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Protection from Acute Cellular Injury Using *Sleeping Beauty* Mediated Telomerase Gene Transfer

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Abstract

We developed a *Sleeping Beauty* (SB) transposon mediated hTERT gene delivery system for *in vitro* use. We have constructed telomerase or luciferase gene expressing SB-transposons with a SV40 enhancer (pT3.hTERT.Con and pT3.Con, respectively) or without an enhancer (pT3.Pro). Using the SB transposon system *in vitro* hTERT gene overexpression has protective effects from acute cellular injury by tert-butyl hydroperoxide (tBH), carbon tetrachloride (CCl₄) and D-galactosamine (D-GalN) in normal human cells IMR-90. pT3.hTERT.Con vector and helper plasmid co-transfection resulted in a ~3 fold increase in telomerase activity which was maintained for 14 days. Trypan blue and Cell Death Detection Assays showed the protective effects of telomerase gene against toxic agents. 14 days after co-transfection with pT3.hTERT.Con vector and helper plasmid, IMR-90 cells were incubated with 1.2mM tBH for 50min, 5mM CCl₄ for 1.5hr or 30mM D-GalN for 24hr. Cell viability of SB-mediated telomerase over-expressing cells significantly increased by 48% (tBH), 43% (CCl₄) and 25% (D-GalN) in comparison to mock treated cells. Cell Death Detection ELISA showed a decrease in the rate of apoptosis by 47%. In summary, SB transposon mediated telomerase gene transfer may have a protective effect against tBH, CCl₄ or D-GalN induced acute cellular injury and this results suggested SB-mediated telomerase therapy for tissue engineering.

Keywords

telomerase; Sleeping Beauty transposon; acute cellular injury

Telomere shortening has been linked to replicative senescence, suppressed cell division and regeneration (1,2). Telomerase reverse transcriptase (hTERT) has been shown to promote proliferation of resting cells in addition to maintaining telomere length and preventing cellular senescence (3~5). New genetic material may be introduced into chromosomes of all organisms via viruses or transposons. *Sleeping Beauty* (SB) transposon system, a type of mobile element that belongs to the Tc1/mariner class of transposons, is a promising tool for gene delivery because of the ability to stably express therapeutic genes as well as to infect resting cells (6). At specific TA sequences, SB moves in a simple, cut-and-paste manner. Although a popular tool for gene therapy, viral vectors are not ideal due to immunogenic problems, potential RCV (Replication Competent Virus) outbreaks, and difficulties in mass production. The advantages of the *Sleeping Beauty* transposon system include prolonged transgene expression without

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eliciting an immunogenic response, no possibility of RCV and ease of construction. tert-butyl hydroperoxide (tBH), carbontetrachloride (CCl₄) and D-galactosamine (D-GalN) are well known hepatotoxic agents that used for animal acute liver injury model. The aim of this study is to increase hTERT gene expression in human normal cells using the SB transposon system in order to show the protective effect of telomerase gene from acute cellular injury by tBH, CCl₄ or D-GalN.

Material and Methods

Cell culture

HepG2 and IMR-90 (normal human lung fibroblast) cells were grown in DMEM or EMEM (Gibco BRL, Germany) supplemented with 10% FCS (HyClone, Logan, U.S.A), penicillin (50 units/ml), and streptomycin (50 µg/ml) in the presence of 5% CO₂. All cell lines were obtained from the American Type Culture Collection (Manassas, VA).

Plasmid construction

Sleeping Beauty-transposon system plasmids, pT3.MSC, pCMV.HSB3 and pCMV.mSB were kindly provided by Dr. Mark A. Kay (Stanford University, Stanford, CA). Three SB-transposon vectors, pT3.Pro, pT3.Con and pT3.hTERT.Con were constructed (Fig. 1). Briefly, SV40 promoter-luciferase-SV40 PA cassette of the pGL3-Control or pGL3-Promoter (Promega, Madison, WI) was cut with *Bgl* II and *Sal* I and cloned into the pT3.MSC vector, cut with *Bgl* II and *Xho* I, resulting in the pT3.Con and pT3.Pro vectors with or without the SV40 enhancer. Human telomerase catalytic subunit gene hTERT was cut from pGRN145 (ATCC, Manassas, VA) with *Kpn* I and *Not* I and cloned into pcDNA (Invitrogen, USA), resulting pcDNA-hTERT. This plasmid was cut with *Hind* III and *Xba* I and the telomerase gene was replaced with luciferase gene of pGL3.Con vector, resulting pGL3.hTERT.Con. Telomerase expression cassette was cloned into pT3.MSC vector, resulting pT3.hTERT.Con vector as same way mentioned above.

In vitro studies

The three plasmids were co-transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with *Sleeping Beauty* transposase plasmid (helper plasmid) pCMV.HSB or its mutated form pCMV.mSB plasmid into cells for 7 or 14 days.

Luciferase assay

Luciferase assays (Promega, Madison, WI) were performed according to the manufacturer's protocols using AutoLumat LB953 (Berthold, Wildbad, Germany). Briefly, 10⁵ cells seeded in a 35-mm tissue culture dish were exposed to a transfection mixture containing 2 of luciferase reporter plasmids and 0.5 of pSV-β-galactosidase control plasmid vector (Promega, U.S.A). 48 h after transfection, luciferase assays were performed according to the manufacturer's protocols (Promega, U.S.A) and measured. The Simian virus 40 (SV40) promoter (pGL3-Promoter or pGL3-Control) was used as a positive control. A β-galactosidase assay was also performed with the same cell extracts to standardize for transfection efficiency. All of the data shown in this study were obtained from at least three independent experiments.

