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In vitro anti-tumor immune response induced by dendritic cells transfected with hTERT recombinant adenovirus ☆

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Abstract

Transduction with recombinant, replication-defective adenoviral (Ad) vectors encoding a transgene is an efficient method for gene transfer into human dendritic cells (DC). Several studies have demonstrated that epitopes of the human telomerase reverse transcriptase gene (hTERT) can produce CTLs specific for malignant tumors. In this study, we constructed an hTERT recombinant adenovirus (rAd-hTERT) using DNA recombination. We found that human dendritic cells transduced with rAd-hTERT could effectively induce hTERT-specific cytotoxic T lymphocytes (CTLs) *in vitro* against various tumor cell lines, which were hTERT-positive and HLA-A2 matched. We also found that these hTERT-specific CTLs could not lyse autologous lymphocytes with low telomerase activity. Further studies revealed that rAd-hTERT transduced DCs could increase secretion of IFN-γ by effector cells when they were co-cultured with hTERT-positive and HLA-A2 matched tumor cell lines. These data suggest that an hTERT vaccine can induce anti-tumor immunity against various tumor cells expressing hTERT in a HLA-A2-restricted fashion *in vitro*. The transduction of DCs with rAd-hTERT offers a great opportunity in cancer immunotherapy.

Keywords: Dendritic cells; Human telomerase reverse transcriptase (hTERT); Tumor-associated antigens; Cytotoxic T lymphocyte (CTL); Tumor vaccine

Cellular adoptive immunotherapy with specific cytotoxic T lymphocytes (CTLs) has been used to treat malignant tumors. Antigen presentation is critical for the initiation of an adoptive immune response. Dendritic cells (DCs) are considered the most potent professional antigen presenting cells and have the most powerful antigen presenting capacity [1,2]. They present tumor antigens to the immune system and initiate a specific immune response. The presentation of tumor-associated antigen (TAAs) by DCs, recognition by CTLs and the induction of a specific anti-tumor immune response has been and continues to be an area of significant research in the tumor therapy field. DCs pulsed with various TAAs has proven to be effective at producing specific anti-tumor effects *in vitro* and *in vivo* [3–6]. At present, identification of TAAs in cancer patients has been limited to a few cancers, such as MART-1 (melanoma antigen recognized by T cell-1), which is specific for melanoma, [7] and CEA, which is specific for gastrointestinal tumors [8,9]. These tumor-specific antigens, which are also called autoantigens, are shared among patients of similar tumor type. Therefore, immunization with these autoantigens can only induce an immune response for the same tumor type that expresses the self antigens, but cannot induce an immune response against other tumor types that do not express these autoantigens. As a result, identification of universal TAAs for immunotherapy of multiple tumors continues to be a challenging problem for clinicians.

Abbreviations: hTERT, human telomerase reverse transcriptase; TAA, tumor-associated antigen; DCs, dendritic cells; CTLs, cytotoxic T lymphocytes; E/T, effector-to-target; MOI, multiplicity of infection.

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Ideal universal TAAs need to [10]: (1) be expressed in the vast majority of human cancers, but rarely expressed in normal tissues, (2) be indispensable to the process of tumorigenesis to avoid antigenic variation or depletion, (3) include peptide sequences that bind to MHC molecules, and (4) be recognized by the T-cell repertoire in an MHC-restricted fashion to elicit a specific T-cell response. Telomerase is a unique ribonucleoprotein that mediates RNA-dependent synthesis of telomeric DNA, the distal ends of eukaryotic chromosomes that stabilize the chromosomes during replication [11]. Although telomerase is not an oncogene *per se*, upregulation of telomerase is necessary to maintain immortality of tumor cells [11,12]. It has been reported that telomerase could be detected in more than 85% of all human tumors but few normal somatic cells [13]. Human telomerase reverse transcriptase (hTERT), an important subunit of telomerase, is the rate-limiting component in the telomerase complex and is most closely correlated with telomerase activity [14]. The widespread expression of telomerase in tumors indicates that peptide fragments of hTERT could serve as tumor-specific antigens and this has been confirmed in several reports [15,16]. Although many epitopes of TAAs have been identified, several studies have also demonstrated that the endogenous processing and presentation of TAA peptides might be more efficient for cell surface presentation than exogenous loading of synthetic TAA peptides. Transduction of DCs with a TAA-encoding transgene offers several potential advantages over peptide-pulsing [17,18]. In this paper, we used hTERT-transfected DCs to trigger hTERT-specific CTLs and demonstrated that these CTLs could produce specific anti-tumor immunity against various hTERT-expressing cancer cell lines in a MHC-restricted fashion *in vitro*.

