Article

CTLA4 Silencing with siRNA Promotes Deviation of Th1/Th2 in Chronic Hepatitis B Patients

Yongsheng Yu[1, 2, 3], Hao Wu[1, 2], Zhenghao Tang[1] and Guoqing Zang[1, 3]

To determine whether RNA interference (RNAi) could block cytotoxic T-lymphocyte antigen 4 (CTLA4) in human lymphocytes *in vitro* and promote IFN-γ and IL-2 secretions, three small interfering RNAs (siRNAs) were selected based on target specificity sequences of human CTLA4 and transfected into human lymphocytes of chronic HBV patients. As a result, the expression of human CTLA4 mRNA was efficiently suppressed by all the three siRNAs. Compared with negative control (siRNA-co), siRNA-1 inhibited the expression of CTLA4 most efficiently and was used in the further study. The expressions of IFN-γ and IL-2 were upregulated and the level of IL-4 was almost unchanged in lymphocytes transfected with siRNA-1 compared with the blank control. These results indicated that siRNA-1 led to IFN-γ and IL-2 secretions, which is a main response of Th1/Th2. In a conclusion, RNAi significantly suppressed the expression of human CTLA4 mRNA in human lymphocytes *in vitro*, and could induce Th1/Th2 response. It could be a new therapeutic strategy for chronic HBV infection. *Cellular & Molecular Immunology*. 2009;6(2):123-127.

Key Words: CTLA4, siRNA, lymphocyte, Th1, Th2

Introduction

Cytotoxic T-lymphocyte antigen 4 (CTLA4) molecule is a homolog of CD28. Both CTLA and CD28 along with their common ligands (B7-1:CD80 and B7-2:CD86) participate in the B7/CD28-CTLA4 co-stimulatory pathway for T cell activation. The CTLA4 ligands have an inhibitory role on T cell activation and may contribute to peripheral tolerance (1). Upon B7-1 or B7-2 engagement, CTLA4 expressed on the surface of activated T lymphocytes signals for cell-cycle arrest and attenuating the function of effectors (2, 3). Previous studies have demonstrated that blockage of signaling transduction between CD28 and CTLA4-Ig inhibited the response of T cells and prolonged the allograft survival in several rodent and organ transplantation models (4-7). On the other hand, it was found that the blockage of CTLA4 in previously vaccinated cancer patients increased T cell response and was associated with objective tumor regression (8-10), although in some cases it was accompanied by serious side effects, including hypophysitis and enterocolitis.

Synthetic small interfering RNA (siRNA) mediating RNA interference (RNAi) quickly emerged as a powerful method to reduce specific gene expression both *in vitro* and *in vivo* (11-15), and it has rapidly become a powerful tool to analyze the functions of gene in association with tumors, infectious diseases and genetic abnormalities (16). Up till now, there were only very limited and partly contradictory data available regarding the potential roles of CTLA4 on helper T cell (Th) responses. There were few studies about CTLA4 expression inhibited by RNAi-mediating silencing in primary human peripheral blood lymphocytes. In this study, we first tested the efficacy of RNAi against CTLA4 in human peripheral blood lymphocytes of chronic HBV patients. Secondly, we determined whether there was any difference in the Th responses after siRNA transfection. The responses were measured by enzyme-linked immunosorbent assay (ELISA) for interleukin (IL)-2, interferon-gamma (IFN-γ) and IL-4.

Materials and Methods

Study population

The data were collected between December 2007 and August 2008 at The Sixth People Hospital of Shanghai Jiaotong University. One hundred and twenty-one chronic hepatitis B patients were enrolled in this study, including 91 males and 30 females aged between 19 and 65 years (mean: 37.1; SD:

[1]Department of Infectious Diseases, Sixth People's Hospital, Shanghai Jiaotong University, Shanghai 200233, China;

[2]These authors contributed equally to this work;

[3]Correspondence to: Dr. Guoqing Zang, Department of Infectious Diseases, Sixth People's Hospital, Shanghai Jiaotong University, Shanghai 200233, China. E-mail: zangguoqin@126.com; or Dr. Yongsheng Yu, Department of Infectious Diseases, Sixth People's Hospital, Shanghai Jiaotong University, Shanghai 200233, China. E-mail: yuyongsheng@medmail.com.cn

Received Jan 12, 2008. Accepted Mar 30, 2009.

