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EXPERIMENTAL STUDIES ON RADIATION-INDUCIBLE HUMAN TNF GENE THERAPY FOR CANCER

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Tumor necrosis factor (TNF) has certain radioprotective effect on host tissue and is capable of enhancing the antitumor effect of radiotherapy. In addition, the transcriptional regulation of the promoter region of Egr-1 gene is activated by ionizing radiation. So we fused Egr-1 promoter with hTNF-α cDNA, and resultantly constructed a double-copy and radiationinducible retroviral vector named as pETDC. After packaged with Psi-2 and Crip cells *in vitro*, the hTNF recombinant retroviruses were in the titers of 4×10^5 CFU/ml. By infection of murine fibroblast cell line NIH3T3 and murine melanoma cell line B16.F10 with the recombinant retroviruses and followed by G418 resistant selection, two positive clones secreting TNF at the levels of 2.1 ng/ml and 1.1 ng/ml respectively were generated. After exposure to 20 Gy ionizing radiation, TNF secretions from the two positive clones were elevated to 13.8 ng/ml (6.6-fold) and 5.7 ng/ml (5.2-fold) respectively. Furthermore, hTNF-α expression in pETDC-transfected cells was confirmed by RT-PCR. These data provide an experimental bases for the application of TNF gene therapy combined with local radiotherapy in cancer patients.

Key words: Tumor necrosis factor, Retrovirus, Gene therapy, Radiotherapy, Melanoma.

Presently, most of tumor patients need to receive radiotherapy, but the lethal radiation dosage for tumor cells usually surpass the tolerance dosage of normal tissues greatly. So it's imperative to seek the effective approach to decrease radiation dosage while improving the radiotherapeutic effects. Recently, tumor necrosis factor (TNF) was found to be potential in cancer radiotherapy.[1] In addition, TNF gene therapy is one of the promising approaches to cancer treatment. In order to investigate radiation-inducible TNF gene therapy to exhibit the synergistic antitumor effects of TNF and radiation, we placed the radiationinducible promoter of early growth response gene 1 (Egr-1) into the upstream of human TNF cDNA, and constructed the double-copy retrovirus vector of human TNF, whose expression is greatly enhanced by ionizing radiation.

MATERIALS AND METHODS

Reagents

The plasmid pE425.TNF carrying fused gene of Egr-1 promoter and human TNF cDNA and the double-copy retrovirus vector pN2A were kindly provided by Dr. Thomas Blankenstein (MDC Molecular Medicine Center, Germany). Lipofectin and G418 were purchased from Gibco, polybrene from Sigma, Taq DNA polymerase from Perkin-Elmer, reverse transcription kit from Gibco, human TNF specific primers from Clontech. Mouse β-actin primers were

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synthesized in Shanghai Institute of Cell Biology. Recombinant human TNF-α was from Genzyme Co. with the specific activity of 2×10^7 U/mg.

Mouse fibroblast cell line NIH3T3 and L929, mouse melanoma cell line B16.F10, and the retrovirus packaging cell lines Psi-2 and Crip were cultured in our laboratory.

Construction of pETDC Vector

The constructing procedure is demonstrated as Figure 1. pE425.TNF plasmid was digested with HindIII, and the resultant Egr-1-TNF fusion gene in size of 1.4 kb was recovered with low melting agarose gel, purified, and blunted with Klenow DNA polymerase. pN2A vector was cut with BglII enzyme, blunted with Klenow DNA polymerase, dephosphated with CIAP, and finally ligated with the insert of Egr-1-TNF fusion gene. Subsequently, the ligation react was transformed into HB101 competent cells, and the resultant positive clones were screened and confirmed with appropriate restrictive digestions. The recombinant retrovirus vector with the rightward insertion was named as pETDC.

***In Vitro* Package and Titeration of Recombinant Retroviruses**

pETDC retrovirus vector was transfected into Psi-2 cells with lipofectin, following the instructions of the manufacturer to perform the first-round package. After resistant screening with G418, limiting dilution and titeration, the resultant retrovirus supernatants were utilized to infect Crip cells, and the resultant clone with the highest virus titers was termed as Crip-ET. The control vector pN2A was also packaged as the same procedure. The titeration of retroviruses was performed according to a standard protocol, using NIH3T3 cells as target cells.