Materials and methods

Animals and cell culture. The osteogenic sarcoma cell line U2OS (hTERT$^-$, HLA-A2$^+$) was purchased from Beijing Xiehe Medical University. The gastric cancer cell lines KATO-III (hTERT$^+$, HLA-A2$^+$) and SGC-7901 (hTERT$^+$, HLA-A2$^-$) and liver cancer cell line HepG2 (hTERT$^+$, HLA-A2$^-$) were maintained in our laboratory. U2OS cells were cultured in McCoy's 5A medium (Life Technologies, Inc., Gaithersburg, MD, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. KATO III cell line, SGC-7901 cell line, and HepG2 cell line were all cultured in RPMI-1640 medium containing 10% FBS, penicillin (200 U/ml), and streptomycin (100 μg/ml). All cell lines mentioned above were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Construction of recombinant adenovirus encoding hTERT. The recombinant adenovirus vector encoding hTERT (rAd-hTERT) was constructed using the Adeno-XTM Expression System (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Briefly, the hTERT cDNA was digested out of pGRN145 (A kind gift from Geron Inc., US). The hTERT cDNA was cloned into the *Eco*RI site of shuttle vector pDC315 and sequenced to identify possible *Taq* polymerase-induced errors. The desired replication-deficient adenovirus containing the full length cDNA of hTERT was generated by homologous recombination through co-transfection of plasmids pDC315-hTERT and pBHGloXΔE1, 3Cre in HEK 293 cells using the DOTAP liposome reagent (Roch, Germany). After several rounds of plaque purification, the adenovirus containing the hTERT gene was amplified and purified from cell lysates by banding twice in CsCl density gradients as previously described [19]. Viral products were desalted and stored at −80 °C in phosphate-buffered saline (PBS) containing 10% glycerol (v/v). The infectious titer was determined by a standard plaque assay. A second recombinant, E1-, E3-deleted Ad-5 virus carrying the LacZ protein under the control of CMV promoter (rAd-LacZ), was obtained from Dr. Zhou Q of the Institute of Immunology, Third Military Medical University, and was used as a control vector for DC transduction.

DC generation from human peripheral blood precursors. DCs from peripheral blood mononuclear cells (PBMCs) were generated using the procedure described by Romani et al. [20]. Briefly, PBMCs were isolated from healthy HLA-A2$^+$ donors' buffy coats by Ficoll–Hypaque density gradient centrifugation and were then seeded into culture flasks in RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS. After monocytes adhered (incubation for 2 h), the non-adherent cells were collected and frozen in freezing media (60% RPMI-1640 and 30% FBS, 10% dimethylsulfoxide (DMSO)) for later use in CTL assays. The adherent cells were cultured for 5 days in RPMI-1640 containing 1000 U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D Systems, Inc., USA) and interleukin-4 (IL-4, R&D Systems, Inc., USA) and then were cultured for additional 2 days in the presence of 1000 U/ml of tumor necrosis factor-α (TNF-α, R&D Systems, Inc., USA) to induce final maturation. After 7 days of culture, the mature DCs were harvested and analyzed for DC typical phenotypes using fluorescence-activated cell sorter (FACS) analysis or they were co-cultured with T-cells for sensitization assays.

Flow cytometric analysis of cell populations. DCs were collected and resuspended in cold FACS buffer (phosphate-buffered saline with 0.2% BSA and 0.09% sodium azide). Cells were immunostained with fluorescein isothiocyanate (FITC) conjugated mouse anti-human CD 1a, CD80, CD83, and CD86 antibodies (eBioscience, USA). The corresponding FITC immunoglobulin G (IgG) isotype control antibody (eBioscience, USA) was used. A total of 1×10^6 cells were incubated overnight at 4 °C with antibodies. The cells were then washed once with FACS buffer, resuspended, and phenotyped on a FACScan (Becton–Dickinson, USA).

Adenovirus-mediated gene transfer and expression in DCs. Transduction of human mature DCs with Ad vector was conducted in six-well plates with 1×10^6 DCs/well in 3 ml of RPMI 1640 medium containing 10% FBS. Virus was added to the wells at a multiplicity of infection (MOI) of 200 and the DCs were harvested after 18–24 h of incubation.

For immunohistochemistry, the rAd-hTERT transduced DCs were grown on a slide coated with polylysine and fixed for 10 min in 4% paraformaldehyde (Sigma, St. Louis, USA). The slides were then washed with 0.2% Triton X-100 and anti-hTERT MAb and negative control sera were added to each slide. After incubation for 30 min at 4 °C and thorough washing in PBS (three times for 5 min), the slides were similarly treated with secondary antibody. Finally, the cells were incubated for 15 min with an avidin–biotin enzyme reagent. Staining was developed by immersing slides in DAB/H_2O_2 solution. PBS was used as a negative control in place of the primary antibody.

