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Full Length Research Paper

Effects of deoxycycline induced lentivirus encoding FasL gene on apoptosis of Th1 cells

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Fas/Fas ligand (FasL)-mediated apoptosis plays a critical role in deletion of activated T cells. This study aimed to construct the lentivirus encoding FasL gene induced by deoxycycline and evaluate its effects on apoptosis of Th1 cells. A plasmid expression system encoding FasL was constructed through utilizing the controlling gene and target gene of the Tet-on system controlled by the PBI plasmid containing the bidirectional promoter. The lentiviral vector that consisted of the vector plasmid, the packaging plasmid and the envelop plasmid was isolated and purified. 293 T cells were cotransfected with three plasmids using Lipofectamine 2000. 48 h post transfection, the viral supernatant was collected to determine the virus titre. The FasL protein expression in 293T cells was detected by Western blotting. Spleen lymphocytes of rats were transfected with the lentiviral vector system encoding FasL gene and 5 days later were induced by doxycycline for 24 h, followed by detection of FasL mRNA. The apoptosis index of Th1 cells was measured through both Annexin V-FITC flow cytometry and TUNEL. Additionally flow cytometry was adopted to determine changes of the quantity of Th1 cells. The FasL protein was expressed in 293T cells transfected by the expressing FasL lentiviral vector following deoxycycline induction, while it was not expressed in 293T cells without deoxycycline induction. The virus titre was 4×10^8 u/l for 293T cells. The deoxycycline induced lentiviral vector system encoding FasL gene was successfully transfected into spleen lymphocytes of rats. The FasL RNA expression significantly increased in the deoxycycline plus lentivirus group, compared to that in the control group and the deoxycycline group. A higher apoptosis index and an obviously smaller quantity of Th1 cells were obtained in the deoxycycline plus lentivirus group than in the other two groups. The lentivirus system encoding FasL induced by deoxycycline promotes apoptosis of Th1 cells.

Key words: FasL, lentiviral vector, Th1 cell, apoptosis.

INTRODUCTION

Disequilibrium of Th1/Th2 cells, caused by abnormal increase of Th1 cells, is considered to be relevant to several autoimmune diseases, such as rheumatoid arthritis (RA), multiple sclerosis (MS) and type 1 diabetes mellitus (DM). Th1 cells are involved in onset and severity of organ-specific autoimmune diseases. One of the popular research fields is to reduce abnormal increase of Th1 cells so as to maintain the balance of Th1/Th2 cells. This study aimed to evaluate whether it was feasible to treat relevant diseases through deoxycycline induced apoptosis of Th1 cells using the lentiviral system encoding FasL gene.

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MATERIALS AND METHODS

Materials

The RNA extraction kit, reverse transcriptase-polymerase chain reaction (RT-PCR) kit, plasmid extraction kit, DNA purification kit and enzymes were purchased from Promega (Fitchburg, Wisconsin, USA). Deoxycycline was bought from Sigma (St. Louis,

Missouri, USA).The PBI –rtTA vector was obtained from Clontech (Mountain View, California, USA). The lentiviral system (transfer plasmid, pWPLXd; packaging plasmid, pMD2G; envelope protein plasmid, psPAX2) was kindly provided by Dr. Tian Xue-bi (Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology). Lewis rats were purchased from Vital River (Beijing, China). RPMI1640 medium and fetal bovine serum was purchased from Invitrogen (Carlsbad, California, USA). The TUNEL kit and the Annexin V-FITC apoptosis kit were purchased from Jingmei Bio Co.(Shenzhen, Guangdong, China). Tim-3 and the flow cytometer were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA) and BD (Franklin Lakes, New Jersey, USA), respectively

Design of primers and extraction of total rat mRNA

Pair of primers was designed regarding the rat FasL cDNA sequences and requirements for the pBI-rtTA vector: upper stream 5'–CAGGGTGGGTCTACTTGC - 3' and downstream 5' – GGTGG GCTCAGAAAACAT - 3'. After introduction of BamH I and EcoRI restriction sites, the primers were synthesized by Biodoor Co. (Shanghai, China). Fresh spleen tissues were obtained from Sprague Dawley (SD) rats. Total mRNA was extracted according to instructions for the RNA extraction kit from Promega

Amplification by RT-PCR

The RT-PCR reaction system was established in accordance to instructions for the RT-PCR kit from Promega. The total volume 100 µl included 6 µl template RNA, 2 µl dNTP, 10 µl upper and downstream primers respectively, 1 µl Avian Myeloblastosis Virus (AMV) reverse transcriptase and 2 µl DNA polymerase. PCR procedure consisted of 33 cycles of reverse transcription at 48°C for 45 min, initial denaturation at 95°C for 2 min, denaturation at 94°C for 1 min, annealing at 57 °C for 30 s, and extension at 68.5°C for 2 min and additional extension at 68.5°C for 7min.

