Experience with lentivirus-mediated CD40 gene silencing in a mouse model of Graves’ disease

Feng Ye, Bingyin Shi, Xiaoyan Wu, Peng Hou, Lei Gao, Xiaodan Ma, Li Xu and Liping Wu
Department of Endocrinology, First Affiliated Hospital of Xi’an Jiaotong University School of Medicine, Xi’an 710061, People’s Republic of China

Abstract

CD40 plays an important role in the pathogenesis of Graves’ disease (GD). Inhibition of CD40 expression may be a promising treatment for GD. In this study, we used an animal model to investigate whether lentivirus expressing siRNA for CD40 (LV-CD40-siRNA) could be useful for the therapy of GD. BALB/c mice were injected with PBS alone (PBS group), negative lentivirus (control siRNA group), or LV-CD40-siRNA (CD40 siRNA group), 3 days before being treated with adenosine virus expressing human TSHR-A subunit (Ad-TSHR289) three times at 3-week intervals to induce GD model. Sera thyroxine (T4) levels were assayed by RIA. The expression of CD40 was detected at the mRNA level by real-time PCR and protein level by flow cytometry. The expression of CD40, CD80, and CD86 was significantly decreased in the CD40 siRNA group (P<0.05), while FOXP3 expression was increased compared to the control siRNA group (P=0.05). Mean T4 levels were decreased 14% in the CD40 siRNA group compared to the control siRNA group. The rate of disease induction was similar among the three groups injected with Ad-TSHR289. LV-CD40-siRNA is a useful tool to inhibit the expression of CD40 in vivo, but it cannot decrease the incidence of hyperthyroidism in a limited period of time.

Introduction

In general population, the prevalence of Graves’ disease (GD) is ~0.5%, and is the underlying cause of 60–80% of hyperthyroidism (Weetman 2000, Brent 2008). It is an organ-specific autoimmune disease in which thyroid-stimulating antibody stimulates the TSH receptor (TSHR) leading to overproduction of thyroid hormones and diffuse hyperplasia of the thyroid gland. The mechanisms for breakdown of immune tolerance to the TSHR remain obscure as described in Saitoh et al. (2007). The present treatment modalities include antithyroid drugs, radioactive iodine, and thyroidectomy. Although these are useful, they have limitations and have remained essentially unchanged for 50 years as described by Gilbert et al. (2006). These authors showed reduced hyperthyroidism in mice with on-going GD using decoy molecules of tumor necrosis factor (TNF) family ligand inhibitors to target B lymphocyte proliferation or survival factors. These studies indicate the importance of investigating additional novel therapeutic approaches.

RNA interference is a posttranscriptional mechanism of gene silencing, in which 19–23 nucleotide double-stranded RNA duplexes promote specific endonucleolytic cleavage of mRNA targets called small interfering RNA (siRNA) as described in Raoul et al. (2005) and Suzuki et al. (2008). Gene silencing using siRNA is an extremely efficient, specific, long-lasting, and simple method for blocking the expression of target genes as described by Hill et al. (2003). It has been successfully used in animal models of various diseases and is currently in clinical trials (de Fougerolles et al. 2007). However, the utility of gene silencing as a treatment for hyperthyroidism has not yet been reported.

CD40 is a member of the TNF receptor superfamily. It is expressed by a wide variety of cell types, including B cells, dendritic cells (DCs), fibroblasts, endothelial cells, macrophages, and thyrocytes (Smith et al. 1999). The interaction between CD40 and its cognate ligand CD40L (CD154) provides a costimulatory signal that mediates T-cell maturation, B-cell proliferation, immunoglobulin production, isotype class switching, and generation of memory cells and provides an additional maturation signal to antigen presenting cells (APCs; Sempowski et al. 1998). Blocking the interaction between CD40 and CD154 is beneficial in animal models of autoimmune diseases (Durie et al. 1993, Gerritte et al. 1996, Carayanniotis et al. 1997). It is also reported that the CD40/CD154 interaction may represent an important mechanism in the regulation of the autoimmune humoral response in GD (Mysliwiec et al. 2007). Thus, inhibition of CD40 expression may be a promising treatment for GD.

