Background and Objectives. The tumor suppressor genes p53 and p16INK4a, both of which act in tumor surveillance, are homozygously deleted in the human leukemia cell line K562. This study was performed to assess whether co-transfection of the p16INK4a and p53 genes could inhibit K562 cell proliferation.

Design and Methods. p16INK4a and p53 genes were co-transfected into K562 cells with liposome, and the expression of the transfected genes was detected by Western-immunoblotting and immunocytochemistry. The effect of the p16INK4a and p53 transfected cell culture was quantified by trypan blue staining, and the number of recovered viable cells was assessed every day after transfection. Cells were analyzed for expression of annexin V in order to detect apoptosis. Differentiation of transfected K562 cells was measured by the benzidine oxidation test, and the cell cycle was analyzed by flow cytometry.

Results. After co-transfection, there were 23% and 28% p53 and p16INK4a positive cells respectively. Co-transfection with p16INK4a and p53 genes significantly inhibited cell proliferation when compared to transfection with either p16INK4a or p53 gene. The percentage of cells expressing the apoptosis-related cell surface antigen annexin V was significantly higher in p53 and p16INK4a transfected cells than in p53 or p16INK4a transfected cells (6.24±0.37% vs 4.88±0.17%, p<0.05 and vs 2.78±0.26%, p<0.05, respectively). p16INK4a and p53 co-transfection significantly increased the number of cells in G1 phase and decreased that in S phase.

Interpretation and Conclusions. Expression of wild-type p16INK4a and p53 genes in K562 cells results in reduced proliferation and apoptosis. Introduction of exogenous p16INK4a and p53 genes into K562 cells might contribute to the clinical treatment of leukemia.

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liferation will be enhanced.\textsuperscript{2,15}

Previous reports demonstrated homozygous deletions of p16\textsuperscript{INK4a} and p53 genes in the human erythroleukemic cell line K562.\textsuperscript{19,20} Some studies showed that transfer of p16\textsuperscript{INK4a} or p53 gene alone could inhibit K562 cell proliferation.\textsuperscript{21-23} However, no study on the effect of co-transfection of p53 and p16\textsuperscript{INK4a} genes into K562 cells has been reported so far. In this study, we have assessed the effects of co-transfection of both p53 and p16\textsuperscript{INK4a} genes into K562 cells.

Design and Methods

Cells and culture conditions

The human erythroleukemic cell line K562 (a gift from the Fujian Institute of Hematology) was cultured at 37°C in RPMI-1640 medium (Gibco BRL), supplemented with 10% fetal calf serum (FCS) in 5% CO\textsubscript{2} humidified atmosphere.

Plasmid constructions

A human p16\textsuperscript{INK4a} cDNA (kindly provided by Dr. Gordon Peters, Imperial Cancer Research Fund, London, UK), flankered by BamH1 and EcoR1 restriction sites, was transferred into the mammalian expression vector pcDNA3 (Introngene, San Diego, USA) under the cytomegalovirus (CMV) promoter to obtain plasmid pcDNA3p16. Plasmid pcDNA3p53-SN3 (a gift from Dr. Ming Liang, Fujian Institute of Hematology, P. R. of China) contains human p53 cDNA under the CMV promoter. Plasmid pcDNA3LacZ was constructed by putting the 3.4kb Bam H1 fragment of β-galactosidase (β-gal) gene from plasmid pAdCMVβgalZ (a gift from Dr. Liya Yi, Hubei Medical University, P.R. of China) into the unique Bam H1 site of pcDNA3. Plasmid pcDNA3LacZ and 0.5 μg of proteins and markers of molecular weight were run on a 12.5% SDS-PAGE and electrophoretically transferred to a sheet of nitrocellulose paper. p16\textsuperscript{INK4a} was detected using the rabbit polyclonal antibody to p16\textsuperscript{INK4a} protein (Maxim Biotech Inc.), and p53 using the mouse monoclonal antibody to p53 protein (Maxim Biotech Inc.). Protein detection was visualized by the streptavidin/biotin/peroxidase technique.

Expression of p16\textsuperscript{INK4a} and of p53 in cells was detected by immunocytochemistry using the UltraSensitive\textsuperscript{TM} S-P Kit (Maxim Biotech Inc.). Cells were harvested 48 hours after transfection and placed on silane-coated slides; the slides were then fixed with 4°C acetone. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase for 10 min. Non-specific conjugation was blocked with non-immune rabbit serum in Tris buffered saline for 10 min, following which the mouse monoclonal antibody to p53 or rabbit polyclonal antibody to p16\textsuperscript{INK4a} protein (Maxim Biotech Inc.) was layered on the slides. After incubation at 4°C overnight, the slides were washed with PBS and processed with streptavidin/biotin/peroxidase reagents to detect and amplify the signal.

