell survival, but LY294002 significantly decreased cell survival. Wortmannin and LY294002 have been used as potent inhibitors of mammalian PI3, but the half-life of wortmannin is known to be very short. This might be the reason for the discrepancy between the above two compounds. However, repeated addition of wortmannin did not induce apoptosis. Therefore, it might be also possible that LY294002 induced apoptosis by mechanisms other than the inhibition of PI3.

Troglitazone is a new class of antidiabetic agents that markedly improves insulin action, hyperlipidemia, and glucose utilization in diabetic patients (12, 43, 44). Troglitazone has been reported to be a noncompetitive inhibitor of mitochondrial and microsomal acyl-CoA synthase (45). The energy source is limited to either amino acids or fatty acids, derived from its own components once all nutrients and serum are depleted. Therefore, it is possible that inhibition of β-oxidation in mitochondria by inhibiting mitochondrial acyl-CoA synthase might be critical for the inhibition of tolerance. In the present study, we showed clearly that troglitazone inhibits phosphorylation of Akt in response to glucose and serum starvation; although the inhibition was transient, it might be responsible for the cell death. However, we are still not clear about how troglitazone blocks the tolerance. In recent studies, successful results were reported in growth suppression of breast (46, 47) and prostate (48) cancers, as well as liposar-

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4 Unpublished data.
5 H. Esumi, unpublished data.
6 K. Kato, unpublished data.
comata (49, 50), by troglitazone. The mechanisms might be partially related to our present findings.

Concerning nutrient deprivation therapies, embolization therapy of hepatic artery for hepatocellular carcinoma is the most successful example. The liver has a dual blood supply, with 75% of parenchymal blood flow coming from the portal vein and 25% from the hepatic artery (51). Anatomical and physiological studies using radiolabeled amino acids, florodexoyouridine, and albumin demonstrated that blood supply of malignant tumors in the liver, including metastatic cancer, is mainly derived from the hepatic artery (51–54). However, hepatic arterial embolization therapy for metastatic cancer is not effective and not always sufficient, even for primary liver cancer. Our results might be able to provide a possible explanation and may help to suggest new therapeutic strategies designed to prevent metabolic adaptation of cancer cells. We believe that our present findings open a new field of research to prevent metabolic adaptation of cancer cells.

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