Effects of the suppression of lactate dehydrogenase A on the growth and invasion of human gastric cancer cells

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Abstract. Lactate dehydrogenase A (LDH-A), which regulates glycolytic flux by catalyzing pyruvate to lactate in the cytoplasm, is believed to be one of the highly attractive therapeutic targets for cancers. Firstly, we detected the expression of LDH-A in gastric cancer (GC) cells. LDH-A inhibitor oxamate was then used to suppress the LDH-A activity in GC cells. Cell proliferation, lactic acid production, Transwell migration assay and apoptosis were assessed, respectively. The results showed that inhibition of LDH-A by oxamate decreased the lactate production. In the presence of glucose, oxamate inhibited cell proliferation in a dose-dependent manner. Flow cytometry assay further confirmed a pro-apoptotic effect of oxamate, and this was likely through increased expression of Bax, activated caspase-3, and decreased expression of Bcl-2. Therefore, we believe that oxamate inhibits cell growth, suppresses tumor invasion, and induces apoptosis in GC cells. LDH-A may be a potential therapeutic target for GC.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; 5-FU, 5-fluorouracil; GC, gastric cancer; LDH-A, lactate dehydrogenase A; MTX, methotrexate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Key words: stomach carcinoma, gastric cancer, aerobic glycolysis, LDH-A, oxamate

Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide (1). Although the incidence has decreased in developed countries, it still remains an important public health burden in China (2). Globally, more than two-thirds of GC patients have unresectable disease at the time of diagnosis and 60% of resectable cases eventually relapse (3). The prognosis for advanced GC remains poor, with a 5-year survival rate of 26% (4). Therefore, more effective therapeutic approaches are urgently needed for GC patients.

Altered metabolism is one of the critical hallmarks of cancers (5). Even in the presence of enough oxygen, tumor cells prefer to metabolize glucose by glycolysis rather than oxidative phosphorylation (6). In tumor cells, a substantial amount of pyruvate is reduced to lactate instead of being directed into the mitochondrion. Key alterations in metabolic pathways in tumor cells may create opportunities for the design of new anticancer approaches (7). In fact, agents targeting tumor metabolism have been proven useful in cancer therapy. In this aspect, traditional antimetabolites, such as methotrexate (MTX) and 5-fluorouracil (5-FU), have been used as chemotherapeutic agents for several decades (8). Most of these traditional antimetabolites target the final stages of the nucleotide synthesis and competitively inhibit the functioning of key enzymes involved in nucleic acid synthesis. However, these traditional anticancer agents only offer limited therapeutic benefits, and most of them are associated with severe adverse effects.

Recent studies have indicated that deprivation of tumor cells of an energy supply can be a promising approach for cancer therapy. In mammalian cells, glucose is the key energy source for all tissues. Physiologically, glucose is converted to pyruvate via the glycolytic pathway, which is then either metabolized to lactic acid by lactic dehydrogenase (LDH) or enters the citric acid cycle in the matrix of the mitochondria (9). LDH-A is a cancer-specific isoform of LDH which converts cytoplasmic pyruvate to lactate, an important energy-producing step for cancer cells (10,11). Oxamate is a pyruvate analogue that can competitively inhibit the activity of LDH-A.

Previous studies have revealed that blocking LDH-A only inhibits aerobic glycolysis in tumor cells while the normal cells are largely spared (12-15). It is due to this characteristic that LDH-A is believed to be one of the highly attractive therapeutic targets for cancers. However, the biological significance of LDH-A expression and function in GC remains unknown.

In the present study, we aimed to evaluate the expression pattern and the biological significance of LDH-A in GC cells.

