Dendritic cells transfected with lentiviral vector-encoding hTERT peptide augment antitumor T cell response *in vitro*

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Abstract. HIV-1 derived lentiviral vectors (LVs) have emerged as a powerful tool for gene delivery. hTERT is an ideal tumor-associated antigen with which to develop a potential dendritic cell (DC) vaccine. The purpose of this study was to construct a recombinant lentivirus vector of the hTERT peptide and to determine the hTERT-specific cytotoxic T lymphocyte response elicited by DCs transfected with hTERT-lentivirus vectors *in vitro*. LVs encoding the hTERT peptide were constructed, DCs from cord blood were prepared, their morphology was observed and phenotype was analyzed by flow cytometry. Lenti-hTERT was transfected into DCs to construct the DC vaccines. T lymphocytes stimulated with DC vaccines and HepG2 cells ($hTERT^+$) or 293T cells ($hTERT^-$) were co-cultured for 24 h, respectively. The ability to stimulate proliferation of allogeneic T lymphocytes and the killing activity of CTLs activated by these DCs were determined using the MTT method. According to our results, the recombinant vector lenti-hTERT and lenti-hTERT-DC vaccine were successfully constructed. The stimulatory capacity of the lenti-hTERT DCs in the allogeneic T lymphocyte reaction was markedly enhanced compared with the DC control group ($P<0.01$). Inhibition rates in HepG2 cells of CTLs stimulated with lenti-hTERT-DCs (CTL_T) were significantly higher than CTLs stimulated with the control DC group (CTL_N) ($P<0.01$). Inhibition rates in 293T cells of CTL_T and CTL_N were low and there was no difference between the different DC groups ($P>0.05$). DCs transfected with the hTERT peptide were capable of eliciting a stronger hTERT-specific CTL response *in vitro*. Our data indicate that lenti-hTERT-DCs may potentially be used as an effective approach for cancer immunotherapy.

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Introduction

Cellular adaptive immunotherapy with specific cytotoxic T lymphocytes (CTLs) has been used to treat malignant tumors. Antigen occurrence is critical for the initiation of the adaptive immune response. It is well known that dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) in the induction of primary immune responses. Because of their critical role in controlling cell-mediated immune responses, DCs hold promise as cellular adjuvants in therapeutic cancer vaccines.

Telemeres are located at the distal ends of chromosomes and are shortened with successive cycles of cell division, leading to cellular senescence and death. Telomerase is a specialized DNA-protein complex, consisting of 2 core components: the telomerase reverse transcriptase (TERT) and an RNA template. In cells where telomerase is activated, hTERT synthesizes a TTAGGG sequence based on the RNA template, which is then added to the end of the shortening chromosome, thus preventing the cells from death. The above mechanism is exploited by tumor cells to maintain their immortality. hTERT is overexpressed in most human tumors, but not in most adult somatic cells, except germline cells, activated lymphocytes and certain stem cell populations (1). hTERT represents an ideal tumor-associated antigen (TAA) to develop potential vaccines, which specifically target cancer cells without impairing normal tissues in human cancer immunotherapy. Some researchers have applied hTERT in DC vaccines. Anti-leukemic cytotoxic T cells were identified in telomerase-positive patients when therapeutic vaccination was introduced with the DC vaccine pulsed with the hTERT-peptide, and there was no response in telomerase-negative patients and healthy controls (2). A DC vaccine pulsed with the hTERT peptide was injected into separate lymph node draining regions of patients with advanced cancer every 2-3 weeks (3). Peptide-specific $CD8^+$ T cell responses were induced, and 25% of patients experienced partial clinical responses during vaccination. Anti-tumour immunity of a DC vaccine pulsed with the hTERT peptide was found to be well tolerated with minimal toxicity, but was transient (2,3). Many studies have been performed using hTERT mRNA or peptides transfected into DCs with different vectors, in which the adenovirus was the most popular vector employed (4-6). Until recently, HIV-1 derived lentiviral vectors (LVs) have emerged as powerful

tools for gene delivery to a variety of dividing or non-dividing cells including DCs due to the high transfection rate (7-12). To our knowledge, there are no studies available concerning DC vaccines transfected with the lentiviral vector encoding hTERT peptides. In this study, we first constructed lentiviral vectors encoding the hTERT peptide and transfected them into DCs. Furthermore, we examined the capacity of lenti-hTERT-DCs in stimulating lymphocyte proliferation and generation of anti-tumor cytotoxic T lymphocytes (CTLs). We found that the lenti-hTERT-DCs efficiently stimulated the proliferation of naive allogeneic T lymphocytes and generated hTERT-specific CTLs.

