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Therapeutic gene expression in transduced mesenchymal stem cells can be monitored using a reporter gene

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ABSTRACT

Aim: We constructed a recombinant adenovirus construct Ad5-sr39tk-IRES-VEGF$_{165}$ (Ad5-SIV) that contained a mutant herpes viral thymidine kinase reporter gene (HSV1-sr39tk) and the human vascular endothelial growth factor 165 (VEGF$_{165}$) gene for noninvasive imaging of gene expression. The recombinant adenovirus Ad5-SIV was transfected into rat bone marrow-derived mesenchymal stem cells (MSCs), and we measured the expression of HSV1-sr39tk and VEGF$_{165}$ to evaluate the feasibility of monitoring VEGF$_{165}$ expression using reporter gene expression.

Methods: The MSCs were infected with Ad5-SIV at various levels of infection (MOI), ranging from 0 to 100 infectious units per cell (IU/cell). The mRNA and protein expression levels of the reporter and therapeutic genes were determined using real-time RT-PCR, Western blot, ELISA and immunofluorescence. The HSV1-sr39tk expression in the MSCs was also detected in vitro using a cellular uptake study of the reporter probe ^{131}I-FIAU. Gene expression was also evaluated *in vivo* by micro-Positron Emission Tomography/Computed Tomography (micro-PET/CT) imaging 1 day after injecting Ad5-SIV-tranfected MSCs into the left foreleg of the rat. The right foreleg was injected with non-transfected MSCs and served as an internal control.

Results: The real-time RT-PCR results demonstrated a good correlation between the expression levels of HSV1-sr39tk mRNA and VEGF$_{165}$ mRNA ($R^2=0.93$, $P<0.05$). The cellular uptake of ^{131}I-FIAU increased with increasing viral titers ($R^2=0.89$; $P<0.05$), and in the group that received an MOI of 100, a peak value of 30.15%±1.11% was found at 3 hours of incubation. The uptake rates increased rapidly between 30 and 150 minutes and reached a plateau after 150 minutes. The uptake rates of ^{131}I-FIAU by the Ad5-SIV-infected cells were significantly higher than by the Ad5-EGFP-infected cells for all time points ($t=18.43$-54.83, $P<0.05$). Moreover, the rate of VEGF$_{165}$ protein secretion was highly correlated with the uptake rate of ^{131}I-FIAU ($R^2=0.84$, $P<0.05$). The radioactivity on the micro-PET/CT images was significantly higher in the left foreleg (which received the transfected MSCs) compared with the control foreleg.

Conclusions: These results suggest that radionuclide reporter gene imaging may be used to monitor gene expression *in vivo*.

1. Introduction

Gene therapy and stem cell transplantation are promising novel therapeutic strategies for treating ischemic diseases [1,2]. Several ischemic disease studies that used a combined cell transplantation and gene therapy strategy have shown positive results [3,4]. However, there are no perfect methods for non-invasively monitoring and quantifying the location, magnitude and duration of transgene expression and stem cells in ischemic tissues over time [5–9].

Consequently, imaging strategies that can effectively and noninvasively monitor the location, magnitude and duration of transgene expression in stem cells are urgently needed [7–9]. Reporter gene imaging has emerged as an innovative strategy for non-invasively visualizing the biological processes associated with therapeutic gene expression and stem cell delivery [10]. Reporter genes can be co-expressed with therapeutic genes [11,12] and can be expressed in transfected cells [13]. The principle behind using reporter genes in nuclear medicine imaging is to choose reporter genes that are normally absent in the target tissue and can be detected using a radioactively labeled reporter gene imaging probe (either as a substrate or ligand). The therapeutic gene can then be indirectly tracked by measuring the reporter gene expression using positron emission tomography (PET) or single-photon emission computed tomography (SPECT) [14].

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The mutant form of the herpes simplex virus 1 thymidine kinase (HSV1-sr39tk) is widely used in reporter gene imaging to track the expression of a therapeutic gene and to image the location, survival and engraftment of stem cells. The HSV1-sr39tk gene is translated into the HSV1-sr39TK protein, which efficiently phosphorylates a radiolabeled reporter substrate and converts it to a metabolite that is selectively trapped in the transduced cells [12,15,16]. After phosphorylation, the radiolabeled reporter substrate cannot be transported out of the cells. Therefore, the radiolabeled probes accumulate within the cells and can be visualized using SPECT or PET. FIAU (2′-fluoro-2′-deoxy-1-β-D-arabinofuranosyl-5-iodouracil) and ^{18}F-FHBG [9-(4-[^{18}F] fluoro-3-hydroxymethylbutyl guanine)] are the most widely used out of the spectrum of available radiolabeled reporter probes.

Mesenchymal stem cells (MSCs) are easily harvested from bone marrow and are commonly used for cellular therapy [17]. Under suitable culture conditions, MSCs have the potential to differentiate into osteocytes, chondrocytes, adipocytes, tenocytes, myotubes, astrocytes, hematopoietic supporting stromal cells and endothelial cells [18]. Although these characteristics are advantageous for cellular therapy, the mechanisms by which MSC therapy contributes to cardiac repair and improves cardiac function remain largely unknown [15]. Vascular endothelial growth factor (VEGF) is an important initiator of angiogenesis and has commonly been used in animal and clinical trials. Furthermore, VEGF improves the survival of MSCs in ischemic regions [19].

