

Inhibitory effect of N-oxalylglycine on gastric cancer cells

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Abstract

Some surveys have shown the possibility of controlling the growth of cancer cells by decreasing the content of 2OG oxygenase with NOG. This research aims to supply the blank of whether the anti-cancer ability of NOG exists in vitro and xenografted mice and how this ability is formed. Use human gastric cancer cell lines, SGC7901. They have brought NOG, MTT, and cisplatin from different companies separately. Choose 60 male BALB/c Nude mice aged four weeks with an average weight of 15 – 20g as the experimental subject. Measure killing by MTT assay and tumor shrinkage in an SGC-7901 xenograft mouse and 2OG oxygenase by western blot. The possible results are: (1) NOG restrains SGC7901 cells' growth by inhibiting the abundance of 2OG oxygenase. (2) NOG can restrain the growth of SGC7901 cells, but the principle differs from inhibiting the abundance of 2OG oxygenase. (3) NOG cannot restrain the growth of SGC7901 cells.

Keywords: gastric cancer, N-oxalylglycine (NOG), 2-oxoglutarate (2OG) oxygenase, orthotopic xenograft, cell culture, western blot

1. Introduction

Gastric cancer (GC) is risky cancer, the third most common cause of cancer death globally. Western treatment of advanced GC usually uses medicines like platinum, fluoropyrimidine doublet, and monoclonal antibodies[1]. For early GC, doctors prefer taking operations. However, considering the large number of side effects that may occur after surgery and the main population of GC is elder, treatments like surgery seem to be too drastic. Medical treatment on GC may need more attention. Traditional Chinese Medicine (TCM) could be a suitable treatment for its conservative characteristics. But there is less than enough research about TCM.

Rheum rhabarbarum (rhubarb, also named Rhei or Dahuang) is a classical type of TCM. Also, there is a record that it was used as a medicine in ancient Europe and Arabia. Rhubarb has played the role of laxative for about 5000 years. Chinese herbalists insist rhubarb can “qin re xie huo” (清热泻火, vent the redundant heat) and “huo xue zhu yu”(活血逐瘀, make blood flow normally). In previous research, it has been proved that Rhubarb has main pharmacological activities including antitumor[2], regulation of gastrointestinal flora[3], protection of the intestinal mucosal barrier[4,5], anti-inflammatory[6], and inhibition of fibrosis[7]. Most of the research on rhubarb has focused on two chemical components emodin and sennoside which inhibit fibrosis, and purgation yet.

These years, a new active substance called N-oxalylglycine (NOG, Figure 1) was found in rhubarb. In addition, N-oxalylglycine is also found to exist in spinach leaves[8].

NOG is a competitive broad-spectrum 2-oxoglutarate (2OG) oxygenase inhibitor. 2OG oxygenase catalyzes 2OG into CO₂ and succinate with Fe (II) as a cofactor[9]. According to existing research, the content of 2OG oxygenase increases in cancer cells[10]. Moreover, Fe(II)/2OG-dependent oxygenase has diverse biochemical roles in the regulation/control of transcription, chromatin protein modification, chromatin DNA modification, etc. [11]. These all show the possibility of controlling the growth of cancer cells by decreasing the content of 2OG oxygenase. However, there is no evidence to certify that the abundance of 2OG oxygenase in a certain amount of rhubarb is enough for treatment.

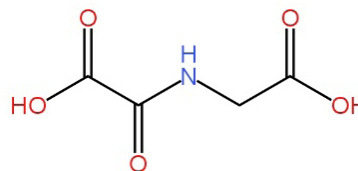


Figure 1.N-oxalylglycine[12] (Molecular mass: 147.086g/mol)

There are two possibilities for NOG being anti-tumor. The first one is, by competing with 2OG to be combined with 2OG oxygenase, NOG can restrain the oxidation of 2OG. Since the progress of 2OG being oxidated into succinic acid is part of the TCA cycle, the decreasing of active 2OG oxygenase will dampen the whole TCA cycle. Furthermore, the rate of cellular respiration in cancer cells will reduce, which means less ATP can be produced. ATP takes part in most biochemical effects in cells. The reduction of ATP causes the rate of effects that need ATP, including those that decide the growth of cells, decreases.

