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Original Article

Down-regulation of nectin-4 inhibits apoptosis in systemic lupus erythematous (SLE) through targeting Bcl-2/Bax pathway

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Abstract: Purpose: The purpose of this study was to investigate the potential role of nectin-4 in systemic lupus erythematous (SLE) cell apoptosis during the disease development and its potential mechanism. Methods: Human peripheral blood mononuclear cells (PBMCs) were obtained for the isolation of monocytes and T lymphocytes. siRNA-nectin-4 plasma was constructed for the transfection into T cells using Lipofectamine 2000 reagent. siRNA with no nectin-4 sequence was transfected into T cells for the control group. mRNA expression of nectin-4 in cells was analyzed using RT-PCR method. Effect of netin-4 expression on T cell apoptosis was analyzed with Annexin V-FITC cell apoptosis kit. Moreover, effects of nectin-4 expression on cell apoptotic-related proteins expressions were detected using western blotting analysis. Results: Nectin-4 was significantly overexpressed in cells from SLE group compared with healthy control (HC) group ($P<0.05$). When T cells were transfected with sinectin-4, nectin-4 slicing increased cell apoptosis in HC group but significantly decreased apoptosis in SLE group ($P<0.05$). Nectin-4 slicing significantly decreased CD40L and CD17 expressions in SLE ($P<0.05$), but performed no effect on CD11a expression. Moreover, nectin-4 down-regulation could significantly decrease Bcl-2, Bcl-XL, and caspase-6 expressions but increase Bax level in SLE group. Conclusion: The data presented in this study suggested that nectin-4 may be a therapeutic target for SLE through affecting the cell apoptosis.

Keywords: Systemic lupus erythematous (SLE), nectin-4, cell apoptosis, Bcl-2/Bax pathway, caspase

Introduction

Systemic lupus erythematous (SLE), an organ-specific autoimmune disease, which is characterized by the abnormal activation of self-reactive T and B cells and self-antibodies and immune complex production, leads to irreversible damage to many kinds of organs and tissues in human body [1]. Complications such as urinary system diseases and pseudo obstruction which resulted from SLE have brought huge damages for patients' life [2, 3]. Solutions on SLE treatment still remain exploration due to the complicate and undefined pathogen mechanism [4, 5]. Thus, to investigate the pathogenesis and to find several treatment solutions for SLE will be necessary in clinical.

Recent papers mentioned that cell apoptosis have played pivotal roles in contributing SLE development, and variety kinds of factors such as apoptosis related genes defects or abnormal expressions and cytokines [6, 7]. Nucleosome produced during cells apoptosis has been reported to be the main auto-antigen for SLE, because of its high specificity [8], and many kinds of apoptotic cells including lymphocyte, neutrophil, and monocyte macrophage have become the accelerators for cell apoptosis in SLE progression and then result in complications such as cardiovascular disease [9, 10]. Many studies have referred the possible mechanism for cell apoptosis in SLE development, such as cytokines of interleukin (IL)-1 and tumor necrosis factor (TNF)-α, and apoptotic related proteins of Bcl-2 and Bax [11], but the mechanism of cell apoptosis in SLE remains unclear.

Nectin-4 (poliovirus receptor-related 4, PVRL4, a 66 KD cell adhesion molecule) is a member of nectin family protein that plays curial roles in many biological processes including cell migra-

tion, proliferation, and invasion [12, 13]. Recently, increasing evidences report that the major reports for nectin-4 are the association between nectin-4 expression and tumors metastasis, prognosis and progression, such as nectin-4 is a serological marker for breast cancer [14], acts as a diagnostic and therapeutic target for lung cancer [15], and the potential marker application in ovarian cancer [16]. Although previous articles have demonstrated that nectin family protein were widely expressed on the surface of epithelial cells, endothelial cells, hematopoietic cells, and nerve cells, but nectin-4 can express in embryo and placenta tissues [17]. In spite of many reports have demonstrated the correlations of nectin-4 and many diseases, the underlying role of nectin-4 in SLE cell apoptosis still remains incomplete described.