Construction of pBI-rtTA/FasL plasmids

PCR amplification products were digested by restriction enzymes Pst I and Not I. The DNA straps of interest were reclaimed through low melting-point agarose gel electrophoresis (AGE), purified through the DNA purification kit, and finally identified through AGE. Subsequently, pBI-rtTA and FasL were digested by restriction enzymes Pst I and Not I, and linked together by T4 ligase. Products from the mentioned reactions (pBI-rtTA/FasL) were delivered to Biodoor Co. for DNA sequencing purposes.

Construction of transfer plasmids for the lentiviral vector

Restriction enzymes BamH I and EcoR I used to digest the lentiviral vectors pWPLXd and pBI-rtTA/FasL. Digestion products were linked together and transferred into JM101 competent cells. Recombinant clones were selected by ampicillin resistance screening, cultured, and extracted by the Plasmid DNA Midi Purification Kit.

Construction of the lentiviral vector

293T cells in good condition were selected and went through passage until 80% cell confluence was achieved. The medium was removed and cells were rinsed with Dulbecco's modified Eagle's medium (DMEM). 20 ul liposome lipofeetamine 2000, 20 µg transfer plasmid pWPLXd, 16 µg packaging plasmid pMD2G and 5 µg envelope protein plasmid psPAX2 were added into the 500 µl DMEM medium. Five minutes (5 min) later, liposome and the lentiviral system were mixed uniformly. Following 20 min standing, the mixture was moved to the culture flask containing 293T cells. All media were discarded and changed to new after 8 h. Viral supernatant was collected to determine the virus titer 72 later.

Transfection of the Lentiviral vector into 293T cells

293 T cells in good condition were selected and categorized into three groups: the Doxplus lentivirus group, the lentivirus group and the control group. One milliliter (1 ml) lentiviral vector and 4 µl polybrain (promoting cell transfection) were respectively put together with 293 T cells in the former two groups without addition of medium until 70% cell confluence was achieved. The cells were not treated in the control group. DMEM medium containing 10% fetal bovine serum was added in the three groups 2 h later. After five-day cell culturing, deoxycycline (final concentration, 1 µg/ml) and an equal dose of medium were added in the Dox plus lentivirus group and the lentivirus group, respectively.

Detection of FasL protein expression by Western blotting

Cells were collected from the three groups 24 h later. After a series of treatment including centrifugation, homogenation, ultrasonic cell disintegration and protein denaturation, they underwent the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (100 to 200 V and steady flow 130 mA). Samples were transferred to the NC filter through the semidrying process until the blue strap vanished. The NC filter was blocked 60 min later and slowly shaken for 4 h on the shaking bed. Rat FasL monoclonal antibody was added after the samples were rinsed, the samples were slowly shaken again for 40 min and stayed overnight at 4°C. All samples were added with AP-labeled goat anti-rabbit IgG, the next day, shaken slowly for 2 h and were rinsed for coloration treatment. The procedure was repeated three times for independent samples.

Treatment and grouping of lymphocytes

Spleen lymphocytes were obtained from Lewis rats in a germ-free environment for cell counting. The cells were cultured in a 6-well plate, (5×10^6 per well) and divided into three groups including the deoxycycline plus lentivirus group, the deoxycycline group and the control group. The supernant was discarded after centrifugation. Five milliliters (5 ml) lentivirus was added into the samples in the deoxycycline plus lentivirus group for 1 h incubation at 37°C. In addition, complete RPMI 1640 Medium and deoxycycline (final concentration, 1ug/ml) were added in the samples in the deoxycycline plus lentivirus group and the deoxycycline group 5 days later.