For Western blot analysis, proteins in the cell extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) through an 8% polyacrylamide gel and were then transferred onto a nitrocellulose membrane. The membrane was incubated with 5% non-fat milk in PBS and later with anti-hTERT MAb for 2 h at room temperature. After washing, the membranes were incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Amersham Biosciences, Buckinghamshire, England) for 1 h at room temperature. Immunoreactive bands were detected using the ECL Western blot analysis system (Amersham Biosciences, Buckinghamshire, England).

Induction of hTERT specific CTLs in vitro. hTERT-specific cytotoxic T lymphocytes (CTLs) were generated *in vitro* by weekly stimulation of non-adherent peripheral blood lymphocytes with irradiated autologous DCs, which were transduced with rAd-hTERT (DC-hTERT). In brief, PBMC-derived DCs were transduced with rAd-hTERT at an MOI of 200 and cultured for 2 days in fresh cytokine-supplemented medium containing

1000 U/ml of TNF-α. They were then irradiated with 40 Gy which entirely prevented outgrowth in the control cultures. The cells were then seeded into 24-well plates at 5×10^4 cells per well. The non-adherent autologous peripheral blood lymphocytes were added at 1.5×10^6 per well. After 7 days of co-culture with stimulators, the lymphocytes were harvested and resuspended at 5×10^5 per well. The T-cell lines were then re-stimulated with 1×10^5 cells per well of irradiated DC-hTERT. After 3 days, the cells were fed with 50 U/ml of IL-2. The cells were harvested 4 days later and then re-stimulated. On the 21st day, after harvesting the T-cell lines, their specificities were evaluated by a 4-h ^{51}Cr release assay. Effector cells that were generated from rAd-LacZ transduced DCs or T lymphocytes stimulated by only IL-2 were used as a control.

Chromium release assays. To evaluate levels of CTL activity, a standard 4-h ^{51}Cr-release assay was used as previously described [9]. Briefly, target cells were incubated with ^{51}Cr (100 μCi per 1×10^6 cells) for 2 h in a 37 °C water bath. Following incubation with ^{51}Cr, target cells were washed three times with PBS, resuspended in RPMI-1640 medium and mixed with effector cells at a 5:1, 10:1, 20:1, or 40:1 effector-to-target (E/T) ratio. Assays were performed in triplicate for each sample at each ratio in a 96-well round-bottomed plate. After a 4 h incubation, the supernatants were harvested and the amount of released ^{51}Cr was measured with a gamma counter. The percent-specific lysis was calculated according to the following formula:

$$\text{Specific lysis} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100\%$$

Enzyme-linked immunosorbent assay (ELISA) for INF-γ. An ELISA was performed for INF-γ following the kit manual. In brief, target cells were added to a 96-well microplate at a density of 1×10^4 cells/well in triplicate. Effector cells were then added to each well with 2×10^5 cells/well. After a 24 h incubation at 37 °C and centrifugation at a speed of 1000*g* for 10 min, 100 μl of each supernatant was collected to examine the level of IFN-γ with an ELISA kit (Jing-Mei Biotech., China) according to the manufacturer's instructions.

Statistics. All experiments were run in triplicate and the results are depicted as the means ± SD of triplicate determinations. Statistical analysis was performed using the Student's *t* test. The difference was considered statistically significant when the *P* value was less than 0.05. All statistical analyses were conducted with SPSS 11.5 software.

Results

Construction and identification of hTERT adenovirus recombinant

hTERT cDNA was digested from plasmid pGRN145 with *Eco*RI restriction endonuclease and then inserted into the pDC315 vector. The orientation of the recombinant DNA was determined by *Bam*HI restriction endonuclease and sequencing. After digestion with *Bam*HI, the sense hTERT vector formed two fragments of 6.4 kb and 0.9 kb, which was named pDC315-shTERT. The orientation of the recombinant DNA was further confirmed by sequencing (data not shown). A recombinant hTERT adenovirus (rAd-hTERT) was successfully generated after pDC315-shTERT homologously recombined with pBHGloXΔE1, 3Cre in HEK 293 cells.

