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CONSTRUCTION OF EXPRESSION VECTOR OF HTERT- HIL18 FUSION GENE AND INDUCTION OF CYTOTOXIC T LYMPHOCYTE RESPONSE AGAINST HTERT

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Abstract

Aim: Recombiant human telomerase reverse transcriptase (hTERT)-human IL-18(hIL18) was constructed to investigate its expression and biological function in eukaryotic cells. DCs transfected with hTERT-IL18 acquired stronger telomerase activity and were able to elicit an hTERT-specific cytotoxic T lymphocyte CTL response *in vitro*.

Methods: hIL-18 gene fragment was amplified by polymerase chain reaction (PCR) and TA cloned. The hIL-18 gene was then subcloned into eukaryotic expression vector pcDNA3.1(+) containing human TERT via a linker. The sequence of gene fusion was confirmed using both restrictive enzyme digestion and DNA sequencer. The expression vector with gene fusion was transfected into 3T3 cell line with Lipofectamine 2000. ELISA, Western blot, immunofluorescence stain were performed to determine the expression properties of hTERT-hIL18 in 3T3 cells. Its biological effect on the anti-apoptosis was measured by Flow cytometry and its effect on INF-γ expression was determined using ELISA. After preparation of dendritic cells, hTERT-hIL18, hTERT, hIL-18 expression vectors were transfected into DCs respectively by electroporation to generate hTERT-specific DCs lines. The peripheral blood mononuclear cells PBMCs were stimulated with different DCs lines to create specific CTL. The response of target cell (leukemia cell line-K562 cell) to hTERT-specific CTL was evaluated by LDH release assay.

Results: The human IL-18 gene fragment was amplified from the human mononuclear cells and was inserted into pcDNA3.1(+)/hTERT vector successfully. The correct sequence was proved by both restrictive enzyme digestion and sequencing. The correct open reading frame was also verified. Fusion protein of hTERT-hIL18 was effectively expressed in eukaryotic cells, which was detected by both Western-blotting and immunofluorescence stain. The expressed recombinant fusion protein induced similar levels of INF-γ to that of native IL-18 protein. FCM assay showed that the transfected fusion protein inhibited the apoptosis, which was consistent with the effects of hTERT as a universal tumor associated antigen. CTL assay shows that hTERT- hIL18 and hTERT gene-transfected DCs stimulated T-cell responses that recognized and lysed tumor target cells of high hTERT expression, whereas DCs transfected with hIL-18 gene didn't induced the response of tumor targets lyses.

Conclusion: The Recombinant hTERT- hIL18 fusion protein had both biological activity of hTERT and hIL-18, indicating that this rationally designed protein can be further developed as novel tumor therapeutics. DCs transfected with hTERT-IL18 gene were capable of eliciting a stronger hTERT-specific CTL response *in vitro*.

Key words: interleukin-18; telomerase reverse transcriptase; fusion gene; dendritic cells

INTRODUCTION

Human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, plays a pivotal role in the maintenance of telomeres and cell proliferation [1]. The hTERT maintains cell survival and proliferation via both telomerase enzymatic activity-dependent telomere lengthening and enzymatic activity-independent intermolecular interactions involving p53 and PARP. Telomerase is associated with cell immortality and cancer, which may relate to the ability of TERT to prevent apoptosis by stabilizing telomeres [2]. So hTERT is also an attractive target for novel immunotherapies against cancer. Data from both human and murine systems demonstrate that cytotoxic T-lymphocytes (CTL) can recognize peptides derived from TERT and kill TERT-positive tumor cells of multiple histological lines.

Dendritic cells (DCs) are important for the initiation of immune response to foreign antigens because of their professional competence to capture and present antigen to T cells. Numerous animal studies have demonstrated that DC pulsed *in vitro* with tumor antigens or transfected by tumor peptide encoding DNA or mRNA are capable of generating protective antitumor immune response [3, 4]. Immunization of mice with TERT RNA-transfected dendritic cells (DC) stimulated cytotoxic T lymphocytes (CTL), which lysed melanoma and thymoma tumor cells and inhibited the growth of three unrelated tumors in mice of distinct genetic backgrounds [5].

The first two authors contributed equally to this study.

Interleukin (IL-) 18 is important immune regulating factor that can induce generation of helper T-cells and enhance the specific anti-tumor cytotoxic T-cell response *in vitro*. IL-18 is fully competent to induce IFN-gamma production [6]. Tatsumi have evaluated the ability of DCs engineered to secrete the potent Th1-biasing cytokine interleukin-18 to promote enhanced antitumor immunity in a mouse sarcoma model [7]. These studies support that IL-18 gene transfer enhances the capacity of DCs to drive broadly reactive Th1-type therapeutic immunoresponse.

