Antitumor activity of interleukin-18 on A549 human lung cancer cell line

ABSTRACT

Objective: In this study, we investigated the antitumor activity of interleukin (IL)-18 on A549 human lung cancer cell line and evaluated the potential of IL-18 therapy in lung cancer.

Materials and Methods: We generated a human IL-18 lentiviral expression vector and examined three groups of A549 cells, including nontransduced cells and cells transduced with either IL-18 or an empty lentiviral expression vector. IL-18 expression, cell proliferation, and apoptosis were examined using Western blotting, methylthiazolyldiphenyl-tetrazolium bromide assay, and flow cytometry, respectively. The expression of the Th helper 1 (Th1) cytokine interferon-γ (IFN-γ) and Th2 cytokine IL-4 was analyzed by enzyme-linked immunosorbent assay (ELISA).

Results: Compared to the other groups of cells, A549 cells transduced with the IL-18 lentiviral expression vector exhibited significant increases in IL-18 expression, apoptosis, and the fraction of cells in G0 and G1 phases of the cell cycle and significant decrease in proliferation. Furthermore, ELISA results showed that IFN-γ expression increased significantly and IL-4 decreased in A549 cells transfected with IL-18 lentivirus expression vector.

Conclusion: Using a lentiviral expression vector, IL-18 was expressed stably and efficiently in A549 cells, which showed attenuated proliferation and tumor cell growth, and enhanced tumor cell apoptosis. IL-18 expression also induced the secretion of IFN-γ, while decreasing the production of IL-4, therefore restoring the balance between Th1/Th2 cell subsets. These findings further demonstrated the antitumor activity of IL-18 and might open new therapeutic avenues for the prevention and treatment of lung cancer.

KEY WORDS: Apoptosis, interleukin-18, lentiviral vector, lung cancer, proliferation

INTRODUCTION

In recent years, cancer biotherapies, mainly cancer gene therapies and immunotherapies, have made significant progress, driven by the rapid development of modern cell biology and recombinant DNA technology. Interleukin (IL)-18 (IL-18) is a multifunctional cytokine, which has been shown to exhibit antitumor, anti-infection, and immunoregulatory activities. While studies investigating the potential applications of IL-18 therapy in lung cancer treatment have been limited in China, this area of research has been actively pursued in other parts of the world. Nevertheless, further expanding these studies may have far-reaching significance in developing new strategies for the prevention and treatment of lung cancer. In this study, we investigated the effects of IL-18 expression in lung cancer cells and the underlying mechanism. To this end, we examined the proliferation, growth rate, and apoptosis of A549 human lung cancer cell line after transduction with a lentiviral expression vector expressing IL-18.

MATERIALS AND METHODS

Cells and reagents

The A549 human lung adenocarcinoma epithelial cell line was purchased from NeuronBiotech (Shanghai, China). The lentiviral plasmid, also encoding the enhanced green fluorescent protein (EGFP), was purchased from Hanbio (Shanghai, China). Polymethylene, pancreatin, methylthiazolyldiphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide were purchased from Sigma (St. Louis, MO, USA). The Roswell Park Memorial Institute (RPMI) 1640
culture medium was purchased from Gibco (Grand Island, NY, USA). The human interferon gamma (IFN-γ) and human IL-4 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Western blotting (WB) reagents were purchased from Abcam (Cambridge, MA, USA). Additional reagents were purchased from Chinese suppliers.

Construction of the interleukin-18 lentiviral expression plasmid and cell lines used in this study
In-Fusion Cloning (TaKaRa, Beijing, China) was used to construct a human IL-18 lentiviral expression plasmid also carrying EGFP as a reporter of transduction efficiency. The lentiviral plasmid construction was performed by NeuronBiotech (Shanghai, China).

Subsequent experiments were performed on three groups of A549 cells: the IL-18 intervention group (Group A) corresponded to A549 cells transduced with the IL-18 lentiviral expression vector, the empty lentivector group (Group B) corresponded to A549 cells transduced with the parental lentiviral expression vector (i.e., not carrying the human IL18 gene), and the blank control group (Group C) corresponded to A549 cells without any intervention.

