HIF-1α activation under glucose deprivation plays a central role in the acquisition of anti-apoptosis in human colon cancer cells

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Abstract. A poor vascular network development in a tumor mass leads to poor oxygen and nutrient supply. To adapt to a hypoxic microenvironment, it is well-known that cancer cells activate the transcription factor hypoxia-inducible factor-1α (HIF-1α). HIF-1α plays a central role in hypoxia-induced metabolic switching, anti-apoptosis, angiogenesis and drug resistance. Glucose deprivation, another major stressful microenvironment, protects cancer cells from drug-induced apoptosis. However, the molecular mechanisms by which cancer cells adapt to poor nutrient conditions remain poorly understood. In this study, we focused on HIF-1α, signal transducer and activator of transcription 3 (STAT3) and transcription factor 4 (TCF4), which are involved in cell survival, anti-apoptosis and drug resistance. We examined their activities and the relationships among these transcription factors under glucose deprivation. Our results showed that glucose deprivation increased HIF-1α, STAT3 and TCF4 DNA-binding activity, as well as the expression levels of their target genes OCT4, BCL-2 and VEGF. HIF-1α knockdown significantly increased poly(ADP-ribose) polymerase 1 (PARP-1) cleavage at higher levels than STAT3 knockdown under glucose deprivation. Furthermore, HIF-1α knockdown led to a significant decrease in the expression levels of both STAT3 and TCF4, although STAT3 knockdown decreased only HIF-1α expression level. Our data indicated that activation of the HIF-1α signaling pathway under glucose deprivation leads to the acquisition of anti-apoptotic properties in human colon cancer cells, and targeting the HIF-1α signaling pathway may provide an effective avenue for treating cancers resistant to conventional therapy.

Introduction

It is widely acknowledged that most cancer cells are exposed to hypoxia. Distinct from normal cells, most cancer cells predominantly produce energy by glycolysis, which is irrespective of oxygen availability, rather than oxidative phosphorylation via the tricarboxylic acid cycle (Warburg effect) (1). This is a reason why cancer cells can adapt to hypoxic condition and survive under hypoxia. Furthermore, these findings indicate that hypoxia in tumor is caused not by the excessive oxygen consumption of cancer cells, but rather the inadequate blood supply resulting from structurally and functionally defective angiogenesis. Consequently, poor vascularization in solid tumors leads to a poor supply of nutrients besides oxygen. Indeed, recent study has revealed that glucose concentration in tumor is lower than that in normal tissue (2).

When cancer cells are exposed to low oxygen condition, hypoxia-inducible factor-1α (HIF-1α) is activated, and its activation leads to cell survival under hypoxic condition through inducing transcriptions of a series of genes for anaerobic glycolysis, angiogenesis, and anti-apoptosis (3). Hypoxia can also enhance the activity of other transcription factors, including c-Jun, nuclear factor-kB (NFkB), SP1, activator protein 1 (AP-1), signal transducer and activator of transcription 3 (STAT3), and STAT5 (4-6), which are known to be critical for cell survival and anti-apoptosis. On the other hand, glucose deprivation, which is another stressful microenvironment, enhances expression of the multidrug resistance-1 (MDRI) gene through c-Jun activation (7) and protects cancer cells from cisplatin-induced apoptosis by enhancing expression of asparagine synthetase (8). However, the network of transcription factors activated by glucose deprivation has not been fully elucidated compared with that of hypoxia.

In this study, we investigated how glucose deprivation affects the HIF-1α, STAT3, and transcription factor 4 (TCF4) signaling pathways involved in cell survival, anti-apoptosis, and drug resistance (9-11). We also further examined their roles in glucose deprivation-induced anti-apoptosis and the relationships among these transcription factors.

Materials and methods

Cell line and antibodies. The human colon cancer cell line COLO-320 was purchased from RIKEN Bioresource Center
Cell culture under glucose deprivation. For glucose deprivation, COLO-320 cells were incubated for 48 h in RPMI-1640 medium containing 2 g/l glucose (control condition) or glucose-free RPMI-1640 medium, which was supplemented with 5% FCS at 37°C in a humidified atmosphere containing 5% CO₂.