Establishment of pETDC-transfected Cells

NIH3T3 and B16.F10 cells were infected with freshly-prepared Crip.ET retrovirus supernatants in the presence of polybrene (0.8 μg/ml), followed by G418 resistant screening (400 μg/ml and 1000 μg/ml respectively for NIH3T3 and B16.F10), limiting dilution and determination of TNF levels in the culture supernatants. The resultant positive clones were named as 3T3.ET and F10.ET. The control cell clones of 3T3.N2A and F10.N2A were also established by infection with control retroviruses.

Determination of Human TNF

TNF was determined according its cytotoxic activity on L929 cells in presence of Actomycin D, and standardized according to standard hTNF-α.

TNF Expression after Exposure the Cells to Radiation

The cells growing logarithmically were digested with trypsin, washed 3 times with HBSS, and resuspended in RPMI-1640 at 1×10^5/ml. After exposure to ionizing radiation (20 Gy), the cells were incubated in 5% CO_2 at 37 ℃ for 24 h, and the culture supernatants were harvested, sterilized and restored at -20 ℃, while the cells were also collected for RT-PCR analysis.

RT-PCR Analysis for hTNF Expression

Total cellular RNA was extracted with guanidine thiocyanate, digested with RNase-free DNase I to degrade the potentially contaminated DNA templates, and determined for the concentrations. Reverse transcription was performed following the manufacture's instruc-tion. 5 μg total RNA was mixed with 1 μl (0.5 μg) oligo-dT and dNTP, incubated at 70 ℃ for 10 min, subsequently added Superscript-II reverse transcriptase 200 U, and incubated at 42 ℃ for 50 min. After terminated at 70 ℃ for 10 min, the transcripts were digested with 1 μl RNaseH to degrade RNA templates. 2 μl reverse transcripts or total RNA were used as templates for amplifying mouse β-actin (internal control) and human TNF. The upstream primer for mouse β-actin is 5'GACCTGAC AGACTACCTCA-T3', and the downstream primer 5'CTCATCGTACTCCTGCTTGCTGATCC3', with the amplified product of 555 bp. The upstream primer for human TNF is 5'TGAGCACTGAAAG-CATGATCCGG3' and the downstream primer 5'CAATGATCCCAAAGTAGACCTGCCC3', with the expected product in size of 695 bp. PCR was performed in the volume of 50 μl containing 30 pmol upstream and downstream primers each, 1.5 mM $MgCl_2$, and 2 U Taq DNA polymerase. The parameters of PCR amplification were 96 ℃ 1 min, 55 ℃ 1 min, 72 ℃ 2 min. The reverse transcripts added in

PCR reaction were balanced according to β-actin yields. After 20 cycles of amplification, the PCR products were subjected to electrophoresis on 1.5% agarose gel.

RESULTS

Construction and *In Vitro* Package of pETDC

As demonstrated in Figure 1, the fused gene of Egr-1-hTNF was inserted into the U3 region of 3'LTR of pN2A vector to create the double-copy retrovirus vector expressing hTNF under the control of Egr-1 promoter. The resultant vector pETDC was packaged *in vitro* with ecotropic packaging cell line Psi-2 at first, yielding the recombinant retroviruses with the titers of 5×10^3 CFU/ml. After second-round package with the amphotrophic packaging cell line Crip, the hTNF recombinant retroviruses were generated with higher titers of 4×10^5 CFU/ml, indicating cross-infection with ecotropic and ampho-tropic packaging cells was capable of improving the titers of packaged retroviruses. After two rounds of package for pN2A vector, the control retroviruses were generated with the titers of 8×10^4 CFU/ml.

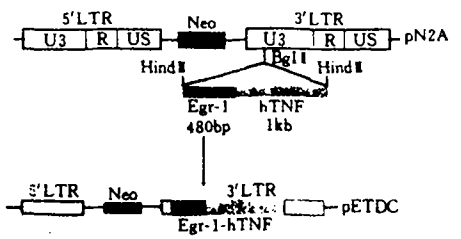
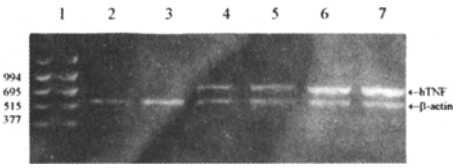


Fig. 1. Skematic demonstration for the construction of pETDC recombinant retrovirus vector.

