Basic Study

Glucose deprivation induces chemoresistance in colorectal cancer cells by increasing ATF4 expression

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AIM: To investigate the role of activating transcription factor 4 (ATF4) in glucose deprivation (GD) induced colorectal cancer (CRC) drug resistance and the mechanism involved.

METHODS: Chemosensitivity and apoptosis were measured under the GD condition. Inhibition of ATF4 using short hairpin RNA in CRC cells under the GD condition and in ATF4-overexpressing CRC cells was performed to identify the role of ATF4 in the GD induced chemoresistance. Quantitative real-time RT-PCR and Western blot were used to detect the mRNA and protein expression of drug resistance gene 1 (MDR1), respectively.

RESULTS: GD protected CRC cells from drug-induced apoptosis (oxaliplatin and 5-fluorouracil) and induced the expression of ATF4, a key gene of the unfolded protein response. Depletion of ATF4 in CRC cells under glucose deprivation conditions sensitized cells to oxaliplatin and 5-fluorouracil treatment, suggesting the involvement of ATF4 in glucose deprivation-induced drug resistance.

CONCLUSION: Activating transcription factor 4 (ATF4) plays a critical role in glucose deprivation-induced drug resistance in colorectal cancer cells.
the GD condition can induce apoptosis and drug re-resistant. Similarly, inhibition of ATF4 in the ATF4-overexpressing CRC cells reintroduced therapeutic sensitivity and apoptosis. In addition, increased MDR1 expression was observed in GD-treated CRC cells.

CONCLUSION: These data indicate that GD promotes chemoresistance in CRC cells through up-regulating ATF4 expression.

Key words: Glucose deprivation; ATF4; Oxaliplatin; 5-Fluorouracil; Chemoresistance

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Core tip: In this work, we demonstrated that glucose deprivation induces chemoresistance in colorectal cancer (CRC) cells through up-regulating ATF4 expression, and ATF4 is an attractive therapeutic target to combat therapeutic resistance in CRC cells.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer death worldwide[1]. Chemotherapy is one of the basic treatments for CRC. However, more than half of CRC patients did not respond to conventional chemotherapy due to drug resistance. Multiple factors contribute to the failure of CRC chemotherapy, including multidrug resistance (MDR) and tumor heterogeneity[2]. Cancer cells with MDR phenotype simultaneously become resistant to multiple drugs with different structures or cellular targets[3]. The development of MDR is commonly mediated by multiple factors, including accelerated drug efflux, drug activation and inactivation, alterations in the drug target, repair of drug-induced damage, and escape from apoptosis[4].

Recent data showed that tumor microenvironment plays a key role in tumor MDR[3]. In the tumor microenvironment, the abnormal development of vasculature results in insufficient blood supply, which is a key reason for the tumor progression and has been associated with glucose deprivation (GD), chronic hypoxia and other nutrient stress. Increasing evidence indicates that GD promotes tumor cell survival and angiogenesis and induces drug resistance by inducing complex signaling pathways, including unfolded protein response (UPR)[6-11]. However, the molecular mechanisms by which cancer cells adapt to GD condition and inhibit drug-induced apoptosis remain poorly understood. Recent studies have indicated that the activating transcription factor 4 (ATF4) pathway, a key player in UPR signaling, is important in regulating malignant phenotypes in various types of human cancers, including breast cancer[12], CRC[13], and head and neck squamous cell carcinoma[14]. In cellular adaptation to tumor GD, the GD activates cell survival through PERK-dependent ATF4 expression. In addition, our previous work revealed that GD and amino acid deprivation promote tumor angiogenesis through activating ATF4[15]. Accumulated data strongly suggest that ATF4 is an important gene in regulating tumor survival under stress conditions, but the functional relationships among cell drug resistance, ATF4 and GD in CRC have not been fully elucidated.

