Effect of hydroxyurea and etoposide on transduction of human bone marrow mesenchymal stem and progenitor cell by adeno-associated virus vectors[1]

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ABSTRACT

AIM: To study the effect of hydroxyurea and etoposide on transduction of human marrow mesenchymal and progenitor stem cells by adeno-associated virus (AAV). **METHODS:** Isolated human bone marrow mesenchymal stem and progenitor cells (hMSCs) were cultured in DMEM containing 10 % FBS or 5 % FBS and dexamethasone 1 μmol/L respectively. After being treated with hydroxyurea and etoposide, hMSCs were transduced by AAV-LUC. After two days luciferase activity (relative light unites per second or RLU/s) were tested, which indirectly reflected the relative transduction efficiency of different groups, and virus DNA was isolated by Hirt extraction for Southern hybridization. **RESULTS:** Transduction luciferase activity and transduction efficiency in cultures treated with hydroxyurea and etoposide were significantly higher than that in control cultures. Dividing cells had about 20-fold higher transduction efficiency compared with control cells. Transduction efficiency in stationary cells was about 50 times higher than that in control cells. Southern analysis showed that hydroxyurea and etoposide enhanced second-strand DNA synthesis by rAAV. **CONCLUSION:** Hydroxyurea and etoposide could increase transduction efficiency of hMSCs by AAV vectors, and stationary cells were more sensitive to these drugs than dividing cells.

INTRODUCTION

Adeno-associated virus (AAV) is a nonpathogenis human parvovirus that contains a single-stranded DNA genome and belongs to the genes dependovirus. In the absence of helper virus, the wild-type AAV establishes a latent infection by integrating site-specifically into human chromosome 19[1-3]. Although first isolated from rhesus monkey's kidney cell cultures, many evidences proved that a wide variety of cell types can be transduced by AAV, including cultured normal human cells[4,5]. AAV shows promise as gene transfer because it is capable of long-term gene expression *in vivo*, and since it does not encode viral genes, it does not elicit a cell-mediated immune response[6-9]. Thus, these properties have been instrumental in the development of AAV as a vector for human gene therapy. Recombinant AAV (rAAV) vectors have been shown to transduce certain cell types well, such as brain and muscle. However there is much difference in transduction efficiency among various cell types, and transduction efficiency in S-phase cells by AAV vector was higher than that in

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non-S-phase cells[5]. These difference stems in large part from the counts of AAV receptors and coreceptors in the apical membrane[10,11] and an immediate-early event of life cycle of AAV, namely, second-strand DNA synthesis. Based on these results, many investigators have taken some methods to enhance transduction efficiency, including γ- and UV-irradiation[12]. However these methods will reduce the colony-forming ability of treated cells. In this study, we examined whether hydroxyurea (DNA synthesis inhibitor) and etoposide (topoisomerase inhibitor) could increase transduction efficiency in human bone mesenchymal stem and progenitor cells by AAV with less cytotoxicity.

MATERIALS AND METHODS

hMSC preparation and cell culture Human bone marrow aspirates 10 mL, taken from the iliac crest of normal donors, were diluted 1:1 with phosphate-buffer saline (PBS) and centrifuged at 900×*g* for 10 min at room temperature. The washed cells were resuspended in PBS to a final volume of 10 mL and layered over an equal volume of 1.073 kg/L Percoll solution. After centrifugation at 900×*g* for 30 min, the mononuclear cells were recovered from the gradient interface and washed with PBS. hMSCs were suspended in low glucose Dulbecco's modified Eagle's medium(DMEM-LG) supplemented with 10 % fetal bovine serum (FBS). All cells were plated in 7-mL medium in a 25-cm^2 plastic culture flast at a density of 2×10^4 per cm^2. The cultures were maintained at 37 ºC in 5 % CO_2 in air, with an initial medium change on the 7th day and then medium changes every 3 d.