The inability to monitor the delivery and expression of therapeutic genes in target organs and tissues limits the application and optimization of clinical gene therapy protocols. Noninvasive imaging of therapeutic gene expression would benefit many ongoing and future clinical gene therapy trials by defining the location, magnitude and persistence of transgene expression over time [20]. To this end, we constructed a recombinant adenovirus vector encoding both the HSV1-sr39tk and the $VEGF_{165}$ genes to evaluate the feasibility of using recombinant adenoviral vectors for noninvasive imaging during gene-based therapy. This preliminary study provides an experimental basis for monitoring therapeutic gene expression with non-invasive reporter gene nuclide imaging.

2. Materials and methods

2.1. Isolation, culture and identification of rat bone marrow-derived MSCs

MSCs were prepared from rat bone marrow, as described previously [21]. Briefly, 6-week-old male Sprague–Dawley (SD) rats (120–150 g) were obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). The bone marrow cells were collected by flushing the femur and the tibia with phosphate-buffered saline (PBS). The monocyte layer was then collected by centrifuging the cells using a Ficoll density gradient (TBD, Tianjin, China) and resuspending the appropriate layer in Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (DMEM/F12; Gibco, Grand Island, NY, USA) supplemented with 100 U penicillin, 100 g/ml streptomycin and 15% fetal bovine serum (FBS, Gibco). The cells were cultured at a density of 1×10^6 cells/cm^2 in T-25 culture flasks and were incubated at 37 °C in a humidified atmosphere containing 5% carbon dioxide. The culture medium was replaced after the first 24 hours and every 3 days thereafter. The non-adherent hematopoietic cells were removed each time the media was changed. Two weeks after primary cultivation, the adherent cells reached almost 80% confluence. The cells were then removed from the culture surface using a trypsin-EDTA solution (0.25 g/L; Invitrogen, Carlsbad, CA, USA) and were replated at a density of 1×10^4 cells/cm^2 in T-75 flasks for expansion. The MSCs were used at passages 3–8 for the experiment. MSCs were harvested at passage 4 for immunophenotype identification. The MSCs were placed on glass coverslips and were washed three times with PBS before permeabilizing and fixing them for 10 minutes in 4% paraformaldehyde. The cells were incubated with rabbit polyclonal anti-CD34 and anti-CD44 antibodies (Boster, China) at 4 °C overnight, followed by incubation with a secondary antibody (goat anti-rabbit IgG) at room temperature for 1 hour. The cells were then visualized by diaminobenzidine (DAB) staining.

2.2. Construction, purification and characterization of recombinant adenovirus

The replication-defective adenovirus (Ad5-CMV-HSV1-sr39tk-IRES-$VEGF_{165}$, Ad5-SIV) had a cytomegalovirus (CMV) early promoter to drive the expression of a mutant herpes simplex virus type 1 thymidine kinase (HSV1-sr39tk), the reporter gene, and vascular endothelial growth factor 165 ($VEGF_{165}$), as the gene used for therapy, which included an internal ribosomal entry site (IRES). It was constructed, purified and characterized by Vector Gene Technology (China). The HSV1-sr39tk and $VEGF_{165}$ genes in this recombinant adenovirus vector were identified using polymerase chain reaction (PCR). The viral titer of Ad5-SIV was determined to be 7.9×10^9 IU/ml using the tissue culture infective dose (TCID50) method. For the control experiments, a replication-defective adenovirus type 5 carrying the enhanced green fluorescent protein (Ad5-EGFP) was constructed, purified and characterized using a titer of 1.4×10^{10} IU/ml.

2.3. Recombinant adenovirus infections

Adenovirus infection with Ad5-SIV and Ad5-EGFP was performed as previously described [14]. Briefly, the MSCs were incubated with Ad5-SIV and Ad5-EGFP at various multiplicities of infection (MOI) (0, 10, 25, 50, 75 or 100 IU/cell). The incubation was continued for approximately 2 hours before the transfection medium (opti-MEM (Invitrogen, USA)) was removed and replaced with culture medium. Adenovirus-infected MSCs were always used 48 hours after infection.

2.4. Effect of Ad5-SIV on MSCs differentiation potential

We then determined whether the Ad5-SIV infection could have an impact on MSCs differentiation potential into the osteogenic lineage. MSCs infected with Ad5-SIV at a MOI of 100 for 48 hours were cultured on 6-well plates. To induce the osteogenic differentiation, we used the basic culture medium supplemented with 0.1 mM dexamethasone, 50 mM ascorbic acid, and 10 mM β-glycerophosphate. The cells were subsequently incubated for 3 weeks; during this differentiation period the culture medium was replaced every 3 days. After completion of the differentiation protocol, von Kossa staining was used to demonstrate the presence or absence of calcium deposits in MSCs. Non-transfected MSCs were used as control cells.

2.5. Radioiodination of FTAU

We used 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-(trimethylstannyl)-2,4-(1H,3H)-Pyrimidinedione (FTAU; ABX, Germany) as a precursor for FIAU [22]; thus, we prepared ^{131}I-FIAU using FTAU. Briefly, we added 100 μl of $Na^{131}I$ (185 MBq) (no carrier, no reducer; Beijing Atom High Tech, Beijing, China) in 40 μl of 0.1 N HCl and 50 μl of FTAU (5 mg/ml) in methanol in a 1.5 ml Eppendorf tube. After vortexing for 20 seconds at room temperature in a 3:1 (v/v) mixture solution of acetic acid and 30% hydrogen peroxide (H_2O_2/H_3CO_2H, 1:3), we added approximately 1/5 volumes of $Na^{131}I$, and the mixture was vortexed again for 30 seconds at room temperature. The