Construction of Recombinant Adenoviral Vector Overexpressing Human HIF-1α Gene

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Abstract Hypoxia inducible factor 1 (HIF-1) is a heterodimeric transcription factor that plays an important role in oxygen homeostasis. In response to low level of oxygen, subunit HIF-1α expression is upregulated and transactivates its target genes essential for energy metabolism, erythropoiesis and vascular development. HIF-1α is thought to be able to protect hypoxic cells from apoptosis or necrosis under ischemic and anoxic conditions, the major trauma factors that affect the recovery of brain and spinal cord injury. Here we report the construction of recombinant adenovirus vector overexpressing HIF-1α intended for gene therapy against desired neuronal injuries. The recombinant vector could be packaged and yielded significantly high viral titers at 2×10^{13} CFU in HEK293T cells and good expression levels of HIF-1α when superinfected in Hela cells.

Key words HIF-1α, adenovirus vector, gene therapy

Oxygen homeostasis plays an important role in human development and physiology. This delicate balance is disrupted in heart disease, cancer, cerebrovascular disease, and chronic obstructive pulmonary disease, which represents the most common causes of mortality in the world[1]. Hypoxia inducible factor (HIF-1) is a basic helix-loop-helix PAS (Per/Arnt/Sim) protein consisting of HIF-1α and HIF-1β subunits[2]. HIF-1β is constitutively expressed, and can heterodimerize with the ary1 hydrocarbon receptor nuclear translocator (Arnt)[2,3]. Whereas HIF-1α mRNA is constitutively expressed in tissue culture cells, it is markedly induced by hypoxia or ischemia in vivo[4,5]. On the other hand, HIF-1α protein expression is negatively regulated in normal cells by ubiquitination and proteasomal degradation[6-8]. The induction of HIF-1α may account for the steady level of VEGF expression in hypoxic cells in angiogenesis[9]. Other target genes transactivated by HIF-1α are those involved in glucose/energy metabolism, erythropoiesis and vascular development/ remodeling[1].

There is a good body of evidence suggesting that HIF-1α may protect hypoxic cells from apoptosis or necrosis. First, ischemic preconditioning experiments have shown that cerebral preconditioning is blocked by NOS inhibitors[10], the gene product upregulated by HIF-1α. Second, significant protection can also be achieved by injecting the rats with cobalt chloride or desferrioxamine[11], which are known inducers of HIF-1α activity[12]. Third, when adult rats are subjected to permanent middle cerebral artery occlusion, HIF-1α mRNA is induced in the penumbra or viable tissue surrounding the infarction[5], which consequently upregulates the glycolytic metabolism to promote the survival of neurons within the penumbra.

Ischemia and anoxia are two major factors that affect the recovery of the wounds in brain and spinal cord. We reasoned that overexpression of HIF-1α might make the injured tissue reconstruct blood circulation and supply nutrition through VEGF, EPO and iNOS. As a first step to explore this possibility, we constructed recombinant adenoviral vector expressing HIF- 1α and analyzed pseudovirus packaging and overexpression profiles of HIF-1α. The results indicated that the vector might be a promising gene therapeutic tool in application to brain and spinal cord injuries.

1 Material and Methods

1.1 Cell culture and reagents

HEK293T cell line was a kind gift from Dr. G.X. Gao (IOM,

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CAS), and Hela cell line was obtained from ATCC. These lines were maintained in MEM (Hyclone), supplemented with 10% fetal bovine serum (Hyclone), 100 units/mL of penicillin, and 100 mg/mL of streptomycin at 37℃ in 5% CO_2. Restriction enzymes were all obtained from New England Biolabs, italic DNA polymerase was purchased from Invitrogen, and Lipofectamine was from GIBCO. All other fine chemicals were purchased from Fluka or Sigma.

