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Autophagy is an important metabolic pathway to determine leukemia cell survival following suppression of the glycolytic pathway

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ABSTRACT

Most cancer cells predominantly produce energy by glycolysis, even in the presence of adequate oxygen. Therefore, inhibition of glycolysis is a promising cancer treatment target. Recently, it has been recognized that to conduct thorough treatment of cancer, comprehensive understanding of cancer metabolism is essential, not only focusing on glycolysis. Here, we investigated the supporting mechanism of autophagy, which is a catabolic process that recycles intracellular components, for energy supply in the glycolysis-inhibited condition. Autophagy is thought to be highly activated in cancers and to promote their growth or progression by adapting to the harsh surrounding microenvironment. We found that cancer cells positively promoted autophagy to overcome the energy shortage from glycolysis by maintaining mitochondrial activity for ATP production essential for survival. Conclusively, autophagy plays a role in determining whether cancer cells live or die, and autophagic ability in cancer cells is a promising target for therapy.

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1. Introduction

Cancer cells including leukemia cells mainly obtain their energy for survival, ATP, from glycolysis instead of mitochondrial oxidative phosphorylation (OXPHOS) even in the presence of available oxygen [1]. Although mitochondrial impairment and subsequent defective OXPHOS are frequently found in cancers, most cancer cells have normal mitochondrial function including an ability to perform OXPHOS, indicating that they are equipped with specific mechanisms to regulate enhanced glycolysis. This metabolic switch involves specific oncogenes, which mostly activate hypoxia-inducible factor (HIF)-1 α and/or the phosphoinositide 3-kinase/Akt signaling pathway. HIF-1 α stimulates glycolysis by trans-activation of glucose transporters and many glycolytic rate-limiting enzymes, such as hexokinase and pyruvate kinase [2]. HIF-1 α also

upregulates pyruvate dehydrogenase kinase, resulting in decreased flux through the tricarboxylic acid (TCA) cycle and OXPHOS [2]. Akt, a serine/threonine kinase, is a key molecule for promoting cancer growth, and mammalian target of rapamycin (mTOR) is a direct effector of Akt [3]. Akt-mTOR is a major signaling pathway involved in cancer development and progression, and has a pivotal role in the regulation of aerobic glycolysis and tumor growth, for example, by activation of HIF-1 α and c-Myc [3].

For cancer therapies, it is not surprising that the main focus is on molecules or machineries interfering with the energy level in cancer cells, especially the glycolytic pathway. Several small molecules such as 2-deoxyglucose and 3-bromopyruvate, which are inhibitors of hexokinase, exhibit promising anti-cancer activity *in vitro* [4] and have been clinically tested. However, there are no ongoing trials with these compounds as a single agent because of the lack of effects *in vivo* [5]. The exact reason for this failure is unclear; however, a combination of chemotherapeutic agents and/or other inhibitors of several energy metabolic pathways along with glycolytic inhibitors should be sufficient to obtain anti-cancer effects.

Autophagy is a self-digestion system for recycling damaged proteins and organelles. During this process, cytoplasmic constituents, including abnormal mitochondria or unfolded proteins, are surrounded by autophagosomes and degraded in lysosomes via fusion [6]. Emerging data suggest that autophagy plays a critical

Abbreviations: 6-AN, 6-aminonicotinamide; CQ, chloroquine diphosphate; DMSO, dimethyl sulfoxide; HIF, hypoxia-inducible factor; mTOR, mammalian target of rapamycin; OXPHOS, oxidative phosphorylation; PPP, pentose phosphate pathway; TCA, tricarboxylic acid.

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role in maintaining cellular homeostasis and survival under various stressed conditions including nutrient starvation [7]. Generally, it is believed that cancer cells depend more heavily on autophagy for survival than normal cells because of the higher metabolic demands associated with excessive proliferation and exposure to more stresses [7,8]. In particular, Soga's group observed that most amino acids were strongly accumulated in colon and stomach tumor tissues, and they discussed the possibility that the amino acids are derived from autophagy [9]. On the other hand, multiple studies have suggested that autophagy also leads to cell death in some types of cancers, and autophagy inhibition is associated with prolonged survival of the cells [10]. However, whether mammalian cell death can occur by autophagy has not been answered definitively, and it should be noted that at least some of the proteins that are essential for autophagy might also participate in signaling pathways that lead to autophagy-unrelated lethal processes. Therefore, it is unclear whether autophagy is involved in cell death or survival because of a lack of knowledge.

