Original Article

Anti-tumor Effects of Lentivirus-mediated and hTERT Gene Modified DC Vaccine*

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ABSTRACT **Objective:** Human telomerase reverse transcriptase (hTERT) is the catalytic subunit of telomerase, the aim of this study is to construct a novel DC vaccine, which is lentivirus –mediated and hTERT gene modified, and analyze the killing activity of cytotoxic T lymphocyte (CTL) activated by this kind of DC vaccine. **Methods:** Constructed a lentiviral vector encoding hTERT gene (lenti-hTERT), infected dendritic cells (DCs) with lenti-hTERT, then the ability of stimulating proliferation of allogeneic T lymphocytes and the kill activity of CTL activated by these DCs were detected. **Results:** The titration of lentiviral vector was 5.88×106IU/ml, and the infecting efficiency was about 90%. Transgene could express in host cells for more than two months. The DC vaccine modified by hTERT gene demonstrated fine ability of stimulating mixed lymphocyte reaction and anti-tumor effects.

Conclusion: These findings suggested that DC vaccine which is lentivirus-mediated and hTERT gene modified might represent an effective strategy for therapy of cancer.

Keywords: Human telomerase reverse transcriptase (hTERT); lentiviral vector; DC Vaccine; Tumor; Immunotherapy

INTRODUCTION

DCs are powerful antigen-presenting cells (APCs) in the generation of a primary immune response against a variety of tumors[1-3]. Because DC culture from blood or bone marrow-derived progenitors is now clinically applicable. DC has become useful tools for immunotherapy in malignant disease. One of these strategies is DC vaccine, which could present tumor antigens or tumor-associated antigens (TAAs) to T cell and generate antigen-specific CTL responses. While, a great majority of the antigens that are highly expressed on tumor cells have also been found on normal cells, albeit to a lesser extent. Moreover, the TAAs identified so far are usually specific to a particular tumor type and found only in a subset of tumor patients[4]. So identification of a universal tumor antigen would have obvious remarkable impact on the application of cancer immunotherapy.

Telomerase is expressed in human germ tissues and in the most of primary human tumors. Physiologically renewing tissues have weak telomerase activity, which is assumed to be derived from proliferating stem cells[5]. Human telomerase reverse transcriptase (hTERT) is the catalytic component of a functional telomerase complex. Telomerase activity has been shown to correlate with expression of hTERT, which is the rate-limiting subunit of the telomerase [6-7] .These data indicated that peptide fragments of hTERT could serve as tumor specific antigen and this has been confirmed in several reports [8-10] .

Gene transfering is a central tenet of construction of DC Vaccine. Many techniques have been tested for gene transfer. Nonviral methods, such as liposomes are inefficient and only achieve transient expression of the transgene. Vectors derived from retroviruses, such as Moloney murine leukemia virus (MLV) require proliferation of the target cells for integration and stable express. Consequently, the use of retroviral vectors is limited to ex vivo gene transfer. Several other viral vectors, including

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herpes simplex virus, adenovirus and adeno-associated virus vectors, have been developed for gene delivery to nondividing cells. However, their clinical use might be limited by some vector related toxicity as well as short-term expression of the transgene, because of the non-intergration of the therapeutic gene in the tumor cell DNA and to the clearance of adenoviral vector-carrying cells by the immune response[11-12]. Recently, a kind of novel vector, which is lentiviral vector system, was reported. It could transduce dividing and nondividing cells, in vitro and in vivo, as well as showed efficient gene transfer and sustained long-term expression for the transgene. Thus, the lentiviral vector delivery system offers substantial promise for gene therapy on cancer [11-14]. In this study we constructed a lentiviral vector encoding hTERT gene, which was used to infect DCs to constitute DC Vaccine. Our results indicated that lenti-hTERT Vector could transfer hTERT gene to DCs effectively and hTERT gene could be expressed in DCs persisted for more than two months with no apparent decrease. Moreover, the DC vaccine could activate T-cells and show a noticeable anti-tumor activity.

