Functional characteristics of autophagy in pancreatic cancer induced by glutamate metabolism in pancreatic stellate cells

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Abstract
Objective: To observe the effects of glutaminase (GLS) inhibitors on autophagy and proliferation of pancreatic stellate cells, and to explore their functions in pancreatic cancer.

Methods: Pancreatic cancer cells were divided into two groups. Group A was the control untreated group, and group B cells were treated with GLS inhibitors. Western blotting was used to detect the expression of Atg5, Bcl-2, Bax, and Bid proteins. The bromodeoxyuridine assay and scratch test were employed to investigate cell proliferation and invasion, respectively. The expression of E-cadherin, vimentin, cell adhesion molecule 2 (CADM2), and Snail protein was investigated by immunofluorescence.

Results: The expression of Atg5, Bax, and Bid was higher in group A than in group B, while Bcl-2 expression was lower in group A than in group B. Group A cells demonstrated greater proliferation and invasion than group B cells. The expression of E-cadherin was lower in group A cells than group B cells, while vimentin, CADM2, and Snail were expressed at higher levels in group A than group B cells.

Conclusion: The inhibition of glutamine isozymes reduces autophagy and apoptosis in astrocytes, and inhibits pancreatic cancer cell proliferation and metastasis, while reducing their invasiveness.

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Keywords
Autophagy, pancreatic cancer, glutamate metabolism, glutaminase, cell adhesion molecule 2, pancreatic stellate cells

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Introduction
The prognosis of pancreatic cancer is extremely poor, and the survival rate of patients is low. Therefore, actively exploring the pathogenesis of pancreatic cancer and its new treatment methods are important directions of clinical research. In recent years, increasing attention has been paid to the dynamic microenvironment of pancreatic cancer. Pancreatic stellate cells (PSCs), extracellular matrices, inflammatory cells, and various growth factors and cytokines interact with each other and with pancreatic cancer cells. Moreover, PSCs play an important role in the development of pancreatic cancer and chronic pancreatitis.

In the healthy pancreas, PSCs are located around the pancreatic acinus and within the interlobular space, accounting for 4% of the total number of cells. However, when the pancreas is damaged, adenocarcinoma cells specifically activate surrounding PSCs, which promote the synthesis of matrix metalloproteinase 2 (MMP2). MMPs are closely associated with the formation of tumor connective tissue. PSCs have been proven to promote cancer cells by increasing their proliferation, migration, and invasion.

Glutaminase (GLS) is an important enzyme that catalyzes the breakdown of glutamate, and studies have shown that GLS is highly expressed in most tumors and has a cancer-promoting effect. For example, biosignal analysis showed that GLS is highly expressed in pancreatic tissue. GLS is a potential target for anti-tumor metabolism therapy, and its inhibitors could become new anti-tumor metabolism drugs. However, the role of GLS and PSCs in the development and progression of pancreatic cancer has not been investigated. Therefore, in this study, we observed the autophagy effect of PSCs and the proliferation of cancer cells under the action of GLS inhibitors, and explored their functional characteristics in pancreatic cancer.

Materials and methods

Materials
The human pancreatic cancer cell line SW1990 was purchased from American Type Tissue Collection (Manassas, VA, USA) and Transwell chambers were from Becton, Dickinson and Co. (Franklin Lakes, NJ, USA). The following antibodies were from Vector Laboratories Inc. (Burlingame, CA, USA): primary rabbit anti-human polyclonal antibodies against ATG5, Bcl-2, Bax, and Bid (1:200); a secondary biotinylated goat anti-rabbit antibody (1:500); and a mouse anti-BrdU monoclonal antibody (1:500). The DAB color development kit was from Beijing Zhongzhuang Jinqiao Biotechnology Development Company (Beijing, China), and antibodies against actin were from Sigma-Aldrich (St Louis, MO, USA).

