Preferential expression of B7.2 (CD86), but not B7.1 (CD80), on B cells induced by CD40/CD40L interaction is essential for anti-DNA autoantibody production in patients with systemic lupus erythematosus


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Abstract

Objective

B7 (CD80/CD86) molecules are over-expressed in patients with SLE. However, it is not clear whether CD80/CD86 molecules are involved in the pathogenic autoantibody production specifically or in the polyclonal antibody production in human SLE. The present study was carried out to characterize B7 molecules on B cells in autoantibody production.

Methods

Expression of costimulatory molecules was analyzed by RT-PCR and two-color immunofluorescence staining. Purified B cells were co-cultured with T cells in the presence of anti-costimulatory molecule antibody.

Results

Excessive expression of CD86 and CD80 molecules was evident on freshly isolated B cells in patients with SLE. Normal B cells did not express CD86 molecules spontaneously and expressed it after co-culture with activated T cells. CD86 expression on normal and SLE B cells induced by the activated T cells was inhibited by the addition of anti-CD40L into the cell culture. Furthermore, CD40L expression on T cells upon activation was enhanced in SLE patients. Anti-DNA antibody production by SLE B cells in the presence of activated T cells was markedly inhibited by anti-CD86, but not anti-CD80. Anti-CD86 treatment inhibited polyclonal Ig and anti-SS-A antibody production of SLE B cells, suggesting the preferential involvement of CD86 in polyclonal antibody production.

Conclusion

SLE T cells express CD40L excessively, and the CD40/CD40L pathway is involved in the CD86 over-expression of SLE B cells; thus T cell abnormality is at least partially involved in B cell hyperactivity. Enhanced CD86 expression of B cells by CD40L is essential for polyclonal antibody production.

Key words

CD40L, B7, SLE, anti-DNA autoantibody.

CD86 on B cells and polyclonal antibody production / H. Nagafuchi et al.

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Received on May 29, 2002; accepted in revised form on November 25, 2002. © Copyright CLINICAL AND EXPERIMENTAL RHEUMATOLOGY 2003.

Introduction

Systemic lupus erythematosus (SLE) is characterized by polyclonal B cell activation with the production of various autoantibodies (1,2). Although intrinsic B cell abnormalities have been noted, excessive T-B cell interactions have also been implicated in autoantibody production in SLE (3).

T cell activation requires the interaction of the T cell receptor with the MHC molecule complexed with the antigen in the presence of additional signaling through costimulatory molecules. CD28-CD80/CD86 and CD40-CD40L interactions appear to play pivotal roles. Because CTLA4Ig, a fusion protein that blocks B7/CD28 interactions, induces antigen-specific T cell unresponsiveness (4). CTLA4Ig has been used successfully in vivo to inhibit B cell differentiation into Ig-secreting cells (5). CD40L is expressed on activated but not on resting helper T cells. CD40L binds to CD40 on B cells and other APC cells and provides a critical stimulus for B cell proliferation, Ig production, isotype switching, and up-regulation of other costimulatory molecules, including B7 (6).

SLE lymphocytes overexpress costimulatory molecules on their surface (7-14). It has been reported that CD86 is overexpressed on B cells in patients with SLE. However, their roles in B cell activation and pathogenic anti-DNA autoantibody production remain to be fully clarified. In particular the relationship between CD40/CD40L and CD80/CD86 pathway in pathogenic antibody production is not clear (14-16). The present study was carried out to characterize the costimulatory molecules of T cells and B cells essential for pathogenic autoantibody production. Our study suggests that aberrant CD86 expression on SLE B cells through the CD40/CD40L pathway is implicated in polyclonal B cell activation, including the anti-DNA antibody production in patients with SLE.

Patients and methods

Patients and healthy donors

We studied 32 patients (1 male and 31 females) who fulfilled the 1982 revised criteria for the classification of SLE by the American Rheumatism Association (17). The mean age ± SD of these patients was 33.8 ± 10.4 yr. (range 14-55). Thirty healthy volunteer blood donors served as control subjects; their mean age ± SD was 36.2 ± 6.3 yr. (range 25-52). Patients who had been treated with intermediate to high doses of corticosteroids (more than 15 mg prednisolone/day) or immunosuppressive therapy were excluded from the study cohort. Informed consent was obtained from the patients and all procedures were carried out in accordance with the Helsinki Declaration of 1975/83.