Morphological and phenotypic characteristics of PBMC-derived DCs

On day 7 of cell culture, mature DCs displaying typical morphological characteristics were harvested from monocytes cultured in medium containing GM-CSF, IL-4, and TNF-α. When viewed by phase-contrast microscopy, these mature cells were loosely suspended, exhibited irregular cell shapes, and displayed many fine processes at their edges. The phenotypes of mature transduced and untransduced DCs were analyzed by FACS. The results showed that expression of CD1a, CD83, CD86, CD80 and HLA-DR was 60.5%, 72.7%, 76.1%, 93.1%, and 84.5%, respectively, before rAd-hTERT transfection, and 68.5%, 70.3%, 80.8%, 95.1%, and 90.2%, respectively, after rAd-hTERT transfection.

Adenoviral transduction of human DC in vitro and the expression of hTERT

Transduction efficiency of adenovirus was determined with rAd-LacZ after 12–48 h of incubation following infection at MOIs of 50–200. We found that the transduction efficiency increased in an MOI-dependent manner. At an MOI of 200, the rAd-LacZ transduction rate was almost 100% from 12 to 48 h (data not shown). Subsequently, DCs were transfected with rAd-hTERT at an MOI of 200. Expression of the hTERT protein in DCs before and after rAd-hTERT transfection was evaluated by immunohistochemistry staining and Western blot. As seen in Fig. 1, expression of the hTERT protein was significantly increased after Ad-hTERT transfection. In addition, we also found that the hTERT protein expression levels increased during the maturation of DCs.

Induction of hTERT-specific CTL activity in vitro

As mentioned above, DCs from HLA-A2 positive donors were transduced with rAd-hTERT or rAd-LacZ vector at an MOI of 200. As shown in Fig. 2, these hTERT-specific CTLs caused greater than 50% lysis of KATO-III gastric cancer cells, which were both hTERT and HLA-A2 positive, at an E:T ratio of 40:1 as compared to 20% lysis using rAd-LacZ transduced DCs and 15% lysis using T lymphocytes only stimulated by IL-2. However, the induced effectors could not lyse SGC-7901 gastric cancer cells or HepG2 liver cancer cells, which were hTERT-positive but HLA-A2 negative. Even at the highest E:T ratio, the lysis rate was less than 10%. Taken together, these results indicated that rAd-hTERT transduced DCs were capable of generating specific CTLs against HLA-A2 matched and hTERT-positive tumor cells.

Specificity of CTLs directed against hTERT

To further confirm CTL specificity, we took the advantage of the HLA-A2 positive, telomerase-negative osteogenic sarcoma cell line, U2OS. U2OS cells were transduced with pIRES2-EGFP-hTERT plasmid, an eukaryotic fluorescent expression vector of hTERT, by the DOTAP lipofection method according to the manufacturer's protocol. After 24 h of transfection, 400 μg/ml of G418

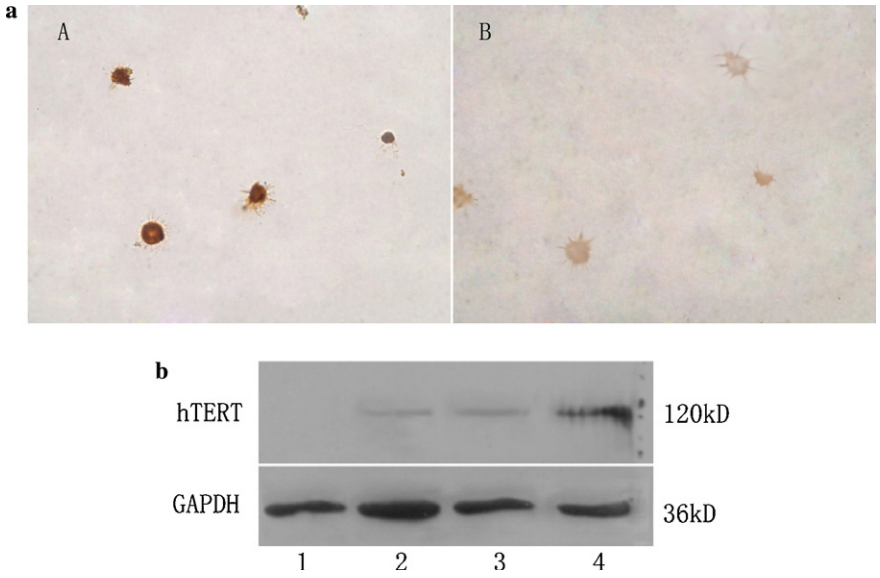

Fig. 1. Expression of hTERT protein in rAd-hTERT-transfected DCs. (a) Immunohistochemical staining of DCs for hTERT expression (200× magnification). Expression of the hTERT protein increased in rAd-hTERT transduced DCs (A) compared with untransduced DCs (B). (b) Western blot analysis of DCs for analysis of hTERT expression. The results show that expression of the hTERT protein significantly increased in rAd-hTERT transduced DCs compared with untransduced DCs or rAd-LacZ transduced DCs. Lanes 1, immature DCs; 2, mature DCs; 3, mature DCs transduced with rAd-LacZ; 4, mature DCs transduced with rAd-hTERT.

