Non-essential amino acids attenuate apoptosis of gastric cancer cells induced by glucose starvation

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Abstract. Energy and nutrition are essential requirements for all living cells, including cancer cells. In the initiating stage of cancer in organs, cancer cells grow fast and have inadequate supplies of glucose, oxygen and other nutrients due to deficient angiogenesis. Anaerobic conditions cause cancer cells to rely on glycolysis, which produces pyruvate and ATP that can be used by cancer cells to survive. However, glucose starvation may result in apoptosis or necrosis of cancer cells. It has been reported that autophagy is a consequence of glucose starvation and that amino acids are products of autophagy. The present study investigated whether amino acids may represent an alternative energy source for cancer cells undergoing glucose starvation. With non-essential amino acids, growth inhibition and apoptosis of gastric cancer cells induced by glucose starvation were attenuated compared with that of cells undergoing glucose starvation without amino acids, as measured by cell viability, apoptosis rates, membrane potential of mitochondria, and apoptosis-related genes. Meanwhile, both mitochondrial DNA copy number and amino acid transporter genes were increased compared with those in control cells. Non-essential amino acids prevented gastric cancer cells from glucose starvation-induced apoptosis.

Introduction

Deregulating cellular metabolism is one of the hallmarks of cancer, first observed by Otto Warburg in the 20th century, and now known as the Warburg effect (1-4). The Warburg effect in cancer cells is partially induced by rapid cell division, proliferation and anaerobic conditions as well as the requirement of small molecules for the subcellular construction of new cells (1). Under anaerobic conditions, cancer cells convert glucose to pyruvate and then the pyruvate is transformed to lactic acid for the production of ATP and other cellular construction materials.

High glycolytic activity and inadequate glucose intake are contradictory factors in cancer cells. To handle this stress, the expression of glucose transporters, glucose-metabolizing enzymes and pyruvate dehydrogenase kinase are upregulated to promote glucose uptake; however, there is insufficient adaptive angiogenesis and blood supply (5). To attenuate nutrient starvation, cancer cells utilize several compensatory approaches, such as autophagy (6). Autophagy may be induced by high temperature, hormonal stimulation, low oxygen or nutrient starvation (7,8). In cancer cells, a lack of nutrient sources triggers autophagy, which can sustain the survival of malignant cells (6). Cancer cells may undergo autophagy for back-up energy reserve to support self-survival (6,9), although autophagy may increase apoptosis and cell death (10-12). The non-vascularized and metabolically stressed sites of tumors are usually accompanied by autophagy (9).

During the early neonatal stages of newborns, starvation occurs in most mammals (13). To overcome this life-threatening problem, autophagy is activated and self-holding proteins are degraded by autophagy to produce a pool of amino acids with which to attenuate nutrient starvation (13). Meanwhile, nitrogen deprivation also triggers autophagy-dependent amino acid production (14). Amino acids are enzymatically degraded and become nutrient molecules, which may sustain cancer cell survival while cancer cells are under conditions of low oxygen and blood supply.

To investigate the relationship between amino acids and cancer cells under low glucose conditions, we designed and performed a series of experiments. The present study determined whether amino acids can attenuate nutrient starvation of gastric cancer cells.

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Materials and methods

Cell lines and materials. Gastric cancer cells SGC7901, AGS and MGC803 were purchased from Shanghai Cell Collection (China) and cultured in RPMI-1640 medium (Gibco, USA) containing 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and supplemented with 10% fetal bovine serum (FBS; HyClone, USA). The cell culture flasks were maintained in a humidified incubator at 37˚C with a 5% CO_2 atmosphere until they reached appropriate confluence. RPMI-1640 [-] (no glucose, cat. 11879-020), standard RPMI-1640, MEM [100X, non-essential amino acids (NEAs); Invitrogen, cat. 11140-050], and MEM amino acid solution (cat. 11130-051) were obtained from Life Technologies (USA). The JC-1 kit was obtained from Beyotime Biotech Inc. (China).

Cell viability assay. When grown to 60-80% confluence, gastric cells were trypsinized and seeded on 96-well plates at a density of $1x10^4$ cells/well. The next day, complete medium containing 2,000, 1,000 or 500 mg/ml glucose was added into microplate wells, and a NEA solution was added into corresponding wells. Twenty microliters of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) solution (5 mg/ml) were added to each well at intervals of 24, 48, 72 and 96 h after NEA infection. Plates were incubated at 37˚C for 4 h, and 100 μl of a lysis buffer DMSO was then added to each well and mixed thoroughly for 10 min. Finally, absorbance values were determined at 570 nm by a microplate reader (Bio-Rad, USA) (15).

