Downregulation of c-MYC Protein Levels Contributes to Cancer Cell Survival under Dual Deficiency of Oxygen and Glucose

Hiroaki Okuyama¹, Hiroko Endo¹, Tamaki Akashika², Kikuya Kato², and Masahiro Inoue¹

Abstract

The c-MYC protein participates in energy-consuming processes such as proliferation and ribosome biosynthesis, and its expression is often dysregulated in human cancers. Cancer cells distant from blood vessels in solid tumors are in short supply of oxygen and nutrition yet can adapt to the microenvironment and survive under metabolic stress. The role and regulation of c-MYC protein in the tumor microenvironment of limited energy sources are poorly understood. Here, we show that c-MYC protein levels in cancer cells are strikingly reduced in the area distant from the blood vessels in vivo and also under oxygen- and glucose-deprived conditions in vitro. The rapid reduction of c-MYC protein levels requires low levels of both oxygen and glucose, and under these conditions, downregulation is mainly achieved by enhanced degradation. Suppression of c-MYC protein levels by small hairpin RNA decreases the necrotic cell death induced by oxygen and glucose deprivation. Thus, the environmental milieu regulates c-MYC protein levels, and downregulation of c-MYC might be a strategy for cancer cells to survive under conditions of limited energy sources.

Introduction

Most solid tumors contain substantially large hypoxic regions. Hypoxic cancer cells are known to be resistant to chemotherapy and radiation, leading to a high risk of local recurrence and distant metastasis, which are associated with a poor clinical prognosis (1).

Under hypoxic conditions, a transcription factor, hypoxia inducible factor-1α (HIF-1α), accumulates dramatically through inhibition of ubiquitination and proteasomal degradation (2). HIF-1 is a heterodimeric protein consisting of HIF-1α and HIF-1β subunits (3) and regulates the expression of hundreds of genes to promote angiogenesis, proliferation, and survival of hypoxic tumor cells (2). Cancer cells show a high glycolytic rate even under aerobic conditions (4), and hypoxic cancer cells are challenged by a compound deficiency in energy substrates, including oxygen and glucose (2, 5). Nonetheless, cancer cells can survive under energy-poor conditions, and the characteristics of cancer cells in the hypoxic microenvironment within a solid tumor are still poorly understood.

The oncoprotein c-MYC is a basic helix-loop-helix/leucine zipper (bHLH/Zip)-type transcription factor. c-MYC forms a heterodimer with the bHLH/Zip protein MAX. After dimerization, this complex binds specific DNA sites, at a CACGTG sequence known as the E-box motif (6), to activate and repress transcription of target genes as well as to modulate chromatin. In many human cancers, the expression level of c-MYC is increased. On the other hand, overexpression of c-MYC is known to induce apoptosis in normal cells as well as in cancer cells (7, 8). Thus, the expression levels of c-MYC must be tightly regulated in cancer cells.

c-MYC contributes to the promotion of cell proliferation, ribosomal biosynthesis, glycolysis, mitochondrial functions, and differentiation. Notably, these are all energy-consuming processes. It is reasonable to speculate that c-MYC helps cancer cell proliferation only when the energy source supply is abundant. Meanwhile, high levels of c-MYC might be harmful for cancer cells in regions with a poor energy supply. Indeed, it has been reported that glucose deprivation enhances induction of cell death by c-MYC overexpression (9). Therefore, we hypothesized that cancer cells have an adaptive system of reducing c-MYC levels to survive under conditions of deficient energy sources.

In this study, we show that tumor cells in a region distant from blood vessels in vivo are metabolically inactive and that c-MYC protein levels are dramatically reduced in this area. The metabolically inactive status with low levels of c-MYC conditions, and the characteristics of cancer cells in the hypoxic microenvironment within a solid tumor are still poorly understood.
protein can be reproduced in vitro by culturing of cells under oxygen- and glucose-deprived conditions. These low levels of c-MYC are mainly mediated by enhanced protein degradation. Suppression of c-MYC protein protects cancer cells from the necrotic cell death that would result from the energy source deficiency.

