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Induction of interleukin 2 expression in the liver for the treatment of H22 hepatoma in mice☆

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ABSTRACT

Background and aims: We designed this study to evaluate the ability of a plasmid carrying an RU486 regulatory system to induce expression of interleukin-2 (IL-2) gene and to examine the antitumour efficacy of the induced IL-2 gene.
Methods: The plasmid pRS-mIL-2,which contains an RU486 inducible system and IL-2 gene was injected into mice. Sera and tissues from liver, spleen, lungs and kidneys were taken to test the properties of the plasmid. To examine the antitumour efficacy of pRS-mIL-2, tumours were established in the liver by direct inoculation of H22 hepatoma cells.
Results: The IL-2 levels in serum correlated with the dose of plasmid and RU486. High and sustained IL-2 levels could be achieved by administration of RU486 every day. The mRNA of transgene IL-2 was found only in the liver. Treatment of mice with pRS-mIL-2 plus RU486 resulted in the significant reduction in tumour volume compared with control groups.
Conclusions: Tight temporal and spatial control of transgene IL-2 expression can be achieved by a plasmid containing an RU486 inducible system driven by liver specific promoter. pRS-mIL-2 exhibited strong antitumour efficacy following consecutive induction with RU486.

1. Introduction

Animal experiments and clinical research indicate that interleukin-2 (IL-2) exhibits antitumour effects on liver cancer [1–3]. The common administration routes of IL-2 involve systemic or local injection of recombinant protein [1–4]. However, frequent injection of recombinant IL-2 protein can have local or systemic toxic side effects [1,5,6]. Gene therapy is one of the promising strategies for therapy of liver cancer [7]. Nevertheless, targeting and regulating the vector are key problems, because unrestricted gene expression is non-physiological [8], and potentially harmful.

An ideal vector for gene therapy should be regulated, tissue-specific, and have low immunogenicity [9]. The RU486 regulatory system regulates transgene expression by controlling transcription under exogenous small molecular drug RU486 administration [8]. It has been proven to be an attractive gene switch because of its low immunogenicity, low background expression, fast response to induction and powerful inducibility [10–17].

Based on the advantages of the RU486 regulatory system, in the present study, we constructed a murine IL-2 (mIL-2) expressing plasmid pRS-mIL-2 containing the RU486 regulatory system driven by a liver-specific promoter. This was injected into mice by hydrodynamic injection [18,19]. The ability of the plasmid to deliver and regulate transgene expression was investigated. The antitumour efficacy of the plasmid was explored in mice bearing an orthotopic hepatic tumour.

2. Materials and methods

2.1. Construction of the plasmid

Plasmid pRS17 was a gift from Dr. Cheng Qian (Navarra University, Spain). The pRS-17 contains a GLp65 transactivator expression cassette controlled by a liver-specific promoter transthyrin (TTR) and a target gene expression cassette controlled by a TATA box. Plasmid pUC57 containing the mIL-2 gene was constructed by Sangon (China). The mIL-2 gene was excised by digesting pUC57 with *Cla I* and was subcloned into the *Cla I* site of the target gene expression cassette of pRS17 to generate pRS-mIL-2. The LacZ gene

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from pCMVβ (Clontech) was inserted into the *Cla I* site of the target gene expression cassette of pRS17 to obtain a new plasmid named pRS-LacZ.

2.2. Cells and animals

The murine H22 hepatoma cell line was obtained from the China Center for Type Culture Collection (Wuhan, China) maintained in DMEM medium supplemented with 10% FCS and cultured at 37 °C in 5% CO_2. Female Balb/c mice, 6–8 weeks of age, were purchased from the animal centre of Norman Bethune College of Medicine of Jilin University (China). All animal procedures were conducted in compliance with the guidelines approved by the China Association of Laboratory Animal Care and the Institutional Animal Care Committee.

2.3. Technique of plasmid DNA injection

The plasmids were delivered into the mice by a hydrodynamics-based procedure [18,19]. Briefly, plasmids diluted in 1.7 ml saline were injected into the tail vein over a period of 8 s. The mice had an average weight of 17 g. RU486 (Sigma) was dissolved in sesame oil. RU486 or sesame oil was given to the mice by intraperitoneal injection at indicated time points.

2.4. Measurement of mIL-2 and IFN-γ

Serum concentration of mIL-2 and IFN-γ were measured by an OptEIA™ Mouse IL-12 (p70) Set or OptEIA™ Mouse IFN-γ ELISA Set (BD Biosciences Pharmingen) according to the instructions of the manufacturer.