Flow cytometry for apoptosis. To measure low-glucose-induced apoptosis, gastric cancer cells were pelleted in 6-well tissue plates and 16 h later, complete medium containing 2,000, 1,000 or 500 mg/ml glucose or complete medium containing 2,000, 1,000 or 500 mg/ml glucose with 2X NEAs (20 μl of 100X NEAs in 1 ml of experimental treatment medium) was added. Forty-eight hours later, gastric cancer cells were washed with phosphate-buffered saline (PBS), trypsinized with no-EDTA trypsin, centrifuged at 1,000 rpm for 5 min, repeatedly washed with PBS, and resuspended in 500 μl binding buffer/10^6 cells. Cells were then incubated with FITC-labeled Annexin V for 15 min on ice, followed by incubation with propidium iodide (PI) for another 15 min (BD Biosciences, USA) (16). The apoptosis of gastric cancer cells was determined by flow cytometry.

Mitochondrial membrane potential detection by JC-1 staining. Gastric cancer cells were seeded in 6-well tissue plates at a density of $1x10^5$ cells/well and grown to 60-70% confluence. Cells were then stimulated by experimental treatment medium for 48 h. The cells were washed twice with serum-free medium and incubated with a final concentration of 1 μM JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) in DMEM medium for 30 min at 37˚C and 5% CO_2 (17,18). After the medium was removed, each treatment group of cells was washed with PBS 3 times, and 100 μl of PBS was added to each well. Finally, the changes in the mitochondria membrane potential were determined by fluorescence-activated cell sorting (FACS; BD Biosciences) at excitation and emission wavelengths of 544 and 590 nm, respectively.

Table I. Primers for amino acid transporter and apoptosis-related genes used in qRT-PCR.

Gene names	Primers
LAT1	F: TGTGCTGGCATTATACAGCG
LAT1	R: AGGTGATAGTTCCCGAAGTC
LAT2	F: TTTCCAGGAACCTGACATCG
LAT2	R: ACATTGCAGTGACATAAGCG
h4F2hc	F: CTCAGGCAAGGCTCCTGACT
h4F2hc	R: GGCAGGGTGAAGAGCATCA
Bax	F: GATGCGTCCACCAAGAAGCT
Bax	R: CGGCCCCAGTTGAAGTTG
Bcl-xL	F: ACCCCAGGGACAGCATATCA
Bcl-xL	R: TGCGATCCGACTCACCAATA
Bcl-2	F: TCCGCATCAGGAAGGCTAGA
Bcl-2	R: AGGACCAGGCCTCCAAGCT
Bik	F: CTTGATGGAGACCCTCCTGTATG
Bik	R: AGGGTCCAGGTCCTCTTCAGA

Total RNA isolation and quantitative real-time PCR. Total RNA from different treatment groups was extracted using TRIzol reagent (Invitrogen, USA), and the quality and concentration of RNAs were determined by UV spectrophotometry (Bio-Rad). Complimentary DNA (cDNA) was obtained, and quantitative real-time PCR (qRT-PCR) was then performed using a StepOne Plus instrument (Applied Biosystems, USA) with the following procedures: 95˚C for 10 min, followed by 95˚C for 15 sec, and 60˚C for 1 min for 40 cycles. The relative expressions of the mRNAs of amino acid transporter genes (Lat1, Lat2 and h4F2hc) and cell apoptosis genes (Bcl-2, Bcl-xL, Bik and Bax) were detected by qRT-PCR, and the GAPDH gene was used as an internal control. The comparative CT method was used to calculate the relative expression level of these genes, and qRT-PCR was performed according to a previously described protocol (19). Primers for these genes are listed in Table I.

Mitochondrial DNA copy number detection. mtDNA copy number from experimental treatment medium-stimulated gastric cancer cells was determined by a standard protocol, as described in Current Protocols in Human Genetics (20). Total genomic DNA was isolated with a QIAamp kit (Qiagen, Germany), according to the manufacturer's instructions. The method for mtDNA copy number detection based on qRT-PCR involved the utilization of a 107-bp-sized amplicon of mtDNA $tRNA^{Leu(UUR)}$ (forward primer, CACCCAAGAACAGGGTT TGT and reverse primer, TGGCCATGGGTATGTTGTTA); and an 86-bp-sized amplicon of β2-microglobulin (forward primer, TGCTGTCTCCATGTTTGATGTATCT and reverse primer, TCTCTGCTCCCCACCTCTAAGT) of nuclear DNA (nDNA) was used as an internal control (20). The PCR procedure was as follows: 95˚C for 10 min, followed by 95˚C for