Western blotting
Proteins from each group of cells were isolated 24 h and 96 h after lentiviral expression vector transduction. The protein concentration was determined using the bichinchoninic acid protein assay. After adjusting the protein concentration of each sample, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide gel) and transferred to a polyvinylidene fluoride membrane following electrophoresis. The membrane was blocked at 4°C with 5% nonfat dry milk for 2 h and then incubated overnight at 4°C on a rocking platform with IL-18 (1:1000) or internal reference (β-actin, 1:1000) primary antibodies. Next, the membrane was washed with Tris-Buffered Saline and Tween (TBST) and incubated at room temperature for 2 h with horseradish peroxidase-conjugated secondary antibodies (1:5000). Finally, the membrane was treated with Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and exposed to films to detect the protein bands.

Methylthiazolyldiphenyl-tetrazolium bromide assay
Cells from each group were seeded into 96-well plates at a density of 3 × 10^{4} cells/well using 100 µl of medium per well. The plates were incubated at 37°C, 5% CO₂, and saturated humidity. The MTT assay was performed after 1–4 days of incubation. After incubation with the MTT reagent, the absorbance was measured at a wavelength of 490 nm optical density (OD₄₉₀) using a microplate spectrophotometer. The final OD₄₉₀ was determined as the mean value of six independent wells. A growth curve was drawn, with the time of incubation as the horizontal axis and the OD₄₉₀ as the vertical axis, to examine cell proliferation. The inhibition rate for cell proliferation was calculated using the following formula: inhibition rate = 1 − (OD₄₉₀ value for Group A or B)/OD₄₉₀ value for Group C × 100%.

Flow cytometry assays
All cells were incubated for 96 h at 37°C, 5% CO₂, and saturated humidity. After washing two times with ice-cold phosphate-buffered saline (PBS), 1 × 10⁶ cells from each group were digested with trypsin and centrifuged for 5 min at 1000 × g, and the supernatant was discarded. For apoptosis, 500 µl of binding buffer was used to gently resuspend the cells. Then, 5 µl each of annexin V Fluorescein Isothiocyanate-FITC and propidium iodide (PI) was added to the cell suspension. After mixing, the cells were stained for 15 min at room temperature and analyzed using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). For cell cycle progression, the cells were washed with ice-cold PBS, fixed with 70% ethanol, centrifuged, and collected. The pelleted cells were washed with PBS and resuspended in 500 µl of PI staining solution. The cells were stained for 30 min at 4°C avoiding light and analyzed using a BD FACSCalibur flow cytometer. The data were analyzed with ModFit 4.0 (Verity Software House, Washington, USA).

Enzyme-linked immunosorbent assay
The levels of IFN-γ and IL-4 present in the cell supernatants were measured using commercial sandwich ELISA kits with 100 µl of diluted supernatant (1:10). Briefly, the samples were added to plates precoated with the IFN-γ or IL-4 monoclonal antibody and incubated for 2 h at 37°C and 5% CO₂. Next, the biotin-conjugated rabbit polyclonal antibody was added to each well, and the plate was incubated for 1 h at 37°C and 5% CO₂. After three washes, 100 µl of avidin conjugated to horseradish peroxidase was added to each well, and the plate was incubated for 30 min at 37°C. After five washes, 100 µl of the substrate solution was added to each well, and the plate was incubated for 30 min at 37°C in dark, before the addition of 100 µl of stop solution to each well. The absorbance was then measured at a wavelength of 450 nm using a fully automated enzyme mark instrument (Thermo Scientific, USA). The values for the test samples were calculated based on a standard curve.

Statistical analysis
All data were processed using the IBM SPSS Statistics 19.0 software (IBM Corp.). All quantitative data were expressed as mean values ± standard deviation. A one-way ANOVA was performed to analyze the differences between the groups. P < 0.05 indicated a statistically significant difference.

RESULTS
Interleukin-18 expression in A549 lung cancer cells following lentiviral vector transduction
WB analysis performed 24 h after lentiviral vector transduction did not show significant differences in the levels of IL-18

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expression among the three groups of cells examined. In contrast, 96 h posttransduction, IL-18 expression was significantly higher in the cells from Group A, transduced with the IL-18 lentiviral expression vector, than in the cells from Groups B and C ($P < 0.05$). In contrast, the levels of IL-18 expression remained comparable between the cells from Groups B and C [Table 1 and Figures 1 and 2].

