Divergent Actions of Pyruvate and ATP in Glucose-mediated Irinotecan Chemoresistance in Colorectal Cancer

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Research Article

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Abstract

Background: Altered glucose metabolism is associated with chemoresistance in colorectal cancer (CRC). The aim of this study was to illustrate the molecular mechanisms of glucose-mediated chemoresistance against irinotecan, a topoisomerase I inhibitor, focusing on the distinct roles of metabolites such as pyruvate and ATP in modulating cell death and proliferation.

Methods: Four human CRC cell lines, tumorspheres, and mouse xenograft models were treated with various doses of irinotecan in the presence of high concentrations of glucose, pyruvate or ATP-encapsulated liposomes. Cell apoptosis was measured by DNA fragmentation and caspase activities, and necroptosis was evaluated by immunoprecipitation of receptor-interacting protein kinase (RIP) 1/3 complex. Cell cycles were assessed by flow cytometric analysis.

Results: Human CRC cell lines treated with irinotecan in the presence of high glucose displayed increased cell viability and larger xenograft tumor sizes in mouse models compared to those treated in the presence of normal glucose. Irinotecan induced apoptosis and necroptosis, both of which were mitigated by high glucose. Liposomal ATP prevented irinotecan-induced apoptosis, while it had no effect on necroptosis. In contrast, pyruvate attenuated the RIP1/3-dependent necroptosis via free radical scavenging, without modulating apoptotic levels. Regarding the cell cycle, liposomal ATP aggravated irinotecan-induced G0/G1 shift whereas pyruvate diminished the G0/G1 shift, showing opposite effects on proliferation. Last, tumorsphere structural damage, an index of solid tumor responsiveness to chemotherapy, was determined. Liposomal ATP increased tumorsphere sizes while pyruvate prevented the deformation of spheroid mass.

Conclusions: Glucose metabolites confer tumor chemoresistance via multiple modes of action. Glycolytic pyruvate attenuated irinotecan-induced necroptosis and potentiated drug insensitivity by shifting cells from a proliferative to quiescent state. On the other hand, ATP decreased irinotecan-induced apoptosis and promoted active cell proliferation, which might contribute to tumor recurrence.

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality [1]. Irinotecan, in combination with 5-fluorouracil and folinic acid (FOLFIRI), is one of the best established first-line regimens for CRC management [2]. Irinotecan functions as a DNA topoisomerase I inhibitor by stabilizing intermediate cleavage complexes formed between the enzyme and untangled single-stranded DNA, thus preventing DNA religation during cell proliferation [3, 4]. By inhibiting this religation, irinotecan interferes with DNA replication and transcription, leading to cell senescence and programmed cell death, including apoptosis and necroptosis [3, 4]. Apoptosis is characterized by cell shrinkage, caspase-3 activation and endonuclease-dependent DNA fragmentation, while necroptosis is a type of programmed necrotic cell death executed by receptor-interacting protein (RIP) kinase-dependent signaling and synthesis of mitochondrial free radicals [5]. Nevertheless, chemoresistance to irinotecan-based therapy may develop in

some patients, whose progression-free survival in clinical studies was reported to range from six to nine months [6].

Early work has focused on genetic changes in association with drug metabolism and efflux pathways for chemoresistance [7, 8]. Accumulating evidence indicates that an altered cellular bioenergetic status also causes tumor hyperproliferation and resistance to cell death [9, 10]. Normal cells rely on oxidative phosphorylation (OXPHOS) to produce energy for fundamental cellular functions, while cancer cells depend on glycolysis as their primary energy source for rapid tumor growth in hypoxic microenvironments or even aerobic conditions, a phenomenon known as the Warburg effect [11, 12]. Previous work by our group and others showed abnormal expression of glucose transporters (GLUT-1 to -4) and sodium-dependent glucose transporter 1 (SGLT1) associated with upregulation of glycolytic enzymes in human CRC specimens [13-15]. Patients with diabetes mellitus and hyperglycemia are at increased risk of developing CRC, and a positive correlation was observed between altered glycolytic metabolism and tumor recurrence in patients after preoperative chemotherapy [16-18]. In addition, enteric malabsorption, as a chemotherapeutic side effect, leads to colonic retention of dietary sugars, which accounts for an alternative route for high glucose delivery to CRC cells [19, 20]. Despite the irrefutable roles of high glucose uptake and altered glucose metabolism contributing to tumor growth, the underlying mechanisms of glucose-mediated tumor chemoresistance remain incompletely understood.

