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Serum concentrations of soluble 4-1BB and 4-1BB ligand correlated with the disease severity in rheumatoid arthritis

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Abbreviations: BD, Behcet's disease; RA, rheumatoid arthritis; s4-1BB, soluble 4-1BB; s4-1BBL, soluble 4-1BB ligand; SLE, systemic lupus erythematosus

Abstract

Rheumatoid arthritis (RA) is a multifactorial autoimmune disease whose etiopathogenesis is not well understood. Although soluble (s) forms of 4-1BB (s4-1BB) and 4-1BB legand (s4-1BBL) have been detected in the sera of RA patients, their significance is not known. We compared the serum levels of s4-1BB and s4-1BBL in RA patients with those in systemic lupus erythematosus (SLE) and Behcet's disease (BD) patients. Serum levels of s4-1BB and s4-1BBL were significantly higher in RA patients compared with healthy controls, SLE or BD patients, and the abundance was correlated with disease severity in patients with RA. The serum levels of s4-1BB in RA patients were inversely corroborated with 4-1BB expression levels on activated T lymphocytes. In addition, there was a correlation between serum levels of s4-1BB and s4-1BBL. The augmented secretion of s4-1BB and s4-1BBL levels into the serum may reflect the clinical symptoms of RA and levels of s4-1BB and s4-1BBL in sera at the time of diagnosis may be indicative of the severity and outcome of RA.

Keywords: autoimmune diseases; Behcet syndrome; biological markers; lupus erythematosus systemic; rheumatoid arthritis; serum

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by radiographic joint destruction, severe functional deterioration, and work disability (American College of Rheumatology Ad Hoc Committee on Clinical Guidelines, 1996). It is thought to involve autoimmune recognition of self-antigens by autoreactive T lymphocytes. The cause of this breakdown of tolerance to self-antigens is not known, but probably involves impairment of apoptosis of potentially pathogenic and autoreactive T lymphocytes (Pender, 1998). As a consequence, the elimination of autoreactive cells and down-regulation of lymphocyte responses are defective.

4-1BB (CD137), a member of the tumor necrosis factor (TNF) receptor family, is expressed on activated T lymphocytes (Kwon and Weissman, 1989; Schwarz *et al*., 1995). It generates either co-stimulatory signals leading to T cell activation and proliferation, or death signals (Alderson *et al*., 1994), and may promote tumor rejection *in vivo* (May *et al*., 2002). A recent report suggests that cross-linking of the membrane-bound 4-1BB induces apoptosis of activated T lymphocytes by a Fas-independent pathway (Michel *et al*., 1999).

4-1BB ligand (4-1BBL) is expressed on activated APCs, including IFN-g-activated macrophages, Ig or CD40 ligand-activated B cells (Pollok *et al*., 1994), primary B cells and B-cell lines (Zhou *et al*., 1995) or monocytes, splenic dendritic cells (Futagawa *et al*., 2002). There are reports, indicating that 4-1BBL can argument T cell proliferation, cytokine production and cytolytic effector function and prevent activation- induced cell death (DeBenedette *et al*., 1997; Gramaglia *et al*., 2000).

Soluble forms of receptors among the members of the TNF receptor family have been discovered. These include CD27, CD30 and CD95 (Nozawa *et al*., 1997; Metkar *et al*., 2000; Lienhardt *et al*., 2002). These soluble receptor forms are generated by proteolytic cleavage (Matsuno *et al*., 2000) or alternative splicing (Inaba *et al*., 1999), leading to the inhibition of biological activities of TNF. A soluble form of 4-1BB (s4-1BB), released by activated lymphocytes has

been shown to involve in negative feedback control of inflammation in autoimmune disease like rheumatoid arthritis (Setareh *et al*., 1995). The level of s4-1BB is inversely correlated with lymphocytes proliferation, and positively reflects the degree of activation-induced cell death caused by mitogen stimulation (Michel and Schwarz, 2000). High levels of s4-1BB have been detected in sera of patients with rheumatoid arthritis (Michel *et al*., 1998) and multiple sclerosis (Sharief, 2002) probably reflecting prolonged abnormal immune responses. The precise role of s4-1BB or s4-1BBL in autoimmune diseases is, however, not completely understood.