Materials and Methods

Cell lines and cell culture

HCT116 and DLD1 were obtained from the American Type Culture Collection in 2001 to 2009. We monitored the cell morphology of these cell lines by microscopy and confirmed to maintain their morphologic images in comparison with the original morphologic images. No Mycoplasma contamination was confirmed in cultures of all of both cell lines, using a Mycoplasma Detection kit (Takara Bio) in 2009. To determine the effect of oxygen and glucose deprivation, the cells were placed in Dulbecco’s modified Eagle’s medium without glucose supplemented with 10% FBS and incubated under hypoxia in an Invivo2 400 hypoxia workstation (Ruskinn). The cells were cultured at 1% oxygen tension for hypoxic conditions. Cell viability was assessed using trypan blue exclusion.

Reagents

Pimonidazole (Hypoxprobe-1 Plus Kit) was purchased from Natural Pharmacia International, Inc. 5-Bromo-2'-deoxyuridine (BrdU), 4-hydroxytamoxifen, cycloheximide, and propidium iodide (PI) was purchased from Invitrogen Molecular Probes.

Construction of c-MYC-ERT and IRES/c-MYC

DNA encoding amino acids 1–829 of human c-MYC was excised from pMX/hc-MYC (Addgene Inc.) at the BamHI and the ClaI sites; DNA encoding amino acids 829–1,365 of human c-MYC was excised from pGEM/hc-MYC at the ClaI and the XhoI sites; and DNA encoding amino acids 282–595 of the murine estrogen receptor, which includes 3 point mutations (G400V, M543A, and L544A), was excised from pBLC/Cre-ERT2, which was derived from pCre-ERT2 (kindly gifted by Dr. Pierre Chambon), at the XhoI and the NotI sites (10–12). Subsequently, all 3 fragments containing the full length of c-MYC and ERT2 were cloned in the correct orientation into the BamHI and the NotI sites of the retrovirus vector pMXpuro-stuffer X to form pMXpuro/c-MYC-ERT. DNA encoding the full length of IRES that is derived from the encephalomyocarditis virus was excised from pMX/IRES/EGFP (kindly gifted from Dr Toshio Kitamura, Tokyo University) at the BamHI site. This fragment containing the full length of IRES was cloned in the correct orientation into the BamHI site of pMX/he-MYC to form pMX/IRES/c-MYC.

Establishment of cell lines

The short hairpin RNA (shRNA) vector targeting c-Myc, pSUPER.retro Myc shRNA (#15662) (corresponding sequence: 5'-GATGAGGAAGAATCGATG-3'), was purchased from Addgene. A shRNA targeting HIF-1α (corresponding sequence: 5'-GACATACAGGATGCTTGC-3') was inserted into the retrovirus vector pSUPER.retro.puro. Puromycin-resistant clones were selected in the presence of 1.0 μg/mL puromycin.

Laser microdissection

The regions around CD31-positive areas and the regions adjacent to pimonidazole-stained areas were microdissected using Application Solutions Laser Microdissection System version 4.1 (Leica; ref. Fig 1D). The pimonidazole-staining regions were not collected, because pimonidazole detects nearly anoxic area (13). Furthermore, both quality and quantity of RNA from the area were quite poor, probably because of substantial amount of dead or dying cells in the area (data not shown). Total RNA was extracted by RNeasy Micro Kit (Qiagen).

Microarrays

One microgram of total RNA was reverse transcribed by MMLV-RT (Agilent Technologies), using an oligo-dT primer that incorporated a T7 promoter sequence. The cDNA was then used as a template for in vitro transcription using T7 RNA polymerase and Cy3-labeled RNA. The Cy3-labeled RNA was hybridized to Agilent 4× 44K microarrays and scanned as directed by the manufacturer. Data were extracted using Feature Extraction Software v8.1 (Agilent Technologies) under default settings for normalization.