1.2 Construction of recombinant shuttle plasmid pAdTrack-CMV- HIF-1α

Oligo DNA with *Kpn* I and *Eco*RV restriction sites in forward and reverse primers, respectively, were synthesized (top primer: 5′-GAGGTACCACCAGTGAGGGCGCCGG-3′ and bottom primer: 5′-TGCTTACGTAGATATCCACCAGTG-3′). After add-on PCR amplification of HIF-1α encoding sequence using pWAV-1 plasmid as the template (constructed by Yu *et al*, unpublished data), the PCR product and shuttle vector plasmid pAdTrack-CMV (gift from Dr. Bert Vogelstein) were then simultaneously digested with *Kpn* I and *Eco*R V (for plasmid maps, see He *et al*.[13]). Agarose gel purified DNA fragments were ligated using T4 DNA ligase (NEB) and transformed into DH5α according to protocols previously described[14]. Positive clones were identified by restriction endonuclease digestion with *Kpn* I and *Eco*R V and further confirmed by DNA sequencing analysis (GeneCore, China). The resulting shuttle plasmid was designated as pAdTrack-CMV/HIF-1α.

1.3 Generation of Recombinant Adenoviral Plasmid pAd/HIF-1α by Homologous Recombination in *E. coli*

The shuttle plasmid pAdTrack-CMV/HIF-1α and its parental vector pAdTrack-CMV (for plasmid maps, see He *et al*.[13]) were linearized with *Pme* I, purified by phenol-chloroform extraction and ethanol precipitation, and mixed with the supercoiled receptor plasmid pAdEasy-1 (gift from Dr. Bert Vogelstein). The DNA mixture was electroporated into E. coli BJ5183 cells (2.0 mm cuvette, 2,500 V, 200 ohms, and 25 Mf, Gene Pulser, Bio-Rad). Positive clones were screened first by analyzing the sizes of the supercoiled plasmid DNA by pulse field electrophoresis in 0.8% agarose gel (Biowest Inc.). Potential recombinant clones were then confirmed by PCR amplification, followed by PmeI digestion. Once confirmed, supercoiled plasmid DNAs were electroporated into DH10B bacterial cells for maxi plasmid DNA preparation (Qiagen).

1.4 Package of recombinant adenoviruses in HEK293 cells

5×10^5 HEK293T cells were plated in 10cm plates 12 hours to reach 50% ~ 70% confluency. Recombinant adenoviral vector pAd/HIF-1α and its parental control pAd were linerized with Pac I, ethanol precipitated, and then transfected into HEK293T using Lipofectamine according to manufacturer's manual. Transfection efficiency was monitored by GFP green fluorescence microscopy. Transfectants were collected 7 ~ 10 days later, and the recombinant adenoviruses, Ad/HIF-1α or Ad, were harvested by 4 cycles of freeze-and-thaw of the treated cells.

PCR method was used to identify the recombinant adenoviruses. Briefly, 200 μL of Ad/hif-1α and Ad viral lysates were digested with 15μL protease K (Merck) at 65℃ for 6h. Viral DNA was purified by phenol-chloroform extraction and isopropanol precipitation followed by boiling at 95℃ for 5 min.

1.5 Measurement of infection titers of recombinant viruses

The infection efficiency of recombinant adenoviruses of pAd/HIF-1α was conveniently followed by GFP expression levels. In brief, 50% confluent HEK293T cells in a 96-well plate were super-infected with 200 μL of ten-fold serial dilutions of pAd/HIF-1α or its control pAdEasy-1. After 18hours of incubation, cells that expressed GFP were counted under a fluorescence microscope (Nikon). The Viral titer is calculated by using the equation: Viral titer = Number of cells expressing GFP × dilution ratio, colony forming unit/mL, CFU/mL).

The infection ability of recombinant adenoviruses was determined by analyzing ectopic expression levels of HIF-1α in a Western blot assay. In brief, 3.0×10^5 Hela cells were seeded into 35mm plate to reach 75% confluency before 4.0×10^6 CFU/mL of pAd/HIF-1α or pAd were added. After indicated time of incubation, cells were collected and lysed, and HIF-1α was detected by its monoclonal antibody (NeoMarker) using the Supersignal ECL system (Pierce).