A previous study has shown that blocking CD40/CD154 interaction by adenovirus expressing CD40-Lg could reduce the incidence of hyperthyroidism (Chen et al. 2006). However, the exact mechanism was not clarified and neither was the mechanism for the inhibition of hyperthyroidism in...
the Ad-TSHR289-induced GD animal model by the adenovirus vector. The effect of CD40 inhibition on GD thus needs further investigation. Hence, in this study, we have used lentivirus as a gene delivery vector to investigate whether LV-CD40-siRNA-induced posttranscriptional CD40 gene silencing could reduce the incidence of hyperthyroidism in Ad-TSHR289-induced GD animal model and the possible mechanisms.

**Materials and Methods**

**Construction of TSHR adenoviruses**

Construction of Ad-TSHR289 was performed as previously described (Chen et al. 2003, Saitoh & Nagayama 2006). Briefly, adenoviruses (Ad-TSHR289) were propagated in HEK293 cells and purified by ion-exchange column chromatography. The concentration of viral particles was determined by measuring the absorbance at 260 nm, and they were stored at −80 °C.

**Construction of LV-CD40-siRNA**

The lentivector used in this study was designed to coexpress green fluorescent protein (GFP). The sequence of the siRNA-targeting murine CD40 was gaTGGTAAGA-GAGTCGCATC. Pairs of complementary oligonucleotides containing these sequences were synthesized and cloned into the PGCL-GFP Lentivector; 293T cells were cotransfected with plasmid pHelper1.0, pHelper2.0, and the PGCL-GFP Lentivector containing the shRNA sequences using Lipofectamine 2000 (Invitrogen). Viral supernatants were harvested after 48 h, and the titers were determined with serial dilutions of concentrated lentivirus.

**Immunization and treatment**

Thirty-two 6–8-week-old female BALB/c mice were purchased and housed in a specific pathogen-free facility at the Experimental Animal Center of the Fourth Military Medical University (Xi’an, P R China). Room temperature (23 ± 1 °C), humidity (50 ± 10%), and a 12 h light:12 h darkness cycle were strictly controlled. Mice were randomly divided into four groups and provided with food pellets and water *ad libitum*. One group of mice was injected with 50 µl PBS as the PBS-treated mice and the other three groups of mice were injected i.v. with PBS alone (PBS group), negative lentivirus (control siRNA group), or LV-CD40-siRNA (CD40 siRNA group) 3 days before being treated with Ad-TSHR289, which was injected i.m. three times at 3-week interval to induce hyperthyroidism. Blood, spleen, and thyroid tissues were obtained 4 weeks after the third immunization. All experiments were conducted in accordance with the principles and procedures outlined in the Guideline for the Care and Use of Laboratory Animals at Xi’an Jiaotong University.

**Thyroxine and TRAb measurements**

Total serum thyroxine (T₄) was measured with an RIA kit (Beijing Atom High Tech Co., Ltd, Beijing, China). The normal range was defined as mean ± 3 S.D. of the PBS-treated mice. TRAb values were determined using human recombinant TSHR protein (a TRAb kit, BRAHMS Diagnostica GmbH, Berlin, Germany).

**Histology**

Thyroid tissues were removed and fixed in 4% formalin. Sections were examined at 3–5 µm intervals and were stained with hematoxylin and eosin. Analysis was performed using an Olympus Cue-2 image analysis system connected to an Olympus compound microscope.

**Flow cytometry analysis**

To analyze surface expression of CD40, CD80, and CD86, 1 × 10⁶ splenocytes were stained with PE, FITC, or APC-conjugated Ab or the appropriate isotype-controlled Ab respectively, in a final volume of 100 µl for 60 min in dark (eBioscience, San Diego, CA, USA), followed by washing twice in 1 ml of cold flow cytometry wash buffer (PBS with 1% BSA). The cells were then resuspended in 300 µl PBS, and protein expression was analyzed using a FACScan apparatus (BD Biosciences, San Jose, CA, USA). To analyze FOXP3 expression, 1 × 10⁶ splenocytes were stained with anti-CD4-PerCP-cy5.5 and anti-CD25-APC antibodies (eBioscience) for 30 min and incubated with fixation/permeabilization solution. After 30 min incubation, cells were washed twice with permeabilization buffer and incubated with anti-FOXP3-PE for 30 min.