MATERIALS AND METHODS

Cell lines

Human liver cancer cell line HepG2, human umbilical vascular endothelial cells UE, human cervical cancer cells HeLa (preserved in pathology department of Qianfoshan Hospital) and 293T human kidney cells (kindly provided by Department of Haematology and oncology,university of Alabama) were cultured in DMEM and RPMI –1640 respectively supplemented with 10% fetal bovine serum and 100 units of penicillin. These cell lines were maintained at 37℃ in 5% CO2 humidified atmosphere.

Preparation of hTERT gene

Total RNA of HepG2 cells were extracted with Trizol method. After cDNA synthesis, the PCR was performed. We searched hTERT gene from NCBI-Gene Bank and chose 420bp (743-1162) as target DNA fragment. The primers which contain BamHI/XhoI sites for PCR were as follows, forward primer: 5'CG GGATC CATGAGCCGAAGTCTGCCGTT3'; reverse primer: 5'CCG CTCGAG TCA GGAACCCAGAAAGATGGT 3'. PCR was followed by 40 cycles at 95℃ for 1 min, 62℃ for 45 seconds, and 72℃ for 1minites. PCR product was visualized by running agarose gel electrophoresis containing ethidium bromide. Then T-A clone was performed to amplify hTERT DNA fragment. Finally the DNA fragment was sequenced to make sure that we got the correct product.

Generation of Lentiviral Vector, infection and titration

The three-plasmid based lentivector system including L166, L205, L311 was generously provided by Department of Haematology and oncology, university of Alabama, which was reported to has high gene transfer efficiency[15]. Of them, L166 contains green fluorescent protein (GFP) with BamHI/XhoI sites lying both sides. The L166–hTERT plasmid was constructed by ligating the hTERT DNA fragment into the BamHI/XhoI sites of L166 plasmid, replacing the existing GFP sequence. The Lentiviral vectors (Lenti-hTERT) were produced by co-transfecting 10ug of pL166-hTERT, 10ug pL205 and 2.5ug pL311 into subconfluent monolayer cultures of 293T cells by the calcium phosphate precipitation method. For comparison, Lenti-GFP was generated in the same way. Supernatants were harvested after 3 days, clarified by low-speed centrifugation(1000g, 20min), filtered through 0.45-um pore-size filters, aliquoted, and frozen at –80℃. To determine vector titers and infection efficiency, 1× 105 HeLa cells per well seeded in 24-well plates were infected with serial dilution of Lenti-GFP Vector stocks, and GFP-positive (green) cell colonies were counted 2 days later with a fluorescence microscope. Each GFP positive cell colony was measured as a single infectious Unit (IU).

Generation and culture of DC

Monocytes were isolated form cord blood (donated by healthy neonate of Qianfoshan Hospital) by Ficoll-Hypaque density gradient centrifugation. DCs were generated from monocytes in the presence of GM-CSF, IL-4 and TNF-α. Monocytes were washed twice with cold PBS, resuspended and cultured in PRMI1640 medium for 2h. Nonadherent cells were removed and frozen in freezing media for later use in CTL assays. The adherent cells were cultured for 7 days in PRMI1640 containing 100ng/ml rhGM-CSF and IL-4 (30ng/ml), 2/3 of the medium was replaced every 2 days, and 20ng/ml rhTNF-α were added into the medium on day 7. DCs were harvested on the 8th day of culture. Cell surface markers were analyzed by flow cytometry. For flow cytometry we used the following antibodies: CD14-FITC; CD83-FITC; and CD86-FITC and anti-rat IgG-fluorescent as isotype control. Cells(1×106) were incubated with antibody for 30 minutes on ice, then washed with phosphate-buffered saline and fixed with 5% paraformaldehyde in PBS. Flow cytometric analysis was performed on the FACScan flow cytometer (Becton Dickinson) using CELLQuest program (Becton Dickinson).

Construction of hTERT peptide–loaded DC vaccine

Dendritic cells were cultured as described above and pulsed with Lenti-hTERT to construct DC Vaccine on day 5. Peptide loaded DC obtained on day 8 were used to stimulate fresh T cells.

