Attenuation of specific CTL responses by highly efficient transduction of the recombinant adenovirus expressing His-tag-ICP47 fusion gene

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Abstract: Hepatocyte transplantation (HT) has been proposed as an alternative therapy to orthotopic liver transplantation (OLT) for patients with acute liver failure and metabolic disorders and hepatocytes are attractive targets for gene therapy. Adenovirus vector is considered a safe and efficient way to introduce foreign genes into several kinds of cells and is widely used in the various fields of gene therapy. But the response of host immune systems against gene products expressed by genetically modified cells is a major obstacle to successful gene therapy. Major histocompatibility complex (MHC) class I antigen presenting pathway is very important in acute allograft rejection and blocking MHC I antigen expression is becoming a research hotspot of inducting immune tolerance. Infected cell protein 47 (ICP47) expressed by herpes simplex virus type 1 (HSV-1), inhibits MHC I antigen presentation pathway by binding to host transporter associated with antigen presentation (TAP), and thereby attenuates of specific cytotoxic T lymphocytes（CTL）responses by virus-infected cells and evades the host immune clearance. This subject was designed to construct a recombinant adenovirus expressing His-tag-ICP47 fusion protein to investigate further the role of ICP47 in the elimination of transgene expression.
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1. Introduction

Organ transplantation is one of the most important treatments of end-stage organ failure, yet graft rejection and shortage of organ donors are the two major problems persisting in the treatments. Hepatocyte transplantation (HT) may serve as an alternative to orthotopic liver transplantation (OLT) for patients with end-stage liver disease because the most important advantage of its decreasing mortality in the waiting list and allowing more patients to be treated[1-2], but immune rejection of HT is still an important problem to be solved[3]. With recent advances in transgenic technology, the availability of transgenic HT evading the clearance of host immune systems could be a critical subject of HT.

Adenovirus vector is considered a safe and an efficient way to introduce foreign genes into several kinds of cells and is widely used in the various fields of gene therapy[4]. However, the response of host immune systems against foreign gene products expressed by genetically modified cells and/or vector-encoded proteins is a major obstacle to successful gene therapy [5]. Therefore, prevention of an immune response against the product of introduced genes and transplanted cells could be as critical as circumventing allograft rejection for the long-term success of gene therapy[6].

On the cellular level, major histocompatibility complex (MHC) class I antigen presenting pathway is very important in acute allograft rejection and has been an attractive target for immune rejection, and blocking MHC I antigen expression is becoming a research hotspot of inducting immune tolerance[7].

Many viruses have evolved mechanisms to evade clearance of host immune systems by blocking MHC I antigen presentation pathway. ICP47, an 88–amino acid cytosolic polypeptide, which is an immediate-early protein expressed by herpes simplex virus type 1(HSV-1), inhibits MHC I antigen presentation pathway by binding to host transporter associated with antigen presentation (TAP), and thereby attenuates of specific CTL responses by virus-infected cells and evades the host immune clearance[8].

Based on these studies, this subject was designed to construct a recombinant adenovirus vector expressing His-tag-ICP47 fusion protein to investigate further the role of ICP47 in the elimination of transgene expression. We expect this finding should have important implications for analyzing the

mechanisms of immune tolerance as well as human gene therapy.

2. Material and Methods

Construction and purification of recombinant adenovirus

The adenovirus vector AdEasy-1 system was used to prepare the recombinant adenovirus expressing His-tag-ICP47 fusion gene or the control empty recombinant adenovirus r-Track as previously described[9]. Firstly, His-tag-ICP47 fusion gene was cloned into the pAdTrack-CMV vector, then the gene fragments digested by restriction endonuclease Pme I were co-transformed in E.coli BJ5183 cells with adenoviral backbone vector pAdEasy-1 to produce recombinant adenovirus vector pAdEasy-H-ICP47. The His-tag-ICP47 fusion gene nucleotide sequence of final constructs was determined to confirm that no mutation was introduced. Linearized with Pac I, the recombinant adenovirus vector was subsequently transfected into human embryonic kidney 293 cells to product r-H-ICP47. Meanwhile, the control empty recombinant adenovirus r-Track was generated in the same way. Finally, the viruses were amplified, purified by ultracentrifugation on a cesium chloride (CsCl) step gradient, dialyzed, and stored in -80 ℃

Efficiency of transfection of HL-7702 Cells In Vitro.