**Real-time PCR**

Total RNA was isolated from the spleens using RNAfast200 purification kit (Fastagen Biotech, Shanghai, China) according to the manufacturer’s protocol. The RNA was subsequently reverse transcribed using a RevertAid First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). The primer sequences were designed using Primer Express (Applied Biosystems, Foster City, CA, USA) (shown in Table 1). Quantitative PCR assay was performed in triplicate for each sample in a final reaction mixture of 20 µl containing SYBR Premix Ex TaqTM II PCR Master Mix (TakaRa, Dalian, China) and 0.25 µM of each primer. After an initial denaturation step at 95 °C for 10 s, 40 cycles of 5 s at 95 °C, 15 s at 55 °C, and 15 s at 72 °C were run on an IQ5 Real-Time PCR Detection System (Bio–Rad). Mouse β-actin mRNA was used for normalization to ensure equal amounts of starting RNA. The specificity of real-time quantitative PCR for all these genes was confirmed by running the PCR products on a 2% agarose gel to show single specific bands at the expected sizes (data not shown).
Lenivirus did not influence the expression of CD40 disease induction among these three groups, mean T4 levels in oid. Although there was no significant difference in the rate of LV-CD40-siRNA (CD40 siRNA group) became hyperthyr-and treated with negative lentivirus (control siRNA group) or three percent (5/8) of mice immunized with Ad-TSHR289 treated with PBS (PBS group) became hyperthyroid. Sixty-mice, 75% (6/8) of mice immunized with Ad-TSHR289 and hyperthyroid. In this study, compared with the PBS-treated (Fig. 2A). Mean T4 levels were similar between the PBS group and the CD40 siRNA group were much lower than the other two (Fig. 2A). Mean T4 levels were similar between the PBS group and the control siRNA group (Fig. 2A) and there was a 14% decrease in the CD40 siRNA group compared to the control siRNA group, although this difference was not statistically significant. We subsequently measured TRAb in all of the mice and found that mice injected with Ad-TSHR289, no matter hyperthyroid or euthyroid, had significantly higher TRAb compared with the PBS-treated mice (Fig. 2B). Next, we investigated whether the hyperthyroid BALB/c mice as judged by T4 levels displayed histological changes. Unlike euthyroid or control mice, all the hyperthyroid BALB/c mice had diffuse goiter and hyperplastic follicles with cuboidal or columnar thyroid epithelial cells, but no lymphocytic infiltration as previously reported (Saitoh & Nagayama 2006). Statistical analysis All comparisons between the groups were performed using either Student’s t-test or ANOVA, and P values <0.05 were considered statistically significant.

Results

Silencing CD40 in vivo using LV-CD40-siRNA

During in vitro experiments, we observed that mature DCs (mDCs) expressed high levels of CD40 and these were significantly decreased by LV-CD40-siRNA (data not shown). We subsequently investigated the ability of LV-CD40-siRNA to suppress CD40 expression of splenocytes in vivo. Mice were killed 4 weeks after the third immunization with Ad-TSHR289. The silencing effects of LV-CD40-siRNA were detected at the mRNA level by real-time PCR and protein level by flow cytometry as compared with negative lentivirus. As shown in Fig. 1, CD40 expression was significantly lower in the CD40 siRNA group than the control group (P<0.05), at both RNA and protein levels. CD40 expression did not differ significantly between the PBS groups and the control siRNA groups (Fig. 1). Our data thus showed the feasibility and efficacy of LV-CD40-siRNA on downregulating the expression of CD40 in splenocytes in vivo. Lenivirus did not influence the expression of CD40 in vivo.

Thyroid function after virus immunizations

Serum T4 levels were measured 4 weeks after the third injection of Ad-TSHR289. Mice with serum T4 levels higher than mean ± 3 s.d. of PBS-treated mice were considered hyperthyroid. In this study, compared with the PBS-treated mice, 75% (6/8) of mice immunized with Ad-TSHR289 and treated with PBS (PBS group) became hyperthyroid. Sixty-three percent (5/8) of mice immunized with Ad-TSHR289 and treated with negative lentivirus (control siRNA group) or LV-CD40-siRNA (CD40 siRNA group) became hyperthyroid. Although there was no significant difference in the rate of disease induction among these three groups, mean T4 levels in the CD40 siRNA group were much lower than the other two (Fig. 2A). Mean T4 levels were similar between the PBS group and the control siRNA group (Fig. 2A) and there was a 14% decrease in the CD40 siRNA group compared to the control siRNA group.