In this study, we analyzed the potential effects of nectin-4 on SLE immune cell apoptosis based on the gene slicing method. Variety experimental methods were used to investigate the influences of nectin-4 expressions on cell apoptosis and cell apoptotic associated proteins expressions. This study aimed to illustrate the potential effect of nectin-4 in immune cell apoptosis during SLE development and progression and to investigate its potential mechanism. This study may provide basis for the possibility of nectin-4 being a therapeutic target for SLE in clinical application.

Materials and methods

Cell isolation and cell transfection

Human peripheral blood mononuclear cells (PBMCs) were obtained from standard buffy-coat preparations from routine healthy donors (collected Children's hospital affiliated in Shanghai Jiaotong University) and then isolated from Ficoll-Hypaque gradients (Sigma, USA) with centrifugation. T lymphocytes and monocytes were isolated based on a Percoll gradient as previously described [18]. The T cells population percentage collected from the gradient was more than 96% surface $CD14^+$, while monocytes was approximately 85% surface $CD13^+$, which was determined by flow cytometry. This study was approved by the local ethics committee from the Children's hospital affiliated in Shanghai Jiaotong University.

siRNA (synthesized by Sangon Biotech, Shanghai, China) was used for nectin-4-specific slicing with the following target sequence: 5'-CAGAGCAGTATTAATGATGCA-3'. Control siRNA with no slicing sequence was transfected into the T cells as the control group. Cell transfections were conducted using Lipofectamine 2000 reagent (Invitrogen, USA) based on manufacturer's protocol. G418 (Sigma, USA) was used for the stable sinectin-4 transfectants selection [19].

Cell apoptosis assay

Influence of nectin-4 expression on T cells apoptosis was assessed using Annexin V-FITC cell apoptosis kit (Invitrogen, USA) according to manufacturer's protocol [20]. Briefly, T cells transfected with sinectin-4 or blank siRNA plasmas for 24 h, followed by the replacement of cell medium. Total cells in each group were harvested and washed with PBS buffer (PH 7.4) for three times, and then resuspended in the staining buffer. After that, 5 μL of annexin-V-FITC (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.4) and 5 μL of propidium iodide (PI) were mixed with the cells. After being cultivated at room temperature for 10 min, mixtures were analyzed using the FACScan flow cytometry (BD, USA). Annexin V-positive and propidium iodide-negative cells were considered to be apoptotic cells.

RT-PCR analysis

Total RNA isolated from T cells collected at 48 h was conducted with TRIzol Reagent (Invitrogen, USA) as previously described [20] and was treated with RNse-free Dnase I (Sigma, USA). Next, concentration and purity of isolated RNA were measured using SMA 400 UV0VIS (Merinton, Shanghai, China). Purified RNA at density of 0.5 μg/μL with nuclease-free water was used for cDNA synthesis with the PrimerScript 1st Strand cDNA Synthesis Kit (Invitrogen, USA). Expressions of targets in T cells were detected in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-qPCR Kit (Takara, China). The total reaction system of 20 μL volume was as follows: 1 μL cDNA from the above PCR, 10 μL SYBR Premix EX Taq, 1 μL each of the primers (10 μM), and 7 μL ddH_2O. The PCR program was as follows: denaturation at 50°C for 2 min; 95°C for 10 min; followed by 45 cycles of 95°C for 10 s, and 60°C for 1 min. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and det-

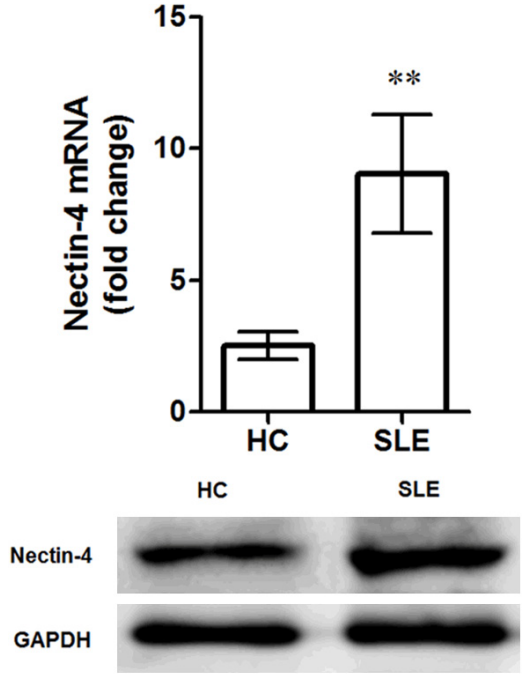

Figure 1. Expression of nectin-4 in systemic lupus erythematous (SLE) and healthy control (HC) tissues. ***P*<0.01, compared with HC group.

