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Study on the proliferation of human gastric cancer cell AGS by activation of EGFR in H_2O_2

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Abstract. – OBJECTIVE: This study is to investigate the effect of low concentration hydrogen peroxide (H_2O_2) on the proliferation of gastric cancer AGS cell line *in vitro* and the mechanism.

MATERIALS AND METHODS: AGS cells were treated with different low concentrations of H_2O_2 (1, 0.1, 0.01, and 0.001 μm) for 48 hours. The effect of H_2O_2 concentration gradient on the activity of AGS cell activities was detected by methyl thiazolyl tetrazolium (MTT) method. The expression of the epidermal growth factor receptor (EGFR) and its downstream signaling pathway extracellular signal-regulated kinase (ERK) protein in H_2O_2 was detected by Western blot method; moreover, the effect of H_2O_2 on intracellular reactive oxygen species (ROS) in AGS cells was observed under the fluorescence microscope and quantitative analysis by flow cytometry. The effect of H_2O_2 on the level of c-myc mRNA in AGS cells was also detected by reverse transcription polymerase chain reaction (RT-PCR).

RESULTS: MTT detection results showed that 1 μm and 0.1 μm H_2O_2 at 48h can effectively promote the proliferation of AGS cells ($p<0.05$). The Western blot results showed that after 48 hours of low concentration of H_2O_2 treatment of AGS cells, the EGFR protein levels and ERK protein phosphorylation levels increased significantly ($p<0.05$). Fluorescence microscopy and flow cytometry showed that the low concentration of H2O2 increased the intracellular reactive oxygen species (ROS). RT-PCR results showed the levels of c-myc mRNA in AGS cells treated with a low concentration of H_2O_2 were significantly increased ($p<0.05$) and EGFR blocking agent AG1478 can resist the above effects.

CONCLUSIONS: Low concentration of H_2O_2 can significantly promote the proliferation of AGS cells by activating EGFR/ERK signaling pathway.

Key Words:
Gastric cancer, H_2O_2, AGS cell, EGFR, Proliferation.

Introduction

In recent years, although the incidence of gastric cancer worldwide has declined, it is still a major cause of cancer death and ranked as the second highest incidence of all cancers after lung cancer because of the poor prognosis and limited treatment options[1]. In China, the mortality rate of gastric cancer was the highest in the first place[2]. At present, the treatment of gastric cancer is mainly based on surgery, radiotherapy, and chemotherapy. However, the majority patients were diagnosed at the advanced stage, and the effect of chemotherapy was not obvious. H_2O_2 is an endogenous oxidant, which has been considered as a signal molecule in recent years. Low concentration H_2O_2 can be involved in the intra-and inter-cellular information transfer[3]. H_2O_2 has made great progress in the research of incidence and development of tumor by inhibition of DNA damage repair, genome stability and the activity of fat, protein and other biological macromolecules[4,5]. Epithelial growth factor receptor (EGFR) is a membrane receptor that has been expressed in a variety of malignant tumors. Recent studies have shown that EGFR was highly expressed in gastric cancer, which was closely related to the incidence and development of gastric cancer, and was expected to be a new target for gastric cancer gene therapy[6,7]. To this end, through the culture of AGS *in vitro*, the effects of low concentration H_2O_2 on the growth and proliferation of AGS were observed, while the role of EGFR/ERK signaling pathway in cell survival and proliferation was also observed, providing sufficient theoretical basis for the low concentration H_2O_2 to promote the occurrence of gastric cancer

Materials and Methods

Major Reagents

Roswell Park Memorial Institute – 1640 culture medium (GE Healthcare Life Sciences HyClone Laboratories, Logan, UT, USA). Phosphate buffered saline (PBS) powder (Xinmai Biotech Co. Ltd., Fuzhou, Fujian, China). Fetal bovine se-

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rum (FBS) (GE Healthcare Life Sciences HyClone Laboratories, Logan, UT, USA). Pancreatin (Sichuan Biosyn Pharmaceutical Co., Ltd. Chengdu, Sichuan, China). Ethylene diamine tetraacetic acid (EDTA, Gibco BRL, Grand Island, NY, USA). Bovine serum albumin (BSA) (Gibco BRL, Grand Island, NY, USA). EGFR antibody (Cell Signaling Technology, Danvers, MA, USA). ERK and p-ERK antibody (Santa Cruz Biotechnology Inc., Dallas, TX, USA). c-myc primer (Shanghai Shenggong Inc., Shanghai, China). Enhanced chemiluminescence reagent for horseradish peroxidase labeling (Beyotime Biotechnology, Nanjing, Jiangsu, China). Methyl thiazolyl tetrazolium (MTT) (Beijing Dingguo Changsheng Biotechnology Co. Ltd., Beijing, China).