Growth inhibition assay

The effect of the p16\textsuperscript{INK4a} and p53 transfected cell culture was quantified by trypan blue staining. The number of recovered viable cells was assessed every day after transfection. Results were expressed as the mean cell number of three wells.

Apoptosis detection

Cells were analyzed for expression of annexin V in order to detect apoptosis. Cells were washed once in cold PBS and then resuspended to 1×10\textsuperscript{6}
Next, 100 µL of the cell suspension were incubated for 15 min at room temperature in the dark with 5 µL of annexin V-FITC (PharMingen, San Diego, CA, USA) and 10 µL propidium iodide (PI), and then taken to flow cytometric analysis in a FACScan flow cytometer (Becton Dickinson, USA).

**Assessment of differentiation**

The benzidine oxidation test was performed as described previously. Briefly, 0.5×10⁶ transfected cells were incubated with 5µM hemin for 4 days. Cells were then washed twice in 1xPBS, and finally resuspended in 0.9% NaCl. After 30 min incubation with benzidine, reagent solution (to 1 mL of 0.2% tetramethylbenzidine in 0.5 M acetum, 20 µL of 30% H₂O₂ are added just prior to use) was added to start the reaction. After a 30 min incubation in the dark at room temperature, 200 cells were counted and the number of cells containing oxidized tetramethylbenzidine (visualized as cells containing blue crystals) was taken as indicative of peroxidase activity and thus reflecting hemoglobin production.

**Cell cycle analysis**

Cells were washed with PBS, then 0.2 mL nuclear isolation medium containing propidium iodide was added (50 µg/mL propidium iodide, 0.6% NP40, 100 µg/mL RNase, in PBS). The cells were incubated at room temperature in the dark for 60 min before the addition of 0.4 mL PBS and then taken to flow cytometric analysis in a FACScan flow cytometer (Becton Dickinson, USA). Cell-Fit software (Becton Dickinson) was used for cell cycle analysis.

**Statistical analysis**

Chi-squared tests were used for analysis of categorical variables. All the results were expressed as mean ± standard deviation and all p values were two-tailed and were considered statistically significant when they reached the probability level <0.05.

**Results**

**Transfected exogenous p16INK4a and p53 expression in K562 cells**

p16INK4a and p53 expression was detected 48 hours after transplantation. Immunocytochemistry results showed that p16INK4a and p53 proteins were located in the nucleus, and only partially in the cytoplasm. p53 and p16INK4a expressing cells were, respectively, 23% and 28%. After co-transfection 23% and 28% of cells expressed respectively (Figure 1). In the transfections with p53 vector alone, 25% of cells expressed the protein, while in the cells transfected with p16INK4a vector, 31.5% expressed the protein. In mock experiments, pcDNA3LacZ and pCNeo-SN3 co-transfected cells expressed neither p53 nor p16INK4a protein. By immunoblotting, the cells co-transfected with the two tumor suppressor genes showed detectable levels of p16INK4a and p53 protein, while mock-transfected cells showed none (Figure 2).

**Inhibition of cell growth**

The effects of co-transfection on cell growth were assessed by enumerating the viable cells every day after transfection. As shown in Figure 3, cell growth of p53 and p16INK4a vector co-transfected cells was significantly inhibited compared with that of cells co-transfected with control vectors. For cells transfected with p53 alone, moderate inhibition was observed. However, in p16INK4a transfected cells, only a mild inhibition was seen.
Co-transfection of p16INK4a and p53 genes into the K562 cell line

No significant inhibition was seen in cells transfected with control vectors.

Wild-type p16INK4a and p53 induce cell apoptosis

To determine whether the cell growth inhibition was attributable to apoptosis, the cells were analyzed for expression of annexin V by FACS analysis concomitantly with PI. As shown in Table 1, cells transfected with p53 and p16INK4a vectors showed higher rates of apoptosis.

Wild-type p16INK4a and p53 promote the capacity for hemoglobin production

After 4 days with 5µM hemin, the transfected cells were subjected to a benzidine oxidation test. This test is performed to determine the peroxidase activity of the cells, which reflects their content of hemoglobin. As shown in Figure 4, p16INK4a and p53 increased the differentiation sensitivity of the K562 clones 7-fold compared with control vectors, 1.6-fold compared with p16INK4a and 1.3 fold compared with p53 alone.