Adenovirus efficiency of transfection was quantified by monitoring the expression of GFP in r-H-ICP47 or r-Track infected cells. Briefly, HL-7702 cells were plated on 12-well plates at a density of 1×10^5 cells/ml, allowed to adhere overnight and removed nonadherent cells by gentle washing with phosphate-buffered saline (PBS). Subsequently, wells were then randomly assigned to one of four experimental groups: multiplicity of infection (MOI) of 0, 50, 100, 200 in triplicate, respectively. HL-7702 cells exposed to viruses at various MOI of r-H-ICP47 or r-Track in 1mL RPMI1640 and incubated for 48 h at 37℃ under air plus 5% CO_2 conditions.

The total cell number was calculated using a standard haemacytometer following cell detachment with 0.1% trypsin plus 1 mM ethylenediamine tetraacetic acid (EDTA) in PBS and the GFP-positive cells were counted with a fluorescent light microscope. Efficiency of transfection (ET) was calculated according to the following formula: ET= number of GFP-positive cells/ number of total cell ×100%.

Western blot analysis

The expression of the proteins produced by recombinant adenovirus was analyzed by Western blot analysis. Briefly, the total proteins were extracted from r-H-ICP47-infected, r-Track-infected, and mock-infected HL-7702 cells, respectively. The proteins were separated by SDS-PAGE, and at the end of the run, polypeptide bands in the gel were electrophoretically transferred to a PVDF membrane (Bio-Rad). The membrane was incubated for 1 h at room temperature with rabbit anti-6×His antibody, rabbit anti-GFP antibody or rabbit anti-β-tubulin antibody (Bioss Inc.), respectively. On the membrane, the binding of antibody to the specific protein band was detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (Bioss Inc.) and an ECL Western blotting detection system.

Growth curves of HL-7702 cells with or without adenovirus infection

HL-7702 cells were plated on 96-well plates at a starting number of 1000 cells/well, allowed to adhere overnight and removed nonadherent cells by gentle washing with phosphate-buffered saline (PBS). Subsequently, HL-7702 cells were infected with 100 µl of r-H-ICP47 or r-Track at a MOI of 100 in triplicate and incubated at 37℃ under air plus 5% CO_2 conditions. The cell proliferation rate was determined every other day for 7 days after infection by an 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously. Briefly, 20 µl MTT solution (5 mg/ml) were added to the culture medium. After 4 h at 37℃ the medium was removed and 150 µl DMSO was added to each well. The colour was allowed to develop for 5 min and optical density at 570 nm was determined.

CTL assay

Briefly, HL-7702 cells were plated on 12-well plates at a density of 1×10^6 cells/ml, allowed to adhere overnight and removed nonadherent cells. On the following day, HL-7702 cells were infected with r-H-ICP47 or r-Track at a MOI of 100 in triplicate and incubated for 48 h at 37℃ under air plus 5% CO2 conditions. Subsequently, r-H-ICP47-infected, r-Track-infected, and mock-infected HL-7702 cells were treated with mitomycin C (50 µg/ml) at 37°C for 45 min to inhibit proliferation, respectively and then washed three times to remove residual mitomycin C.

Lymphocytes were generated from PBMCs. Briefly, human PBMCs were isolated from healthy donors obtained from first affiliated hospital of Zhengzhou University, China, upon ethical approval for the use of such materials. Subsequently, PBMCs were allowed to differentially adhere to 6-well plates by culturing 5×10^6cells /mL in 1 ml of complete RPMI-1640medium/well for 4h at 37°C. Nonadherent cells were then removed by gentle rinsing and lymphocytes were harvested.

The lymphocytes were stimulated in vitro by Mitomycin C-treated cells at an effector: stimulator ratio of 10:1 in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 20U/mL IL-2. Seven days later, The effector cells

were harvested, counted, and mixed with target cells at an effector: target ratio of 20:1 and incubated overnight at 37℃.