Experimental Method

Cell Culture

Human gastric cancer cell line AGS was cultured in 10% FBS, 1% penicillin, and double anti streptomycin 1640 culture medium, and incubated in the condition of 5% CO_2, 37°C. The cells were grown up to 80% or so for cell subculture; then, a proper amount of PBS was added to remove the cells without sticking to the wall. 1 mL of 0.25% pancreatin was added for room temperature digestion. After adding fresh culture medium by the elbow dropper into the culture medium, the cells were re-suspended when the round shapes appeared, and carried on the passage for the subsequent experiment at 1:3 proportion. In accordance with the requirements of the cell volume of the experiment, the cells were transferred to the culture bottle, the 96-well plate, the 24-well plate or the 6-well plate.

Detection of the Effect of Low Concentration of H_2O_2 on the Proliferation of AGS Cells by MTT Method

The cells in the logarithmic growth phase were inoculated on 96-well plate with a number of 2×10^3 cells per well (100 μl) for 24 hours. H_2O_2 with different final concentrations (1, 0.1, 0.01 and 0.001 μm) were added to each well, as well as H_2O_2 and AG1478 (0.1 m H_2O_2, 0.1 m H_2O_2, 0.1 m H_2O_2+300 μm AG1478, 1 m H_2O_2+300 μm AG1478) in incubation added under condition of 37°C, 5% CO_2 for 48 hours. After the cells had been processed, 20 μl of 5 g/L MTT was added to each well. Continue the culture for 4 hours then the culture medium was sucked out; then 150 μl dimethyl sulfoxide (DMSO) (Shanghai Airui Biotechnology Co. Ltd., Shanghai, China) was added to form formazan. When the formazan was fully dissolved, the absorbance (A490) value was available at the wavelength of 490 nm by using the microplate reader (ELX800, Bio-Tek, Winooski, VT, USA).

Detection of the Effect of Low Concentration H_2O_2 on Protein Expression of EGFR/ERK Signaling Pathway in Cells by Western Blot Method

After the AGS cell had been stimulated by 1 and 0.1 μmol/L H_2O_2 for 48 hours, the protein concentration was determined by BCA method. 50 μg proteins were taken in total from each group and then were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Beyotime Biotechnology, Nanjing, Jiangsu, China). The film was transferred to the PVDF film (Professional Plastics, Fullerton, CA, USA), and the 80V transfer film was 90 min. 5% skim milk PBST (PBS Tween-20) was closed for 2 hours. The following first antibody, including EGFR (1:1000), ERK (1:800), p-ERK (1:1000) and β-actin (1:5000 dilution), was added. Then, it was incubated overnight on shaker at 4°C condition. The film was washed the next day, followed by adding the second antibody goat-anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA). Then, it was incubated at the room temperature for 2-3 hours. After cleaning, the proteins were colored with enhanced chemiluminescence method and were labeled with horseradish peroxidase. After, the proteins were visualized on X-ray (Scienscope, Chino, CA, USA). LabWorks 4.6 software was used to analyze the gray value of the target protein and the β-actin bands.