In this study, we investigated whether and how GD affects drug resistance and apoptosis in CRC cells, and revealed that GD induces drug resistance and apoptosis inhibition by activating PERK/ATF4 signaling pathway.

MATERIALS AND METHODS

Cell lines

Human CRC cell lines HCT116 and LoVo were obtained from ATCC. All cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 23.05 mmol/L glucose (Hyclone) supplemented with 10% fetal bovine serum (FBS; Gibco) and penicillin/streptomycin at 37 °C in a humidified incubator containing 5% CO₂.

GD treatment

To mimic the GD condition of tumor microenvironment, HCT116 and LoVo cells were incubated for 48 h in DMEM containing 1.5 mmol/L glucose and 10% FBS containing about 0.5 mmol/L glucose at 37 °C in a humidified atmosphere containing 5% CO₂.

Assessment of cell proliferation and chemotherapy sensitivity

For the cell proliferation assay, 1000 CRC cells were plated in 96-well plates and incubated for different time periods (24, 48, 72, and 96 h), and then the cell growth was detected with the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacturer’s instructions. For the cell chemotheraphy sensitivity assay, 2000 LoVo or HCT116 cells were plated in 96-well plates and treated with oxaliplatin (LOHP; range, 0-16 μg/mL) and 5-fluorouracil (5-FU; range, 0-1.6 μg/mL) for 48 h, and cell inhibition was then assessed by the CCK-8 assay.

Hoechst staining

Hoechst Staining was performed according to the
manufacturer’s protocol (Beyotime, China). Cells were visualized with a DP70 inverted immunofluorescence microscope (Olympus). Cells with condensed and fragmented nuclei were judged to be apoptotic.

Quantitative real-time RT-PCR
Total RNA was prepared from cultures on day 10, using the RNAiso reagent (TaKaRa, Japan) according to the manufacturer’s instructions. Complementary DNA (cDNA) was synthesized using the HiFiScript cDNA Kit (CWBio, China). Quantitative real-time RT-PCR analysis was performed to detect mRNA expression using UltraSYBR Mixture (CWBio), with β-actin as an internal control. The sequences of primers used in this study were as follows: 5’-CGTGGCTTTGTGGG TG-3’ and 5’-TGCGGTCTTTTGGCTGGAAT-3’ for ATF4; 5’-GACCATTGGGTTCTTCG-3’ and 5’-CTCAGCGGTCTTTTATC-3’ for Grp78; 5’-ATAATGATAAAGTGGTTTGT-3’ and 5’-ACAGGAGTTTCTGGAAGGAG-3’ for PERK; 5’-AGTGTGACGTGGACATCGCCAAG-3’ and 5’-ATCCATCTCCTGGAAGGTGG AC-3’ for β-actin.

Western blot
Cells were lysed with RIPA buffer and incubated on ice for 30 min. After centrifugation, protein concentration was measured with BCA Protein Assay Reagent (CWBio). Cell lysates dissolved in sample buffer were separated using SDS-PAGE and transferred to a polyvinylidene fluoride membrane. After blocking with Tris-buffered saline containing Tween-20 containing 5% milk, the membrane was immunoblotted with appropriate primary antibodies, including anti-Grp78 (Santa Cruz, United States), anti-PERK (Cell Signaling, United States), anti-ATF4 (Santa Cruz), anti-MDR1 (Santa Cruz) and anti-β-actin (Abcam, United States), followed by incubation with goat anti-mouse immunoglobulin (Ig) or anti-rabbit Ig conjugated with horseradish peroxidase. After washing, the membrane was developed using Chemiluminescent Substrate (CWBio).

