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Adeno-associated virus mediated expression of human erythropoietin *in vitro*

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KEY WORDS dependovirus; erythropoietin; gene expression; gene therapy

ABSTRACT

AIM: To investigate the expression of human erythropoietin (hEpo) in 293 cell line using adeno-associated virus (*AAV*) vector, and provide information for its potential application in human gene therapy. **METHODS**: The human Epo cDNA was inserted into the *AAV* cassettes, and recombinant adeno-associated virus (*rAAV*-EPO) vectors were generated by cotransfection methods without helper virus. The expression of hEpo was detected by RT-PCR and ELISA methods. **RESULTS**: A recombinant adeno-associated virus expressing hEpo was constructed and generated successfully. Positive signal of mRNA was detected from 293 cells transduced with *rAAV*-EPO, and the hEpo level in supernatant reached 500 U/L. **CONCLUSION**: *rAAV*-EPO expressed biologically active hEpo effectively, suggesting that it is a suitable gene expression tool for further study *in vivo* experiments.

INTRODUCTION

Erythropoietin (Epo) is a glycoprotein that serves as the primary regulator of red blood cell production in mammals. Repeated intravenous or subcutaneous injection of recombinant human Epo (rhEpo) is now widely used for long-term treatment of anemia associated with chronic renal failure, cancer, and human immunodeficiency virus infections. Although they are largely effective, such therapies are both expensive and inconvenient.

Gene therapy is ideally suited for long term delivery of therapeutic proteins, since, in principle, gene therapy can deliver genes to cells or tissues that serve as sites for the synthesis and secretion of proteins that have effects elsewhere in the body. So gene therapy offers the potential for increased efficacy and more cost-effective production and administration for therapeutic proteins.

The human parvovirus adeno-associated virus (*AAV*) is currently considered as one of the most promising viral vectors for *in vivo* gene delivery, because of a unique combination of attractive properties[1]. *AAV* is regarded as being naturally defective, and this, together with the fact that, the virus has never been associated with any human disease, gives *AAV* one of the highest biosafety ratings among all gene transfer vectors of viral origin. An additional intriguing feature of *AAV* vectors is their capacity to infect both mitotic and postmitotic cells of varying cell type. The most appealing property of *AAV* vectors is, probably, their potential to mediate long-term gene expression, based on transformation of the recombinant genomes into stable episomal forms and/or integration into the host genome. Moreover, the feasibility of peroral administration renders *AAV* vector as a palatable choice compared with current invasive pharmacological treatments[2-4].

In this study, we constructed a recombinant *AAV* (*rAAV*) expressing hEpo gene, and examined the *in vitro* expression in 293 cells.

MATERIALS AND METHODS

Cell lines The 293 cells (purchased from Institute of Virology, Chinese Academy of Preventive Medicine) was cultured in Dulbecco's modified Eagle's medium (Hyclone, Utah, USA) containing 10 % fetal bovine serum.

Vectors and plasmids The plasmid pUC-EPO containing the hEpo cDNA was kindly provided by Prof HU Yun-Long in Nanjing Huadong Medical Institute. The *pAAV-lac*Z (Constructed by Prof XU Rui-An in

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Hong Kong University) contains an eukaryotic expression cassette controlled by the cytomegalovirus (CMV) promoter and the simian virus 40 (SV40) polyadenylylation signal, and flanked by the *AAV* 145-bp inverted terminal repeats (ITRs). The *pAAV*-EPO was made by inserting an *Eco*R V/*Xho*I fragment of hEpo cDNA into *pAAV-lac*Z cassette after *lac*Z was detected.

PCR PCR amplification was performed using PCR Amplification Kit R011CA (TaKaRa Biotechnology Co, Ltd, Dalian - China). The 598-bp hEpo cDNA fragment was derived by PCR from pUC-EPO, using primers as follows: P1 5'-CCG CTC GAG ATG GGG GTG CAC GAA TG (containing *Xho* I site); P2 5'-CCG GAT ATC ATC TGT CCC CTG TCC TGC (containing *Eco*R V site). The 100 μL PCR mixture contained 0.2 μg template DNA in a volume of 1 μL, 10 × buffer 10 μL, dNTPs 20 μmol, each primers 8 pmol, and *Taq* DNA polymerase 5 U. After one cycle of pre-heating at 94 ℃ for 5 min, the cycle program was set to denature at 94 ℃ for 30 s, to anneal at 55 ℃ for 30 s, and to extend at 72 ℃ for 1 min. After 25 cycles were finished, 1 μL of aliquot was analyzed on a 1.5 % agarose gel.