Detection of Levels of Reactive Oxygen Species (ROS)

We obtained AGS cells in logarithmic phase. After digestion and counting, $3x10^4$ cells were inoculated in each well of 24-well plate, and adherent cells were cultured for 24 hours. 0.1 m H_2O_2, 0.1 m H_2O_2+300 μm AG1478 were added in the experimental group and cultured for 14 hours; meanwhile, the control group was also set up. The culture solution was discarded and the cells was washed with PBS for twice. In accordance with 1:1000 diluted DCFH-DA probe with serum-free 1640 culture solution, the final concentration as 10 ml/L was set, 500 ml diluted probe in each well were added and were incubated for 20 minutes. Then, the probe was discarded and the cells

with serum-free 1640 were rinsed for three times. The 2',7'-dichlorofluorescin diacetate (DCFH-DA) probe outside of the cells should be fully rinsed. Under the fluorescence microscope (Nikon, Tokyo, Japan), the cell activity oxygen level was detected qualitatively, and the cell flow cytometry (FACSVerse, BD Biosciences, San Jose, CA, USA) was used to detect the level of reactive oxygen species.

Effect of RT-PCR Detection of H_2O_2 Concentration of c-myc mRNA Expression in AGS Cells

AGS cells with good growth in logarithmic phase were obtained and 10^5 cells were inoculated in 6-well plates after digestion and cultured for 24 hours. 0.1 m H_2O_2, 0.1 m H_2O_2+300 μm AG1478 were added to the experimental group; meanwhile, a control group for contrast was set up. The instructions of RNA extraction kit were followed to extract RNA and the content of RNA was detected. The ratio of OD_{260}/OD_{280} was between 1.8-2.0 with high purity. The reverse transcription reaction system was: 2×TS Reaction Mix (10 μl) (TransGen Biotech, Beijing, China), TransScript RT/RI Enzyme Mix (1 μl) (TransGen Biotech, Beijing, China), Anchored Oligo $(dT)_{18}$ (1 μl) (TransGen Biotech, Beijing, China), total RNA (500 μg) (TransGen Biotech, Beijing, China), RNase-free Water to 20 ml (Thermo Fisher Scientific, Waltham, MA, USA). Reaction conditions were at 42°C for 30 min and 85°C for 5 min. Primers were as follows:

GAPDH upstream: 5- GAAGGTGAAGGTCGGAGTC-3;

GAPDH downstream: 5-GAAGATGGTGATGGGATTTC-3;

c-myc upstream: 5-CAGGACTGTATGTGGAGCGGTTTC-3,

c-myc downstream: 5-TGCTGTCGTTGAGCGGGTAG-3.

The PCR circular system was as follows: 1) pre-denaturation at 94°C for 5 min; 2) denaturation at 94°C for 1 min; 3) annealing at related temperature for 1 min; 4) extending at 72°C for 1 min; 5) returning to step; 2) for 30 cycles; 6) termination at 72°C for 1 min; 7) extending at 4°C; 8) after removal, the RNA expression was detected by agarose gel electrophoresis.

Statistical Analysis

All of the above experiments were repeated more than three times and the data in each group were in the form of mean ± SD. The data were analyzed by SPSS17.0 statistical software (SPSS Inc., Chicago, IL, USA). t-test was used to compare the measurement data between the two groups and the $p<0.01$ was statistically significant.

Results

Effect on AGS Proliferation of Testing H2O2 Stimulation by MTT Method

To compare with the control group (0 H_2O_2 concentration), the cell viabilities were increased after the presence of H_2O_2. The cell viabilities were 1.24±0.001 ($p<0.05$), 1.38±0.001 ($p<0.05$), 0.88±0.01 ($p<0.05$) and 0.51±0.01 ($p<0.05$) times of the controls respectively. With the decrease of the H_2O_2 concentration gradient, the cell viability was decreased. The cell viability was the highest when 0.1 μm H_2O_2 was added; therefore, the follow-up experiment selected 0.1 μm H_2O_2 as the optimum concentration. The cell viability was significantly decreased after addition of EGFR blocking agent AG1478, as shown in Figure 1.

