Original Research Article

Alantolactone exerts anti-proliferative and apoptotic effects on BGC823 and SGC7901 cells via activation of p38MAPK and inhibition of NF-κB signaling pathway

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Abstract

Purpose: To investigate the anti-proliferative and apoptotic influences of alantolactone on gastric carcinoma (GC) cell lines, and the mechanism(s) involved.
Methods: Human gastric cancer cell line (BGC823) and gastric adenocarcinoma lymph node metastasis cell line (SGC7901) were maintained in Ham's F12 medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS). In each group of cancer cell line, 5 groups of cells were used: control and four alantolactone groups which were treated with increasing concentrations of alantolactone (5 - 30 µM) for varying periods. Proliferation was determined using MTT assay, while real-time quantitative polymerase chain reaction (qRT-PCR) was used to assay the expressions of apoptosis- and metastasis-related genes. The expressions of p38MAPK and nuclear transcription factor-κB (NF-κB) in BGC823 and SGC7901 cells were measured with Western blotting.
Results: Phosphorylated protein (p-p38 protein) expression was significantly higher in both groups of GC cells, relative to control (p < 0.05). The expressions of NF-κB in plasma protein were markedly higher in both groups of GC cells than in control group, but the corresponding expressions in nuclear protein were significantly lower in both groups of GC cells, relative to control (p < 0.05).
Conclusion: Alantolactone exerts anti-proliferative and apoptotic effects on BGC823 and SGC7901 cells via mechanisms involving activation of the p38MAPK, and inhibition of the NF-κB signaling pathways. Thus, alantolactone may be a new and effective anti-gastric cancer drug.

Keywords: Gastric cancer, Alantolactone, Proliferation, Metastasis, Apoptosis

INTRODUCTION

Gastric cancer (GC), a malignant tumor of the gastric mucosa is characterized by high incidence and mortality. In China, the incidence of GC increases on yearly basis due to aging of the population, changing lifestyles, and *Helicobacter pylori* infection [1]. Clinical studies have shown that lymphatic metastasis of advanced GC is as high as 70 %, and proliferation and metastasis of the cells are the main factors responsible for postoperative recurrence of GC [2].

An understanding of the metastasis and apoptosis of GC cells is important in achieving improved prognosis. Alantolactone, a sesquiterpene lactone isolated from *Inula helenium* L., possesses anti-inflammatory, antibacterial and anti-tumor properties, and its anti-tumor effect is particularly prominent [3]. Alantolactone is effective against cancers such as breast cancer, glioma, and multiple myeloma, but studies on its anti-GC activity and possible mechanism of action are scanty [4-6].

The present study was aimed at investigating the anti-proliferative and apoptotic influences of alantolactone on GC cell lines, and the underlying mechanism(s).

EXPERIMENTAL

Materials and chemicals

The BGC823 and SGC7901 cell lines were obtained from China Medical University; 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) assay kit was purchased from Guangzhou Baiyunshan Pharmaceutical Co., Ltd., while alantolactone was product of Nanjing Jingzhu Biotechnology Co. Ltd. Dimethylsulfoxide (DMSO) was obtained from Tianjin Saifurui Technology Co. Ltd.; ethanol was purchased from Shanghai Youyu Biotechnology Co., Ltd., while Bax, Bcl-2, p53, matrix metallopeptidases 2, 7 and 9 primers were products of Shanghai Jingjing Reagent Co., Ltd. Acrylamide was purchased from Shanghai Yanjin Biotechnology Co. Ltd; ammonium persulfate and bicinchoninic acid (BCA) protein kit were products of Beijing Zhongke Ruitai Biotechnology Co. Ltd, while ECL chemiluminescence liquid was obtained from Beijing Meikewande Biotechnology Co., Ltd. Phosphate buffered saline (PBS) was purchased from SGM Biotech and RIPA protein lysis kit was obtained from Shanghai Spark Crystal Molecular Biotechnology Co. Ltd.

Cell lines and culture

The BGC823 and SGC7901 cells were maintained in Ham's F12 medium supplemented with 10 % heat-inactivated FBS in a humidified incubator at 37 ^{o}C and 5 % CO_2 atmosphere. Cells in exponential growth phase were used for this study. In each group of cancer cell line, the cells were divided randomly into five groups: control group and four alantolactone groups which were treated with increasing concentration of alantolactone (5 - 30 µM) for varying periods.

