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An experimental Study on the mechanism for IL-8 promoting migration of A549 cells.

Jiang Yuequan, Cai Huarong, Zhou Hong, Zhang Zhi

Department of Thoracic Surgery, Chongqing Cancer Institute, Sha Ping Ba district, Han Yu Street 181#, Chongqing 400030, China.

Abstract

The effect and mechanism of IL-8 on A549 cells migration were investigated. The appropriate concentration of IL-8 was chosen by MTT assay. The Western-blot assay, Scratch test and Transwell assay were used in this study to analyse the effect and mechanism of IL-8 on lung adenocarcinoma A549 cells migration. The results of Western-blot assay showed that: (1) IL-8 can promote the expression of MMP-2 protein and has no effect on the expression of MMP-9. (2) IL-8 can promote the expression of JNK/SAPK phosphorylated protein. (3) Inhibitor (SP600125) can block the effect of IL-8 on the expression of MMP-2 protein. Scratch test proved from the opposite side that the low expression of MMP-2 could inhibit the migration of A549 cells. These results suggest that IL-8 can regulate MMP-2 protein expression through the JNK/SAPK signaling pathway and further promote the migration of lung adenocarcinoma A549 cells.

Keywords: IL-8; MMPs; SP600125; A549 cell; cancer cell migration

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Introduction

Lung cancer is one of the most common malignancies. The early invasion and metastasis of tumor cells is the major obstacle to achieving successful lung cancer treatment. It is also the most important reason for the death of patients with lung cancer [1]. Early invasion and metastasis of tumor cells are associated with the degradation and destruction of extracellular matrix and basement membrane, which requires the involvement of the corresponding lytic enzyme. During the matrix metalloproteinase (MMPs) family members [2,3], especially MMP-2 and MMP-9 play an important role. Interleukin-8 (IL-8) is a glycoprotein with molecular weight of about 8KD, which was extracted by Yoshimural from mononuclear cell culture medium stimulated with lipopolysaccharide (LPS) and phytohemagglutin in (PHA) [4]. As an inflammatory factor, IL -8 can be involved in the immune and inflammatory responses. In addition, IL-8 hasa chemotactic effect that can promote cell migration. A growing number of studies have showed that IL-8 is closely related to tumor cell migration. Luca et al. [5] found that IL-8 can up regulate the expression of MMP-2 in human melanoma cells and further promote cell migration. Wang et al. [6]. Reported that IL-8 secreted by ovarian cancer cells promotes malignant behavior of these cells via inducing intracellular molecular signaling. Rafrafi A et al. [7] also demonstrated that the IL-8 promoter polymorphism is associated with NSCLC risk. Although there are many studies showed that IL-8 is related to tumor cell migration, the effect and mechanism of IL-8 on lung cancer cell migration is unclear. Therefore, in this study the specific effects and possible mechanism of IL-8 on lung cancer cell migration were investigated. This may provide valuable experimental data for the clinical treatment of lung cancer.

Material and Methods

Materials

Main reagents

Recombinant human interleukin-8 (IL-8) was from Hangzhou Sijiqing Biological Engineering Materials Co, Ltd. (China). Anti-MMP-2 antibody, anti-MMP-9 antibody, anti-Phospho-JNK/SAPK (c-Jun NH2-terminal kinase/-stress-activated protein kinase) antibody and anti-β-actin antibody were obtained from Beyotime Institute of Biotechnology (China). C-JNK inhibitor (SP600125) was purchased from Invitrogen (USA).

Cell culture

Human lung adenocarcinoma A549 cell line was obtained from the Cell Bank of Institute of Life Sciences, Chong-

qing Medical University. RPMI-1640 medium containing 10% inactivated FBS, 100 U/ml penicillin and 100 mg/L streptomycin was cultured at 37°C in a 5 % CO^2 incubator. Cells were digested with 0.25% trypsin (containing 0.02% EDTA) and passaged every 2 to 3 days. Cells in logarithmic growth phase were used.