RT and real-time PCR

Real-time PCR was done using the iQ SYBR Green Supermix and iCycler Real-Time PCR Detection System (Applied Biosystems) as previously described (14). Primer sequences are tabulated in the supplementary information. The expression of each target mRNA relative to 18 S rRNA or β-actin was calculated on the basis of threshold cycle (Ct) as r = 2−ΔΔCt, where ΔCt = Ct target − Ct control and ΔΔCt = ΔCt experimental − ΔCt control.

Immunoblot assays

Antibodies used for immunoblot assays were anti–HIF-1α (H1067; Novus Biologicals), anti-HIF-1α (clone54; BD Bioscience), anti-c-MYC (Millipore), and anti-p-S6 (Cell Signaling).

Measurement of glucose and lactate

The concentrations of glucose and lactate were measured with a colorimetric kit according to the manufacturer’s instructions (Wako and Roche, respectively). The value of glucose consumption and lactate production was normalized to the cell number.

Oxygen consumption assay

Oxygen tension was measured using a Clark-type oxygen electrode system (Model 203, Instech Laboratories). Oxygen consumption was calculated as previously described (15).
Polysome assay

The cytoplasmic extracts were layered over 10%–50% sucrose gradients and centrifuged for 90 minutes at 33,400 rpm at 4 °C in a HITACHI RPS-50 rotor. After determination of polysomal and monosomal fractions by 1% agarose gel electrophoresis, total RNA was isolated from each fraction using the RNeasy Mini Kit (Qiagen), and cDNA synthesis was carried out on pooled RNA fractions using SuperScript II Reverse Transcriptase (Invitrogen). Subsequently, real-time PCR was done to quantify c-MYC, HIF-1α, and β-actin mRNA.

Measurement of ATP

ATP levels were measured using an ATP assay kit (Sigma) according to the manufacturer’s instructions. Luminescence was measured using a Wallace microplate luminescence reader (Perkin Elmer) and normalized to the protein concentration.

Figure 1. Cancer cells are inactive in the region distant from blood vessels in the tumor. Immunohistochemistry of a xenograft tumor derived from HCT116 cells. Pimonidazole (Pimo; green) and CD31 (red; A); BrdU (red; B); phosphorylated S6 protein (pS6; red; C); N, necrotic region; T, live tumor cell region. D, mapping of A for the microdissection. D, distal region from blood vessels not including pimonidazole stained areas. P, proximal region from blood vessels. Scale bar, 100 μm. E, microarray analysis. Relative expression of HIF target genes under hypoxic conditions against normoxic conditions in vitro (white bars) and in the distal region versus the proximal region for 24 hours in vivo (black bars). ADM, adrenomedulin; GLUT3, glucose transporter 3; IGFBP3, insulin-like growth factor binding protein 3; GAPDH, glyceraldehyde 3-P-dehydrogenase; PDK3, pyruvate dehydrogenase kinase 3; ALDOA, aldolase A; PGK1, phosphoglycerate kinase 1. F, validation of gene expression by real-time PCR.
Flow cytometry
Cells were labeled with 10-mmol/L bromodeoxyuridine (BrdU) for the last 1 hour of treatment. The cells were stained with PI (10 mg/mL) and AlexaFluor-488–conjugated anti-BrdU antibody (Invitrogen Molecular Probes). Apoptotic cells were detected using Annexin V-FITC Apoptosis Detection Kit (Biovision). The cells were analyzed using FACScalibur (Becton Dickinson). Data analysis was done with FlowJo (TreeStar Inc.).

Xenograft
All animal experiments were approved by the Osaka Medical Center Animal Care and Use Committee. One million cells were injected subcutaneously into the flanks of 6- to 8-week-old female athymic nude mice (Charles River). One month later when the tumor diameters were about 10 mm, the mice were sacrificed and the tumors removed, followed by subsequent histologic evaluation.