In the present study, we construct the hTERT–IL-18 fusion gene and investigate whether the fusion protein possessed the biological function of hTERT and IL18 or not in 3T3 cell. We transfected the fusion gene into DCs and investigated its effects on the immune response of DC-induced hTERT-specific CTLs. It may provide a research platform for the future study of therapeutic DCs as tumor vaccine.

Materials and Methods

Cell Line and Vectors

The 3T3 cell line (mouse fibroblast cell line), KG-1 cell line (human myelomonocytic cell line) and K562 cell line (human acute erythroid leukemic cell line) from ATCC (Rockville, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, N.Y., USA), high glucose media and generously supplemented with 100 ml/L fetal bovine serum (Gibco BRL, N.Y., USA), penicillin, streptomycin and nonessential amino acids. *E. coli DH5α* used for cloning experiments was grown in LB medium containing 50 mg/L ampicillin (Invitrogen Corporation, California, USA) per liter. pcDNA3.1(+)/hTERT was constructed previously by our own lab (pcDNA3.1 vector from Invitrogen Corporation, California, USA). hTERT full length cDNA was inserted into the EcoR□/Not□site of eukaryotic expression vector pcDNA3.1(+) previously.

Amplification of IL-18 Gene Fragment

Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by Ficoll-Hypaque (Amersharm Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. Total cellular RNA was isolated using Trizol Reagent (Gibco BRL, N.Y., USA) in accordance with the manufacturer's instructions. Complementary DNA (cDNA) was prepared by reverse transcription (RT) of 2ug total RNA using oligo-dT18 and 200U superscript□ reverse transcriptase (Invitrogen, California, USA) at 42□ for 70 min according to the manufacturer's instruction. Based on the complete DNA sequence of hIL-18 published and multiple clone sites of pcDNA3.1(+), the primers to amplify IL-18 containing *Nhe*□restriction enzymes site in sense primer and *Hind*□ restriction enzymes site in anti-sense were designed: sense, 5' CTG *GCT AGC* ATG GCT GCT GAA CCA GTA GAA G 3', anti-sense, 5' GGG *AAG CTT* GTC TTC GTT TTG AAC CAG TGA 3'. Amplification was carried out in a total volume of 50μl under conditions: at 95□ for 5 min, then 35 cycles at 94□ for 40 s, at 50□ for 50 s and at 72□ for 50s, followed by 10 min at 72□. The PCR products were analyzed on 2.0% agarose gels stained with ethidium bromide.

Confirmation of IL-18 PCCR Product

PCR products were separated using a QIAquick gel extraction kit (QIAGEN, CA, USA). Purified IL-18 DNA fragments were subcloned into TA cloning vector pUCmT (Takara, Dalian, China), and then the IL-18 mRNA was confirmed by agarose gel electrophoresis analysis.

Construction of Recombinant pcDNA3.1(+)/hTERT+IL18

Fragments of Nhe□ and Hind□ digested pUCmT-IL18 were inserted into the Nhe□/ Hind□ site of eukaryotic expression vector pcDNA3.1(+)/hTERT, through a series of enzyme digestion and ligation reactions. Then the recombinant fusion pcDNA3.1(+)/hTERT+IL18 was confirmed by DNA sequence test and restriction enzyme digestion.

The Recombinant pcDNA3.1(+)/hTERT+IL18 Sequence was Confirmed by Restrictive Enzyme Digestion and DNA Sequence

Recombinant pcDNA3.1(+)/hTERT+IL18 was used to transform E.coli DH5α in LB medium with calcium chloride, then the large scales of recombinant plasmid was prepared. The plasmid was digested by restrictive enzyme Nhe□/Not□, Nhe□/Hind□, EcoR□/Not□ (Takara, Dalian, China). The products of digestion were analyzed on 1.0% agarose gels stained with ethidium bromide. DNA sequence of recombinant pcDNA3.1(+)/hTERT+IL18 was tested by BOYA (Shanghai, P.R.China).

In vitro Transfection

To detect the protein expressed by recombinant plasmid, pcDNA3.1(+)/hTERT+IL18 was transfected into 3T3 cells. 3T3 cell line was cultured at 37 °C, 5 % CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Gibco-BRL, UK), 100 U/ml penicillin and 100μg/ml streptomycin, 15 mmol/L HEPES, and 2 mmol/L L-glutamine. Twenty four hours before transfection, 1×10^5 3T3 cells were seeded into six-well plates, and the mixture of pcDNA3.1(+)/hTERT+IL-18 vector and Lipofectamine 2000 (Invitrogen, California, USA) were added to the cells. Forty-eight hours after transfection, cells were washed with PBS, three wells 3T3 cells were assayed by immunofluorescence stain and the remaining cells were extracted using protein extraction reagent (Pierce, Illinois, USA) for Western-blot assay.