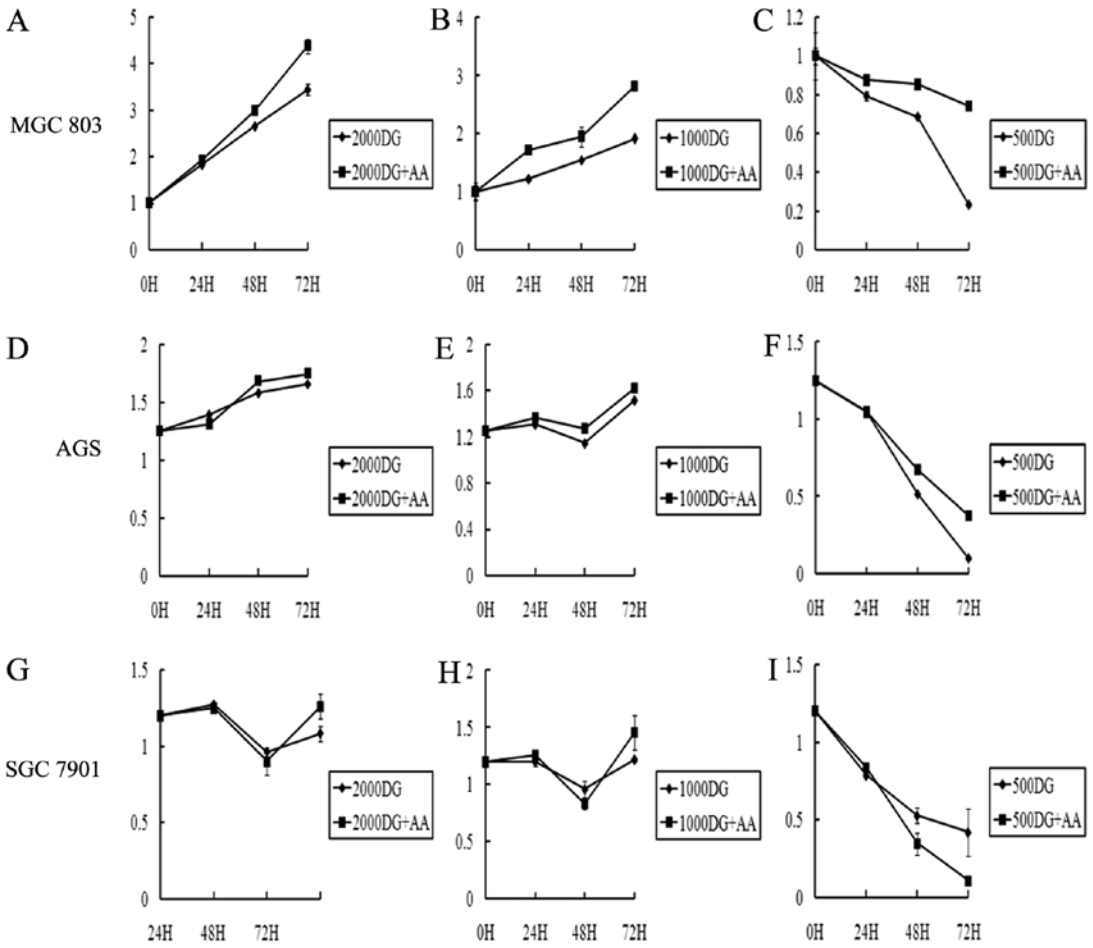

Figure 1. Non-essential amino acids promote gastric cancer cell survival under conditions of glucose starvation. Non-essential amino acids may help MGC803, AGS and SGC7901, three gastric cancer cell lines, resist low glucose. Cell growth in the 500 DG group was significantly greater than that in the 500 DG with NEA group ($p<0.05$) (C, F and I). Others were not as high as that in the 500 DG with NEA group (A, B, D, E, G and H).

15 sec, and 60˚C for 1 min for 40 cycles. PCR assays were performed in triplicate for each DNA sample. The expression of mtDNA copy number relative to the expression of nDNA was determined using the formula: $2x2^{(\Delta CT)}$, where ΔCT is the difference in C_T values between the β2-microglobulin gene and $tRNA^{Leu(UUR)}$ (20).

Statistical analysis. The statistical significance of the data between different groups was evaluated by performing an analysis of variance and Student's t-test using the SPSS 1 statistical software. $p<0.05$ was considered to indicate a statistically significant difference.

Results

Amino acids help gastric cancer cells survive during glucose starvation. In solid tumors, low blood supply, glucose starvation and hypoxia are common due to the rapid proliferation of malignant cells (21). Therefore, glucose starvation leads to apoptosis or necrosis of fast-growth cancer cells. Gastric cancer cells MGC803, AGS and SGC7901 were cultured in medium containing glucose concentrations of 2,000, 1,000 or 500 mg/ml in the presence or absence of 2X MEM amino acids. Cell viability detection assays were performed 24, 48 and 72 h later. As shown in Fig. 1, the cell viability at concentrations of 2,000 and 1,000 D-glucose (DG) was not significantly different between samples with and without NEA (Fig. 1A, B, D, E, G and H), but cells in 500 DG medium with NEA had a significantly higher survival rate compared with those in medium without NEA (Fig. 1C, F and I). Amino acids, therefore, promote gastric cancer cell survival under glucose starvation.