Immunohistochemistry
The tumor was placed in OCT compound and frozen. Ten-micrometer sections were affixed to a slide glass, and sections were incubated with a 1:100 dilution of anti–HIF-1α antibody (BD Biosciences) and anti-c–MYC antibody (Millipore) and a 1:200 dilution of anti-phosphorylated S6 antibody (Cell Signaling) and anti-BrdU (BD Biosciences).

Statistics
The values presented as bar graphs are means ± SD. The differences between groups were examined for significance with Student’s t test, with P values < 0.05 considered to be significant.

Results

Cancer cells are in an inactive metabolic status in the region distant from blood vessels in the tumor
To evaluate the proliferation and metabolic activity of cancer cells located in the hypoxic region of the tumor, we examined xenograft tumors derived from the human colon cancer cell line, HCT116. Pimonidazole, a hypoxia marker, was detected in the area distant from the tumor vessels (Fig. 1A). Proliferative cancer cells showing BrdU incorporation were found only in the area proximal to the tumor vessels, including the pimonidazole-positive zone (Fig. 1B). Similarly, phosphorylated S6 (p-S6), downstream of mTOR and involved in ribosomal biogenesis, was detected only in the area proximal to the tumor vessels but not in the area distant from the tumor vessels, including the pimonidazole-positive zone (Fig. 1B). Similarly, phosphorylated S6 (p-S6), downstream of mTOR and involved in ribosomal biogenesis, was detected only in the area proximal to the tumor vessels but not in the area distant from the tumor vessels (Fig. 1C). These results indicate that cancer cells proximal to the blood vessels are active in proliferation and metabolism, whereas cancer cells distant from the blood vessels are inactive.

To assess cancer cell activity further, we laser microdissected the xenograft tumor, followed by microarray analysis, to compare the gene expression pattern of cancer cells under 4 different conditions: 1) distant from or 2) proximal to the blood vessels in vivo (Fig. 1D), and cultured under 3) normoxic or 4) hypoxic conditions in vitro. Expression of some HIF target genes was increased in cells cultured under hypoxic conditions compared with normoxic conditions and in cells at a distance from blood vessels compared with those in the proximal area. In contrast, except for GLUT3 genes, which are glycolysis-related genes and which are also HIF targets, were not induced in the area distant from the vessels in vivo, although these genes were induced under the hypoxic condition in vitro (Fig. 1E). Furthermore, we confirmed by real-time PCR that glycolysis-related genes were not induced in the area distant from the vessels in vivo (Fig. 1F). These results suggest that cancer cells distant from blood vessels are inactive in terms of glucose metabolism.

Cancer cells cultured under oxygen- and glucose-deprived conditions are metabolically inactive in vitro
In this part of the experiment, we attempted to mimic the inactive status of cancer cells in vivo. The cell cycle arrest at G0/G1 as well as the G2/M phase was markedly induced by oxygen- and glucose-deprived conditions (Fig. 2A). When the cells were cultured under the dual-deprivation conditions, cancer cell growth was dramatically suppressed (Supplementary Fig. S1A).

Next, we assessed metabolic status, especially translational signal activity, by detecting phosphorylation of S6. Under hypoxia/high-glucose conditions, phosphorylation of S6 was slightly inhibited, whereas under dual-deprivation conditions, phosphorylation of S6 was dramatically inhibited (Fig. 2B). Similarly, in the DLD1 human colon cancer cell line, phosphorylation of S6 was completely inhibited under the dual-deprivation conditions (Fig. 2B).

Hypoxia with high glucose-induced expression of glycolytic enzyme genes, such as GLUT1, PFK-P, PGK1, and GAPDH (Fig. 2C), a finding consistent with the previous reports (3, 9). In contrast, the dual-deprivation conditions inhibited hypoxia-induced gene expression of glycolytic enzymes, including PFK-P, PGK1, and GAPDH, although GLUT1 genes were still upregulated (Fig. 2C). Lactate production was increased in hypoxia compared with normoxia when the glucose concentration was high (Supplementary Fig. S1B). In contrast, when cells were cultured under glucose-deprived conditions, lactate production was low both under normoxic and hypoxic conditions (Supplementary Fig. S1B). To rule out latent cell death, we examined the colony-forming capacity and found no differences in plating efficiency among these 4 conditions (Supplementary Fig. S1C).

