Inhibition of the hypoxia-induced factor-1α and vascular endothelial growth factor expression through ginsenoside Rg3 in human gastric cancer cells

ABSTRACT

Objective: The aim of this study is to probe in the inhibitory effects of ginsenoside Rg3 on the expression of hypoxia-induced factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) in human gastric cancer cells.

Materials and Methods: Human gastric cancer BGC823 cells were divided into the control group and experiment group, and expression levels of HIF-1α and VEGF were detected by immunocytochemistry and Western blot after cells were cultured under hypoxia for different durations.

Results: Under hypoxia, expression of HIF-1α and VEGF in human gastric cancer BGC823 cells showed an increasing trend, and that was remarkably lower in experiment group than in the control group after applying Rg3, which was obvious at 12 and 24 h (P < 0.05).

Conclusion: Rg3 can inhibit expression of HIF-1α and VEGF in human gastric cancer cells and may influence abdominal implantation metastasis of gastric cancer through inhibiting its expression.

KEY WORDS: Ginsenoside Rg3, human gastric cancer cell, hypoxia-induced factor-1α, vascular endothelial growth factor

INTRODUCTION

Gastric cancer is one of the most common causes of cancer-related deaths in the world with high rates of metastasis and recurrence. Tumor metastasis is a multi-step complicated process involving multiple factors, and changes in tumor microenvironment (including extracellular matrix, growth factor, chemokine, and matrix metalloprotein, etc.) play an important role in tumor cell metastasis. It is indicated in research that hypoxia can promote persistently high expression of hypoxia-induced factor-1α (HIF-1α) in tumor cells, while HIF-1α can promote the overexpression of vascular endothelial growth factor (VEGF), thus enhancing proliferation, migration, and vascular engineering of vascular endothelial cells, as well as influencing blood flow volume.

Ginsenoside Rg3 is one of the most active ingredients extracted from Ginseng, a traditional Chinese medicine. In recent years, it has been used extensively in the research field and clinical use of cancer treatment. Accumulating findings suggest that Rg3 can inhibit cancer cell growth, invasion, and metastasis, it also exhibits an anti-cancer effect in various tumors, such as lung cancer, hepatocellular carcinoma, breast cancer, colorectal cancer, ovarian cancer, and bladder cancer. Preclinical studies have indicated that Rg3 inhibits tumor growth and angiogenesis through downregulating VEGF expression and targeting hypoxia-induced multiple signaling pathways and is also able to induce apoptosis in cancer cells.

This article focused on probing into the problem that whether Rg3 could inhibit HIF-1α and VEGF in human gastric cancer cells under hypoxia, to provide the foundation for the point that Rg3 could inhibit intra-abdominal implantation metastasis of gastric cancer.
MATERIALS AND METHODS

Reagents
Ginsenoside Rg3 was purchased from Sigma and dissolved in distilled water at a concentration of 100 mmol/L. Rabbit polyclonal antibody to human HIF-1α and VEGF were bought from Proteintech; mouse anti-rabbit β-actin antibody was purchased from Elabscience; goat anti-rabbit IgG secondary antibody kit and DAB developing kit were obtained from Beyotime Biotech, China; RPMI1640 medium was purchased from HyClone; trypsin-EDTA and penicillin-streptomycin were provided by Solarbio; fetal bovine serum was provided by Gibico; and total protein extraction kit was purchased from Omega.

Cell culture
Human gastric cancer cell line BGC823 was purchased from the tumor cell bank of Beijing Jinzijing Company. The cells were cultured in complete medium in 5% CO2 incubator at 37°C, and the passage was conducted every 3 days. Cells at the exponential phase were collected, counted, and transferred to a 60 mm culture dish to continue the culture under normoxia for 24 h. Cells were divided into control group and experiment group after stable cell adherence. PBS and Rg3 were added (with the final concentration of 100 μmol/ml), and cells were cultured in a hypoxic incubator (at 37°C, 1% CO2 and 5% CO2) for 2, 6, 12, and 24 h. Total proteins at different time points were reserved for subsequent use. Cells were grown on a 24-well plate, and the time and drug concentration were the same as above.

Immunocytochemistry
Cells plated on coverslips were fixed in 4% paraformaldehyde, incubated with Triton-10 for 20 min and with hydrogen peroxide for 15 min, blocked with serum working solution for 20 min, and incubated with primary antibody at 4°C overnight. The primary antibody was replaced by PBS as the negative control, and cells were incubated with secondary antibody for 30 min, followed by DAB developing, hematoxylin counterstaining, and mounting. HIF-1α expression located in the nucleus and cytoplasm, while VEGF expression was localized in cytoplasm and cell membrane, and distribution characteristic of positive cells was observed. Image-Pro Plus image automatic analysis system was employed; blank space calibration, together with five representative fields of view was selected under magnification of ×40; optical density (OD) values were determined for calculation, and the mean OD value of the selected fields of view was thus obtained.