**Effects of interleukin-18 expression on the proliferation of A549 cells**

The MTT assay was used to examine the proliferation of A549 cells 24 h, 48 h, 72 h, and 96 h after transduction with the IL-18 lentiviral expression vector, and the corresponding growth curves were generated. The comparison of the growth curves showed that the growth rate of the cells from Group A was significantly lower than that of the cells from Groups B and C. This observation strongly suggested that IL-18 inhibited the proliferation of A549 cells. Meanwhile, the cells in Groups B and C exhibited comparable growth rates, which indicated that transduction with an empty lentiviral expression vector did not affect the proliferation of A549 cells [Figure 3].

The MTT assay was also used to measure the proliferative capacity of A549 cells at 24 h, 48 h, 72 h, and 96 h posttransduction. While the proliferative capacity of the three groups of cells was comparable at 24 h, the proliferative capacity of the cells from Group A was significantly lower than that of the cells from Groups B and C at 48 h, 72 h, and 96 h ($P < 0.05$). In contrast, the proliferative capacity of the cells from Groups B and C remained comparable throughout the experiment. Moreover, at 48 h, 72 h, and 96 h, the inhibition rate of the cells from Group A was statistically significantly higher than that of the cells from Group B ($P < 0.05$) [Tables 2 and 3 and Figure 4].

**Interleukin-18 expression-induced apoptosis in A549 cells**

Cells from each group examined were cultured for 96 h, stained with Annexin V-FITC/PI, and analyzed by flow cytometry to

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**Table 1: The expression of interleukin-18 after 24 h and 96 h in the culture of cells of three groups (mean±standard deviation, n=6)**

| Groups | 24 h       | 96 h       |
|--------|------------|------------|
| Group A| 0.521±0.047| 0.774±0.061*** |
| Group B| 0.490±0.044| 0.490±0.057 |
| Group C| 0.468±0.042| 0.488±0.054 |

Compared with Groups B and C. ***$P<0.001$

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**Figure 1:** The Western blotting test results. 1: Group B at 96 h; 2: Group C at 96 h; 3: Group A at 96 h; 4: Group B at 24 h; 5: Group C at 24 h; 6: Group A at 24 h

**Figure 2:** Expression of interleukin-18 in three groups shown by Western blotting. Compared with Groups B and C, ***$P < 0.001$**

**Figure 3:** The growth curve of cells of the three groups

**Figure 4:** Inhibitory rate of Group A cells at different times compared with Groups B and C, *$P < 0.05$**
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determine the rate of apoptosis. Whereas the rate of apoptosis was similar for the cells from Groups B and C, the rate of apoptosis was statistically significantly higher for the cells from Group A \( (P < 0.05) \) [Table 4 and Figures 5 and 6].

Effects of interleukin-18 expression on the cell cycle progression of A549 cells
Flow cytometry was used to analyze the cell cycle distribution 96 h after transduction with the IL-18 lentiviral expression vector. The results showed that there was no difference in the cell cycle distribution of the cells from Groups B and C, demonstrating that transduction with an empty lentiviral expression vector did not affect the cell cycle progression of A549 cells. However, the number of cells in G0 and G1 phases of the cell cycle was statistically significantly higher in Group A \( (85.87 \pm 0.09) \) than in Groups B \( (84.17 \pm 0.43) \) and C \( (83.76 \pm 0.91) \) \( (P < 0.05) \). Concomitantly, the number of cells in S and M phases of the cell cycle was statistically significantly lower in Group A \( (14.13 \pm 0.09) \) than in Groups B \( (16.83 \pm 0.43) \) and C \( (16.24 \pm 0.91) \) \( (P < 0.05) \) [Table 5 and Figure 7]. Collectively, these data suggested that IL-18 expression in lung cancer cells inhibited mitosis and therefore cell growth.

Changes in T-cell cytokine production following interleukin-18 expression in A549 cells
The production of the T helper 1 (Th1) cytokine IFN-\( \gamma \) and the Th2 cytokine IL-4 was examined by ELISA. At 24 h posttransduction, the production of IFN-\( \gamma \) and IL-4 was statistically significantly higher in the cells from Group A than in the cells from Groups B and C \( (P < 0.05) \). However, the IFN-\( \gamma \)/IL-4 ratio remained similar for the three groups of cells. Furthermore, at 96 h posttransduction, the production of IFN-\( \gamma \) and IL-4 was statistically significantly higher in the cells from Group A than in the cells from Groups B and C \( (P < 0.05) \). Remarkably, the IFN-\( \gamma \)/IL-4 ratio was statistically significantly higher for the cells from Group A than for the cells from Groups B and C \( (P < 0.05) \). Importantly, the cells from Groups B and C exhibited no difference in the production of IFN-\( \gamma \) and IL-4 and in the IFN-\( \gamma \)/IL-4 ratio [Tables 6-8].

