Rapid Construction of EGFP Labled Recombinant Adenovirus Containing hVEGF165 and Its Expression in Haematopoietic Cells*

ZHONG Zhaodong (仲照东)[1], ZOU Ping (邹 萍)[1], HUANG Shiang (黄士昂)[1], HU Zhongbo (胡中波)[1], LIU Lingbo (刘凌波)[1], LU Yunping (卢运萍)[2]

[1] *Institute of Haematology, Xiehe Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022*

[2] *Biological Center of Cancer, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022*

Summary: By using AdEasy system, which is based on the homologous recombination in bacteria, an EGFP labeled recombinant adenovirus vector containing hVEGF165 was constructed quickly and efficiently expressed in mouse haematopoietic cells. First, hVEGF165 coding sequence was subcloned into shuttle plasmid pAdTrack-CMV, then cotransformed with adenoviral backbone vector pAdEasy-1 into E. coli strain BJ5183. The recombinant adenoviral plasmid was transfected into HEK293 cells to assembly replication-defective adenovirus Ad-EGFP/hVEGF165. The expression of EGFP could be easily detected. The rate of EGFP positive mouse bone marrow mononuclear cells by flow cytometric analysis was 27.3 % (MOI=100), and the expression of hVEGF165 protein in the supernatant was (1385±332) pg/10^6 cells. These results suggest that the construction of adenovirus vector by homologous recombination in bacteria features high efficiency and simplicity. The prepared high titer Ad-EGFP/hVEGF165 can be used an efficient helpful vector to infect hematopoietic cells.

Key words: EGFP; vascular endothelial growth factor; adenovirus; homologous recombination; haematopoietic cells

Vascular endothelial growth factor (VEGF) is an endothelial cell specific mitogen that plays an important role in normal and pathological angiogenesis. More and more reports have revealed the significant regulation of embryonic and postnatal hematopoiesis by VEGF[1-4]. Though the application of recombinant VEGF in many studies has made some inspiring results, there still exists problems such as the expensive price and difficulties in procession of delivery. However, by using adenoviral vector, the expression of hVEGF can achieve sufficient level and duration[3]. A simplified system (the AdEasy System) expedites the process of generating recombinant adenoviruses[5].

1 MATERIALS AND METHODS

1.1 Plasmids, Bacterial Strains and Cell Lines

The plasmid pCD-hVEGF$_{165}$ (Amp^r) containing hVEGF$_{165}$ coding sequence provided by Doctor Yang Shuhua and Yang Cao (Xiehe Hospital, Tongji Medical College); BJ$_{5183}$ strains, shuttle plasmid pAdTrack-CMV (containing EGFP gene) (Kan^r) and pShuttle-CMV (Kan^r), adenoviral backbone plasmid pAdEasy-1 (Amp^r) provided by Doctor He Tongchuan (Johns Hopkins Oncology Center); DH5α strains, HEK293 cells preserved in our laboratory.

1.2 Generation, Purification and Titter of Recombinant Adenovirus EGFP/hVEGF165

hVEGF$_{165}$ segment was subcloned from pCD-hVEGF$_{165}$ (Amp^r) into pAdTrack-CMV, then the positive recombinant clone pAdTrack/hVEGF$_{165}$ (Kan^r) was picked up and 1.0 μg plasmid was linearized by endonuclease PmeI and electrotransformed with adenoviral backbone plasmid pAdEasy-1 (Amp^r) into *E. coli* strain BJ5183 (2500 V, 200 Ω, 25 μF). Homologous recombinant adenoviral plasmid pAd EGFP/hVEGF165 was identified on electrophoresis gel by restriction endonuclease digestion with PacI and by PCR analysis for hVEGF165. The upstream primer was 5′-TGC CTT GCT GCT CTA CCT CC-3′ and the downstream primer was 5′-TCA CCG CCT CGG CTT GTC AC-3′. PCR was performed for 30 cycles as follows: 94 ℃ 30 s, 64 ℃ 30 s, 72 ℃ 30 s. Four μg of recombinant pAd EGFP/hVEGF165, digested with PacI, were used for transfection of 293 cells by using Lipofectamine 2000 (GIBCOBRL, USA). Transfected cells were monitored for EGFP expression or cell pathogenic effect (CPE) to test the production of recombinant viruses about 5—7 days post-transfection. After 3 cycles of freezing/thawing, the supernatant of viral lysate was used to infect 293 cells for the preparation of high titer viruses. The total lysate supernatant was transferred to CsCl band (density of 1.35 g/ml) and purified after centrifugation (Beckman, 32 000 r/min, 10 ℃, 18 h). The purified viruses were diluted with PBS (pH 7.4, GIBCO, USA) and stored at 80 ℃. The viral titer was checked by EGFP expression or by traditional plaque assays[5].