MTT assay

Cell proliferation was determined using MTT assay. The cells (1 × 10^5 cells/mL) were seeded into 96-well plates and pretreated with varied concentrations of alantolactone (5 - 30 µM). This was followed by the addition of 20 mL of 0.5 % MTT solution within 4 h, after which the culture medium was changed. Then, DMSO (150 µL) was added in drops to each well, and the wells were placed for 10 on an oscillator for 10 min so as to ensure dissolution of the formazan crystals. The control wells contained culture medium, MTT solution and DMSO only, and were treated same way as the sample wells. Each well was incubated in the dark for 2 h and absorbance was measured at 570 nm using Sunrise enzyme labeling instrument. The procedure was performed in triplicates and cell proliferation was calculated as shown in equation 1.

$$C (\%) = \{(1 - As)/Ac\} 100 \ldots\ldots\ldots (1)$$

where As = absorbance of sample well, and Ac = absorbance of control well.

qRT-PCR

The treated and control cells were lysed using radioimmunoprecipitation assay (RIPA) buffer. Their total RNAs were extracted using Trizol reagent, and reverse-transcribed to cDNAs, which were then quantified using qRT-PCR. The reaction conditions were: pre-denaturation at 95 ^{o}C for 30 s, denaturation at 95 ^{o}C for 5 s, annealing at 60 ^{o}C for 34 s, and 40 cycles. β-Actin gene was used as internal reference.

Western blotting

The expressions of p38MAPK), nuclear transcription factor-κB (NF-κB) in BGC823 and SGC7901 cells treated with alantolactone (20 µM, 24 h) were measured using Western blotting. The supernatant was assayed for protein content with BCA assay. A portion of total tissue protein (20 µg) from each sample was subjected to SDS-PAGE, followed by transfer to fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, 3 % BSA in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at room temperature and incubated to block non-specific binding of the blot. Thereafter, the blot was incubated with the appropriate primary antibodies (1: 500) at 4 °C overnight. Then, the membrane was rinsed thrice with TBS-T and further incubated at room temperature with horseradish peroxidase-conjugated secondary antibody for 2 h. The blot was developed using

an x-ray film. ImageJ software was used for grayscale assessment of the resultant bands. The respective protein expression levels were normalized to that of β-actin which was used as a standard reference.

Statistical analysis

Data are expressed as mean ± SEM, and were statistically analyzed with SPSS (22.0). Group comparisons were carried out with Student *t*-test. Statistical significance was fixed at $p < 0.05$.

RESULTS

Effect of alantolactone on proliferation of GC cells

The proliferations of BGC823 and SGC7901 cells were significantly inhibited in a time- and concentration-dependent manner by alantolactone, relative to control group ($p < 0.05$). These results are shown in Figure 1.
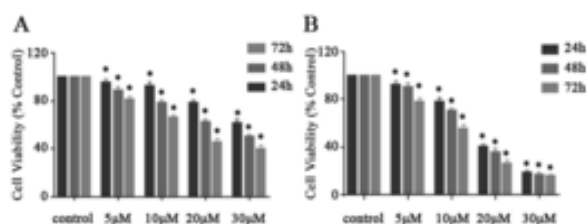

Figure 1: Effect of alantolactone on the proliferation of BGC 823 and SGC 7901 cells. A: BGC823 cells; B: SGC7901 cells. [*]$p < 0.05$, relative to control

Levels of expressions of apoptosis-related genes in GC cells

Figure 2 shows that when compared with control, Bax was expressed markedly higher in BGC823 cells, and its expression increased with time of incubation ($p < 0.05$). In contrast, Bax expression levels in the alantolactone groups and control group were comparable ($p > 0.05$). The expression of Bcl-2 was significantly lower in both groups of GC cells than in control group, and was significantly reduced with time of incubation ($p < 0.05$). The expression of p53 was markedly upregulated in both groups of GC cells, relative to the control group, and was significantly increased with duration of incubation ($p < 0.05$).

Influence of alantolactone on metastasis of GC cells

As shown in Figure 3, the expressions of the matrix metallopeptidases were significantly lower in both groups of GC cells than in control group,
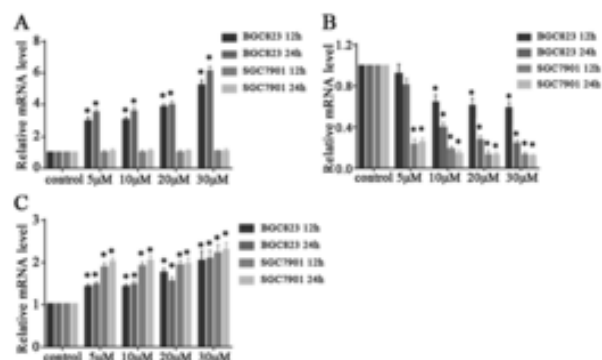
and were significantly reduced with time ($p < 0.05$).