In the present study, we investigated how cancer cells acquire survival energy when glycolysis, the major energy source in cancer cells, is suppressed, using leukemia cells as model cancer cells that are highly dependent on glycolysis in a steady-state. In particular, we clearly showed that autophagy rescues the shortage of energy by maintaining OXPHOS in mitochondria for ATP production required for survival. Our findings suggest that to fully understand the energy supply in cancer cells, we need to consider not only glycolysis, but also autophagy, as a hidden factor that supports the survival of cancer cells.

2. Materials and methods

2.1. Materials

Chloroquine diphosphate (CQ), oligomycin, and 6-aminonicotinamide (6-AN), all purchased from Sigma–Aldrich (St. Louis, MO, USA), were dissolved in milliQ water or dimethyl sulfoxide (DMSO). The final DMSO concentration in every cell culture did not exceed 0.5% (v/v).

2.2. Cell culture and transfection

All examined cell lines were purchased from RIKEN Cell Bank (Tsukuba, Japan). CCRF-CEM, Jurkat, and L1210 cells were maintained in RPMI 1640 (Nacalai Tesque, Kyoto, Japan) and HeLa cells were maintained in MEM (Nacalai Tesque), with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA) plus antibiotics (Nacalai Tesque) at 37 °C in a humidified atmosphere of 5% CO₂ in air. In each experiment, cells were cultured in glucose (2 g/L)-, galactose (2 g/L)-, or pyruvate (2.4 g/L)-based medium.

2.3. Plasmid construction and transfection

LC3 cDNA was cloned from CCRF-CEM cells and inserted into the pAcGFP1-C1 plasmid (Clontech, Palo Alto, CA, USA), and the resultant plasmid was named GFP-LC3. For transfection of GFP-LC3 into HeLa cells, Lipofectamine™ 2000 (Life Technologies) was used according to the manufacturer's protocol. After 48 h of transfection, images were obtained using a Carl Zeiss LSM700 laser scanning confocal microscope (Prenzlauer, Berlin, Germany).

2.4. Flow cytometric detection of dead cells

Collected CCRF-CEM cells were stained with the Zombie NIR™ Fixable Viability kit (BioLegend, San Diego, CA, USA) to distinguish dead cells from live cells according to the manufacturer's

instruction. Data acquisition of fluorescence was performed using an EC800 cell analyzer (SONY, Tokyo, Japan).

2.5. Measurement of cellular lactate release

The amount of cellular lactate release was measured as described previously [11], with minor modifications. Briefly, the supernatant of the collected cell culture medium was deproteinized with perchloric acid and neutralized with potassium hydroxide. For lactate assessment, the supernatant was mixed with nicotinamide adenine dinucleotide and glutamate pyruvate transaminase (Roche, Mannheim, Germany). The enzymatic reaction was started by adding lactate dehydrogenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to each sample and then incubating at 37 °C for 30 min. Absorbance was measured at a wavelength of 340 nm.

2.6. Western blot analysis

Cells were harvested and lysed on ice with cell lysis buffer containing phosphate-buffered saline (pH 7.4), 1% Triton X-100, and a protease inhibitor cocktail (Roche). Identical amounts of protein from each sample were loaded and run on SDS-PAGE gels and then transferred to polyvinylidene difluoride membranes (Merck Millipore, Berlin, Germany). After membrane blocking, the blots were probed with specific primary antibodies (Cell Signaling Technologies, Beverly, MA, USA) according to the manufacturer's instructions. Immunolabeled proteins were detected using a horseradish peroxidase-labeled donkey anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ECL prime detection reagent (GE Healthcare, Buckinghamshire, UK).