Another possibility for NOG being anti-tumor is very complex and vague. Because the types of 2OG oxygenase are diverse. Moreover, almost all these different 2OG oxygenases are relevant to a different part of the central dogma. Although there has been massive research on the relationship between 2OG oxygenase and the central dogma[11], it still cannot be declared which part takes the most important role. Also, some of the researches noticed above focuses on microorganisms. There is no evidence to explain if the results of these researches still work in GC cells.

The meaning of the research is to supply the blank in NOG treatment on GC. In addition, testing the suitable content of NOG for treating GC may contribute to certifying if the NOG in a certain amount of rhubarb is enough for GC treatment. If the content of NOG in rhubarb is enough for GC treatment, changing the content or ratio may promote many Chinese potions. In addition, this research may be useful for surveying how NOG works as an anti-tumor medicine in vivo.

1.1 Hypothesis

I predict that increasing concentrations and increasing durations of treatment with NOG can kill SGC-7901 gastric cancer cells by decreasing the 2OG oxygenase. Measure killing by MTT assay and tumor shrinkage in an SGC-7901 xenograft mouse and 2OG oxygenase by western blot. The positive control is cisplatin, and the negative control is PBS/DMSO.

2. Materials

2.1 Cells Culture

Use human gastric cancer cell lines, SGC7901, purchased from ATCC. SGC7901 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). The culture medium was changed three times per week. The cell was kept at 5% CO₂ and 90% O₂ concentrations. The temperature was held around 37°C. Differed the cells which were cultured for two weeks into six different groups. Every group had the same GC cells in mass, type, and number.

2.2. N-oxalylglycine

The NOG was brought from APExBIO (Apexbio, a company). Coupling of activated oxalic acid and glycine is the way to synthesize naturally. It should be stored at -20°C and be kept dry. Heated NOG in the tube at 37 °C and shook for 5min in an ultrasonic bath. Made the NOG solution with water as a solvent separately in 1μM, 10μM, 100μM, and 1000μM concentrations. Divided them into 4 different groups including NOG of different concentrations and saline mixed solution.

2.3. Cisplatin

Cisplatin (CDDP) was bought from MCE (MedChem-Express). The anti-tumor activity of cisplatin is generally from its formation of DNA adducts, which induce structural DNA distortion. Made a solution of 50μM CDDP concentration. Took 10ml saline and CDDP mixed solution as a positive group.

2.4. MTT solution

MTT (Thiazolyl Blue) was brought from MERCK (a company). The application solution is configured with PBS to a final 5 mg/ml concentration.

2.5. Mice

Choose 60 male BALB/c Nude mice aged 4 weeks with an average weight of 15 – 20g as the experimental subject. Mice were fed with a routine rodent diet and water ad libitum. The mice were housed in a sterile environment and allowed to acclimatize for 15 days before any experiments. All experimental animals were cared for by the guidelines of institutional authorities.

3. Methods

3.1 MTT assay

SGC7901 cells were digested by trypsinization, digestion of serum was aborted, and pipette until cells were in suspension. SGC7901 cell suspension was centrifuged at 1000 rpm for 5 min, the supernatant was discarded, and the pellet was resuspended to make a cell suspension. Adjusted the SGC7901 cell count to 5×10^4 /ml and implanted SGC7901 cells in 96-well cell cultured plates. SGC7901 gastric cancer cells cultured for 2 weeks with the same growth and biological activity were divided into 6 groups named A, B, C, D, E, and F. Each experimental group has a column (6 complex wells) with 100ul cell suspension added to each well. The CO₂ concentration in the environment was maintained at 5% and the oxygen concentration at 90%. Cultured SGC7901 cells to adherence. Groups A, B, C, and D were added 100μl of NOG and PBS solution at 1 μM, 10 μM, 100 μM, and 1000 μM. 100μl of 50 μM cisplatin mixed with PBS/DMSO was added to group E and 100μl of PBS/DMSO was added to group F. Half of each group (chosen at random) was treated for 48h, and the other half was treated for 96h. Added 10~20μl MTT solution (5mg/ml MTT) to each well, and continued to culture for 5h. Aspirated the supernatant, added 150μl DMSO per well and shook at low speed in a shaker for 10 min to fully dissolve the crystals. The ELISA measured the absorbance of each well at OD490 nm and OD570 nm respectively.