Glucose-mediated chemoresistance is generally assumed to be related to ATP, which serves as the primary energy source for cell division and drug efflux via ATP-binding cassette transporters [21, 22]. One model simulated that the rate of ATP production could be 100 times faster with glycolysis than with OXPHOS in cancer cells [23], and the increased amount of ATP was linked to more rapid cell proliferation [23, 24]. Nevertheless, other studies have presented contradictory data showing that administration of exogenous ATP augmented the chemosensitivity of tumor cells [25, 26]. Emerging evidence indicates that the glycolytic end-product pyruvate is involved in biological functions other than energy production by acting as a free radical scavenger [27]. One study showed that pyruvate scavenged free radicals by directly coupling with ROS, and the conversion of pyruvate to acetate may serve as an alternative carbon source for metabolism [28]. We previously demonstrated that glycolytic pyruvate, uncoupled to ATP synthesis, rescued epithelial cells from hypoxia- and 5-fluorouracil-induced necroptosis via suppression of the mitochondrial superoxide load, suggesting an energy-independent mechanism of death resistance [15, 29, 30].

In the current study, we aimed to investigate the molecular mechanisms of glucose-mediated chemoresistance against irinotecan, focusing on the regulatory roles of glucose metabolites (i.e., pyruvate and ATP) on the different programmed death pathways, including apoptosis and necroptosis, as well as on cell cycle shifts between proliferative and quiescent status.

Materials And Methods

Cell cultures

The human colorectal adenocarcinoma cell lines HT29, HCT116, SW480, and T84 were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5 mM glucose without pyruvate (Life Technologies, Carlsbad, CA) [30-33]. Cells were exposed to various concentrations of irinotecan for 48 hours in culture medium containing 5 or 25 mM glucose or ethyl pyruvate, a cell-permeable pyruvate derivative [30, 31]. Glucose concentrations of 5 mM mimic the normal glucose levels in blood (euglycemia), and glucose concentrations of 25 mM represent high-glucose levels. In other experiments, cells were exposed to irinotecan in the presence or absence of ATP-encapsulated or empty liposomes (500 μ M) in glucose- and pyruvate-free culture medium [29].

Additional experiments included pretreatment of cells with necrostatin-1 (a specific inhibitor of receptor-interacting protein kinase 1), ZVAD-FMK (a pancaspase inhibitor), iodoacetate (a glycolytic enzyme inhibitor), UK5099 (a specific mitochondrial pyruvate carrier inhibitor), or N-acetyl L-cysteine (NAC, an antioxidant) before irinotecan exposure in glucose-containing medium. The reagents were all purchased from Sigma-Aldrich (St. Louis, MO).

Xenograft mouse models

The human colorectal adenocarcinoma cell line HT29 (ATCC#HTB-38) was injected into immunodeficient mice to establish a xenograft tumor model as previously described [10, 15]. Animal protocols were approved by the Institutional Animal Care and Use Committee of NTUCM. Briefly, HT29 cells (2×10^6) were suspended in a 1:3 ratio of Matrigel (Corning #354248) to culture medium containing irinotecan (0.02 mM) with normal or high glucose concentrations, before they were subcutaneously injected into the right and left flanks, respectively, of immunodeficient NOD.CB17-Prkdc(scid)/JNarl mice (NOD/SCID). For the control groups, HT29 cells suspended in the Matrigel medium containing saline at normal or high-glucose concentrations were injected in the same manner. The tumor size and body weight were monitored every 2-3 days until the tumor volume reached 2500 mm³ as the end point. The tumors were then resected and fixed in 4% paraformaldehyde for tissue embedding followed by histological sectioning to confirm the dysplastic morphology.

Spheroid cultures

Cells were plated as three-dimensional spheroid cultures based on previous protocols [31, 34, 35]. The cell monolayer was trypsinized and resuspended in DMEM containing 25 mM glucose. The cells were then mixed with ice-cold Matrigel (Corning #354234) and cell culture medium at a 3:1 ratio for seeding. Spheroids were cultured for four days and exposed to irinotecan for 48 hours. Twenty-four hours prior to irinotecan treatment, the overlaying medium was changed to DMEM containing 5 mM glucose. The spheroids were then treated with irinotecan by replacing the overlaying medium with cell culture medium containing various concentrations of glucose, pyruvate, or ATP-encapsulated liposomes.

The spheroid structures were observed and images viewed under a microscope were captured. Spheroids lacking a circular shape and displaying invagination or crevices on the spheroid mass was classified as structurally damaged. A total number of 100-120 spheroids were quantified per experimental group, and extent of damage was reported as a percentage.

Cell viability assays

Cell viability was evaluated with the tetrazolium dye (MTT) assay, and the half maximum inhibitory concentration (IC₅₀) of irinotecan was calculated.