2.7. Autophagic flux assay

Autophagic flux was evaluated by monitoring turnover of the autophagic marker LC3-II using western blot analysis in the presence and absence of CQ, a lysosomal degradation inhibitor, according to a previous report [12]. Autophagic flux was measured as the difference in LC3-II protein between CQ-treated samples and CQ-untreated samples in the respective group.

2.8. Measurement of the cellular ATP content

Suspensions of CCRF-CEM cells cultured in various conditions were collected and used for measurement. The cellular ATP concentration was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

2.9. Measurement of cellular oxygen consumption rate

The oxygen consumption of cells cultured in glucose-, galactose-, or pyruvate-based medium was measured using a fluorescent oxygen probe, PreSens Sensor Dish Reader (Regensburg, Germany). The oxygen tension was continuously monitored every minute and the concentration at time 0 was set to 100%.

2.10. Statistical analysis

All data were expressed as the mean \pm SD of at least three independent experiments unless indicated. Statistical analysis was performed using the Student's *t* test or an analysis of variance followed by the Bonferroni test where applicable.

3. Results

3.1. Autophagy is enhanced under the glycolysis-suppressed condition

First, to examine the effect of glycolysis inhibition on cancer cell survival, the sugar source in the culture medium of CCRF-CEM cells, leukemia model cells, was changed from glucose to galactose or pyruvate. It is reported that oxidation of galactose to pyruvate, which is the final glycolytic product, via glycolysis yields no net ATP [13]. By changing the sugar source from glucose to galactose or pyruvate, production of lactate, which is converted from pyruvate in the final step of anaerobic glycolysis, was markedly decreased, meaning ATP supply by glycolysis was suppressed (Fig. 1A). We next evaluated the viability of CCRF-CEM cells cultured in medium containing each sugar source. While culture in galactose- or pyruvate-based medium significantly inhibited cell proliferation (Fig. 1B), it rarely led to noticeable cell death (Fig. 1C). These results suggest that CCRF-CEM cells can survive by well-regulating the metabolic system, even if the ATP supply from glycolysis is inhibited.

Next, we monitored autophagy when glycolysis was inhibited. For analysis of autophagy, CCRF-CEM cells were subjected to immunoblotting to evaluate flux of the specific marker LC3-II as mentioned in the Materials and methods section. CQ is an inhibitor of the later autophagic steps via the fusion between autophagosomes and lysosomes. Compared with cells grown in glucose-based medium, autophagy was continuously induced in cells grown in galactose- or pyruvate-based medium (Fig. 1D), suggesting that glucose depletion in the culture medium promoted autophagy in live cells.

3.2. Autophagy contributes to cancer cell survival to overcome glycolysis suppression

To investigate whether increased autophagy during glycolysis inhibition contributes to the survival of cancer cells, the viability of CCRF-CEM cells grown in glucose-, galactose-, or pyruvate-based medium with the autophagy inhibitor CQ was evaluated. While

cells cultured in glucose-based medium were barely affected by CQ treatment, the death rate of cells grown in pyruvate-based medium was significantly increased by the same treatment and that of cells grown in galactose-based medium was slightly increased, indicating that such cells were reliant on autophagy for survival (Fig. 2A). Cancer cells in which the glycolytic pathway is inhibited seem to induce autophagy for survival to avoid cell death due to energy shortage. When the sugar source in the culture medium was replaced with pyruvate, the cellular ATP content was significantly reduced by CQ treatment (Fig. 2B).