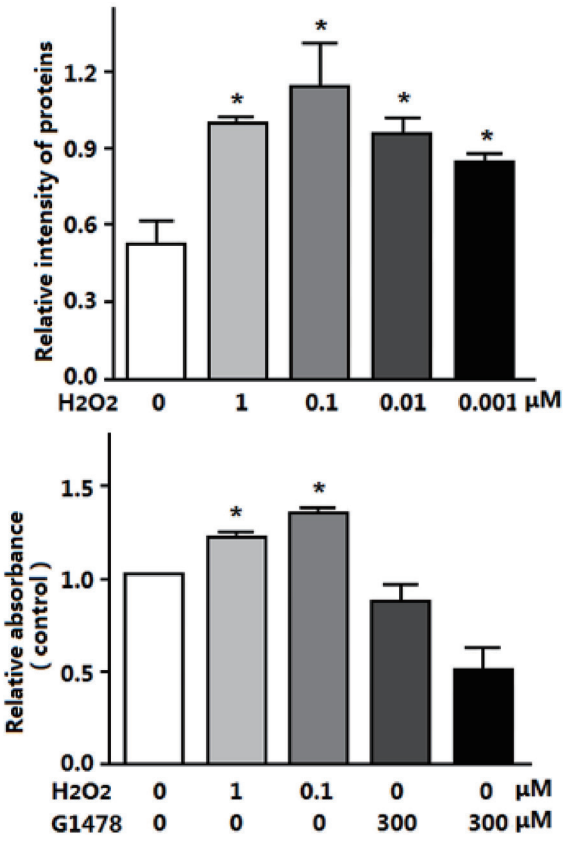

Figure 1. The proliferation of cells by H_2O_2 stimulation on AGS for 48 hours after addition of AG1478 by MTT method.

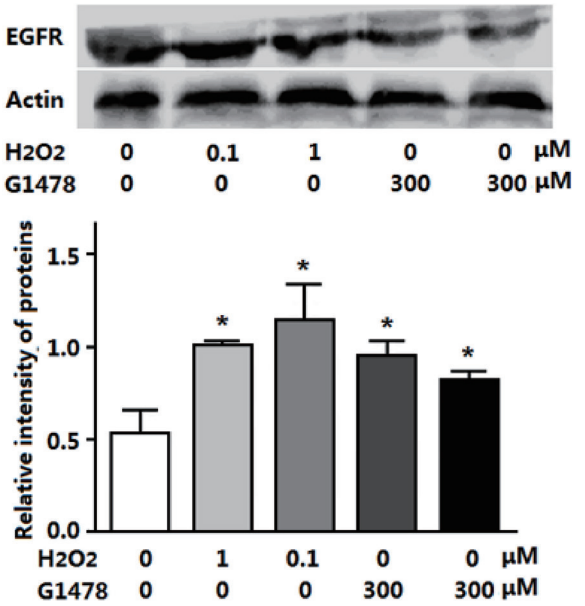

Figure 2. The expression of EGFR by H_2O_2 stimulation on AGS after addition of AG1478 by Western blot method.

Effect on EGFR/ERK Signaling Pathway Protein Expression by Low Concentration H_2O_2

Effect on EGFR Protein Expression by Low Concentration of H_2O_2

After stimulation on AGS cells by different low concentrations of H_2O_2 for 48 hours, in contrast with the control group (0 H_2O_2 concentration), the cellular EGFR protein expressions were significantly increased when 1 and 0.1 μm of H_2O_2 was added. The expressions of EGFR protein were lower than that in the H_2O_2 group after addition of EGFR blocking agent AG1478. In comparison with the protein ratio of EGFR/β-actin, 1 and 0.1 μm H_2O_2 were increased by 1.03 and 1.32 times of the control group, and decreased by 0.82 and 0.67 times after addition of 300 μm AG1478. The data of each group were 0.72±0.03 (p<0.05), 0.95±0.03 (p<0.05), 0.63±0.03 (p>0.05), 0.05±0.03 (p<0.05), as shown in Figure 2

Effect on ERK Protein Phosphorylation by Low Concentration of H_2O_2

After stimulation on AGS cells by different low concentrations of H_2O_2 for 48 hours, in contrast with the control group (0 H_2O_2 concentration), the phosphorylated ERK protein expressions were significantly increased by 1 and 0.1 μm H_2O_2; however, the expressions of phosphorylated ERK protein were lower than that in the H_2O_2 group after addition of EGFR blocking agent AG1478. In comparison with the protein ratio of p-ERK/ERK, the data of each group were 1.00±0.02 (p<0.05), 1.14±0.17 (p<0.05), 0.95±0.06 (p<0.05), 0.84±0.04 (p<0.05) respectively, as shown in Figure 3.