2.5. Polymerase chain reaction (PCR) and reverse-transcription PCR (RT-PCR) analysis

Total DNA and RNA from tissues of mice were isolated with TRI Reagent (Sigma). For the detection of plasmid DNA of pRS-mIL-2 or mRNA of GLp65 and mIL-2 in tissues, PCR and RT-PCR was used with the following primers: GLp65 forward: 5′-AGCCAGATCTGAAGCTAC-3′, GLp65 reverse: 5′-TGCTTGATATCTCGTCGA-3′; mIL-2 forward: 5′-TCTACAG-CGGAAGCACAG-3′, mIL-2 reverse: 5′-TCATCGAATTGGCACTCA-3′; and β-actin forward: 5′-ACTGCGCTTCTTGCCGC-3′, reverse: 5′-CATGACGCCCTGGTGTC-3′. The amplification products were separated on agarose gel. The predicted size for GLp65, mIL-2 and β-actin was 1103 bp, 344 bp and 182 bp, respectively.

2.6. Tumour model and treatment procedure

Hepatic tumours were established by direct liver implantation of H22 cells (10^6 cells/mouse, in 50 μl saline) at day 1. The mice were divided into four groups: IL-2 group (pRS-IL-2 plus RU486), oil group (pRS-IL-2 plus sesame oil); Lac Z group (pRS-LacZ plus RU486) and saline group (saline plus RU486). The last three groups were employed as controls. Ten micrograms of the plasmids were injected into the mice at day 3. RU486 (250 μg/kg) or 50 μl sesame oil was administered daily by intraperitoneal injection for 6 days from day 7. The mice were sacrificed at day 14 and the tumour sizes were measured by using a calliper. The edge of the tumour mass was defined based on the colour of the mass. The tumour mass was grey-white in colour and irregular in shape, and the liver tissue was dark red in colour. The longitudinal axis of the mass was measured as length, and the vertical axis was measured as width. Tumour volume was calculated using the formula: tumour volume (mm^3) = [length (mm) × width $(mm)^2$] × 0.5 [14].

2.7. Liver histology and immunohistochemical staining

Liver samples were fixed in formalin and embedded in paraffin for H&E and immunohistochemical staining. The sections were stained with H&E according to standard procedures. For mIL-2 and proliferating cell nuclear antigen (PCNA) detection, the primary antibody mIL-2 (Boster, China) and PCNA (Santa Cruz) was applied overnight at 4 °C. Phosphate-buffered solution (PBS) was the negative control instead of the primary antibody. Detection was performed using UltraSensitive™ S-P kit (Maixin, China). Sections were counterstained with haematoxylin. Positive expression was indicated by brownish-yellow granules in the cytoplasm of hepatocytes for mIL-2 or in the nucleus of tumour cells for PCNA.

2.8. Flow cytometric analysis

Liver and spleen leukocytes were harvested following the method of Gonzalez-Aparicio et al. [15]. Leukocytes were stained with the following fluorochrome-conjugated antibodies: CD8-PE, CD4-FITC, CD25-APC, FoxP3-PE, CD49b-APC, and CD3-FITC from BioLegend. Intracellular staining for FoxP3 was performed using FOXP3 Fix/Perm Buffer Set (BioLegend). Cells were acquired on FACSCalibur and the data were analyzed using CellQuest software (BD Biosciences).

2.9. Statistical analysis

Data are reported as means ± SEM. Differences between groups were analyzed by a two-tailed unpaired *t*-test (GraphPad InStat). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Structure of plasmids

The structure of plasmid pRS-IL-2 and pRS-LacZ is shown in Fig. 1A. The transgene mIL-2 or Lac Z was cloned downstream of the Gal4 DNA-binding site and upstream of the GLp65 expression cassette under the TATA minimal promoter followed by the BGH polyadenylation signal. The RU486 responsive transactivator GLp65 was under control of TTR and followed by the SV40 polyadenylation signal. When the GLp65 was activated by RU486, it induced transgene expression by binding to the Gal4 site.

3.2. Kinetics and RU486 or plasmid dose-dependent expression of mIL-2 in serum

The mice were divided into two groups 7 days after injection of pRS-mIL-2. One group was given a single intraperitoneal injection of RU486 at a dose of 250 μg/kg. Another group was given 50 μl of sesame oil as a control. Serum mIL-2 levels were measured at different time points. As shown in Fig. 1B, the kinetics study indicated that the peak concentration of mIL-2 was detected 4 h after administration of RU486. It then declined abruptly to baseline levels at 10 h, and was undetectable by 24 h. The kinetic pattern was similar regardless of the plasmid dose. MIL-2 was undetectable in mice treated with sesame oil alone.

In mice receiving 2 μg, 5 μg or 10 μg plasmid, 4 h after RU486 induction, the mIL-2 level was respectively 46, 320 and 479 fold-greater than the values before induction. The maximum level of mIL-2 was found in the mice receiving 10 μg plasmid ($P < 0.05$ *vs* 5 μg, $P < 0.001$ *vs* 2 μg) (Fig. 1B).

In mice receiving 0–500 μg/kg RU486, at days 7 after injection of 5 μg pRS-mIL-2, a clear correlation was observed between the dose of RU486 and the serum concentration of mIL-2. MIL-2 was undetectable in the absence of RU486. In comparison to the 500 μg/kg