Immunofluorescence

Adherent cells were fixed in 70% ice-cold ethanol and processed for indirect immunofluorescence. Background binding of the antibodies was reduced by a

preincubation with normal goat serum (10% in PBS). Cells were then incubated with primary antibodies (hTERT or IL-18 Santa Cruz) for 2 hours at room temperature, washed in PBS, and incubated with the secondary antibodies-fluorescence conjugated. After several washes, the specific binding and cellular distribution of the antigens was assessed by fluorescence microscopy (LSM 410, Zeiss, Germany).

Expression of hTERT and IL-18 Protein Detected by Western Blot

40 ug whole-cell protein extracts were prepared and resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with antibodies against hTERT (1 : 500, Santa Cruz Biotech, California, USA)or IL-18 (1 : 800, R&D System, Minnesota, USA) for 2 hour. Thereafter, the blot was washed, exposed to HRP-conjugated secondary antibodies for 1 hour, and finally detected by chemiluminescence (ECL; Amersham Pharmacia Biotech, Illinois, USA).

Determination of IFN-γ Protein in KG-1 Cells Stimulated by hTERT+IL18 Fusion Protein

100 μl cell-free supernatants were collected from 3T3 cells transfected with hTERT+IL18 fusion gene, cocultured with 1×10^5 KG-1 cells in 96-well plates for 18 hours, diluted with different ratio (1 : 1, 1 : 2, 1 : 5, 1 : 10, 1 : 20). Supernatants from 3T3 cells without transfected were set as negative control group. Standard IL-18 (R&D System, Minnesota, USA) stimulate aliquots was used as positive control group. Equal volume of PBS was used as blank control group. INF-γ contents were determined by enzyme-linked immunosorbent assay (ELISA) kit (R&D systems, Minnesota, USA).

The Anti-apoptosis Function of Fusion Protein was Assayed by Flow Cytometry

To determine the effect of anti-apoptosis function of fusion protein, 3T3 cells were treated with Methotrexate (MTX, Calbiochem, California, USA), a strong apoptosis inducing drug. The 3T3 cells transfected with or without hTERT+IL18 gene cocultured with various concentrations of MTX (0-100nm) for 24 h. And these cells were washed in phosphate-buffered saline, resuspended in 100 μl binding buffer containing 5 μl Annexin V-FITC (BD Pharmingen, California, USA) and 5 μl propidium iodide (PI) for 15 min at the room temperature, then analyzed by flow cytometry (Becton Dickinson, CA, USA). Unstained treated cells were also analyzed in parallel.

Preparation of Human Blood Cells

The peripheral blood mononuclear cells PBMCs were isolated and cryopreserved in aliquots for later use as stimulators and responder cells. DCs were generated by plating thawed PBMCs in six-well plates at 10^7 cells/well in X-VIVO 10 (BIO-Whittaker, Walkersville, MD) supplemented with 2% heat-inactivated human pool serum. The cells were allowed to adhere for 1.5 h in 5% CO_2 at 37°, and the nonadherent cells were removed. The adherent cells were washed three times and suspended in X-VIVO 10 supplemented with 2% heat-inactivated human pool serum, 800 U/ml GM-CSF, 500 U/ml IL-4, 10 ng/ml TNF-α (R&D systems, Minnesota, USA) and continually incubated (CO_2 at 37°) for 5∼7 daysto facilitate the differentiation of DCs. The phenotype of differentiated cells was evaluated by staining with fluorochrome-labelled antibodies against the MHC-II, CD80, CD83, CD86 and CD14 cell surface markers and analysed by flow cytometry using the FACSCalibur flow cytometer (Becton Dickinson, CA, USA). Differentiation of mature DCs, as measured by up-regulation of MHC II, CD80, CD83 and CD86, and down-regulation of CD14, was complete on day 5 (results not shown).

Transfection of DCs

DCs were washed once and suspended in X-VIVO 10 and placed on ice. DNA transfection, 0.1 ml 10^4 –10^5 cells was mixed with 2 ug DNA (1μg/μl). The cells were transfected to a 2-mm-gap cuvette and pulsed with a BTX ECM 830 square-wave electroporator (Genetronics, San Diego, CA, USA) using parameter settings as specified in the text. After incubation on ice for 1 min, the cells were seeded in maturation medium and incubated at 37°. Expression of hTERT and IL-18 after transfection with pcDNA3.1(+)/ hTERT+IL18 was analysed using the methods described above.