Vectors rAAV-LUC was obtained from Institute of Virology, Chinese Academy of Preventive Medicine. It contains cDNA sequence encoding luciferase under the control of human cytomegalovirus immediate early gene promoter (CMV-P) and the simian virus 40 (SV40) polyadenylylation singal, whose titer is 2×10^{18} particles/L.

Transduction assay hMSCs were treated with trypsin (0.25 %) and resuspended in DMEM containing 10 % FBS (dividing culture) or 5 % FBS and dexamethasone 1 μmol/L (stationary culture)[5]. The cells were counted accurately and added to 6-well plate with 1×10^5 cells per well, and then incubated for 24 h. To infect cells, the culture medium in every well was removed, and 0.5 mL serum-free DMEM and 10 μL of a suspension with a multiplicity of infection of 1×10^2-5×10^5 vector particles were added to each culture. Then cells were incubated for 2 h, and then DMEM with 10 % or 5 % FBS and dexamethasone 1 μmol/L were added to wells respectively. At d 2 cells were collected and carried out for luciferase activity assay.

Drug treatment Hydroxyurea and etoposide were diluted into DMEM with various concentrations for use in experiments. The concentrations of hydroxyurea were 0.2-200 mmol/L and etoposide were 0.05-50 μmol/L, which were adequate to inhibit DNA synthesis[13]. Drug treatments were the 12-14 h overnight incubations. After treatment, cultures were washed twice with DMEM, then AAV vectors were added for transduction, and the cultures were maintained in DMEM containing 10 % FBS (dividing culture) or DMEM containing 5 % FBS and dexamethasone 1 μmol/L (stationary culture).

Luciferase activity assay Two days after transduction, luciferase activity assay were performed. Cells were washed twice with PBS, and then 300 μL luciferase lysis buffer was added to the wells. After 15 min at room temperature, cells were collected to centrifuge tube and centrifuged at 2000×*g* for 15 s. According to the Promega assay system, 20 μL supernatant and 100 μL reagent buffer were mixed, then relative light unites per second (RLU/s) was detected immediately by luminometer. The RLU/s of untreated hMSCs was regarded as base, and the transduction efficiency was indirectly reflected by counting the relative values of luciferase activity in various groups. The relative transduction efficiency was the number of RLU/s in drug treated cultures divided by the number in untreated cultures. The bigger the RLU/s, the higher the transduction efficiency.

Southern blot Stationary cells were treated with hydroxyurea of 0, 0.2, 2, 20 mmol/L and etoposide of 0, 0.05, 0.5, 3 μmol/L, respectively. Then cells were transducted by rAAV-LUC at a MOI of 1×10^5 vector particles per cell as described above. Two days after transduction, cells were collected and counted. Low-molecular-weight genomic DNA was isolated by the method of Hirt[17]. Cells 1×10^7 were lysed by 1 mL of 0.6 % sodium dodecyl sulphate-Tris-hydrochloride (10 mmol/L)-edetic acid (10 mmol/L, pH 7.4). After 20 min at room temperature the viscous lysate were harvested into centrifuge tube. Then 0.25 mL NaCl 5 mmol/L was added to make a final concentration of 1 mmol/L, and the solution was gentlely mixed by inverting the tube 10 times. Then the tube was left on ice for 1 h. The sample was mixed again and kept at 4 ºC

overnight. The tubes were centrifuged at 17000 ×*g* for 30 min at 4 ºC. The supernatant was removed, and extracted with equal volume of phenol for two times and 10 min with each time. Then it was extracted with equal volume of chloroform/isoamyl alcohol (v/v= 24:1) for 10 min. Two volumes of ethanol were added into the sample and the sample was stored at -20 ºC overnight. Then the sample was centrifuged at 12 000 r/min for 20 min at 4 ºC. The precipitated pellet was suspended in 50 μL distilled water, and 5 mL of this solution of DNA was electrophoresed through a 0.8 % alkaline agarose gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled luciferase sequence.

Statistical analysis The data shown were mean values of at least 8 experiments and expressed as mean±SD. Student's *t*-test was used. $P<0.05$ was considered statistically significant.