12.9). The selection criteria for patients were based on their carrying both HBsAg and anti-HBc antibodies for at least 6 months. A quantitative HBV DNA was performed for all the patients and those with a detectable viremia were submitted to a liver biopsy. Patients who were excluded from this study included the ones who had received antiviral or immunosuppressive medications lately, who were tested positive for antibodies to hepatitis D virus, hepatitis C virus, or human immunodeficiency virus, or who had a history of alcoholism with alcohol ingestion of more than 20 g/d for women and of more than 40 g/d for men. Patients who did not participate in all phases of the study protocol were also excluded.

Detailed information was given about the treatment protocol and the disease, and all enrolled patients provided written informed consent. The study was carried out in accordance with the Declaration of Helsinki and its amendments and was approved by the local ethics committee.

Design and construction of siRNA

Three siRNA sequences were selected and synthesized specifically for human CTLA4. Specificity was tested using the BLAST search engine (www.ncbi.nlm.gov/BLAST). Excluding orthologous identity, none of the siRNAs had matches of greater than 13 nucleotides to known human or murine mRNAs or expressed sequence tags. The target sequences of siRNAs (Table 1) and negative control siRNA were obtained from Ambion, Inc. The experiments described in this study were performed with the most efficient siRNA-duplex.

HBV DNA and CTLA4 level detection

All of the serum samples were collected before medicine administration, and HBV DNA and CTLA4 levels were detected immediately. HBV DNA level was measured using a real-time polymerase chain reaction (PCR) assay at one of clinic services, and the detection range was $1 \times 10^3 \sim 5 \times 10^8$ copies/ml. CTLA4 level was also measured by real-time PCR as described below.

Cell preparation

Human peripheral blood mononuclear cells (PBMCs) were isolated from blood samples using Ficoll-Hypaque (Sigma) density separation. PBMCs were carefully transferred into a hemolysis buffer (154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 8.0) and incubated at room temperature for 10 min. After washing with PBS, cells were suspended at a density of 1×10^7 cells/ml and cultured in 6-well plates (Corning, NY) with RPMI medium containing 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM glutamine and 10% (v/v) heat inactivated fetal bovine serum (Life Technologies, Grand Island, NY). For T cell activation, PBMCs were stimulated with 20 μg/ml phytohemagglutinin (PHA, Sigma). Cells were incubated for 36 h at 37°C in a humidified incubator with 5% CO_2.

Table 1. The siRNA sequences for human CTLA4

siRNA	Sequences	Length
siRNA-1	5'-CCC AAA UUA CGU GUA CUA C-3' 5'-GUA GUA CAC GUA AUU UGG G-3'	19
siRNA-2	5'-CGG AAC CCA GAU UUA UGU A-3' 5'-UAC AUA AAU CUG GGU UCC G-3'	19
siRNA-3	5'-GGU GGA GCU CAU GUA CCC A-3' 5'-UGG GUA CAU GAG CUC CAC C-3'	19

Small interfering RNA transfection

Thirty-six hours after incubation, cells were resuspended at a density of 2.0×10^7 cells/ml in 400 μl of transfection buffer (Ambion, Inc.) with 80 nM siRNA and were transferred into a sterile Eppendorf tube. Electroporation was carried out using Gene Pulser Xcell (Bio-Rad, USA). Immediately after electroporation, cuvettes were incubated for 10 min at 37°C and then the cells were seeded into six-well plates for further experiments.

Real-time PCR analysis of CTLA4 expression

After siRNA treatment, the total RNA was extracted from PBMCs using Trizol reagent (Invitrogen, USA). The purified total RNA from each sample was subjected to reverse transcription (RT) using ReverTra Ace-α-^{TM} kit (TOYOBO BioTech Co., Ltd.) according to manufacturer's guidelines. One μg of the total RNA was mixed with 20 μl of RT mix containing dNTPs, 5× Reverse Transcription Buffer, RNase inhibitor, Oligo(dT)20 and ReverTra Ace. RT reaction was carried out in a thermal cycler for 20 min at 42°C, then 5 min at 99°C, followed by 5 min at 4°C. Real-time quantitative PCR was performed by employing the Real Time PCR Master Mix (TOYOBO Biotech Co., Ltd.) following the protocols. Genes encoding human CTLA4 and β-actin (control) were amplified in parallel. Primer sequences were as follows: human CTLA4, F: 5'-CTA CCT GGG CAT AGG CAA CG-3' and R: 5'-TCA CAT TCT GGC TCT GTT GGG-3'; β-actin, F: 5'-CAT GGG TCA GAA GGA TTC CTA TGT G-3' and R: 5'-ATA GCA CAG CCT GGA TAG CAA CGT A-3'. The following amplification cycles were used: 95°C for 3 min (heat inactivation), 35 cycles of 95°C for 20 s, 61°C for 20 s, and 72°C for 30 s. After the final cycle, we used a 5 min extension period at 72°C. All samples were amplified in triplicate from the same RNA preparation and the mean value was calculated. The expression level of target genes was normalized to internal β-actin. Data were analyzed using Microsoft Excel and calculated using the relative standard curve method.

