The Cyclin Dependent Kinase Inhibitor Protein 21 Cytoplasmic Expression Depressing the Apoptosis of Human Bronchial Epithelial Cell

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**Abstract:** AIMS: To investigate the cytoplasmic P21 expression regulating the apoptosis of human bronchial epithelial cell. METHODS: The relationship of the cytoplasmic P21 expression with the apoptosis of 16HBE cells was studied after the plasmid pEGFP-N1-p21 was transfected into the 16HBE cells. After the 16HBE cells was stimulated by the TGF-β1, the cytoplasmic and nucleic P21 expression and the apoptosis of 16HBE cell was detected, then the relationship of the P21 expression with the apoptosis of 16HBE cells was studied. RESULTS: The 16HBE cell had the basic low cytoplasmic and mainly high nucleic P21 protein expression, the plasmid pEGFP-N1-p21 could express P21 protein only in the cytoplasm of 16HBE cell and did not affect the nucleic P21 protein level. The apoptosis of 16HBE cells after transfection of the pEGFP-N1-p21 decreased. The apoptosis of 16HBE cells decreased as the time of the pEGFP-N1-p21 transfection increased, but the apoptosis of 16HBE cells increased without the pEGFP-N1-p21 transfection. The stimulation by TGF-β1 led to the expression of the cytoplasmic and nucleic P21 proteins, but mainly the cytoplasmic P21 protein expression, as the stimulation concentration of TGF-β1 increased, the cytoplasmic P21 expression decreased, but the nucleic P21 did not change. The apoptosis of 16HBE cells increased as the cytoplasmic P21 expression decreased after the concentration of TGF-β1 stimulation increased. CONCLUSIONS: The apoptosis of 16HBE cell was inhibited by the high cytoplasmic P21 expression through the transfection of pEGFP-N1-p21. TGF-β1 stimulation promoted the apoptosis of 16HBE cell by inhibiting the cytoplasmic P21 expression. The cytoplasmic P21 expression depresses the apoptosis of 16HBE cells.

**Keywords:** Cyclin Dependent Kinase Inhibitor P21, Transforming Growth Factor-β1, Apoptosis

1. Introduction

The apoptosis and the inflammation interact with each other to lead to occurrence, development of lung diseases [1]. The apoptosis is a complex procedure regulated by many factors including the cyclin dependent kinase inhibitor protein 21 (P21) and cytokine as transforming growth factor-β1 (TGF-β1) [2, 3]. In cancer and other premature cells P21 inhibits the cell cycles to make cells apoptosis and death when highly expressing in the nuclei of these cells and inhibits the cells apoptosis when highly expressing in the cytoplasm of these cells [4]. In earlier researches P21 was found highly expressing in the smoker, alveolar macrophages, airway epithelial cells and asthmatic bronchial epithelial cells and the expression of P21 correlated with the apoptosis these cells [3, 5]. The earlier researches did not study the relationships of cell apoptosis with the P21 expression in cytoplasm or in nuclear in the lung cells. Transforming growth factor (TGF)-β family proteins are multifunctional cytokines that have been implicated in the pathogenesis of diverse biologic processes including cell growth and survival, cell and tissue differentiation, development, inflammation, immunity, hematopoiesis, and tissue remodeling and repair [6]. TGF-β1 can induce apoptosis through P21 [7]. There haven’t studies about the relationship of the cytoplasmic P21 expression and the cell...
apoptosis under the stimulation of TGF-β1. This research has studied the relationship of the P21 cytoplasmic expression with the apoptosis in the human bronchial epithelial cell 16HBE and that under the stimulation by TGF-β1.

2. Materials and Methods

2.1. Cell Recovery and Culture

The human epithelial cell line 16HBE (The Experimental Medical Research Center, Guangzhou Medical University, China) was recovered from the liquid nitrogen vessel and cultured in the Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal bovine serum (Gibco) at 37°C and 5% CO2.