The levels of c-MYC protein decrease under oxygen and glucose deprivation
Next, we investigated the status of c-MYC in cancer cells identified as inactive. Forced induction of c-MYC resulted in elevated glucose consumption, lactate production, and oxygen consumption (Fig. 3A and Supplementary Fig. S2A). These results indicate that c-MYC contributes to energy-consuming processes.

We next evaluated the expression pattern of c-MYC protein both in vivo and in vitro. Immunohistochemistry revealed detection of c-MYC expression in the area proximal to the...
blood vessels but not in the area distant from blood vessels, coinciding with the inactive area described above (Fig. 3B). The levels of c-MYC protein were dramatically downregulated under oxygen- and glucose-deprived conditions in vitro (Fig. 3C). We observed the same pattern of c-MYC expression in DLD1 cells (Fig. 3C), with downregulation of c-MYC protein within 3–4 hours (Fig. 3D and Supplementary Fig. S2B). Meanwhile, c-MYC protein levels were gradually downregulated when the cancer cells were cultured under hypoxic conditions with high glucose, but this response required 48 hours to become prominent (Fig. 3E), consistent with previous reports (16, 17), probably as a result of the glucose consumption. Then, we checked whether glucose deprivation is critical for rapid downregulation of c-MYC protein under hypoxic conditions. The repression of c-MYC protein levels depended on glucose concentration (Fig. 3F), and addition of glucose rescued c-MYC expression levels to basal levels (Fig. 3G). These results indicate that glucose levels determine c-MYC protein levels under hypoxic conditions.

Protein degradation contributes to the downregulation of c-MYC protein levels under oxygen and glucose deprivation

We analyzed the mechanism of how c-MYC protein levels decrease under oxygen- and glucose-deprivation conditions and found that c-MYC mRNA levels did not change under these conditions (Fig. 4A). Next, we examined the translation of c-MYC mRNA by assessing the association of c-MYC mRNA with polysomal rRNA. Regarding specific gene translation, b-actin mRNA translation was completely inhibited under the dual-deprivation conditions, consistent with inhibition of global mRNA translation (Fig. 4B and Supplementary Fig. S3). In contrast, translation of c-MYC and HIF-1α mRNA was not inhibited under these conditions (Fig. 4B). Next, we examined degradation of the c-MYC protein, which is a rapid turnover protein because of its rapid degradation by the ubiquitin–proteasome system (18, 19). We assessed c-MYC protein levels over time after inhibition of de novo protein synthesis and found that oxygen and glucose deprivation...
The levels of c-MYC protein are decreased under oxygen and glucose deprivation. A, glucose consumption (left), lactate production (middle), and oxygen consumption (right) in the medium in which HCT116 MYC-ERT cells were cultured without serum for 24 hours, followed by stimulation with 4-OHT or ethanol (Et) for 24 hours. The values for glucose consumption, lactate production, and oxygen consumption were normalized to the cell number, mean ± SD, n = 3; *, P < 0.05.

B, immunohistochemistry of a xenograft tumor derived from HCT116 cells using antibodies against pimonidazole (green) and c-MYC (red). Scale bar, 100 μm.

C, Western blot of c-MYC and β-actin from 2 colon cancer cell lines, HCT116 (left) and DLD1 (right). The cells were cultured under the indicated conditions for 6 hours.