The level of CTLA4 expression was measured using Ct (threshold cycle) (17). The ΔΔCt method for relative gene expression quantification was used to determine CTLA4 expression levels. The ΔCt was calculated by subtracting the Ct of the β-actin mRNA from the Ct of the CTLA4 mRNA. The ΔΔCt was calculated by subtracting the ΔCt of the reference sample (cells without transfection) from the ΔCt of each sample. The fold change was generated using the equation of $2^{-\Delta\Delta Ct}$. The relative CTLA4 mRNA level in each

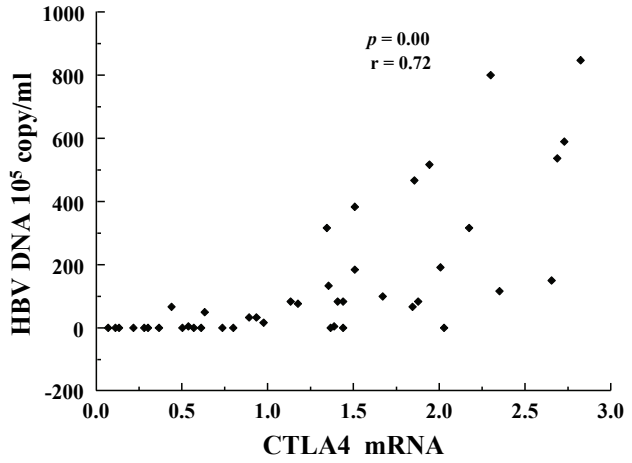

Figure 1. Positive correlation between HBV DNA and CTLA4 mRNA levels. The mRNA levels of HBV DNA and CTLA4 were detected by real-time PCR. HBV DNA less than 1×10^3 copies/ml were normalized to zero. The significance of the correlation was determined by Pearson's test.

sample was expressed as the percentage of the mRNA level compared to that of the cells without transfection, which was defined as 100%.

Cytokine assay by ELISA

PBMCs were incubated as described above in four separate groups. All the assays were performed in duplicate in 12-well plates at a density of 5×10^5 cells/ml (800 μl per well) for 72 h. Then the supernatants were collected for IFN-γ, IL-2 and IL-4 determinations. The cytokine production was assessed with ELISA kits (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Absorbance was measured at 450 nm on a microtiter plate photometer (SPECTRA, SLTInc., Australia).

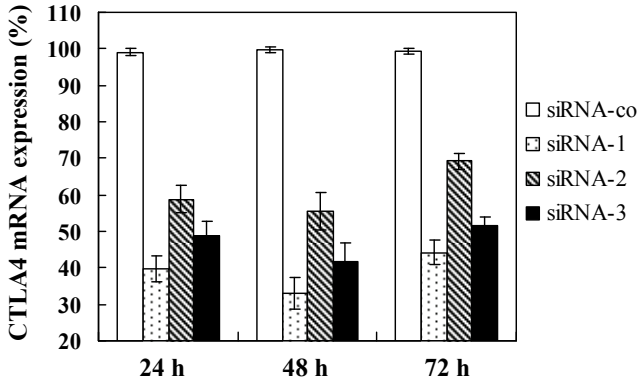

Figure 2. Suppression of CTLA4 mRNA expression by siRNAs. PBMCs were transfected with siRNA-1, siRNA-2, siRNA-3 or siRNA-co at a final concentration of 80 nM in the culture medium, with cells without siRNA transfection as blank control. PBMCs were harvested at 24 h, 48 h and 72 h after transfection and total RNA was extracted for real-time PCR. The CTLA4 mRNA levels were corrected for loading differences performed on the β-actin results. The mean values of RNA levels from blank control were set as 100%, and the mean ± SD of the different siRNA transfections were presented as the percentages of the blank values ($p < 0.05$, analysis of variance). The experiment was performed in triplicate.