DISCUSSION
Many studies have shown that cytokines play an important role in the development of non-small cell lung cancer (NSCLC). In 2016, Zhang et al.\(^{[4]}\) found that the serum level of IF-20 increased in patients with lung adenocarcinoma, and...
chemotherapy could inhibit the increase of IF-20. In 2018, Vinocha et al.\cite{5} found that the overexpression of IF-6 was related to the occurrence and development of lung cancer. Studies have shown that the detection of programmed cell death protein-1, programmed death-ligand 1, tumor mutational burden, and other factors in serum plays an vital part in guiding the treatment of NSCLC.\cite{6,7} Our researches on NSCLC have been involved in targeting molecule,\cite{8} the reversal of drug resistance in NSCLC\cite{9,10} and risk assessment after NSCLC recovery.\cite{11} Our previous study of targeting molecules\cite{12} detected estrogen receptor (ER) expression in tumor tissues and normal lung tissues of 67 NSCLC patients by immunohistochemistry. We found that ER-positive rate in NSCLC tissues was higher than that in normal lung tissues. ER expression was associated with tumor differentiation, but not with gender, age, tumor histologic type, tumor size, lymph node metastasis, or clinical tumor, node, metastasis staging.

Numerous studies have shown that IL-18 exhibits a variety of biological activities, while its powerful antitumor activity has attracted significant attention in the field of cancer research. Indeed, IL-18 can activate Th cells, which produce cytokines that interact with activated natural killer cells and mediate the antitumor activity of IL-18. Furthermore, IL-18 has anti-angiogenic and pro-lymphangiogenesis properties, which contribute to its antitumor activity. Therefore, the clinical applications of IL-18 therapy show high potential for cancer treatment.\cite{12,13} Moreover, Lu et al.\cite{14} have demonstrated that the SMMC-7721 human hepatocellular carcinoma cell line transfected with the IL18 gene could stably secrete the IL-18 protein. In this study, the IL18 gene was directly cloned into a lentiviral plasmid, and lentiviral expression vectors encoding IL-18 were obtained using 293T cells transfected with three lentiviral plasmid systems. The resulting IL-18 lentiviral expression vector was then

| Table 4: The apoptosis rate of cells of three groups after 96 h (percentage, mean±standard deviation, n=3) |
|-----------------|-----------------|
| Groups          | Apoptosis rate  |
| Group C         | 11.01±0.64      |
| Group B         | 10.70±0.56      |
| Group A         | 17.89±1.30***   |
| Compared with Group B and C, ***P<0.001 |

| Table 5: The cell cycle of cells of three groups after 96 h (percentage, mean±standard deviation, n=3) |
|-----------------|-----------------|
| Groups          | G0/G1          | S/M          |
| Group C         | 83.76±0.91     | 16.24±0.91   |
| Group B         | 84.17±0.43     | 16.83±0.43   |
| Group A         | 85.87±0.09*    | 14.13±0.09*  |
| Compared with Groups B and C, *P<0.05 |

| Table 6: Secretion of interferon-γ in cells of three groups (pg/ml, mean±standard deviation, n=6) |
|-----------------|-----------------|
| Groups          | IFN-γ 24 h     | IFN-γ 96 h   |
| Group C         | 109.93±1.46    | 124.09±3.01  |
| Group B         | 108.71±1.64    | 130.11±0.59  |
| Group A         | 129.01±1.76*** | 155.58±0.43*** |
| ***P < 0.001. IFN-γ=Interferon-γ |

Figure 7: The cell cycle of cells of three groups after 96 h. (a) Group C. (b) Group B. (c) Group A
Table 7: Secretion of interleukin-4 in cells of three groups (pg/ml, mean±standard deviation, n=6)

| Groups   | IL-4          |
|----------|---------------|
|          | 24 h          | 96 h |
| Group C  | 63.34±0.73    | 79.42±3.17 |
| Group B  | 62.76±0.96    | 82.00±3.04 |
| Group A  | 74.59±1.22*** | 84.80±1.26* |

***P < 0.001. IL=Interleukin

Table 8: Secretion of interferon-γ/interleukin-4 in cells of three groups (mean±standard deviation, n=6)

| Groups   | IFN-γ/IL-4   |
|----------|--------------|
|          | 24 h          | 96 h |
| Group C  | 1.74±0.13     | 1.56±0.04 |
| Group B  | 1.73±0.007    | 1.59±0.05 |
| Group A  | 1.79±0.01     | 1.84±0.03*** |

Compared with Groups B and C ***P<0.001; IFN-γ=Interferon-γ, IL=Interleukin

successfully used to express IL-18 stably and efficiently in A549 lung cancer cells.