Figure 2: Effect of alantolactone on the expressions of apoptosis-related genes in GC cells. A: Bax; B: Bcl-2; C: p53. [*]$p < 0.05$, relative to control
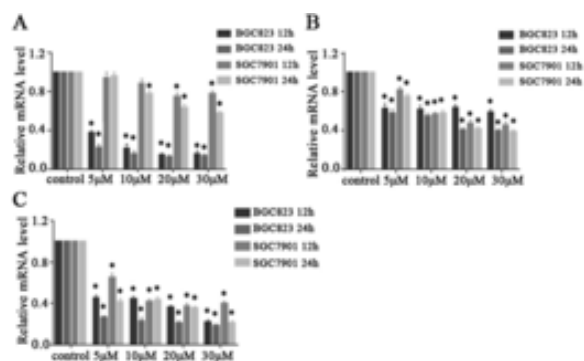

Figure 3: Influence of alantolactone on metastasis of GC cells. A: MMP-2; B: MMP-7; C: MMP-9; [*]$p < 0.05$, relative to control

Expressions of p38MAPK and p-p38 in GC cells

The expression levels of p38 protein in the alantolactone groups and control group were comparable ($p > 0.05$). However, phosphorylated protein (p-p38 protein) expression was significantly upregulated in both groups of GC cells, when compared to control ($p < 0.05$). These results are depicted in Figure 4.

NF-κB expression in GC cells

The NF-κB protein expression was significantly higher in both groups of GC cells than in the control group, but, compared with control, the corresponding expression of NF-κB in nuclear protein was significantly lower in both groups of GC cells ($p < 0.05$) (Figure 5). However, NF-κB protein expressions in both groups of GC cells were comparable with that of control group ($p > 0.05$).
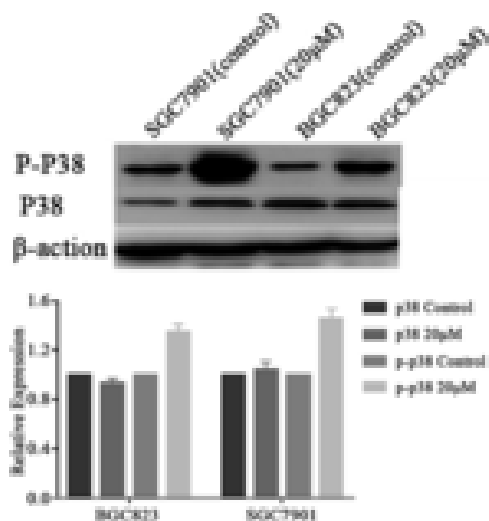
Figure 4: Effect of alantolactone on expressions of p38 protein and p-p38 in GC cells
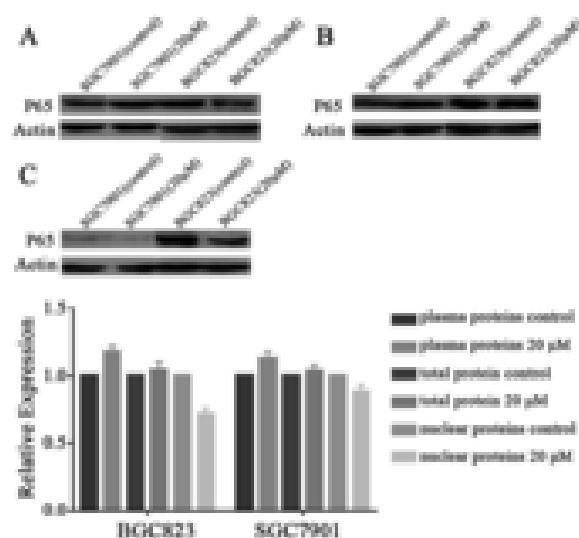

Figure 5: Effect of alantolactone on the expression of NF-κB in GC cells. A: NF-κB expression in plasma proteins of GC cells; B: NF-κB expression in total protein of GC cells; C: NF-κB expression in nuclear proteins of GC cells