Generally, mammalian cells can generate ATP through two metabolic pathways, glycolysis and OXPHOS, and autophagy is not a direct pathway for supplying ATP. We focused on OXPHOS for producing cellular ATP when the glycolytic pathway is inhibited. As expected, CCRF-CEM cells cultured in galactose- or pyruvate-based medium exhibited high sensitivity to treatment with oligomycin, an inhibitor of ATP synthase in mitochondria, because ATP production was significantly suppressed (Fig. 2C), suggesting that these cells relied on OXPHOS to acquire energy for survival. Next, to examine whether autophagy functions as a regulator of OXPHOS, we evaluated the change in the oxygen consumption rate when autophagy was inhibited by CQ exposure in CCRF-CEM cells. In every culture condition, the rate of oxygen consumption was reduced by autophagy inhibition, and, interestingly, while cells cultured in galactose-based medium consumed more oxygen than those cultured in glucose-based medium, cells cultured in pyruvate-based medium did not show accelerated oxygen consumption (Fig. 2D). These data suggest that basal or upregulated ATP production from OXPHOS is utilized for survival, as a small energy source to overcome glycolysis suppression, and autophagy functions as a positive regulator of OXPHOS.

3.3. Metabolic shift from glycolysis to the pentose phosphate pathway (PPP) partially helps to drive OXPHOS

While inhibition of OXPHOS in CCRF-CEM cells cultured in

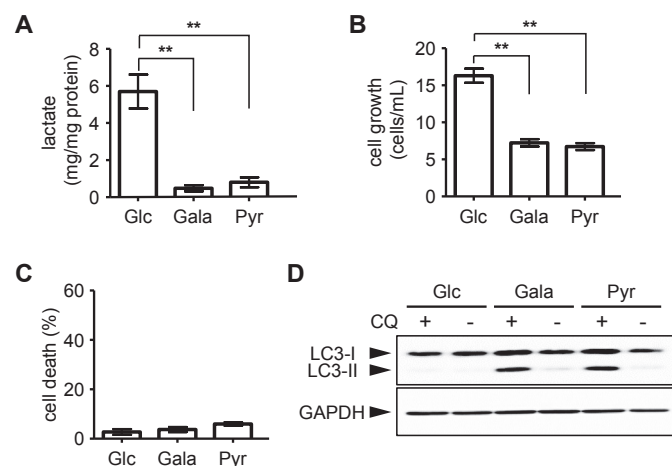


Fig. 1. Autophagy is enhanced under the glycolysis-suppressed condition. (A) CCRF-CEM cells were cultured in glucose (Glc)-, galactose (Gala)-, or pyruvate (Pyr)-based medium for 5 days, and the amount of lactate released was evaluated. ** $P < 0.01$. (B) The number of cells grown in (A) was counted. ** $P < 0.01$. (C) The death rate of the cells in (A) was evaluated using flow cytometry. (D) CCRF-CEM cells pre-cultured in Glc-, Gala-, or Pyr-based medium for 48 h were additionally treated with or without CQ (5 μ M) for 72 h. The cell lysates were subjected to western blotting with anti-LC3 and anti-GAPDH antibodies.

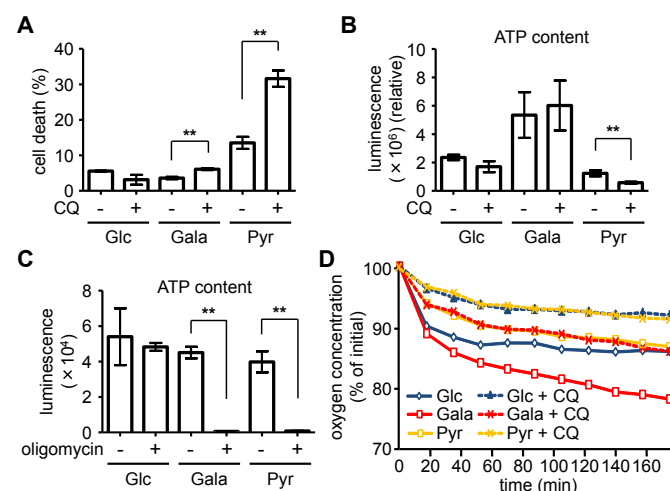


Fig. 2. Autophagy contributes to cancer cell survival to overcome glycolysis suppression. (A) CCRF-CEM cells pre-cultured in glucose (Glc)-, galactose (Gala)-, or pyruvate (Pyr)-based medium for 48 h were additionally treated with or without CQ (50 μ M) for 72 h, and the death rate of the cells was evaluated. ** $P < 0.01$. (B) The ATP concentration in the cells in (A) was quantitated and normalized by the amount of protein. ** $P < 0.01$. (C) CCRF-CEM cells pre-cultured in Glc-, Gala-, or Pyr-based medium for 48 h were additionally treated with or without oligomycin (50 ng/mL) for 24 h, and the total ATP content was quantitated. ** $P < 0.01$. (D) The oxygen concentration in the culture medium of cells cultured in Glc-, Gala-, or Pyr-based medium with or without CQ (20 μ M) was measured over time. The data are the average of duplicate samples.