RESULTS

The effects of various MOI of AAV vectors on transduction of untreated cultures We measured the transduction efficiency of hMSCs infected with a multiplicity of infection of 1×10^2-5×10^5 vector particles per cell and found that there was a dose-dependence between the MOI of AAV vector particles and transduction efficiency within a certain range (Fig 1). When AAV vectors were 1×10^5 per cell transduction efficiency changed into the "platform phase", and cells were shown to be at a "saturation status". If vector particles increased continuously, transduction efficiency could not be markedly enhanced. So MOI of 1×10^5 vector particles per cell was optimal for transduction experiment. In addition, we found that transduction efficiency of dividing cells were 7-9-fold higher than that of stationary cells, which was accordance with previous reports[5].

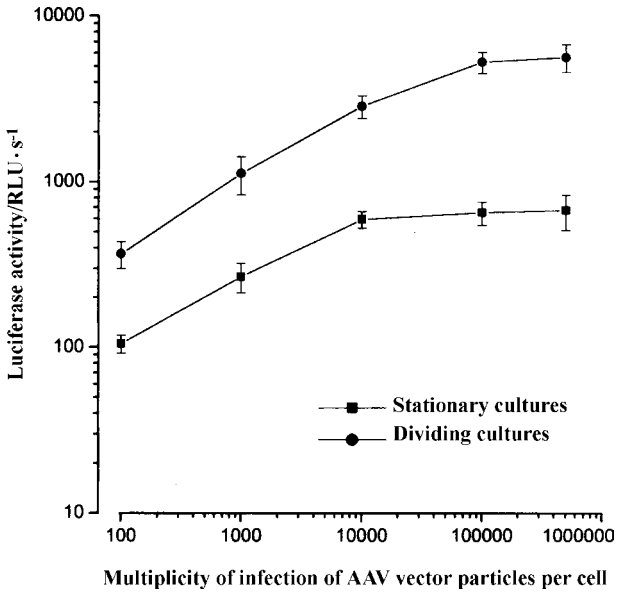

Fig 1. Effect of various MOI of AAV vector particles per cell on luciferase activity of stationary and dividing cultures. *n*=8. Mean±SD.

Hydroxyurea increases transduction by AAV vectors Hydroxyurea is a DNA synthesis inhibitor, which prevents DNA synthesis by inhibiting ribonucleotide reductase and depleting deoxynucleotide pools. Cultures pretreated with hydroxyurea had 20-50-higher transduction efficiency compared with untreated control cultures, and the optimal concentration of hydroxyurea was 20 mmol/L (Tab 1). These results suggest that prior exposure to drugs that inhibits DNA synthesis can increase transduction by AAV vectors. Moreover, the transduction efficiency increased in both dividing and stationary cultures. However, the increased times of transduction efficiency in dividing cultures was approximately 20, and transduction efficiency in stationary cultures was about 50 times higher than that in control cultures (Fig 2).

The effects of etoposide on transduction We also tested etoposide that inhibits type II topoisomerase and enhances enzyme-mediated DNA cleavage, which affects many aspects of DNA metabolism, including replication, recombination, and repair. The transduction efficiency of dividing and stationary cultures exposed to etoposide increased very much (Tab 1). The effect of etoposide on dividing and stationary cultures transduced by AAV vectors was similar with the effect of hydroxyurea. Stationary cultures had about 40-fold higher transduction efficiency compared with control cultures and transduction efficiency in dividing cultures was about 16 times higher than that in control cultures (Fig 2).

Drug treatment enhance second-strand DNA synthesis by rAAV To test the mechanism that drug treatment enhanced transduction by rAAV, we analysed the molecular state of the rAAV genome in infected cells. Virus DNA in infected cells of various groups was isolated by Hirt extraction[19] and applied for Southern hybridization. Analysis of results as shown in Fig 3 revealed a direct correlation between formation of double-stranded forms of the rAAV genome and enhancement of rAAV transduction. In all samples we all detected rAAV-LUC single-stranded DNA. However, the duplex replicative form of rAAV molecules were

Tab 1. Effect of various concentration of hydroxyurea, etoposide, and various MOI on luciferase activity in stationary and dividing cells. *n*=8. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control. $^{f}P<0.01$ *vs* stationary cultures.