Measurement of apoptosis and necroptosis

Apoptosis as characterized by DNA fragmentation was measured with a Cell Death Detection ELISA kit (Roche, Basel, Switzerland) [31]. Necrosis/Necroptosis was measured using a lactodehydrogenase (LDH) leakage assay [29, 30]. Moreover, RIP1/3 signaling, a hallmark of necroptosis, was assessed by immunoprecipitation of the RIP1-RIP3 complex [30]. Briefly, cell lysates were immunoprecipitated with anti-human RIP1 (BD Bioscience, Franklin Lakes, NJ) and incubated with protein G agarose beads followed by heat denaturation. The immune complex was then subjected to reducing gel electrophoresis before the proteins were transferred to membranes, which were incubated with anti-RIP1 (1:1000, BD Bioscience) or anti-RIP3 (1:1000, Abcam, Cambridge, UK) for immunoblotting.

Mitochondrial free radical assays

Mitochondrial-derived reactive oxidative species (ROS) generation was measured using MitoSOX™ [Red Mitochondrial Superoxide Indicator](#) (Invitrogen). The irinotecan-treated cells were incubated with MitoSOX reagent (5 µM) for 20 min and then subjected to fluorometric readings [30].

Measurement of ATP, pyruvate, and acetate content

The levels of ATP (Invitrogen), pyruvate (Biovision, Milpitas, CA, USA), and acetate (Biovision) in cell lysates were measured using corresponding commercial assay kits.

Evaluation of cell cycle progression

Cells treated with irinotecan were harvested for cell cycle analysis. Briefly, the cells were incubated with anti-Ki67 primary antibody (1:500, LSBio, WA) followed by Alexa488-conjugated goat anti-mouse secondary IgG (1:1000, Molecular Probes, OR). The cells were then incubated with propidium iodide (PI)

for 30 min at room temperature. A minimum of 10,000 PI-stained cells were analyzed by flow cytometry, and the percentages of cells in the G0, G1, S, and G2–M phases were determined [31, 34].

Statistical analysis

All values are expressed as the mean $\pm$ SEM, and the means were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison as a post-hoc test to determine the *P* value. Student's *t* test was used when comparing two groups of samples (Sigma Stat). A *P* value less than 0.05 was considered significant.

Detailed methods are described in the Supplementary Material and Methods

Results

High glucose attenuates irinotecan-induced cytotoxicity in colon cancer cells

Four human colorectal cancer cell lines (HT29, HCT116, SW480, and T84) were exposed to various doses of irinotecan. Dose-dependent cytotoxicity by irinotecan was confirmed based on the decreased viability in all four cell lines under normal glucose concentrations (5mM) (Figure 1A-D). Increased viability of irinotecan-treated cells was observed in the presence of high glucose (25 mM), with significantly higher IC50 values for HT-29, HCT116, and SW480 cells (Figure 1A-C). T84 cells treated with irinotecan showed a trend of increased cell viability under high glucose compared to normal glucose but without statistical significance (Figure 1D). The IC50 value of irinotecan (0.02 mM) in HT29, SW480, and HCT116 cells was similar to the mean plasma concentration of irinotecan in cancer patients receiving intravenous chemotherapy infusion [36], and was adopted for subsequent experiments to investigate the roles of glucose metabolites in chemoresistance. Moreover, the increase in glucose uptake in cells incubated in high-glucose medium was confirmed in our previous work using radiolabeled sugar assays [37, 38].

Glucose promotes tumor chemoresistance in xenograft mouse models

The effect of high glucose on tumor chemoresistance was assessed using a xenograft mouse model. Mice were randomized to receive either saline control or irinotecan treatment (Figure 1E), and subcutaneously injected with HT29 cells suspended in normal and high glucose culture medium into the right and left flanks, respectively, of each mouse. Reduced tumor volumes were observed in the irinotecan group compared to the saline control group on day 22 postinjection. In the irinotecan-treated group, the tumor sizes of HT29 cells cultured in high glucose were larger than those cultured in normal glucose

(Figure 1F and 1G). There was no significant difference in the tumor sizes or histological morphology of saline-treated cells cultured in normal and high glucose medium (Figure 1F and 1G).

ATP played a more dominant role than pyruvate in preventing irinotecan-induced apoptosis

Glycolysis generates ATP and pyruvate which are further consumed in oxidative mitochondrial respiration to increase ATP synthesis. Here, the relative roles of glucose metabolites (i.e., ATP and pyruvate) in modulating cell death and proliferation were investigated in irinotecan-treated cells. Under normal glucose concentrations, HT29, HCT116, and SW480 cells exposed to irinotecan displayed an increase in apoptosis, as evidenced by increased DNA fragmentation and caspase 3/7 activities (Figure 2A and 2B, and Suppl Figure 1A). In the presence of high glucose, reductions in apoptotic DNA fragmentation and caspase 3/7 activities were observed (Figure 2A and 2B, and Suppl Figure 1A). Pretreatment with ZVAD-FMK (a pancaspase inhibitor), but not necrostatin-1 (Nec-1, a specific inhibitor of receptor-interacting protein kinase 1 (RIP1)), blocked irinotecan-induced DNA fragmentation (Figure 2C).