Induction of hTERT-specific CTL Response *in vitro*

DCs were transfected with pcDNA3.1(+)/hTERT+ IL18 and incubated for 24 h in maturation medium. They were then washed and mixed with thawed autologous PBMCs at a stimulator to responder ratio of 1:10 in X-VIVO 10 supplemented with 10% heat-inactivated human pool serum and 50 U/ml recombinant interleukin-2 (R&D systems, Minnesota, USA). The bulk cultures were stimulated weekly with freshly transfected DCs (prepared by incubation for 10 days with GM-CSF and IL-4 of transfectedly immature DCs, then transferring to maturation medium for another 2 days before stimulation of T cells). Partial replacement of medium was done twice a week. We collected live cells as the effecter cells, mixed them with K562 cells (acute erythroid leukemic cell line, with high hTERT expression) as target cells (1°¡104 K562) at different effecter cell / target cell ratios 10:1, 20:1 and 50:1. The cell mixture was put in a 96 well-round bottom culture plate with its total volume reached to 200 mL and incubated for 4 h. After incubation, 0.9% NaCl was added to stop effecter/target reaction. The plate was centrifuged at 1500 r/min for 5 min and 100 mL supernatant was taken to test LDH release rate. The killing activity of CTL cells against tumor cells was calculated with the following formula: Killing activity (%) = (sample release group A value / natural release group A value) / (the greatest release group A value - natural release group A value) ×100%.

Statistical Analysis

Student's t test was used to determine the difference among groups. P values of less than 0.05 were considered statistically significant.

Results

Confirmation of IL-18 PCR Product

PCR products of human IL-18 were cloned into TA cloning vector pUCmT. A 510 bp fragment of IL-18 PCR product was confirmed by agarose gel electrophoresis analysis. (Fig. 1).

Construction of Recombinant pcDNA3.1(+)/hTERT+IL-18, Restriction Enzyme Confirmation and DNA Sequence

After pUCmT-IL-18 and pcDNA3.1(+)/hTERT were digested by both Nhe□ and Hind□, a 510 bp fragment of IL-18 was directly cloned into site of Nhe□ and Hind□, resulting in a recombinant plasmid pcDNA 3.1(+)/hTERT+IL-18. pcDNA3.1(+)/hTERT+IL-18 was digested by Nhe□/Not□, Nhe□/Hind□, EcoR□/Not□. The analysis of the products on agarose gel (Fig. 2) showed that the recombinant plasmid contained IL-18 and hTERT gene. The DNA sequence test confirmed there was a correct open reading frame and two objective gene are consistent with that of hTERT and IL-18 published in the *Gene Bank of Pub Med* (the coincident rate of hIL-18 and hTERT were 99% and 97 respectively)(part sequence show in Fig. 3).

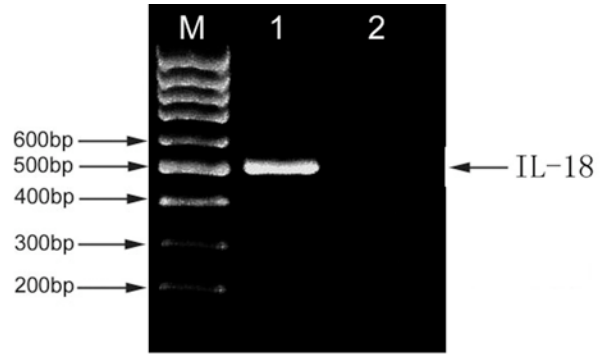

Fig. 1. Agarose gel electrophoresis analysis. Lane 1: PCR products of hIL-18. Lane 2: a negative control; M: DNA Marker.

Expression of hTERT and IL-18 Protein Detected by Western Blot and Immunofluorescence

The results of Western blot analysis showed fusion protein expression in cells transfected with pcDNA3.1(+)/hTERT+IL18 plasmid. After probed with antibodies against hTERT and IL-18 respectively, the molecular weight of objective band are all 127 KD (the IL-18 protein is about 17KD and the hTERT protein is about 110 KD) (Fig. 4). To investigate expression pattern of hTERT and IL-18 in 3T3 cells, immunofluorescence analysis was performed on 3T3 cells using mouse anti- hTERT polyclonal serum or anti-IL-18 monoclonal antibodies respectively as previously described. The result revealed that more than 40% of the fusion gene-transfected cells stained green in the nuclear (incubated with primary antibody hTERT) (Fig. 5). The same percentage of stained cells was also detected when incubated with anti-IL-18 antibody, which diffusely distributed in the cytoplasm (figure not shown). In contrast, none of 3T3 cells without transfected fusion gene were stained green.

