Basic Research Paper

Secretory expression vector V-pLNCX-s-hri inhibits the growth of mouse B16 melanoma

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Background and Objective: Human ribonuclease inhibitor (hRI) extracted and purified from human placenta has been shown to remarkably inhibit some solid tumors in mice. This study was to construct V-pLNCX-s-hri, a secretory expression vector, and explore its inhibition effects on the growth of mouse B16 melanoma cells.

Methods: The hRI gene sequence conjugated with the synthesized signal peptide of mouse IgG was cloned into the retroviral vector V-pLNCX to construct V-pLNCX-s-hri. The PA317 cells were used for viral package and NIH3T3 cells were employed to determine the viral titer. The expression of hRI gene was detected by RT-PCR and Western blot. The content of hRI was determined by enzyme-linked immunoabsorption assay (ELISA). The model of B16 melanoma-carrying mouse was established and received different treatments. The tumor weight and microvessel density (MVD) were assessed. Normal saline (NS), V-pLNCX, and V-pLNCX-hri were used as controls.

Results: The infection efficiency of V-pLNCX-s-hri in cultured B16 cells reached 38.5%. mRNA and protein levels of hRI were detected in B16 cells infected with V-pLNCX-s-hri. The hRI content in the supernatant of infected B16 cells reached 0.228 μg/mL. The hRI content in the peripheral blood of mice was significantly higher in the V-pLNCX-s-hri group (0.249 μg/mL) than in the NS group (0.035 μg/mL), V-pLNCX group (0.028 μg/mL) and V-pLNCX-hri group (0.169 μg/mL) (p < 0.01). The tumor weight and MVD were significantly lower in the V-pLNCX-s-hri group compared with those in the NS, V-pLNCX and V-pLNCX-hri groups (p > 0.01). The content of hRI in the peripheral blood of mice was significantly higher in the V-pLNCX-s-hri group (0.249 μg/mL) than in the NS group (0.035 μg/mL), V-pLNCX group (0.028 μg/mL) and V-pLNCX-hri group (0.169 μg/mL) (p < 0.01). The tumor weight and MVD were significantly lower in the V-pLNCX-s-hri group compared with those in the NS, V-pLNCX and V-pLNCX-hri groups (p > 0.01). Conclusions: V-pLNCX-s-hri can effectively infect B16 cells and induce high expression of hRI. V-pLNCX-s-hri is superior to V-pLNCX-hri in inhibiting the growth of B16 cells.

Ribonuclease inhibitor (RI), the inhibitor of ribonucleases A (RNase A), is an acidic cytoplasmic glycoprotein, with an isoelectric point of 4.7 and molecular weight of about 50kDa. RI can effectively inhibit the hydrolysis of RNA by RNase A.1,2 Angiogenin (Ang) is the homologue of RNase A, which shares highly conservative amino acid sequences and similar structures with RNase A.3 The RI molecule, which has a symmetric horseshoe-shaped structure, can bind to Ang and RNase. The affinity of RI to Ang (Ki=7.1 × 10^{-16}mol/L) is far stronger than to RNase A (Ki=4.0 × 10^{-14}mol/L).4 Many experiments have demonstrated that the formation of new blood vessels is necessary for the growth and metastasis of tumor. Inhibition of tumor angiogenesis would significantly retard tumor growth, or even result in tumor reduction.

RI is a cytoplasmic protein under physiological conditions, which makes its inhibition on Ang, a secretory protein, to be limited to some extent. In this study, the N terminal of human RI (hRI) was conjugated with the signal peptide of mouse IgG and then cloned into the retroviral vector V-pLNCX to construct V-pLNCX-s-hri. The constructed vector was transfected into the package cell line PA317 using the liposome method. The toxicigen strain with high titer was screened out to observe the effect of V-pLNCX-s-hri on the number of new vessels and the growth rate of tumors, in order to investigate the role of hRI in anti-angiogenic gene therapy of cancer.