DISCUSSION

More than 50 % of GC occur in the antrum of the stomach. It affects the anterior and posterior walls, and small and large curvatures of the stomach. More than 70 % of early GC cases have insidious onset, and as the disease progresses, non-specific symptoms such as upper abdominal fullness, dull pain, pantothenic acid indigestion, nausea and vomiting, and black stool become more prominent [7- 9]. The prognosis of GC is poor due to its complicated pathological mechanism, easy diffusion and metastasis, and single treatment method. Alantolactone is a sesquiterpene lactone isolated from *Inula helenium* L. It possesses biological

activities such as hemodynamic effect, antifungal, anti-tumor, hypoglycemic, and anti-inflammatory properties [10]. *In vitro* studies have shown that alantolactone inhibits the growth of *Mycobacterium tuberculosis* at a concentration of 0.1 μg/mL [11]. It has been reported that high concentration of alantolactone promotes microvascular perfusion in stimulated rat skeletal muscle [12]. It has also been shown that alantolactone inhibits the proliferation of multiple myeloma RPMI-8226 cells and induces their apoptosis by blocking the activity of ERK signal pathway [13]. In a previous study, it was reported that it inhibits the proliferation and metastasis of mammary cancer MDA-MB-231 cells by blocking the activation of STAT3 signal pathway [14]. Research has shown that alantolactone markedly suppresses proliferation of drug-resistant chronic myeloid leukemia cell lines (K562/ADR and K562), and promotes apoptosis of K562/ADR cells via a mechanism involving mitochondrial apoptosis [15]. Alantolactone produces weak inhibitory effect on the expression of tumor testis antigen TSP50 in normal hepatocytes [16].

In the present study, the proliferations of BGC823 and SGC7901 cells were significantly inhibited, relative to control group, and the inhibition increased with time and increase in the concentration of alantolactone. These results suggest that alantolactone may significantly inhibit the proliferation of GC cells in a concentration- and time-dependent manner, and are in agreement with those previously reported [13-16]. It appears that alantolactone exerts anti-GC effect via the inhibition of proliferation and metastasis of the cells.

In this study, Bax expression was markedly upregulated in BGC823 cells, when compared with the control group, and increased with time of incubation. In contrast, Bax expression levels in the alantolactone groups and control group were comparable. The Bcl-2 expression was markedly lower in both GC cell groups than in control group, and the expression was significantly reduced with time of incubation. However, p53 expression was markedly higher in both groups of GC cells than in control group, and was significantly increased with time of incubation. These results suggest that alantolactone may promote apoptosis in GC cells by down-regulating Bcl-2 while simultaneously up-regulating the expressions of Bax and p53 genes. The expressions of the matrix metallopeptidases were significantly lower in both groups of GC cells than in control group, and the expressions were significantly reduced with time. This appears to suggest that alantolactone may inhibit the metastasis of GC

cells by down-regulating the expressions of the three metallopeptidase genes.

It has been reported that p38MAPK is intimately associated with tumor survival and apoptosis, and that it promotes apoptosis in U937 cells by stimulating Bax translocation and enhancing p53 gene expression [17]. In a previous study, it was shown that NF-κB is vital for tumor cell proliferation, metastasis and apoptosis because it controls the expressions of genes for Bax and Bcl-2 [18]. In this study, the results of Western blotting revealed that p38 protein expressions in the alantolactone groups and control group were comparable. However, phosphorylated protein (p-p38 protein) expression was significantly higher in both groups of GC cells than in control group. It is possible that p38MAPK plays an important role in alantolactone-induced apoptosis of GC cells, and alantolactone may promote the phosphorylation of p38MAPK pathway via activation of downstream apoptosis-related genes p53 and Bax.

In this study, NF-κB expression in plasma protein was significantly higher in both groups of GC cells than in control group, but the corresponding expressions of NF-κB in nuclear protein were significantly lower in both groups of GC cells than in control group. However, the expressions of NF-κB in total protein in both groups of GC cells were not significantly different from that of control group. It is possible that alantolactone prevented the activation of NF-κB by inhibiting its migration to the nucleus of GC cells, thereby blocking the expressions of the matrix metallopeptidase mRNAs, and inhibiting GC cell metastasis.

CONCLUSION

Alantolactone exerts anti-proliferative and apoptotic effects on BGC823 and SGC7901 cells via mechanisms involving activation of the p38MAPK pathway and inhibition of the NF-κB signaling pathway. Thus, alantolactone can potentially be developed into a new and effective anti-gastric cancer drug.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

This work was done by the authors named in this manuscript, and the authors accept all liability resulting from claims which relate to this manuscript and its contents. The study was conceived and designed by Xie JunFeng; Tang JianHua, Liu YouShun, Wei YingFeng, Gu QiuPing, Luo QingTian, Zhu FangQin collected and analysed the data; Xie NingSheng wrote the manuscript. All authors read and approved the manuscript prior to publication.

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