galactose-based medium significantly reduced ATP production (Fig. 2C), these cells exhibited relatively low sensitivity to the autophagy inhibitor (Fig. 2A) and high oxygen consumption (Fig. 2D) compared with those cultured in pyruvate-based medium. To interpret these results, we focused on involvement of the PPP, a sub-pathway of glycolysis, because galactose can be converted to glucose 6-phosphate, a starting material of the PPP. We hypothesized that NADPH generated from the PPP can excessively activate OXPHOS by buffering oxidative stress generated by OXPHOS [14]. To prove this hypothesis, we exposed CCRF-CEM cells grown in galactose-based medium to 6-AN, which is an inhibitor of glucose-6-phosphate dehydrogenase, the rate-limiting step enzyme of the PPP. Indeed, 6-AN exposure reduced the NADPH/NADP⁺ ratio in the cells as reported previously (data not shown) [15]. In addition, these PPP-inhibited CCRF-CEM cells had more enhanced autophagic activity (Fig. 3A), suggesting that the PPP helps to generate energy for survival in cancer cells as well as autophagy. To confirm this possibility, CCRF-CEM cells grown in galactose-based medium were co-treated with CQ and 6-AN. This co-treatment significantly increased cell death (Fig. 3B). Furthermore, we compared the oxygen consumption ratio between 6-AN-treated CCRF-CEM cells grown in galactose-based medium and those cultured in pyruvate-based medium. 6-AN suppressed oxygen consumption in cells cultured in galactose-based medium and the ratio was close to that in cells cultured in pyruvate-based medium (Fig. 3C), suggesting that not only autophagy but also the PPP can positively drive OXPHOS in specific conditions when a sugar source such as galactose can be utilized in the PPP.

3.4. Autophagy contributes to survival of other cancer cells in the glucose-depleted condition

The above examinations using CCRF-CEM cells revealed that autophagy plays an important role in energy supply when the energy supply from glycolysis is insufficient. It is worthwhile to clarify whether this machinery is also applicable to other leukemia and/or cancer cells. First, we investigated the impact of autophagy inhibition on the survival of other leukemia cell lines, Jurkat and L1210. Indeed, autophagy inhibition with CQ increased the death rate of both these cell lines, especially in galactose- or pyruvate-based medium (Fig. 4A and B). Next, using solid cancer HeLa cells, autophagic flux was evaluated in each culture condition. Autophagy was active in HeLa cells grown in galactose- or pyruvate-