Effect of Low Concentration of H_2O_2 on AGS Intracellular ROS

As shown in Figure 4, the green fluorescence intensity showed a significant increase when the AGS cells were processed by 0.1 μm H_2O_2 for 12 hours, indicating that H_2O_2 can cause the increase of intracellular reactive oxygen species; however, the H_2O_2 induced fluorescence was significantly decreased after addition of EGFR blocking agent AG1478, indicating that AG1478 can effectively alleviate the increase of H_2O_2 induced reactive oxygen species.

As shown in Figure 5, the ROS content increased significantly to 1.86±0.17 (p<0.05) in comparison with the control group when the AGS cells were processed by 0.1 μm H_2O_2 for 12 hours. However, by adding 300 μm AG1478, it can significantly alleviate the H_2O_2 induced increase in ROS, and decrease to 0.90±0.18 (p<0.05) times when compared to the control group, the differences had statistical significance.

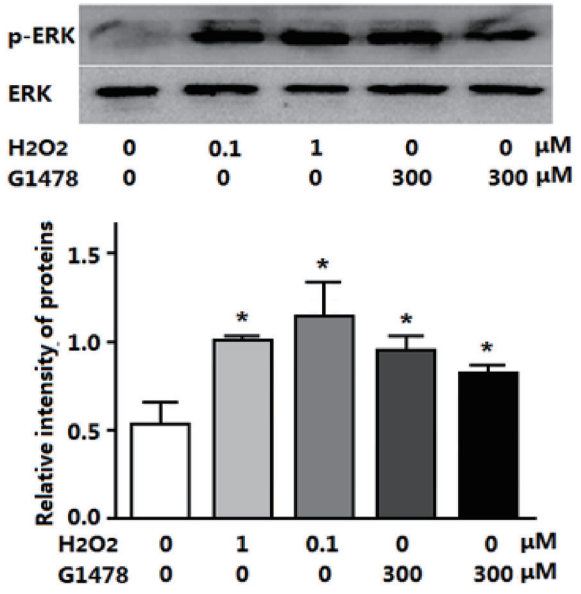

Figure 3. The expression of ERK phosphorylation by H_2O_2 stimulation on AGS after addition of AG1478 by Western blot method.

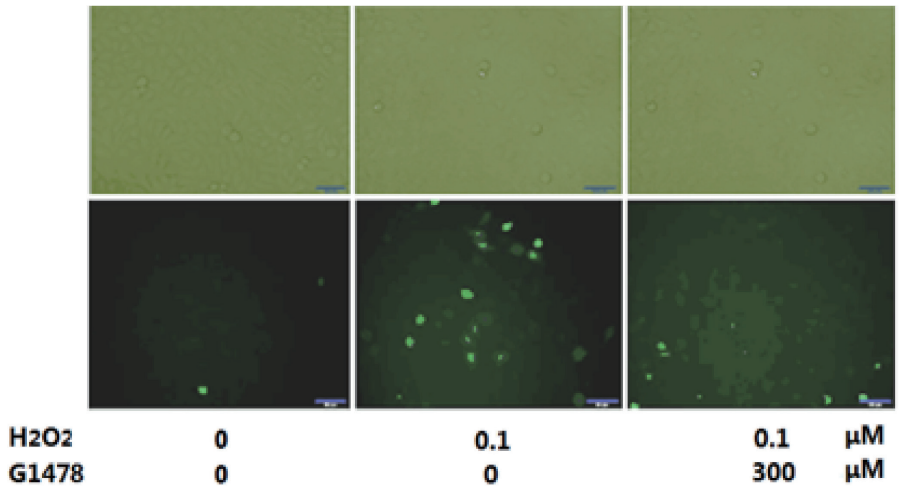

Figure 4. Qualitative detection of intracellular ROS accumulation induced by H_2O_2 and AG1478 under fluorescence microscopy.