D, Western blot of c-MYC from HCT116 cells, cultured under the indicated conditions for 1 to 4 hours. E–G, Western blot of c-MYC and β-actin from HCT116 cells, cultured in the medium with high glucose conditions under the indicated conditions for 24, 48, or 72 hours (E); with the indicated glucose concentrations under normoxic or hypoxic conditions for 6 hours (F), and under the indicated conditions with or without glucose for 6 hours, or without glucose followed by addition of glucose at the concentration for the high-glucose conditions for 5 hours (G).
enhanced degradation of c-MYC protein levels (Fig. 4C, D). In addition, treatment with a proteasome inhibitor rescued the decreased c-MYC protein levels under the dual-deprivation conditions (Fig. 4E). Furthermore, c-MYC protein levels failed to increase even with the forced expression of c-MYC with an expression vector containing the translational initiation site or the internal ribosomal entry site (Fig. 4F).

Downregulation of c-MYC protein levels under oxygen and glucose deprivation is independent of HIF-1α

HIF-1α reportedly counteracts c-MYC-mediated mitochondrial biogenesis and oxygen consumption in renal cell carcinoma cells via transcriptional activation of the MXI1 gene, which encodes a protein that represses the transcriptional activity of c-MYC; furthermore, HIF-1α boosts proteasomal degradation of the c-MYC protein (16). Immunohistochemistry revealed HIF-1α expression in the tumor, but not in the pimonidazole-positive area or in the center of the tumor cord, which corresponds to the area adjacent to the blood vessels (Fig. 5A). In vitro, under oxygen and glucose deprivation, HIF-1α protein levels were reduced but still detectable (Fig. 5B).

Next, we examined the role of HIF-1α in the suppression of c-MYC protein levels under oxygen and glucose deprivation. The HCT116 cells were transiently transfected with a constitutively active HIF-1α (CA5)-expressing vector and a wild-type HIF-1α-expressing vector. Overexpression of the active or wild-type HIF-1α had no effect on c-MYC protein levels under normoxic conditions (Fig. 5C). We further generated HIF-1α knockdown HCT116 cancer cells by stable transfection with shRNA for HIF-1α. Although HIF-1α protein levels were remarkably decreased by the knockdown, c-MYC protein levels still decreased under the dual-deprivation conditions (Fig. 5D and Supplementary Fig. S4).

Downregulation of c-MYC protein levels rescues cancer cells from necrosis induced by prolonged culture under oxygen and glucose deprivation

To assess the functional role of c-MYC downregulation, we examined cell death under various culture conditions. After a prolonged culture of 48 hours, flow cytometry showed prominent PI-positive and Annexin-V-negative cells under conditions of oxygen and glucose deprivation (Fig. 6A and
Supplementary Fig. S5A). In addition, the cleavage of PARP and caspase-3 was detected under glucose-deprived conditions in normoxia, whereas they were hardly detectable under dual-deprivation conditions (Fig. 6B and Supplementary Fig. S5B). Meanwhile, the cancer cells dramatically decreased levels of ATP (Fig. 6C) and HMGB1 (Supplementary Fig. S6A) under oxygen- and glucose-deprivation conditions. These results support the inference that the type of cell death under the dual-deprivation conditions is mainly necrosis.

To examine the functional role of c-MYC suppression in cell death under dual-deprivation conditions, we modulated c-MYC expression in the HCT116 cells (Supplementary Fig. S6B). First, we checked the cell cycle profiles by flow cytometry and confirmed that knocking down of c-MYC did not affect the cell cycle status in this experimental setting (Supplementary Fig. S6C). Knocking down of c-MYC inhibited cell death under glucose-deprived conditions in normoxia and more remarkably under the dual-deprivation conditions (Fig. 6D and Supplementary Fig. S6D). Thus, downregulation of c-MYC contributes to avoiding necrotic cell death under oxygen and glucose deprivation.

Discussion

We showed that c-MYC levels were downregulated in regions distant from blood vessels in solid tumors derived from a colon cancer cell line, HCT116. In vitro, oxygen and glucose deprivation also reduced c-MYC protein levels through increased degradation of c-MYC protein. Downregulation of c-MYC protein contributes to survival of cancer cells under the dual-deprivation conditions, as the forced reduction of c-MYC expression by shRNA reduced necrotic cell death under these conditions.