2.2. Preparation of the Plasmid

The plasmid PEGFP-N1-p21 purchased from the Shanghai Invitrogen Corporation was amplified in the Escherichia coli and extracted according to the structures of the endotoxin free Plasmid Extraction kit (OMEGA Corporation).

2.3. Transfection and Analysis of the Expression of the Plasmid PEGFP-N1-p21

The experiment groups included the groups of the blank, the empty plasmid and the plasmid PEGFP-N1-p21. The transfection processes were done according to the structures of the Lipofactamine 2000 transfection kit (American Invitrogen Corporation) with some modifications. The 12 holes transfection plate was used and each experiment group had 4 holes. The 5×10^5 16HBE cells were planted in the each transfection hole. The quality of the plasmid was 8 µg.

After the transfection, the expressions of PEGFP-N1-p21 were checked by the analysis of the mRNA and the protein of P21 in the nuclear and the cytoplasm. The total RNA was extracted according to the structures of the RNA Extracted Kit and the c-DNA was synthesized with the primer 5’CCGTGGGAAGGTAGAGCTTG 3’. The 20ml reaction mixture included the total RNA 5µl, 2×TS Reaction Mix 10µl, RT/RI Mix 1µl, ribonuclease free H2O 4µl. It was incubated at the 42°C for 30mins and heated at the 85°C for 5mins. After the c-DNA synthesized, the RT-PCR of P21 mRNA was conducted together with the control of β-actin. The P21 primer was 5’GACTGTGA TGCGCTAA TGGC 3’and the reverse primer was 5’CCGTGGGAAGGTAGAGCTTG 3’. The 50µl TR-PCR reaction mixture included cDNA 2µl, P21 primers 1µl, β-actin primers 1µl, 10×buffer 5µl, dNTPs 4µl, DNA-polymerase 0.5µl, ddH2O 29.5µl. The PCR reaction conditions were pre-denaturation at 94°C 30s, splitting at 94°C 5min, annealing at 60°C 30s, extending at 72°C 30s, 35circles, extending at 72°C 10min. The PCR reaction products were electrophoresised, the bands of P21 and β-actin cDNA were scanned by BIO-RAD, the ratio of the gray levels of the bands of P21 and β-actin cDNA represented the levels of the P21 mRNA.

2.4. Stimulation to the Human Epithelial Cell Line 16HBEs by TGF-β1

The 1-5×10^6 16HBE cells were cultured in the 6 holes culture plates. Before the stimulation, the cells were cultured in the DMEM with 0.5% FBS for 12 hours. The TGF-β1 at the concentrations of 0ng/ml, 3ng/ml, 10ng/ml were added into the different 5×10^5 16HBE cells for 12h, 24h.

2.5. Analysing the Cell Apoptosis by Flow Cytometry

The single cell suspension of 16HBE cells was centrifuged at 2000rpm for 5min. According to the structures of apoptosis kit, the cell aggregations were mixed with 500ul Binding Buffer, 5ul Annexin-FITC, 5ul PI light. The mixtures reacted at room temperature for 10minutes. The apoptosis was detected by flow cytometry. The excitation light wave length of the fluorescent dye was 488nm, and the emission light wave length was 530nm. The results showed that the lower left quadrant was living cells, the lower right quadrant was early apoptotic cells, the right upper quadrant was late apoptotic and dead cells, and the left upper quadrant was dead cells.