Pretreatment with iodoacetate (IA, a glycolytic enzyme inhibitor) or UK5099 (UK, a mitochondrial pyruvate carrier inhibitor) prior to irinotecan exposure prevented the glucose-mediated reduction in DNA fragmentation and caspase 3/7 activities (Figure 2D). This suggests that glycolysis and mitochondrial respiration are involved in the resistance to irinotecan-induced apoptosis. Since pharmacological inhibition of glycolysis cannot distinguish the individual roles of pyruvate and ATP, we resorted to exogenous addition of a cell-permeable pyruvate derivative and ATP-encapsulated liposomes, respectively. Liposomal ATP reduced the levels of DNA fragmentation and caspase 3/7 activities in irinotecan-treated cells (Figure 2E). On the other hand, cell-permeable pyruvate had no effect on DNA fragmentation or caspase 3/7 activities (Figure 2F). The results validated the role of ATP as the main effector for rescuing cells from apoptotic death.

Pyruvate uncoupled to ATP inhibited irinotecan-induced necroptosis

Necrotic cell death was also observed in irinotecan-treated cells, as evidenced by LDH leakage in human colorectal cancer cells, which was prevented by the presence of high glucose (Figure 3A and Suppl Figure 1B). Pretreatment with Nec-1 but not ZVAD-FMK decreased irinotecan-induced LDH leakage (Figure 3B). The immunoprecipitation results confirmed the formation of the RIP1/3 complex in irinotecan-treated cells (Figure 3C), suggesting activation of programmed necrotic cell death (i.e., necroptosis). As expected, high glucose also attenuated the level of RIP1/3 complex formation induced by irinotecan (Figure 3C).

Pretreatment with IA but not UK reversed the glucose-mediated protection against LDH leakage (Figure 3D), suggesting that glycolysis but not mitochondrial respiration was involved in rescuing cells from

necroptosis. In contrast to its role in apoptosis, liposomal ATP did not prevent irinotecan-induced LDH leakage (Figure 3E). The presence of pyruvate reduced both LDH leakage and RIP1/3 complex formation caused by irinotecan (Figure 3F and 3G). The collective data demonstrated that programmed necroptosis was reversed by glycolytic pyruvate in an ATP- and a mitochondria-independent manner.

We also assessed the intracellular levels of pyruvate and ATP to ensure the uptake and metabolism of glucose and liposomal ATP in the culture medium. The high-glucose medium showed increased cellular pyruvate content (Suppl Figure 2A), confirming that glucose uptake led to intracellular pyruvate synthesis. The addition of ATP-encapsulated liposomes but not empty liposomes significantly increased the intracellular ATP levels (Suppl Figure 2B).

Glycolytic pyruvate suppresses free radical-dependent necroptosis without modulating apoptosis

Our previous work demonstrated that pyruvate acted as a scavenger for free radicals [15], while others reported that reactive oxygen species (ROS) were involved in the machinery of necroptosis [5]. Here, elevated levels of mitochondrial superoxide were observed after irinotecan exposure, and high levels of pyruvate significantly reduced ROS levels (Figure 4A). Pretreatment with the antioxidant N-acetylcysteine (NAC) decreased irinotecan-induced cellular LDH leakage (Figure 4B), confirming that ROS was involved in necroptosis pathways. On the other hand, NAC had no effect on the apoptotic caspase 3/7 activities induced by irinotecan (Figure 4C).

Previous studies have shown that pyruvate is oxidized to acetate via direct coupling to ROS in human colorectal cancer cells [28]. Therefore, we determined the cellular acetate levels, as they are a byproduct of pyruvate oxidation. Significantly elevated levels of acetate were observed in irinotecan-treated cells compared to the untreated controls in the presence of normal concentrations of pyruvate (Figure 4D), which correlated with increased superoxide levels under genotoxic stress. In the presence of high levels of pyruvate, the acetate amount was further augmented in the irinotecan-treated cells (Figure 4D), indicating more oxidative byproducts of pyruvate. No differences in the levels of redox enzymes, such as superoxide dismutase, were observed in any treatment group regardless of the concentration of pyruvate (Suppl Figure 3), suggesting that pyruvate-mediated ROS scavenging was independent of enzymatic activity. Taken together, the findings supported an antioxidant role for pyruvate by direct coupling to free radicals.