The tumor microenvironment is heterogeneous, and one of the determinant factors of the heterogeneity is the distance from the vessels (20). Although cancer cells around the vessels receive sufficient oxygen, cells distant from the vessels (about 100 μm) are hypoxic because of the oxygen consumption by the amassed cancer cells closer to the blood supply (21). Tumor hypoxia is strongly associated with malignant progression, poor prognosis, and resistance to chemotherapy and radiation (22). Some hypoxic cancer cells are quiescent or dormant, that is, not proliferative and metabolically inactive (23). These cells might be a reservoir of therapy-resistant cancer cells, although neither the character nor the mechanism of the switching between active and inactive status has been elucidated. Work with an inducible c-MYC transgenic mouse model has shown that c-MYC inactivation results in tumor dormancy in liver whereas c-MYC reactivation restores neoplastic features (24–26). Thus, it is reasonable to speculate that c-MYC is a regulator between the active and inactive status of the cancer cells.
HIF-1 plays a central role in the hypoxia-induced malignant phenotype (2). Recent studies have highlighted the importance of the interaction between HIF and MYC (27, 28). First, HIF-1 functionally regulates c-MYC (16, 29), and second, HIF-1 regulates c-MYC expression at the transcriptional level (30). However, our results suggest that the decrease in c-MYC under dual-deprivation conditions is not regulated at the transcriptional level and is independent of HIF-1.

c-MYC is known as a rapid turnover protein (31) with an extremely short half-life (approximately 30 minutes) in proliferating cells. The c-MYC protein is rapidly ubiquitinated and degraded by the proteasome (18, 19). Phosphorylation of Thr-58 promotes c-MYC ubiquitination through interaction of the ubiquitin ligases Fbw7 and Skp2 (32–35). In addition, a deubiquitinating enzyme of c-MYC, USP28, contributes to degradation of the c-MYC protein (36). The degradation of c-MYC is reportedly enhanced in hypoxia through various mechanisms. In a VHL-deficient renal cell carcinoma cell line, HIF-1 promotes proteasome-dependent degradation of c-MYC (16). Furthermore, HIF-2 promotes c-MYC degradation through induction of Skp2 (37). Hypoxia increases phosphorylation of c-MYC at Thr-58 and subsequent ubiquitination of c-MYC by the ubiquitin ligase Fbw7 (38). In addition, hypoxia decreases the deubiquitinating enzyme USP28, leading to promotion of c-MYC degradation, and expression of both HIF-1 and HIF-2 is necessary for hypoxia-induced c-MYC degradation (38). We found that the mechanism of c-MYC degradation under the dual-deprivation conditions differed from these reported mechanisms. The shRNA knockdown of HIF-1Δ did not recover c-MYC protein levels, although the role of HIF-2α remains an open question. In some human cancer cells, c-MYC plays critical roles in their carcinogenesis through deregulated expression by gene amplification and/or chromosomal translocation (39). Therefore, we examined the

Figure 6. Downregulation of c-MYC protein levels rescues cancer cells from necrosis induced by prolonged culture under oxygen and glucose deprivation. A, cell death analysis by flow cytometry using antibodies against Annexin-V and PI. HCT116 cells were cultured under the indicated conditions for 48 hours. B, Western blot of cleaved PARP, total PARP, and β-actin. HCT116 cells were cultured under the indicated conditions for 48 hours. C, ATP levels in HCT116 cells cultured under the indicated conditions for 48 hours, mean ± SD, n = 3; *, P < 0.05. The value of ATP was normalized to the protein concentration. D, cell death analysis by the trypan blue exclusion test. HCT116 cells stably transfected with the shc-MYC construct (black bars) or the empty vector (white bars) were cultured under the indicated conditions for 48 hours. Mean ± SD, n = 3; *, P < 0.05.
deregulated c-MYC protein in human Burkitt lymphoma cell line, Raji cells, in which c-MYC is translocated to a switch region of the gamma heavy chain locus, as well as the HCT116 cells in which we forced overexpression of c-MYC by MYC-ERT system. In both cases, deregulated c-MYC protein was down-regulated under dual-deprivation conditions (Supplementary Fig. S7A and B). Further investigation is required to elucidate the molecular mechanism of c-MYC degradation under the dual-deprivation conditions.