3.2 CDX (Cell Derived Xenograft)

Cryopreserved SGC7901 cells were removed from liquid nitrogen, quickly placed in a 37°C water bath, rapidly thawed, added DMEM/High medium containing 10% FBS, seeded in a T25 flask, and passaged in a 37°C, 5% CO₂ incubator. Discarded the old culture medium in the culture flask, rinsed twice with sterile PBS, added trypsin-EDTA 1 ml to the culture flask to digest the cells, observed under the inverted phase contrast microscope, poured out the pancreatic enzyme when the cells appear screen-like voids under the microscope, added DMEM/High medium containing 10% fetal bovine serum to cover the bottom of the flask, pipetting to suspend the cells, transferred the cell suspension into a centrifuge tube for 1000r/min centrifugation for 5min, discarded the supernatant, and passage according to 1:4. Culture in a 37°C, 5% CO₂ incubator, changed the solution for 2~3 days, and select the cells of the logarithmic stage for the experiment. Added sterile PBS, gently pipetted the cell pellet, mixed into a cell suspension, and counted the cells to achieve a cell density of 1×10^7 pcs/ml. In an ultra-clean stage, disinfected the back of the nude mouse with 75% alcohol, and inject 0.1 ml of cell suspension subcutaneously. Divided these mice into 6 groups named A, B, C, D, E, and F. Groups A, B, C, and D were injected with 10ml of NOG and PBS solution at 1 μ M, 10 μ M, 100 μ M, and 1000 μ M. 10ml of 50 μ M cisplatin mixed with PBS/DMSO was injected into group E and 10ml of PBS/DMSO was injected into Group F. Half of each group (chosen at random) was administered for 48h, and the other half was administered for 96h. After 21 days,

measured and compared the mass of tumors.

3.3 Western Blot

Isolated gastric cancer cell tissue obtained from MTT and CDX with pre-cooled tools and placed on ice as much as possible to prevent 2OG oxygenase hydrolysis. Placed the GC cells tissue block in an EP tube with a round bottom, poured liquid nitrogen to freeze the tissue, and ground it homogeneously on ice. Added protease and phosphatase inhibitors to GC cell tissue. Added about 200 μ l of pre-cooled lysate per 10 mg of tissue, homogenized in an ice bath, and placed at 4 °C with shaking for 2h. Centrifuged at 12,000 rpm at 4°C for 20 min, gently pipetted the supernatant, and transferred to a freshly pre-cooled microcentrifuge tube. Then, added protease inhibitor. The concentration of the 2OG oxygenase content in the resulting supernatant was quantified using BCA Protein Assay Kit to make sure equal loading of samples. Electrophoresed 2OG oxygenase through sodium dodecyl sulfate/polyacrylamide gel (SDS-PAGE). Then transferred it to a polyvinylidene difluoride (PVDF) membrane. After incubating for 1h with Tris-buffered saline and Tween-20 including 5% BSA solution to block non-specific binding, rinsed the membrane and stirred with the first antibody at 4°C until the next day. TBST was washed 3 times for 5 min each and incubated with HRP-labeled secondary antibody for 1h at 25°C. After 3 washes with TBST for 10 min each, the strips were visualized by the ECL system[13]. Analyzed the data got from the results. (see Table 1).