Materials and methods

Cell lines. The 293T cell line was a kind gift from the laboratory of the Department of Medicine and Microbiology, University of Alabama, USA and was used for the generation of recombinant lentiviral vectors. HepG2 (human hepatocellular carcinoma cell line) and 293T were used as targets for the lentiviral titration and CTL assay.

PCR amplification of the hTERT cDNA fragment. HepG2 cell mRNA was used as the PCR template. The immunogenic potential of the peptide fragments according to their hydrophilicity and antigenic index were determined with DNAstar sequence analysis software. The peptide, p228-p368, had the most appropriate immunogenic potential. The PCR primers were designed based on the gene sequence (accession no. NM-198253), and extra restriction enzyme sites, *Bam*HI and *Xho*I, were added at the 5' end and 3' end (underlined) of the primers. Their sequences were as follows: forward primer, 5'-GCGGATCCATGTGGCTGCAGAGCCTGCT-3'; reverse primer, 5'-GCCTCGAGTGACTCCTGGACCTGGCTCC-3'. PCR parameters were 95˚C for 30 sec, 62˚C for 30 sec, and 72˚C for 30 sec for 40 cycles. The PCR product was 443-bp long. The product was cloned into the T-easy vector and was confirmed by sequencing.

Plasmid DNA and the cloning of the hTERT cDNA fragment into the lentiviral vectors. The lentiviral vector was obtained from the Department of Medicine and Microbiology, University of Alabama as a kind gift. The vector was constructed to ensure the greatest predictable level of safety for the clinical application of retroviral vectors (13). The lentiviral vector system consisted of the delivery vector L166, packing vector L205 and envelope protein L311. L166 plasmid carried the green fluorescent protein (GFP) gene. Restriction enzyme sites, *Bam*HI and *Xho*I, were inserted into the upper and lower segment of GFP, respectively. The cDNA of hTERT and lentiviral vector L166 was purified and cut with *Bam*HI and *Xho*I. The hTERT cDNA was then ligated into the L166 at *Bam*HI and *Xho*I sites.

Generation of the recombinant lentiviral vectors. To produce recombinant lentiviral vectors, 293T cells were seeded at $3x10^6$ cells/10-mm plate in DMEM (Gibco Laboratories, Grand Island, NY, USA) and grown for 24-30 h. Plasmid DNA L166-hTERT together with packaging plasmids, L205 and L311, were co-transfected into 293T cells using the calcium phosphate precipitation method. Cells were cultured, and DMEM medium was replaced on day 3. On day 5, viral supernatant was collected and concentrated by centrifugation at 966 g for 20 min at 4˚C. A second recombinant, lenti-GFP virus was used as a control vector for transduction and titer determination. Immunochemical staining was performed to determine the expression properties of hTERT in 293T cells. Immunochemcal staining was conducted according to the protocol supplied with the hTERT UltraSensitive™S-P kit (Fuzhou Maixin Biotechnology Development Co., Ltd, Fuzhou, China).

DC isolation and transfection. DCs were generated from PBMCs in the presence of GM-CSF and rhIL-4 (Jingmei Bio-Tech Co., Ltd, Beijing, China). PBMCs were seeded into culture flasks in RPMI-1640 medium (Gibco Laboratories). After monocytes became adherent (incubation for 2 h), the non-adherent cells were removed and frozen in freezing media [60% AIM-V, 30% fetal bovine serum (FBS), 10% DMSO] for later use in the CTL assays. The adherent cells were cultured for 7 days in RPMI-1640 medium containing 10 ng/ml human recombinant GM-CSF (50 ng/ml) and IL-4 (100 ng/ml) (both from Jingmei Bio-Tech Co., Ltd.). DCs were harvested on the 7th day of culture. Cell surface markers were analyzed by immunostaining and flow cytometry. For immunophenotyping we used the following antibodies: CD14, CD80 and CD86 (Jingmei Bio-Tech Co., Ltd.). CD80 and CD86 are critical co-stimulatory molecules of DCs, and CD14 is a characteristic molecule of monocytes, which are progenitors of DCs. Cells ($1x10^6$) were incubated with antibody for 30 min on ice, then washed with phosphate-buffered saline (PBS) and fixed with 1% paraformaldehyde in PBS. Flow cytometric analysis was performed on the FACScan Flow Cytometer (Becton, Dickinson and Co. Immunocytometry Systems, San Jose, CA, USA). Lenti-hTERT vectors were transfected into DCs using a calcium phosphate-DNA co-precipitation method. Non-treated DCs were used as the control. Total RNA of DCs were extracted, and RT-PCR was conducted to confirm whether the transfected DCs expressed the hTERT fragment. The primers and parameters were the same as those mentioned above.