Plasmid and lentivirus production
Green fluorescent protein-expressing lentiviral plasmids expressing short hairpin RNA (shRNA) against human ATF4 (Vehicle-shATF4) were obtained from Open Biosystems (Carlsbad, CA), and ATF4 lentiviral plasmid (Vehicle-ATF4) was constructed as described in our previous work[8]. The Vehicle, Vehicle-shATF4, Vehicle and Vehicle-ATF4 plasmids were cotransfected into HEK-293T cells along with the packaging plasmid ps-PAX2 and the envelope plasmid pMD2G using Lipofectamine 2000 (Invitrogen). Virus particles were harvested 48 h after cotransfection. Then, the particles were individually used to infect HCT116 and LoVo cells. The cells were then incubated for 3 d after infection for Western blot and qRT-PCR validation.

Apoptosis detection
LoVo or HCT116 cells were plated in 6-well plates and treated with LOHP (0.1 μg/mL) and 5-FU (0.05 μg/mL) for 48 h. The cells were then harvested and subjected to apoptosis analysis using an Annexin V/7-AAD Apoptosis Detection Kit (CWBio).

Statistical analysis
Each experiment was repeated at least three times. The data are presented as the mean ± SD. Differences between groups were analyzed with Student’s t test. All statistical analyses were performed using GraphPad Prism 5 software. The significance level was set at 0.05.

RESULTS
GD decreases sensitivity of CRC cells to chemotherapy and inhibits drug-induced apoptosis
To investigate whether the surviving CRC cells under GD could acquire drug resistance, we assessed the potential effect of GD on the sensitivity of CRC cells to LOHP and 5-FU, two of the most commonly used drugs for CRC treatment[15]. The results revealed that the IC50 values of GD-treated HCT116/LoVo cells were significantly higher than those of their corresponding control cells (Figure 1A and Figure 2), suggesting that GD strongly decreases the sensitivity of CRC cells to LOHP and 5-FU. These data indicate that GD induces a MDR phenotype in CRC cells. Next, to determine whether GD inhibits chemotherapy-induced apoptosis in CRC cells, we used Hoechst staining to investigate the apoptotic rates. After incubation under GD condition for 24 h, CRC cells were treated with LOHP or 5-FU for subsequent 48 h under normal culture conditions. These cells were then subjected to Hoechst staining. The results revealed that the apoptotic rates were much lower in the GD-treated CRC cells than in the control cells (Figure 1B). To confirm the MDR phenotype of the GD-treated CRC cells, we examined the expression levels of multidrug resistance gene 1 (MDR1), a major marker of MDR. As shown in Figure 1C and D, both the mRNA and protein expression levels of MDR1 were increased in the GD-treated CRC cells as compared to the control cells. Taken together, these observations suggest that GD, through inhibiting apoptosis, significantly decreases the sensitivity of CRC cells to chemotherapy.

Grp78/PERK/ATF4 pathway is activated in GD-induced CRC cells
Our previous work showed that GD induces tumor growth and angiogenesis by activating PERK/ATF4 arm of UPR signaling. To investigate the role of PERK/ATF4 pathway in GD-induced MDR in CRC cells, we examined the mRNA and protein expression of UPR markers (Grp78, PERK and ATF4), which are well-known to be induced by stressful microenvironments such as GD and hypoxia[8,16]. As expected, the
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Figure 1 Glucose deprivation promotes drug resistance of colorectal cancer cells. A: GD decreased drug susceptibility to CRC cells. LoVo cells were treated with the indicated doses of the different drugs for 48 h under GD or normal culture condition. The in vitro drug sensitivity was tested by the CCK-8 assay; B: GD inhibited LOHP- and 5-FU-induced apoptosis. LoVo cells were treated with 0.1 μg/mL LOHP or 0.05 μg/mL 5-FU for 48 h. Hoechst 33258 nuclear staining and Annexin V/7-AAD staining assays were performed to detect apoptosis. C and D: GD promoted the expression of resistance gene MDR1. The mRNA and protein levels of MDR1 were examined by qRT-PCR and Western blot, respectively. *P < 0.05, **P < 0.01, ***P < 0.001, control vs GD. GD: Glucose deprivation; CRC: Colorectal cancer.