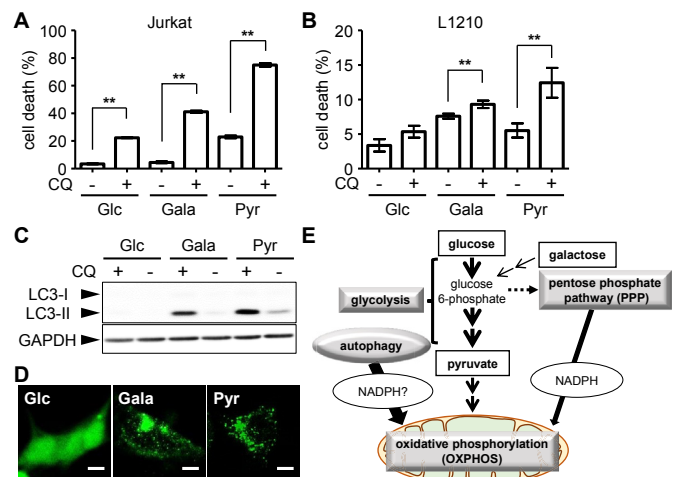


Fig. 4. Autophagy contributes to survival of other cancer cells in the glucose-depleted condition. (A) Jurkat cells pre-cultured in glucose (Glc)-, galactose (Gala)-, or pyruvate (Pyr)-based medium for 48 h were additionally treated with or without CQ (50 μ M) for 72 h, and the death rate of the cells was evaluated using flow cytometry. ** $P < 0.01$. (B) A similar examination as described in (A) was performed using L1210 cells treated with CQ (20 μ M). ** $P < 0.01$. (C) HeLa cells pre-cultured in Glc-, Gala-, or Pyr-based medium for 48 h were additionally treated with or without CQ (5 μ M) for 72 h. The cell lysates were subjected to western blotting with anti-LC3 and anti-GAPDH antibodies. (D) HeLa cells were transiently transfected with GFP-LC3 and cultured in Glc-, Gala-, or Pyr-based medium for 72 h. The subcellular localization of GFP-LC3 was observed using confocal microscopy. Scale bar indicates 10 μ m. (E) A schematic diagram of the energy-acquiring network between the examined typical pathways, glycolysis, OXPHOS, autophagy, and the PPP.

based medium (Fig. 4C). In addition, enhancement of autophagosome formation in HeLa cells grown in galactose- or pyruvate-based medium was visualized by observing the subcellular localization of introduced GFP-LC3 (Fig. 4D). Taken together, these results suggest that autophagy is key to maintaining cellular energy, especially in the glucose-depleted condition, in almost all cancer cells tested. In the light of these results, we proposed a scheme indicating the connection of several metabolic pathways (Fig. 4E).

4. Discussion

In this study, we clearly proposed the importance of autophagic ability for cancer cell survival, especially under the glycolysis-suppressed condition. In addition, we showed that autophagy is a driving force for entry into OXPHOS. Furthermore, we found that the PPP, which is a sub-pathway of glycolysis, helps to maintain stable OXPHOS activity as well as autophagy.

Emerging data suggest that autophagy plays a critical role in maintaining cellular homeostasis and survival under stressed conditions [7]. Here, we clearly showed for the first time that autophagy is key to generating cellular energy by maintaining OXPHOS in mitochondria, and that energy is essential for survival especially when glycolysis is suppressed in cancer cells. It is reported that the oncogenic gene Ras upregulates basal autophagy, which is required for tumor cell survival in starvation conditions and tumorigenesis [16]. Furthermore, they revealed that in Ras-overexpressing cells, defective autophagosome formation causes accumulation of abnormal mitochondria and reduced oxygen consumption. Another group showed that pancreatic ductal adenocarcinoma, with activation of KRAS and loss or mutation of p53, INK4A, and SMAD4, has a distinct dependence on autophagy, and inhibition of autophagy leads to a decrease in OXPHOS [17]. As argued in these reports, several specific conditions such as overexpression of oncogenes may accelerate autophagy for

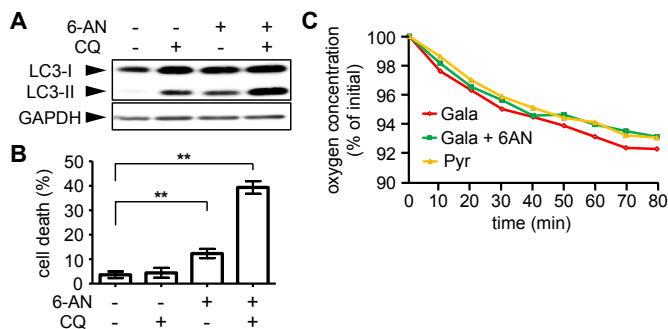


Fig. 3. Metabolic shift from glycolysis to the PPP partially helps to drive OXPHOS. (A) Lysates of cells grown in galactose (Gala)-based medium with or without 6-AN (200 μ M) or CQ (5 μ M) for 72 h were subjected to western blotting with anti-LC3 and anti-GAPDH antibodies. (B) The death rate of the cells cultured in Gala-based medium with or without 6-AN (200 μ M) or CQ (50 μ M) for 72 h was evaluated using flow cytometry. ** $P < 0.01$. (C) The oxygen concentration in the culture medium of cells cultured in Gala or pyruvate (Pyr)-based medium with or without 6-AN (200 μ M) was measured over time. The data are the average of duplicate samples.