Western blot analysis
The total protein extraction kit was adopted, and lysis buffer was prepared 30 min before extraction and was placed at 4°C for 5 min, followed by 30 s of fierce oscillation for five loops. Cell debris and impurities were removed through centrifugation, the protein was subpackaged, and the OD value was determined by protein content detection kit; the protein was boiled at 10°C for 10 min, followed by loading for sodium dodecyl sulfate-polyacrylamide gel electrophoresis electrophoresis. ECL chemiluminescence developer was adopted and exposed. Band gray value was measured using Image J (National Institutes of Health, Bethesda, USA), with β-actin being the internal reference, and gray value ratio of the two at different time points was treated as the relative protein content.

Statistical analysis
SPSS version 17.0 software (SPSS Inc., Chicago, USA) was adopted for statistical analysis, means of two samples were compared with t-test, and means of multiple samples were compared using Chi-square test analysis. Statistical significance was defined as P < 0.05.

RESULTS

Expression of hypoxia-induced factor-1α and vascular endothelial growth factor in gastric cancer cells under hypoxia
The expression of HIF-1α and VEGF at different time points in the experiment group and control group showed that positive HIF-1α expression located in the nucleus and cytoplasm, which was claybank (data not shown). Protein expression quantity in the experiment group was remarkably reduced compared with that in control group after 2, 6, 12, and 24 h of culture under hypoxia, and the difference was of statistical significance [P < 0.05, Table 1]. Positive VEGF expression located in cytoplasm and cell membrane, which was claybank and dominated by cytosolic expression (data not shown). Protein expression quantity in experiment group was notably reduced compared with that in control group after 2, 6, 12, and 24 h of culture under hypoxia, and the difference was of statistical significance [P < 0.05, Table 2].

Ginsenoside Rg3 inhibits expression of hypoxia-induced factor-1α and vascular endothelial growth factor by western blot analysis
Protein expression of HIF-1α and VEGF at different time points in the experiment group and control group was analyzed using

Table 1: Expression levels of hypoxia induced factor-1α determined by immunocytochemistry in experiment and control groups (±s)

| Categories          | n   | 2 h     | 8 h     | 12 h    | 24 h    | F       | P      |
|---------------------|-----|---------|---------|---------|---------|---------|--------|
| Control group       | 3   | 0.32±0.006 | 0.36±0.005 | 0.36±0.004 | 0.43±0.011 | 177.231 | 0.000  |
| Experiment group    | 3   | 0.25±0.010 | 0.31±0.007 | 0.31±0.009 | 0.29±0.012 | 28.102  | 0.000  |
| t-test              |     | 10.321  | 8.401   | 9.821   | 15.634  |         |        |
| P                   |     | 0.002   | 0.003   | 0.002   | 0.000   |         |        |

HIF-1α=Hypoxia-induced factor-1α
Li and Qu: Rg³ inhibiting HIF-1α and VEGF expression

It could be discovered that protein expression quantity of HIF-1α in experiment group after 2, 6, 12, and 24 h of culture under hypoxia was markedly lowered than that in control group, with the difference being of statistical significance \[P < 0.05, \text{Figure 1a}\]; while that of VEGF in experiment group after 2, 6, 12, and 24 h of culture under hypoxia was distinctly lowered than that in control group, with the difference being of statistical significance \[P < 0.05, \text{Figure 1b}\].

## DISCUSSION

Metastasis and recurrence account for the major reasons for the high mortality of gastric cancer.\[18\] The roles of key factors in metastasis have been increasingly recognized with the gradual deepening of research on tumor cell metastasis,\[19\] which has provided a new direction for targeted and individualized drug therapy. It is found in research that novel tumor molecular markers are of crucial importance to the diagnosis and prognosis of gastric cancer patients.\[20\] Patients with metastasis and recurrence are still associated with poor prognosis\[21\] despite the increasingly improved surgical and drug chemotherapy levels for gastric cancer, as well as the continuously updated systemic chemotherapeutic regimens.\[22-24\] Therefore, whether first-line and second-line adjuvant chemotherapy drugs can better improve survival rate has become a key to treat gastric cancer metastasis.\[25\]

In this study, it is found that the expression of HIF-1α and VEGF showed an increasing trend accompanied by the extension in culture time under hypoxia, indicating that VEGF expression under hypoxia is closely associated with HIF-1α. Protein expression quantities of HIF-1α and VEGF in the experiment group show decreasing trends as time prolongs, which is more obvious at 12 and 24 h particularly. Furthermore, the low expression of the two shows time dependency, demonstrating that Rg³ can reduce the expression of HIF-1α and VEGF, and it is speculated that Rg³ can influence VEGF expression through inhibiting HIF-1α expression. Meanwhile, Rg³ may also influence VEGF expression through other pathways. Results of this experiment are similar to those in previous research, which indicates that Rg³ contributes to inhibiting the expression of HIF-1α and VEGF suggesting that Rg³ probably downregulates VEGF expression through suppressing HIF-1α, and decreases angiogenesis, thus influencing abdominal implantation metastasis of gastric cancer.