The Biological Function of hTERT+IL18 Fusion Protein was Tested using ELISA and FCM

As shown in Figure 6, 3T3 cells transfected with

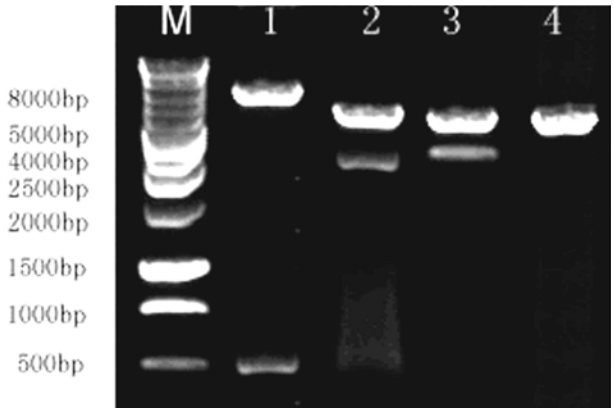

Fig. 2. Agarose gel electrophoresis analysis of recombinant pcDNA3.1(+)/ hTERT+IL-18. lane M: DNA Marker; lane 1: pcDNA3.1(+)/ hTERT+IL-18 after digestion with NheI and HindIII (IL-18 cDNA 510bp); lane 2: after digestion with EcoRI and NotI (hTERT cDNA 3400bp); lane 3: after digestion with NheI and NotI (IL-18+hTERT 3910 bp); lane4: pcDNA3.1(+) plasmid (5428bp).

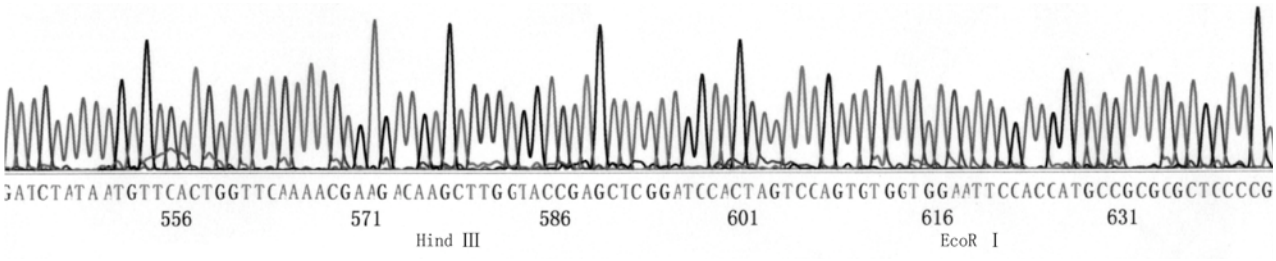

Fig. 3. The nucleotide sequence of the linker region in hTERT/hIL-18 gene tested by DNA sequence analysis. Site of enzyme digestion HindIII : AAGCTT; EcoRI: GAATTC.

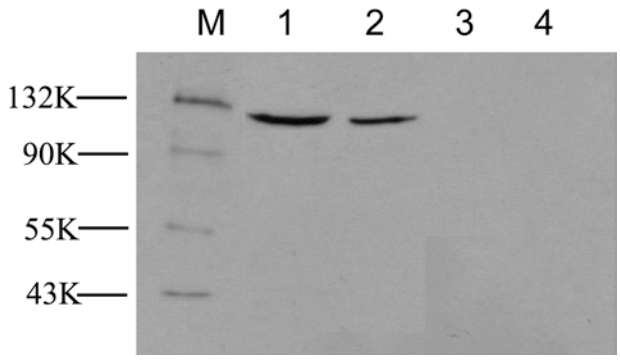

Fig. 4. Western blotting of analysis of expressed pcDNA3.1(+)/hTERT+IL-18 products with IL-18 antibody. Lane M: protein marker; Lane 1, 2: 3T3 cells transfected by pcDNA3.1(+)/hTERT+IL-18. The molecular weight of objective band are all 127 KD (the IL-18 protein is about 17KD and the hTERT protein is about 110 KD); Lane 3, 4: 3T3 cells blank control.