TRAP assay

The telomerase activity was examined by a telomeric repeat amplification protocol (TRAP) assay with TeloTAGGG Telomerase PCR ELISA (Roche Diagnostics, Germany).

Cell death detection ELISA

Cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) were determined by Cell Death Detection ELISA^{PLUS} (Roche, Germany). Briefly, cell lysates were placed in a streptavidin-coated micro-plate. A mixture of anti-histone-biotin and anti-DNA-POD was added and incubated for 2 h at 15–25. After removal of unbound antibodies by a washing step, POD was determined photometrically at 405 nm with ABTS as substrate.

SA- β -galactosidase staining

Cells were fixed and stained for SA- β -galactosidase were performed according to the manufacturer's protocols using Senescent Cells Staining Kit (Sigma)

Results and Discussion

To see if SV40 enhancer could augment the expression in SB delivery system, SB vectors with or without SV40 enhancer were used. HepG2 cells co-transfected with pT3.Con with SV40VE and helper plasmid gave rise to a ~2.8 and ~3.4 -fold higher level of luciferase activity for 2 and 7 days after transfection, in comparison to the pT3.Pro without a SV40 enhancer (Fig. 2A). pT3.hTERT.Con vector with SV40VE and helper plasmid were co-transfected into HepG2 cells. ~2 fold increased telomerase activity was maintained for 7 days after transfection using the TRAP assay as compared to the mock control (Fig. 2B).

IMR-90 cells co-transfected with pT3.Con and helper plasmid gave rise to a 6-fold higher level of luciferase activity for 14 days after transfection, in comparison without helper plasmid (Fig. 3A). pT3.hTERT.Con vector and helper plasmid were co-transfected into IMR-90 cells. ~3 fold increased telomerase activity was maintained for 14 days after transfection using the TRAP assay as compared to the mock control without transferase (Fig. 3B).

Up-regulation of telomerase showed preventive effects from CCl₄ (7,8), stress-induced apoptosis and necrosis (9) and ischemic wound (10). To investigate the protective effect of telomerase gene against toxic agents, IMR-90 cells were treated for 50 min with 1.2mM tBH, 1.5 h with 5mM CCl₄ or 24 h with 30mM D-GalN. Expression of telomerase gene prevented acute cellular injury by tBH, CCl₄ or D-GalN. Cell viability of SB-mediated telomerase overexpressing cells significantly increased by 48% (tBH), 43% (CCl₄) and 25% (D-GalN) in comparison to mock treated cells (Fig. 4). Cell Death Detection ELISA showed a decrease in the rate of apoptosis by 47% (Fig. 5). However, overexpression of telomerase induced the senescence phenotype revealing by SA- β -galactosidase staining (11 and Fig. 5). To investigate the protective effect of telomerase by using the SB system, we constructed an SB vector expressing human telomerase gene (hTERT) controlled by the SV40 promoter and enhancer. Increased expression of hTERT and luciferase were observed in normal human fibroblast using a *Sleeping Beauty* transposon system *in vitro*. Although overexpression of telomerase induced the senescence phenotype, SB transposon mediated telomerase gene transfer may have a protective effect against tBH, CCl₄ or D-GalN induced acute cellular injury.

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Figure 1.
Vectors for SB-mediated transposition

Fig. 2.

Luciferase assay and telomerase activity assay for SB-mediated transgene integration in HepG2 cells transfected with donor plasmid with (pT3.Con, pT3.hTERT.Con) or without (pT3.Pro) the SV40 enhancer. pCMV.HSB3 (SB transposase); pCMV.mSB (mutated SB transposase). Telomerase activity of HepG2 was measured by TeloTAGGG Telomerase PCR ELISA. For negative control, cell extract was treated with RNase (293+RNase). 293 cells were used for positive control. The S.D. are shown by the lines extending above the bars.

Fig. 3.

Luciferase assay and telomerase activity assay for SB-mediated transgene integration in IMR-90 cells transfected with donor plasmid. pCMV.HSB3 (SB transposase); pCMV.mSB (mutated SB transposase). Telomerase activity of IMR-90 was measured by TeloTAGGG Telomerase PCR ELISA. For negative control, cell extract was treated with RNase (293 +RNase). 293 cells were used for positive control. The S.D. are shown by the lines extending above the bars.

Fig. 4.

Trypan blue assay. Cells were incubated for 50 min with 1.2mM tBH, 1.5 h with 5mM CCl₄, or 24 hr with 30 mM D-GalN 14 days after transfection and assayed with Trypan blue for cell viability.

Fig. 5.

Cell Death Detection ELISA Detection of nucleosomes in cytoplasmic fractions treatment with tBH, CCl₄, and GalN. After cell lysis the cytoplasmic fractions of the samples were prediluted 1:10 with incubation buffer and tested in the immunoassay.

Fig. 6. Effect of hTERT overexpression in IMR-90 normal human fibroblast by SA-β-galactosidase staining. 14 days after transfection, cells were stained for SA-β-galactosidase (β-gal) activity.