Statistics

Data analysis was performed using Microsoft Excel (2003) and SPSS for Windows (SPSS 11.0). Statistical significance was assessed with the Student's *t* test. Differences between experimental groups were analyzed using two-sided *t* test or by two-way ANOVA for experiments with more than two subgroups. The significance of the correlation was determined by Pearson's test. Results were expressed as mean ± SD. Differences were considered statistically significant if $p < 0.05$.

Results

Correlation between mRNA levels of HBV DNA and CTLA4

The correlation between mRNA levels of HBV DNA and CTLA4 was determined using univariate analysis. The results showed that there was significant positive correlation between CTLA4 mRNA and HBV DNA mRNA levels ($r = 0.721$, $p = 0.000$, Figure 1). This indicated that increased CTLA4 mRNA level was correlated with increased concentration of HBV DNA.

Suppression of CTLA4 mRNA expression by siRNAs

The efficiency and quality of the synthesized siRNAs were compared to a randomized control siRNA using real-time quantitative PCR. PBMCs without transfection were used as blank control, and cells transfected with randomized control siRNA (siRNA-co) were used as the negative control. The results showed that mRNA levels of CTLA4 from PBMCs transfected with siRNA-1, siRNA-2, and siRNA-3 for 24 h, 48 h and 72 h were significantly lower than that of the negative control ($p < 0.05$) (Figure 2). It indicated that all the three synthesized siRNAs could efficiently suppress the mRNA expression of CTLA4. Among the three siRNAs, siRNA-1 was the most potent CTLA4 silencer, giving an overall inhibition of 67.07 ± 4.36% at 48 h. Therefore, siRNA-1 was selected for further studies.

The relative expression ratio of mRNA was calculated by real-time PCR, and the exact inhibitory rate was calculated by the formula (1 - TS/TO) (TS, transfected with siRNA-1, siRNA-2 or siRNA-3; TO, transfected with siRNA-co).

CTLA4 siRNA upregulated the secretions of IFN-γ and IL-2

Compared with blank control, the expressions of IFN-γ and IL-2 in PBMCs were significantly upregulated after siRNA-1 transfection (569.42 ± 84.06 pg/ml and 211.87 ± 75.07 pg/ml, respectively) or PHA activation (523.64 ± 40.16 pg/ml and 258.93 ± 76.15 pg/ml, respectively) (Figure 3). The levels of IFN-γ and IL-2 in PBMCs treated with siRNA-1 transfection and PHA stimulation simultaneously were 657.13 ± 93.49

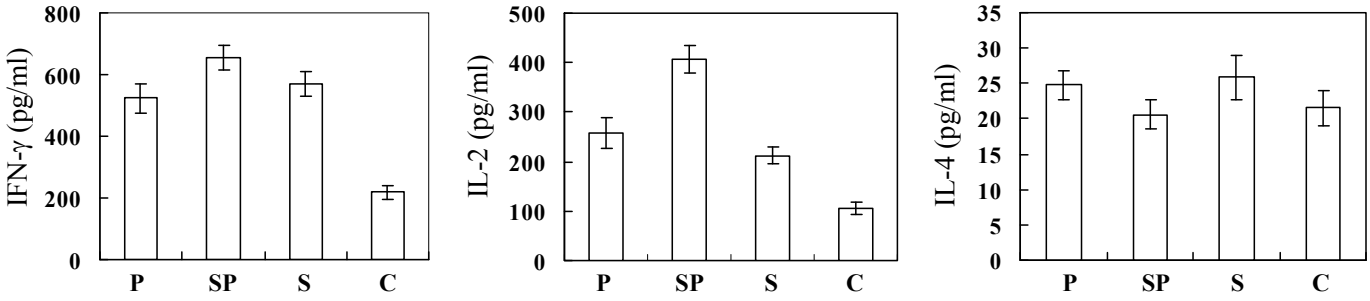

Figure 3. Detection of cytokine expression after siRNA-1 transfection. Cells were harvested at 72 h after different treatments (P, PHA stimulation; SP, siRNA-1 transfection + PHA stimulation; S, siRNA-1 transfection; C, blank control without siRNA-1 transfection and PHA stimulation). The cytokine levels were examined by ELISA. Data were shown as mean ± SD, n = 91.

pg/ml and 407.23 ± 62.00 pg/ml respectively, which showed an enhanced effect compared to single treatment ($p < 0.05$). But there was no significant difference between PHA stimulation and siRNA-1 transfection in the IL-2 or IFN-γ production ($p > 0.05$). Interestingly, the IL-4 concentration was not significantly affected by any treatment ($p > 0.05$) (Figure 3).