Although c-MYC is a proto-oncogene and stimulates proliferation, overexpression of c-MYC activates apoptotic and senescence pathways (7, 40–42). Glucose deprivation enhances c-MYC–induced apoptosis in c-MYC–transformed fibroblasts, lymphblastoïd, or lung carcinoma cells (9, 43, 44). Indeed, under glucose-deprived conditions in normoxia, we observed that activation of c-MYC by MYC-ERT induced apoptosis in the HCT116 cells. In contrast, the type of death under the dual-deprivation conditions in HCT116 cells was likely not apoptosis. Neither PARP nor caspase-3 was cleaved under these conditions, and the number of Annexin-V–positive cells did not increase throughout the death process. Moreover, intracellular levels of ATP and HMGB1 decreased under the dual-deprivation conditions. Taken together, these findings suggest that the type of death under the dual-deprivation conditions is necrosis, which is usually associated with structural changes such as swelling and cell lysis (45). Functionally, it is accompanied by ATP depletion, HMGB1 release, and failure of the plasma membrane ionic pumps. Necrosis is a common feature of human cancers and often related to poor prognosis, especially in glioblastoma (46), but how necrosis arises in human cancers is not understood. We speculate on this subject as follows: Given that c-MYC stimulates energy-consuming processes, rapid depletion of ATP levels resulting from activation of c-MYC under dual-deprivation conditions might drive the cancer cells to necrosis. The initial high level of c-MYC protein might exhaust intracellular ATP rapidly by maintaining regular levels of energy-consuming processes. Once ATP was depleted, the cancer cells could not undergo either apoptosis or autophagy because both processes require ATP (47, 48). There might be other factors contributing c-MYC down-regulation in the in vivo hypoxic regions. Glutamine is critical for various cancer cells to proliferate and survive (49). Glutamine deprivation induces apoptosis by c-MYC activation (50). Therefore, we examined impacts on c-MYC expression by glutamine deprivation in vitro. Glutamine deprivation down-regulated c-MYC expression under both normoxic and hypoxic conditions (Supplementary Fig. S8), suggesting that glutamine deprivation might also be a factor for cancer cells to down-regulate c-MYC expression in the hypoxic area of in vivo tumor. Here, we show that high levels of c-MYC expression could be a disadvantage for survival of cancer cells under oxygen- and glucose-deprived conditions. In this context, disturbing the inactivation of c-MYC under these dual-deprivation conditions could be a therapeutic strategy for targeting hypoxic cancer cells. However, because c-MYC is an oncogene that stimulates proliferation, it would be difficult to use a c-MYC activator in the clinic. Further elucidation of the degradation mechanism of c-MYC under oxygen- and glucose-deprived conditions will contribute to find ways to activate c-MYC and eradicate the cancer cells specifically under these dual-deprivation conditions.

Disclosure of Potential Conflicts of Interest

There is no conflict of interest.

Acknowledgments

We express our thanks to Momoko Shiozaki for technical support, Toshiko Yasuda for administrative support, and Takaaki Takeda for critical discussion.

Grant Support

This work was supported by a grant-in-aid for the Creation of Innovations through Business-Academic-Public Sector Cooperation and Open Competition for the Development of Innovative Technology, from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 07/27/2010; revised 09/21/2010; accepted 09/27/2010; published OnlineFirst 10/27/2010.

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Cancer Res 2010;70:10213-10223. Published OnlineFirst October 27, 2010.

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