Role of pathogenic auto-antibody production by Toll-like receptor 9 of B cells in active systemic lupus erythematosus


Objectives. Toll-like receptor 9 (TLR9) is a pattern-associated receptor functioning in innate immunity that may be involved in the recognition of self-antigens and the production of pathogenic auto-antibodies. Therefore, we examined the expression of TLR9 in systemic lupus erythematosus (SLE) to determine whether TLR9 is involved in the production of pathogenic auto-antibodies.

Methods. B cells were collected from patients with active SLE, and subjected to analysis of the TLR9 molecule using flow cytometry. Subsequently, TLR9 mRNA expression was measured by reverse-transcriptase polymerase chain reaction. SLE B cells were stimulated with CpG-ODN, and subsequent cytokine and anti-dsDNA antibody production was measured by enzyme-linked immunosorbent assay.

Results. The expression and mRNA level of TLR9 on B cells were up-regulated in SLE patients, and SLE disease activity index (SLEDAI) and CH50 were correlated with TLR9 expression on CD20+ B cells. Moreover, TLR9–CpG interaction enhanced the production of anti-dsDNA antibody and IL-10.

Conclusions. The present study demonstrated that higher expression of TLR9 on peripheral blood B cells from patients with active SLE was significantly correlated with CH50 and SLEDAI to TLR9, and induced the production of anti-dsDNA antibody and IL-10. These results suggest that an abnormality of innate immunity plays a crucial role in the pathology of SLE, and that blockade of CpG–TLR9 interaction may be a new therapeutic approach for SLE.

Keywords: SLE, B cells, Toll-like receptor 9, CpG-DNA, Anti-dsDNA antibody, IL-10, SLEDAI, CH50, Innate immunity.

Introduction

Autoimmune diseases are associated with various immunological abnormalities, such as an increased number of activated B cells and auto-antibody production, and in patients with systemic lupus erythematosus (SLE) B-cell hyperactivity is a central feature [1, 2]. Although it is considered that autoimmune disease is mainly related to acquired immunity, a recent study has shown that some abnormality of the innate system may also be involved [3].

The Toll-like receptor (TLR) contributes to innate immunity, and its gene has been cloned in humans. The human TLR has 10 subunits, each recognizing a different causative factor [4, 5]. When these causative factors react with TLR, MyD88 present in the cytoplasmic compartment is activated, and nuclear localization of nuclear factor-kappa B is promoted. This in turn leads to production of inflammatory cytokines such as tumour necrosis factor-α or interleukin-6 (IL-6) [4]. TLR9 is present only in plasmacytoid dendritic cells (pDCs) and B cells, and is a receptor for microbial CpG-DNA [5]. It recognizes a single-stranded ‘CpG motif’, consisting of unmethylated CpG dinucleotides flanked by particular bases and it is not generally present in mammalian cells, including those of humans [6, 7]. However, our previous study has shown that methylation of DNA is decreased in patients with SLE, suggesting that CpG-DNA is related to the SLE pathogenesis [8]. Furthermore, a recent study has shown that anti-DNA auto-antibody production is impaired in TLR9 gene-knockout lupus-prone mice [9]. However, it has not been clarified whether TLR9 contributes directly to the pathogenesis or clinical features of human SLE.

B cells play an important role in auto-regulation of humoral immune responses, and B cells from patients with active SLE and from lupus-prone mice have an intrinsic tendency to over-react to immunological stimulation during anti-genomic challenge. This has led to a novel hypothesis regarding therapeutic approaches that might interfere with the development and progression of SLE [10]. Since TLR9 recognizes unmethylated CpG motifs characteristic of bacterial DNA and is involved in the immediate response to a wide range of microbial organisms, B cells may, in addition to their role as antibody-producing cells during the adaptive immune response, respond to pathogens in a manner associated with the innate branch of immune defence. Inducible expression of TLR9 in B cells may thus provide a link between the innate and adaptive branches of the immune system [11].

Furthermore, it has been reported that CpG-ODN can enhance TLR9 mRNA expression in activated human B cells [12]. In addition, it has been reported that TLR9 controls anti-DNA auto-antibody production in murine lupus [9].

In the present study, we examined the level of TLR9 expression on peripheral blood B cells from patients with active SLE and its correlation with clinical parameters. We also investigated whether CpG ligation of TLR9 is involved in pathogenic auto-antibody production in active SLE.

Patients and methods

Patients

We obtained samples of peripheral blood from 19 SLE patients (Table 1), age- and sex-matched healthy donors and patients with rheumatoid arthritis (RA) as a disease control. Informed consent was obtained from all patients and healthy donors. Eleven blood samples were drawn from SLE patients before treatment. Eight patients had been receiving steroid therapy with prednisolone at 5–15 mg/day. All SLE patients fulfilled the 1997 revised criteria of the American College of Rheumatology (ACR) [13]. Disease activity in the SLE patients was assessed using the ACR SLE disease activity index (SLEDAI) [14].