In this study, we investigate the levels of surface expressions of 4-1BB and 4-1BBL on the surface of PBMC of both normal healthy donors and RA patients. Additionally correlations were drawn between the kinetics and abundance of serum content of s4-1BB and s4-1BBL and serological parameters of RA.

Materials and Methods

Patients

RA patients were randomly selected from the patients treated in the Department of Allergy and Rheumatology, Ulsan University Hospital, Ulsan, Korea. The RA patients selected for the present investigation were classified as clinically active according to the American College of Rheumatology criteria revised in 1987 (Arnett *et al*., 1988). A total of thirty randomly selected patients (twenty-five females and five males) ranged in age from 34 to 70 years with a mean age of 48±11 (mean±SD) years with rheumatoid factor-positive. Peripheral blood samples were obtained from the patients with RA before the start of immunosuppressive therapy. The samples were also obtained from healthy donors presenting with non-specific disorders, to serve as normal controls. Disease severity was evaluated using RA diagnosis serological parameters such as rheumatoid factor (RF), erythrosedimentation rate (ESR), C reactive protein (CRP), and white blood cell count (WBC). Six patients of RA who responded to the immunosuppressive therapy, were particularly analyzed over several months.

Surface expression of 4-1BB and 4-1BBL

In order to study the surface expression of 4-1BB and 4-1BBL on PBMC, the frequency of 4-1BB or 4-1BBL positive cells as well as the intensity of 4-1BB and 4-1BBL expression were analyzed by flow cytometry in twenty patients with RA and compared with healthy controls.

PBMC were prepared by Ficoll gradient density centrifugation, and 2×10^6 cells were stimulated with or without 1 ㎍/ml anti-human CD3 (OKT3) monoclonal antibody (mAb) or 5 ㎍/ml PHA for 24 h. They were washed and resuspended in 500 ㎖ FACS buffer (1×PBS, 2% BSA, 0.1% NaN_3) and stained for 30 min at 4°C with appropriate concentrations of fluorochrome-labeled monoclonal antibodies; markers for T cells (CD4 and CD8), B cells (CD19), monocytes (CD14) were used. After two washes with FACS buffer, cells were analyzed by flow cytometry using FACScan and Lysis II software (Becton Dickinson, Mountain View, CA). Samples were analyzed by setting appropriate SSC/FSC gates around the lymphocyte populations. All antibodies were purchased from BD Biosciences (PharMingen, San Diego, CA).

Detection of s4-1BB and s4-1BBL in serum

Serum levels of s4-1BB and s4-1BBL were measured by an enzyme-linked immunosorbent assay (ELISA). Serum samples were frozen immediately following collection and thawed just before assay (Kim *et al*., 2002). All assays were performed in a blind fashion. ELISA microtiter plates were coated overnight at 4°C with 1 ㎍/ml of anti-4-1BB mAb (AB 4815, Immunomics, Co. Ulsan, Korea) or 1 ㎍/ml of anti-4-1BBL mAb (AB 5G11; Immunomics). Purified human recombinant 4-1BB-Fc or 4-1BBL-Fc (PeproTech, Inc. Rocky Hill, NJ) were used as standards. Non-specific binding was blocked by addition of 100 ㎖/well of 4% BSA in phosphate buffered saline (PBS) for 2 h at 37°C; the samples were then diluted, and added to the wells in duplicate. The microtiter plates were washed and incubated with 100 ㎖/well of detecting anti-4-1BB mAb (AB 4785-biotin, Immunomics) or anti-4-1BBL mAb (AB 4H3-biotin, Immunomics) in 1% BSA-PBS for 2 h at 37°C, then washed before the addition of 100 ㎖/well of a 1/5,000 solution of HRP-straptavidin in 1% BSA-PBS. They were incubated further for 1 h at 37°C, washed, and developed using the TMB peroxidase substrate system (Endogen, BD Bioscience). Absorbance was measured at 450 nm.