ected. Phosphoglyceraldehyde dehydrogenase (GAPDH) was chosen as the internal control. Primers used for targets amplification were as follows: nectin-4 sense, 5'-ATGAACCCTGCCGATATTG-3' and nectin-4 anti-sense, 5'-GCTGACATGGCAGACGTAGA-3'; and GAPDH sense, 5'-TATGATGATATCAAGAGGGTAGT-3', and GAPDH anti-sense, 5'-TGTATCCAAACTCATTGTCATAC-3'.

Western blotting

T cell apoptosis related proteins that isolated from SLE and HC tissues were prepared for Western blotting analysis as previously described [21]. The procedures were briefly as follows, T cells transfected with sinectin-4 plasma and cultured at 48 h were lapped in radioimmunoprecipitation assay (RIPA, Sangon Biotech) lysate containing phenylmethanesufonyl fluoride (PMSF), and then were centrifuged at 12,000 rpm for 5 min at 4°C. Supernatant was collected for the measurement of protein concentrations using bicinchoninic acid (BCA) protein assay kit (Pierce, Rochford, IL). Consequently, a total of 20 μg protein per cell lysate was subjected onto a 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then

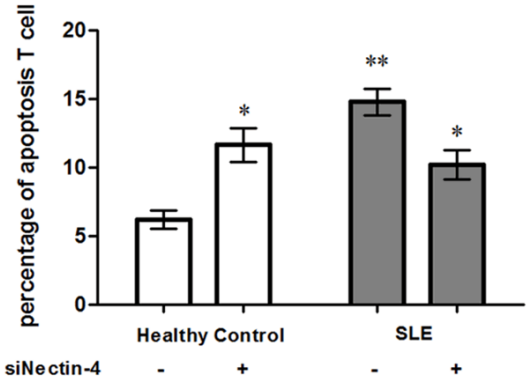

Figure 2. Effect of netcin-4 expression on T cells apoptosis in SLE and HC tissues. **P*<0.05, and ***P*<0.01, compared with HC group.

were transferred onto a polyvinylidencefluoride (PVDF) membrane (Mippore). After that, the membrane was blocked in Tris Buffered Saline Tween (TBST) mixed with 5% non-fat milk for 1 h, and then incubated with rabbit anti-human antibodies (Bcl-2, Bax, Bcl-XL, and caspase-6, 1:100 dilutions, Sigma, USA) overnight at 4°C, followed by incubation with hoseradish peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. PVDF was washed with 1×TBST buffer for 10 min with 3 times. Finally, detection was performed using the development of X-ray after chromogenic substrate with an enhanced CEL (chemiluminescence) method. In addition, glyceraldehyde phosphate dehydrogenase (GAPDH, Invitrogen, USA) was chosen as the internal control.

Statistical analysis

All experiments were conducted for 3 times independently. All data in this study were presented as mean ± standard deviation (SD). Statistical analyses were performed using graph prism 5.0 software (GraphPad Prism, San Diego, CA). ANOVA (analysis of variance) was used to calculate the difference for more than 3 groups. *P*<0.05 was defined as statistically significant.