2 Results

2.1 Construction of the recombinant adenoviral vector pAd/HIF-1α

Add-on PCR amplification yielded a DNA fragment with the size of 2.5 kb, similar to the length of HIF- 1α gene (Fig. 1A). The corresponding fragment was purified from the gel and ligated into pAdTrack-CMV. After transformation into DH5α, the positive insertion clones were first identified by PCR reaction using primers flanking the MCS site (see Material and Methods, Fig. 1B). Clones containing 2.5 kb inserts were further analyzed by restriction digestion with *Pme* I, *Kpn* I and *Eco*R V, respectively (Fig. 2). The resulting

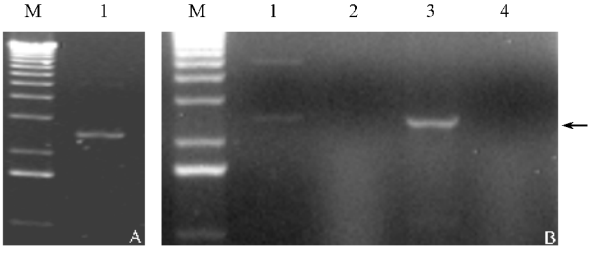

Fig.1 Construction of pAdTrack-CMV/HIF-1α

(A) The product of add-on PCR amplification of HIF-1α was resolved in agarose gel (lane 1). (B) Subclones of pAdTrack-CMV/HIF-1α were screened by PCR reactions using primers flanking the MCS, clones #1 and #3 (lanes 1 and 3) yielded the amplified insert with correct size. Lane M is 1 kb DNA ladder

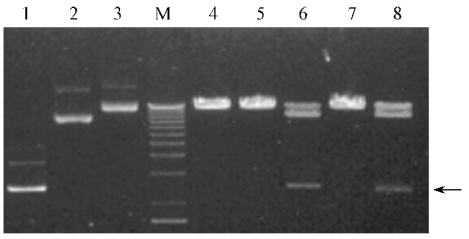

Fig.2　Restriction digestion of pAdTrack-CMV/HIF-1α

DNA fragments were resolved in a 1% Agarose gel. Lane 1 was the PCR product using pET30α(+)-*hif*-1α as a positive control and lane 2 was the parental plasmid preparation of pAdTrack-CMV. Uncut plasmid of the putative positive clone #3 was loaded in lane 3. Restriction digested products by Pme I, *Kpn* Ⅰ, *Kpn* Ⅰ plus *Eco*RⅤ, and *Bgl* Ⅱ plus *Eco*RⅤ were loaded in lanes 4, 5,6,7 and 8, respectively. Note that the plasmid pAdTrack-CMV-*hif*-1αwas digested partially in lanes 6 and 8. Lane M is 1 kb DNA ladder

2.5 kb and 2.4 kb fragments matched exactly the anticipated length of the inserted HIF- 1α gene and the vector, respectively, suggesting that clones contained the right inserts. The positive clones were further confirmed by DNA sequence analysis.

Plasmid pAdTrack-CMV/HIF-1α or the empty vector pAdTrack-CMV was co-transformed into BJ5183 with adenoviral backbone plasmid pAdEasy-1 (see Material and Methods). To verify whether homologous recombination had indeed occurred in the bacteria, restriction digestion of purified plasmids from BJ5183 was performed using Pac I. The anticipated fragments, with the size of 4.5 kb and 21.5 kb, respectively, were detected in the pulse field electrophoresis analysis (Fig. 3, lane 2), indicating the desired recombination events had occurred. The clone was designated pAd/HIF-1α or pAd.