Determination of the apoptosis index using the Annexin V-FITC

Cells from all three groups were collected, respectively, rinsed by 4°C phosphate-buffered saline (PBS) twice and the total volume was adjusted to 400 µl finally. Then apoptosis index was determined by flow cytometer using Annexin V-FITC / PI.

Determination of the apoptosis index by TUNEL

Cells from all three groups were collected, respectively, after centrifugation and the supernatant was discarded. 50 µl PBS was added

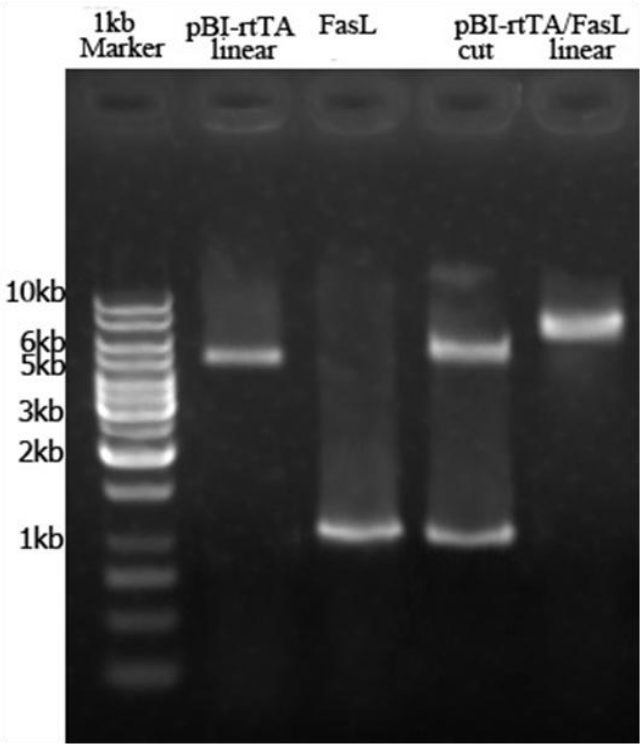

Figure 1. Identification of pBI-rtTA/FasL plasmid digested by enzymes NheI and EcoR V. A clear strap of about 870bp was consistent with the targeted gene.

on the smear, followed by fixation by 4% paraformaldehyde for 1 h and other steps according to instructions for the TUNEL kit. The cells with yellowish-brown granules in nucleus were defined as positive cells, that is, apoptotic cells. The apoptosis index was determined as the percentage of apoptotic cells per 1000 cells under the light microscope. The mentioned steps were performed repeatedly for five times.

Detection of FasL mRNA expression by RT-PCR

Total RNA was extracted from cells of three groups using Trizol. After reverse transcription into cDNA, FasL mRNA was obtained by PCR, with β–action as internal reference. These were the upper and downstream primers, respectively. The PCR procedure consisted of 32 cycles of initial denaturation at 94°C for 3 min, 94°C for 45 s and 72°C for 45 s, followed by extension at 72°C for 5 min. All PCR products were identified using 1% AGE.

Detection of Th1 cells

Cells from three groups were collected and incubated with the monoclonal antibody Tim-31 for 1 h and rinsed with PBS. After co-incubation with FITC-labeled goat anti-rabbit IgG, the cells were rinsed with PBS. The quantity was determined using flow cytometer.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and the ratio was tested using the Chi-square test. A statistical significance was considered if P was <0.05.

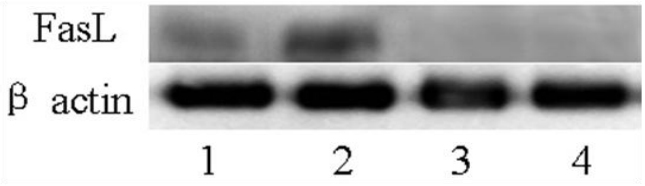

Figure 2. Expression of FasL protein in 293T cells transfected with deoxycyclin--induced lentiviral vector pBI-rtTA/FasL. Western blotting exhibited a significant increase of FasL protein in 293T cells transfected with 1μg/ml deoxycycline-induced lentiviral vector. 1. Transfection with pBI-rtTA/FasL; 2. transfection with pBI-rtTA/FasL plus induction with 1 μg/ml deoxycycline; 3. transfection with pBI-rtTA; 4. transfection with lipofectine.