Hypoxia is a major influencing factor of high HIF-1α expression. Hypoxia exists in numerous tumors. The continuous increase in tumor volume will lead to changes in the tumor microenvironment, and insufficient blood supply in tumor region results in the growth state of hypoxia within tissues while oxygen enrichment within surrounding tissues.\[26\] HIF-1α is a kind of cytoplasmic protein that promotes the activation of multiple genes and is involved in processes such as angiogenesis, cell proliferation, and energy metabolism. In eukaryotic cells, HIF-1α mainly regulates oxygen balance in the body and is affected and regulated by oxygen. HIF-1α is shown to express at a high level persistently as hypoxia within malignant tumor becomes more and more severe, which continuously promotes tumor growth and migration. In contrast, HIF-1α loss will give rise to reduced tumor growth and migration.\[27\] It is currently believed that HIF-1α is closely associated with peritoneal

| Categories       | n  | 2 h     | 8 h     | 12 h    | 24 h    | F       | P      |
|------------------|----|---------|---------|---------|---------|---------|--------|
| Control group    | 3  | 0.16±0.007 | 0.23±0.006 | 0.25±0.012 | 0.23±0.008 | 52.185  | 0.000  |
| Experiment group | 3  | 0.14±0.003 | 0.16±0.004 | 0.19±0.009 | 0.21±0.002 | 83.132  | 0.000  |
| t-test           |    | 3.101   | 16.586  | 5.839   | 4.478   |         |        |
| P                |    | 0.032   | 0.002   | 0.004   | 0.041   |         |        |

VEGF = Vascular endothelial growth factor

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**Figure 1:** Expression levels of hypoxia-induced factor-1α and vascular endothelial growth factor protein determined by Western blot in experiment and control groups. (a) Relative expression of hypoxia-induced factor-1α; (b) Relative expression of vascular endothelial growth factor. β-actin was used as internal control. Three independent experiments performed in duplicate; \[^\ast P < 0.05\]
metastasis of gastric cancer.[28-30] HIF-1α expression levels at different time points under hypoxia are detected by immunocytochemistry and Western blot; in this research, the results of which indicate that the application of Rg3 renders gradually reduced HIF-1α expression in a time-dependent manner.

VEGF is a kind of glycosylated mitogen that acts on endothelial cells, the high expression of which during metastasis may induce endothelial barrier destruction, thus promoting migration of endothelial cells.[31,32] It has been discovered that the removal of HIF-1α during angiogenesis could significantly reduce diffusion and chemotaxis of endothelial cells, extracellular matrix permeability, and wound healing.[33] However, the blood vessel size and number in the gastric tumor after transfection with HIF-1α apparently increased, suggesting that HIF-1α could regulate VEGF expression, which was closely related to angiogenesis. It is reported in the literature that high HIF-1α expression in the tumor can promote angiogenesis through upregulating VEGF.[34] The application of Rg3 leads to the gradual decrease of VEGF expression, which is closely associated with HIF-1α expression, as is found in this experiment.

Recent advances have indicated that ginsenoside Rg3 acts to reduce growth, invasion, and metastasis of cancer cells, and the mechanisms involve with VEGF and hypoxia associated multiple signaling pathways.[17,35] Recent studies reported that Rg3 suppresses the phosphorylation cascade of the VEGF-dependent p38/ERK signaling.[36,37] In patient with acute leukemia, ginsenoside Rg3 inhibits HIF-1α and VEGF expression through blocking the activation of PI3K/Akt and ERK1/2 pathways.[38] Furthermore, Rg3 increases the susceptibility of patients to chemotherapy,[6,7] therefore, Rg3 has synergistic effects in clinical trials in combination with chemotherapy regimens. For instance, the survival rate of patients with advanced gastric cancer could be improved when Rg3 was applied in combination with adjuvant chemotherapy.[8] The previous study revealed that Rg3 is also able to induce apoptosis in gastric cancer cells.[39] Therefore, Rg3 could be promising in cancer management.

**CONCLUSION**

Rg3 can obviously inhibit expression of HIF-1α and VEGF in human gastric cancer cells under hypoxia, the mechanism of action of which may be to downregulate VEGF expression though reducing HIF-1α, thus lowering angiogenesis in tumor and affecting tumor growth. Results in this experiment have provided theoretical support for the mechanism of action of Rg3 inhibiting implantation metastasis of gastric cancer; in addition, providing novel evidence for the target drugs for inhibiting tumor angiogenesis.

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**Conflicts of interest**

There are no conflicts of interest.

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