| Table 1  Primer sequences used in this study for real-time quantitative RT-PCR analysis |
|---------|-----------------|------------------|------------------|
| Gene    | Forward          | Reverse          | Product size (bp) |
| CD40    | GCCATCGTGGAGGTACTGGT | CTCGGATGGTGCTTTCGTCT | 110           |
| CD80    | GCCAAGGCAAGCAATACCTAA | CTCCTTGCTGCTGATCG | 94            |
| CD86    | TCTCCAGGAAACACATCT | CTTACGGAAGCACCATGAT | 100           |
| FOXP3   | CTCGCTGAAAGGAGTGGCA | TGGCAGAGGGATTTGAGGG | 106           |
| β-actin | CCAGGACATTGCTGACAGG | GCTGGAAGGTGGACAGTG | 142           |

Table 1. Primer sequences used in this study for real-time quantitative RT-PCR analysis.

FOXP3, the forkhead family transcription factor.

Statistical analysis

All comparisons between the groups were performed using either Student’s t-test or ANOVA, and P values <0.05 were considered statistically significant.

Alterations of other costimulatory molecules by CD40 silencing

DCs play an essential role in immune homeostasis. mDCs act as potent APCs, stimulating T-cell activation, whereas immature DCs (iDCs) characterized as low expression of costimulatory molecules (CD40, CD80, and CD86) induce
Peripheral T-cell tolerance (Chorny et al. 2006). In vitro experiments showed that mDCs expressed high levels of CD80 and CD86, and they were significantly decreased by CD40 downregulation (data not shown). In this study, we used an siRNA approach to explore the effect of CD40 downregulation on the expression of CD80 and CD86 in splenocytes in vivo. As shown in Fig. 3, the expressions of CD80 and CD86 were significantly decreased in the CD40 siRNA group compared to the control siRNA group (P < 0.05). There was no significant difference in the expression of CD80 between the control siRNA group and the PBS group (Fig. 3A). However, a significant difference was found in the expression of CD86 between the control siRNA group and the PBS group (Fig. 3B), suggesting that lentivirus can affect the expression of some costimulatory molecules through certain unknown molecular mechanisms. Moreover, we also analyzed the expression of CD80 and CD86 on the surface of splenocytes by flow cytometry. Fewer CD80 and CD86 positive cells were found in the CD40 siRNA group compared with the other two groups, which was consistent with the results of mRNA, although no significant difference was noted (data not shown).

Alteration of immune response by CD40 silencing

T regulatory cells (Tregs) are essential for maintaining immunological self-tolerance and immune homeostasis (Wing et al. 2008), which play an important role in autoimmune diseases. It has been reported that a blockade of the CD40/CD40L interaction actively generates Treg cells (Suzuki et al. 2009). We thus used our own mouse model to explore the effect of CD40 siRNA on the expression of FOXP3 in splenocytes in vivo using real-time PCR. As shown in Fig. 4, the treatment of CD40 siRNA dramatically increased FOXP3 gene expression compared with the control siRNA (P = 0.05) or PBS group (P < 0.05). We also used flow cytometry to determine whether treatment of CD40 siRNA can increase the number of Treg cells in vivo. The proportion of Treg cells in CD40 siRNA group were also increased compared with the other two groups although there were no significant differences (data not shown).

Discussion

The GD animal model, induced by immunization with adenovirus expressing the TSHR, is the most widely used model to study the pathogenesis and therapy of GD. In this study, six of eight BALB/c mice (75%) immunized with...
reflected by T4 level. siRNA attenuated disease severity in a GD animal model, as treatment for GD. In this study, we found that LV-CD40-siRNA inhibited the expression of CD40 both at RNA and protein levels. This may be a new method for exploring the feasibility of gene therapy by CD40 gene silencing. CD40 over expression plays a role in the pathogenesis of GD through stimulation of T-cell maturation, activating B cells, and inhibiting thyrocyte apoptosis (Faure et al. 1997, Kie et al. 2001, Tomer et al. 2002, Jacobson & Tomer 2007). Inhibition of CD40 expression may be a prospective treatment for GD. In this study, we found that LV-CD40-siRNA attenuated disease severity in a GD animal model, as reflected by T4 level.