Finally, specific cytotoxic activity was determined by an MTT assay and optical density at 490 nm was determined. Specific cytotoxic activity was calculated as follows: specific lysis (%)=(OD_{target}-OD_{mock}-$OD_{control}$)/ OD_{target}×100%.

Statistical analysis

Quantitative results were expressed as mean ± standard error of the mean ($\bar{x} \pm s$). Means between two groups were compared using a two-tailed, unpaired Student's *t* test, Statistical analysis was performed by SPSS 10.0 software. *P* value of 0.05 or less was considered statistically significant.

3. Results

Sequencing identification of recombinant adenovirus and fluorescence photomicrograph observation

The results of sequencing identification of recombinant adenovirus and fluorescence photomicrograph observation was reported in our previous study[9]. The results verified that His-tag-ICP47 fusion gene fragment had been correctly cloned into the recombinant adenovirus vector. And the recombinant adenoviruses of r-H-ICP47 and r-Track were successfully constructed and successfully transducted into 293 cells.

Western blot analysis

Proteins producted by mock-infected, r-Track-infected or r-H-ICP47-infected HL-7702 cells were confirmed by Western blot analysis (Fig.3). In all cells extracts, the blots probed with anti-β-tubulin antibody were detected at approximately 55 kDa molecular mass. Bands of extracts of r-Track-infected and r-H-ICP47-infected HL-7702 cells were recognized at approximately 27 kDa molecular mass when the blots were probed with anti-GFP antibody, but no bands were recognized in the extracts of mock-infected HL-7702 cells. When blots were probed with anti-6×His antibody, the identical band of His-tag-ICP47 fusion protein (11kDa) was recognized in extracts of r-H-ICP47-infected HL-7702 cells, but no bands were recognized in the extracts of mock-infected and r-Track-infected HL-7702 cells.

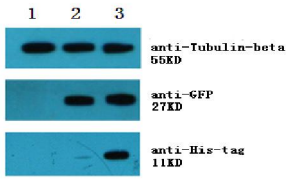
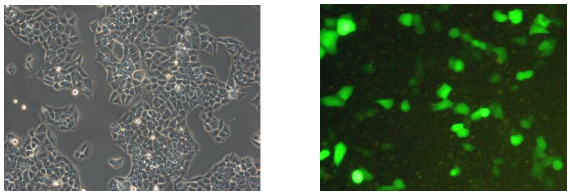


Fig.1 Western-blot assay

Lane1: mock-infected HL-7702 cells; Lane 2: r-Track-infected HL-7702 cells; Lane 3: r-H-ICP47-infected HL-7702 cells

Efficiency of transfection of HL-7702 Cells In Vitro

HL-7702 cells were exposed to viruses at various MOI of r-H-ICP47 or r-Track in 1mL RPMI1640 and incubated for 48h and the green fluorescence could be seen under a fluorescence microscope (Fig. 4). The results of fluorescence photomicrograph observation verified that the recombinant adenoviruses of r-H-ICP47 and r-Track were successfully transducted into HL-7702 cells. Adenovirus efficiency of transfection was quantified by monitoring the expression of GFP in r-H-ICP47 or r-Track infected cells. As MOI increased, efficiencies of transfection were increasing (Fig.5). There was no difference in efficiencies of transfection with r-H-ICP47 between MOI 100 group and MOI 200 group, which were 86.87±3.14% and 88.53±3.69%, respectively (*P*>0.05). Whereas they were significantly higher than the transfection efficiency of MOI 50 group (29.52±5.22%) (*P*<0.05). Similarly, 32.12±2.27% r-Track infected HL-7702 cells expressing *GFP* with a MOI of 50, 90.32±2.25% at a MOI of 100 and 90.64±3.65% at a MOI of 200. On the basis of these studies, all subsequent studies were carried out with a MOI of 100.