Effect of Low Concentration of H_2O_2 on c-myc mRNA in AGS Cells

As shown in Figure 6, it showed the effect of up-regulation of genes because of blocking agent on H_2O_2 tested by the RT-PCR method. The figure indicated that the c-myc gene expressed the up-regulation in the H_2O_2 group after 36 hours, and it was 1.14±0.00 (p<0.05) times when compared with the control group. Nevertheless, the addition of AG1478 can significantly inhibit c-myc up-regulation as induced by H_2O_2. In comparison with the control group, the c-myc mRNA expressions from the blocking group were 0.95±0.00 (p<0.05) times of the control group. There was a significant difference between the blocking agent and the H_2O_2 group compared with the single H_2O_2 group.

Discussion

H_2O_2 is one of the main ROS products in the cell. Studies have confirmed that the appropriate level of ROS in the cells was the prerequisite for the maintenance of growth and migration of stem cells and endothelial cells[8]. Researches also suggested that the low concentration of ROS had similar characteristics as the growth factors. Low levels of H_2O_2 and O_2 can increase the synthesis of cell DNA and induce up-regulation of cell c-fos and c-myc gene expression. These genes are related to the cell proliferation[9]. Also, H_2O_2, as a type of small molecule compound with membrane permeability, can stimulate the intracellular signaling pathways and play an important role in

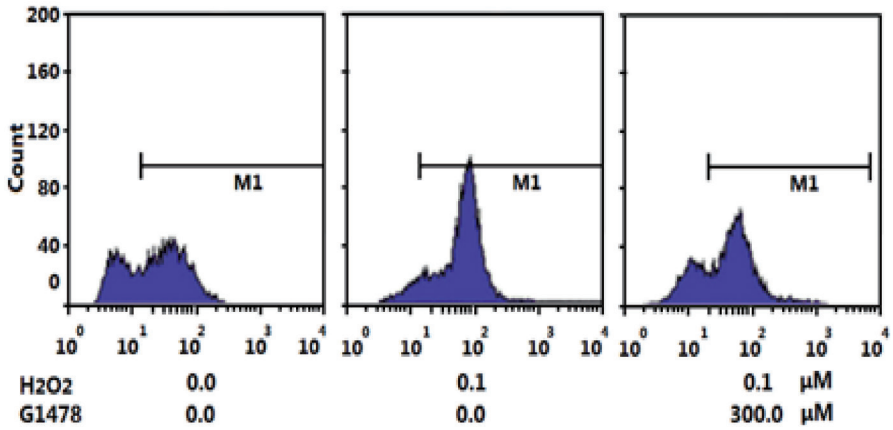

Figure 5. Qualitative detection of ifntracellular ROS accumulation induced by H_2O_2 and AG1478 under flow cytometry.

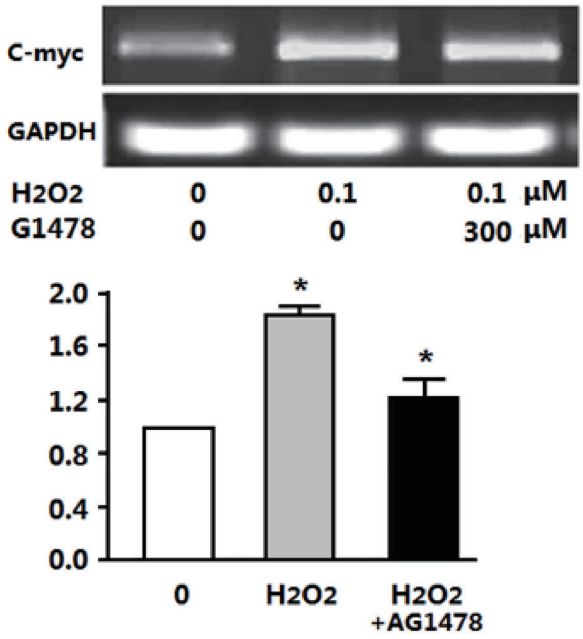

Figure 6. RT-PCR detection of H_2O_2 and AG1478 induced intracellular mRNA c-myc levels.