2.6. Checking the Expression of P21 in the Cytoplasm and the Nuclear

The cytoplasm and nuclear proteins were extracted according to the structures of the cytoplasm and nuclear protein extraction kits (Sigma). The expression of P21 was detected by the Western-blot. The electrophoretic gel was made up with 15% separation gel and 5%concentration gel. The denatured P21 samples mixed with 5× SDS buffer and the pre-stained protein Makers of 7ul at one side and 3ul at the other side were added into the electrophoretic gel pores. The electrophoresis was conducted at 80V before the dye got into the concentration gel and conducted at 120V when the dye got into the separation gel. After the end of electrophoresis, the concentration gel was removed and the separation gel was soaked in the transfer buffer. The PVDF membrane was soaked in the methanol for 30s, placed in the transfer buffer. The transfer membrane sandwich of the filter paper -PVDF film - gel - filter paper from the anode to the cathode stacked was placed in the wet transfer instrument board. The electrical transfer was conducted at 276mA constant current for 90min. After electrical transfer, the PVDF film was immersed and slowly swayed in the sealing solution containing 5% skim milk powder for 2 hours. After sealing, the PVDF film was taken out and immersed into the solution with the mouse anti-P21 monoclonal antibody (1: 1000) and mouse anti-β-tubulin monoclonal antibody (1: 1200), swayed at 4°C overnight. At the second day the PVDF film was taken out and washed with the TBST solution10minfor 3 times, then immersed into the solution with goat anti-mouse fluorescence antibody (1:5000) and swayed at room temperature for 2h. The PVDF film with the antibodies was washed with TBST 10min for three times. The PVDF film with the antibodies was added with ECL fluorescent agent and exposed with machine. The results of Western blot were...
analyzed by image J analysis system, the ratios of gray value of target protein band to the reference protein band represented the quantities of the target protein.

2.7. Statistical Analysis

SPSS17.0 statistical software package was used for the data analysis. The measurement data were expressed by mean±standard deviation (M±S). Paired t test was used for paired data and the single factor variance analysis (one-way-ANOVA) was used to compare the multiple sample means. The means of within and among groups was compared with LSD method. The difference between the groups was statistically significant by P < 0.05.

3. Results

3.1. The Gene Expression of the Plasmid PEGFP-N1-p21 Being Transfected Into 16HBE

After the transfection, the expression of the PEGFP-N1-p21 was checked by RT-PCR. The electrophoresis results of PEGFP-N1-p21 and β-actin CDNA was presented in the Figure 1 and the table 1. This result presented that the 16HBE cell had the basic low cytoplasmic and mainly high nucleic P21 protein expression, the plasmid PEGFP-N1-p21 could express P21 protein only in the cytoplasm of 16HBE cell and did not affect the nucleic P21 protein level.

![Figure 1. The electrophoresis bands of the PEGFpN1-p21 (1, 4), the PEGFP (2, 5) and the blank (3, 6). the left band: 100bp DNA mark.](image)

Table 1. The quantitative analysis of the mRNA expressions of the PEGFP-N1-p21 after transfection.

<table>
<thead>
<tr>
<th>groups</th>
<th>protein P21 expression</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>cytoplasmic</td>
<td>1.74±0.102</td>
<td>20.859</td>
<td>0.002</td>
</tr>
<tr>
<td>nuclear</td>
<td>0.41±0.096</td>
<td>11.981</td>
<td>0.007</td>
</tr>
<tr>
<td>blank</td>
<td>0.19±0.073</td>
<td>5.349</td>
<td>0.033</td>
</tr>
<tr>
<td>P</td>
<td>0.000</td>
<td>1.74±0.102</td>
<td>0.41±0.096</td>
</tr>
</tbody>
</table>

Table 2 The difference of the cytoplasmic protein levels of the PEGFP-N1-p21 group, the PEGFP group and the blank was statistically significant (F=383.989, P < 0.05), and the difference of the nucleic protein levels of the PEGFP-N1-p21 group, the PEGFP group and the blank was not statistically significant (F=0.049, P > 0.05). In the PEGFP-N1-p21 group the cytoplasmic P21 level was higher than the nucleic protein level (t=20.859, P < 0.05). In the PEGFP and the blank group the cytoplasmic P21 protein level was lower than the nucleic protein level (t=5.349, P < 0.05; t=11.981, P < 0.05).

3.2. The Expression of the Protein of Plasmid PEGFP-N1-p21 Being Transfected Into 16HBE

After the transfection, the cytoplasmic and nuclear P21 proteins of the plasmid PEGFP-N1-p21, the plasmid PEGFP and the blank were extracted respectively and checked by Western-blot. The Western-blot results of P21 proteins were presented in the Figure 2. The comparisons of the relative quantities of the cytoplasmic and nuclear P21 proteins were presented in the Table 2. These results showed the 16HBE cell had the basic low cytoplasmic and mainly high nucleic P21 protein expression, the plasmid PEGFP-N1-p21 could express P21 protein only in the cytoplasm of 16HBE cell and did not affect the nucleic P21 protein level.