Proliferation assay

Primary allogeneic mixed lymphocyte reactions were performed with MTT method. DCs were incubated for 30min at 37℃ in complete medium with mitomycin 25ug/ml. Serial dilutions of DCs were mixed with a constant amount (1×105) of allogeneic T cells in 96-well culture dishes at different stimulator-to-reactor (S:R) ratios (1:10,1:100,1:1000). At the same time, T cells alone were used as controls. Cultured these cells with 10μL MTT (5mg/mL) for 4h, then added 100μL hydrochloric acid-isopropanol keeping in dark place for 10 minutes. Finally detected OD value with Enzyme-labelled meter. T cell proliferation was measured through calculate stimulate index (SI).

Cytotoxicity assay

T cells were incubated with autologous DC (DC:T cell ratio 1:10) in 24-well plate. After that, the T cells were restimulated with peptide-loaded DC on days 7 and 14 and utilized as cytotoxic effectors on day 21. HeLa cells/UE cells (target cells) and effector cells (CTL) were seeded in 96-well plates at different effector-to-target (E:T) ratios (20:1,10:1,5:1). Coincubation of effector and target cells as well as target cells alone was performed in complete medium at 37℃ for 24h. Cytotoxicity was assessed by MTT method.

Statistics

Differences between groups were tested for significance with t test using SPSS 10.0 software.

RESULTS

Identification of gene product

Agarose gel electrophoresis proved the hTERT DNA fragment of the anticipated size (approximately 440bp) was PCR amplified. The recombinant plasmid L166-hTERT was extracted using EndoFree Maxi Plasmidkit (Qiagen), and the OD260/OD280 was 1.93, bio-digestion used BamHI and XhoI showed that we got the correct product. Adds to gene sequence, all our data demonstrated that we have constructed the just recombinant plasmid L166-hTERT successfully.

Packaging of Lentivirus, infection and titration

293T cells producing virus particles seemed injuried and swell. In control group almost 100% of the cells expressed GFP fluorescence. The intensity of GFP fluorescence varied from cell to cell. The titer and infection of Lentivirus vector was determined by infecting HeLa cells. The titer was 5.88×106IU/ml through confocal laser scanning microscopy analyzing, and the infecting efficiency was about 90%. GFP expression had no obvious effects on the appearance of the cultured HeLa cells. The cells could be maintained for many months, without evidence of toxicity.

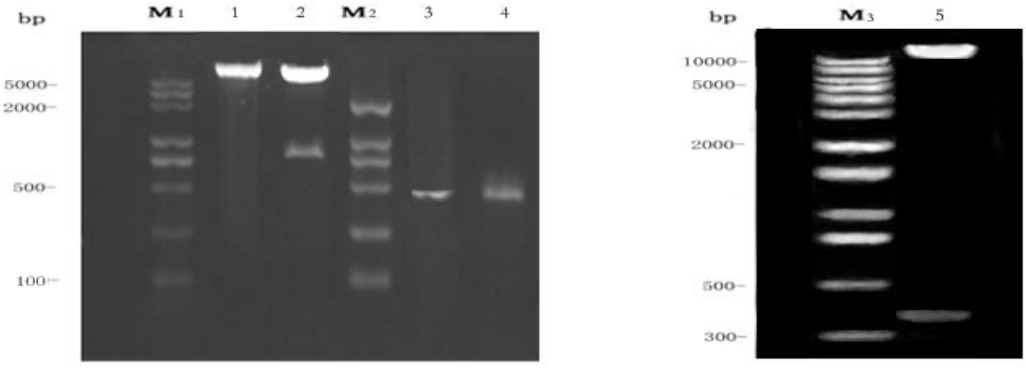


Fig.1 Gene and their products after digestion

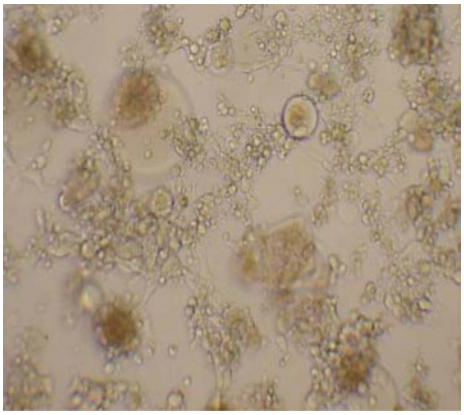
A:Plasmid L166,amplification fragment of hTERT gene and their restriction map