A B

Fig. 2 Morphological identification of HL-7702 cells

A. Normal HL-7702 cells before transfected with r-H-ICP47/r-Track（200×）

B. HL-7702 cells transfected with r-H-ICP47/r-Track at the 48th h（200×）

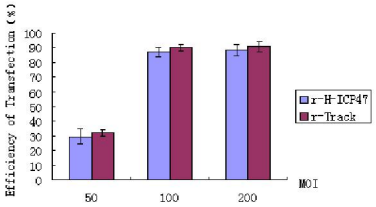


Fig. 3 Efficiency of transfection with r-H-ICP47/r-Track in HL-7702 cells（%, $\bar{x} \pm s$, n=3）

Growth curves of HL-7702 cells with or without adenovirus infection

The cell proliferation rate was determined every other day for 7 days after infection by an MTT assay and the results showed that the kinetics of cell proliferation in HL-7702 cells after exposure to r-H-ICP47 or r-H-ICP47 were similar to that in mock-infected HL-7702 cells as Fig.6 showed（*P*>0.05）.

Moreover, the accelerated phase of the growth curve was initiated from the 3rd day after transduction in three groups of HL-7702 cells. The results verified that the growth of HL-7702 cells was not inhibited by either of the two recombinant adenoviruses.

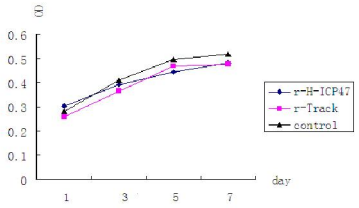


Fig. 4 Growth curves of HL-7702 cells with or without recombinant adenovirus infection.

CTL assay

After HL-7702 cells were transfected with either r-H-ICP47 or r-Track at a MOI of 100, the cytotoxic activity of CTL was determined by using MTT assay. The results showed that the percent cytolysis of HL-7702 cells transfected with r-H-ICP47 by CTL （20.67±3.54%）was reduced comparing with those of the r-track group （36.91±5.36%）and the control group （34.84±4.59%）as Tab.1 showed (P<0.05) .

Tab. 1 Cytotoxicity of CTL activated by HL-7702 cells transfected with r-H-ICP47/r-Track (%, $\bar{x} \pm s$, n=3）

Group	Cytotoxicity of CTL
r-H-ICP47	20.67±3.54*▲
r-Track	36.91±5.36
Control	34.84±4.59

Compared with the control group,* P <0.05; compared with the r-Track group, ▲P <0.05

4. Discussions

Liver pathologies affect hundreds of millions of patients worldwide and can lead to progressive liver injury, liver fibrosis, cirrhosis, and ultimately liver failure, and in some instances, cancer[10]. One of the most common causes of hepatopathy is chronic hepatitis virus infection and one of the most curative therapy is orthotopic liver transplantation (OLT)[11]. But the shortage of organ donors and the high costs are worldwide problems of OLT, and nearly 15% of adult patients with life threatening liver diseases are going to die while on the waiting list[1-2]. Thus, the development of human hepatocyte transplantation (HT) for the treatment of end-stage hepatic diseases is currently under investigation all over the world. Compared with OLT, HT is less expensive, less invasive and relieving shortages of donor organs and it has been proposed as an alternative or a “bridge” therapy for patients with acute liver failure and metabolic disorders[12]. However the procedure of HT is still limited by the immune rejection and the lifelong immunosuppressive treatments, which are not always effective and associated with the risk of infectious complications, and other regimen-related toxicities[13-14].

Adenovirus vectors offer many advantages, including efficient gene transfer, high titer, limited pathological potential, broad range of infectivity, and feasibility for delivery in vivo compared to plasmid vector [4,15]. They have become versatile tools for gene delivery and expression and been used extensively in the treatments of genetic disease[16] and cancer[17]. However, the response of host immune systems against foreign gene products expressed by genetically modified cells and/or vector-encoded proteins is a major obstacle to successful gene therapy. Therefore, prevention of an immune response against the product of introduced genes and transplanted cells could be as critical as circumventing allograft rejection for the long-term success of gene therapy,.