signal transduction and messenger molecules[10]. At the physiological level of low concentration, H_2O_2 is used as a second messenger or intracellular signaling molecule to initiate a variety of signaling pathways, including: (1) increase of the intracellular calcium levels, which may cause the ribosomal protein phosphorylation and activation of calmodulin; (2) activation of protein kinase (MAPK) by exogenous or endogenous H_2O_2 induced mitogen, especially the phosphorylation of extracellular signal regulated kinase (ERK), and increase of the expression of MAPK dependent genes such as e-jun and e-fos, etc.; (3) activation of a variety of transcription factors NF-kappa B and early gene JunD, c-myc, AP-1, etc. to promote cell proliferation, migration, growth, differentiation, apoptosis, autophagy and other physiological and pathological processes. H_2O_2 induced the phosphorylation of EGFR/PDGF-R and activation of ERK phosphorylation to increase the expression of Gi-α protein, and the content of cAMP was decreased; thus, the cell proliferation was induced[11-14]. EGFR, belonging to the tyrosine kinase type I receptor family, had the tyrosine kinase activity. EGFR binding with ligand EGF or TGF-α activates tyrosine protein kinase pathway, promoted the cell growth, DNA synthesis and gene expression in cancer. EGFR high expression can promote angiogenesis and tumor cell proliferation, adhesion, invasion and metastasis, inhibition of tumor cell apoptosis[15]. In recent years, researches have suggested that EGFR was highly expressed in gastric cancer, which was closely related to the incidence and development of gastric cancer and considered to be an ideal target for tumor therapy of gastric cancer[16].

This study founds that the low concentration of H_2O_2 (1, 0.1, 0.01 and 0.001 μm) was added for 48 hours, the proliferation of AGS cells was significantly promoted. However, with the decrease of the H_2O_2 concentration gradient, the cell proliferation was decreased as well. The effect of cell proliferation reached the highest when 0.1 μm H_2O_2 was added. It was suggested that the low concentration of H_2O_2 was more effective in promoting the proliferation of AGS cells. In the works of gastric cancer, EGFR was highly expressed[17]. Since the EGFR/ERK signaling pathway is playing a core role in the cell growth, it is involved in the regulation of cell growth, cell cycle and DNA damage repair and other processes. To clarify whether the effect of low concentration H_2O_2 on the proliferation of AGS depended on the activation of EGFR/ERK signaling pathway, we added the EGFR protein inhibitor AG1478 in the cell culture system. Results showed that AG1478 could resist the proliferation effect of AGS induced by H_2O_2, which suggested that H_2O_2 could promote the proliferation of AGS cells, which may depend on the activation of EGFR/ERK signaling pathway. To this end, we further observed the effect of low concentration of H_2O_2 on the expression of key proteins in EGFR/ERK signaling pathway and the activation of phosphorylation. The results suggested that low concentration of H_2O_2 (0.1 μmol/L) could significantly up-regulate the phosphorylation of key protein EGFR and ERK in the EGFR/ERK signaling pathway; after addition of EGFR inhibitor AG1478, the phosphorylation level of ERK was decreased. Studies indicated that exogenous H_2O_2 can promote the proliferation of cells by increasing the endogenous ROS induced by EGFR[18]. In this paper, we discussed the effect of H_2O_2 on the ROS of AGS cells by EGFR. As a result, 0.1 μm H_2O_2 can increase the intracellular ROS and the level of ROS decreased after the addition of EGFR protein inhibitor AG1478. C-myc gene was closely correlated with the cell proliferation. The effects of H_2O_2 on the level of mRNA c-myc were also discussed. RT-PCR results showed that 0.1 μm H_2O_2 could increase the level of intracellular mRNA c-myc, and the level of mRNA c-myc decreased after addition of EGFR protein inhibitor AG1478. The above

experiments proved that exogenous H_2O_2 could promote the proliferation of AGS cells through EGFR-ROS-ERK-c-myc signaling pathway[19].

Conclusions

The development of gastric cancer is a multi-factor and multi-step complex process. EGFR is involved in the tumor growth, the tumor cell proliferation, the angiogenesis, the tumor invasion, the metastasis and the apoptosis by downstream signal transduction pathways. In this paper, the experimental results showed that exogenous H_2O_2 could promote the proliferation of AGS cells through the EGFR-ROS-ERK-c-myc signaling pathway, which provided a new signal pathway for the development of gastric cancer.

Conflict of interest

The authors declare no conflicts of interest.

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