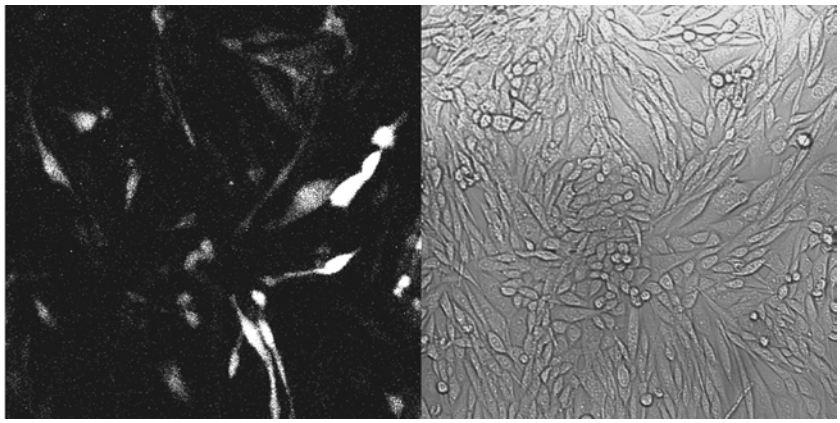

Fig. 5. Expression of hTERT in 3T3 cells transfected hTERT/hIL-18 fusion gene. Expression were determined by immunofluorescence. Left: 40% positive cells stained in nuclear(green). Right: same contrast images(red).

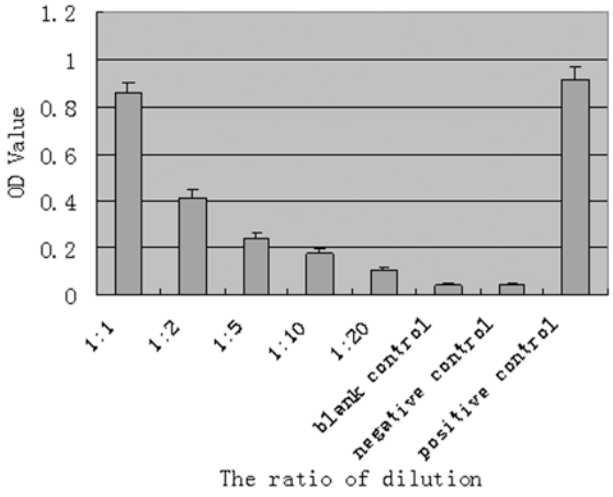

Fig .6. Bioactivity of IL-18. 3T3 cells transfected with hTERT+IL18 fusion gene stimulate KG-1 cells produced a significant amount of INF-γ protein in a concentration-dependent manner. Supernatants were harvested after 18 hours, and levels of interferon-_ were assayed by ELISA. Supernatants from 3T3 cells without transfected as negative control group. Standard IL-18 stimulate aliquots was used as positive control group. Equal volume of PBS was used as blank control group.

hTERT+IL18 fusion gene stimulate KG-1 cells produced a significant amount of INF-γ protein in a concentration-dependent manner whereas neither 3T3 cells(negative control) nor PBS(blank control) produced any detectable amount of INF-γ as measured by the ELISA method. The level of INF-γ induced by highest concentration of supernatants is similar to that of standard sample.

We investigated whether expression of hTERT in 3T3 cells leads to anti-apoptosis. The MTX-induced apoptosis was examined by the Annexin V method. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, a characteristic feature of cells entering apoptosis. This allows for live cells to be discriminated from apoptotic cells. To check this, 3T3 cells with or without transfected fusion gene were treated for 24 hours with different concentrations of MTX(0 nm, 5 nm, 10 nm, 50 nm, 100nm) and then stained with Annexin V–FITC. Results show a dose-dependent increase in 3T3 cells without fusion gene stained with Annexin V(0.71%, 2.46%, 4.19%, 6.68%, 10.81%), indicating the onset of apoptosis in MTX-treated cells. However, the hTERT/ IL18 transfected cells significantly decreased the positive stain for Annexin V at the same dose of MTX (0.82%, 1.90%, 2.13%, 2.72%, 2.94%). (Figure not showen).