Materials and methods

Reagents and cell lines. The GC cell lines (AGS, BGC-823, MGC-803 and SGC-7901) were purchased from the Cell Research Institute of the Chinese Academy of Sciences (Shanghai, China). The normal gastric mucosa endothelial cell line GES-1 was purchased from Beijing Cancer Institute (Beijing, China). All primary antibodies were purchased from Bioworld Technology (St. Louis Park, MN, USA). The secondary antibodies were purchased from Beijing Zhongshan Biotechnology Co., Ltd. (Beijing, China). RIPA lysis buffer and protease phenylmethanesulfonyl fluoride (PMSF) were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The Super ECL kit was purchased from Applygen Technologies Inc. (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) without glucose and pyruvate was purchased from Gibco-BRL (Gaithersburg, MD, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). L(+)-Lactic Acid assay kit and D-galactose and glucose powder were purchased from Sigma-Aldrich (St. Louis, MO, USA). Neutral red staining solution was purchased from Beyotime Institute of Biotechnology. Transwell migration chamber, Annexin V-FITC and propidium iodide (PI) were purchased from Invitrogen Corporation (Carlsbad, CA, USA). The PCR primers, reverse transcription kit, and qPCR reaction kits were purchased from Takara Biotechnology Co. Ltd. (Dalian, China). The primer sequences used were: LDH-A forward, 5'-GGTTGGTGCTGTTGGCATGG-3' and reverse, 5'-TGCCCCAGCCGTGATAATGA-3'; β-actin forward, 5'-TGGCACCCAGCACAATGAA-3' and reverse, 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'.

Cell culture and treatment. Two different culture media were used: DMEM (containing 25 mM glucose) and glucose-free DMEM (supplemented with 10 mM galactose, for the neutral red staining assay). Both media were deprived of sodium pyruvate, supplemented with 10% FBS, 100 IU/l penicillin and 100 IU/l streptomycin. All cells were cultured under standard conditions in humidified 5% CO_2 at 37˚C.

The effect of LDH-A inhibitor oxamate on GC cells was tested in glucose-free condition in which 10 mM of galactose was added to the glucose-free DMEM medium.

RNA isolation, reverse transcription and PCR. Total RNA was extracted with TRIzol reagent according to the manufacturer's instructions. Approximately 30 ng of total RNA was reverse-transcribed into cDNA. The synthesized cDNA samples were subjected to qPCR using a SYBR-Green quantitative PCR kit. Amplification was carried out in a total volume of 20 μl for 40 cycles of 15 sec at 95˚C, 20 sec at 60˚C and 30 sec at 72˚C. Samples were run in triplicate and the relative expression of target genes was determined by normalizing expression of each target to that of β-actin. The amplification was monitored on a Rotor-Gene real-time PCR apparatus (Rotor-Gene, Australia).

Cell proliferation assay. The effect of modulating LDH-A activity on cell proliferation was tested in AGS and SGC-7901 cells by neutral red staining assay (16). Briefly, ~$5x10^4$ cells suspended in 200 μl of DMEM containing 25 mM glucose or 10 mM galactose were seeded into each well of a 96-well plate and incubated under standard growth conditions overnight. Cells were treated with various concentrations of oxamate (0-100 mM) for 24 h, incubated with neutral red, and the absorbance was read at 490 nm in a microplate reader. The IC_{50} value (dose needed for 50% growth inhibition) was determined using CalcuSyn software (17).

Cell morphology. For morphological analysis, ~$1x10^6$ cells/well were grown in a 6-well plate and were treated with oxamate (0, 20, 40, 60, 80 and 100 mM) for 24 h. Cells were observed under an inverted phase contrast microscope (CHK-213; Olympus, Japan).

Lactic acid quantitation. Approximately $5x10^4$ cells in 200 μl culture medium containing 25 mM glucose were seeded into each well of a 96-well plate and incubated overnight. Cells were then treated with various concentrations of oxamate (0, 20, 40, 60 and 80 mM). Lactic acid secreted into the culture medium was measured at the start of the experiment and 4 h after oxamate treatment. The concentration of lactic acid was determined by an enzyme assay kit, and the absorbance of NADH formation was measured in a microplate reader at 450 nm. The amounts of lactic acid were calculated by substracting the value measured at the start time from that of the 4-h treatment and the final concentration of lactic acid was normalized against the cell number. Inhibition rate (%) of lactic acid production was defined as (control group - experimental group)/control group x 100%.