For comparison, serum s4-1BB and s4-1BBL were determined in the patients with systemic lupus erythematosus (SLE), a prototypic autoimmune disease and Behcet's disease (BD), a chronic relapsing multisystem inflammatory disorder.

Serological parameters

Total serum lactate rheumatoid factor (RF; IU/mL), C reactive protein (CRP; mg/dL), and erythrosedimentation rate (ESR; mm/h) were determined by standard methods (Noel *et al*., 1992). White blood cell counts (WBC; cells/㎖) were made with a haematology analyzer.

Statistical analysis

Student's *t* test was used to analyze 4-1BB and 4-1BBL data, which are expressed as means±SD. Correlation between s4-1BB or s4-1BBL levels within patient groups and in normal controls was assessed by one-way ANOVA performed with the Microcal™ Orijin™ software package (MicrocalSoftware, Inc, Northampton). *P*-values of less than 0.05 are considered statistically significant.

Results

Surface expression of 4-1BB and 4-1BBL on PBMC

PBMC isolated from twenty patients at the disease onset of RA were cultured for 24 h in 24-well plates in 1 ml RPMI-1640 with or without OKT3 or PHA stimulation and the levels of surface expression of 4-1BB and 4-1BBL were determined by FACS. OKT3 stimulation induces 4-1BB on T cells and PHA stimulation provokes 4-1BBL on B cells and monocytes. Isotype-matched and unrelated mAbs were used for each fluorescence parameter to correct for non-specific binding of each indicated mAb.

Surface expression of 4-1BB on PBMC from RA patients was much less than that on PBMC from healthy donors (Figure 1). Approximately 5.59% of PBMC (mean±SD; 5.59±3.81%) from RA patients expressed low levels of 4-1BB compared with 16.6% in healthy donors (16.6±8.16%) upon OKT3 stimulation. The surface expression levels of 4-1BB on activated PBMC ranged between 0.05 and 11.5% in RA patients, and between 3.06 and 32.47% in healthy donors, respectively. The expression level of 4-1BB was significantly lower ($P < 0.0001$) on PBMC of RA patients than that of healthy controls in OKT3 stimulation. 4-1BB was not detected on resting PBMC of both healthy donors and RA patients.

On the other hand, PBMC from RA patients expressed high levels of 4-1BBL (18.19±10.63%) after OKT3 stimulation compared with those of healthy donors (9.9±8.22%) (Figure 1). Surface expression levels of 4-1BBL on activating PBMC ranged between 5.86 and 42.59% in RA patients, and between 4.09 and 22.73% in healthy donors, respectively. The expression level of 4-1BBL on activating PBMC was significantly high on that of patients with RA ($P < 0.05$) compared with the healthy donors. Particularly, 4-1BBL expression was significantly high levels ($P < 0.001$) on resting PBMC (9.9±8.22%) with RA patients compared with healthy control group (2.54±1.75%).

Thus, 4-1BB expression was observed on both $CD4^+$ and $CD8^+$ T cells from RA patients after activation but not present on resting T cells from RA patients (Figure 2A) or those from healthy donors (Figure 2B). On the other hand, 4-1BBL was constitutively expressed on antigen presenting cells (APCs) such as $CD19^+$ B cells and $CD14^+$ monocytes from RA patients but not on T cells further induced after activation (Figure 2A) compared with healthy donors (Figure 2B).