Figure 2 Glucose deprivation promotes drug resistance of HCT116 cells to LOHP and 5-FU. HCT116 cells were treated with the indicated doses of the different drugs for 48 h under GD or normal culture condition. The in vitro drug sensitivity was tested by the CCK-8 assay. *P < 0.05, **P < 0.01, ***P < 0.001, control vs GD. GD: Glucose deprivation.

mRNA levels of Grp78 and ATF4 were significantly increased in GD-treated CRC cells. Although the mRNA and protein expression of PERK was not significantly increased as that of Grp78 and ATF4, the phosphorylation (activation) of PERK (upward shift in the bands) was clearly observed in GD-treated CRC cells (Figure 3A and B). These data suggest the activation of UPR upon GD treatment and the potential key role of Grp78/PERK/ATF4 pathway in GD-induced MDR phenotype in CRC cells.
ATF4 pathway contributes to GD-induced drug resistance in CRC cells

To explore whether the acquisition of anti-apoptotic property in glucose-depleted CRC cells was due to the activation of ATF4, we silenced the expression of ATF4 using shATF4 in the GD-treated LoVo and HCT116 cells (Figure 4A). The results showed that silencing ATF4 expression counteracted GD-induced drug resistance of CRC cells to both drugs (LOHP and 5-FU) compared with the control cells (Figure 4B and Figure 5A). Moreover, both Hoechst nuclear staining (Figure 5B) and Annexin V/7-AAD staining assays (Figure 5C) showed that ATF4 knockdown significantly increased apoptotic rates of GD-treated CRC cells compared with the control cells. These results suggest that GD inhibits apoptotic activity in CRC cells by activating ATF4 expression. In addition, down-regulation of MDR1 was observed in the ATF4-depleted CRC cells treated with LOHP compared with the control cells, suggesting that ATF4 may mediate GD-induced MDR effect in CRC cells by up-regulating MDR1 expression (Figure 5D). Collectively, these results suggest that the activation of ATF4 plays a crucial role in the GD-induced MDR phenotype in CRC cells.

ATF4 knockdown increases the sensitivity of CRC cells to chemotherapy and counteracts drug-induced apoptosis

To further investigate the role of ATF4 in the drug resistance of CRC cells, forced expression of ATF4 was induced in LoVo and HCT116 cells (LoVo-ATF4 and HCT116-ATF4) using lentivirus transduction. ATF4-overexpressing CRC cells were co-treated with shATF4 and therapeutic drugs, and the results demonstrated that inhibition of ATF4 increased the sensitivity of LoVo-ATF4 and HCT116-ATF4 cells to chemotherapy (Figure 6A and Figure 7A). Moreover, we detected the apoptosis in ATF4-overexpressing cells treated with shATF4, and revealed that the apoptotic rates were much higher compared with the control cells (Figure 6B and Figure 7B). Meanwhile, qRT-PCR and Western blot results also showed the decreased expression of MDR1 (Figure 6C and D). These findings further demonstrate that ATF4 contributes to the induction of chemoresistance in CRC cells.

ATF4 promotes proliferation of CRC cells

Previous studies have proved the role of ATF4 in tumor proliferation. To investigate the proliferation-prompting function of ATF4 in CRC, we overexpressed ATF4 in LoVo and HCT116 cells and then inhibited ATF4 in these cells or their control cells, respectively (As shown in Figure 8A). ATF4 overexpression significantly increased the growth rates of HCT116 and LoVo cells compared to the vector control (Figure 8B). In contrast, inhibition of ATF4 in the ATF4-overexpressing CRC cells significantly decreased the growth rates compared to the control cells (Figure 8C). The results suggest that ATF4 may play multiple roles in CRC progression.