Methods

Cell growth experiments

A549 cells in logarithmic growth phase were seeded in the 96-well plate (1.0×10^4 cells/well) and incubated for 24 hours. In each experimental group, 120 μl different concentrations of IL-8 (25, 50, 100, 500 μg/L) were added respectively. The same volume of normal medium was added in the control group. To each well was then added 20 μl of 5 mg/ml MTT and 80 μl of normal medium at 37ºC to culture for 3 hours. Then to each well was added with 100 μl of DMSO, and shaken at the shaking table for 10 minutes until the blue crystal was completely dissolved. The absorbance value (D) was measured at 492 nm using an ELISA reader. Each group was given five wells, and the experiment was done in triplicate. The appropriate concentration of IL-8 was evaluated by cell viability. The value of cell viability was calculated by the following formula: cell viability value=(average D value of experimental group/average D value of control group)× 100 %.

Scratch test

A549 cells in logarithmic growth phase were seeded in the 24-well plate at 1.0×10^5 cells/well. After the cells grow up to 80%~90%, 10 μl of sterile pipette was used to draw a straight line in the center of each well. To each well was added 400 μl IL-8 of different concentrations prepared by serum-free medium as the experimental group (40 μl serum-free medium was added alone as the control group). Both the experimental and control groups were then incubated for 24 h. Under TE2000-U invert fluorescence microscope (Nikon, Japan), images were taken to observe the remaining distance after the cells in each group migrated from the scratch edge to the scratch center.

Transwell migration assay

The logarithmic growth phase A549 cell were taken to adjust cell concentration with serum-free medium to 1.0×10^5 cells/ml, and inoculated at 100 μl/well in the upper Transwell chamber (polycarbonate membrane with a pore size of 8μm). Six hundred μl of IL-8 at different concentrations was added in the lower Transwell chamber as experimental group (600 μl of normal medium was added as the control group) and cultured at 37ºC for 24 hours. The cells on the top of filter membrane were removed with a cotton swab and washed with PBS twice. The upper chamber was placed in 4% paraformaldehyde fixed for 15 minutes, and stained with hematoxylin for 20 minutes. Under TE2000-U inverted fluorescence microscope (Nikon, Japan), the averaged numbers of cells penetrating the membrane were measured from 5 different fields of view.

Western blot assay

The cells of each experimental group were collected, centrifuged at 1000 r/min for 10 minutes, lyzed with CST lysate, and centrifuged at 4ºC at 12000 r/min for 15 minutes. The supernatants were collected to measure protein concentration with BCA method. Protein samples were diluted with 5×loading buffer and boiled in water for 10 minutes at 100°C. Polyacrylamide gel electrophoresis (carrying 70 μg protein) was conducted, and the membrane was translocated with 0.45 μm PVDF membrane and sealed with 5% defatted dry milk for 1 hour. The primary antibodies of MMP-2 (1:800), MMP-9 (1:800), p-JNK/SAPK (1:1000) and β-actin (1:300) were incubated respectively at 4ºC overnight and rinsed with PBS three times. The horseradish peroxidase conjugated secondary antibodies (1: 2500) were incubated for 1 hour and rinsed. The results were analyzed with ECL (Electrochemiluminescence) reagent under ChemiScope 2650 Fluorescence/Chemiluminescence imaging system (Bio-Rad, USA).

Statistical analysis

SPSS12.0 software was used for statistical analysis. Values were expressed as mean±SD. The results were compared by t test analysis between the two groups. *P* values of < 0.05 were regarded as significant.

Results

Effect of IL-8 on A549 cell growth

To determine the most appropriate concentration of IL-8on cells in the experiment, the effect of different concentrations of IL-8 on cell growth was analyzed first. The results showed that after the action with different concentrations of IL-8 (25, 50, 100 and 500μg/L) for 24 hours, the mean values of cell vitality were: 98.18%, 97.24%, 99.05% and 89.55% respectively (Fig. 1). Compared with the control group, IL-8 at concentration of 25, 50 and 100 μg/l had no significant effects on cell growth, but at concentration of 500g/l can significantly inhibited cell growth ($P<0.05$). Therefore, the three different concentrations of IL-8 (25, 50, 100 μg/L) were used in the following experiments.

Effect of IL-8 on A549 cell migration

To evaluate the effect of IL-8 on A549 cell migration, a straight line were scratched in the center of each well seeded the cells and treated with different concentrations of IL-8 (25, 50, 100 μg/l) for 24 hours. After the cells in the control group and different IL-8 concentrations group migrated from the scratch edge to the scratch center. The remaining distances were (13 ± 1.95) mm, (7.3 ± 1.1) mm, (3.3 ± 0.5) mm and (4.8 ± 0.72) mm respectively.