Materials and Methods

Materials. Reagents. Restriction enzymes Spe I, Cla I, Hind III, DNA ligation kit, DNA blunting kit, BAP kit, PCR kit, and DNA molecular weight standard (DL2000) were purchased from Takara Bioengineering Co. Ltd (Dalian, China). Lipofectamine and G418 were the products of GIBO BRL (NY, USA). B16 melanoma cells and PA317 cells were provided by Tianjin Institute of Hematology, China. Low carbohydrate DMEM was purchased from Clontech. Trizol was purchased from Sigma. Protein molecular weight standard was the product of Shanghai Lizhu Bioengineering Co. Ltd (China). Mouse IgG signal peptide was synthesized by Takara Bioengineering Co. Ltd. (Dalian, China). Rabbit anti-human RI antibody was prepared in the Department of Biochemistry & Molecular Biology, Dalian Medical University. Horseradish peroxidase labeled goat anti-rabbit secondary antibody was purchased from Shanghai Shiyeh Kehua Biochemicals Co, Ltd.
Establishment of a highly toxigenic cell line. DH5α positive colonies were picked, transferred into 100mL LA liquid culture medium, and cultured at 37°C overnight. Plasmid DNA was extracted and transfected into PA317 cells using the liposome method. G418 was added to a final concentration of 800 μg/mL to sort out positive clones. Positive clones were subjected to Hind III and Cla I double enzyme digestion, and the Cla I restriction enzyme cutting site ATCGAT was introduced into the N terminal; and the Spe I restriction enzyme cutting site ACTAGT was introduced into the N terminal; respectively. The size of the synthesized signal peptide was 69bp 5'-AAGCTTATGAAATGCAGCTGGGTCAATTCAACTAGT-3', and the SpeI restriction enzyme cutting site ACTAGT was introduced into the N terminal; and the SpeI restriction enzyme cutting site ACTAGT was introduced into the N terminal; respectively. Then the cells were cultured in complete medium for 24 h, followed by culture in selective medium containing G418 for 12 h for three times successively. Then the cells were cultured in complete medium for 24 h, followed by culture in selective medium containing G418 for three weeks. Subsequently, cells were cultured in selective medium containing 1 mg/mL G418 for two weeks, and G418 resistant clones were selected for later amplification. Non-transfected B16 cells in selective (2 mg/mL G418) and non-selective culture medium were counted respectively. The infection efficiency was calculated.

Expression analysis of V-pLNCX-s-hri in B16 melanoma cells in vitro. B16 cells were seeded into a six-well plate. When cells reached 50–80% confluence, the culture medium was replaced by 3 mL culture supernatant from toxigenic PA317 cells infected with V-pLNCX-s-hri, supplemented with 8 mg/L polyrene. The B16 cells were infected every 12 h for three times successively. Then the cells were cultured in complete medium for 24 h, followed by culture in selective medium containing G418 for three weeks. Subsequently, cells were cultured in selective medium containing 1 mg/mL G418 for two weeks, and G418 resistant clones were selected for later amplification. Non-transfected B16 cells were used as control. Total RNA of B16 cells was isolated using Trizol after viral infection. RI mRNA was amplified by one-step RT-PCR. The primer sequences of RI were as follows: (upstream), 5'-ACTAGTATGAGCCTGGAGATGACCCTCAG-3', and the SpeI restriction enzyme cutting site ACTAGT was introduced into the N terminal; (downstream) 5'-AAATCGATTCAGGAGATGACCCTCAG-3', and the Cla I restriction enzyme cutting site ATCGAT was introduced into the N terminal; respectively.

Detection of helper virus. NIH3T3 cells were transfected with V-pLNCX-s-hri, and G418 resistant cells were maintained by serial subcultivation for three weeks. The supernatant was collected and filtered through a 0.45 μm filter membrane. NIH3T3 were transfected with 1ml of the filtered fluid and cultured for two weeks with G418. The virus titer was calculated.

Analysis of virus infection efficiency in vitro. B16 cells were infected with high-titer supernatant from toxigenic PA317 cells and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, 100 U/mL ampicillin at 37°C 5% CO₂ for 14 d. Colonies formed from transfected and non-transfected B16 cells in selective (2 mg/mL G418) and non-selective culture medium were counted respectively. The infection efficiency was calculated.
Figure 2. Sequencing analysis of cloned human ribonuclease inhibitor (hRI) DNA
non-transfected B16 cells was detected by western blot using rabbit anti-human placenta RI antibody prepared in our laboratory.

Inhibition of recombinant virus V-pLNCX-s-hri on mouse B16 melanoma. B16 melanoma cells (1 × 10^6) were injected into each mouse via the right armpit. The mice were randomly assigned to five groups four days later. Ten mice, half males and half females, were included in each group, and treated with NS (control) and V-pLNCX, V-pLNCX-hri, V-pLNCX-s-hri, and cyclophosphamide, respectively.

The virus (0.1 ml) was administered via retrobulbar injection with a titer of 1 × 10^5 cfu/mouse. The dose of cyclophosphamide was 1mg/kg. After 28 days, mice were sacrificed by cervical dislocation. The tumor tissue was removed and weighed. The tumor inhibition rate was calculated using the following equation: tumor inhibition rate = (tumor weight of the control group - tumor weight of the treatment group) / tumor weight of the control group × 100%. Sections of tumor tissues were stained by the HE method, and the number of vessels was counted in ten visual fields under microscopy.