Allogeneic mixed leukocyte reaction (MLR). Mononuclear cells were isolated from the peripheral blood of healthy subjects. After incubation for 2 h, the non-adherent cells were collected as T lymphocyte cells. T lymphocytes were added as responder cells into a 96-well plate at a concentration of $1x10^5$/ml. DCs were collected as stimulator cells at a concentration of $1x10^6$/ml and were incubated with mitomycin C (25 μg/ml) for 30 min. Effector and target cells (E:T) were co-cultured at different ratios (1:10, 1:100, 1:1000). T lymphocytes with lenti-hTERT-DCs and untreated DCs, T lymphocytes alone without stimulators and blank wells without cells were set as sextuple wells. After being cultured for 3 days, the absorbance (A) at 492 nm (A492) was assayed by MTT and the stimulator index (SI) was calculated using the formula: SI = (A of stimulated T cells - A of blank cells)/(A of non-stimulated T cells - A of blank cells).

Generation of anti-tumor CTLs in vitro. HepG2 ($hTERT^+$) and 293T ($hTERT^-$) cells in the log growth stage were added to a

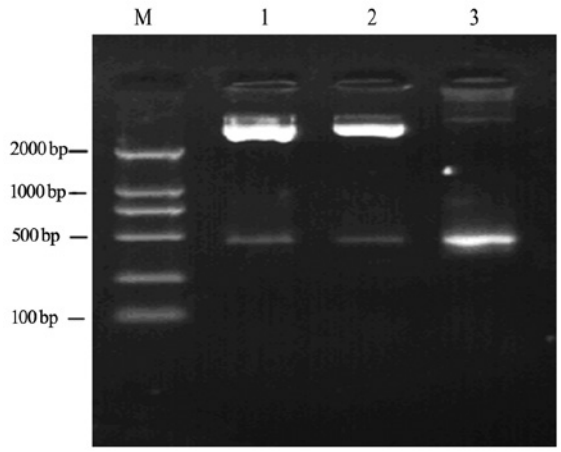

Figure 1. Gel electrophoretic profile of restriction enzyme digestion and PCR products. Lane 1, hTERT-T easy vector digested with *Bam*HI and *Xho*I; 2, L166-hTERT recombinant lentiviral vector digested with *Bam*HI and *Xho*I; 3, PCR product; M, marker. After digestion and gel electrophoresis a fragment <500 bp was observed. The fragment was confirmed by sequencing.

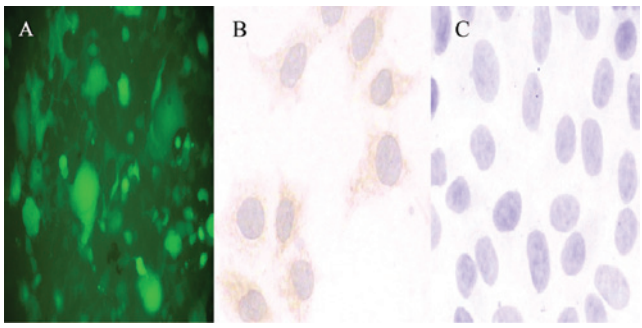

Figure 2. Recombinant lentiviral vector transfection. (A) As a control, lenti-GFP was transfected into 293T cells. Lenti-GFP viruses were used to infect 293T cells at serial diluted concentrations of the virus. The results showed lentiviral virus transduction efficiency reached 90% and the titer reached 3.2×10^6 TU/ml. (B) Immunochemical staining of hTERT showed that hTERT was expressed in the 293T cell plasma. (C) Immunochemical staining failed to detect any signals in the negative 293T control cells.

96-well plate at a concentration of $1x10^4$/ml and cultured 24 h as target cells. T lymphocytes stimulated with lenti-hTERT-DCs and DCs, respectively, were collected as effector cells. Non-stimulated T lymphocytes were used as the control. Target cells and effector cells were co-cultured at different effector-to-target (E:T) ratios (20:1, 10:1, 5:1). Each group was set as sextuple wells. After being cultured for 24 h, the absorbance (A) at 490 nm (A490) was detected by MTT. The inhibition rate (IR) was calculated using the formula: IR (%) = (1 - A experimental/A control) x100%.