was added to the RPMI-1640 medium. Following a G418 selection of 4 weeks, the drug resistant individual clones were randomly collected from the transfected cultures. For controls, the drug resistant clones were selected from the cultures transfected with the empty vector pIRES2-EGFP. The selected clones were named U2OS-hTERT and U2OS-EGFP, respectively. Immunohistochemistry and Western blot demonstrated that the hTERT protein was highly expressed in U2OS-hTERT but not in U2OS-EGFP (data not shown). CTLs were generated using a rAd-hTERT transduced DC population generated from a normal HLA-A2 positive PBMC sample. After three stimulations, CTL activity was tested against U2OS, U2OS-EGFP, and U2OS-hTERT cells. As shown in Fig. 3a, these hTERT-specific CTLs could lyse 38% of U2OS-hTERT at an E/T of 40:1, while no obvious lysis effect on U2OS-EGFP or U2OS was detected even at the highest E/T ratio. These results clearly demonstrated that the majority of CTLs specifically targeted telomerase peptides that were presented in the context of HLA-A2 by rAd-hTERT-modified tumor cells.

Killing effect of hTERT specific CTLs on autologous lymphocytes

Although telomerase expression is largely restricted to cancer cells, certain hematopoietic progenitor cells, activated lymphocytes and tissue-specific stem cells in the skin, intestinal tissues, and germ cells are known to possess telomerase activity. Theoretically, immunotherapy aimed at hTERT may elicit immune system side effects. To investigate the effect of hTERT-specific CTLs on immunologic-activated lymphocytes, hTERT specific CTLs were also employed to lyse autologous lymphocytes. The results indicated that hTERT vaccination had no detectable lysis effect on these cells *in vitro* (Fig. 3b).

Inspection of human INF-γ by ELISA

Since CTLs are known to produce the Th1 cytokine, IFN-γ, in a non-antigen-specific manner, we also assayed the presence of tumor-specific CTLs using the IFN-γ ELISA. As shown in Fig. 4a, when different effector cells were co-cultured with KATO-III cells, IFN-γ secretion was significantly increased by effector cells induced by DC/rAd-hTERT compared with effector cells induced by DC/rAd-LacZ or lymphocytes stimulated with only IL-2 ($P < 0.05$). Additionally, the concentration of IFN-γ was significantly increased accompanied with an increase of the E/T ratio ($P < 0.05$). Moreover, we also studied the level of IFN-γ when hTERT-specific CTLs were co-cultured with various target tumor cells. As shown in Fig. 4b, IFN-γ secretion by effectors was significantly higher when they were co-cultured with U2OS-hTERT target cells than when they were co-cultured with U2OS/EGFP cells, HepG2 cells or autologous lymphocytes. These results indicated that an hTERT-specific DC vaccine could increase IFN-γ secretion by effectors and enhanced the Th1 immune response.

Discussion

Human telomerase reverse transcriptase (hTERT) represents an attractive target for a broad range of tumors because it is undetectable in most somatic cells, but is