Transwell migration assay. The effect of LDH-A inhibition by oxamate on the migratory effect of SGC-7901 cells was examined by the Transwell migration assay. Briefly, cells were placed onto 8-μm pore sized Transwell filters at a concentration of $2x10^5$ cells/well in 250 μl of FBS-free DMEM medium. To the lower chamber, 1 ml of medium with 10% FBS and various concentrations of oxamate were added. After 24 h, the cells that transmigrated through the membrane to the lower side of the filter were stained and counted under a microscope. Incorporated dye was extracted with cell detachment buffer, and the absorbance of the released dye was read in a microplate reader at 550 nm.

Flow cytometry for apoptosis. The effect of oxamate on apoptosis was detected by flow cytometry following Annexin V-FITC and PI staining. Briefly, cells were incubated with various concentrations of oxamate for 24 h, washed and resuspended in 0.5 ml PBS, and stained in Annexin V-FITC and PI buffer. Cells were then analyzed by flow cytometry.

Western blotting. Whole-cell extracts were prepared from the treated cells with 2 ml of RIPA buffer containing protease

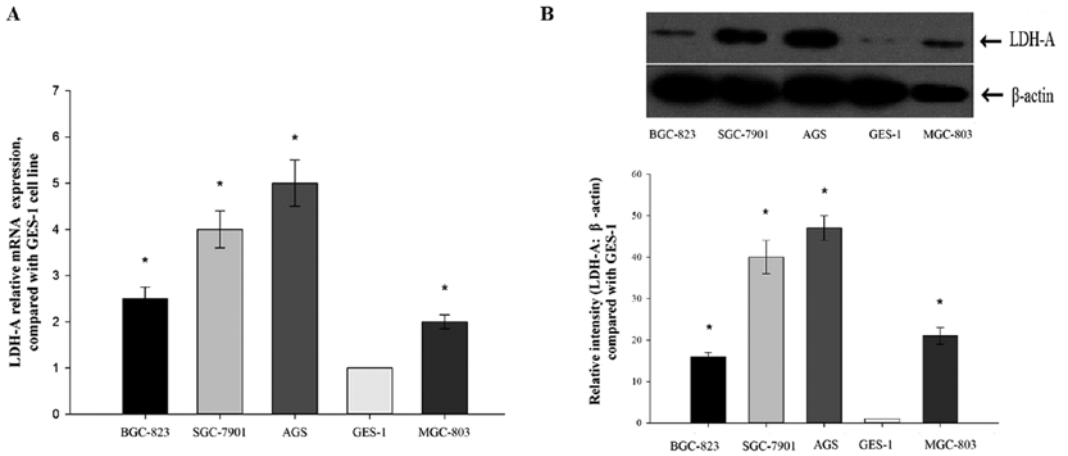

Figure 1. Basal expression of LDH-A in GC cells and a normal gastric endothelial cell line. The expression of LDH-A was examined at the mRNA and protein levels by (A) qPCR and (B) western blotting, respectively. Increased expression was found in GC cells compared with that noted in the GES-1 cells. Among the four GC cell lines, AGS showed the highest expression level of LDH-A at both the mRNA and protein levels. $^{*}p<0.05$; $^{**}p<0.01$. All comparisons were made between treated and untreated cells. LDH-A, lactate dehydrogenase A; GC, gastric cancer.