RESULTS

Construction of pBI-rtTA/FasL plasmids

Results of AGE following digestion of enzymes, NheI and EcoR V, showed a clear strap of about 870 bp consistent with the targeted gene, indicating successful establishment of the lentiviral system (Figure 1).

DNA sequencing

DNA sequencing results of FasL gene following enzyme digestion, reclamation and purification agreed with the sequence from the GenBank (U03470).

FasL protein expression after transfection with pBI-rtTA/FasL

The quantity of FasL protein in the group transfected by 1 μg/ml deoxycycline-induced lentiviral vector (42.8 ± 2.6) is 2.2 times as much as that in the control group (20.3 ± 1.4). Results of Western blotting exhibited a significant increase of FasL protein in 293T cells transfected with 1μg/ml deoxycycline-induced lentiviral vector (Figure 2).

FasL protein expression after transfection with pWPL/FasL

The quantity of FasL protein in the group transfected by 1 μg/ml deoxycycline-induced lentivirus (46.3 ± 4.6) is 2.8 times as much as that in the control group (16.7 ± 1.4). The result showed a significant increase of FasL protein in 293T cells transfected with 1 μg/ml deoxycycline-induced lentivirus (Figure 3).

Effect of lentivirus transfection on FasL mRNA

Analysis of GE images of RT-PCR products revealed that

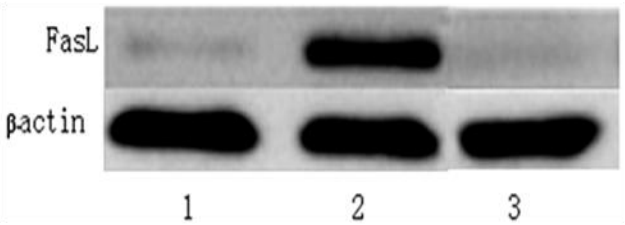

Figure 3. Expression of FasL protein in 293T cells transfected by deoxycyclin-induced lentiviral plasmid pWPL/FasL. The result showed a significant increase of FasL protein in 293T cells transfected with1 μg/ml deoxycycline-induced lentivirus. 1. Transfection with pWPL /FasL plasmid; 2. transfection with pWPL /FasL plasmid plus induction with 1 μg/ml deoxycycline; 3. no transfedtion with pWPL /FasL.

β–action and FasL were respectively 186 and 400 bp. The gray scale ratios of the two were 0.42 ± 0.11 in the control group, 0.63 ± 0.16 in the deoxycycline group and 1.17 ± 0.15 in the deoxycycline plus lentivirus group (n = 5). Results exhibited that FasL mRNA expression increased significantly in the deoxycycline plus lentivirus group compared with that in the other two groups (Figure 4) ($P < 0.05$).

Apoptosis index of cells determined by flow cytometer

The apoptosis indexes determined by flow cytometer were (26.29 ± 3.18)%, (5.86 ± 1.47)% and (4.92 ± 2.05)%, respectively in cells from the three groups (Figure 5). Results demonstrated that the apoptosis index of cells from the deoxycycline plus lentivirus group was significantly higher than that from the other two groups ($P < 0.05$), indicating that apoptosis of lymphocytes is promoted by deoxycycline-induced lentivirus transfection.

Apoptosis index of cells determined by TUNEL

The apoptosis indexes determined by TUNEL were (16.2 ± 2.1)%, (3.6 ± 1.4)% and (2.9 ± 1.5)%, respectively in cells from the control group, the deoxycycline plus lentivirus group and the deoxycycline group. Results demonstrated that the apoptosis index of cells from the deoxycycline plus lentivirus group was significantly higher than that from the other two groups ($P < 0.05$), suggesting that apoptosis of lymphocytes is promoted by deoxycycline-induced lentivirus transfection.

Alteration of Th1 cells quantities

Quantities of Th1 cells prior to treatment were, respectively (5.54 ± 0.47) × 10^5, (5.27 ± 0.53) × 10^5 and (5.83 ± 0.56) × 10^5 from the control group, the deoxycycline plus lentivirus group and the deoxycycline group. Quantities of Th1 cells post treatment were, respectively (5.36 ± 0.48) × 10^5, (4.90 ± 0.54) × 10^5 and (2.78 ± 0.62) × 10^5 from the control group, the deoxycycline plus lentivirus group and the deoxycycline group. Th1 cells were significantly fewer in the deoxycycline plus lentivirus group, compared with the other two groups ($P < 0.05$), indicating that Th1 cells may be reduced as a result of apoptosis promoted by Dox-induced lentivirus transfection (Figure 6).