Cell cycle arrest mediated by p53 and p16INK4a

As shown in Table 2, p53 and p16INK4a co-transfection significantly increased the number of cells in G1 phase and decreased that in S phase. Either p53 or p16INK4a transfection also appreciably increased the number of cells in G1 phase. In contrast, no G1 arrest was observed in the control group. These results suggest that the p53 and p16INK4a proteins suppress the growth of the tumor cells by mediating G1 arrest in cell lines that do not express p53 and p16INK4a.

Discussion

p53 and Rb proteins are tumor suppressors which negatively regulate different steps in cell cycle progression. In turn, their activities are modulated by p19ARF and p16INK4a proteins encoded by the INK4a/ARF locus. Together, the loci encoding these four proteins are probably the most commonly inactivated in cancer. Because the p16INK4a protein is a CDK4 inhibitor, deletion of this gene may increase the activity of CDK4, resulting in hyperphosphorylation of the Rb protein, thus driving the cell cycle from G1 to S phase and enhancing cell proliferation. Restoring the expression of p16INK4a in p16INK4a negative cell lines arrests the cells at G1 phase, thus inhibiting cell proliferation. One of the functions of p53 is cell cycle arrest at the G1/S boundary to allow repair of damaged DNA before DNA replication or induction of apoptosis, when DNA damage is too severe. Expression of wild-type p53 in p53-deficient tumor cell lines renders them more susceptible to induction of apoptosis by radiation or DNA-damaging chemotherapeutic drugs.

p53 mutations, accompanied by p16INK4a muta...
tions, have been found in many cancer cell lines. Though restoration of wild-type p16\(^{INK4a}\) or p53 function alone could inhibit the proliferation and colony formation of some human cancer cell lines, tumors with both p16\(^{INK4a}\) and p53 mutations might also display aggressive characteristics owing to defect in the p53 and Rb pathway. Our study showed that both p53 and p16\(^{INK4a}\) expression in K562 cells, a human leukemia cell line with homozygous deletion of p53 and p16\(^{INK4a}\) genes, induced obvious growth arrest, as indicated by the decrease in the percentage of cells in the S phase of the cell cycle. The expression of annexin V implied that the cell growth inhibition was attributable to apoptosis. Either p53 or p16\(^{INK4a}\) expression alone also inhibited K562 cell growth, whereas p16\(^{INK4a}\) protein showed mild inhibition. A role of p53 or p16\(^{INK4a}\) protein in G1 arrest is consistent with previous studies.\(^ {15,29-31}\)

Our data have important implications for the multi-gene therapy of tumors.

In these experiments, we used liposome-coated DNA for p16\(^{INK4a}\) and p53 gene transfer. Cationic liposome-DNA complexes have been used in vitro and in vivo as gene delivery vehicles as an alternative to viral vectors.\(^ {32,33}\) Some of the advantages of liposomes are that they can carry large pieces of DNA, they are not immunogenic, they are safe relative to viral vectors, and large scale production of liposomes is relatively straightforward.\(^ {33}\) The efficiency of transfection, assessed by measuring the percentage of cells expressing p16\(^{INK4a}\) or p53 protein, was found to be too low to enable the therapeutic plasmid to inhibit all cell growth. No more than 30% of all transfected K562 cells expressed both p16\(^{INK4a}\) and p53. Even considering either p16\(^{INK4a}\) or p53 transfected cells, there were still no more than 35% positive cells. Because of the low transfer efficiency, most K562 cells did not take in these two genes, so that the effect of cell growth suppression was not satisfactory. Further studies are required to investigate whether the stable expression of both functional p53 and p16\(^{INK4a}\) in mismatch deficient cells might improve the role of multi-gene therapy in cancer. These studies indicate that the transfection efficiency of liposomes needs to be significantly increased before they can be used successfully in vivo. Fortunately, novel liposomes improved transfection efficiencies and activities are being developed.\(^ {34,35}\) In previous reports of p16\(^{INK4a}\) gene transfer using retroviruses, the viral titer obtained was low.\(^ {21}\) It was reported that murine NIH3T3 cells, from which most amphotropic packaging cell lines are

### Table 1. Expression of the apoptosis-related cell surface antigen annexin V in p16\(^{INK4a}\) and p53 transfected K562 cells (mean±SD). Values shown are percentage of cells expressing Annexin V.