Table 1. Combination of Possible Results (PR)

		Decrease the growth of GC cells in vitro?	Decrease the growth of GC cells in vivo?	Decrease the abundance of 2OG oxygenase?	Support Hypothesis?
Possible Results (PR)	1	+	+	+	Yes
	2	+	+	-	P
	3	+	-	+	P
	4	-	+	+	p
	5	+	-	-	P
	6	-	+	-	p
	7	-	-	+	P
	8	-	-	-	NO

Note: “+” represents the result of the group being more efficient than the negative control on retraining GC cells’ growth, “-” represents the result is less efficient or similar to the negative control, and “P” represents that the hypothesis can be partially proved.

PR1: This PR1 was the most optimistic result. The experimental groups' results of the MTT assay claimed that the final value of OD (absorbance) did fall below about 0.8~1.2, which meant that increasing the treating time and the concentration of the NOG could decrease the growth of SGC7901 in vitro. The tumors were shrinking when the mouse was treated with a higher concentration of the NOG or for a longer time. It explained the effectiveness of the NOG in vivo. Also, on the western blot, the band of 2OG oxygenase was lighter for the NOG-treated groups than for the negative control group. The decrease of active 2OG oxygenase in experimental groups indicated the method of the NOG being anti-tumor was possibly like the hypothesis.

PR2: The experimental groups' results of the MTT assay claimed that the final value of OD did fall below about 0.8~1.2, which meant that increasing the treating time and the concentration of the NOG could decrease the growth of SGC7901 in vitro. The tumors were shrinking when the mouse was treated with a higher concentration of NOG. It explained the effectiveness of the NOG in vivo. But the band of 2OG oxygenase was thicker for the NOG-treated groups than for the negative control group. Or the bands of the NOG-treated groups, the positive control groups, and the negative control groups had little difference. There was no or little difference between the experimental groups and the controlled groups, which means the NOG may be anti-tumor in a way different from the hypothesis. With NOG-treated time getting longer, the SGC7901 cells' growth was restrained more completely and tumors in mice became smaller. Same as the variable, concentration of NOG, it cannot prove the method of NOG working.

PR3: The experimental groups' results of the MTT assay claimed that OD's final value fell below about 0.8~1.2, which meant that increasing the treating time and the concentration of the NOG could decrease the growth of SGC7901 in vitro. But there was not much difference between the tumors when the mice were treated with a higher concentration of the NOG. It could not partially explain the effectiveness of the NOG in vivo. Also, on the western blot, the band of 2OG oxygenase was lighter for the NOG-treated groups than for the negative control group which indicated the method of the NOG being anti-tumor may be like the hypothesis.

The treatment time with NOG played a role in vitro environment, but there was no difference between negative control groups and experimental groups in vivo. In addition, the band of 2OG oxygenase was lighter for the NOG-treated groups than for the negative control group.

PR4: The experimental groups' results of the MTT assay claimed that the final value of OD did not fall

below about 0.8~1.2, which meant that increasing the treating time and the concentration of the NOG could make no or little difference for the growth of SGC7901 in vitro. The tumors were shrinking when the mouse was treated with a higher concentration of NOG. It explained the effectiveness of the NOG in vivo. Also, on the western blot, the band of 2OG oxygenase was lighter for the NOG-treated groups than for the negative control group. The shrinkage of active 2OG oxygenase happened in experimental groups. The treatment time with NOG played a role in vivo environment, but there was no difference between negative control groups and experimental groups in vitro. In addition, the band of 2OG oxygenase was lighter for the NOG-treated groups than for the negative control group.

PR5: The experimental groups' results of the MTT assay claimed that OD's final value fell below about 0.8~1.2, which meant that increasing the concentration of the NOG could decrease the growth of SGC7901 in vitro. But there was not much difference between the tumors when the mice were treated with a higher concentration of the NOG. It could not partially explain the effectiveness of the NOG in vivo. Also, the band of 2OG oxygenase was thicker for the NOG-treated groups than for the negative control group. Or the bands of the NOG-treated groups, the positive control groups, and the negative control groups had little difference. There was no or little difference in the abundance of the 2OG oxygenase between the experimental groups and the control groups. Increasing NOG-treated time, the SGC7901 cells were controlled. However, there was no diversity between negative control groups and experimental groups in vivo. Moreover, the working method of NOG cannot be explained by this experiment.