![Figure 2. The Western-blot results of P21 proteins after transfection: 1 the cytoplasmic P21 protein of plasmid PEGFP-N1-p21; 2 the cytoplasmic P21 protein of the plasmid PEGFP; 3 the cytoplasmic P21 protein of the blank. 4 the nucleic P21 protein of plasmid PEGFP-N1-p21; 5 the nucleic P21 proteins of the plasmid PEGFP; 6 the nucleic P21 protein of the blank.](image)

Table 2 The quantitative analysis of the protein expressions of the PEGFpN1-p21 after transfection.

<table>
<thead>
<tr>
<th>groups</th>
<th>protein P21 expression</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>cytoplasmic</td>
<td>1.74±0.102</td>
<td>20.859</td>
<td>0.002</td>
</tr>
<tr>
<td>nuclear</td>
<td>0.41±0.096</td>
<td>11.981</td>
<td>0.007</td>
</tr>
<tr>
<td>blank</td>
<td>0.19±0.073</td>
<td>5.349</td>
<td>0.033</td>
</tr>
<tr>
<td>P</td>
<td>0.000</td>
<td>1.74±0.102</td>
<td>0.41±0.096</td>
</tr>
</tbody>
</table>

3.3. Analyzing the Apoptosis of 16HBE After the Transfection of Plasmids

After the transfection by the plasmids for 24 hours and 48 hours, the 16HBE cells were collected to analyze apoptosis by the flow cytometry. The percentages of apoptosis were present in the right lower quadrant of the results in the Figure 3 and Figure 4. The comparisons of the percentages of apoptosis were presented in the Table 3. These results showed that the apoptosis of 16HBE cells after transfection of the PEGFP-N1-p21 decreased. Combining the results of 3.2, it was found that the apoptosis of 16HBE cells decreased as the time of the PEGFP-N1-p21 transfection increased, but the apoptosis of 16HBE cells increased without the PEGFP-N1-p21 transfection.
3.4. Analyzing the Apoptosis of 16HBE After the Stimulation by TGF-β1

After the stimulation by TGF-β1 for 12 hours and 24 hours, the 16HBE cells were collected to analyze apoptosis by the flow cytometry. The percentages of apoptosis were present at the right lower quadrant of the results in the Figure 5, Figure 6, and Figure 7 (partly). Through the comparison of the percentages of apoptosis in the Table 4, it was found that at the same stimulation time the percentages of apoptosis increased as the stimulation concentrations of TGF-β1 increased and that at the same stimulation concentrations of TGF-β1 the percentages of apoptosis increased as the stimulation time increased. These results suggested that the stimulation of TGF-β1 promoted the 16HBE cells apoptosis.

Table 3. The quantification analysis of the apoptosis of 16HBE cells after the transfection of PEGFP-N1-p21.

<table>
<thead>
<tr>
<th>groups</th>
<th>Transfection time 24h</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEGFP-N1-p21</td>
<td>0.22±0.031*</td>
<td>17.889</td>
<td>0.036</td>
</tr>
<tr>
<td>PEGFP</td>
<td>0.35±0.010</td>
<td>19.000</td>
<td>0.033</td>
</tr>
<tr>
<td>blank</td>
<td>0.31±0.008</td>
<td>17.333</td>
<td>0.037</td>
</tr>
</tbody>
</table>