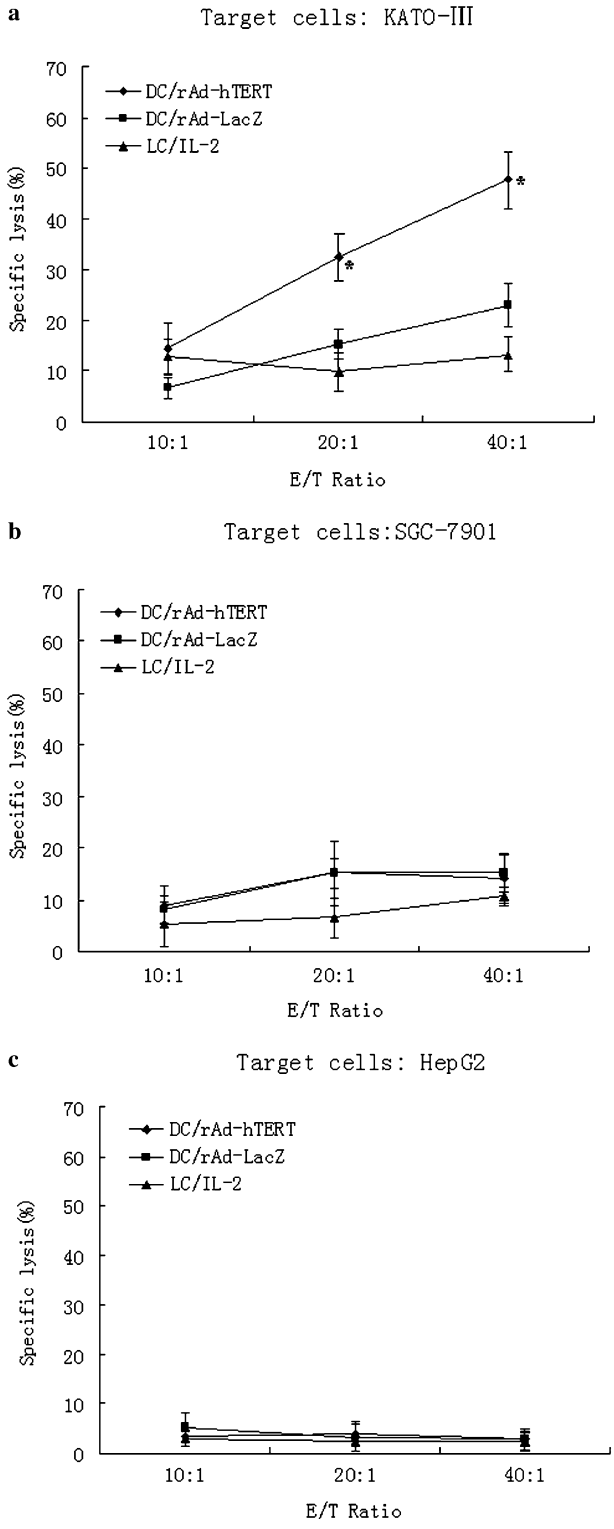

Fig. 2. Specific lysis of various tumor cell lines *in vitro* by CTLs generated from rAd-hTERT transduced DCs. hTERT-primed CTLs were tested against KATO-III cells (a, hTERT$^+$, HLA-A2$^+$), SGC-7901 cells (b, hTERT$^+$, HLA-A2$^-$) and HepG2 cells (c, hTERT$^+$, HLA-A2$^-$). Effector:target ratios are presented on the *x*-axis while the *y*-axis represents the percent of specific lysis. CTLs generated from rAd-LacZ transduced DCs and effector cells generated from T lymphocytes stimulated by only IL-2 were used as controls. Results are given as the means ± SD. *Statistically significant values at $P < 0.05$ using a paired Student's *t* test compared with the corresponding controls.