Cell culture
SW1990 cells were cultured in DMEM medium containing 10% newborn calf serum in a 37°C incubator. When cells reached 70% to 80% confluency, they
were subcultured with 0.25% trypsin and divided into two groups. Group A was set as an untreated control group. Group B was treated with the glutamine isoenzyme inhibitor CB-839 1 μM. Pancreatic cancer cells Panc-1 (upper layer) and human pluripotent stem cell stellate cells (lower layer) were isolated by the outgrowth method, as described previously.

**Western blot detection of Atg5, Bcl-2, Bax, and Bid expression in lower stellate cells**

Lower layer cells were collected and positive clones were grown to sub-fusion. Total protein was routinely extracted, and its concentration was determined by the BCA method. Denatured protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, with 50 μg loaded per well, and transferred to polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk for 2 hours, then incubated with primary antibody at 4°C overnight. It was washed three times with Tris-buffered saline (TBS) with Tween 20, then incubated with the corresponding secondary antibody for 2 hours. Detection was achieved using enhanced chemiluminescence, scanned using the Gene Gel Imaging System, and analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA).

**Bromodeoxyuridine (BrdU) detection of upper pancreatic cancer cell proliferation**

Upper layer cells were collected and BrdU was incorporated into cells during their exponential growth phase. Cells were seeded at $1 \times 10^4$ mL$^{-1}$ in six-well plates with coverslips. After culturing for 48 hours, BrdU (final concentration, 10 g/mL) was added and cultured for 2 hours. The primary mouse anti-BrdU monoclonal antibody (1: 500, Vector Laboratories Inc.) was incubated with the cells at 4°C overnight and was detected by ABC immunohistochemistry according to the manufacturer’s protocol. The primary antibody was replaced with BxPC3 and TBS without BrdU as a negative control. The appearance of brown particles in the nucleus was taken as a positive result. Ten high power fields of view were randomly selected, and the index (L1) was calculated and averaged. The marker index (u) was the number of labeled cells/total number of counted cells.

**Invasion of the lower layer by upper layer pancreatic cancer cells**

Logarithmic growth phase cells were taken and adjusted to a density of $2 \times 10^5$ mL$^{-1}$, then 1 mL cell suspension was added to each well of six-well plates. A sterile pipette tip was used to make a scratch on the cell monolayer growing on the bottom plate. Cell debris was washed away with phosphate-buffered saline, and culturing was continued for 48 hours. Three parallel wells were set up for each group. Cell mobility was calculated as follows: (initial scratch width value – corresponding point scratch width value)/initial scratch width value ×100%. The experiment was repeated three times.

**Fluorescent in situ hybridization (FISH) analysis of GLS expression in lower stellate cells**

Cells on slides underwent conventional xylene dewaxing, gradient ethanol hydration, sodium sulfite treatment, protease digestion, and HCl soaking. They were then dehydrated in an ethanol gradient, fixed in acetone, and baked at 56°C for 5 minutes. adding Lo probe working solution from the SensiFAST probe Lo-Rox kit (Bioline, London, UK) on cells, denatured at 73°C for 5 minutes, hybridized in a wet box at 42°C for 16 hours, then rinsed with
50% formamide, citrate buffer, 0.1% NP-40, and 70% ethanol before being naturally dried in the dark. They were then counterstained with 4’,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), sealed, placed in the dark for 20 minutes, and observed under a fluorescence microscope. A total of 30 cells were examined microscopically, and the ratio value was calculated as follows: ratio value = total number of red signals in 30 nuclei/total number of green signals in 30 cell nuclei. A ratio <1.8 indicated a negative result, suggesting that the sample had no HER-2 gene amplification, a ratio >2.2 was a positive result suggesting HER-2 gene amplification, while a ratio 1.8 to 2.2 suggested that HER-2 gene expression was slightly increased. In these cases, the ratio value calculation was determined in up to 100 cells or FISH was repeated to determine the final result.