Detection of RI content by ELISA. The samples were diluted 16,000-fold with the coating solution. The diluted sample (50 μl) was added to each well on the microplate in quadruplicate. The microplate coated with samples was rinsed in a plate washing machine for five times. Blocking solution (100 μl) was added to each well and the plate was incubated at 37°C for 1.5 h. After a rinse, 50 μl primary antibody (1:4000 dilution) was added to two of the four wells for each treatment, while 50 μl antibody buffer solution was added to the other two wells as the control. The plate was incubated at 37°C for 1 h. After another rinse, 50 μl secondary antibody (1:1000 dilution) was added to each well, and incubated at 37°C for 40 min. After the third rinse, 50 μl substrates A and B were added to each well, and incubated at 37°C for 13 min. After termination of the reaction by adding 50 μl stop buffer, the absorbance of each well was measured at 580 nm (A580).

Statistical analysis. The tumor weight and the vessel density were assessed, and recorded as mean ± SD. Data analyses were performed using SPSS 13.0 software, and single factor variance analysis was conducted. p < 0.05 was considered statistically significant.

Results

Construction of V-pLNCX-s-hri. The recombinant retroviral vector V-pLNCX-s-hri was double digested by Hind III and Cla I. A 6.6 kb and a 1.4 kb bands were separated by 1% agarose gel electrophoresis. The sequence of the 1.4 kb band was consistent with that published in GenBank (Figs. 1 and 2), confirming successful construction of V-pLNCX-s-hri.

Measurement of virus titer, identification of highly toxigenic cell line and detection of helper virus. Culture supernatant of V-pLNCX-s-hri was double digested by Hind III and Cla I. A 6.6 kb and a 1.4 kb bands were separated by agarose gel electrophoresis. The sequence of the 1.4 kb band was consistent with that published in GenBank (Figs. 1 and 2), confirming successful construction of V-pLNCX-s-hri.

Measurement of virus titer, identification of highly toxigenic cell line and detection of helper virus. Culture supernatant of V-pLNCX-s-hri was collected from positive PA317 cells infected with V-pLNCX-s-hri. Virus was purified by high speed centrifugation. NIH3T3 cells were infected by diluted virus and the virus titer was as high as 4.2 × 10^5 cfu/ml.

NIH3T3 cells were infected by V-pLNCX-s-hri, and G418 resistant cells were maintained by serial subcultivation for three weeks. The supernatant was collected and filtered through a 0.45μm membrane. NIH3T3 cells were infected by 1ml of filtered solution and cultured for two weeks in the presence of G418. No resistant clone grew, indicating that no helper virus was produced and the gene transfer system was safe.

Infection efficiency of V-pLNCX-s-hri for B16 cells and detection of hRI mRNA expression. Non-infected B16 cells did not form clones in selective culture medium, while formed 182 clones on non-selective culture medium. B16 cells infected with V-pLNCX-s-hri formed 66 and 171 clones on the selective and non-selective culture medium, respectively, with an infection efficiency of 38.5%. A 1.4 kb band was stably present in infected B16 cells as shown by 1% agarose gel electrophoresis (Fig. 3), suggesting that the hRI gene was stably integrated into the genome of infected B16 cells.

Protein expression of hRI in infected B16 cells. Protein contents of hRI in V-pLNCX-s-hri infected and non-infected B16...
Secretory expression vector V-pLNCX-s-hri inhibits the growth of mouse B16 melanoma cells and the corresponding culture supernatants were detected by Western blot. A 50 ku protein band was present in the supernatant of non-infected B16, confirming successful construction of V-pLNCX-s-hri.

Inhibition of secretory hRI on the growth of B16 melanoma cells in mice. After treatment, the mice in each group were routinely fed for 28 days. When tumors were formed in all mice, the mice were sacrificed by cervical dislocation. The tumor tissues were removed and weighed. There was no significant change in the average body weight after the removal of tumor in each treatment group, compared with that in NS group. The weight of tumor tissues was significantly decreased in V-pLNCX-hri and V-pLNCX-s-hri groups compared with the NS and V-pLNCX groups (p < 0.01). In addition, the weight of tumor tissues in V-pLNCX-s-hri group was lower than that in V-pLNCX-hri group, and the tumor inhibition rate was 56.9% in V-pLNCX-s-hri group (Table 1). The population doubling time of B16 cells was 34. h, 33.5h, 33.2h and 33.6h in V-pLNCX transfection group, V-pLNCX-hri transfection group, V-pLNCX-s-hri transfection group and non-transfection group, respectively, and the differences among the groups were not significantly different.