Generation of *rAAV* vectors The *rAAV*-EPO vector was generated using the helper-free packaging system with cotransfection methods[1,5,6]. At eight hours after transfection, the medium was replaced with fresh Dulbecco's modified Eagle's medium (Gibco, New York, USA) containing 10 % fetal bovine serum. The cells were harvested at 48 h postinfection using sonication. After low-speed centrifugated on a tabletop centrifuge, the cell pellets were resuspended in 2 mL of NaCl 100 mmol/L- Tris-HCl 10 mmol/L (pH 8.5), subjected to 4 cycles of freeze-thaw and cell debris were removed from it. The *rAAV*-EPO stocks were stored at -20 ℃ before use.

***In vitro* transduction of 293 cells** Exponentially growing 293 cells were seeded in triplet in 6-well tissue culture plate at a density of 7.5×10^5/well 24 h before transfection and transduction. The 293 cells were transfected *rAAV*-EPO plasmids with calcium phosphate precipitation in order to examine this construct. While for transduction efficiency, the cells were transduced with 20 μL *rAAV*-EPO stocks per well.

RT-PCR Total RNA was extracted using Catrimox-14™ RNA Isolation Kit (TaKaRa Biotechnology Co, Ltd, Dalian, China). RT-PCR amplification was performed using One Step RNA PCR Kit RR024A CA (TaKaRa Biotechnology Co, Ltd, Dalian, China) with the same primers as in PCR. The temperature profile was as follows: One strand cDNA reverse-transcription at 50 ℃ for 30 min, reverse transcriptases inactivation at 94 ℃ for 2 min, 30 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 1 min. Aliquot 1 μL was run on a 1.5 % agarose gel for analysis.

Epo assays Epo levels were measured in 293 cell culture supernatants at different time points after transduction by ELISA kit (human erythropoietin Quantikine IVD, R&D Systems Inc, USA) according to manufacture's recommendations. Aliquots of media were taken and analyzed at the different time points after transfection and transduction.

RESULTS

Construction of *pAAV*-EPO plasmid To construct a plasmid expression vector that could program high-level production and secretion of Epo, we cloned the hEpo cDNA into *pAAV-lac*Z, a plasmid vector that had been used to generate *rAAV* and showed to program high-level *lac*Z gene expression after peroral administration of the *rAAV* into rats in the previous experiments[2]. The *pAAV-lac*Z was digested with both *Xho* I and *Eco*R V. The large fragment was ligated to the *Eco*R V/*Xho* I fragment of hEpo cDNA PCR product (Fig 1).

Analysis of *pAAV*-EPO plasmid After double digestion of *pAAV*-EPO with *Xho* I and *Eco*R V, two DNA fragments about 0.6 kb and 4 kb were released as expected (Fig 2). The same analysis of *pAAV-lac*Z was also performed at the same time.

RT-PCR To determine transduction efficiency of *rAAV*-EPO vectors, total RNA was extracted from 293 cells post transduction with *rAAV*-EPO vectors for 48 h, and then subjected to RT-PCR. Clearly positive signal of mRNA was detected on the gel (Fig 3), while no any positive signal was found in the control, *rAAV-lac*Z group.

***In Vitro* analyses of hEpo** The transfection experiment on 293 cell lines revealed that this construct worked functionally as high Epo level was obtained. Analysis of media taken from hEpo 293 cells culture at various times after transduction showed that levels of hEpo increased gradually, and high level of hEpo secretion was observed at 48 h post transduction (Fig 4). In contrast, levels of hEpo were under detected in the media from 293 cells transduced with *rAAV-lac*Z.

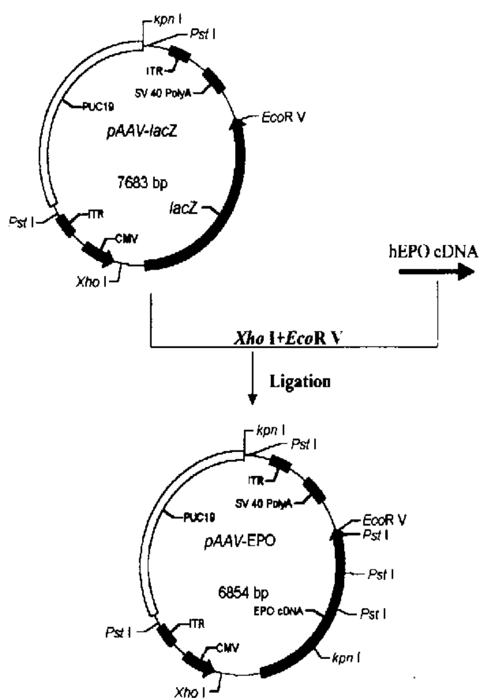

Fig 1. Construction of *pAAV*-EPO expressing human erythropoietin. Human erythropoietin cDNA obtained by PCR was cloned into *pAAV-lacZ* which contains a eukaryotic expression cassette controlled by the CMV promoter and the SV40 polyadenylylation signal, and flanked by the *AAV* 145-bp inverted terminal repeats (ITR).