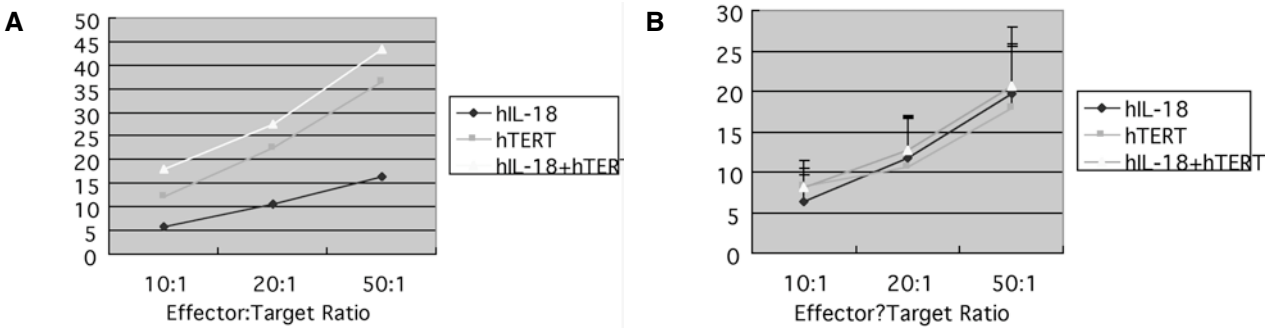

Fig. 7. K562 cells (with high hTERT expression) as targets to assess the extent of non-MHC restricted cytotoxity of CTL s were tested in cytotoxicity assays after stimulation with the DCs transfected with hTERT–IL-18, hTERT and IL-18 gene inducing hTERT-specific CTL response *in vitro* (7A). On the contrary, there were no difference in cytotoxity to target 3T3 cells (with low hTERT expression) among three DCs groups (7B).

Induction of hTERT-specific CTLs

DCs were transfected with pcDNA3.1(+)/hTERT+ IL-18 plasmid. PBMCs were processed as outlined in Materials and Methods, stimulated with hTERT-IL-18, hTERT and IL-18 positive DCs respectively. After the stimulation, testing of its toxicity against hTERT cell line K562 was performed with three randomly selected samples in a conventional LDH release assay. According to the effect/target ratios 10:1, 20:1 and 50:1, the killing rate of 18.1%, 27.6% and 43.3% respectively was detected on target cells K562 cells(with high hTERT expression), a specific release with hTERT-IL-18 positive DCs. hTERT–positive DCs inducing CTL response was 12.2%, 22.5%, 36.4% respectively. However very low cytotoxicity were observed in CTL stimulated with DCs transferred IL-18 gene (6.8%£ª10.6%£ª17.3%) (Fig. 7). As shown in Figure 7, Compared to hIL-18 transfected DCs, the level of CTL responses was increased significantly after induced by hTERT-IL-18 positive DCs and hTERT positive DCs. The datas show that hTERT-IL-18 positive DCs induce higher CTL response than that of hTERT positive DCs, however, there were no statistical difference between the two group. For further study, we select the 3T3 cells(with low hTERT expression) as target cell for CTL toxicity. The figure shows there was no difference in terms of CTL stimulation in hTERT-IL-18, hTERT , hIL-18 positive DCs groups (Fig. 7).

Discussion

In all proliferate somatic tissues, including stem cells of renewal tissues, telomerase are shorter compared to younger individuals. The idea, that telomerase was a aiming or a clocking mechanism, was now well recognized. Telomerase is up-regulated or re-activated as part of cancer progression [8]. The human telomerase RNA (hTR), together with thehuman telomerase reverse transscriptase, constitute the core components of telomerase that is essential for telomere maintenance. The telomerase has a independent effect on tumor inducing [9]. However, it is generally thought that human telomerase reverse transcriptase (hTERT) is the limiting component of the telomerase holoenzyme and that it is expressed in most cancer cells, resulting in the activation or up regulation of telomerase [10].

Therefore, the polypeptide component of telomerase (TERT) is an attractive candidate for a broadly expressed tumor rejection antigen because telomerase is silent in normal tissues but is reactivated in more than 85% of cancers [2]. The immunological properties of the telomerase reverse transcriptase hTERT suggest that the enzyme is also an attractive target for novel immunotherapies against cancer. Given the vast overexpression of hTERT in human tumors and its low-level expression in rare normal tissues, clinical trials have begun that test the credentials of hTERT as a broadly applicable target for immunotherapy of cancer [11]. Several Phase I studies of hTERT immunotherapy have been completed in patients with breast, prostate, lung and other cancers, and clinical and immunological results are encouraging. Immunotherapy induces functional, antitumour T cells in patients in the absence of clinical toxicity. The opportunity for vaccinating individuals as an immunoprevention strategy can also be envisioned for hTERT-based therapies [12]. Nair's [13] study shows that immunization against TERT induces immunity against tumors of unrelated origin. In recent years, data from both human and murine systems demonstrate that cytotoxic T-lymphocytes (CTL) can recognize peptides derived from TERT and kill TERT-positive tumor cells of multiple histologies [1].