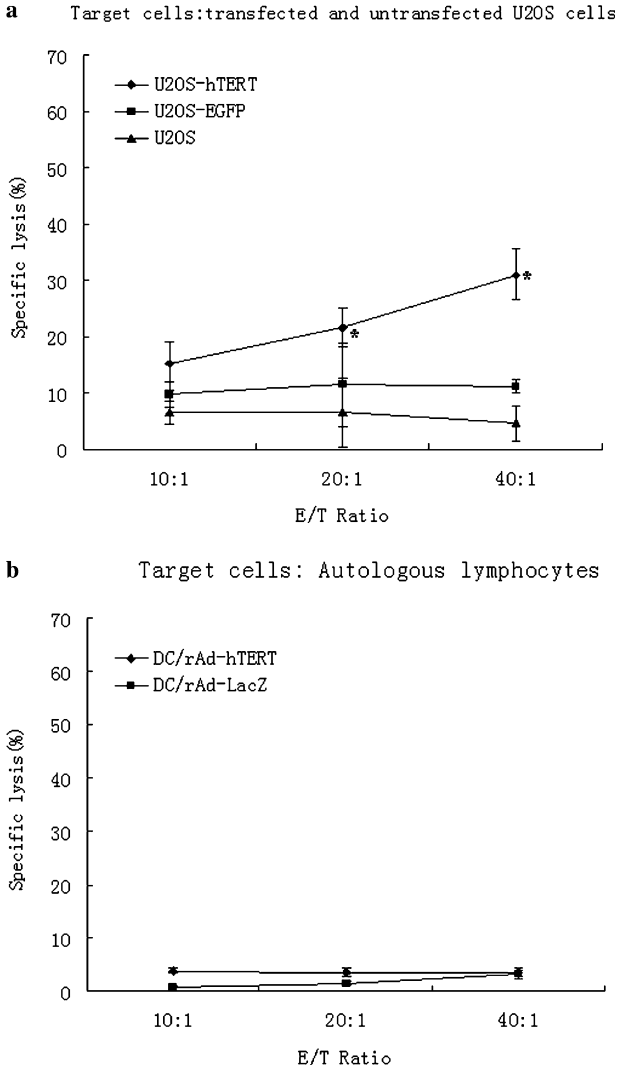

Fig. 3. Specific lysis of CTLs generated from rAd-hTERT transduced DCs on U2OS cells or autologous lymphocytes *in vitro*. (a) Killing effect of hTERT specific CTLs on transduced and untransduced U2OS cells. (b) Killing effect of hTERT specific CTLs on autologous lymphocytes. Effector:target ratios are presented on the *x*-axis while the *y*-axis represents the percent of specific lysis. CTLs generated from rAd-LacZ transduced DCs and effector cells generated from T lymphocytes stimulated by only IL-2 were used as controls. Results are given as the means ± SD. *Statistically significant values at $P < 0.05$ using a paired Student's *t* test compared with the corresponding controls.

reactivated and over-expressed in more than 85% of human tumors [13]. Telomerase expression has been directly linked to the ability of tumor cells to replicate indefinitely and the inhibition of telomerase in tumor cells has been shown to promote cell death [21,22]. Therefore, if a T-cell response could be directed against peptide epitopes processed from telomerase, it seems that any immune escape variants that did not express this protein would not be able to survive. Thus, telomerase is considered as an attractive candidate antigen for the development of immunotherapies in the treatment of patients with malignant tumors. Vonderheide et al. [15] have shown that hTERT is processed for class I presentation in a broad range of human tumors. Their

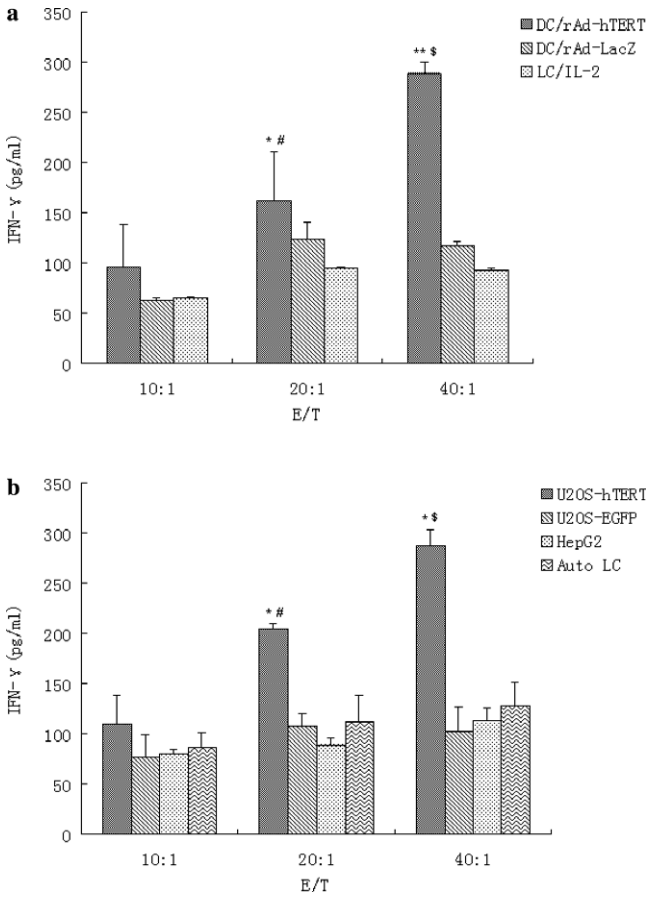

Fig. 4. Inspection of human INF-γ from effector cells by ELISA assay. (a) Secretion of IFN-γ when different effector cells co-cultured with KATO-III gastric cell line. Compared with the corresponding control groups (DC/rAd-LacZ or LC/IL-2) $^{*}P < 0.05$; $^{**}P < 0.01$; compared with E/T = 10:1 $^{\#}P < 0.05$; compared with E/T = 20:1 $^{\$}P < 0.05$. (b) Secretion of IFN-γ when different target cells co-cultured with hTERT specific CTLs generated from rAd-hTERT modified DCs. Compared with the corresponding control groups (U2OS-EGFP, HepG2 or autologous lymphocytes group) $^{*}P < 0.05$; compared with E/T = 10:1 $^{\#}P < 0.05$; compared with E/T = 20:1 $^{\$}P < 0.05$. Auto LC: autologous lymphocytes.

findings suggested that hTERT could serve as a target for widely applicable immunotherapy against various cancers. In fact, several studies have demonstrated that DCs pulsed with hTERT peptide or transduced with hTERT mRNA stimulated CTL responses *in vitro* [22,23] and in advanced cancer patients [4,6,24].