Cell viability under glucose depletion. To investigate cell viability, cells were seeded in 6-well plates (3x10⁴ cells/well) under control condition or glucose deprivation. Following incubation for 48 h, the cells were harvested using a cell scraper and suspended in phosphate-buffered saline (PBS). The cell suspension was mixed with an equal amount of trypan blue solution and the number of living cells was counted.

Western blotting. Cells were lysed with 1% NP-40 lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% (v/v) Nonidet P-40 and 1X protease inhibitor] and incubated on ice for 30 min. After centrifugation, cell lysates containing 20 µg protein were dissolved in sample buffer solution with reducing agent (Nacalai Tesque, Kyoto, Japan) and incubated at room temperature for 20 min. Cell lysates dissolved in sample buffer were separated by electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with Tris-buffered saline containing Tween-20 (TBST) containing 0.3% milk, the membrane was immunoblotted with the appropriate primary antibody, followed by goat anti-mouse immunoglobulins (Igs) or anti-rabbit Igs conjugated with horseradish peroxidase (HRP). After washing, immune complexes were detected using Amersham ECL Prime Western Blotting Detection reagent (GE Healthcare, Little Chalfont, UK).

Preparation for nuclear extracts and cytoplasmic extracts. Nuclear extracts and cytoplasmic extracts were prepared from COLO-320 cells cultured under control condition or glucose deprivation using the Nuclear Complex Co-IP kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol.

Gel shift assay. Nuclear extracts were prepared from COLO-320 cells cultured under control condition or glucose deprivation. A gel shift assay was conducted using a double-stranded, biotin-labeled oligonucleotide probe containing the consensus binding site for STAT3 (sense strand, 5'-GATCCCT CTGGGAATTCCTAGATC-3'), HIF-1α (sense strand, 5'-TCT GTACGTTGACCACACTCACC-3') or TCF4 (sense strand, 5'-GGCTTTGAAGATGA-3') using the Gelshift Chemiluminescent EMSA kit (Active Motif) according to the manufacturer's protocol. Protein-DNA complexes were resolved on a non-denaturing polyacrylamide gel, transferred to a positively charged nylon membrane, and cross-linked to a membrane using the UV-light cross-linker. After blocking, the membrane was incubated with blocking buffer containing streptavidin conjugated to HRP. After washing, protein-DNA complexes were detected using a chemiluminescent substrate (Active Motif).

Reverse transcription-polymerase chain reaction. Total RNAs were purified from COLO-320 cells cultured under control condition or glucose depletion using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Synthesis of cDNA and subsequent PCR were conducted using PrimeScript One Step RT-PCR Kit Ver. 2 (Takara, Shiga, Japan) according to the manufacturer's protocol. The sequences of primers used in this study were as follows: OCT4, 5'-ACACCTGGCTTCCGAGTTCG-3' and 5'-GGGCG ATGTTGGTACGTGCT-3'; NANO, 5'-AACATGAGTTG GATCCAG-3' and 5'-TCACTCTACCTTCACCTGGTCTC AGGTTG-3'; BCL-2, 5'-AGATGGTCCAGGAGGTCGACCT GAC-3' and 5'-ATAGGCCCCAGGTGTGACAGTCT-3'; VEGF, 5'-TCGGGCTCTCCGAAACCATGA-3' and 5'-CCT GGTGAGATCTGTTCT-3'; GAPDH, 5'-GGAAAGGT AAGGTCGAGTC-3' and 5'-GAAGATGTGGTGGAGGAT TTC-3'. Reaction conditions for each primer set were 50°C for 30 min and 94°C for 2 min followed by 20 cycles (GAPDH), 24 cycles (OCT4, NANO), 25 cycles (VEGF) or 29 cycles (BCL-2) of the following reaction: denaturing step at 94°C for 30 sec; annealing at 55°C (NANO, GAPDH), 58°C (VEGF), 65°C (OCT4) or 70°C (BCL-2) for 30 sec; and extension at 72°C for 30 sec. PCR products were analyzed on 1% agarose gel containing ethidium bromide and detected using a UV transilluminator.