Results

mRNA and protein expression in T cells

RT-PCR and western blotting analyze were used respectively to detect the mRNA and protein expression of nectin-4 in T cells that isolated

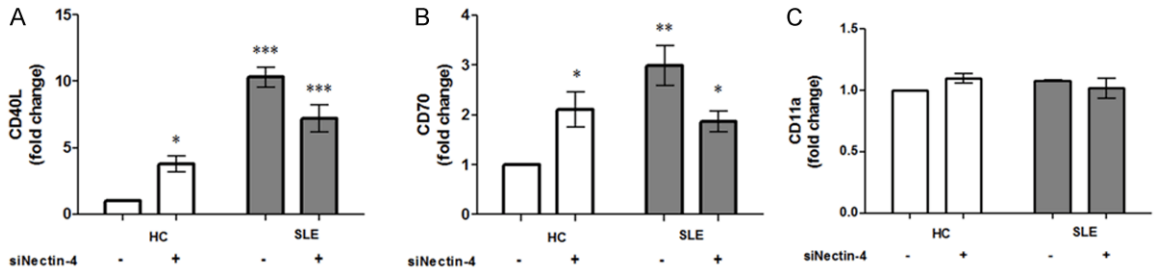

Figure 3. Expressions of SLE development associated proteins expression. A: Nectin-4 slicing significantly increased CD40L expression in HC but decreased CD40L level in SLE; B: Nectin-4 slicing significantly increased CD70 expression in HC but decreased CD70 level in SLE; C: There was no significant difference between nectin-4 expression and CD11a expression among groups. *P<0.05, and **P<0.01, compared with HC group.

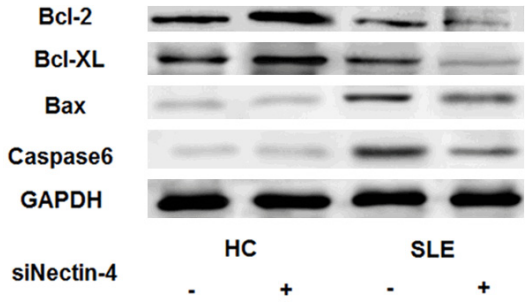

Figure 4. Analysis of cell apoptotic proteins expressions in T cells. Nection-4 slicing decreased Bcl-2, Bcl-XL and caspase-6 expressions but increased Bax expression in SLE group.

from human SLE tissues and HC tissues (**Figure 1**). The results performed that nectin-4 mRNA in SLE tissues was significantly increased compared with that in HC tissues (P<0.01). As well as the protein level among two groups, suggesting that nectin-4 expression may be correlated to SLE.

Cell apoptosis assay

Effects of nectin-4 expression on T cells apoptosis was analyzed using cell apoptosis assay (**Figure 2**). After being transfected with siRNA-nectin-4, apoptotic T cells in HC group were significantly increased (P<0.05), but was significantly declined in SLE group (P<0.01). However, apoptotic T cells in SLE group were significantly increased compared with HC group (P<0.05), suggesting that nectin-4 slicing may be correlated with suppressing T cell apoptosis in SLE.

Expressions of CD40L, CD70 and CD11a in T cells

Western blotting analysis was used to detect whether nectin-4 expression was correlated with CD40L, CD70 and CD11a expression in SLE or not (**Figure 3**). The results showed that nectin-4 slicing could significantly increase CD40L and CD70 expressions in HC group (P<0.05), but significantly declined the two proteins expression in SLE group (P<0.05, **Figure 3A** and **3B**). However, CD40L and CD70 expressions in SLE group were significantly increased compared with that in HC group (P<0.01), indicating that down-regulation of nectin-4 may play roles in affecting CD40L and CD70 expressions in SLE. Besides, the data showed that there was no significant difference for CD11a expression between two groups (**Figure 3C**).

Cell apoptosis associated proteins expressions

Association between nectin-4 expression and cell apoptosis related proteins expressions was analyzed using western blotting analysis (**Figure 4**). In this study, the results showed that Bcl-2, Bcl-XL, Bax and caspase-6 expressions were significantly declined in SLE group when nectin-4 was sliced. However, Bcl-2 and Bcl-XL expression were increased by nectin-4 slicing, but there was no effect of nectin-4 down-regulation on Bax and caspase-6 expressions in HC group.