1.3 Identification of Recombinant Ad EGFP/hVEGF165

Purified Ad EGFP/hVEGF165 were observed and photographed under a electron microscope. 5 μl of supernatant of normal 293 cells or infected 293 cells and purified Ad EGFP/hVEGF165 were digest-

ZHONG Zhaodong, male, born in 1971, M. D., Ph. D., Doctor in Charge

* This project was supported by the Overseas Scholar Cooperative Foundation (No. 39928010).

ed with 10 μl proteinase K (20 mg/ml) at 55 ℃ for 5 min. Each 2 μl production was used as PCR template for amplification of hVEGF165 fragment in adenoviral genomic DNA (conditions as above).

1.4 Recombinant Adenovirus Mediated EGFP and hVEGF165 Expression in Mouse Bone Marrow Mononuclear Cells

BALB/c mice (6—8 week-old) bone marrow were obtained by flushing both separated femoral bones with cold RPMI 1640 containing 10 % FCS. Bone marrow mononuclear cells (BMMNCs) were collected after centrifugation over continuous gradient using Ficoll-Hypaque (1.084), washed twice with RPMI 1640 to a final density of 1×10^7 cells/ml. 1×10^6 cells were incubated with 100 μl purified Ad EGFP/hVEGF165 (MOI=100) for 2 h at 37 ℃ 5 % CO_2, then cultured continuously up to 24 h after each well was supplemented with complete media (RPMI 1640 containing 10 % FCS). The expression of EGFP was tested by fluorescent microscope and flow cytometry. The hVEGF165 levels in the supernatant of cultured BMMNCs were evaluated using commercial ELISA Kit (Jing Mei Biological Engineering Company, Co Ltd) following the manufacturer' instructions. The control group was normal BMMNCs from BALB/c.

2 RESULTS

2.1 Identification of Recombinant pAd EGFP/hVEGF165

The products of homologous recombinant pAd EGFP/hVEGF165 digested with PacI were a 30 kb fragment and a 4.5 kb fragment (Lane 3 in fig. 1), while only a 30 kb fragment (Lane 1) for adenoviral backbone plasmid pAdEasy-1, a 4.5 kb and a 3.0 kb fragments (Lane 2) for recombinant shuttle plasmid pAdTrack/hVEGF$_{165}$. The recombinant shuttle plasmid pShuttle-hVEGF$_{165}$ and adenoviral plasmid pAd hVEGF$_{165}$ without EGFP were reconstructed at the same time, and the results of restriction endonuclease analysis were shown in Lanes 4 and 5 (fig. 1). PCR analysis for hVEGF$_{165}$ sequences inserted in recombinant plasmid were demonstrated in fig. 2 (Lanes 1 and 6).

2.2 Identification of Recombinant Adenovirus

HEK293 cells swelled and turned round 5—7 days post-transfection. The expression of EGFP located in the plasma and nucleus under the inverted fluorescent microscopy. Under electron microscopy, the purified viral particles were homogenous hexagon. The amplification of exogenous hVEGF$_{165}$ sequence inserted in adenovirus genome by PCR showed a 540 bp band on 2 % gel (Lanes 2 and 3 in fig. 2).