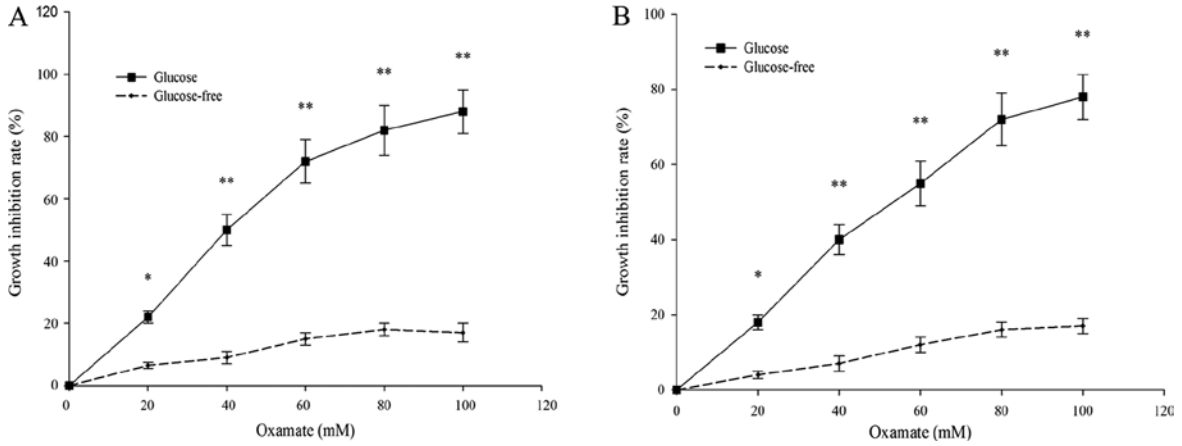

Figure 2. Oxamate inhibits proliferation of GC cells only under a glucose-containing condition. (A) AGS and (B) SGC-7901 cells were treated with various concentrations of oxamate for 24 h. In the presence of glucose, oxamate inhibited cell proliferation in a dose-dependent manner. In contrast, in the absence of glucose, oxamate barely suppressed the growth of GC cells (only by 0-18%). $^{*}p<0.05$; $^{**}p<0.01$. All comparisons were made between treated and untreated cells. GC, gastric cancer.

inhibitors. Cell lysates were centrifuged at 8,000 rpm for 10 min, and the total protein concentrations in the supernatant were determined. An equal amount of total protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% fat-free milk at room temperature for 1 h, incubated with the respective primary antibodies against LDH-A and the apoptosis-associated protein, followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies (all diluted at 1:1,000) for 2 h at room temperature. β-actin was used as a loading control. The membranes were detected using an enhanced chemiluminescence (ECL) system.

Statistical analysis. All experiments were repeated for three times. Data are presented as means ± standard deviation (SD). Statistical comparisons were performed using analysis of variance (ANOVA) with a *post-hoc* test. A p-value <0.05 was considered to indicate a statistically significant result. All data were analyzed using SPSS software (version 11.0).

Results

Increased expression of LDH-A in GC cells. Initially, LDH-A mRNA expression was examined in four GC cell lines (AGS, BGC-823, MGC-803 and SGC-7901) and a normal gastric mucosal cell line (GES-1) by qPCR and western blotting. As shown in Fig. 1, a significantly increased expression of LDH-A mRNA (Fig. 1A) and protein (Fig. 1B) were found in the GC cells compared with these levels in the GES-1 cells, with the highest expression level of LDH-A being found in AGS and SGC-7901 cells. Thus, most of the following experiments were performed in these two cell lines.

Inhibition of LDH-A by oxamate reduces the proliferation of GC cells. To test the effect of LDH-A blockade on cell proliferation, AGS and SGC-7901 cells were treated for 24 h with various concentrations of oxamate in the presence or absence of glucose. As shown in Fig. 2, in the presence of glucose, oxamate inhibited cell proliferation in a dose-dependent manner. The IC_{50} value of oxamate for AGS cells was

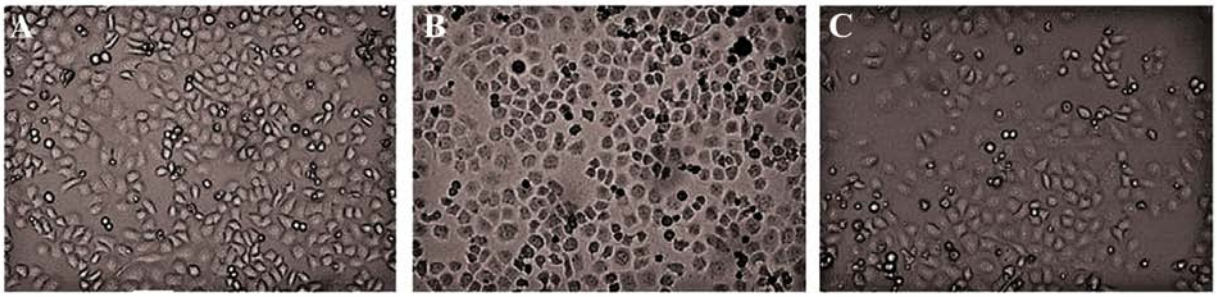

Figure 3. Effect of oxamate on the morphology of SGC-7901 cells. (A) In the absence of oxamate, SGC-7901 cells showed a round-shape. (B) Following treatment with 40 mM of oxamate, some SGC-7901 cells detached from the culture plate. (C) Treatment with a low dose of oxamate (20 mM) only resulted in a slight reduction in cell number without apparent morphological changes. Magnification, x200.