DISCUSSION

FasL is a type-II membrane protein that belongs to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family that induces apoptosis by specific binding to and activating the Fas ligand (FasL), a transmembrane protein expressed in activated lymphocytes (Nagata and Golstein, 1995; Algeciras-Schimnich et al., 2002; Dockrell, 2003). The Fas/FasL system plays a pivotal role in the process of activation induced cell death (AICD) and in maintaining immune homeostasis. Abnormal cells can escape from immunologic surveillance due to apoptosis deficiency, as a result of down-regulated expression or malfunction of Fas/FasL system. This is considered as one of the main mechanisms underlying the pathogenesis of autoimmune diseases (Dong et al., 2002; Dominguez and Rodriguez, 2002; Scholz and Cinatl, 2005). Therefore, intervention of apoptosis of lymphocytes through the Fas/FasL system becomes a potential measure to prevent or even treat diseases of this type.

Apoptosis has an important role in cellular homeostasis. In the immune system, especially in lymphocytes, apoptosis functions to maintain the T cell repertoire and delete autoreactive lymphocytes, thus regulating the immune response (Osborne, 1996; Nagata, 1996). The conduction of apoptosis signals is enhanced by Fas/FasL interaction to induce T cell apoptosis. Cross-linking of surface monomeric Fas molecules by Fas ligand or agonistic anti-Fas antibody activates the apoptotic death programme *in vitro* and *in vivo*. The interaction between Fas and FasL or activation of anti-Fas antibodies results in the trimerization of Fas, followed by the assembly of other intracellular proteins to form the death-inducing signal complex (DISC). Caspase-8 recruitment to the DISC causes its proteolytic activation, which, in turn, activates other members of the caspase family, eventually ending in apoptosis (Peter et al., 1997).The recombinant lentiviral system is one of the most commonly used vector systems in scientific community focusing on gene therapy and other related fields (Lois et al., 2002). In addition, the tet-on system provides an alternative to regulate exogenous gene expression in the lentiviral system according to requirements of the host organism, indicative of validity and safety of gene therapy. On the basis of structural features of the tetracycline resistance operon of *Escherichia coli*, the resistance operon was

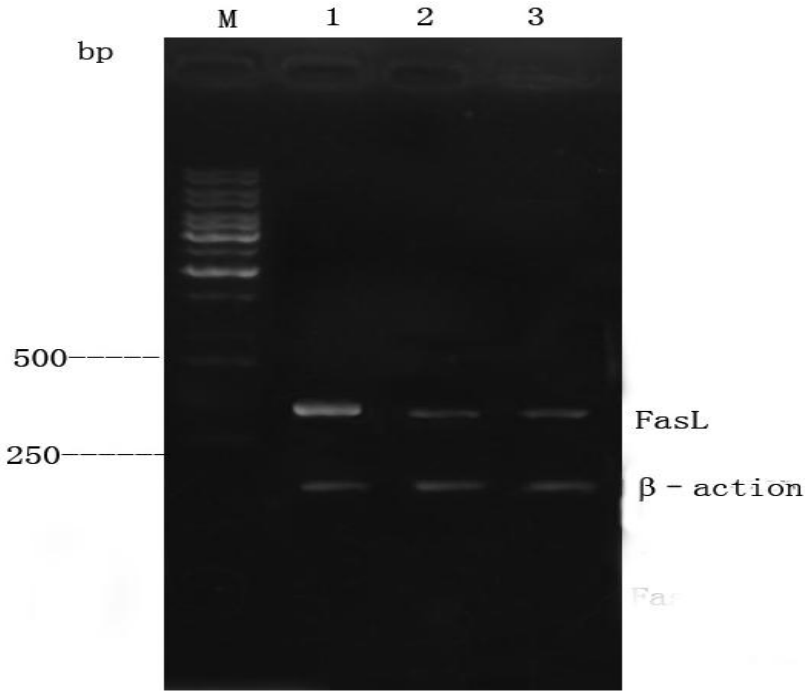

Figure 4. Expression of FasL mRNA. Analysis of GE images of RT-PCR products revealed that β–action and FasL were respectively, 186 and 400bp. The gray scale ratios of the two were 0.42 ± 0.11 in the control group, 0.63 ± 0.16 in the deoxycycline group and 1.17 ± 0.15 in the deoxycycline plus lentivirus group (n = 5). FasL mRNA expression increased significantly in the deoxycycline plus lentivirus group, compared with that in the other two groups ($P < 0.05$). 1. The deoxycycline plus lentivirus group; 2. the deoxycycline group; 3. the control group.