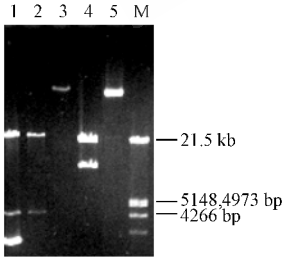

Fig.3　Pulse-field electrophoretic analysis of recombinant pAd/HIF-1α viral vectors

Two independent clones (#1 and #3 in lanes 1 and 2) were subjected to Pac I digestion. The uncut plasmid was loaded in the lane 3 as a control. Recombination acceptor plasmid pAdEasy-1 before and after EcoR I digestion was run in lanes 4 and 5, respectively. Lambda DNA/EcoR I + Hind Ⅲ MW marker was in lane 6

2.2　Packaging of the recombinant adenoviral containing HIF-1α gene

To estimate the infection efficiency by the adenoviral vector, transient transfection of HEK293T cells with pAd/HIF-1α and its mock vector pAdEasy-1, was performed. 48 hours after transfection, about less than 1% cells expressed GFP counted under a fluorescent microscopy. About 80% cells 9 days post transfection lit up indicating a maximal GFP expression (Fig. 4).

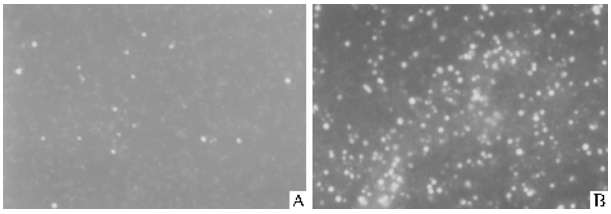

Fig.4　Fluorescent imaging analysis of GFP expression by pAd/HIF-1α in HEK293T cells (×100)

Adenovirus-producing foci (stained in green) were formed 2 days (A) and 9 days (B) after transfection

Supernants from the above transfection were collected and HEK293T or Hela cells were super-infected at indicated titers. 10 hours after infection, cells began to express GFP, detected by the appearance of the green fluorescence. 24 hours post transfection, a few cells began to swell and the number of swollen or necrotic cells increased as the incubation time prolonged. The presence of recombinant adenoviruses was confirmed by PCR analysis using the viral supernatant lysates (Fig. 5). The specific DNA fragment could be amplified from pAd/HIF-1α super-infection (lane 3), but not from that of pAd (lane 4). The viral titers were subsequently calculated as approximately Ad/HIF-1α at 2×10^{13} CFU, and Ad at 8×10^{12} CFU.

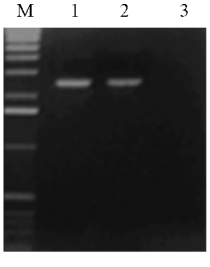

Fig.5　PCR detection of HIF-1αintegration in the packaged virus

Amplified HIF-1α fragment from pAd/HIF-1α super-infection (lane 2) was not detected in the control supernatant of pAdEasy-1 mock infection (lane 3). Lane M was 1kb DNA ladder and lane 1 was positive PCR control using pET30α(+)-hif-1α as the template

2.3　Expression of HIF-1α protein

To investigate whether HIF-1α had been successfully expressed in the superinfected Hela cells, Western blot analysis was done using HIF-1α specific monoclonal antibody. During the time course (Fig. 6A), a protein product with apparent molecular weight about 120 kD was detected, indicating the correct HIF-1a expression (lanes 4,5 and 6). Mock infection with the adenoviruses pAd (lane 2) or untreated Hela cells (lane 7) showed no expression of HIF-1α. HIF-1α level was peaked at 6 hours post-infection (lane 6), and gradually decreased along the time course, while there was almost no detectable HIF-1α by 24 hours. If the viral titer was lowered to 4×10^{5} CFU/10^{6} cells, the expression of HIF-1α could be prolonged even to 72 hours (Fig.6B).