	MOI=10^2:1 Luciferase activity/RLU·s^{-1}		MOI=10^4:1 Luciferase activity/RLU·s^{-1}		MOI=10^5:1 Luciferase activity/RLU·s^{-1}	
	Stationary cultures	Dividing cultures	Stationary cultures	Dividing cultures	Stationary cultures	Dividing cultures
Hydroxyurea/ mmol·L^{-1}						
0 (Control)	105±13	367±69	593±68	2859±451	651±103	5286±763
0.2	194±28^{c}	405±95	1205±151^{c}	5248±881^{cf}	1495±252^{c}	7238±1052^{bf}
2	1068±201^{c}	2762±431^{cf}	4011±428^{c}	21569±3492^{cf}	5743±486^{c}	44523±1961^{cf}
20	4137±692^{c}	7837±963^{cf}	20627±2194^{c}	77352±7261^{cf}	30013±2509^{c}	119728±7493^{cf}
50	3398±445^{c}	6651±783^{cf}	18372±2631^{c}	63578±7064^{cf}	25726±4127^{c}	105637±8017^{cf}
100	2994±308^{c}	5437±659^{cf}	10095±1162^{c}	55317±4641^{cf}	17572±904^{c}	95146±6538^{cf}
200	Cells death	Cells death	Cells death	Cells death	Cells death	Cells death
Etoposide/μmol·L^{-1}						
0 (Control)	105±13	367±69	593±68	2859±451	651±103	5286±763
0.05	337±52^{c}	607±131^{cf}	1802±186^{c}	5836±592^{cf}	2047±289^{c}	9238±1183^{cf}
0.5	1251±195^{c}	2602±325^{cf}	7612±902^{c}	13291±1957^{cf}	8719±1135^{c}	24519±4864^{cf}
3	4129±399^{c}	7989±836^{cf}	19327±2068^{c}	44567±5043^{cf}	25428±2150^{c}	86158±6003^{cf}
5	3521±468^{c}	7025±902^{cf}	16549±1884^{c}	39812±4565^{cf}	19859±2720^{c}	78721±8657^{cf}
50	Cells death	Cells death	Cells death	Cells death	Cells death	Cells death

RLU: relative light units.