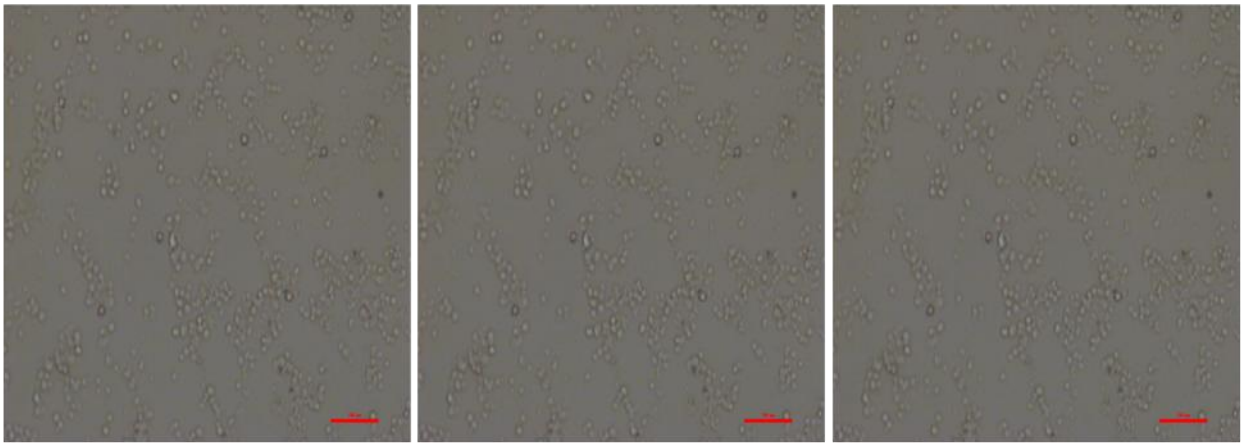

Figure 5. Apoptosis of lymphocytes determined by TUNEL. The apoptosis indexes determined by flow cytometer were (26.29 ± 3.18)%, (5.86 ± 1.47)% and (4.92 ± 2.05)%, respectively in cells from the three groups and the apoptosis index of cells from the deoxycycline plus lentivirus group was significantly higher than that from the deoxycycline group and the control group ($P < 0.05$). 1. The deoxycycline plus lentivirus group; 2. the deoxycycline group; 3. the control group.

composed of tetR, tetO and a specific binding site of DNA (Buchschacher and Weng-Staal, 2000; Hendriks et al., 2004; Ruitenberg et al., 2002). Tetracycline or deoxycycline can combine with tetR, change the formation of the latter and dissociate the combination of tetR and tetO and the expression of targeted genes can be controlled