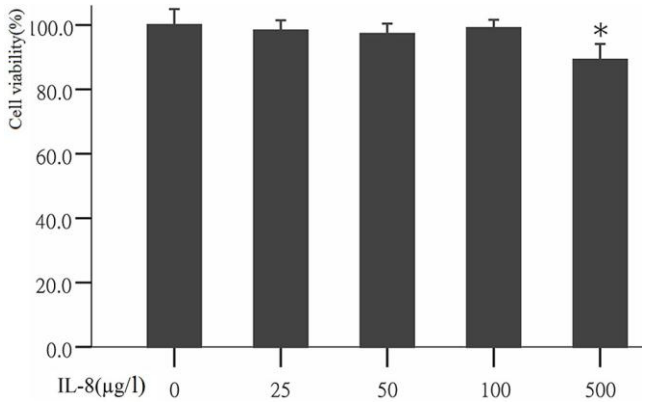

Figure 1.*Effect of IL-8 on A549 cells viability at different concentrations.*
**P<0.05 vs. control group (mean ± SD, n = 15).*

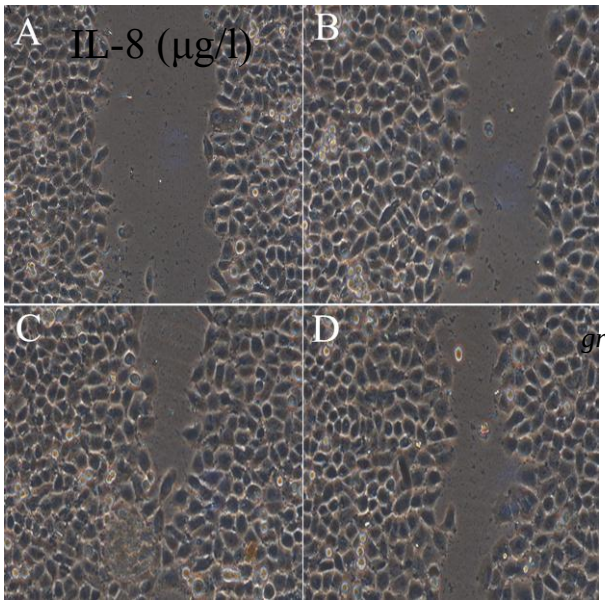

Figure 2. *Representative images of the effect of IL-8 on cell migration measured by Wound-healing experiment (200×).*
A: control group, B: 25 μg/l IL-8 group, C: 50 μg/l IL-8 group, D: 100 μg/l IL-8 group.

Compared with the control group, the migration distance of cells in different IL-8 concentrations group from the scratch edge to the scratch center increased significantly (P<0.05).

To further validate the effect of IL-8 on the migration of A549 cell, the cells were treated by the same method and determined with Transwell migration assay. Finally, the average counts of cells penetrating the Transwell chamber in the control group and different concentrations in the IL-8 group were 12 ± 1.8, 48 ± 7.2, 108 ± 16.2, 60 ± 9 cells/field respectively. Compared with the control group, the counts of cells penetrating the Transwell chamber in different concentrations of IL-8 increased significantly (P <0.05). This result was consistent with the result of the scratch test and further demonstrates that IL-8 can promote the migration of A549 cells.