Clinical colorectal cancer specimens. Patients with colorectal cancer who underwent surgical treatment at Yamaguchi University and affiliated hospitals between April, 2012 and September, 2012, were enrolled in this study. Resected tumors specimens were immediately taken from resected colons and kept at -80°C until RNA extraction. These samples were used in accordance with institutional guidelines and the Declaration of Helsinki after obtaining informed consent from all patients.

Knockdown of target gene expression by siRNA transfection. For siRNA transfection, scrambled (control), STAT3 or HIF-1α siRNA (Thermo Scientific Dharmacon, Lafayette, CO, USA) was mixed with Lipofectamine RNAiMAX Reagent (Life Technologies, Carlsbad, CA, USA) in serum-free RPMI-1640 medium, and then the siRNA solution was incubated for 20 h at room temperature to form the siRNA-cationic lipid complex. Trypsinized COLO-320 cells were suspended with RPMI-1640 medium containing 10% FCS and mixed with the siRNA solution (reverse transfection). Following incubation with siRNA at concentrations of 100 nM for 2 days, siRNA-transfected COLO-320 cells were spread and incubated for 3 more days. After that, the cells were harvested using a cell scraper and resuspended in PBS. Following the centrifugation...
at 1200 rpm for 3 min, the supernatants were removed and the siRNA-transfected cell pellets were used to prepare total cell lysates, nuclear extracts or cytoplasmic extracts for western blotting.

Statistical analysis. Data are expressed as means ± standard deviation (SD). Statistical comparisons between groups were conducted using Student’s t-test. Values of p<0.05 or p<0.01 were considered statistically significant.

**Results**

**Human colon cancer cells adapted to glucose deprivation acquire resistance to doxorubicin-induced apoptosis.** Following cell culture under glucose deprivation for 48 h, the cell number of the survived COLO-320 cells was approximately half compared with the control (Fig. 1A and B). To confirm that COLO-320 cells adapt to glucose deprivation, we examined the mRNA expression level of glucose-regulated protein 78 (GRP78), which is well-known to be induced by stressful microenvironments such as glucose deprivation and hypoxia (12). As shown in Fig. 1C, the expression level of GRP78 was increased under glucose deprivation. These results indicate that the survived COLO-320 cells can adapt to glucose deprivation. Next, to investigate whether the survived COLO-320 cells under glucose deprivation could acquire drug resistance, the cells were treated with 10 µM doxorubicin for 24 h, followed by examination of PARP-1 cleavage, which is an indicator for apoptosis. As shown in Fig. 1D, PARP-1 cleavage was induced by doxorubicin treatment in cells cultured under control condition, and doxorubicin-induced PARP-1 cleavage was lower in cells cultured under glucose deprivation than that in the control cells. This result suggests that glucose deprivation confers resistance to doxorubicin-induced apoptosis.

Glucose deprivation increases DNA-binding activity of HIF-1α, STAT3, and TCF4 as well as the expression levels of their target genes. Gel shift assays using nuclear extracts revealed that HIF-1α, STAT3 and TCF4 binding activity in glucose-depleted COLO-320 cells were higher than those in the control cells (Fig. 2A-C). To further investigate the effect of glucose deprivation on the expression levels of HIF-1α, STAT3 and TCF4 target genes, we prepared total RNA from cells cultured under control condition or glucose deprivation, followed by RT-PCR. We selected HIF-1α, STAT3 or TCF4 target genes, which were previously demonstrated to be involved in anti-apoptosis and drug resistance, including OCT4 as HIF-1α target gene (13), BCL-2 as STAT3 target gene and BIM as TCF4 target gene.
gene (14), and VEGF as HIF-1α, STAT3, and TCF4 target gene (15-17). Our results showed that glucose deprivation significantly increased the expression levels of these target genes (Fig. 2D-G) at 1.4-3.25-fold higher compared with those in COLO-320 cells cultured under control condition. Furthermore, we examined the expression levels of GRP78, OCT4 and NANOG which is well-known as OCT4 target gene (18) in clinical colon cancer tissues. GRP78 is induced in the tumor microenvironment such as hypoxia and glucose deprivation, therefore it may be possible to estimate the tumor microenvironment in which clinical colon cancer tissues have grown by means of GRP78 expression level. As shown in Fig. 2H, GRP78 expression level in some clinical colon cancer tissues were higher than that in others, and OCT4 and its target gene, NANOG expression levels tended to correlate with GRP78 expression level.