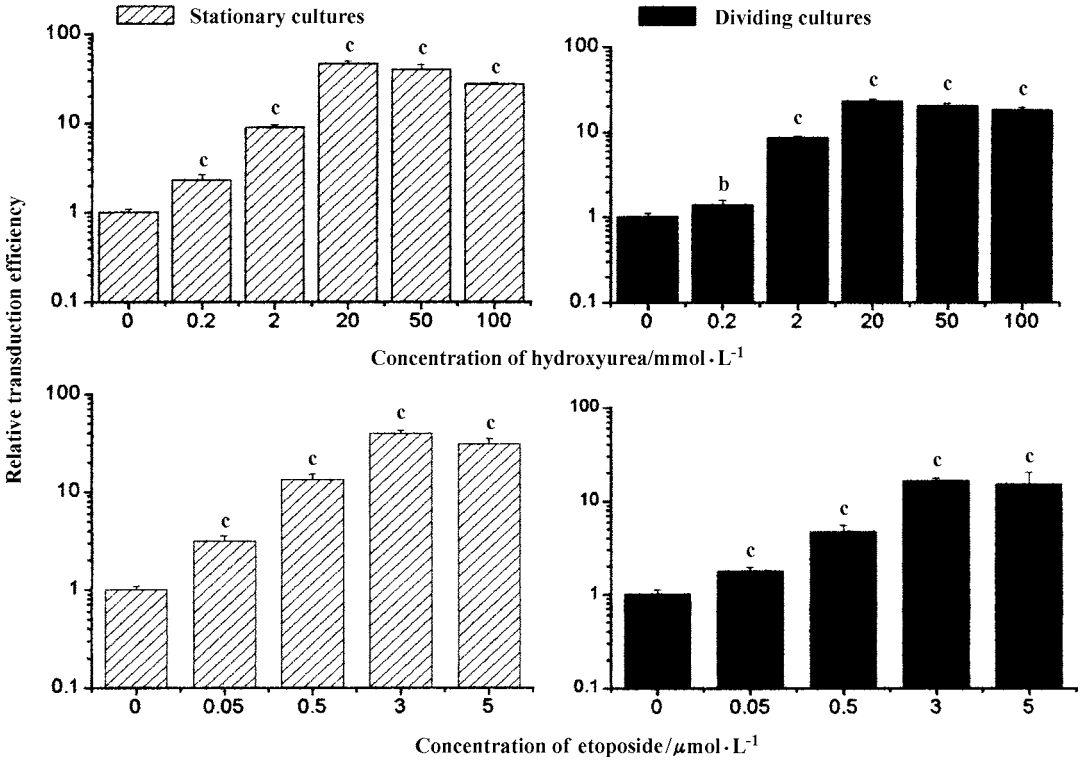

Fig 2. Increased relative transduction efficiency of stationary and dividing cultures treated with hydroxyurea and etoposide. The transduction efficiency of untreated cultures was regarded as 1, and the relative transduction efficiency of other groups is the ratio of treated groups to the corresponding untreated group. *n*=8. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control cultures.

detected at significantly greater levels only in the drug-treated samples, and there exited dose-dependent relation in certain extent (Fig 3). This result indicated that the conversion of rAAV single-stranded DNA to a double-stranded state was enhanced by hydroxyurea and etoposide. Increased transduction by rAAV at different concertrations of hydroxyurea and etoposide (Tab 1) directly was correlated with increased accumulation of duplex rAAV replicative form (Fig 3), and there existed obvious consistency between them. So the conversion of the single-stranded rAAV genome into a double-stranded form is the rate-limiting step of rAAV transduction.

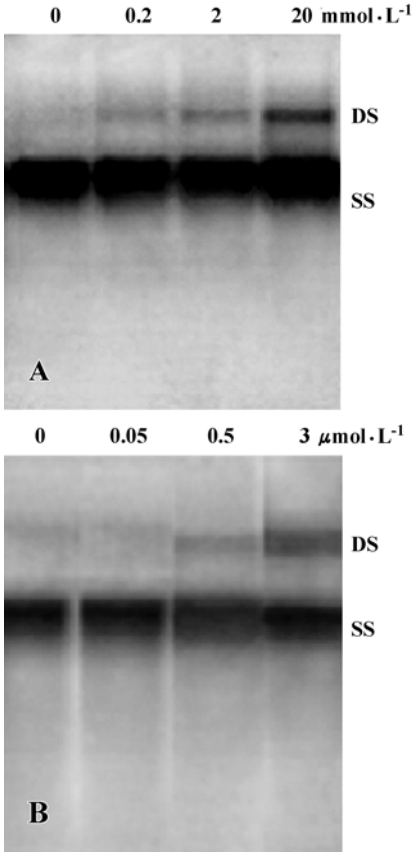

Fig 3. Effect of hydroxyurea (A) and etoposide (B) on rAAV-LUC second-strand DNA synthesis. SS: single-stranded forms of the rAAV genome; DS: double-stranded forms of the rAAV genome.