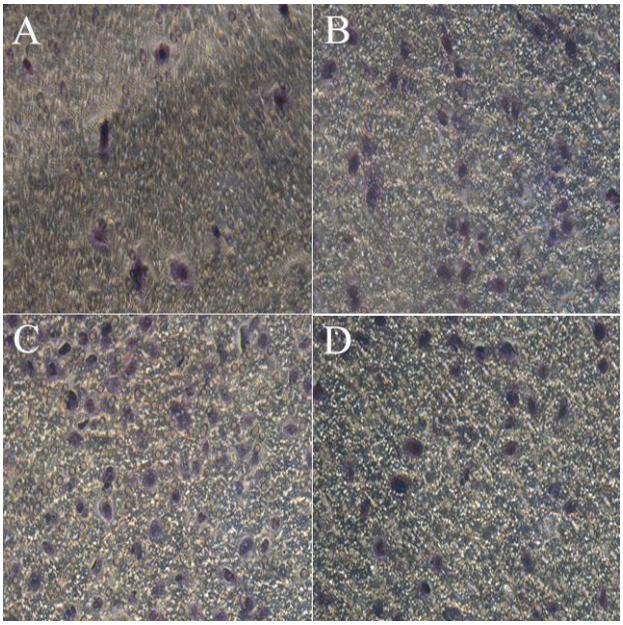

Figure 3. *Representative images of theeffect of IL-8 on cell migration measured by Transwell arrays (200×)*
A: control group, B: 25 μg/l IL-8 group, C: 50 μg/l IL-8, D: 100 μg/l IL-8 group.

Effect of IL-8 on MMP-2 and MMP-9 protein expression
Tumor cells producing hydrolase is an important step in the process of tumor invasion and metastasis. During the process, MMP-2 and MMP-9 were often highly expressed, and could promote tumor cell migration by extracellular matrix degradation. To study the mechanism of IL-8 inducing A549 cell migration, the cells were treated with different concentrations of IL-8 (25, 50, 100 μg/l) for 24 hours, and the proteins were collected to detect MMP-2 and MMP-9. After treatment by different concentrations of IL-8, compared with the control group, MMP-2 expression significantly increased 1.6, 3.1 and 2.4 times that of the control group respectively (P <0.05), and the results were consistent with those of IL-8 on A549 cell migration. By comparing different IL-8 concentrations group with the control group, MMP-9 expression was shown to have no significant change (Fig. 4).

Effect of IL-8 on phosphorylated protein expression in signal pathway JNK/SAPK
Studies have shown that the nuclear transcription factor activator protein-1 (AP-1) can regulate the expression of MMPs. JNK/SAPK is an upstream signal of AP-1. This experiment also studied that whether JNK/SAPK can regulate the expression of MMPs. After the cells were treated by different concentrations of IL-8 (25, 50, 100 μg/l) for 24 hours, the results of Western blot assay showed that

compared with the control group, JNK/SAPK phosphorylated protein expression significantly increased 1.5, 2.8 and 1.9 times that of the control group respectively ($P < 0.05$) (Fig. 5).

Effect of C-JNK/SAPK inhibitor (SP600125) on MMP-2 protein expression

To further validate that signal pathway JNK/SAPK can regulate MMP-2 expression, the cells were treated in the following way: (1) the cells were not treated with any reagent in the control group, (2) treated with 50 μg/l of IL-8 for 24 hours in 50 μg/l IL-8 group, (3) pre-treated with 20 μmol/l of C-JNK/SAPK signal pathway inhibitors (SP600125) for 1 hour and then treated with IL-8 (50 μg/l) for 24 hours in C-JNK/SAPK inhibitor (SP600125)