NEAs inhibit apoptosis caused by glucose starvation. To further investigate the effects of NEA on gastric cancer, we utilized flow cytometry to confirm the results of NEA-assisted gastric cancer cells surviving glucose starvation. Forty-eight hours after stimulation with conditional medium (medium containing glucose at a concentration of 2,000, 1,000 or 500 mg/ml with or without 2X MEM amino acids), cells were trypsinized and stained with PI and FITC-labeled Annexin V (22). An obvious increase in the apoptosis rate was detected in the 500 DG groups (15.9-44.1%) compared with the 500 DG with NEA group in both the FITC/PI-positive (31.2-54.3%) and FITC-positive/PI-negative (19.2-27.6%) cells (Fig. 2A-c and -f; Fig. 2B-c and -f; Fig. 2C-c and -f). In contrast, no significant differences were found between the 2,000 and 1,000 DG groups (Fig. 2A-a, -b, -d and -e; Fig. 2B-a, -b, -d and -e; Fig. 2C-a, -b, -d and -e). NEA therefore decreased the apoptosis rate of gastric cancer cells in glucose-deficient medium.

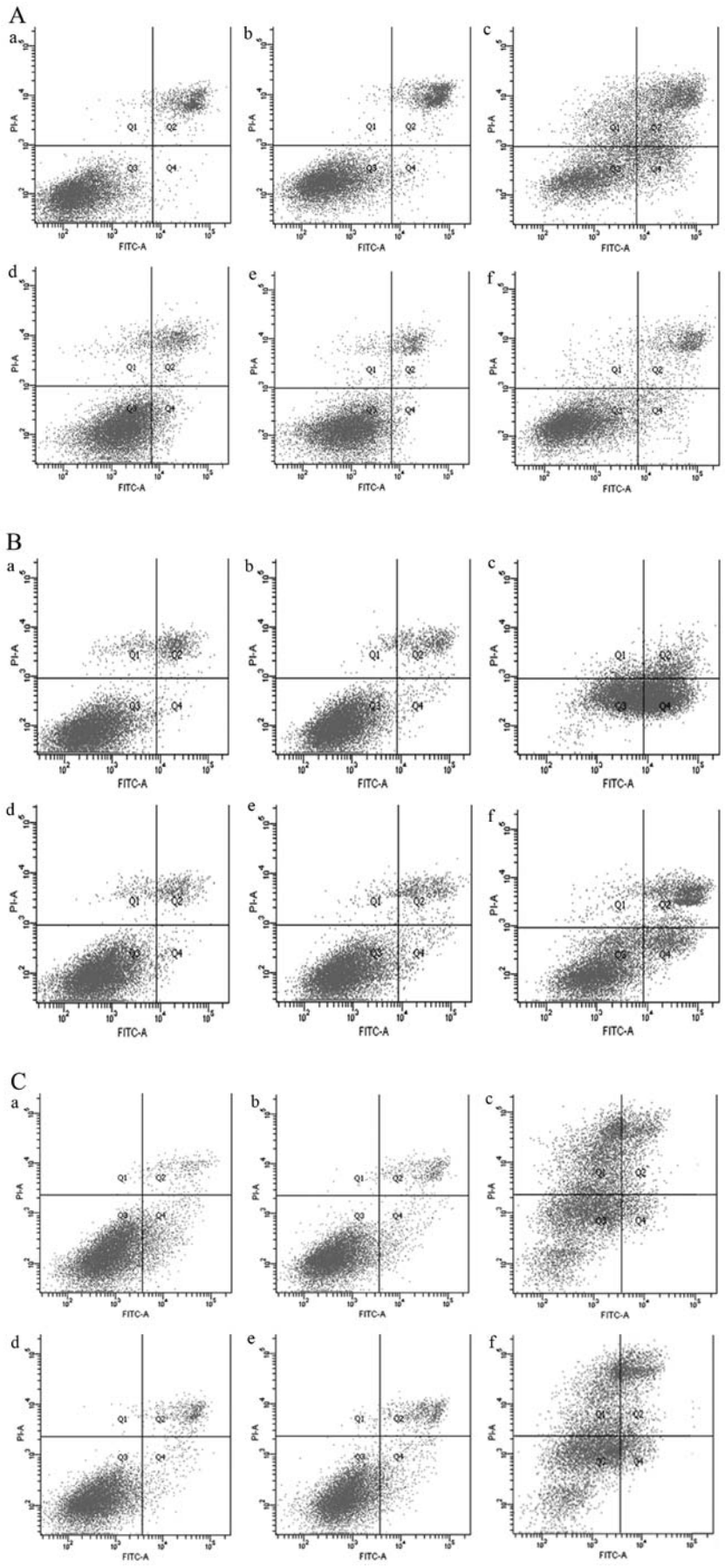

Figure 2. Apoptosis of gastric cancer cells induced by glucose starvation. (A) Apoptosis of MGC803 cells induced by glucose starvation. (B) Apoptosis of AGS cells induced by glucose starvation. (C) Apoptosis of SGC7901 cells induced by glucose starvation. FITC-labeled Annexin V and PI were both used as indices for apoptosis induced by medium containing 2,000, 1,000 or 500 mg/ml glucose (a-c) with or without NEA (d-f). (a, 2,000 DG group; b, 1,000 DG group; c, 500 DG group; d, 2,000 DG-AA group; e, 1,000 DG-AA group; and f, 500 DG-AA group).