Discussion

Immune systems are mainly regulated by cytokines differentially produced by two distinct Th subsets, Th1 and Th2 cells. Th1 cells produce IFN-γ and IL-2 that activate cytotoxic T lymphocytes to promote cell-mediated immunity and killing of virus-infected cells. Indeed, decreased level of these cytokines may facilitate progression of chronic HBV infection (18). Th2 cells produce IL-4, IL-5, IL-9 and IL-13 that contribute to the regulation of humoral immunity against extracellular pathogens, which may play a role in viral persistence (19). Immunopathogenesis analysis of chronic hepatitis B suggested that a preferential shift towards a Th1-polarized phenotype could be associated with a spontaneous viral clearance or a successful control of infection (20). In addition, an early commitment towards a Th2 phenotype plays a significant role in disease progression (21). There is a general agreement about the concept that hyporesponsiveness of HBV-specific T cells is an important determinant of virus persistence in chronic HBV infection (20). Thus, therapeutic strategies that aim to correct this deficiency have been extensively studied (22).

Significantly positive correlation between HBV DNA and CTLA4 was identified in this study. This suggests that the mRNA level of CTLA4 has a tight association with persistence of chronic hepatitis B. This finding is consistent with the emerging role of regulatory T cells in the pathogenesis of disease.

In our study, three sequence-specific siRNAs for human CTLA4 were designed and synthesized. Their inhibitory function was assessed by real-time quantitative PCR. We found that all of the siRNAs could downregulate the mRNA expression of CTLA4, and siRNA-1 had the strongest inhibitory function (Figure 2). The most efficient siRNA-1 was used in further experiments.

The levels of IFN-γ, IL-2 and IL-4 in the supernatants of PBMC culture were measured on day 3. The results indicated that the levels of IFN-γ and IL-2 were increased but that of IL-4 was almost invariant compared with control (Figure 3). In order to assess the effect of siRNA transfection on human lymphocytes of chronic HBV patients, both the blank control without siRNA transfection and PHA stimulation and the positive control with only PHA stimulation were designed. The results showed that siRNA-1 could evidently lead to enhanced IFN-γ and IL-2 secretions without a significant effect on IL-4 secretion. By combined siRNA and PHA, we found that the expressions of IFN-γ and IL-2 were increased significantly compared with each treatment alone. These phenomena indicated that a response of Th1/Th2 occurred. This finding is consistent with some other studies. Damle et al. demonstrated that the activated (B7.2) T cells in the presence of CD28 molecules had an increased ability to synthesize IFN-γ and IL-2 (23). Fan W et al. found that CTLA4Ig could switch T cell responses from Th1 to Th2 in URSA by blocking CD80/CD86 co-stimulation (24).

In the present studies, downregulation of CTLA4 *via* siRNA diminished the interaction between CTLA-4 and B7 that could favor B7-CD28 interaction, and induced enhanced IFN-γ and IL-2 secretions *in vitro*. However, no compromised IL-4 secretion was observed with the treatment. It suggested that CTLA4 might regulate the production of cytokines by Th1 and Th2 cells and have diverse effects on the progress of infectious diseases, which are dependent on pathogens and host factors (25). Our studies provided the experimental basis for transfection of CTLA4-siRNA into primary mammalian cells and brought optimism for the future use of siRNA *in vivo* to regulate the imbalance of cytokines in human lymphocytes of chronic HBV patients. However, because of the apparent short time of effectiveness of the 19-nt siRNA *in vitro*, the application of siRNA *in vivo* may require stable expression by means of a suitable vector (26).

In summary, our studies demonstrated that CTLA4 was associated with viral persistence and CTLA4-siRNA can

promote lymphocyte response by induction of response of Th1/Th2. Whether it could lead a novel strategy to treat chronic HBV infection will be studied in the near future.