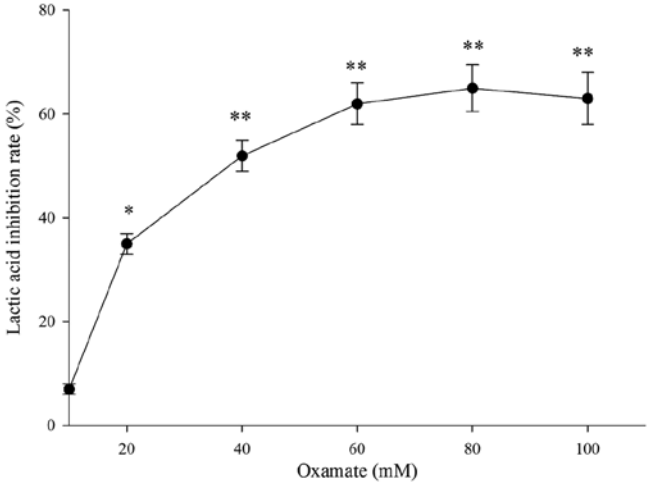

Figure 4. Oxamate inhibits lactic acid production and glycolysis in SGC-7901 cells. Treatment of SGC-7901 cells with 40 mM of oxamate for 4 h reduced the production of lactic acid by 50%. *p<0.05; **p<0.01.

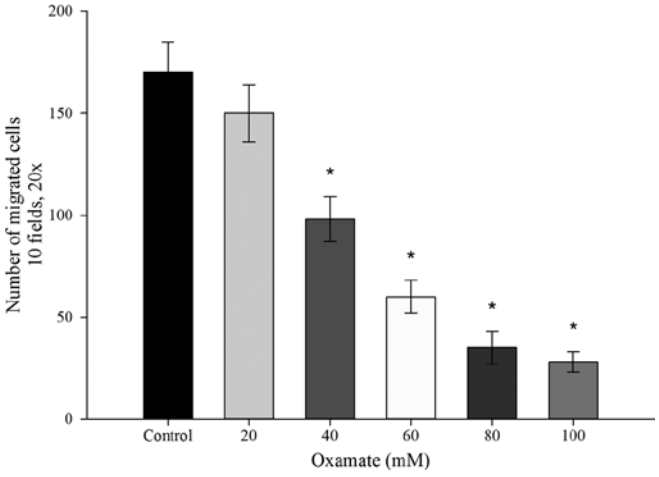

Figure 5. Oxamate inhibits the invasiveness of SGC-7901 cells. Treatment of SGC-7901 cells with 40 mM of oxamate for 24 h resulted in a 50% inhibition of the cell migration. *p<0.05; **p<0.01. All comparisons were made between treated and untreated cells.

38.11 mM (r=0.9990) (Fig. 2A), and 49.26 mM (r=0.9963) for SGC-7901 (Fig. 2B) cells.

Morphological changes in GC cells treated with oxamate. As revealed by phase contrast microscopy, treatment of SGC-7901 cells with 40 mM of oxamate for 24 h led to a significant detachment (Fig. 3B), compared to the control SGC-7901 cells (non-oxamate-exposed cells, Fig. 3A). Cells treated with 20 mM of oxamate only showed a decrease in the cell number without morphological changes (Fig. 3C).

Inhibition of LDH-A by oxamate reduces the production of lactic acid in GC cells. As shown in Fig. 4, treatment of SGC-7901 cells with oxamate led to a reduced production of lactic acid. When exposed to 40 mM (IC_{50} value) of oxamate for 4 h, the production of lactic acid was reduced by 50%. The effective concentration of oxamate to suppress lactic acid production was similar to that previously reported (18,19). Hence, oxamate inhibits glycolysis in SGC-7901 cells.