Cell preparation

For isolation of peripheral blood B cells, 5 ml of peripheral blood was labelled with 40 μl of anti-human CD20 antibody coupled...
with colloidal paramagnetic microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany) and isolated using AutoMACS (Miltenyi Biotech). B cells were isolated at a purity of >93%, as assessed by flow cytometric analysis.

**TLR9 mRNA analysis**

Total RNA was isolated from 1 x 10^6 B cells using an RNeasy Mini kit (QIAGEN, Valencia, CA, USA). Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in a single 50-μl reaction volume containing 25 μl of One-step RT-PCR SYBR SYBR Green Master Mix (Applied BioSystems, Foster City, CA, USA) with 0.25 μM of forward and reverse primers for TLR9 (5'-ATGGGTTTCGTCCGC-3'; 3'-GAAGAGATGCCGCAGGACGATCGTCGGGGG-3') and β-actin (5'-GGACTTGCCGAGAAGAGATGCCGCAGGACGATCGTCGGGGG-3' and 3'-AGCCTGTTTGGCGTACA) and 5 μg of RNA. TLR9 was measured with an ABI PRISM 7500 Sequence Detection System (Applied BioSystems). TLR9 levels were normalized to β-actin for each.

**Flow cytometric analysis**

We carried out cell surface staining by adding 10 μl of anti-human CD20, CD80 and CD86 antibody (PharMingen, San Diego, CA, USA) conjugated with PE to the B cells (5 x 10^7/ml), and incubating them for 30 min on ice. For intracellular staining, we used Intraprep™ (Beckman Coulter, Miami, FL, USA) for fixation and permeabilization. Then, intracellular staining was performed with 5 μl of anti-human TLR9 antibody (26C593) (Imgenex, San Diego, CA, USA) conjugated with fluorescein isothiocyanate (FITC), and incubated for 30 min in accordance with the manufacturer's instructions. Two-colour analysis was then performed using FacsAria (Becton Dickinson, Mountain View, CA, USA).

**Detection of anti-dsDNA antibody and cytokine**

Isolated SLE B cells differentiated for 3 days were re-plated in 96-well round-bottom plates at 1 x 10^5 cells/well, then stimulated with medium, ODN2006 (5 μM) and ODN2216 (5 μM). Complete medium (CM) consisted of RPMI-1640 containing L-alanyl-L-glutamine dipeptide supplemented with 5-10% fetal calf serum (FCS; ICN Biomedicals, Inc.), 5 x 10^{-5} M 2-ME (Sigma) and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, GIBCO BRL). The stimulatory CpG-ODNs were ODN2006 (5'-TCGTCTGTTTGTGGTTCGTT-3') and ODN2216 (5'-GGGGACGATCGTCGGGGG-3') [15].

Anti-dsDNA antibody (Bio-Rad) and cytokine (IL-10, R&D Systems, Minneapolis, MN, USA) concentrations were quantified by enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions.

**Statistical analysis**

Statistical analysis was performed using the Mann-Whitney U-test and Pearson’s correlation coefficient. Statistical significance was defined as a P-value of <0.05.

**Results**

**Expression of TLR9 on B cells from active SLE**

We first examined the expression of TLR9 mRNA on B cells from patients with active SLE, and found that this was higher than on B cells from healthy donors and disease controls (P < 0.01) (Fig. 1A and B). We then examined the surface expression of TLR9 on B cells, but none was detected on B cells from any of the three groups. We then examined intracellular expression of TLR9 in B cells, and found that this was strikingly and significantly (P < 0.01) higher in CD20+ B cells from SLE patients than in those from healthy controls and disease controls by mean fluorescence intensity (MFI) (Fig. 1C and D).

Next, we examined the relationship between TLR9 expression on CD20+ B cells from patients with active SLE and disease activity. The intracellular expression of TLR9 was significantly (P < 0.05) lower in patients whose disease activity had decreased after treatment (Fig. 2). However, the expression of TLR9 mRNA on B cells from patients with active SLE was not significantly decreased.

Moreover, we investigated the relationship between the expression of TLR9 on B cells from patients with active SLE and laboratory parameters (SLEDAI, anti-dsDNA antibody, CH50), and found significant correlations with SLEDAI and CH50 by MFI (Fig. 3A) (P < 0.01, R = 0.929) and CH50 by MFI (Fig. 3B) (P < 0.05, R = 0.631). However, there was no significant correlation between the expression of TLR9 mRNA and anti-dsDNA antibody.

**CpG induces anti-dsDNA antibody and IL-10 production**

We next investigated whether TLR9-CpG interaction was related to SLE activity and its activation. ODN2006 enhanced the expression of TLR9 on B cells from patients with active SLE, and was correlated with production of anti-dsDNA antibody (Fig. 4A) (P < 0.05). Moreover, IL-10 production was significantly increased (Fig. 4B; P < 0.05) by ODN2006 ligation.