B:Restriction map of plasmid L166–hTERT

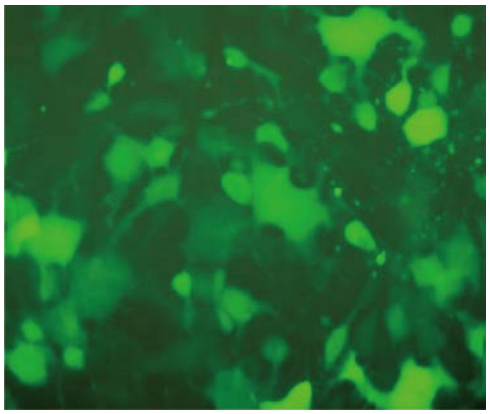
M1: D plus2000 DNA Ladder; M2:DL2000 DNA Ladder; M3:1kb plus DNA Ladder

1: plasmid L166; 2: plasmid L166 digested with BamHI and XhoI 3: the target fragment of hTERT gene

4: PCR product digested with BamHI and XhoI 5: plasmid L166–hTERT digested with BamHI and XhoI



A(x200)



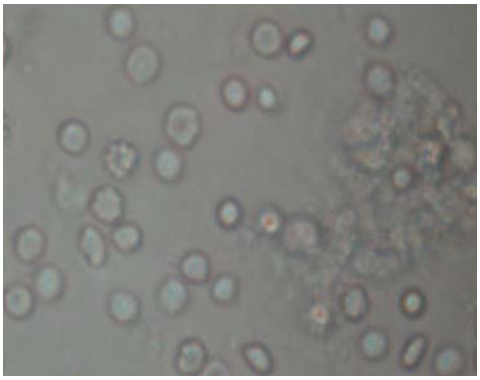
B(x200)

Fig.2 The 3 day 293T cells after virus packaging

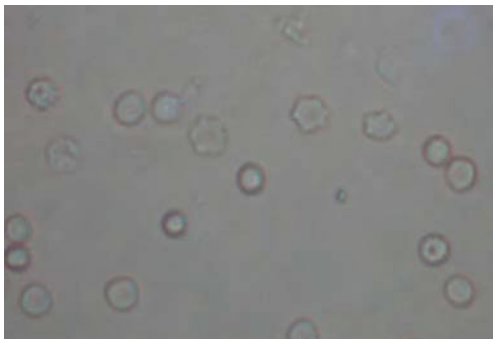
A: 293T cells that produce lentivirus B: GFP widespread expression in 293T cells

Generation of DC Viccine

Monocytes isolated from cord blood became irregular and had plenty of thorns after 3 to 5 days culture, which was a typical form of dendritic cells, and flow cytometry also showed dendritic cells, phenotypes (CD83: 87.2%; CD86: 92.8%; CD14: 3.56%). The DC Vaccine modified by hTERT gene could keep for more than two months without notable change.



A(3days)



B(5days)

Fig.3 Monocytes after 3 and 5 days culture, which show typical form of dendritic cells

Mixed lymphocyte reaction

Data indicated that dendritic cells pulsed with hTERT peptide were able to stimulate autologous T cells, and the SI was more than 1.5, which was higher than that of the negative control significantly (data not shown).

hTERT-specific CTLs against hTERT positve tumor cells

CTLs induced by DC Vaccine modified with hTERT could inhibit hTERT positive tumor cells (HeLa cells)obviously but not telomerase negative cells(UE cells), while CTLs induced by normal DCs show almost the same inhibition of hTERT positive tumor cells and negative cells .