HIF-1α knockdown significantly increases apoptosis in glucose-depleted COLO-320 cells. To investigate whether acquisition of anti-apoptotic property in glucose-depleted COLO-320 cells was due to activation of HIF-1α or STAT3, we performed siRNA transfection studies. Glucose-depleted
COLO-320 cells transfected with HIF-1α or STAT3 siRNA showed a marked decrease in HIF-1α or STAT3 expression level, respectively (Fig. 3A and B). As shown in Fig. 3C, HIF-1α knockdown induced PARP-1 cleavage at the highest levels under both control condition and glucose deprivation, and the amount of cleaved PARP-1 under glucose deprivation was decreased compared with that under control condition, which indicates that anti-apoptotic property is increased under glucose deprivation. Moreover, HIF-1α knockdown significantly increased PARP-1 cleavage compared with STAT3 knockdown under glucose deprivation (Fig. 3D). These results suggest that HIF-1α plays an important role in the acquisition of anti-apoptotic property induced by glucose deprivation.

Cross-talk among HIF-1α, STAT3, and TCF4 in glucose-depleted COLO-320 cells. We further investigated how knockdown of HIF-1α or STAT3 affected the expression levels of two other transcription factors. HIF-1α- or STAT3-silenced COLO-320 cells were cultured under glucose deprivation, followed by western blotting using nuclear extracts and cytoplasmic extracts. Our results showed that HIF-1α knockdown significantly decreased the expression levels of STAT3 and

Figure 3. Comparison of PARP-1 cleavage between STAT3- and HIF-1α-silenced COLO-320 cells incubated under glucose deprivation. COLO-320 cells transfected with scrambled siRNA, STAT3 siRNA or HIF-1α siRNA were incubated under glucose deprivation for 48 h, and total cell lysates were prepared. (A and B) STAT3 and HIF-1α were detected in total cell lysates using western blotting with anti-STAT3 and HIF-1α antibody, respectively. α-tubulin was used to assess the total amount of proteins loaded on the gel. (C) COLO-320 cells transfected with scrambled siRNA, STAT3 siRNA or HIF-1α siRNA were incubated under control condition or glucose deprivation for 48 h and total cell lysates were prepared. PARP-1 and cleaved PARP-1 were detected in total cell lysates using western blotting with anti-PARP-1 antibody. α-tubulin was used to assess the total amount of proteins loaded on the gel. Hash mark (#) in the upper and the lower panels indicates the same band of cleaved PARP-1. (D) The intensity of each band was quantified using Image J software. The ratio of cleaved PARP-1 to α-tubulin under glucose deprivation was normalized to the values in control cells. Each bar represents the mean ± SD of three independent experiments. *p<0.05, **p<0.01, significantly different (n=3).
TCF4 in the nucleus (Fig. 4A-C). In contrast, STAT3 knockdown significantly decreased the expression level of HIF-1α in the nucleus, but did not affect TCF4 expression (Fig. 4D-F). These results indicate that cross-talk among HIF-1α, STAT3, and TCF4 is involved in glucose deprivation-induced anti-apoptosis.

Discussion

Therapeutic resistance remains a major obstacle in cancer therapy. To elucidate the mechanism by which colon cancer cells acquire drug resistance, we focused on the mechanism of the adaptation of colon cancer cells to glucose deprivation.
which leads to the acquisition of resistance to drug-induced apoptosis.