**Discussion**

In the present study, we demonstrated that higher expression of TLR9 on peripheral blood B cells from patients with active SLE was significantly correlated with CH50 and SLEDAI to TLR9, and induced anti-dsDNA antibody and IL-10 production by TLR9-CpG ligation. Although a recent study has shown that apoptosis via TLR9 on B cells may play a role in the pathogenesis of SLE [16], our present study demonstrated that production of anti-dsDNA antibody is related to SLE pathogenesis through ligation of CpG to TLR9 on B cells.

Since the TLR9 molecule is produced by mRNA and usually exists in the cytoplasm, it is difficult to examine by flow cytometric analysis. Using cytoplasmic staining and RT-PCR, the present study confirmed that the expression of TLR9 was
increased on B cells from patients with active SLE, and that this was correlated with disease activity. These results suggest that innate immunity might play a role in the pathogenesis of SLE through TLR9 on B cells. TLR induced the expression of co-stimulatory molecules, as represented by CD80 and CD86, and the expression of TLR9 was correlated with that of CD86 on B cells (data not shown). Since the expression of CD86 on B cells is commonly increased in active SLE, it is possible that the increased expression of TLR9 induces the initial activation of B cells.

It has been suggested that TLR9 expression on B cells plays a role in SLE pathogenesis through the induction of anti-dsDNA antibody. As described previously, the ligand of TLR9 is CpG that is a product of abnormal methylation of DNA, and commonly recognized in SLE. A previous study has shown that methylation of DNA is decreased in SLE patients, suggesting that CpG-DNA is related to the pathogenesis of SLE [8]. Our present study showed that production of anti-dsDNA antibody occurred through ligation of CpG to TLR9 on B cells from patients with active SLE. Although the mechanism responsible for production

**Fig. 1.** Expression of TLR9 on B cells from active SLE patients and healthy donors. (A) Expression of TLR9 mRNA in active SLE patients was significantly higher than in healthy individuals (**P < 0.01). Bars show the mean ± s.d. (B) TLR9 or β-actin samples (10 μl) were used as template for RT-PCR. The products were electrophoresed on 2% Tris base, boric acid and EDTA (TBE) agarose gels containing 0.5 μg/ml ethidium bromide and visualized under ultraviolet (UV). Negative control was 50 μl of total RNA isolated from 1 x 10^6 CD4^+ T cells. (C) Representative staining pattern of TLR9 on peripheral B cells from a healthy donor, a patient with active SLE and a patient with RA. Peripheral blood mononuclear cells were stained with monoclonal antibodies against TLR9 and CD20. (D) TLR9 molecule in B cells from patients with active SLE and healthy controls was analysed by flow cytometry. The MFI of TLR9 in active SLE patients was significantly higher than that in healthy donors and disease controls (**P < 0.01).

**Fig. 2.** Decreased expression of TLR9 on B cells from active SLE patients after treatment. Post-treatment expression of TLR9 on B cells from active SLE patients. The MFI of TLR9 in post-treatment SLE patients was significantly lower than that before treatment (**P < 0.01). Pre-Tx: pre-treatment; Post-Tx: post-treatment.
of anti-dsDNA antibody is heterogeneous, we think that the interaction between CpG and TLR9 plays a pivotal role. The production of anti-dsDNA antibody may not be induced directly by CpG, but may require various processes including the differentiation of B cells. In order to investigate this issue, we examined various cytokines in the culture supernatant. Surprisingly, we found that IL-10 production from patients with active SLE B cells was increased by stimulation with CpG. Although it has recently been established that IL-10 is a regulatory cytokine, it was originally reported to be a factor inducing antibody production in certain diseases, especially SLE. Indeed, injection of an IL-10 blocking antibody has been reported to suppress disease activity in lupus mice [17]. Furthermore, it has been reported that B cells from SLE patients produce IL-10 [18], and that this cytokine induces the production of anti-dsDNA antibody [17]. Abnormality of DNA methylation easily induces the transcription of mRNA from DNA, and it has been shown previously that this abnormality commonly occurs in SLE [8]. This finding suggests that the abnormal methylation induces increased transcription of the TLR9 gene, and that the CpG produced as a result reacts with the highly expressed TLR9.

The role of innate immunity in autoimmune disease is still unclear. We have demonstrated that expression of TLR9 on B cells is increased in human SLE, and that this increased expression is closely related to disease activity. We suggest that abnormality of innate immunity also plays a crucial role in the pathology of SLE, and that blockade of CpG–TLR9 interaction may be a promising new therapeutic approach for SLE.

Rheumatology key messages
- The expression and mRNA level of TLR9 on B cells up-regulated in SLE patients.
- TLR9 expression is strongly associated with activity of SLE.
- In function, SLE B cells were activated interacting to CpG-DNA.

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Expression of TLR9 in SLE

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