Discussion

SLE is an organ-specific autoimmune disease which leads to irreversible damage to many kinds of organs and tissues in human body [1, 22]. Increasing evidences suggest that cell apoptosis may play crucial roles in autoimmune response during SLE development and progression. Necin-4 has been wildly proved to be associated tumor metastasis, invasion, and development, but few reports mentioned its role in cell apoptosis. In the present study, we assessed the effects of nectin-4 expression on

immune cells apoptosis in SLE based on the gene slicing method. Our data presented that mRNA expression of nectin-4 was high in SLE cells compared with HC tissues, and CD14L and CD70 levels were high in HC group than that in SLE group. Moreover, nectin-4 down-regulation could significantly suppress cell apoptosis, as well as significantly increase CD40L and CD17 expressions. Furthermore, nectin-4 slicing showed could suppress the expression of cell apoptotic proteins expressions including Bcl-2, Bax, Bcl-XL, and caspase-6.

The potential effects of nectin-4 expression have been reported in many diseases such as considered as a biomarker for breast cancer, prostate cancer and lung cancer [14]. Our results showed that mRNA expression of nectin-4 in T cells from SLE group was significantly higher than that in HC group, suggesting that nectin-4 overexpression may play some certain roles in SLE self-reaction mechanism. Consequently, the effects of nectin-4 expression in SLE were analyzed using gene slicing method.

Previous evidences have demonstrated that the over-activation of $CD4^+$ T cells played important parts in SLE pathogenesis through overexpression of CD40L, CD70, and CD14a compared with the healthy persons [23, 24]. CD40L is a type-II membrane glycoprotein that belongs to the TNF superfamily, and is expressed on the surface of antigen-presenting cells including B lymphocytes, macrophages, and dendritic cells [25], while CD70 (the ligand for CD27) that play pivotal roles in stimulating T cells activation and B cells activation [26]. It has been proved that both CD40L and CD70 were overexpressed in SLE PBMCs compared with the healthy persons [27, 28]. On the other hand, Patel and his colleagues have proved that nectin-4 has been identified as a receptor for MV viral entry in cancer, while MV replication in dendritic cells has been found following engagement of CD40 through interaction with T cells stably expressing CD40L [29], which suggests the correlation between CD40L expression and nectin-4 [30]. Coincidence with former evidences, our results showed that expressions of CD40L and CD70 were high in SLE tissues but were all low in healthy T cells, however, CD40L and CD70 were all significantly declined when nectin-4 was sliced, suggesting that nectin-4 down-regulation may inhibit CD40L and CD70 expression SLE T cells, and then suppressed the activation of B or T cells in SLE. Interestingly, our data found that there was no significant difference for CD11a expression between two groups. Former evidence showed that CD11a was over-expressed in SLE PBMCs [28]. Associations between necint-4 and CD11a in SLE has not been fully discussed, therefore, we speculated that there may no correlation between nectin-4 and CD11a expressions during SLE development. However, the mechanism may be assessed in our future study.

Meanwhile, our data performed that nectin-4 down-regulation could suppress T cell apoptosis and suppress the cell apoptotic-related proteins such as Bcl-2, Bcl-XL, Bax-2 and caspase-6 in SLE, suggesting the important role of nectin-4 expression in T cell apoptosis. It has been demonstrated that cell apoptosis play crucial roles in SLE pathogenesis. Activation of caspase-6 and overexpression of Bcl-2 are the contributors for accelerating cell apoptosis [31]. Association between nectin-4 expression and cell apoptotic-related proteins of Bcl-2/Bax and caspase-6 has not been fully discussed. However, Bcl-2 and Bcl-XL were increased in SLE, and caspase-6 [32]. Based on our results, we speculated that nectin-4 slicing may play an inhibit role in SLE T cell apoptosis through suppressing the expressions of Bcl-2/Bax and caspase-6.

In conclusion, the data presented in this study suggests that nectin-4 expression may play pivotal roles in suppressing cell apoptosis during SLE development through Bcl-2/Bax signal pathway. Nectin-4 down-regulation inhibits cell apoptosis via suppressing the expressions of Bcl-2/Bax and caspase-6 and restraining $CD4^+$ T cells expressed proteins of CD40L and CD70 expression in SLE. This study provide basis for the potential application possibility for nectin-4 in SLE treatment. However, further studies are still needed to deep the investigate mechanism.

Disclosure of conflict of interest

None.

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