Although a variety of vectors are available for gene transfer to DCs, recombinant adenovirus is the most efficient. The adenoviral vector is a highly efficient and reproducible method of gene transfer. Indeed, several studies have shown that successful adenoviral gene transfer into human DCs resulted in induction of a T-cell response against the tumor [25,26]. In the present study, we used an E1,E3-deleted adenoviral vector containing the full length hTERT gene under the CMV promoter. The results demonstrated that this is an adenovirus vector strategy that provides a highly efficient reproducible method of gene transfer into non-dividing DCs. The transduction efficiency increased in an MOI-dependent manner. At an MOI of 200, the transduction rate was almost 100%. Immunohistochemistry and Western blot also demonstrated that expression of the hTERT protein in rAd-hTERT transduced DCs was significantly increased compared with untransduced DCs or rAd-LacZ transduced DCs. Interestingly, we found that the hTERT protein in immature DCs was undetectable by Western blot. However, the hTERT protein was detectable in mature DCs (Fig. 1). This finding was similar to the study reported by Ping et al. In that study, they found that telomerase activity was dramatically increased during dendritic cell differentiation and maturation [27].

Consistent with the observation reported by Nair et al. [3] using *in vitro* transduced hTERT RNA, we present evidence that DCs can process hTERT proteins that ultimately lead to the expression of HLA-A2-restricted peptides after introducing the telomerase gene. In particular, rAd-hTERT transduced DCs likely have potential advantages. First, DCs transduced with the entire TAA gene may present multiple epitopes including previously unknown epitopes associated with different MHC class I molecules. Second, TAA gene-transduced DCs may possibly present helper epitopes associated with MHC class II molecules [28]. Third, DCs are provided with a renewable supply of the antigen for presentation by transduction with the gene, as opposed to a single pulse of peptide [29]. Fourth, by using viral vectors to modify DCs *in vitro*, there is far less administration of viral proteins, in contrast to direct *in vivo* vaccination with viral vectors, thus minimizing the generation of neutralizing antibodies which prevent repeat treatment [30].

In order to show that hTERT can be used as a universal tumor antigen, we chose four tumor cell lines of diverse tissue origins. The osteosarcoma cell line, U2OS, is negative for the hTERT antigen but positive for HLA-A2. The gastric cancer cell line, KATO-III, is both positive for hTERT and HLA-A2, while SGC-7901 is positive for hTERT and negative for HLA-A2. In addition, HepG2, a liver cancer cell line, is known to be positive for hTERT and negative for HLA-A2. Using rAd-hTERT-transfected DCs, we found that the hTERT-specific CTLs were able to specifically lyse KATO-III cells, whereas these hTERT-specific CTLs could not lyse U2OS, SGC-7901 or HepG2 cells. Interestingly, CTL activity increased after U2OS cells were transfected with the pIRES2-hTERT plasmid, a eukaryotic expression vector containing full length hTERT cDNA. These results indicate that the immune responses are hTERT specific and HLA-A2 restricted and that hTERT is a universal antigen that may have a wide application for cancer immunotherapy.

For the development of cancer vaccines, safety concerns still limit their use in the future. Although telomerase expression is largely restricted to cancer cells, germ cells and some somatic cells, such as hematopoietic progenitor cells and activated lymphocytes, are known to possess telomerase activity [31,32]. Theoretically, any application of a telomerase-based cancer vaccine therapy will require

assessment of the potential adverse effects associated with autoimmunity to cells and organs that are telomerase-positive. *In vitro* studies in human systems suggest that hTERT is a poor autoantigen for telomerase-positive hematopoietic progenitor cells or activated T lymphocytes [15,16,33]. *In vivo* murine studies demonstrated the generation of TERT-specific protective immunity without the development of autoimmunity against TERT expressing cells [3]. In this study, in order to investigate the effect of hTERT specific CTLs on immunologically activated lymphocytes, hTERT-specific CTLs were also used to lyse autologous lymphocytes. The results revealed that hTERT vaccination did not have a remarkable lysis effect on these activated lymphocytes *in vitro*. Of course, the reason why these hTERT-specific CTLs did not lyse autologous lymphocytes remains unclear. One explanation for this finding is that the level of hTERT expression in normal cells is below the threshold needed for recognition by these hTERT-specific CTL populations.

In summary, we demonstrate that the DCs transduced with rAd-hTERT are able to induce a potent anti-tumor immune response *in vitro* against the various tumor cell lines which are hTERT-positive and HLA-A2 matched. These genetically modified DCs did not elicit a specific killing effect on autologous lymphocytes, which indicates that the hTERT vaccine is safe. This study shows that hTERT can serve as a universal TAA target of immunotherapy for various malignant tumors.

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