Opposing effects of ATP and pyruvate in modulating the cell cycle progression of irinotecan-treated cells

The cell cycle progression was next evaluated in irinotecan-treated cells to determine modulatory effects of glucose metabolites (Figure 5A). The different phases of the cell cycle were analyzed by staining with anti-Ki67 and propidium iodide (PI, a nucleic acid intercalating agent) for flow cytometry. The G0 phase

represents the resting quiescent state, during which the cells express low levels of Ki67. The cells in the proliferative cycles are characterized by four phases; gap phase (G1), DNA synthesis (S), growth phase (G2) and mitosis phase (M), during which Ki67 is expressed at high levels. As irinotecan inhibits topoisomerase, we observed a higher percentage of cells in the S phase following irinotecan treatment under normal glucose concentrations (Figure 5A-a and 5B-a). In addition, the S-phase arrest was accompanied by a higher ratio of G1 to G0 in the irinotecan-treated cells under normal glucose (Figure 5C-a), indicating that this G0/G1 shift might be a compensatory response for cells reacting to genotoxic stress. The presence of high glucose did not modulate irinotecan-induced S-phase arrest or the G0/G1 shift (Figure 5B-a and 5C-a).

For glucose metabolites, neither pyruvate nor liposomal ATP reversed irinotecan-induced S-phase arrest (Figure 5B-b and 5B-c). Instead, we found that liposomal ATP aggravated the irinotecan-induced G0/G1 shift, whereas pyruvate attenuated the irinotecan-induced G0/G1 shift (Figure 5C-b and 5C-c). The opposing effects of pyruvate and ATP may account for the insignificant changes in glucose for modulating the irinotecan-induced G0/G1 shift.

Last, tumorsphere structural damage is considered an index of solid tumor responsiveness to chemotherapy, and the tumorsphere sizes and percentage of destroyed spheroids were determined using three-dimensional HT29 cell cultures (Figure 6A). Irinotecan treatment under normal glucose concentrations reduced the tumorsphere size and caused spheroid destruction (Figure 6B-a and 6C-a), whereby irinotecan treatment in high glucose medium resulted in increases in spheroid sizes but no difference in the percentage of destroyed spheroids (Figure 6B-a and 6C-a). The addition of liposomal ATP had a similar effect to that of high glucose on spheroid structural damage (Figure 6B-c and 6C-c). On the other hand, pyruvate prevented the deformation of the spheroid mass but had no effect on tumorsphere sizes (Figure 6B-b and 6C-b). Consistent with the cell cycle and cell death results, the spheroid data suggested that ATP promoted cell proliferation and tumorsphere growth, while glycolytic pyruvate attenuated necroptosis-associated tumor mass deformation (Figure 6D).

Discussion

Resistance to chemotherapy is a critical issue for the clinical management of patients with CRC. Our works demonstrated that glucose metabolites (i.e., pyruvate and ATP) played distinct roles in irinotecan chemoresistance. These results included a novel role of glycolytic pyruvate in reducing irinotecan-induced necroptosis and to potentiating drug insensitivity by causing a shift of cells from a proliferative to quiescent state. On the other hand, ATP reduced irinotecan-induced apoptosis and promoted active cell proliferation, which may contribute to tumor recurrence (Figure 6D).

Clinical findings have indicated that a high dietary glycemic load, fasting hyperglycemia, and diabetes mellitus are all poor prognostic factors in advanced CRC patient undergoing chemotherapy [22, 39, 40]. Preprandial levels of blood glucose could be high (10-30 mM) in patients with diabetes. Moreover, colonic retention of dietary sugars due to enteric malabsorption in patients undergoing chemotherapy allows

additional routes of glucose delivery, further compromising treatment effectiveness[19]. *In vitro* studies and *in vivo* mouse models supported that hyperglycemia increased CRC cell resistance to chemotherapies [41-43]. Despite the longstanding observation of glucose-mediated chemoresistance, most studies have only focused on the role of ATP for drug efflux and protection against cytotoxicity[21, 22]. Repair of irinotecan-induced DNA damage, including ubiquitin/26S proteasome-mediated degradation of topoisomerase I, was dependent on ATP[44]. The ATP-binding cassette transporter ABCG2 contributed to drug efflux of irinotecan, and its expression correlated with the toxicity of irinotecan[45, 46]. There are limited studies on the modulatory effects of glycolytic pyruvate on tumor resistance.

Here, we dissected the individual roles of glucose metabolites (i.e., pyruvate and ATP) that confer chemoresistance, and found that pyruvate and ATP had distinct actions on irinotecan-induced cancer cell death and cell cycle arrest. The current study and our previous work demonstrated that glucose exerted a protective effect against first-line chemotherapeutics, including irinotecan and 5-*fluorouracil* (5-FU)[15], in various colorectal cancer cell lines. The human cell lines tested (HT-29, HCT-116, SW480, Caco-2 and T84 cells) were isolated from individuals at different stages of adenocarcinoma which had different metabolic profiles[47], indicating that glucose-mediated chemoresistance could be a universal phenomenon for refractory colorectal cancers.