Consistent with a previous study (19), ~50% of COLO-320 cells survived following glucose deprivation for 48 h and the survived COLO-320 cells under glucose deprivation acquired resistance to doxorubicin-induced apoptosis. These results suggest that the adaptation of colon cancer cells to glucose deprivation leads to the acquisition of resistance to drug-induced apoptosis. To elucidate the mechanism by which glucose deprivation confers resistance to drug-induced apoptosis, we further investigated how glucose deprivation affects the HIF-1α, STAT3, and TCF4 signaling pathways. The reasons why we focused on these signaling pathways are as follows. The HIF-1α signaling pathway is well-known to be activated in response to hypoxia, which is another major stress microenvironment. The STAT3 signaling pathway is one of the major signaling pathways involved in regulation of cell survival and anti-apoptosis, and the TCF4 signaling pathway is critical for the development of colon cancer through the interaction between TCF4 and β-catenin (20). Interestingly, glucose deprivation activated all the three signaling pathways by increasing their DNA-binding activities as well as increasing the expression levels of their target genes, OCT4, BCL-2 and VEGF. Previous studies have shown that increased expression of BCL-2 and VEGF contributes to drug resistance (21,22). Notably, among the upregulated genes, OCT4 is known to be highly and specifically expressed in normal stem cells. OCT4 is abundantly expressed in pluripotent stem cells, including embryonic stem cells and iPS cells, and has been shown to be necessary for maintaining the ‘stemness’ and pluripotency of stem cells (23). Coexpression of OCT4 and NANOG induces cancer stem-like properties including the ability to form sphere-like shapes, tumorigenicity, and drug resistance in lung adenocarcinoma (24). As cancer stem-like cells are highly resistant to chemotherapy (25), glucose deprivation may enhance drug resistance of cancer cells by changing their phenotype into that of cancer stem-like cells. In agreement with these data, the expression levels of OCT4 and its target gene NANOG tended to correlate with that of GRP78 in clinical colon cancer tissues. The clinical samples in which GRP78 was highly expressed seemed to grow under hypoxia and glucose deprivation. In such clinical samples with high GRP78 expression, OCT4 and NANOG expression levels tended to be higher than those in other samples.

We found that HIF-1α knockdown significantly induced PARP-1 cleavage at higher level than STAT3 knockdown in glucose-depleted colon cancer cells. It has been reported that HIF-1α regulates the transcription of several genes involved in glycolysis (26,27). Furthermore, recent studies have revealed that HIF-1α-mediated autophagy in oxygen- or nutrient-starved cancer cells promotes tumor cell survival and protects cancer cells from drug-induced apoptosis (28). Therefore, HIF-1α knockdown in glucose-depleted colon cancer cells may disrupt glucose metabolism and autophagy induction, leading to apoptosis. Furthermore, we attempted to understand cross-talk among HIF-1α, STAT3, and TCF4 which are activated under glucose deprivation. Previous studies have demonstrated that STAT3 regulates HIF-1α expression (29,30). We also found that STAT3 knockdown significantly decreased the expression level of HIF-1α, but not TCF4, in glucose-depleted COLO-320 cells. Notably, HIF-1α knockdown also significantly decreased the expression levels of STAT3 and TCF4, indicating the role of HIF-1α as an important regulator in both the STAT3 and TCF4 signaling pathways. These results may explain why HIF-1α knockdown induced PARP-1 cleavage at higher levels than STAT3 knockdown.

In conclusion, our data clearly show that glucose deprivation activates multiple transcription factors including HIF-1α, STAT3 and TCF4 in colon cancer cells. Among these transcription factors, HIF-1α plays a central role in the acquisition of anti-apoptotic property under glucose deprivation and targeting the HIF-1α signaling pathway may provide an effective avenue for treating colon cancer cells resistant to conventional therapy. However, glucose-depleted COLO320 cells did not completely undergo cell death by HIF-1α knockdown alone. Further studies are necessary to identify key molecules that enhance the effect of HIF-1α knockdown on anti-apoptosis of colon cancer cells resistant to conventional therapy.

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