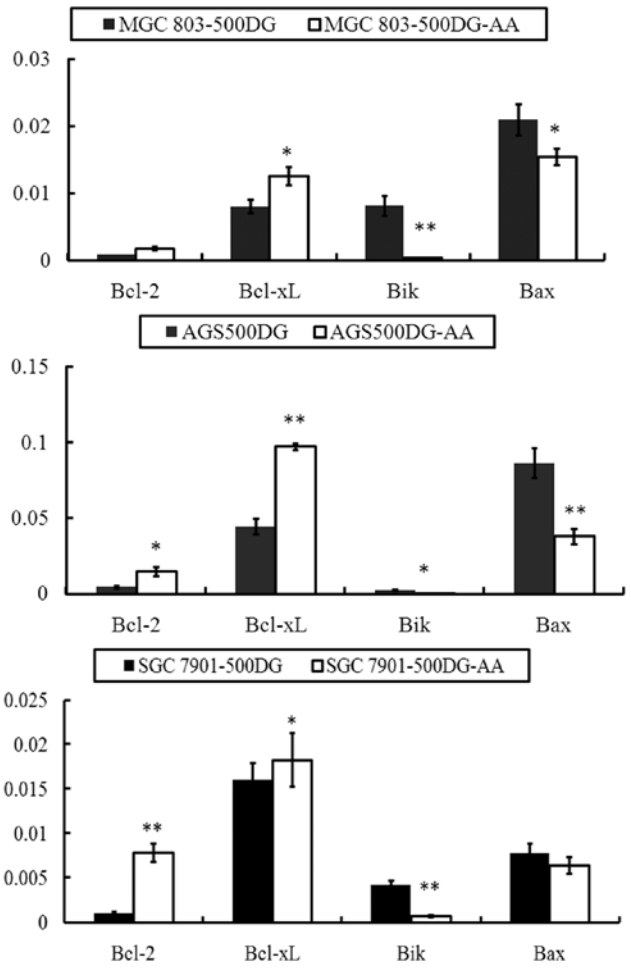

Figure 3. Non-essential amino acids affect the expression of Bcl-2 family genes under glucose starvation conditions. Real-time PCR analysis of Bcl-2 family genes (Bcl-2, Bcl-xL, Bik and Bax) in three gastric cancer cell lines, MGC803, AGS and SGC7901, cultured in 500 DG (with or without NEA) for 48 h. NEA aided the cells by reducing the effects induced by glucose starvation. Data are representative of three experiments. ('*' represents a statistically significant difference, and '**' represents a highly statistically significant difference).

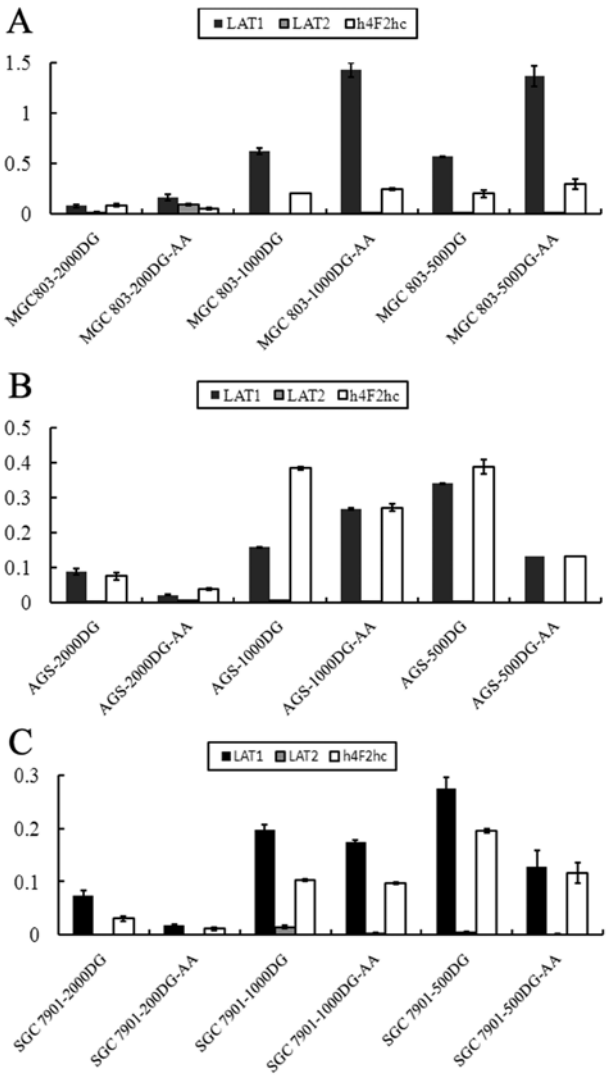

Figure 4. Low glucose concentration upregulates amino acid transporter genes in gastric cancer cells. Amino acid transporter genes in low glucose-induced (A) MGC803 cells, (B) AGS cells, (C) SGC7901 cells.