The results demonstrated that glucose metabolism inhibited irinotecan-induced apoptosis and necroptosis, with ATP and pyruvate playing distinct roles. Our results are in agreement with previous work showing that anaerobic glycolysis but not mitochondrial respiration was involved in the mechanism of glucose-mediated resistance to necroptosis caused by hypoxia[30] and cytotoxic agents[15]. We further confirmed that the anti-necroptotic role of pyruvate was exerted through direct scavenging of ROS coupled to *de novo* acetate production in an ATP-independent manner. Notably, ATP failed to rescue cells from irinotecan- and 5-FU-induced necroptosis[15] but did prevent the irinotecan-induced apoptosis in the current study. The exact mechanisms for ATP-mediated resistance to apoptosis warrant further investigation.

Regarding cell cycle progression, intracellular ATP was linked to enhanced proliferation of cancer cells under irinotecan and 5-FU treatment[15], which might contribute to tumor recurrence. ATP-dependent alterations in the cell cycle, as evidenced by the potentiation of the G0/G1 shift, were consistent with the larger tumorsphere sizes. The G0/G1 shift is considered an adaptive compensatory response to maintain active proliferation under chemotherapeutic genotoxic stress. In contrast, pyruvate increased the percentages of cancer cells in a quiescent state which was protective against cytotoxic damages. This is consistent with previous findings that suppression of cellular ROS levels is related to promotion of stemness properties of cancers[48]. While pyruvate had no effect on tumorsphere sizes, it prevented necroptotic cell death and diminished tumor deformation. Insufficient anticancer drug delivery to the core of solid tumors is one of the main challenges in the clinical management of cancer. Our data further emphasize a key role of glycolytic pyruvate in promoting cell quiescence to reduce chemotherapeutic damage, and in preventing destruction of the tumor mass, which could block the rise of local concentrations of anticancer agents.

Novel cancer interventions targeting glucose metabolism are needed. One promising strategy is the administration of a combination comprising glycolytic enzyme or glucose transporter inhibitors and chemotherapeutic agents. Previous studies demonstrated that 3-bromopyruvate functioned as a chemosensitizer in combination with cisplatin, oxaliplatin, or 5-FU [49, 50]. Another strategy would be targeting GLUT transporters to facilitate drug delivery since these transporters were upregulated on the cell surface of various types of cancer. A recent study revealed that a novel drug conjugate of 5-FU and phloretin (a GLUT inhibitor) exhibited better antitumor efficacy than 5-FU alone, with fewer side effects in animal models [10]. Our data illustrated a proof-of-concept for the synergistic effects of targeting glucose pathways with chemotherapy.

Conclusion

High glucose conferred irinotecan chemoresistance in colon cancers; this was mediated by pyruvate and ATP through distinct mechanisms. Glycolytic pyruvate functioned as an antioxidant, which reverted necroptosis and promoted cell quiescence. On the other hand, ATP attenuated apoptosis and enhanced cell proliferation, which may contribute to tumor recurrence. These findings shed light to our understanding of glucose metabolic modulation on cell death and proliferation and provided insights into the development of novel therapies against recalcitrant cancers.

Abbreviations

BHA, butylated hydroxyanisole; CRC, colorectal cancer; FBS, fetal bovine serum; GLUT, glucose transporter; LDH, lactodehydrogenase; NAC, N-acetyl L-cysteine; OXPHOS, oxidative phosphorylation; RIP, receptor-interacting protein kinase; ROS, reactive oxygen species; SGLT1, sodium/glucose transporter 1; TCA, tricarboxylic acid.

Declarations

• Ethics approval and consent to participate:

Approval for the animal study was granted by institutional *animal* care and use committee of NTUCM (20160359).

• Availability of data and material:

The data are available upon request.

• Consent to publication:

Not applicable.

• Competing interests:

The authors declare that they have no competing interests.

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• Statement of author contributions:

Guarantor of integrity of entire study, LCY; study conception and design: LCY; data acquisition: CYH, YCP; data analysis/interpretation: CYH, LCY; statistical analysis: CYH; funding provider: LCY; manuscript drafting: CYH, LCY; revision for important intellectual content, literature research, manuscript editing, and manuscript final version approval: all authors.