Statistical analysis. Data were analyzed using SPSS Software v10.0 (SPSS, Inc., Chicago, IL, USA). The significant difference between groups was determined by two-tailed t tests. A value of $P<0.05$ was considered to denote statistical significance.

Results

Cloning of hTERT cDNAs into the lentiviral vectors. The hTERT cDNA fragment was amplified by PCR. A band <500 bp was observed in the gel electrophoresis assay of the PCR product. The recombinant construct was confirmed by sequencing. The hTERT cDNA was cut and ligated into lentiviral vector L166 at the *Bam*HI and *Xho*I sites. The recombinant lentiviral vector L166-hTERT was confirmed by restriction enzyme digestion and sequencing (Fig. 1).

Production of high-titer recombinant lentiviral vectors. The lenti-GFP virus was used as a control vector for transduction and titer determination. The lentiviral vector transduction efficiency reached 90% and the titer determined using 293T cells reached $3.2x10^6$ TU/ml (Fig. 2). Immunochemical staining of hTERT demonstrated that it was expressed in the plasma of the 293T cells and lasted for >2 months. The data indicate that the recombinant lenti-hTERT virus was successfully constructed (Fig.2).

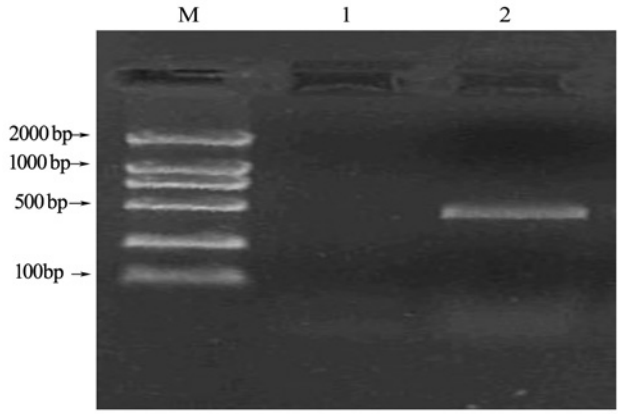

Figure 3. Gel electrophoretic profile of PCR products of DC cDNA. Lane 1, wild-type DCs; 2, lenti-hTERT-transfected DCs; M, marker. No product was noted in the wild-type DCs. A fragment <500 bp was observed in the lenti-hTERT DCs. The fragment was confirmed by sequencing. The results showed that hTERT was expressed in the lenti-hTERT DCs.

Morphological analyses and phenotype of DCs. After 24 h of culture, PBMCs began to grow similar to fully differentiated swarm cells as observed under a microscope. There was an increase in the size and number of DCs after 3 days. On day 5, more salience on the surface of DCs was observed. The phenotypes of DCs were determined by FACS on the 7th day of culture. The expression of phenotypic molecules in the DCs was diversified among groups when treated with different concentrations of LAM. The expression rates of CD14, CD80 and CD86 on DC cells were 3.56, 87.2 and 92.8%, respectively. The results demonstrated that the DCs were successfully induced. The results of the PCR showed that the lenti-hTERT-transfected DCs expressed the hTERT fragment, while no hTERT expression was noted in the wild-type DCs (Fig. 3).

Stimulatory capacity of the DCs on lymphocyte proliferation. In the allogeneic mixed leukocyte reaction (MLR), lymphocyte proliferation reflected the stimulatory function of the DCs. The stimulatory capacity of the lenti-hTERT-DCs in the

Table I. Stimulatory capacity of the different DCs in the mixed leukocyte reaction.

	DC_N + T			DC_T + T		
S:R	1:10	1:100	1:1000	1:10	1:100	1:1000
A492 (λ=492 nm)	0.243±0.015	0.232±0.011	0.223±0.009	0.443±0.008	0.392±0.013	0.321±0.017
SI	1.20	1.14	1.09	2.34[a]	2.05[a]	1.65[a]

DC_N + T, wild-type DCs as stimulators; DC_T + T, T lymphocytes with lenti-hTERT-DCs as stimulators. S:R, stimulator cells:responder cells). A492 (mean ± SD, n=6). SI, stimulator index. [a]Compared with DC_N + T.

Table II. Generation of antitumor CTLs *in vitro* with different DC vaccines.