References

1. Tivol EA, Boyd SD, McKeon S, et al. CTLA4Ig prevents lymphoproliferation and fatal multiorgan tissue destruction in CTLA-4-deficient mice. J Immunol. 1997;158:5091-5094.
2. Doyle AM, Mullen AC, Villarino AV, et al. Induction of cytotoxic T lymphocyte antigen 4 (CTLA-4) restricts clonal expansion of helper T cells. J Exp Med. 2001;194:893-902.
3. Salomon B, Bluestone JA. Complexities of CD28/B7:CTLA-4 costimulatory pathways in autoimmunity and transplantation. Annu Rev Immunol. 2001;19:225-252.
4. Tan P, Anasetti C, Hansen JA, et al. Induction of alloantigen-specific hyporesponsiveness in human T lymphocytes by blocking interaction of CD28 with its natural ligand B7/BB1. J Exp Med. 1993;177:165-173.
5. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA. CTLA-4 is a second receptor for the B cell activation antigen B7. J Exp Med. 1991;174:561-569.
6. Turka LA, Linsley PS, Lin H, et al. T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection *in vivo*. Proc Natl Acad Sci U S A. 1992;89:11102-11105.
7. Sayegh MH, Akalin E, Hancock WW, et al. CD28-B7 blockade after alloantigenic challenge *in vivo* inhibits Th1 cytokines but spares Th2. J Exp Med. 1995;181:1869-1874.
8. Hodi FS, Mihm MC, Soiffer RJ, et al. Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. Proc Natl Acad Sci U S A. 2003;100:4712-4717.
9. Phan GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A. 2003;100:8372-8377.
10. McCoy K, Camberis M, Gros GL. Protective immunity to nematode infection is induced by CTLA-4 blockade. J Exp Med. 1997;186:183-187.
11. Geisbert TW, Hensley LE, Kagan E, et al. Postexposure protection of guinea pigs against a lethal ebola virus challenge is conferred by RNA interference. J Infect Dis. 2006;193:1650-1657.
12. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 1998;391:806-811.
13. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature. 2001;411:494-498.
14. Song E, Lee SK, Wang J, et al. RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med. 2003;9:347-351.
15. Perl M, Chung CS, Lomas-Neira J, et al. Silencing of Fas, but not caspase-8, in lung epithelial cells ameliorates pulmonary apoptosis, inflammation, and neutrophil influx after hemorrhagic shock and sepsis. Am J Pathol. 2005;167:1545-1559.
16. Ren JL, Pan JS, Cheng T, et al. RNA interference inhibits hepatitis B virus gene expression and replication in HepG2-N10 cells. Chin J Dig Dis. 2006;7:230-236.
17. Botta F, Maestroni GJ. Adrenergic modulation of dendritic cell cancer vaccine in a mouse model: role of dendritic cell maturation. J Immunother. 2008;31:263-270.
18. Lechmann M, Woitas RP, Langhans B, et al. Decreased frequency of HCV core-specific peripheral blood mononuclear cells with type 1 cytokine secretion in chronic hepatitis C. J Hepatol. 1999;31:971-978.
19. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol. 1989;7:145-173.
20. Loggi E, Gramenzi A, Margotti M, et al. *In vitro* effect of thymosin-α1 and interferon-α on Th1 and Th2 cytokine synthesis in patients with eAg-negative chronic hepatitis B. J Viral Hepat. 2008;15:442-448.
21. Akpolat N, Yahsi S, Godekmerdan A, Demirbag K, Yalniz M. Relationship between serum cytokine levels and histopathological changes of liver in patients with hepatitis B. World J Gastroenterol. 2005;11:3260-3263.
22. Bertoletti A, Naoumov NV. Translation of immunological knowledge into better treatments of chronic hepatitis B. J Hepatol. 2003;39:115-124.
23. Damle NK, Klussman K, Leytze G, et al. Costimulation of T lymphocytes with integrin ligands intercellular adhesion molecule-1 or vascular cell adhesion molecule-1 induces functional expression of CTLA-4, a second receptor for B7. J Immunol. 1994;152:2686-2697.
24. Fan W, Li SW, Liu XR. Influence of blockade of costimulation on Th1/Th2 cytokines shift in unexplained early recurrent spontaneous. Sichuan Da Xue Xue Bao Yi Xue Ban. 2006;37:773-775.
25. Watanabe K, Murakami K, Sato R, et al. CTLA-4 blockade inhibits induction of *Helicobacter pylori*-associated gastritis in mice. Clin Exp Immunol. 2004;135:29-34.
26. Gao Z, Wang Z, Shi Y, et al. Modulation of collagen synthesis in keloid fibroblasts by silencing Smad2 with siRNA. Plast Reconstr Surg. 2006;118:1328-1337.