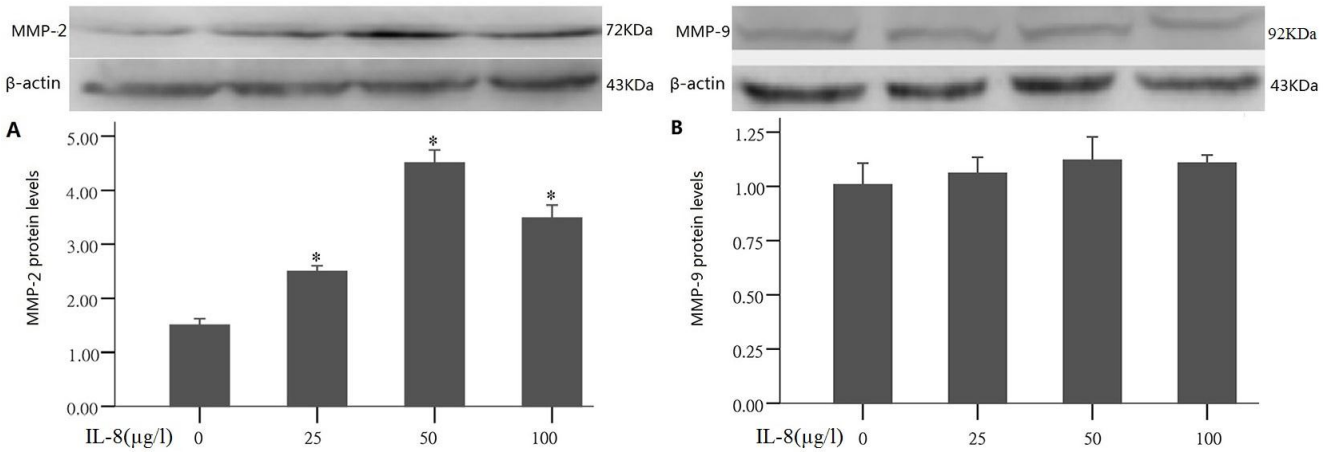

Figure 4. *Effects of IL-8 on protein expressions of MMP-2, MMP-9. A: protein expressions of MMP-2, B: protein expressions of MMP-9. * $P<0.05$ vs. control group (mean ± SD, n = 8).*

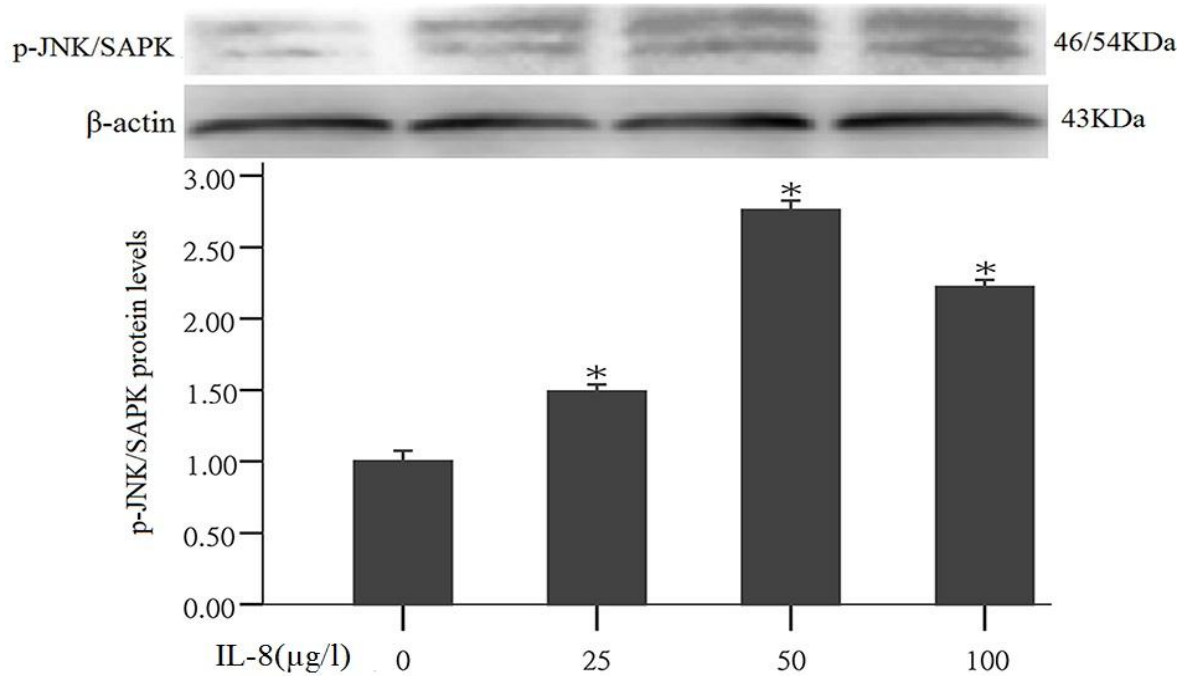

Figure 5. *Effects of IL-8 on protein expressions of p-JNK/SAPK.* *$P<0.05$* vs control group (mean ± SD, n = 8).