Cell separation and cell cultures

PBMC were obtained by Ficoll-Hypaque centrifugation of heparinized blood from patients with SLE and normal healthy adults. Freshly isolated PBMC were suspended in RPMI 1640 medium containing 10% FCS, penicillin and streptomycin (Life Science, Tokyo, Japan). T cells and B cells were purified by means of an SRBC rosette technique, followed by depletion of monocytes by a Petri dish adherence procedure (18). B cells were further purified using magnetic beads coated with anti-CD3 (UCHT-1; Immunotech, Marseille, France) and anti-CD56 mAbs (N901 (NKH-1); Immunotech) according to the manufacturer’s instructions. Cell purity was analyzed by staining B cells with PE-conjugated anti-CD20 mAb: 99.7% of the purified cells were B cells with < 0.2% being CD3+ cells. T cells were treated to deplete NK cells by anti-CD56 mAb.

Antibodies

Purified mAb to human CD40L (TRAP-1), phycoerythrin (PE) conjugated anti-human CD40L, as well as isotype-matched control IgG1 antibody and PE-control IgG1 were obtained from Immunotech (Marseille, France). Anti-CD80 and anti-CD86 mAbs were from Becton Dickinson Japan (Tokyo), FITC-conjugated mAbs to human CD20 (B9E9) and CD3 (UCHT1), and control FITC-mAb were also from Immunotech. For flow cytometry, all mAbs were used at optimal saturating concentrations as recommended by the manufacturer.
Flow cytometry
The cells were harvested and stained as described (19). Briefly, the cells were washed in PBS/5% FCS/0.05% sodium azide (FACS buffer) and incubated with 10 µg of human IgG (Sigma Chemical Co., St. Louis, MO) for 30 min at 4°C to block Fc receptors. Cells were washed to remove excess IgG and were double-stained with either FITC-conjugated mAb against human CD80, CD86, CD40L or FITC-control IgG mAb and PE-conjugated mAb against CD20, CD3 or PE-control IgG mAb for 30 min at 4°C. Cells were washed twice, re-suspended in FACS buffer, and analyzed by flow cytometry.

Cell cultures
To analyze the CD40L expression on CD3+ T cells, the T cells (1 x 10^6/ml) were stimulated in complete RPMI medium with 5 ng/ml PMA (Sigma) and 1 µg/ml PHA (Sigma) for various periods at 37°C. Thereafter, their CD40L expression was analyzed. To induce CD80 and CD86 expression on B cells, the B cells (1 x 10^6/ml) were co-cultured for 24 hours with autologous T cells which had been either kept unstimulated or stimulated with PHA + PMA for 6 hours in advance.

To induce IgG autoantibody production, the highly purified B cells (0.1 x 10^6 cells in 200 µl/microwell) were cultured in the presence of either anti-CD 80, anti-CD86, anti-CD80+anti-CD86, CTLA4Ig (kindly provided by Dr. PS Linsley, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) or isotype matched control IgG1 in complete RPMI with 10% FCS for 6 d at 37°C (17,20). The concentration of the antibodies including CTLA 4Ig was 10 µg/ml. In the preliminary experiments, we found that the antibodies at this concentration were sufficient to inhibit normal T cell responses. Baseline production of autoantibodies secreted by lupus B cells was measured by cultivating enriched B cells alone (0.1 x 10^6 cells/microwell). At the end of 6 days, production of IgG autoantibodies to double-stranded DNA and SS-A antigen was quantitated by ELISA, and total polyclonal IgG was measured by ELISAspot assays (20, 21).

ELISA and ELISAspot assay
IgG class autoantibodies to DNA and SS-A, as well as total polyclonal IgG, were measured as described (17, 20). Standard curves for anti-DNA ELISA were obtained by serial dilutions of one lupus patient’s serum that had a high anti-DNA autoantibody titer (20). Similarly, serum with a high anti-SS-A autoantibody titer was used. The absorbance value (at 405 nm) of a 1/1000 dilution of the reference patient’s serum was considered to be equivalent to 1 U/ml of anti-DNA or anti-SS-A autoantibodies, and the standard curve with the same reference serum was generated with each assay (20).

RNA extraction, reverse transcription and PCR reaction
CD80 and CD86 cDNA were amplified by a PCR based technique (21). In brief, the total RNA of the cells was reverse transcribed and CD80 and CD86 cDNA was amplified by PCR. The primers for CD80 message amplification were 5-ttggggcgtgtctctgtgt-3 (sense) and 5-ccaggagagttgaggca-3 (antisense). Those for CD86 were 5-gctctgacattattacactc-ac-3 (sense) and 5-tttctctattcccttgagg-3 (antisense). PCR amplification of the cDNA was performed using a 35-cycle program consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The DNA fragments were separated on 1.5% agarose gels and visualized by staining with ethidium bromide (21). The internal sequences of the amplified products were confirmed by a TA cloning system and subsequent DNA sequencing. We adopted RT-PCR analysis using limiting dilutions of cDNA to accurately compare the relative amounts of CD80 and CD86 mRNA expression in different samples, as previously reported (22, 23). In the limiting dilution RT-PCR technique, varying dilutions of cDNAs were subjected to PCR amplification. We performed exactly the same 3 PCR reactions, except for the amount of cDNA added to the reaction tubes, in one sample. The amounts of the total cDNA used for PCR amplification were 10%, 5% and 2%, respectively. cDNA from each sample was also subjected to PCR amplification for expression of the housekeeping gene, β-actin (a sense primer 5-gtggggegcccccaggcac-3, and an antisense primer 5-ctctaatgtcaagcagcactg-3). A 1 kb DNA ladder from Life Technologies was used as a DNA marker. Results shown are representative of 8 independent experiments.