Altered expression of the Bcl-2 gene family. Apoptosis is a programmed procedure involving various genes. The Bcl-2 gene family is a member of the apoptosis-associated genes and plays an important role in promoting or inhibiting apoptosis (23,24). The family contains Bcl-2, Bcl-xL, Bak, Bik and other genes (24). The general function of Bcl-2 and Bcl-xL is anti-apoptotic and that of Bax and Bik is pro-apoptotic (24,25). Our data showed that the groups treated with 500 DG and 500 DG-AA medium had significantly different rates of proliferation and apoptosis. Thus, we evaluated the expression of Bcl-2, Bcl-xL, Bak and Bik in gastric cancer cells treated with 500 DG or 500 DG-AA medium. As shown in Fig. 3, Bcl-2 and Bcl-xL genes demonstrated decreased expression when gastric cancer cells were exposed to 500 DG medium compared with 500 DG-AA medium. Conversely, the expression of the Bik and Bax genes increased, which suggested that NEA sustained gastric cancer cells by inducing the expression of anti-apoptotic members of the Bcl-2 gene family and preventing the expression of pro-apoptotic members.

Overexpression of amino acid transporter genes induced by glucose starvation. The data presented above demonstrate that amino acids inhibit apoptosis. We hypothesized that the expression of amino acid transporters in the cell membrane may increase to fulfill the energy requirements of the cell. Thus, we examined the relative expression levels of amino acid transporter genes L-type amino acid transporter 1 (LAT1), LAT2 and h4F2hc. LAT1, as a transporter gene, is reported to be highly expressed in various human cancers (26-32), and its expression is strongly correlated with that of another amino acid transporter gene, h4F2hc (26). We performed qRT-PCR to determine transporter gene expression after 48 h of low-glucose culture and found that the decrease in glucose concentration led to the significant upregulation of amino acid transporters (LAT1 and h4Fhc). As shown in Fig. 4, LAT1 expression in MGC803 cells was upregulated under low-glucose conditions by 8.17- and 7.48-fold (1,000 and 500 DG, respectively) compared with the 2,000 DG group. LAT2 expression was decreased under low-glucose conditions, and the expression of h4F2hc was upregulated by 2.48- and

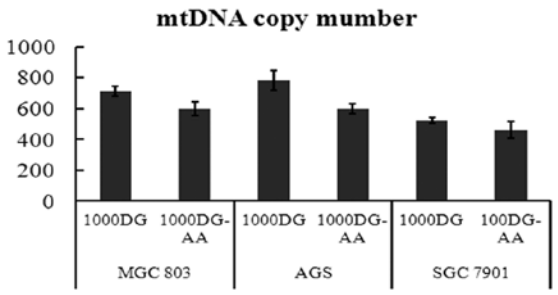

Figure 5. Amino acids stimulate an increase in mitochondrial DNA (mtDNA) copy number in gastric cancer cells cultured in low-glucose medium. MGC803, AGS and SGC7901 cells were stimulated with medium containing 1,000 DG or 1,000 DG with NEA for 48 h, and the genomic DNA of these cells was extracted for real-time PCR detection. We calculated the relative amount of mtDNA using the formula $2x2^{(\Delta CT)}$. The amount of mtDNA increased slightly (599 to 712 in MGC803 cells, 597 to 783 in AGS cells and 461 to 523 in SGC7901 cells). DG, D-glucose; NEA, non-essential amino acids.

2.40-fold (1,000 and 500 DG, respectively; Fig. 4A) compared with the 2,000 DG group. Additionally, the ratios of LAT1, LAT2 and h4F2hc in the 1,000 and 500 DG groups compared with the 2,000 DG group were changed from 1.79 to 3.87, 2.42 to 0.69 and 5.09 to 5.14, respectively, in AGS cells (Fig. 4B). The corresponding ratios were 2.68 and 3.72, 20.23 and 6.20, and 3.41 and 4.50 in SGC7901 cells (Fig. 4C). Thus, glucose starvation was capable of upregulating amino acid transporter genes.