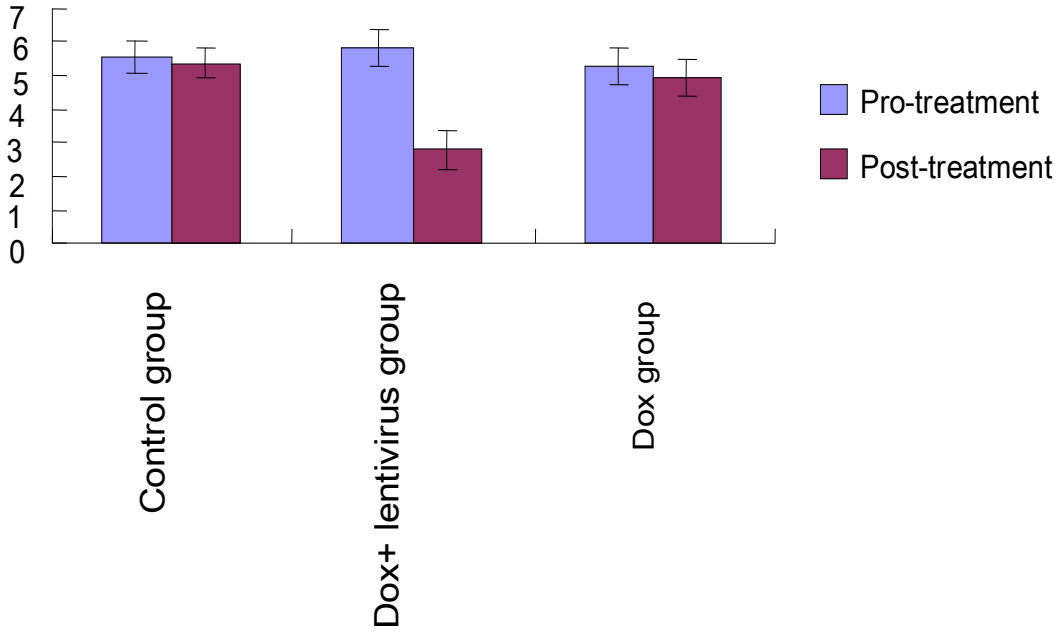

Figure 6. Quantity of Th1 cells determined by flow cytometer ($\times 10^5$). Quantities of Th1 cells prior to treatment were, respectively (5.54 ± 0.47) × 10^5, (5.27±0.53) × 10^5 and (5.83 ± 0.56) × 10^5 from the control group, the deoxycycline plus lentivirus group, and the deoxycycline group. Quantities of Th1 cells post treatment were respectively (5.36 ± 0.48) × 10^5, (4.90 ± 0.54) × 10^5, and (2.78 ± 0.62) × 10^5 from the control group, the deoxycycline plus lentivirus group and the deoxycycline group. Th1 cells were significantly fewer in the deoxycycline plus lentivirus group, compared with the other two groups ($P < 0.05$).

efficiently as a consequence (Markusic et al., 2005; Barde et al., 2006).

The results of the current research suggest that the deoxycycline-induced lentiviral system encoding FasL gene can be expressed in transfected lymphocytes under the regulation of deoxycycline. Due to the capability of transfection into cell strains hardly transfected by other vector systems such as lymphocytes and neurons, the lentiviral system can be probably used in gene intervention of apoptosis of related cell strains and affected positively on the prevention and treatment of autoimmune diseases (Tran and Kung, 2007; Ji et al., 2007; Yasuda et al., 2001).

Th1 cells are generally considered to be involved in cell immunity, delayed allergy, resistance against intracellular pathogens (including viruses and bacteria) and relevant to pathogenesis of organ specific autoimmune diseases (Cao et al., 2007; Sportoletti et al., 2006; Skapenko and Schulze-Koops, 2007). Th1 cytokines, such as IFN-γ, TNF-β, and IL-2, not only have direct effects on histocytes and vascular endothelial cells, but also promote inflammation cells, namely monocytes/macrophagus and neutrophilic leukocytes to secrete mediators of inflamemation (D'Acquisto et al., 2007; Onoé et al., 2007). In a word, Th1 cells are closely related to T cells mediated inflammation reactions. Additionally, Th1 cytokines with cytokines from other inflammation cells build up a complex network of cytokines regulating the development and prognosis of diseases on the basis of mutual influences (Fishman and Perelson, 1999).

In the current research, we demonstrate that the quantity of Th1 cells significantly decreases after transfection with the Dox-induced lentiviral system encoding FasL gene in lymphocytes. This finding underscores theoretically the possibility of gene therapy targeting at imbalance of Th1/Th1 cells due to abnormal increase of Th1 cells and provides a solid foundation for further studies as well.

Abbreviations

RA, Rheumatoid arthritis; **MS,** multiple sclerosis; **DM,** diabetes mellitus; **RT-PCR,** reverse transcriptase-polymerase chain reaction; **SD,** Sprague Dawley; **AGE,** agarose gel electrophoresis; **DMEM,** Dulbecco's modified Eagle's medium; **PBS,** phosphate-buffered saline; **TNF,** tumor necrosis factor; **NGF,** nerve growth factor; **FasL,** Fas ligand; **AICD,** activation induced cell death; **DISC,** death-inducing signal complex.

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