Inhibition of LDH-A by oxamate reduces the invasiveness of GC cells. Treatment of SGC-7901 cells with 40 mM of oxamate for 24 h resulted in a 50% reduction in cell migration (Fig. 5).

Inhibition of LDH-A by oxamate leads to early apoptosis in GC cells. As revealed by flow cytometric analysis (Fig. 6) treatment of SGC-7901 cells with 40 mM of oxamate caused a marked increase in the proportion of early apoptotic cells, compared to the proportion in the control cells (54.29±7.90 vs. 10.95±2.08%, respectively, p<0.001). The pro-apoptotic effect of oxamate was also demonstrated by concomitant changes in the expression of apoptosis-associated proteins, as detected by western blot assay (Fig. 7). As exemplified in SGC-7901 cells, oxamate significantly decreased Bcl-2 expression, yet enhanced the expression of Bax and caspase-3.

Discussion

In the present study, we demonstrated an increased expression of LDH-A in GC cells. Using its specific inhibitor oxamate, we further demonstrated that inhibition of LDH-A in GC cells led to reduced aerobic glycolysis, as shown by the reduced production of lactic acid, an important end-product of glycolysis. These changes were associated with several biological consequences, including altered cell morphology, reduced proliferative and migratory potentials, and enhanced apoptosis.

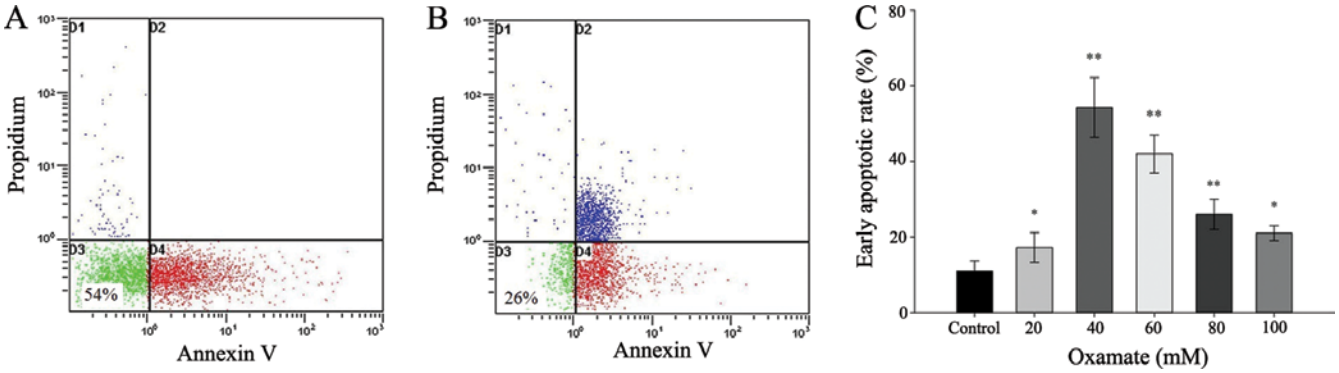

Figure 6. Oxamate-induced apoptosis of SGC-7901 cells. (A) As analyzed by flow cytometry, treatment of SGC-7901 cells with 40 mM of oxamate resulted in early apoptosis. (B) When the dose was increased to 80 mM, oxamate induced late apoptosis/necrosis. (C) Histographical presentation of the effect of oxamate on early apoptosis. When the cells were treated with 20 and 40 mM of oxamate for 24 h, 17.26±3.90 and 54.29±7.90% of the cells, respectively, became early apoptotic. In contrast, only 10.95±2.08% of cells in the control group were apoptotic. *p<0.05; **p<0.01. All comparisons were made between treated and untreated cells.