**Immunofluorescence detection of epithelial-mesenchymal transition (EMT) markers of E-cadherin, vimentin, CADM2, and Snail in upper pancreatic cancer cells**

Upper pancreatic cancer cells were collected and stably transfected cells were dropped onto coverslips. Cells were grown for 24 hours, fixed with methanol-20 for 10 minutes, and washed three times with PBS. Then, 0.5% PBST (Triton X-100) was added and incubated at 37°C for 5 minutes. Cells were washed a further three times with PBS, then an appropriate volume of 3% bovine serum albumin was added for 30 minutes, after which PBS-diluted primary antibody against E-cadherin, vimentin, CADM2, or Snail was added for an overnight incubation at 4°C. After washing with PBS in triplicate, fluorescein-conjugated secondary antibody was then added for a 1 hour incubation at 37°C, cells were washed three times with PBS, incubated with DAPI for 15 minutes (1:500) and mounted with 50% glycerol. Cells were observed under a fluorescence microscope.

**Statistical analysis**

Data were analyzed by SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). Measurement data were expressed as means ± variance. The t-test was used for comparisons between groups. Count data were expressed as percentages (%). Comparisons between groups were performed by the chi-squared test. The analysis was statistically significant at $P < 0.05$.

**Results**

**Expression levels of Atg5, Bcl-2, Bax and Bid proteins in stellate cells**

The expression levels of ATG5, Bax, and Bid in group A were higher than those in group B at 12, 24, and 36 hours. The expression of Bcl-2 protein was significantly lower in group A cells than in group B cells ($P < 0.05$; Figure 1).

**Proliferation of pancreatic cancer cells**

Optical density values were significantly higher in group A cells (0.85 ± 0.03) than in group B cells (0.51 ± 0.04) ($P < 0.05$), indicative of a higher level of cell proliferation (Figure 2).

**Invasion of the lower layer by upper layer cells**

Average cell migration rates at 12, 24, and 36 hours were 55.72%, 83.61%, and 22.58%, respectively, in group A and 38.55%, 70.08%, and 11.49%, respectively, in group B. The effect of group A cells on promoting the invasion of pancreatic cancer cells to the lower layer was greater than that of group B cells at different times.
The proliferative effect at 12 hours was lower than that at 24 hours, and the effect at 36 hours was markedly lower than at 12 and 24 hours (Figure 3).

**Expression of GLS in stellate cells**

GLS expression was significantly higher in group A cells than group B cells ($P < 0.01$) (Figure 4).

**EMT marker expression in pancreatic cancer cells**

The expression of E-cadherin in group A cells was lower than in group B cells. The expression of vimentin, CADM2, and Snail protein was significantly higher in group A cells than in group B cells ($P < 0.05$; Figure 5).

**Discussion**

The autophagy protein ATG5 is involved in the formation of autophagosomes and inhibition of tumorigenesis. During the formation of autophagosomes, ATG5 binds to the outer membrane to promote expansion of the autophagosome membrane structure. Changes in autophagy affect multiple aspects of tumor progression including tumor cell apoptosis, angiogenesis, and anti-tumor therapy. The Bcl-2 family is the most studied regulators of apoptosis. Bcl-2 inhibits apoptosis, and is abnormally expressed in many malignant tumors; for example, it is increased in pancreatic cancer. Bax belongs to the Bcl-2 family and antagonizes Bcl-2 to inhibit apoptosis, while Bax overexpression induces apoptosis. Bid also belongs to the Bcl-2 family and increases the release of mitochondrial CytC and apoptosis-inducing factor. Bid expression is enhanced in some tumors such as gastric cancer and leukemia.