DISCUSSION

Telomerase activity is upregulated in a great majority of human tumors mostly because of an increase in the rate of transcription of hTERT gene that encodes for the catalytic subunit of the

Table1 Cytotoxicity assay result

Groups		E:T	I I(inhibit index)
CTLnormal	Hela	5:1	7. 16 *
		10:1	8. 85 *
		20:1	12. 08 *
	UE	5:1	5. 48 * *
		10:1	6. 60 * *
		20:1	9. 55 * *
CTLhTERT	Hela	5:1	47. 19 #
		10:1	50. 42 #
		20:1	54. 78 #
	UE	5:1	5. 76 # #
		10:1	6. 88 # #
		20:1	10. 25 # #

*Note: * vs # P<0.01, # vs # # P<0.01*

enzyme. Therefore, telomerase has been implicated as a universal tumor antigen for many types of malignancies[4]. A number of studies have been performed testing the ability of different peptides (hTERT 324-332, 461-469, 540-548, 865-873) to induce hTERT-specific CTL against diverse telomerase-expressing tumor cell lines in vitro[9,16-17]. Induction of a specific CTL response by HLA-A2 restricted hTERT 540-548 peptide was also analyzed in vivo in two clinical trials [8,18] .In the present study, we used a 140-amino acid hTERT peptide(229-368) as tumor antigen, which showed strong antigenecity when analyzed with DNAStar software, and Our data also indicated this hTERT peptide actually induced remarkable anti-tumor effection.

Our observations in this work were consistent with other investigators , which demonstrated that lentiviral vectors might retain some advantages compared with other vectors in terms of gene transfer efficiency and long-term expression of the transgene [13,19] . Furthermore, because lentiviral vectors immediately achieved highly efficient levels of gene transfer into the target cells, no selection was necessary to obtain relatively pure populations of transduced cells, and the potential for any selection bias is significantly reduced [14]. Lentiviral vectors hold great promise for gene therapy, and clinical trials to evaluate their safety and efficacy for treating certain human diseases are being considered. In this experiment our findings suggested that the lentiviral transduction of cells, as well as expression of hTERT, did not change the in vitro growth characteristics of DCs. Besides, our lentivector system was improved by Department of Medicine of Alabama University, which belongs to the trans-lentiviral vector and will ensure the greatest predictable level of safety for clinical application [15] .

DC are the most potent stimulators of primary immune responses known thus far, and DC have long been recognized as potential tools for immunotherapy and Vaccination strategies, especially for the therapy of tumors. In our study normal CTLs showed none efficient anti-tumor activity because human monocyte-derived dendritic cells lack telomerase activity[4]. While CTLs induced by hTERT modified DC Vaccine exhibited killing of telomerase positive but not telomerase negative tumor cells. Although telomerase expression is largely restricted to cancer cells, certain hematopoietic progenitor cells, activated lymphocytes and tissue-specific stem cells in the skin and intestinal tissues and germ cells are known to possess telomerase activity. Consequently, any hTERT-based cancer vaccine therapy will have to assess the potential side effects associated with autoimmunity to cells and organs that are telomerase positive. Encouraging results published from two independent groups showed that hTERT-peptide-specific HLA-A2-restricted CTLs were unable to kill telomerase-positive autologous DC34+ hematopoietic stem cells from normal donors or from cancer patients. The CTLs, however, were very effective in causing lysis of telomerase-positive cancer cells targets [8-9]. It is conceivable that the level of telomerase expression in normal cells is below the threshold needed for recognition by these TERT peptide-specific CTL populations. Several other investigators once constructed hTERT modified DC Vaccine using adenoviral or retroviral to test the anti-tumor activity, and they also got ideal results [20-21]. These findings along with the results described in this manuscript and those published earlier suggest that the hTERT –modified DC Vaccine may have useful application for cancer therapy.

To our knowledge, this is the first report to demonstrate a novel DC Vaccine which was lentivirus-mediated and hTERT gene modified .We combined the superiority of lentivirus in gene transfer and the universality of hTERT in tumors in one DC Vaccine, which showed perfect safety and efficiency in against

tumors. This will shed a new light in cancer therapy in future.

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