Statistical analysis
The irregularly distributed data for some subject groups were compared by the Mann-Whitney U test. A p value less than 0.05 was judged to be statistically significant.

Results
We first conducted two-color immunofluorescence analysis using anti-CD20 and anti-CD80 or anti-CD86. The vast majority of normal B cells did not express CD80 or CD86 spontaneously (Fig. 1A). In contrast, SLE B cells expressed CD80 and CD86 brightly (Fig. 1A). This finding was confirmed by the semi-quantitative RT-PCR method (22, 23). SLE B cells expressed CD80 mRNA and CD86 mRNA just after isolation, after a 12-hour culture in vitro, and after a 24-hour culture (Fig. 1B). Normal B cells did not express the mRNAs. We carried out a complete analysis on 22 normal individuals and 32 SLE patients in this experiment, and found that both CD80 and CD86 expression were enhanced in patients with SLE (Fig. 1C).

In order to obtain insight into the mechanisms responsible for the enhanced CD80 and CD86 expression of B cells, normal B cells were co-cultured for 24 hours with autologous T cells and the expression was analyzed. CD80 expression was not significantly affected by co-culturing with unstimulated T cells or activated T cells by PHA + PMA for 6 hours in advance. CD86 expression was not affected by the co-culture with unstimulated T cells. CD86 expression was up-regulated only when cultured with activated T cells, and the up-regulation of CD86 expression was abrogated by anti-CD40L mAb. It was thus suggested that normal B cells up-regulated CD86, but not CD80, expression through CD40/CD40L interaction.

We next compared the induction of CD86 expression of SLE B cells with
 gated CD20+ cells are shown. The figure in each panel shows % positive cells among the B cells.

**CD86 mRNA.**

(B) Limiting dilution RT-PCR analysis of CD80 and CD86 mRNA expression of SLE B cells. Purified B cells from SLE patients were immediately recovered (0 h), or cultured in medium for 12 and 24 hours. The B cells were subjected to limiting dilution RT-PCR analysis to compare their mRNA expression. Normal B cells did not express CD80 and CD86 mRNA. SLE B cells expressed CD80 and CD86 mRNA spontaneously. Even after 24 hours of culturing, SLE B cells sustained expression of CD80 and CD86 mRNA.

(C) Two-color analysis was conducted in 22 normal individuals and 32 SLE patients. CD80 and CD86 expression were enhanced in the B cells of patients with SLE. Mean ± SEM of the groups is shown.

**Discussion**

T cells and B cells interact with each other to elicit immune responses. The roles of costimulatory molecules have been thoroughly studied in T cell activation (14). The CD28/CD80 or CD86 interaction is considered to be one of the most important costimulatory signals for T cell activation. However, studies addressing the roles of the CD80 and CD86 molecules in autoantibody secretion in patients with SLE are rather scanty. In this study we found the following:

1. Freshly isolated SLE B cells expressed CD80 and CD86 molecules, while normal B cells little expressed the molecules without stimuli.
2. The expression of CD86 on B cells was enhanced by co-culturing with activated T cells, and the enhancement was abrogated by anti-CD40L mAb in patients with SLE.
3. In SLE patients, anti-DNA antibody production in the presence of activated T cells was inhibited by the addition of anti-CD86 mAb, but not anti-CD80 mAb.

Our results are in accordance with others showing that CD86+ cell levels are significantly higher in patients with SLE (9). We earlier analyzed CD80 and CD86 expression in patients with rheumatoid arthritis (RA) (24). The enhanced expression of CD80 was noted on some CD4+ T and LeuM3+ cells in patients with RA. Similarly, CD86 was overexpressed on LeuM3+ cells. There have been several similar reports describing elevated CD80/86 expression in patients with RA (25). CD80/86 overexpression was reported in patients with liver diseases (26-28). Thus, enhanced expression of CD80/86 is not SLE-specific. Similarly, there that of normal B cells (Fig. 2B). SLE B cells were co-cultured with autologous T cells for 24 hours. After the 24-hour culture of purified B cells alone, 41.0 ± 26.1% of SLE B cells (n=7; mean ± SD) and 16.7 ± 3.9% of normal B cells (n=6) expressed CD86. When SLE B cells were cultured with autologous activated T cells, CD86 expression was up-regulated and the up-regulation of CD86 was abrogated by the anti-CD40L mAb. Thus, CD40L/CD40 interaction was at least partly responsible for the enhanced CD86 expression of SLE B cells.