Table 3 The difference of the percentages of apoptosis of the PEGFP-N1-p21 group, the PEGFP group and the blank after 24 hours and 48 hours transfection were statistically significant (#F=23.464, P < 0.05; *F=187.265, P < 0.05). The percentages of apoptosis of the PEGFP-N1-p21 group were lower than that of the PEGFP group and the blank group after 24 hours and 48 hours transfection (P < 0.05), but the percentages of apoptosis of the PEGFP group were same as that of the blank group after 24 hours and 48 hours transfection (P > 0.05). In the PEGFP-N1-p21 group the percentages of apoptosis after 48 hours transfection was lower than that after 24 hours transfection (t=17.889, P < 0.05). In the PEGFP group and the blank group the percentages of apoptosis of 48 hours transfection was higher than that of 24 hours transfection (t=19.000, P < 0.05; t=17.333, P < 0.05).
The quantitative analysis of apoptosis of the 16HBE cells after the TGF-β1 stimulation.

<table>
<thead>
<tr>
<th>TGF-β1 (ng/ml)</th>
<th>Stimulation time</th>
<th>12 hours</th>
<th>24 hours</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>6.7±0.47</td>
<td>8.74±1.02</td>
<td>3.64</td>
<td>0.068</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>9.86±0.49</td>
<td>13.51±0.80</td>
<td>20.24</td>
<td>0.002</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>16.00±0.50</td>
<td>27.16±1.60</td>
<td>16.6</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>297.40</td>
<td>194.499</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.5. The Expression of the Protein of P21 After the Stimulation of 16HBE by TGF-β1

After the stimulation by TGF-β1 for 24 hours, the cytoplasmic and nuclear P21 proteins of the groups of 0 ng/ml TGF-β1, 3 ng/ml TGF-β1 and 10 ng/ml TGF-β1 were extracted respectively and tested by Western-blot. The results were presented at the Figure 8. The comparisons of the relative quantities of the cytoplasmic and nuclear P21 proteins of the groups of 0 ng/ml TGF-β1, 3 ng/ml TGF-β1 and 10 ng/ml TGF-β1 were presented in the Table 5. These results showed that the stimulation by TGF-β1 led to the expression of the cytoplasmic and nuclear P21 proteins, but mainly the cytoplasmic P21 protein expression. As the stimulation concentration of TGF-β1 increased, the cytoplasmic P21 expression decreased, but the nuclear P21 did not change.

![Western-blot results of P21 proteins after the stimulation of TGF-β1. The Western-blot results of β-tubulin were the control.](image)

Figure 8. The Western-blot results of P21 proteins after the stimulation of TGF-β1. The Western-blot results of β-tubulin were the control. 1. the result of nuclear P21 protein with the stimulation of 0 ng/ml TGF-β1 2. the result of nuclear P21 protein with the stimulation of 3 ng/ml TGF-β1 3. the result of nuclear P21 protein with the stimulation of 10 ng/ml TGF-β1 4. the result of cytoplasmic P21 protein with the stimulation of 0 ng/ml TGF-β1 5. the result of cytoplasmic P21 protein with the stimulation of 3 ng/ml TGF-β1 6. the result of cytoplasmic P21 protein with the stimulation of 10 ng/ml TGF-β1.

<table>
<thead>
<tr>
<th>TGF-β1 (ng/ml)</th>
<th>p21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclear P21</td>
</tr>
<tr>
<td>0</td>
<td>0.10±0.02</td>
</tr>
<tr>
<td>3</td>
<td>0.26±0.03</td>
</tr>
<tr>
<td>10</td>
<td>0.27±0.02</td>
</tr>
</tbody>
</table>

Table 5. The quantitative analysis of the expression of P21 of the 16HBE cells 24 hours after the TGF-β1 stimulation.

Table 4. The difference of the percentages of apoptosis of the 16HBE cells after the TGF-β1 stimulation.
In the group of T 10ng/ml GF-β1 the cytoplasmic P21 protein level was higher than the nuclear protein level (t=5.24, P < 0.05).

3.6. Analyzing the Relationship of Expression of the Protein of P21 and the Apoptosis of 16HBE After the Stimulation by TGF-β1

Combining the results of 3.4 and 3.5, it was found that the protein P21 mainly expressed in the cytoplasm of the 16HBE cells after the TGF-β1 stimulation, the apoptosis of 16HBE cells increased as the cytoplasmic P21 expression decreased after the concentration of TGF-β1 stimulation increased.