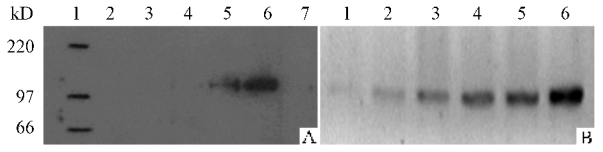

Fig.6 Western blotting analysis of the time course of HIF-1α expression (A) Hela cells infected by pAd/HIF-1 for 6, 12, 24 and 48 hours (lanes 6, 5, 4 and 3, respectively) were lysed and Western blotted with anti-HIF-1 antibody. Mock infection for 6 hours with pAdEasy-1 was loaded in lane 2. Hela lysate was in lane 7. (B) lower dosage of recombinant virus pAd/HIF-1 infection and expression time course of HIF-1 for 6, 12, 18, 24, 36 and 72 hrs post infection indicated in lanes 6, 5, 4, 3, 2, and 1.

3 Discussion

Recombinant adenoviruses provide a versatile system for gene expression studies and therapeutic applications[15, 16]. This unique gene delivery system has been widely used for a variety of purposes, including gene transfer, DNA vaccination[17], and gene therapies[18~20].

Two different approaches are traditionally used to generate recombinant adenoviruses[21, 22]. They both involve direct ligation of homologous recombination inside mammalian cells. However, the low efficiency of large fragment ligation and the scarcity of unique restriction sites have made these approaches difficult in clinical application. The simplified system[13] adopted in this report has three advantageous features to ensure much higher recombination efficiency. (1) It eliminates many unnecessary enzymatic manipulations, (2) the recombinant vector allows much larger gene insertion than the conventional ones, (3) the new vector incorporates a green fluorescent protein (GFP) gene in backbone whose expression is independent of inserted gene. Simultaneous GFP expression permits convenient fluorescent quantification of the transfection efficiency, and (4) the homologous recombination is carried out in *Escherichia coli* rather than in mammalian cells. In our hand as well as in numerous other reports, this system yielded extremely high virus packaging efficiency and high viral titers (10^{12} ~ 10^{13} CFU).

In this study, the recombinant vector pAd/HIF-1α that would potentially used in gene therapy against brain or spinal cord injury has been obtained. When HEK293 cells or Hela cells were super-infected, GFP expression was readily detected 10 hours post infection, indicating that the recombinant viruses could infect HEK293 or Hela cells without the aid of other reagents. Appearance of lytic cells 20 hours after infection indicated that recombinant adenoviruses were able to be packaged and retained infection ability. The expression levels of HIF-1α implied an interesting kinetic pattern, with the maximum at 6 hours after infection. This could be because normal cell culturing conditions do not mimic the O_2-deprived circumstance of HIF-1α functioning. This could also be due to that Hela cells were infected with too high concentration of the recombinant adenovirus (1.2×10^7 CFU/10^6 cells) in our experiments. Over-dosed Hela cells resulted in obvious necrosis that might hinder the expression of HIF-1α. When the viral titer was decreased to 4×10^5 CFU/10^6 cells, HIF-1α could be detected even at 36 hours after infection (Fig. 6B).

Further experiments in determining the correlation between the hypoxic protection and HIF-1α expression by the adenoviral vector *ex vivo* and *in vivo* are underway.

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人 HIF-1α 的腺病毒表达载体的构建与分析

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摘　要　低氧诱导因子 1（hypoxia inducible factor 1, HIF-1）是由 HIF-1α 和 HIF-1β 组成的异源二聚体转录因子，在细胞的氧平衡过程中起重要作用。在应答低氧信号时，HIF-1α 亚基表达水平上调，并通过激活参与细胞能量代谢、红血细胞生成以及血管生成的靶基因表达，达到保护局部缺/贫血细胞免于凋亡或死亡，而后者则是临床上影响大脑和脊椎神经损伤恢复的主要原因。为了达到基因治疗急性神经损伤的目的，我们构建了表达 HIF-1α 的重组腺病毒载体。实验表明，重组腺病毒可以在大肠杆菌中组装，并在 HEK293T 细胞中包装。包装后的 HIF-1α 重组腺病毒载体的病毒感染效率为 2×10^{13} CFU，外源基因 HIF-1α 在 He1a 细胞中的表达 6 h 后达到峰值。目前正在开展建立在此基础上的急性神经损伤动物模型试验。

关键词　HIF-1α，重组腺病毒载体，基因治疗

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