Mitochondrial content determination: DNA copy number. Mitochondrial disorders are complicated heterogeneous diseases that may be caused by molecular or cellular defects (33,34). It is clear that a constant number of mtDNA copies is essential for maintaining cell homeostasis (35-37). Similarly, we investigated whether the copy number of mtDNA varied in gastric cancer cells during glucose starvation. We extracted the total genomic DNA of gastric cancer cells stimulated by 1,000 DG and 1,000 DG with NEA for 48 h and investigated the relative copy number of mtDNA compared to nDNA with real-time PCR. With nDNA as an internal control, the relative mtDNA copy number increased from 599 to 712, 597 to 783, and 461 to 523 in MGC803, AGS and SGC7901 cells, respectively, in the medium with NEA ($p<0.05$) (Fig. 5).

NEAs prevent the loss of the mitochondrial membrane potential of gastric cancer cells during glucose starvation. Depolarization of the mitochondrial inner membrane resulting from ion channel opening is regarded as one of the signs of cell death. To examine whether low glucose and amino acids affected mitochondrial inner membrane potential, we used the voltage-sensitive fluorescent dye JC-1 to stain gastric cancer AGS cells that were treated for 24 h with low-glucose medium or medium with NEA. The ratio of JC-1 red (595 nm)/green (535 nm) represents the percentage of cells undergoing apoptosis. The ratio of JC-1 red/green in AGS cells is shown in Fig. 5. We determined that there were no significant differences in mitochondrial membrane potential between the groups cultured with or without NEA at 2,000 or 1,000 DG (2.45-1.23 and 1.15-3.19%, respectively), but a significant difference was found between the 500 DG groups with and without NEA (78% of the 500 DG group and 15.2% of the 500 DG-AA group) (Fig. 6C and D).

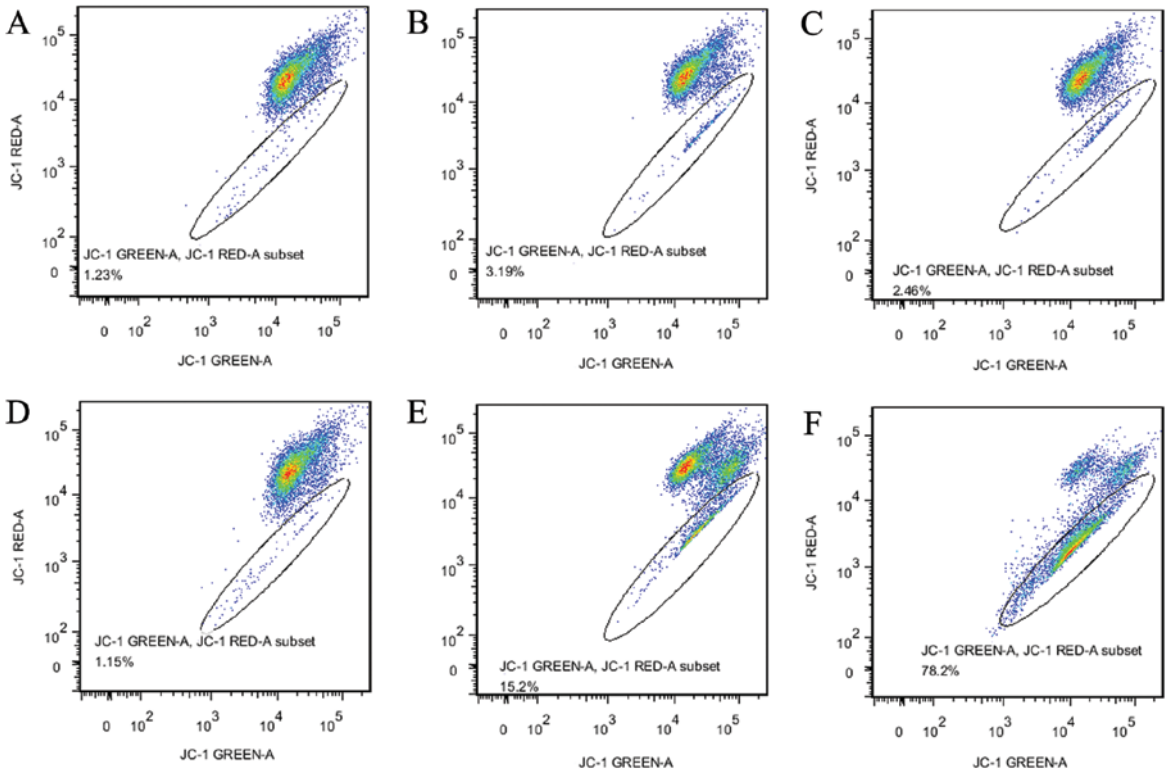

Figure 6. Membrane potential of mitochondria in gastric cancer AGS cells cultured in gradient concentrations of glucose and labeled with JC-1. (A) 2,000 DG group; (B) 1,000 DG group; (C) 500 DG group; (D) 2,000 DG with NEA group; (E) 1,000 DG with NEA group, and 500 DG with NEA group.