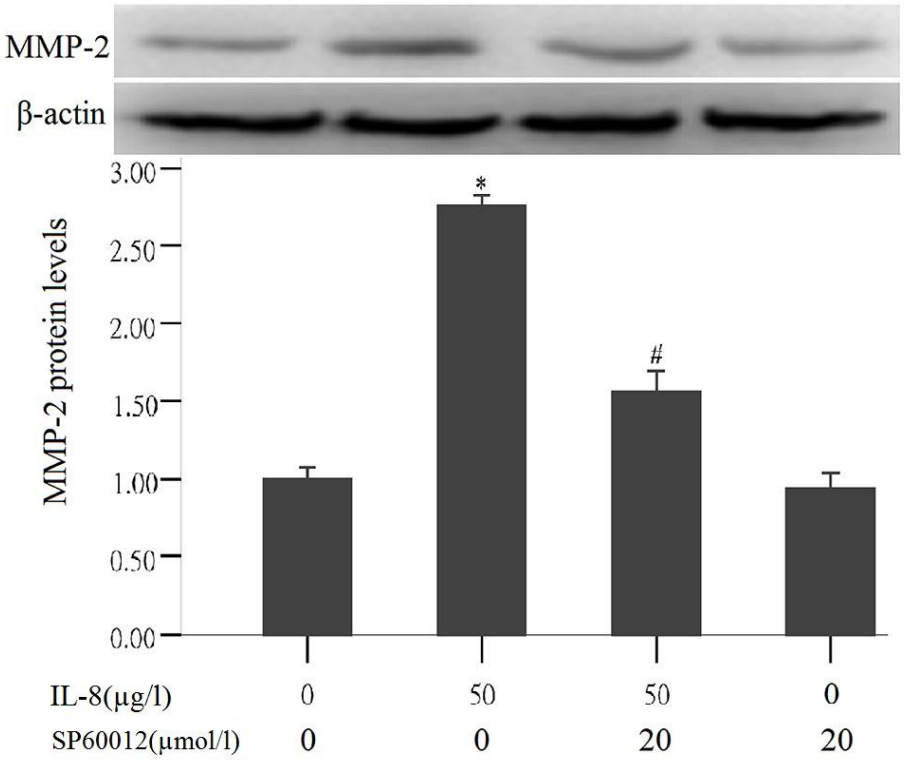

Figure 6. *Effects of C-JNK/SAPK inhibitor SP600125 on protein expressions of MMP-2. * P<0.05 vs. control group, # P<0.05 vs. 50μg/l IL-8 group (mean ± SD, n = 8).*

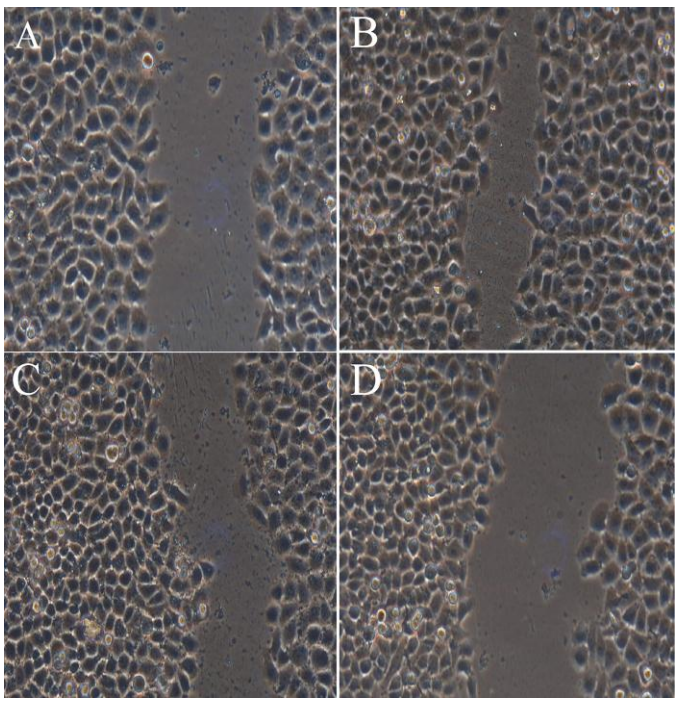

Figure 7. *Effects of lower protein expressions of MMP-2 on cell migration was measured by Wound-healing experiment (200×). A: control group, B: 50 μg/l IL-8 group, C: C-JNK/SAPK inhibitor SP600125+50 μg/l IL-8 group, D: C-JNK/SAPK inhibitor SP600125 group.*

Effect of MMP-2 protein expression on lung adenocarcinoma A549 cell migration

To further validate the relationship between MMP-2 protein expression and lung adenocarcinoma A549 cell migration, the cells were treated in the same way as above and followed with scratch test. The scratch test results showed that the cells in each group migrated from the scratch edge to the scratch center.The remaining distance was (10.5±1.575) mm, (4±0.6) mm, (7.5±1.125) mm and (12±1.8) mm respectively. Compared with MMP-2 protein high expression group (50 μg/l IL-8 group), MMP-2 protein low expression group (C-JNK/SAPK inhibitor SP600125+50 μg/l IL-8 group) can significantly inhibit the migration of A549 cells (P<0.05).