Tumor growth is influenced by numerous factors, such as tumor cell proliferation kinetics, tumor angiogenesis, or tumor progression, to form new and more aggressive subclones. Furthermore, the growth rate of tumor cells depends mostly on the duration of the cell cycle, the proliferative capacity, and the balance between cell proliferation and cell loss.\(^{15-17}\) For example, Nilkaeo and Bhuvanath\(^{18}\) have previously shown that increasing concentrations of IL-18 (0–100 ng/mL) could inhibit the proliferation of the KB human oral carcinoma cell line in a dose-dependent manner, through the induction of cell cycle arrest in the S phase. In line with these findings, our data suggested that lentiviral-mediated expression of IL-18 could inhibit the growth of A549 cell line in a time-dependent manner. In contrast, transduction with an empty lentiviral expression vector did not affect A549 cell growth, indicating that the lentiviral vector had no toxic effect on A549 cells.

Moreover, numerous studies have shown that during cell proliferation, apoptosis is often induced to regulate the development of tissues and organs. However, a disruption of the balance between cell proliferation and apoptosis has been involved in various serious diseases. Accordingly, the abnormal proliferation and uncontrolled growth of tumor cells have been associated with an inhibition of apoptosis. Notably, Nagai et al.\(^{19}\) have reported that in mice, tumors derived from B16F10 melanoma cells transfected with the IL18 gene exhibited delayed growth. The histological analysis of such tumors showed a significant reduction in the number of tumor blood vessels with a concomitant and significant increase in tumor cell apoptosis. Lu et al.\(^{14}\) have also shown that in mice, IL-18 expression in tumors derived from SMMC-7721 cells induced apoptosis. Furthermore, adenovirus-mediated IL18 gene transfer into ZD55 melanoma cells has been reported to induce apoptosis.\(^{15}\) In agreement with these findings, our data suggested that IL-18 expression could induce tumor cell apoptosis and regulate cell cycle progression.

Numerous studies have shown that tumor tissues secrete high levels of Th2 cytokines, which is considered detrimental for cancer patients. Accordingly, the Th1/Th2 shift, an important mechanism in tumor immune escape, has attracted significant attention in the field of cancer research.\(^{20-23}\) Notably, IL-18 can stimulate the differentiation of proliferative Th0 cells into Th1 cells, which, in turn, promotes the secretion of Th1 cytokines, enhances Th1 immune response, and contributes to the antitumor activity of IL-18.\(^{24}\) In 2016, Shaobin et al.\(^{25}\) found that the level of Th1 cells declined in lung cancer patients, after radiofrequency ablation (RFA), the level of Th1 cells and Th1/Th2 increased, whereas the levels of Th2, Th17, and Treg cells declined, indicating an improvement of antitumor immunity. Importantly, our data showed that A549 cells transduced with the IL-18 lentiviral expression vector increased the secretion of the Th1 cytokine IFN-γ and concomitantly decreased the secretion of the Th2 cytokine IL-4. Based on this observation, we propose that IL-18 expression could reverse an imbalance between the Th1/Th2 cell subsets and therefore prevent tumor immune escape.

Collectively, our data showed that an IL-18 lentiviral expression vector could be used to express IL-18 stably and efficiently in transduced A549 cells, which showed attenuated tumor cell growth, enhanced tumor cell apoptosis, and regulated cell cycle progression. Furthermore, IL-18 expression promoted the secretion of the Th1 cytokine IFN-γ and suppressed the production of the Th2 cytokine IL-4, therefore reversing an imbalance between the Th1/Th2 cell subsets and mediating potent antitumor effects.

**CONCLUSION**

This study provides experimental evidence supporting the notion that IL-18 therapy represents a novel approach for the treatment of lung cancer.

**Financial support and sponsorship**

This study was supported by grants from the Natural Science Foundation of Fujian province (No. 2011J01170) and the Nursery Fund of Fujian Medical University (No. 2010MP034).

**Conflicts of interest**

There are no conflicts of interest.

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