DISCUSSION

Therapeutic resistance remains a major cause of tumor chemotherapy failure. Its mechanisms are very complicated. Recently, GD has been reported to promote cell proliferation, migration, invasion, angiogenesis and drug resistance in a variety of human cancers through different mechanisms, suggesting its extensive function in tumor development and progression[6,10,17,18]. UPR is an important mechanism by which GD regulates malignant phenotypes of tumor cells. Our previous work showed that GD contributes to tumor angiogenesis by increasing expression of multiple proangiogenic factors through the PERK/ATF4 signaling, a key signaling pathway in UPR[8]. In this study, we revealed that GD can decrease the sensitivity of CRC cells to two most commonly used chemotherapeutic drugs (LOHP and 5-FU) in CRC cells by activating PERK/ATF4 pathway. Further analysis showed that silencing ATF4 expression could counteract the inhibitory effect of GD on drug-induced apoptosis, suggesting the key role of ATF4 in GD-induced chemoresistance in CRC.

Due to their rapid and uncontrollable growth, tumors are frequently exposed to extracellular environments that are deficient in nutrients and oxygen, resulting in the disruption of homeostasis in the endoplasmic reticulum (ER) and leading to the activation of UPR. UPR serves to decrease the detrimental effects of accumulated unfolded proteins by increasing protein degradation and decreasing protein synthesis. However, UPR can induce apoptosis in normal cells encountering prolonged stress conditions. Accumulating evidence indicates that UPR contributes to the cancer development, affecting angiogenesis, cell growth, cell differentiation, cell migration, and the inflammatory microenvironment. In addition, recent studies also show that UPR activation can alter the sensitivity of tumor cells to a variety of chemotherapeutic agents. As a common stressful microenvironment in tumor, GD can regulate a variety of tumor phenotypes, mainly by activating UPR pathway. In this study, we revealed that GD induced MDR phenotype by inhibiting 5-FU/LOHP-induced apoptosis in CRC cells. A recent study also reported that COLO-320 colon cancer cells adapted to GD could acquire resistance to doxorubicin-induced apoptosis[10]. These data demonstrate the key role of GD microenvironment in regulating the MDR phenotype of CRC.

To elucidate the mechanism by which GD induces MDR phenotype in CRC cell, we checked the PERK/ATF4 arm of UPR pathway and revealed its activation under GD condition. In view of the potential role of ATF4 in the UPR and drug resistance in cancer cells,
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Figure 3  Grp78/PERK/ATF4 pathway is activated in glucose deprivation. A and B: GD promoted the expression of genes involved in UPR. The mRNA and protein expression were examined by qRT-PCR and Western blot, respectively, and β-actin was used as an internal control. The phosphorylation of PERK (upward shift in the bands) indicated its activation in GD-treated CRC cells. *P < 0.05, **P < 0.001, control vs GD. GD: Glucose deprivation; CRC: Colorectal cancer.

Figure 4  Down-regulation of activating transcription factor 4 significantly reverses the glucose deprivation-induced resistance of HCT116 cells to chemotherapy. A: Silencing ATF4 expression using shATF4 in GD-treated LoVo and HCT116 cells; B: Depletion of ATF4 enhanced the sensitivity of HCT116 cells to LOHP and 5-FU. The in vitro drug sensitivity was tested using the CCK-8 assay. *P < 0.05, **P < 0.01, ***P < 0.001, control vs GD. GD: Glucose deprivation.
we tried to reveal its potential influence on the MDR phenotype inducing by GD in CRC cells. As a member of the CREB protein family, ATF4 participates in many intracellular physiological and biochemical processes and has been suggested as an important target of cancer therapy\cite{19-26}. For example, ATF4 is the main transcriptional regulator of the cellular hypoxic response to UPR signaling and activates genes that