In the tests about effect of IL-8 on MMP-2 and MMP-9 protein expression and on phosphorylated protein expression in signal pathway JNK/SAPK, it was the 50μ g/l of IL-8 that always produced the highest results.In the later tests about effect of C-JNK/SAPK inhibitor (SP600125) on MMP-2 protein expression and effect of MMP-2 protein expression on lung adenocarcinoma A549 cell migration, only the 50μ g/l of IL-8were used.

Discussion

In recent years, the incidence of lung cancer has increased significantly in China. Among all the cancers leading to

death, lung cancer ranks first in the mortality rate. Early tumor cell invasion and metastasis is the main cause of death for cancer patients. Therefore, investigating the mechanism of tumor invasion and metastasis will contribute to the treatment of tumors. Thisstudy demonstrated that IL-8 might promote the migration of lung adenocarcinoma A549 cells through regulating MMP-2 expression in signal pathway JNK/SAPK.

During tumor growth and progression, a variety of cells like lymphocytes and neutrophils in the tumor tissues and its surroundings can secrete IL-8. Several studies had shown that IL-8 is associated with tumor angiogenesis, invasion and metastasis. For example, Desai S et al. [8] reported that IL-8/VEGF induced JNK/p38-ATF-2 as a novel pro-invasive pathway, which may be explored as potential therapeutic target to circumvent the invasiveness of lung malignancies. Lin et al [9] reported that the high expression of IL-8 in breast cancer cells was highly related to tumor invasion and angiogenesis. Chen et al [10] reported that the high IL-8 mRNA expression in non-small cell lung cancer was highly correlated with angiogenesis. However, the effect of IL-8 on lung cancer cell migration is still unclear.

Tumor metastasis is a complex multi-step process, in which one of the most critical steps is the degradation of extracellular matrix. In this study, it was found that IL-8 could promote lung cancer cell migration.

For MMP expression mechanism, Storz [12] believed that oxidative stress can promote MMP expression by activating Ras or the MAPK family members (ERK1/2, P38, JNK). Takagi et al [13] indicated that the activation of AP-1 signal pathway could promote MMP expression. For IL-8 up-regulating MMP expression mechanism, Reich, et al [14] reported that IL-8 could induce MMP-2 production by activating phospholipase D (PLD)-mediated signal transduction pathway. However, Luca et al [5] held that IL-8 could promote the combination of MMP-2 promoter and chloramphenicol acetyltransferae (CAT) gene, thus enhancing the expression of MMP-2. Based on the previous studies, we investigated the effect of IL-8 on JNK/SAPK signal pathway in MAPK family, and the results confirmed that IL-8 could regulate MMP-2 expression by increasing JNK/SAPK phosphorylation. JNK/SAPK signal pathway is one of the MAPK signal transduction pathways. The effect of other MAPK signal transduction pathways like ERK1/2 and P38MAPK signal transduction pathways on IL-8 induced MMP-2 expression shall be studied further.

IL -8 is both a chemotactic factor and a pro-angiogenic factor. The results of several studies showed that IL-8 can promote tumor angiogenesis, invasion and metastasis [10,15]. The current study showed that anti-IL-8 monoclonal antibody could inhibit growth, angiogenesis and metastasis of melanomain confirmation of a previous study by Huang et al. [16]. Anti-IL-8 monoclonal antibody can also inhibit tumor growth of bladder-transplantedcells by down-regulating the expression of MMP and NF-κB [17]. Although there are still many unresolved problems in the study of IL-8 as the therapeutic target, investigating the mechanism of IL-8 on lung cancer might provide a new target for comprehensive treatment of lung cancer.

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Correspondence to:

Jiang Yuequan
Department of Thoracic Surgery
Chongqing Cancer Institute
Sha Ping Ba District
Han Yu Street 181#, Chongqing 400030
China