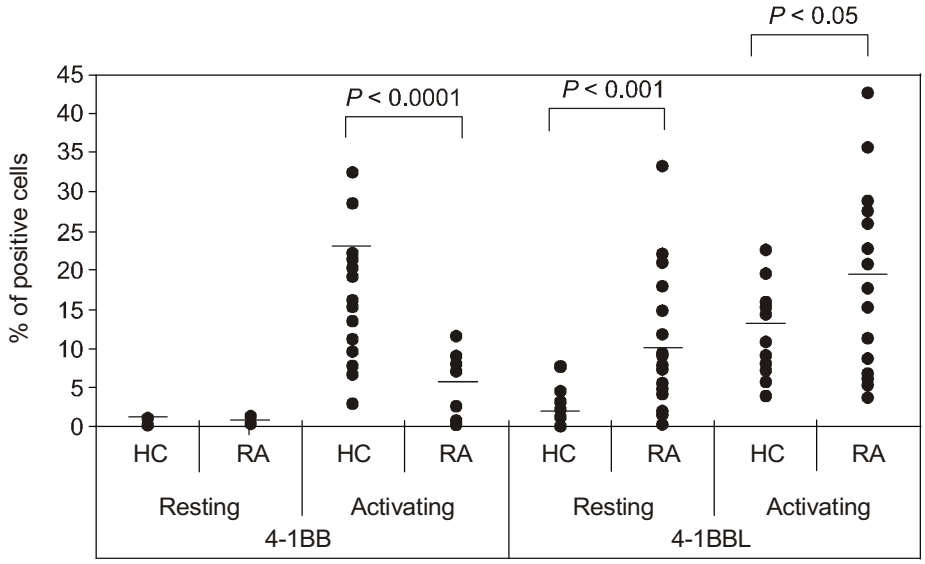

Figure 1. Surface expression levels of 4-1BB and 4-1BBL on PBMC from the patients with rheumatoid arthritis (A) and healthy donors (B). PBMC from twenty RA patients and healthy donors were prepared by Ficoll gradient density centrifugation and 2×10^6 cells were cultured in the absence (resting) or presence (activating) of 1 mg/ml OKT3 or 5 mg/ml of PHA. The surface expression of 4-1BB and 4-1BBL was determined by flow cytometry after 24 h. PE-conjugated anti-4-1BB and anti-4-1BBL mAbs (BD PharMingen) were used to detect PBMC that expressed 4-1BB and 4-1BBL. Horizontal bars depict means ($n = 20$). RA, rheumatoid arthritis and HC, healthy control group.