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Figures

Figure 1

Figure 1

Glucose-mediated chemoresistance was observed in human colorectal cancer cell lines and xenograft mouse models. The cell viability of human colorectal carcinoma cell lines **(A)** HT29, **(B)** HCT116, **(C)** SW480, and **(D)** T84, after treatment with irinotecan at various doses for 48 hours in cell medium containing normal glucose (NG, 5 mM) or high glucose (HG, 25 mM) concentrations. The brackets in each panel indicate the IC₅₀ of irinotecan in the four cell lines under normal and high glucose. Extra-sum-of-

squares F tests were performed for IC50 comparison. Experiments were repeated three times. **(E)** Schema of the experimental design for xenograft tumor models, including saline control (CON) and irinotecan groups. Each mouse was subcutaneously injected with HT29 cells in culture medium containing NG and HG into the right and left flanks, respectively. **(F)** Representative photographs of the excised tumors and histological images were shown for each group. Upper bar: 10 mm. Lower bar: 10 μ m. **(G)** Tumor volume under normal or high glucose conditions in xenograft models. N=6/group. Paired samples *t*-tests were performed. **P*<0.05 vs. respective CON; #*P*<0.05 vs. NG+Irinotecan.

Figure 2

Figure 2

High glucose and liposomal ATP protected colorectal cancer cells from irinotecan-induced apoptotic death. HT29 cells were exposed to IC₅₀ of irinotecan (0.02 mM) under normal glucose (NG) and high glucose (HG) for measurement of cell apoptosis by DNA fragmentation. **(A)** Irinotecan-induced apoptotic levels were decreased in the presence of high glucose. **(B)** Irinotecan increased the caspase 3/7 activity in HT29 cells, which was partially inhibited by high glucose. N=6/group. **P*<0.05 vs. respective CON; #*P*<0.05

vs. NG+Irinotecan. **(C)** Pretreatment with ZVAD-FMK (ZVAD, 120 μ M; a pan-caspase inhibitor) but not necrostatin-1 (Nec-1, 100 μ M; a specific RIP1 inhibitor) reduced the apoptotic levels under NG. N=6/group. * P <0.05 vs. respective CON; # P <0.05 vs. vehicle+Irinotecan. **(D)** Pretreatment with iodoacetate (IA, 100 μ M; a glycolytic enzyme inhibitor) and UK5099 (UK, 10 μ M; a mitochondrial pyruvate carrier inhibitor) prevented the high glucose-mediated inhibition of cell apoptosis. **(a)** DNA fragmentation and **(b)** caspase 3/7 activity. N=6/group. * P <0.05 vs. respective CON; # P <0.05 vs. vehicle+Irinotecan. **(E)** Addition of ATP-encapsulated liposomes (500 μ M) attenuated the irinotecan-induced cell apoptosis compared to those given empty liposome (Empty) or those without liposomes (Media) in glucose- and pyruvate-free medium. **(a)** DNA fragmentation and **(b)** caspase 3/7 activity. N=6/group. * P <0.05 vs. respective CON; # P <0.05 vs. Media or Empty+Irinotecan. **(F)** Presence of high pyruvate (HP, 25 mM) did not prevent the irinotecan-induced cell apoptosis compared to those given normal pyruvate (NP, 5 mM) in glucose-free medium. A cell-permeable pyruvate derivative, ethyl pyruvate, was added to cells. **(a)** DNA fragmentation and **(b)** caspase 3/7 activity N=6/group. * P <0.05 vs. respective CON; # P <0.05 vs. NP+Irinotecan. All experiments were repeated three times. Independent samples t -tests were performed.

Figure 3

Figure 3

Glycolytic pyruvate attenuated the irinotecan-induced necroptosis in an ATP-independent manner. HT29 cells were exposed to IC50 of irinotecan (0.02 mM) under normal glucose (NG) and high glucose (HG) for measurement of necrotic cell death by lactodehydrogenase (LDH) leakage. **(A)** High glucose reduced the LDH leakage caused by irinotecan. N=6/group.* $P<0.05$ vs. respective CON; # $P<0.05$ vs. NG+Irinotecan. **(B)** Pretreatment with necrostatin-1 (Nec-1, 100 μ M; a specific RIP inhibitor) but not ZVAD-FMK (ZVAD, 120