PR6: No evidence that increasing the treating time and the concentration of the NOG could decrease the growth of SGC7901 in vitro for falling beyond about 0.8~1.2 of the OD value. But the tumors were shrinking when the mouse was treated with a higher concentration of the NOG. The band of 2OG oxygenase was thicker for the NOG-treated groups than for the negative control group. Or the bands of the NOG-treated groups, the positive control groups, and the negative control groups had little difference. There was no or little difference in the abundance of the 2OG oxygenase between the experimental groups and the controlled groups. Increasing NOG-treated time, the growth of tumors in mice was controlled. However, there was no diversity between negative control groups and experimental groups in vitro. Moreover, the working method of NOG cannot be explained by this experiment.

PR7: No evidence that increasing the concentration of the NOG could decrease the growth of SGC7901 in vitro for

falling beyond about 0.8~1.2 of the OD value. Also, there was not much difference between the tumors when the mice were treated with a higher concentration of NOG. But on the western blot, the band of 2OG oxygenase was lighter for the NOG-treated groups than for the negative control group. The shrinkage of active 2OG oxygenase happened in experimental groups. When the treatment time of NOG got longer, there was not an obvious difference between the negative groups and experimental groups, or even the growth of GC cells of the negative groups was worse than experimental groups. But the 2OG oxygenase's abundance did shrink.

PR8: PR8 was the most negative result. There was no evidence that increasing the treating time and the concentration of the NOG could decrease the growth of SGC7901 in vitro for falling beyond about 0.8~1.2 of the OD value. Also, there was not much difference between the tumors when the mice were treated with a higher concentration of the NOG or for a longer time. In addition, the result of Western Blotting explained that there was no or little difference in the abundance of the 2OG oxygenase between the experimental groups and the controlled groups. These results possibly meant the hypothesis was wrong.

4. Discussion

In the PR1, it is proved that the hypothesis is true, NOG is anti-cancer by changing the abundance of 2OG oxygenase. But further research about the proper dosage for patients and proper medication cycle is needed for making a personal treatment plan. Also, the efficient abundance of NOG should be measured to discuss how much rhubarb will make an obvious effect on cancer treatment.

PR2, PR5, and PR6 all showed the anti-cancer ability of NOG completely or partially. However, the 2OG oxygenase's abundance did not change which indicated the working pathway of NOG in the hypothesis is wrong. The practical method may be concerned with the oncogene's expression. Through controlling the division of cancer cells, NOG may restrain the growth of tumors. So, further research should keep eye on the practical working method of NOG in the human body.

During the comparison of PR3 and PR4, PR5 and PR6, it is found there is a difference between in vivo and in vitro environments. Especially on the aspect of NOG's delivery, there may be some proteins that can be combined with NOG. Moreover, the concentration of NOG in vivo may be too low to attract an effective result. So, further research should survey the reason why NOG's effects are different in two environments. In PR8, the hypothesis is proved to be false. But there is still a possibility that

SGC7901 cells may have resistance to NOG. Comparing the results of treating other cancer cells by NOG may explain this question. Also, the time of treating cancer cells might be the reason why PR8 happened. Repeating this experiment in the future with a longer treatment time may figure the real cause out.

5. Conclusion

In general, this research is meant to reveal the ability of the NOG to be anti-tumor and anti-cancer in vitro and in xenografted mice. The results of the research can indicate whether the treatment with NOG is able to restrain the proliferation and metastasis of gastric cancer in vitro and in vivo and whether this inhibition is mediated by a change in the abundance of 2OG oxygenase. It may fill the gap of the NOG's effect in the medical field. Also, through a similar way to this research, it is possible to measure the capable content in the medical treatment of NOG. To take a step further, this research can help to adjust the medical value of rhubarb by comparing the natural content of NOG in rhubarb. In addition, this research may give aids in surveying other possible principles of how NOG works as an anti-cancer medicine in the human body.

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