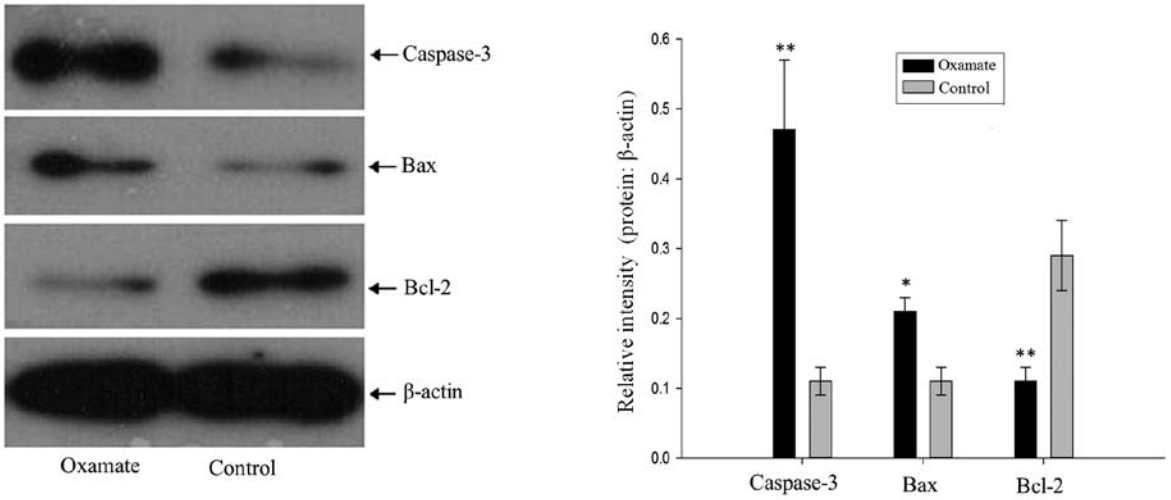

Figure 7. Expression of apoptosis-related proteins in the SGC-7901 cells by western blotting. Treatment of SGC-7901 cells with 40 mM oxamate decreased the expression of Bcl-2, and increased the expression of Bax and caspase-3. The densitometric analysis is shown in the right panel. *p<0.05; **p<0.01. All comparisons were made between treated and untreated cells.

The metabolic properties of tumor cells differ significantly from those of normal cells (20). As early as the 1920's, it was demonstrated that tumors produce ATP via the glycolytic pathway, even in the presence of abundant oxygen, a phenomenon known as the 'Warburg effect' (21). Studies over the recent years have concluded that altered metabolism is one of the most important hallmarks of tumor cells (5). The major metabolic changes in tumor metabolism include high rates of glucose uptake, production of large amounts of lactate, and enhanced aerobic glycolysis (6,22). In the absence of glucose, the tumor cells show little aerobic glycolysis, and their survival and growth are almost exclusively dependent on glutamine oxidation (23).

As such, targeting the glycolytic pathway has become a promising approach for cancer therapy over the recent years (24,25). In particular, key metabolic regulators responsible for the energy supply for cancer cells could prove to be potential therapeutic targets, such as LDH-A, hexokinase-2, pyruvate kinase and glucose transporters (26-29).

Since LDH-A is an essential regulator for the production of lactate, inhibition of LDH-A targets the key point of aerobic glycolysis. LDH-A was initially found to be important for human Burkitt lymphoma clonogenicity (30,31) yet later it was shown to be essential for tumorigenesis (23,32-35). On the other hand, blocking LDH-A activity by its specific inhibitor oxamate or siRNA has been found to inhibit cell proliferation, induce G2/M cell cycle arrest and apoptosis, and sensitize tumor cells to ionizing radiation in several cancer types such as nasopharyngeal and GCs (36). In gastrointestinal cancer cells, reduced LDH-A activity led to reduced tumorigenicity.

Of more clinical importance, oxamate shows cytotoxic effects only on tumor cells while the normal cells are largely spared. In the present study, oxamate had little effect on the growth of AGS and SGC-7901 cells under glucose-free conditions, indicating that this agent only functions to a small extent in the tricarboxylic acid cycle. Instead, oxamate mainly blocks aerobic glycolysis, an important characteristic of tumor cells.

In conclusion, targeting LDH-A holds great promise in cancer treatment. Further mechanistic studies particularly studies in animal models are currently underway.

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