Gene Expression of the Transfected Cell Lines

After infection with human TNF recombinant retroviruses and G418 resistant screening, the positive cell clones derived from NIH3T3 cells (3T3.ET) and B16.F10 cells (F10.ET) secreted human TNF at the levels of 2.1 ng/ml and 1.1 ng/ml respectively. Whereas, there was no detectable TNF in the culture supernatants from parental NIH3T3 cells and B16.F10 cells and that infected with the mock control. Furthermore, the specific fragment in size of about 700 bp was amplified from 3T3.ET and.F10.ET cells by RT-PCR using hTNF specific primers, which confirmed the expression of hTNF in above cell clones, while no such specific fragment detectable from the control cells after PCR amplification (Figure 2).



Lane 1. DNA marker; Lane 2. 3T3.N2A;
Lane 3. F10.N2A; Lane 4. 3T3.ET (0 Gy);
Lane 5. 3T3.ET (20 Gy); Lane 6. F10.ET (0 Gy);
Lane 7. F10.ET (20 Gy)

Fig. 2. RT-PCR analysis for TNF expression of pETDC-transfected cells before and after ionizing radiation.

Enhanced TNF Expression of the Cells after Exposure to Radiation

The parental and control retrovirus-infected NIH3T3 and B16.F10 cells didn't secret any detectable TNF even after radiation. After exposure to ionizing radiation (20 Gy), 3T3.ET and F10.ET cells secreted 13.8 ng/ml and 5.7 ng/ml of hTNF respectively, which were 6.6 and 5.2 times than that before radiation (Figure 3). RT-PCR analysis indicate that exposure to ionizing radiation might improve the transcription of hTNF (Figure 2).

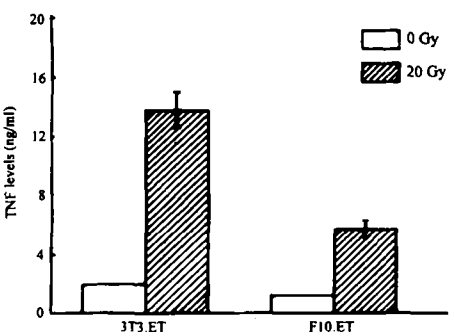


Fig. 3. Comparison for TNF production of pETDC-transfected cells before and after ionizing radiation.

DISCUSSION

It has been observed that the cellular exposure to ionizing radiation results in transcriptional activation of certain genes encoding transcription factors, including NF-κB, Egr-1 etc.[2] X-ray radiation was documented to be capable of inducing Egr-1 transcription via the active motif of CCA(A/T)4AGG(CArG) in the Egr-1 promoter.[3] TNF has significant antitumor activity, and recent researches showed that TNF was capable of enhancing the tumoricidal activity of ionizing radiation *in vitro* and *in vivo*,[4] exhibiting radiosensitizing effect on tumor cells and radioprotective effect on normal cells.[5] To enhance tumor cures by radiotherapy and circumvent the problem of systemic toxicity associated with TNF, Egr-1 promoter was placed at the upstream to TNF gene to construct the radiation-inducible expression vector whose expression could be enhanced by radiation. It has been reported that the human leukemia cells HL-60 transfected with Egr-1-TNF recombinant vector secreted 3-fold TNF after X-ray radiation.[6]

The cytokine gene therapy of cancer is hot in the field of cancer gene therapy.[1] Up to now, there are three kinds of strategies of gene transfer. The first is to transfer the cytokine gene into the immunocompetent cells, including tumor infiltrating lymphocytes (TIL) and cytotoxic T lymphocytes (CTL). After adoptive transfusion, the effector cells could preferentially gather in tumor tissues and kill tumor cells more effectively, but this is limited by low efficiency of gene transfection. The second is to transfer cytokine gene into tumor cells to prepare novel tumor vaccine, which could be used to immunize host to activate antitumor responses. But, the problems are the limited therapeutic effects and there are difficulties in obtaining, cultivating and propagating primary tumor cells *in vitro*. The third one is to introduce the cytokine genes into recipient cells such as fibroblasts, which can easily be available and genetically altered. After implanted into host, the genetically modified recipient cells express constitutively target cytokine *in situ*, capable of augmenting antitumor activity. Our data have demonstrate the fibroblast-mediated IL-2, IL-4, IL-6 and interferon- gene therapy could elicit effective antitumor immunity,[7-10] but their targeting capacity was poor. So, we propose to implant directly the recipient cells carrying certain cytokine gene or their expression vectors into tumor tissue to activate the local antitumor responses directly. In this work, we constructed the radiation-inducible double-copy retroviral vector of human TNF (pETDC), and demonstrated the pETDC-transfected NIH3T3 and B16.F10 cell clones secreted 5.6-fold and 4.2-fold TNF respectively after ionizing radiation. This may be an effective strategy for cancer treatment.

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