Effect of secretory hRI on the number of new vessels in B16 melanoma tissues. Sections of tumor tissues were stained by HE, and the density of new vessels in tumor tissues of each group was observed under light microscopy (Fig. 5). The average number of vessels in 10 visual fields of NS group, V-pLNCX group, V-pLNCX-hri group, V-pLNCX-s-hri group and cyclophosphamide group was 89 ± 6, 87 ± 7, 41 ± 8, 34 ± 4 and 79 ± 5, respectively. Compared with NS group, the density of vessels in tumor tissues was significantly less in V-pLNCX-hri group and V-pLNCX-s-hri groups (p < 0.01), and the vessels were thinner. The content of hRI in peripheral blood of mice was 0.035 μg/mL, 0.028 μg/mL, 0.169 μg/mL, 0.249 μg/mL and 0.031 μg/mL in NS group, V-pLNCX group, V-pLNCX-hri group, V-pLNCX-s-hri group and cyclophosphamide group, respectively. The hRI content in V-pLNCX-s-hri group was significantly higher than that in NS group, V-pLNCX group and V-pLNCX-hri group (p < 0.05).

Discussion

RI is an important multifunctional regulatory protein in living organisms. It can significantly inhibit the biological activity of Ang, an angiogenesis factor. Ang is a single strand alkaline protein (pI > 9.5) which belongs to the ribonuclease superfamily. Ang can hydrolyze 18S and 28S rRNA effectively, and its most important biological function is to enhance the formation of new vessels. Ang and pancreatic RNase
Table 1  Effect of the V-pLNCX-s-hri on the growth of B16 melanoma tissues

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight of tumor tissue (g)</th>
<th>Inhibition rate (%)</th>
</tr>
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<tbody>
<tr>
<td>Normal saline</td>
<td>1.90±1.12</td>
<td></td>
</tr>
<tr>
<td>V-pLNCX</td>
<td>1.77±0.21</td>
<td>6.70</td>
</tr>
<tr>
<td>V-pLNCX-s-hri</td>
<td>1.10±0.46±p</td>
<td>42.0</td>
</tr>
<tr>
<td>V-pLNCX-s-hri</td>
<td>0.82±0.34±p</td>
<td>56.9</td>
</tr>
<tr>
<td>cyclophosphamide</td>
<td>0.43±0.49</td>
<td>77.6</td>
</tr>
</tbody>
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p<0.01, p<0.01, vs. normal saline; p<0.01, p<0.01, vs. V-pLNCX.

A have extremely similar kidney-shaped structures. The angiogenic activity of Ang is intensively inhibited by RI.6

Formation of new vessels in tumor tissues plays a very important role in the process of tumor generation, development and metastasis. Inhibition of new vessel formation in tumor tissues has become a new target for tumor treatment. In addition, such an approach can also decrease metastasis of tumor cells via blood vessels.

Our previous study showed that RI purified from human placenta exerts significant inhibition on the growth of some solid tumors. The growth of B16 cells transfected with nonsecreting vector V-pLNCX-hri was significantly retarded compared with control cells.7-9 Since RI is a cytosolic protein whereas Ang is a secretory one, treatment for tumors using nonsecreting vector is limited to some extent. In this study, secretory vector V-pLNCX-s-hri was constructed to observe the its inhibition effect on the growth of melanoma in mice. We revealed that hRI was highly expressed in the supernatant from V-pLNCX-s-hri infected B16 cells, as high as 0.249 μg/mL. The weight of mouse B16 melanoma tissues and the density of vessels were significantly lower in V-pLNCX-s-hri transfection group than in other groups, while the content of hRI in peripheral blood was significantly higher in V-pLNCX-s-hri group than in other group. Compared with our previous results,10 the inhibition effect of V-pLNCX-s-hri on the melanoma tumor was significantly enhanced. Therefore, secretory RI could obviously enhance the inhibition effect of intracellular RI on Ang.

RI is a multifunctional protein, which not only inhibits Ang, but also has antioxidative properties.11-12 In this study, hRI was directly purified from the culture supernatant of cell clones to avoid tedious processes of purifying RI from tissues, which provides a foundation for further application of RI. RI has potential application values as an anti-tumor and anti-oxidative protein. Different signal peptides have different efficacy in inducing protein secretion. Thus our future work is to screen out the most proper signal peptides which can induce RI secretion. We also wish to obtain cell clones which can produce a high quantity of secretory RI to facilitate industrial production of hRI as an anti-tumor drug.

References