Figure 5 Down-regulation of activating transcription factor 4 significantly reverses the glucose deprivation-induced resistance of colorectal cancer cells to chemotherapy. A: Depletion of ATF4 enhanced the sensitivity of CRC cells to chemical drugs. Vector and shATF4 stably transfected LoVo cells were treated with the indicated doses of the different drugs for 48 h. In vitro drug sensitivity was tested using the CCK-8 assay; B and C: The apoptotic rates were much higher in the ATF4-depleted cells than in the control cells. Hoechst 33258 nuclear staining and Annexin V/7-AAD staining assays were performed to detect apoptosis; D: Depletion of ATF4 by shRNA in CRC cells led to significantly reduced expression of MDR1. The mRNA and protein levels of MDR1 were detected by qRT-PCR and Western blot, respectively, and β-actin was used as an internal controls. *P < 0.05, **P < 0.01, ***P < 0.001, vehicle GD vs shATF4-GD. GD: Glucose deprivation; CRC: Colorectal cancer.
promote restoration of normal ER function and survival under hypoxia condition\(^{23}\). Recently, ATF4 was reported to promote drug resistance in several types of tumors, including breast cancer\(^{27}\), lung cancer\(^{28}\), liver cancer\(^{29}\), and gastric cancer\(^{3}\). Similarly, we revealed that the MDR was reversed when we inhibited the GD-induced up-regulation of ATF4 using shATF4. Moreover, we revealed that inhibition of ATF4 by shRNA in the ATF4-overexpressing CRC cells also reintroduced therapeutic sensitivity and apoptosis in CRC cells.
These data indicate that GD induces MDR mainly by activating ATF4. Nishimoto et al.\[10\] showed that GD could induce acquire resistance to doxorubicin-induced apoptosis in CRC cells, suggesting that multiple downstream targets mediate the MDR-inducing function of GD. Ledoux et al.\[30\] reported that GD enhances expression of MDR1 through c-Jun activation in hepatoma cells. Our data also observed increased MDR1 expression in GD-treated CRC cells. In addition, our data indicated that ATF4 knockdown significantly decreased MDR1 expression in the GD-treated CRC cells compared with the control cells. These results imply that ATF4 contribute to chemoresistance in CRC partly via regulating MDR1.

In conclusion, our data clearly identify that GD induces the MDR phenotype of CRC cells by activating PEKR/ATF4 signaling, and targeting the ATF4 pathway may provide a clinical perspective for treating drug resistance of CRC cells to conventional therapy. Further studies are necessary to identify key molecules that enhance the effect of ATF4 knockdown on CRC cells resistant to conventional chemotherapy. Therefore, interventions based on the disruption of GD-induced ATF4 expression may be effective in reversing drug resistance in CRC cells.

**COMMENTS**

**Background**
Chemoresistance is an important reason for clinical chemotherapy failure. Recent studies suggest that tumor microenvironment is an important determinant of malignant progression and chemoresistance. Changes in the tumor microenvironment, such as hypoxia and glucose deprivation (GD), can prompt tumor progression and drug resistance. However, the role and mechanism of GD in colorectal cancer (CRC) drug resistance are unknown.

**Research frontiers**
GD has been found to be involved in the regulation of multiple pathological processes that contribute to tumorigenesis and metastasis, such as tumor cell proliferation, multidrug resistance, and autophagy. Activating transcription factor 4 (ATF4) is a key player in UPR signaling. Many studies recently revealed that ATF4 participates in drug resistance of cancer. However, the role of ATF4 in GD-induced CRC drug resistance remains unclear and needs further exploration.
Innovations and breakthroughs

The authors clearly identified that GD induces the MDR phenotype of CRC cells by activating PERK/ATF4 signaling and targeting the ATF4 pathway may provide a clinical perspective for treating drug resistant of CRC to conventional therapy.

Applications

ATF4 may be a therapeutic target to combat therapeutic resistance in CRC cells.

Terminology

GD: In the tumor microenvironment, the abnormal development of vasculature results in insufficient blood supply, such as hypoxia and GD. Changes in the tumor microenvironment can promote tumor progression and drug resistance.

Peer-review

This is a very interesting study which explored the role and mechanism of GD in the chemoresistance of CRC. The results suggest that ATF4 may be a new target for overcoming CRC resistance to conventional chemotherapy.

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