DISCUSSION

Human bone marrow mesenchymal stem and progenitor cells (hMSCs) have been found to possess the capacity for self-renewal and multilineage potential to differentiate into osteocytic, chondrocytic, and adipocytic lineages[14,15]. It marks a new era of "stem-cell-based therapy". Stem cells from marrow have also been shown to form other kinds of tissues or cells, including hepatocyte[16], cardiomyocyte[17], neuron, and brain cell[18]. Thus adult hMSCs offer great promise in medicine as they may generate many cell types to repair damaged organs. Now hMSC has become a "seed cell" of many kinds of researches, such as bone and cartilage tissue engineering. To make hMSCs differentiate into the cell types that we need some revulsive agents are required, such as cytokine, certain drugs, or exogenous gene transferred into cells. Plasmid, adenovirus, retrovirus, and adeno-associated virus have all been used as vectors to transfer exogenous gene into cells. AAV is one of the most prospective vectors due to its advantages. However there are some difficulties and problems in transduction efficiency and expression of exogenous gene. In the past many studies were performed to find the reason of the problem. Some researchers showed that transduction efficiency was partly limited by lack of appropriate receptor (heparan sulfate proteoglycan) or coreceptor (fibroblast growth factor receptor 1 and $\alpha V\beta 5$ intergrin) expression on cytomembrane for rAAV binding and entry[10,11]. And other investigations indicated that transduction by rAAV was limited not by transfer of the single-stranded (SS) viral genome but rather by subsequent conversion of the viral genome to a transcriptionally active double-stranded (DS) template[20]. In this study, we showed that hydroxyurea and etoposide increased transduction by AAV vectors. Stationary hMSCs exposed to hydroxyurea and etoposide were transduced at 20-40 fold higher rates, and less increase was observed in dividing cultures. Moreover we found rAAV genomic conversion and subsequent expression of luciferase gene were greatly facilitated by hydroxyurea and etoposide (Fig 2, Tab 1). The formation of double-stranded DNA intermediates was an obligatory first step for rAAV transduction and was the base of subsequent expression of lumiferase gene and protein. By this pathway, transduction by rAAV in hMSC was increased significantly.

The most compelling evidence is the observation that DNA-damaging agents including γ-and UV-irradiation could stimulate replication of wild-type AAV in a helper-independent manner and increase transduction by rAAV[21-23]. Although the responses to these DNA-damaging agents are wide ranging and not fully understood in mammalian cells, the presumed outcome is an intracellular milieu that permits optimal repair of lesions

before the cell reinitiates replicative DNA synthesis[24]. Hydroxyurea and etoposide can also alter chromosome structure by a variety of mechanisms. It is reasonable to suggest that hydroxyurea and etoposide which enhanced transduction by rAAV invoked similar responses. This might involve transient alterations in the expression or availability of cellular proteins, including polymerases, which are needed to initiate leading-strand synthesis of a single-strand rAAV genome. Hydroxyurea and etoposide enhanced transduction by rAAV by activating synthesis of double-stranded AAV.

Drug treatments could increase the transduction of stationary cells in non-S-phase and that of dividing cells in S-phase by inducing unscheduled DNA repair and double-stranded AAV genome synthesis. Dividing cells with the higher level of DNA repair were transduced more efficiently by AAV vectors, and stationary cells with the lower level of DNA repair were more sensitive to agents which could increase transduction. Thus the preferential transduction efficiency of dividing cells may be due to the higher level of DNA repair which was required to correct DNA replication errors, and the greater sensitivity of stationary cells may be explained by the lower level of DNA repair.

Although hydroxyurea and etoposide increased the transduction of AAV vector, most particles failed to transduce hMSCs. Each transduction event required 1×10^3-1×10^4 AAV vector particles, and transduction was limited mainly by the number of hMSCs that are competent for transduction rather than the number of functional vector particals. It is possible that the lower expression of receptors and coreceptors on hMSC could limit the transduction by rAAV. However it is very difficult to enhance the expression level of the receptor because of its inherency. So, to find other effective methods that can increase transduction rates was essential for successful gene therapy with AAV vectors. The present study demonstrated that hydroxyurea 20 mmol/L and etoposide 3 μmol/L was optimal to increase transduction efficiency of hMSC by AAV.

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