On the cellular level, MHC class I–restricted antigen processing pathway is critical for elimination of most tumor surveillance, transplantation rejection, graft-versus-host reactions, virus infections, and has been an attractive target for immune rejection and blocking MHC Ⅰ antigen expression is becoming a research hotspot of inducting immune tolerance[7]. Efficient antigen presentation restricted by MHC class I is associated with TAP[18], which is a member of the ATP binding cassette protein family. TAP plays a critical role in transporting cytosolic peptides across the membrane of endoplasmic reticulum (ER) for combined with MHC class I heavy chain (HC) and β_2-microglobulin (β_2m). In the absence of antigenic peptides and functional TAP, most MHC class I molecules should be eventually redirected to the cytosol and degraded by proteasomes[19-22].

Many viruses have evolved mechanisms to evade clearance of host immune systems by blocking MHC Ⅰ antigen presentation pathway. ICP47, an 88–amino acid cytosolic polypeptide, which is an immediate-early protein expressesd by HSV-1 and binds to the TAP1–TAP2 heterodimer in human cells and inhibits transport of antigenic peptides from mostly cytosolic proteins into ER, where they would be loaded onto freshly synthesized MHC class I molecules. Consequently, the empty MHC class I molecules are retained in the ER, and the presentation of epitopes to $CD8^+$ T cells is abolished in HSV-infected human cells [8-9]. $CD8^+$CTLs are important for viral clearance in many virus systems. And it recognizes MHC class I molecules bound to small peptides 8 to 10 residues in length derived from viral proteins on the surfaces of virus-infected cells. Because priming of CTL responses requires MHC class I-restricted presentation of the relevant antigen,

the HSV-infected human cells are not lysed by CTL and effectively evade the immune response in humans just as during HSV infection in vivo[23-24].

Recently, the immune response to adenovirus has become increasingly important because adenovirus vectors have become versatile tools for gene gene therapy. It is reported that the CTL response to adenovirus vectors contributes to the elimination of transgene expression in vivo[5].

Thereby, the vector-encoded proteins as well as therapeutic proteins in models of gene replacement therapy are a potential problem. Meanwhile, hepatocytes are attractive targets for gene therapy because a variety of genetic disorders play an important role in hepatic metabolic processes. With recent advances in transgenic technology, the availability of transgenic HT evading the clearance of host immune systems could be a critical subject of HT for the long-term success of gene therapy as well as circumventing allograft rejection.

Based on these studies, we constructed an adenovirus vector expressing the His-tag-ICP47 fusion protein to investigate further the role of ICP47 in the elimination of transgene expression. In our study, the shuttle plasmid pAdTrack-CMV of AdEasy-1 system contains a GFP gene allowing direct observation of the efficiency of transduction, which is very convenient for operation[25], and 6×His tag also facilitates detection using biotinylated anti-6×His antibody and enables purification and detection of recombinant adenovirus without affecting tropism or production[26].

Consequently, a recombinant adenovirus expressing the His-tag-ICP47 fusion protein was successfully constructed and the proteins producted by r-H-ICP47-infected HL-7702 cells were confirm by Western blot analysis[9]. Moreover, efficiencies of transduction in HL-7702 with the recombinant adenoviruses at various multiplicity of infection (MOI) were analyzed and the cytotoxic activity of CTL was determined by using a MTT assay. The results verified that recombinant adenovirus expressing His-tag-ICP47 fusion gene could efficiently and safely transfer genes into the HL-7702 cells and the expression of introduced genes was at a desired level without effecting the growth of HL-7702 cells. Most important of all, recombinant adenovirus r-H-ICP47 had the abilities of attenuating of specific CTL responses by HL-7702 cells transfected with r-H-ICP47 comparing with those of the r-track group and the control group.

To some extents, these results could predict an absence of deleterious host immune responses against the transplanted cells or gene products and indicate that recombinant adenovirus expressing His-tag-ICP47 fusion gene and transplanted cells could confer immune tolerance and lead to long-term cell survival in recipients. The experiments lay a good foundation to carry out *in vitro* and animal studies of experimental gene therapy trial for immunological activities of the His-tag-ICP47 fusion protein and we expect those findings should have important implications for analyzing the mechanisms of immune tolerance as well as human gene therapy. Moreover, we expect these studies should open up new horizons for expanding the fields of viral immunology, exploring the interactions between host immune systems and viruses, and enable us to explore more effective preventions and treatments for clinical diseases.

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