<table>
<thead>
<tr>
<th></th>
<th>Annexin V</th>
</tr>
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<tbody>
<tr>
<td>K562/medium</td>
<td>1.75±0.17</td>
</tr>
<tr>
<td>pcDNA3LacZ+pCNeo-SN3</td>
<td>1.81±0.13</td>
</tr>
<tr>
<td>pcDNA3p16</td>
<td>2.78±0.26*</td>
</tr>
<tr>
<td>pC53-SN3</td>
<td>4.87±0.17*</td>
</tr>
<tr>
<td>pcDNA3p16+pC53-SN3</td>
<td>6.24±0.37*</td>
</tr>
</tbody>
</table>

*\(p<0.05\) vs pC53-SN3, pcDNA3p16, *\(p<0.05\) vs pcDNA3-pCNeo-SN3, SD: standard deviation.

### Table 2. Effect of p16\(^{INK4a}\) and p53 co-transfection on K562 cell cycle (mean±SD).

<table>
<thead>
<tr>
<th></th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>K562/medium</td>
<td>37.64±0.52</td>
<td>60.93±2.55</td>
<td>1.43±0.98</td>
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<tr>
<td>pcDNA3LacZ+pCNeo-SN3</td>
<td>37.71±0.78</td>
<td>60.6±0.86</td>
<td>1.60±0.59</td>
</tr>
<tr>
<td>pcDNA3p16</td>
<td>42.10±1.49*</td>
<td>55.39±1.26*</td>
<td>2.52±0.49</td>
</tr>
<tr>
<td>pC53-SN3</td>
<td>46.44±1.99*</td>
<td>51.60±1.73*</td>
<td>1.95±0.29</td>
</tr>
<tr>
<td>pcDNA3p16+pC53-SN3</td>
<td>52.56±1.38*</td>
<td>46.30±1.68*</td>
<td>1.14±0.38</td>
</tr>
</tbody>
</table>

*\(p<0.05\) vs pC53-SN3, pcDNA3p16, *\(p<0.05\) vs pcDNA3-pCNeo-SN3. SD: standard deviation.
derived, have a homozygous deletion of p16\textsuperscript{INK4a} gene, and that ectopic expression of p16\textsuperscript{INK4a} in NIH3T3 cells resulted in G1 phase arrest.\textsuperscript{36} This could explain why high viral titer producing PA317 clones were unable to be obtained, as PA317 clones with low-expression p16\textsuperscript{INK4a} mRNA were probably selected, leading to a weak viral production. This finding will have to be taken into account if retroviruses are to be used as vectors for gene therapy using the p16\textsuperscript{INK4a} gene. There are also several disadvantages associated with retroviral delivery of the p53 gene, such as the potential for insertional mutagenesis, cell division for efficient infection, poor stability and low titer.\textsuperscript{2} All of these limit the use of replication-deficient retroviruses to ex vivo gene therapy.

Adenoviruses have been widely used as vectors in various cell types, and can introduce foreign genes into non-replicating cells at high titer, but do not integrate into the genome.\textsuperscript{1} Several investigators have reported the antitumor effects of adenoviruses encoding wild-type p53 or p16\textsuperscript{INK4a} on various tumor cell lines.\textsuperscript{37,38} However, some studies also reported the poor ability of adenovirus vectors to transduce acute myeloid leukemia cells (AML).\textsuperscript{39,40} A modified adenovirus containing a heparan/heparan sulfate binding domain incorporated into the fiber protein of the adenovirus was investigated. It was reported that retargeting the adenovirus fiber protein to heparan sulfates can overcome the low efficiency of adenovirus in AML blast cells and may provide a useful tool for gene therapy approaches in AML.\textsuperscript{41} Therefore, the adenovirus vector seems suitable for future p53 and p16\textsuperscript{INK4a} gene therapy protocols in acute leukemia cells.

In conclusion, these data indicate that co-transfection of wild-type p53 and p16\textsuperscript{INK4a} can inhibit K562 cell growth. Furthermore, the results suggest a possible role of multi-gene therapy in acute leukemias.

Contributions and Acknowledgments

HBR, JZS: conception and design; HBR: drafting and revisions. All the authors approved the final version of manuscript. The authors thank Dr. Guangshen Zhuo, First Affiliated Hospital, Fujian Medical University, China, for his helpful discussion.

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Disclosures

Conflict of interest: none. Redundant publications: no substantial overlapping with previous papers.

References

Blood 1994; 84:2431-5.