μM; a pan-caspase inhibitor) blocked the irinotecan-induced LDH leakage under NG. N=6/group. * $P<0.05$ vs. respective CON; # $P<0.05$ vs. vehicle+Irinotecan. **(C)** Western blots showing RIP1/3 complex formation by immunoprecipitation as an indicator of necroptosis induced by irinotecan, which was attenuated in the presence of high glucose. The relative densitometric values of RIP3 over RIP1 were shown. N=4/group. * $P<0.05$ vs. respective CON; # $P<0.05$ vs. NG+Irinotecan. **(D)** Pretreatment with iodoacetate (IA, 100 μM; a glycolytic enzyme inhibitor) but not by UK5099 (UK, 10 μM; a mitochondrial pyruvate carrier inhibitor) blocked the high glucose-mediated inhibition of LDH leakage. N=6/group. * $P<0.05$ vs. respective CON; # $P<0.05$ vs. vehicle+Irinotecan. **(E)** Addition of ATP-encapsulated liposomes did not reverse the irinotecan-induced LDH leakage compared to those given empty liposomes in glucose- and pyruvate-free medium. N=6/group. * $P<0.05$ vs. respective CON. **(F)** Presence of high pyruvate (HP, 25 mM) decreased the irinotecan-induced LDH leakage compared to those given normal pyruvate (NP, 5 mM) in glucose-free medium. A cell-permeable pyruvate derivative, ethyl pyruvate, was added to cells. **(G)** RIP1/3 complex formation induced by irinotecan was attenuated under high pyruvate. N=6/group. * $P<0.05$ vs. respective CON; # $P<0.05$ vs. NP+Irinotecan. Experiments were repeated three times. Independent samples t -tests were performed.

Figure 4

Figure 4

Mitochondrial free radicals were involved in the irinotecan-induced necroptosis, which were scavenged by pyruvate. HT29 cells were exposed to irinotecan in the presence of cell-permeable pyruvate or N-acetylcysteine (NAC, an antioxidant). **(A)** Irinotecan-induced mitochondrial superoxide synthesis was reduced by the presence of high pyruvate. N=6/group. * $P<0.05$ vs. respective CON; # $P<0.05$ vs. NP+Irinotecan. **(B)** Pretreatment with NAC (1 mM) decreased the irinotecan-induced cellular LDH leakage

under normal glucose. N=6/group. * $P<0.05$ vs. respective CON; # $P<0.05$ vs. vehicle+Irinotecan. **(C)** Pretreatment with NAC (1 mM) did not suppress the caspase 3/7 activity induced by irinotecan. N=6/group. * $P<0.05$ vs. respective CON. **(D)** High pyruvate led to increased intracellular acetate contents, indicating direct effect of free radical scavenging. The cellular acetate level was measured as a byproduct of pyruvate oxidation. N=6/group. * $P<0.05$ vs. respective CON; # $P<0.05$ vs. NP+Irinotecan. All experiments were repeated three times. Independent samples t -tests were performed.

Figure 5

Figure 5

Irinotecan-induced cell cycle G0/G1 shift was reversed by pyruvate but aggravated by ATP. The cell cycles of HT29 exposed to irinotecan were analyzed by flow cytometry after staining of Ki67 (as FL1) and propidium iodide (PI) (as FL2). The PI binds to DNA by intercalating into nucleotide bases, and the intensity indicates the DNA contents during cell cycles of G1, S, G2, and M phases. Actively proliferating cells express high levels of Ki67 protein, whereas those of low Ki67 levels are resting cells in the G0

quiescent phase. **(A)** Representative dot plot. **(B)** Percentage (%) of cells in each phase, and **(C)** Ratio of cells in G1 to G0 phases. The cells were exposed to irinotecan under normal and high concentrations of **(a)** glucose, **(b)** pyruvate, or **(c)** liposomal ATP. N=4/group. * $P<0.05$ vs. respective CON; # $P<0.05$ vs. respective NG, NP or Empty. Experiments were repeated three times. Independent samples t -tests were performed.

Figure 6

Figure 6

ATP increased tumorsphere sizes while pyruvate attenuated the necroptosis-associated tumor deformation.

Tumorsphere structural damage was considered an index of solid tumor responsiveness to chemotherapy, and the tumorsphere sizes and percentage of destructed spheroids were determined using three-dimensional HT29 cell cultures. **(A)** Representative photoimages of HT29 spheroid cultures after 2 days of irinotecan exposure under normal and high concentrations of **(a)** glucose, **(b)** pyruvate, or **(c)** liposomal ATP. Bar: 10 μ m. **(B)** The percentage (%) of destructed spheroids after irinotecan challenge under normal and high concentrations of **(a)** glucose, **(b)** pyruvate, or **(c)** liposomal ATP. N=6/group. * $P<0.05$ vs. respective CON; # $P<0.05$ vs. NP+Irinotecan. **(C)** The spheroid sizes after irinotecan challenge under normal and high concentrations of **(a)** glucose, **(b)** pyruvate, or **(c)** liposomal ATP. N=6/group. * $P<0.05$ vs. respective CON; # $P<0.05$ vs. NG+Irinotecan or Empty+Irinotecan. Independent samples t -tests were performed. **(D)** Schematic illustration of distinct roles of pyruvate and ATP in glucose-mediated irinotecan chemoresistance. Glycolytic pyruvate reverted necroptosis via free radical scavenging and promoted cell quiescence. On the other hand, ATP attenuated apoptosis and enhanced cell proliferation.

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