The following reasons might explain the observations of this study. First, GD is an organ-specific autoimmune disease and the main organ involved is the thyroid gland. Although we have effectively used LV-CD40-siRNA to inhibit the CD40 expression in spleen, this does not mean that it had an appreciable effect on the thyroid. If we can directly inject LV-CD40-siRNA into the thyroid locally, perhaps it will play a greater role. Secondly, CD80 and CD86 are also required for effective activation of T lymphocytes. In this study, real-time PCR (a very sensitive technique) data demonstrated decreased amounts of CD80 and CD86 mRNA in splenic tissue from mice pretreated with LV-CD40-siRNA compared with PBS or control siRNA, however, no significant differences were obtained by flow cytometry, indicating that the protein levels may not have been significantly reduced due to unknown complicated mechanisms in vivo. It is not sufficient to affect the occurrence of hyperthyroidism. Thirdly, the lack of uniform effect of CD40 silencing on the incidence of hyperthyroidism does not rule out the possibility of a link between these two events. Fourth, it remains to be investigated whether the effect of CD40 silencing on hyperthyroidism may be a long-term consequence. Finally, the current results may be a result of the relatively small number of study animals. Further study with more animals is thus needed.

CD4⁺CD25⁺FOXP3⁺ Treg cells develop in the thymus and peripheral lymphatic or immune organs, and represent 5–10% of the peripheral CD4⁺T-cell compartment (Sakaguchi 2005, Saitoh & Nagayama 2006). These cells suppress the expression of CD80 and CD86, and hamper the proliferation of effector CD4⁺T cells as well as the maturation of DCs (Saitoh & Nagayama 2006, Wing et al. 2008). Recently, Treg cells have been demonstrated to efficiently influence autoimmune disease (Cheng et al. 2008). A previous study showed that regulatory T cells play an important role in the mechanism involved in both susceptibility and severity of GD (Saitoh & Nagayama 2006). In this study, we found that mRNA levels of FOXP3 were dramatically increased and the number of Treg cells was mildly increased in the mice treated with LV-CD40-siRNA. We speculate that increased Treg cells partially dampen the severity of hyperthyroidism in the CD40 siRNA group.

A previous study showed that coinmunization with a second adenovirus, regardless of the nature of its insert, markedly reduced TSHR antibody level and the incidence of hyperthyroidism induced by Ad-TSHR289 (Chen et al. 2006), which seriously limits the use of Ad vectors in such animal models of GD. In this study, our findings showed that re-administration of lentivirus vectors to the GD model did not affect the incidence of hyperthyroidism. Recently, a number of studies showed that Ad-specific effector and memory T cells can be quickly activated following Ad vector administration concomitant with production of proinflammatory cytokines and chemokines, which may be responsible for reduced therapeutic efficacies of this vector (Thacker et al. 2009). However, lentivirus vectors are less likely to stimulate systemic immune responses (Sinn et al. 2008), suggesting that lentivirus is a suitable vector to study the pathogenesis and therapy of GD in animal model induced by Ad-TSHR289, although molecular mechanisms remain unclear.

In summary, we tried a novel immune blocker for preventing the occurrence of GD through lentivirus-mediated CD40 gene silencing in an animal model. We demonstrated that LV-CD40-siRNA is a powerful tool for inhibiting the expression of CD40 in vivo, which can partially lighten the severity of GD in animal model, although it did
not decrease the incidence of hyperthyroidism in a limited period of time. It might be a potential future clinical adjunctive therapy in the prevention and intervention of hyperthyroidism.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Natural Science Foundation of China (30500250.) and the Fundamental Research Funds for the Central Universities.

Acknowledgements

We thank Drs Basil Rapoport, Sandra M McLachlan, and Chunrong Chen (University of California, Los Angeles, CA, USA) for providing us the plasmid expressing human TSHR289 (psv2-neo-ECE).

References


www.endocrinology-journals.org


Received in final form 25 November 2010
Accepted 8 December 2010
Made available online as an Accepted Preprint 8 December 2010