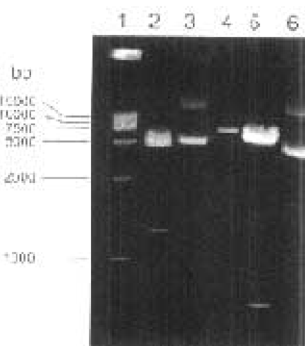

Fig 2. Restriction analysis of recombinant plasmid. 1) DNA Marker. 2) *pAAV-lacZ/EcoR* V + *Xho* I. 3) *pAAV-lacZ*. 4) *pAAV-lacZ/Kpn* I. 5) *pAAV*-EPO/*Eco*RV + *Xho* I. 6) *pAAV*-EPO.

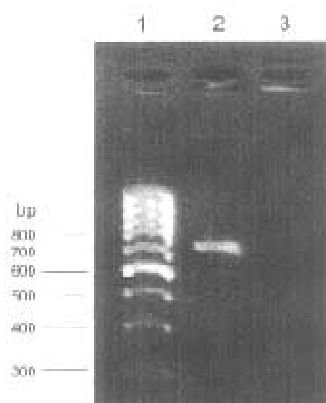

Fig 3. RT-PCR analysis of hEpo mRNA in 293 cells after transducing with *rAAV*. 1) DNA lader. 2) *rAAV*-EPO. 3) *rAAV-lacZ*.

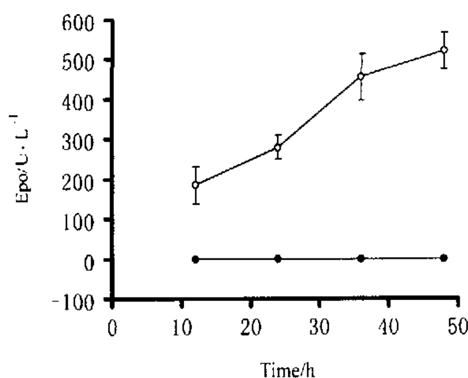

Fig 4. Time course of hEpo secretion after transducing of 293 cells with *rAAV*-EPO (○) and *rAAV-lacZ* (●). Data is representative of 3 experiments. $\bar{x} \pm s$.

DISCUSSION

The availability of rhEpo provided a major advance in the treatment of anemia in renal failure patients. The life quality of these patients was significantly improved. A partial correction of the hemolysis and anemia was also reported after the administration of rhEpo in patients with intermediate β-thalassemia[7]. But the high frequency of the disease and the necessity of life-long treatment with rhEpo would result in unaffordable costs, and 2 or 3 times per week invasive im or sc injection would cause great physical pains. Therefore, looking for a simple and efficient method to cure such a disease become urgent.

The primary objective of a gene therapy approach is to restore metabolism normally. Using gene therapy to approach such a goal may be possible. Furthermore, previous studies have demonstrated that a single peroral administration of the *rAAV* vector resulted in persistent expression of the transgene and long-lasting phenotypic correction in rat model for more than 6 months[2-4],

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indicating that the 'gene pill' strategy using *AAV* vectors might be useful for Epo-responsive anemia. Here we have constructed a novel *rAAV*-EPO vector that directs high-level production and secretion of hEpo from 293 cells into culture media, suggesting that it is an extremely suitable tool for further study *in vivo* experiments. Investigation on the high-level expression of hEpo gene *in vivo* is under way in our laboratory. The feasibility of a long-term systemic delivery of hEpo after peroral administration may have implication for Epo-responsive anemia.

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腺相关病毒介导的人红细胞生成素体外表达

R96 A

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关键词 依赖病毒；红细胞生成素；基因表达；基因治疗

目的：研究腺相关病毒载体介导的人红细胞生成素在 293 细胞的体外表达. **方法**：将人红细胞生成素 cDNA 克隆至 *AAV* 表达载体，采用无辅助病毒共转染法生产重组 *AAV*-EPO 病毒，利用 RT-PCR 和 ELISA 法检测 hEpo 的体外表达. **结果**：成功构建和生产了表达 hEpo 的重组 *AAV*-EPO 病毒. 重组 *AAV*-EPO 病毒感染 293 细胞后，RT-PCR 结果显示有 hEpo 基因表达，细胞上清中 hEpo 的表达水平达 500 U/L. **结论**：所制备的重组 *AAV*-EPO 病毒可以有效表达 hEpo，可以进一步用于动物体内实验.

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