mitochondrial OXPHOS to generate cellular ATP. Our findings give new insights into the important role of autophagy to maintain energy homeostasis by driving OXPHOS in harsh conditions when the energy supply from glycolysis is insufficient.

The reduction in OXPHOS could reflect an accumulation of damaged mitochondria due to suppression of autophagic degradation of mitochondria, so-called mitophagy. Several studies identified a relationship between mitophagy and mitochondrial biogenesis including OXPHOS [18]. Melser et al. revealed that the small GTPase Rheb protein is recruited to the mitochondrial outer membrane upon high OXPHOS activity and regulates induction of autophagy [19]. They also showed that supplementation of the TCA cycle with an analog of α -ketoglutarate, converted from glutamate, is necessary to induce mitophagy. In tumor tissues, strong accumulation of most amino acids, with the exception of glutamine, is observed, probably due to high activation of autophagy [9]. We speculate that conversion of glutamine to glutamate might be enhanced in cancer cells, and a portion of the glutamate pool is necessary for quality control of mitochondria by mitophagy. This hypothesis will be investigated in a future study.

From a series of studies, we propose that autophagy is related to driving of OXPHOS in mitochondria to produce cellular energy. However, while inhibition of OXPHOS in CCRF-CEM cells cultured in galactose-based medium significantly reduced ATP production, these cells exhibited relatively low sensitivity to an autophagy inhibitor. These seemingly contradictory events can be explained by the involvement of the PPP, which is a sub-pathway of glycolysis, resulting in generation of sufficient NADPH to activate stable OXPHOS. By contrast, cells cultured in pyruvate-based medium could not produce NADPH via the PPP, but maintained mitochondrial activity probably only by autophagy. DeBerardinis et al. suggested that transformed cells engage in glutamine metabolism, which finally provides a robust source of NADPH via TCA cycle intermediates [20]. Considering this together with our findings, cells grown in pyruvate-based medium could obtain NADPH, as a driving force of OXPHOS, almost only from highly activated autophagy. With the exception of special circumstances in which the PPP is enhanced, autophagy should be a mainstay for stably driving OXPHOS in mitochondria.

Despite recent advances in cancer treatment, many cancers still exhibit unsatisfactory responses to chemotherapy, resulting in their recurrence or continuation of their growth after treatment. Many researchers believed that the mTOR pathway would be a hopeful cancer therapy target because mTOR regulates cell growth, proliferation, survival, and other processes. However, strikingly, rapamycin, an inhibitor of mTOR, failed in clinical trials. One reason for this was autophagy induced by mTOR inhibition [21]. Owing to this finding, the importance of autophagy, which allows cancer cells to survive, has been noted in cancer research. Recently, accumulating evidence indicated that CQ, which has long been used for anti-malarial effects, sensitizes cancer cells to radiation and other anti-cancer reagents [22]. The underlying mechanism is considered to be the anti-autophagic activity of CQ. Generally, autophagic activity is relatively high in cancers and is commonly upregulated by exposure to cancer therapies, indicating that, when taking our findings into consideration, the cytoprotective effects of autophagy provide therapeutic opportunities, and co-treatment with an autophagy inhibitor and conventional chemotherapies is expected to show synergistic anti-cancer effects.

In conclusion, we found that cancer cells promote autophagy to overcome the energy shortage from glycolysis, the major energy source, by maintaining mitochondrial activity to produce ATP necessary for survival. More evidence of the role of autophagic ability in cancer cell survival with a focus on overall energy

metabolism should provide an appropriate treatment regimen that takes into account the characteristics of the cancer.

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