	CTL_N			CTL_T		
	5:1	10:1	20:1	5:1	10:1	20:1
HepG2 cells						
A490 (λ=490 nm)	0.661±0.009	0.649±0.013	0.626±0.011	0.376±0.012	0.353±0.008	0.322±0.021
IR	7.16	8.85	12.08	47.19[a]	50.42[a]	54.78[a]
293T cells						
A490 (λ=490 nm)	0.673±0.002	0.665±0.017	0.644±0.014	0.671±0.005	0.663±0.023	0.639±0.006
IR	5.48	6.60	9.55	5.76[b]	6.88[b]	10.25[b]

CTL_N, anti-tumor CTLs with lenti-hTERT-DCs; CTL_T, anti-tumor CTLs with wild-type DCs. A490 (mean ± SD, n=6). IR, inibition rate. [a]Compared with HepG2 cells CTL_N, $p<0.01$; [b]Compared with 293T cells CTL_N; $p>0.05$.

allogeneic MLR was markedly enhanced as compared with the wild-type DC group ($P<0.01$) (Table I).

Generation of anti-tumor CTLs in vitro. HepG2 inhibition rates of T lymphocytes when stimulated with lenti-hTERT-DCs were significantly higher than those stimulated with DCs ($P<0.01$). There was no significant difference in 293T inhibition rates between the lenti-hTERT-DC and wild-type DC group ($P>0.05$). The inhibition rate in HepG2 cells was significantly higher than that in the 293T group ($P<0.01$) (Table II). The results indicated that the DC vaccine transfected with lenti-hTERT stimulated specific anti-tumor CTLs to HepG2 cells.

Discussion

Recently, HIV-1 derived lentiviral vectors (LVs) have been proposed as powerful tools for gene delivery to DCs. In contrast to conventional viral vectors, LVs provide several advantages for targeting DCs. First, they can transduce non-dividing, monocyte-derived DCs, as well as bone marrow-derived DCs at high transduction efficiencies (11,12). Second, LVs establish stable, long-term infection with minimal cytotoxicity. Third, LVs express the transgene of interest (up to 9 kb) without viral protein expression (14). Problems of vector-specific immunity are largely reduced with the use of LV because of the absence of preexisting immunity in humans. However, the possibility of generating replication-competent retrovirus (RCR) through genetic recombination raises concerns for safety. Recently, a novel trans-lentiviral vector with enhanced safety profiles has been developed (13). The LV vectors used in this study belong to this type. This HIV-based packaging system (trans-lentiviral) splits gag/gag-pol into 2 parts, in which one expresses gag/gag-pro and another expresses reverse transcriptase and integrase as fusion partners of viral protein R. Since an intact gag-pol structure is absolutely required for retroviral DNA mobilization and RCR, the trans-lentiviral vector design significantly reduces this risk. Therefore, the trans-lentiviral vector design will ensure the greatest predictable level of safety for the clinical application of retroviral vectors, including HIV-based vectors. In this study the lentiviral virus transduction efficiency reached 90% and the titer reached $3.2x10^6$ TU/ml. hTERT was highly expressed in the cells transduced with lenti-hTERT and lasted >2 months.

At present, cancer immunotherapy targeting hTERT has mainly focused on the induction of hTERT-specific T lymphocyte immune responses, among which CTL responses have been shown to specifically kill $hTERT^+$ tumor cells (15-17). To date, a number of such peptides have been identified in hTERT,

and CTL responses against $hTERT^{+}$ cells elicited by those hTERT peptides were also reported (18). In the present study, we chose a peptide (41aa long) using the DNAstar software. The peptide contained P324 (VYAETKHFL), which was described by Arai *et al* (19). The stimulatory capacity of the lenti-hTERT-DCs in the allogeneic mixed leukocyte reaction was markedly enhanced as compared with the wild-type DC group. Furthermore, HepG2 inhibition rates of T lymphocytes when stimulated with lenti-hTERT-DCs were significantly higher than those stimulated with wild-type DCs. There were almost no CTLs to hTERT-293T cells. These results indicated the the selected peptide had powerful immunogenic capacity and that DCs modified by lenti-hTERT can achieve superior tumor-specific CTL effects. DCs transduced with the trans-lentiviral vector encoding the hTERT peptide stimulated specific and long-lasting CTL activity *in vitro*. These findings suggest that DCs transduced with the trans-lentiviral vector encoding the hTERT peptide may have potential for clinical application after strict animal experiments.

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