2.3 The Infection of Mouse Bone Marrow Mononuclear Cells with Ad EGFP/hVEGF165

The EGFP was obviously showed in mouse BMMCs 24 h post-infection with recombinant viruses (MOI=100). The EGFP positive cells were 27 % revealed by flow cytometry (fig. 3).

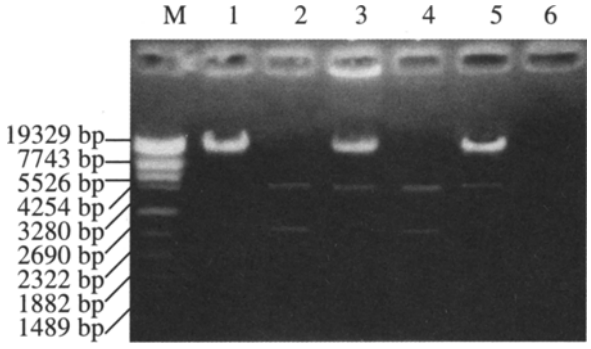

Fig. 1 Identification of recombinant adenoviral plasmid by Pac I digestion
M: Lambda pUC Mix markers;
1: pAdEasy-1;
2: pAdTrack/hVEGF165;
3: pAdEGFP/hVEGF165;
4: pShuttle/hVEGF165;
5: pAd hVEGF165

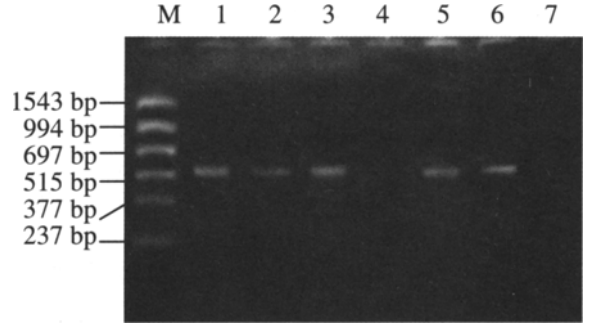

Fig. 2 Identification of hVEGF165 sequence by PCR analysis
M: PCR marker;
1: pAdEGFP/hVEGF165;
2: Ad EGFP/hVEGF165 (purified);
3: Supernatant of infected 293;
4: Supernatant of normal 293;
5: pAdTrack/hVEGF165;
6: pCD-hVEGF165; 7: pAdEasy-1

2.4 The Level of hVEGFf$_{165}$ Protein in the Supernatant of BMMNCs

The expression of hVEGF165 protein in the conditioned medium of mouse BMMNCs was (1385 332) pg/10^6cells ($n=4$) after 24 h incubation with Ad EGFP/hVEGF165. The hVEGF165 protein was not detectable in supernatant of control group.

3 DISCUSSION

Recombinant adenovirus is a versatile tool for gene delivery and expression. The method traditionally used to generate recombinant adenovirus depends on homologous recombination in eukaryotic cells. But the cells grow slowly and the efficiency of homologous recombination is low; Besides, many rounds of