In the present study, the autophagy activity of group B cells was weakened, as shown by the reduced expression levels of ATG5, Bax, and Bid, while apoptosis-inhibiting Bcl-2 expression was up-regulated. With increased treatment time, GLS inhibitors clearly attenuated GLS expression in stellate cells and reduced their autophagy and apoptosis activities.
Glutamate isoenzyme inhibitors were reported to inhibit the proliferation of stellate cells in the liver and increase their apoptosis, while the rapid inhibition of proliferation and apoptosis in malignant tumor cells has not been reported. The observed reduction in autophagy and apoptosis in group B cells suggested that the proliferation activity was also weakened, indicating that GLS inhibition blocked the proliferation of stellate cells.

GLS is crucial for the regulation of tumor redox reactions, which affect gene transcription and hydroxylation, as well as...
autophagy, cell proliferation, and apoptosis.\textsuperscript{14} GLS is highly expressed in a variety of cancers, and promotes cell proliferation and cancer cell metastasis.\textsuperscript{15} Kidney-type glutaminase GLS1 has a cancer-promoting effect, while liver-type glutaminase GLS2 has an anti-cancer effect. The survival of tumor cells depends on GLS. The deprivation of GLS provides a new strategy for the metabolic treatment of tumors,\textsuperscript{16} and a combination of GLS1 inhibitors and other antimetabolites was reported to achieve notable anti-tumor effects. In this study, the proliferation of pancreatic cancer cells in group A was significantly higher than in group B. Moreover, the scratch test revealed that group A upper pancreatic cancer cells were more aggressive than group B cells, suggesting that GLS inhibition blocks pancreatic cancer cell proliferation, consistent with the above findings.

E-cadherin is an important intercellular adhesion molecule that is mainly found in epithelial cells. It is also one of the main molecules that mediates adhesion between cells and the extracellular matrix.\textsuperscript{17} Studies have shown that the functional inhibition or decreased expression of E-cadherin reduces cell adhesion and promotes invasive growth, and the infiltration and metastasis of tumor cells.\textsuperscript{17} Conversely, the enhanced expression of E-cadherin reduces the motility and invasiveness of tumor cells, and inhibits their growth.\textsuperscript{18} Vimentin is a major intermediate filament protein present in mesenchymal cells that plays an important role in the development, invasion, and metastasis of cancer. It regulates the interaction between proteins such as cytoskeletal protein cell adhesion molecules, participates in the adhesion and invasion of tumor cells and cell signal transduction, and is overexpressed in several malignant epithelial tumors such as pancreatic ductal adenocarcinoma. Studies have shown that vimentin inhibition blocks tumor cell growth, invasion, and metastasis.\textsuperscript{19,20} CADM2 regulates intercellular adhesion and is ubiquitously expressed in healthy tissue cells but down-regulated in various tumor cells, while Snail expression is elevated in pancreatic cancer and mirrors the expression of vimentin.\textsuperscript{21,22} In this study, these EMT markers were largely decreased in group B cells, indicating that the inhibition of GLS expression

\textbf{Figure 5.} Protein expression of EMT markers E-cadherin, vimentin, CADM2, and Snail.
reduced the infiltration and metastatic ability of pancreatic cancer cells.

This study revealed that PSCs directly contact pancreatic cancer cells because a more significant effect on cancer cell proliferation was observed in the co-culture system involving direct contact than in the indirect contact co-culture system. This agrees with the previous finding that PSCs have a promoting effect on the migration of pancreatic cancer cells.\textsuperscript{3,4} We showed that under GLS inhibition, both stellate cells and cancer cells showed a decrease in proliferation, while cancer cell invasiveness also decreased. Therefore, we speculate that GLS inhibits the proliferation and metastasis of cancer cells by blocking the proliferation of stellate cells.

In conclusion, the inhibition of glutamine isoenzymes reduces autophagy, apoptosis, and proliferation in stellate cells, which decreases their invasiveness. Further research is needed to explore the mechanism by which glutamine regulates stellate cell proliferation.

Consent
This study was carried out in accordance with approved guidelines by the Ethics Committee of Longhua Hospital Shanghai University of Traditional Chinese Medicine. All patients provided written informed consent for participation in the study.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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