Discussion

Gastric cancer, similar to other types of solid cancer, requires angiogenesis when cancer cells undergo rapid growth and proliferation (38,39). Cancer cells require more energy support compared to normal cells, and glucose is a major source of cellular energy (40). Otherwise, glucose is consumed not for ATP production but for cell growth and the production of new cells. Glycolysis is the major mechanism of glucose metabolism in cancer cells, which is called the Warburg effect, as it was observed by Otto Warburg for the first time in 1956 (3,41,42). Without sufficient angiogenesis, glucose starvation or deprivation is common in fast-growing cancer cells, and autophagy may be an alternative energy source for cancer cells (6,9). Amino acids, as the products of autophagy, could serve as a back-up source of nutrients for rapidly growing malignant cells (13,14). Based on the above-referenced studies, we inferred that amino acids could prevent apoptosis of gastric cancer cells undergoing glucose deprivation.

Here, we presented evidence for the first time supporting the hypothesis that non-essential amino acids can attenuate glucose starvation-induced apoptosis of gastric cancer cells. Survival, apoptosis, the potential of mitochondria, mitochondria DNA copy number, and amino acid transporter proteins are all directly or indirectly regulated by non-essential amino acids added to low-glucose media. These data demonstrated that non-essential amino acids may be an alternative energy source for gastric cancer cells under glucose-starved conditions.

Energy deprivation of somatic or cancer cells could trigger marked reprogramming of cell homeostasis to facilitate the adjustment of cellular metabolism (43,44). To elucidate the bio-function of amino acids in gastric cancer cells undergoing glucose starvation, we adopted a strategy of treating cancer cells with culture medium containing a glucose concentration gradient and investigated whether amino acids performed important effects to attenuate glucose starvation. The results of a series of experiments measuring proliferation, apoptosis, and the expression of related genes showed that low glucose concentration could induce gastric cancer cells to undergo apoptosis, but non-essential amino acids could attenuate that effect. Several studies have mentioned that glucose starvation could result in apoptosis of cells though signaling pathways (43,45).

The amino acid transporter gene LAT1 has been reported to be upregulated in the membranes of many cancer cells (27,28,46-48). This protein transports amino acids into cells and is different from two other amino acid transporter genes, namely LAT2 and h4F2hc (49). Energy deprivation can trigger response systems to adjust the state of cells, and increased amino acid intake may be one of those responses.

Several recent studies have shown that amino acids are associated with proliferation in cancer (50-54). Jain *et al* found that 219 metabolites were released from rapidly proliferating cancer cells, and glycine consumption and the glycine biosynthetic pathway were strongly correlated with the rate of proliferation of almost 60 cell lines (50). Zhang *et al* reported that glycine may be a critical factor associated with tumor-initiating cells and tumorigenesis of non-small-cell lung cancer (54). In *Arabidopsis thaliana*, low energy status induced the upregulation of the basic leucine zipper transcriptional factors bZIP1 and bZIP53, which are crucial factors in proline, asparagine, and branched amino acid metabolism in response to energy starvation (55). Proline and asparagine are included in non-essential amino acids. Increasingly more evidence shows that amino acids may have a critical functional role in proliferation, resistance to anticancer drugs, invasion or metastasis of cancer cells. Amino acids are constructed units of proteins and additionally may serve as an energy resource such as glucose. As shown in the present study, non-essential amino acids were able to mitigate the stress caused by glucose starvation.

All cells and tissues have an amino acid sensor system that can help monitor the state of intercellular amino acids (56,57). In higher eukaryotic cells, mTOR1 is believed to regulate protein synthesis in the amino acid signaling pathway (57). The protein mTOR is a central cell growth regulator and promotes cell growth and cell size by controlling protein synthesis (56). At the same time, mTORC can be stimulated by or control amino acid metabolism through several key components (such as Rag GTPase), and the signaling is described in detail in a review (56).

Mitochondria have important roles in various cellular activities, including apoptosis and energy metabolism (58). The content of mitochondria (mitochondrial DNA copy number) changes with the changed status of cells and may increase or decrease (59-62). In the present study, we found that non-essential amino acids maintained a constant mitochondria content in gastric cancer cells undergoing glucose starvation, which indicates that non-essential amino acids may reduce the decreasing effect on the content of mitochondria that are induced by glucose starvation and thus sustain cell viability (63). The potential of mitochondria is associated with cell apoptosis and mitochondria DNA copy number and is also a marker of cell activity. All the effects that were observed in this study reflected the finding that non-essential amino acids could increase the activity of gastric cancer cells facing glucose starvation.

The results of the present study confirmed the hypothesis that non-essential amino acids and not total amino acid or glutamine levels were capable of promoting gastric cancer survival in cells under glucose starvation. The promoting effects of the non-essential amino acids may be achieved through the increased expression of amino acid transporter proteins, upregulation of the expression of anti-apoptotic genes, and mitochondrial content. Future studies should focus on the signaling pathways by which amino acids are regulated in response to low glucose conditions.

Acknowledgements

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