We then studied CD40L expression of SLE T cells (Fig. 3). SLE T cells were stimulated with PHA + PMA, and analyzed by two-color staining employing anti-CD3 and anti-CD40L. Flow cytometric analysis showed that SLE T cells expressed CD40L excessively in response to PHA + PMA. These results suggest that excessive T cell function was at least partly responsible for the CD86 over-expression of B cells in patients with SLE.

In order to elucidate a role of the excessive CD86 expression on SLE B cells in autoantibody production, we measured the polyclonal IgG production of SLE B cells. As shown in Fig. 4A, freshly isolated normal B cells alone and normal B cells combined with autologous T cells did not induce Ig production. In contrast, SLE B cells supplemented with autologous T cells induced Ig production efficiently, suggesting that the T-B cell interaction led to the polyclonal antibody production in patients with SLE. Addition of anti-CD80 mAb into the cell culture did not inhibit polyclonal IgG production significantly (Fig. 4B). Anti-CD86 mAb and CTLA4Ig inhibited Ig production, suggesting that CD28/CD86 interaction plays an important role in the polyclonal IgG production in SLE patients. Similarly, anti-CD86 mAb inhibited the anti-DNA and anti-SS-A antibody production of SLE B cells (Fig. 4C). Thus, the CD86 molecule expressed on B cells was involved in the polyclonal antibody secretion by B cells.
are several reports describing the dysregulation of CD86 expression by activated T cells and/or the CD40 ligand (29-31). Thus, abnormal CD80/86 expression is evident in patients with SLE and other immune-associated disorders. We found that expression of CD40L on SLE T cells was excessive and prolonged compared with normal T cells. CD40L binds to CD40 on B cells and provides a critical stimulus for B cell proliferation, Ig production, isotype switching, and the up-regulation of other co-stimulatory molecules, including B7 (6). Indeed, we found that the enhanced expression of CD40L on T cells was at least in part responsible for the excessive expression of CD86 on SLE B cells.

It would be intriguing to study whether a representative autoantigen, the nucleosome, may stimulate SLE T cells to overexpress CD40L (32). In a preliminary experiment we found that peripheral blood T cells from Japanese SLE patients failed to show any response to nucleosomes. Thus, at present we are forced to conclude that nucleosomes do not induce increased CD40L expression on T cells in patients with SLE. This could be due to an extremely low frequency of nucleosome-specific T cells in SLE patients, even though the nucleosome-specific T cells themselves were hyper-reactive to the autoantigen. The response to nucleosomes after establishing nucleosome-specific T cell clones in patients with SLE therefore must be more thoroughly studied.

The relationship between the B7/CD28 and CD40/CD40L pathways in B cell activation is not completely understood. B7 co-stimulation appears to be augmented by CD40 signaling in part by increasing B7 expression on antigen-presenting cells (APCs) (33). The B7/CD28 and CD40/CD40L pathways are clearly interrelated in lymphocytes of normal individuals and patients with autoimmune diseases (34). Anti-costimulatory molecule mAbs have prolonged therapeutic effects in animal models of SLE (35, 36). This method may eventually prove beneficial in human autoimmune diseases, as well (37, 38).

In murine lupus the production of anti-DNA antibody seems to be dependent on CD86 costimulation. In one study the injection of CTLA4Ig into NZB/NZWFl mice, which neutralizes both CD80 and CD86, blocked autoantibody production and prolonged life (36). Furthermore, in MRL-lpr/lpr mice treatment with anti-CD86 but not with anti-CD80 inhibited anti-DNA antibody production (39). The T cells may have a defect causing the enhanced and prolonged expression of CD40L (40). This defect may provide abnormal costimulatory signaling to the pathogenic
autoantibody producing B cells in SLE. It has been reported that blockade of the CD40/CD40L pathway is preferentially involved in the production of pathogenic anti-DNA autoantibody (41-43). We found that the CD28/CD86 pathway instead participated in polyclonal B cell hyperactivity in patients with SLE. Interruption of the CD28/CD86 pathway may be beneficial in treating patients with autoantibodies against a broad range of antigen specificities.

References