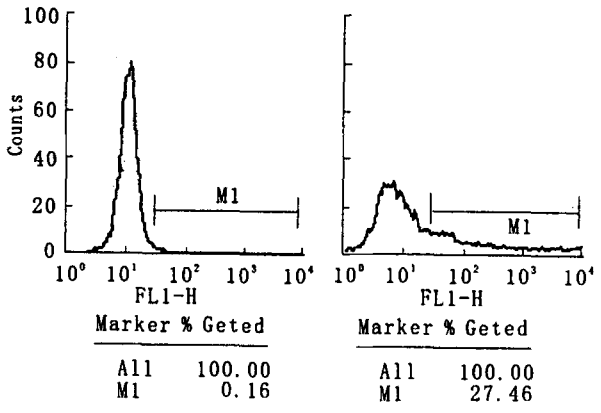

Fig. 3 The percentage of EGFP positive mouse BMMNCs by flow cytometric analysis

plaque isolations are required. All these make the generation of recombinant adenovirus an extremely laborious and time-consuming process. However, bacteria grow rapidly with a high efficiency of homologous recombination and the positive clone is easy to be picked up. The AdEasy system uses a method where homologous recombination is performed in *E. coli*. This method generates recombinant adenovirus vectors that obviate many manipulation in eukayotic cells and significantly simplify the process[5]. By using this method in our research, several recombinant adenoviruses were generated, including Ad-EGFP, Ad-EGFP/hVEGF$_{165}$ and Ad-hVEGF$_{165}$. These facts have confirmed the AdEasy system is a reliable, efficient and convenient method to generate recombinant adenoviruses.

EGFP gene is widely used as reporter gene in various gene transfer system[6]. In the experiment mentioned above, the obvious expression of EGFP in HEK293 cells and BALB/c BMMNCs was observed under fluorecent microscopy. When the viruses were titered by plaque assay, the number of 293 cells which were green could be determined as early as 18 h post-infection. Meanwhile, the infection rate of BALB/c BMMCs was tested in active state by flow cytometry when EGFP is employed as a marker.

In this study, it was found that the result homologous recombinant viruses was titered up to 10^{12} pfu/ml and the infection rate of BALB/c BMMNCs to recombinant adenoviruses was 27.3 % (MOI = 100). The supernatant of BMMNCs cultured contained a high level of hVEGF$_{165}$ proteins (1385 ± 332) pg/10^6 cells.

VEGF has wide bioactivities mediated by two main receptors on the surface of target cells: KDR/Flk-1 and Flt-1. KDR has been recognized as a new marker for hematopoietic stem cells. VEGF and its receptors involve deeply in the prenatal development of hematopoiesis and angiogenesis[4, 7]. VEGF-mobilized PBSCs and EPCs can mediate the recovery of hematopoietic reconstruction successfully[3].

VEGF is a potent mitogen specific for endothelial cells. More studies highlight the role of VEGF on bone marrow microvascular endothelial cells and hematopoietic inductive microenvironment. It has been reported that the release of hematopoietic growth factor was reduced significantly after radiochemotherapy[8]. VEGF has also been shown to exert its pleiotropic effects on tumor immune-escape processes through inhibiting maturation of dendritic cells[1]. Most in vivo experiments assessing the chemoattractant properties of VEGF have been performed with injection of recombinant VEGF. However, the half-life of VEGF in the circulation is too short to evaluate the effects. By using AdEasy system, which is based on the homologous recombination in bacteria, an EGFP labeled recombinant adenovirus vector containing hVEGF165 was successfully generated and efficiently expressed in mouse haematopoietic cells. All these make the following study possible involving the role of VEGF in hematopoiesis and immune response.

REFERENCES

1 Gabrilovich D, Ishida T, Oyama T *et al*. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. Blood, 1998,92: 4150

2 Bautz F, Raffi S, Kanz L *et al*. Expression and secretion of vascular endothelial growth factor-A by cytokine stimulated hematopoietic progenitor cell: Possible role in the hematopoietic microenvironment. Exp Hematol, 2000, 28:700

3 Hattori K, Dias S, Heissig B *et al*. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med, 2001,193:1005

4 Traver D, Zon L I. Walking the walk: Migration and other common themes in blood and vascular development. Cell, 2002,108:731

5 He T C, Zhou S B, Costa L D *et al*. A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA, 1998,95:2509

6 Limon A, Briones J, Puig T *et al*. High-titer retroviral vectors containing the enhanced green flurescent protein gene for efficient expression in hematopoietic cells. Blood, 1997,90:3316

7 Keller G, Lacaud G, Robertson S. Development of the hematopoietic system in the mouse. Exp Hematol, 1999,27:777

8 Gaugler M H, Squiban C, Claraz M *et al*. Characterization of the response of human bone marrow endothelial cells to the in vitro irradiation. Br J Haematol, 1998,10: 980

(Received Oct. 8, 2002)