4. Conclusions

The apoptosis of 16HBE cell was inhibited by the high cytoplasmic P21 expression through the transfection of PEGFP-N1-p21. TGF-β1 stimulation promoted the apoptosis of 16HBE cell by inhibiting the cytoplasmic P21 expression. The cytoplasmic P21 expression depresses the apoptosis of 16HBE cells.

5. Discussions

Katsuyuki Tomita et al found that P21 expressed highly in the alveolar macrophages and airway epithelial cells of smoking patients and localized predominantly to the cytoplasm and protect the apoptosis of these cells, while in the normal macrophages and airway epithelial cells P21 localized predominantly to the nuclear [3]. Sarah M. Puddicombe et al found that P21 expressed highly in asthmatic bronchial epithelial cells, distributed mainly in the nuclear of normal and mild asthmatic bronchial epithelial cells, and in cytoplasm of severe asthmatic bronchial epithelial cells [5]. Thus the sub-cell expression of P21 interacts with the function of human bronchial epithelial cell. P21 is regarded as a modulator of apoptosis, Cytoplasmic p21 leads to an inhibition of multiple caspases and apoptotic effectors including pro-caspase-3, caspase-8, caspase-10, apoptosis signal-regulating kinase 1 and stress-activated protein kinase, Paradoxically, p21 can also promote apoptosis [8-10]. These different functions may be related to phosphorylation of some sites of P21 protein and its sub-cellular localization of cytoplasm and nuclear [4, 11, 12]. In this study, the plasmid PEGFP-N1-p21 was transfected into the human bronchial epithelial cell. It was found that PEGFP-N1-p21 was only expressed in the cytoplasm of normal bronchial epithelial cell and protected the apoptosis of normal bronchial epithelial cell. These results were consistent with other earlier researches, but were more direct to explain the mechanism of the cytoplasm expression of P21 protected the apoptosis of human bronchial epithelial cell and were helpful to study the mechanism in the clinical condition.

Transforming growth factor (TGF-β) family proteins are multi-functional cytokines that have been implicated in the pathogenesis of diverse biologic processes including cell growth and survival, cell and tissue differentiation, development, inflammation, immunity, hematopoiesis, and tissue remodeling and repair [6]. P21 functioning as transcription factor/co-factor is essential for TGF-β mediated breast cancer cell migration and invasion, high P21 expression was correlated with poor overall and distant metastasis free survival of breast cancer patients promoting migration/invasion at the transcriptional level [13, 14]. In accordance with this, another study with a breast cancer mouse model has shown that invasion is accompanied by an up-regulation of P21 pointing to its role in a “reciprocal switching between proliferation and invasion” [15]. Masashi Yamasaki et al demonstrated that P21 inhibited the TGF-β1 induced apoptosis in the mouse airway epithelial cells [7]. Sarah M. Puddicombe et al found that the cell cyclin inhibitory activity of P21 was intimately associated with its nuclear localization upon TGF-β treatment [5]. In our study, we found that after treating the human bronchial epithelial cell with TGF-β, the expressions of nuclear P21, but mainly the cytoplasmic P21 increased and as the increasing of the concentrations of the TGF-β treatment, the expressions of cytoplasmic P21 decreased, the expressions of nuclear P21 did not change. We also found that after treating the human bronchial epithelial cell with TGF-β, the apoptosis was induced and as the increasing of the concentrations of the TGF-β treatment, the apoptosis increased. The apoptosis of 16HBE cells increased as the cytoplasmic P21 expression decreased after TGF-β1 stimulation.

From this research we found that the cytoplasmic expressions of P21 inhibited the apoptosis of human bronchial epithelial and that TGF-β1 could induce the apoptosis of human bronchial epithelium through decreasing the cytoplasmic P21 expression. This is one important mechanism of lung diseases. Cytoplasmic localization of P21 could be a reliable biomarker and a promising intervention target.

References