However, high levels of tumor specific tolerance in some patients may account for a significant barrier to therapeutic vaccination. Therefore, to enhance the immune response against hTERT, human IL-18 gene was fused with hTERT for further studies as tumor vaccine.Interleukin-18 (IL-18) is a proinflammatory cytokine. This protein has a role in regulating immune responses and exhibits significant anti-tumor activities. IL-18 administration promotes innate immunity resulting in significant antitumor effects [14]. Dendritic cells (DCs) are potent antigen-presenting cells that can prime and boost systemic antitumor immunity. DCs transfected with IL-18 can secrete the potent Th1-biasing cytokines and to promote antitumor immunity in a mouse sarcoma model [15]. These results support

the IL-18 gene can enhance the capacity of DCs to drive strongly reactive Th1-type therapeutic immunity.

From these immuny properties, Dendritic cell (DC)-tumor fusion hybrid vaccines that facilitate antigen presentation represent a novel powerful strategy in cancer immunotherapy. Xia [16] investigated the antitumor immunity derived from the vaccination of fusion hybrids between engineered J558/IL-18 myeloma cells secreting Th1 cytokine IL-18 and DCs. In that case DC/J558/IL-18 could secret a higher level of IL-18 than DCs, DCs loaded with total tumor lysate and IL-18 may represent a method for inducing Th1 immunoresponses against the entire repertoire of glioma antigens [17]. Similar phenomenon were found in some studies, IL-18/gp100 were used as vaccine to induce the protective and therapeutic immunity in a B16 melanoma model. IL-18/gp100-DC immunization led to the generation of potent therapeutic immunity that significantly inhibited the tumor growth and improved the survival period of mice bearing established melanoma [18].

These results demonstrate that the engineered fusion hybrid vaccines that combine Th1 gene-modified tumor with DCs may be an attractive strategy for cancer immunotherapy .In this study, we constructed a recombinant human telomerase reverse transcriptase (hTERT) –IL18 vector expressing hTERT and IL-18 protein respectively. Firstly, the complete IL18 gene fragment was amplified from healthy human monocytes; subsequently, purified IL-18 was cloned to eukaryotic expression vector PCDNA 3.1(+)/hTERT. Both the restrictive enzyme digestion and DNA sequence confirmed the successful construction of recombinant plasmid PCDNA3.1(+)/hTERT+IL18. Recombinant fusion gene was successfully transfected into 3T3 cells. The expression of the tumor associated antigen (hTERT) and INF-γ inducing factor (IL-18) are very important for a vaccine; we assessed the protein expression of the recombinant plasmid *in vitro*. It is confirmed by immunofluorescence and western-blotting methods, which reveals the high expression of the recombinant plasmid in the 3T3 cells.

Most importantly, the purified recombinant fusion protein induced similar levels of INF-γ to that of native IL-18 protein [19]. FCM findings show that fusion protein suppresses apoptosis consistent with TERT-universal tumor associated antigen [20]. The results show that the gene-fusion expresses the biological activity of IL-18 and tumor antigen respectively. There are a few published studies describing tumor associated antigen and IL-18 were co-transfected into cells [21], however, there was no paper describing the effect of IL-18 and hTERT genes transfected indendritic cells. In present study, we constructed fusion protein in one eukaryotic expression plasmid as described in the Lu's report [22]. Firstly, it could improve the efficiency of co-transfection. Secondly, hTERT was used as a- broadly applicable target for immunotherapy of cancer. Thirdly, two fusion protein possessed respective biological function and did not disturb each other. Therefore, it provides a research platform for the study of therapeutic vaccine of tumor.

Furthermore, we test the fusion pretein have the capability of eliciting a hTERT-specific CTL response or not. We select K562 cells with high TERT expression as target tumor cells23. The hTERT-positive and hTERT –IL18 positive DCs were in turn used to stimulate T cells and their CTL effectiveness of toxicity was compared with that of IL-18 positive DCs. On the contrary, there was no distinct lysed effection to low-expression 3T3 cells. The experiments show that electrotransfection can be used for antigen loading of DCs and induction of CTL responses. These results suggest that hTERT may serve as a general cancer antigen in humans and initiate the immuneresponse to tumor cells. The results also confirm previous experiments demonstrating that IL-18 enhance the specific anti-tumor cytotoxic T-cell response *in vitro* [24]. However, there have no statistical differences, which maybe result from non-MHC restricted cytotoxity. Therefore, it should be investigated further. Together these results indicate that immunization with DC vaccine co-expressing Th1 cytokine IL-18 and hTERT may be an effective strategy for a successful therapeutic vaccination.

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