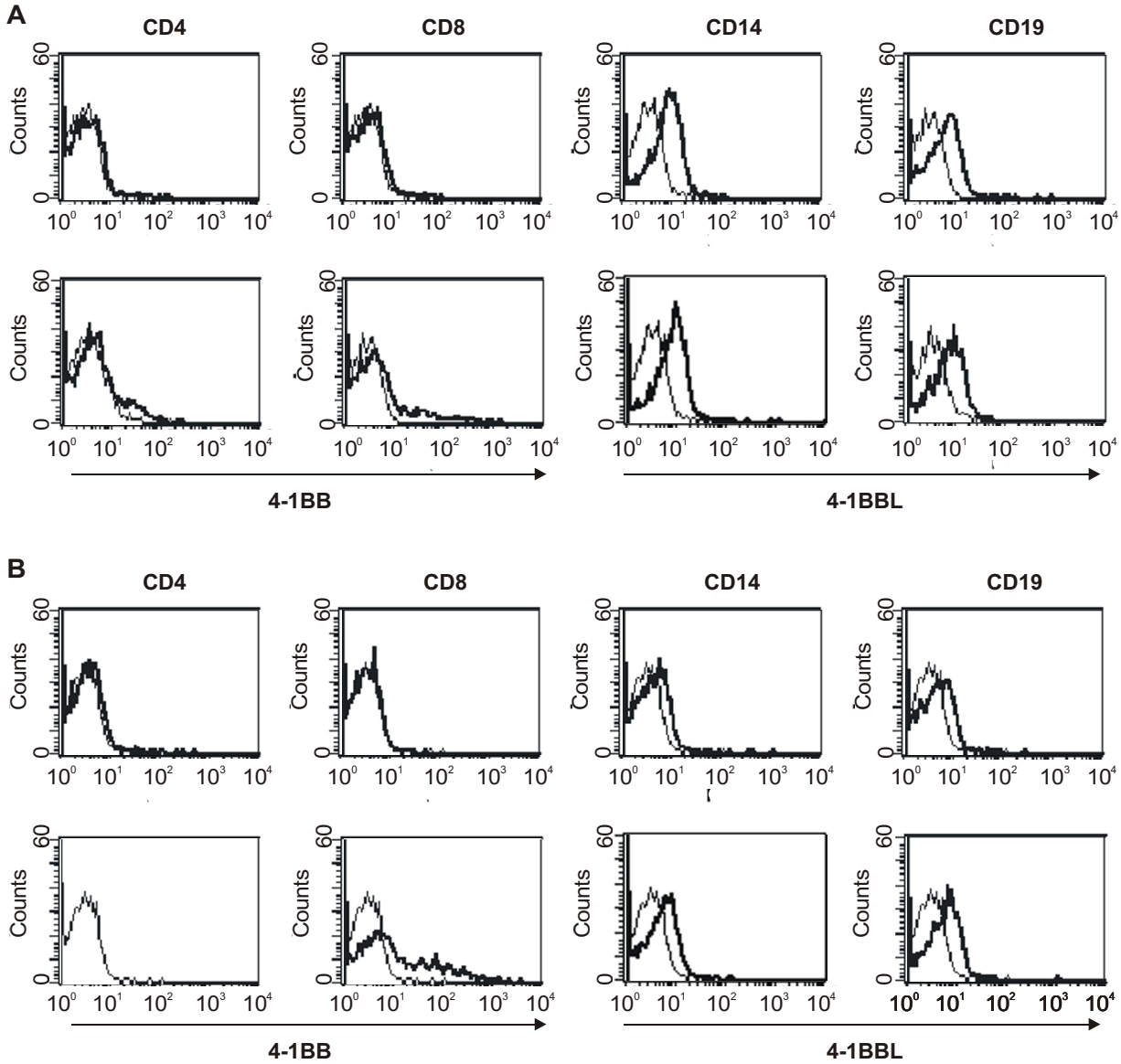

Figure 2. Surface expression of 4-1BB and 4-1BBL on PBMC from the patients with rheumatoid arthritis (A) and healthy donors (B). PBMC cultured in absence (upper panel) or presence (lower panel) of OKT3 or PHA as mentioned in Materials and Methods. PE-conjugated anti-4-1BB, 4-1BBL mAbs and FITC-conjugated anti-CD4, CD8, CD19, and CD14 mAbs (BD PharMingen) were used to detect 4-1BB and 4-1BBL expression (thick lines). Isotype-matched antibodies were used as controls (thin lines). Analysis was performed on a FACscan and analyzed using Lysis II software.

Serum levels of s4-1BB and s4-1BBL

s4-1BB and s4-1BBL were determined by ELISA with the serum samples collected from healthy donors or RA patients and other disorders such as SLE and BD.

The sera of the healthy control group contained low levels of s4-1BB (mean±SD; 272±225 pg/ml) (Figure 3). The levels of s4-1BB ranged between 27 and 681 pg/ml, with a median of 200 pg/ml. Substantially higher serum levels of s4-1BB were detected in patients with RA, SLE, and BD compared to healthy donors. The highest s4-1BB levels were detected in patients with RA (2,964±3,840 pg/ml): the range was between 172 and 16,069 pg/ml, with a median of 957 pg/ml. Values (961±577 pg/ml) in SLE patients were between 190 and 1,802 pg/ml, with a median of 684 pg/ml. In BD patients, s4-1BB levels (1,128±585 pg/ml) were between 474 and 1,996 pg/ml, with a median of 864 pg/ml. The level of s4-1BB was significantly elevated in the sera of patients with RA ($P < 0.0001$), with SLE ($P < 0.001$), and with BD ($P < 0.001$) compared with healthy donors.

The sera of the healthy control group contained

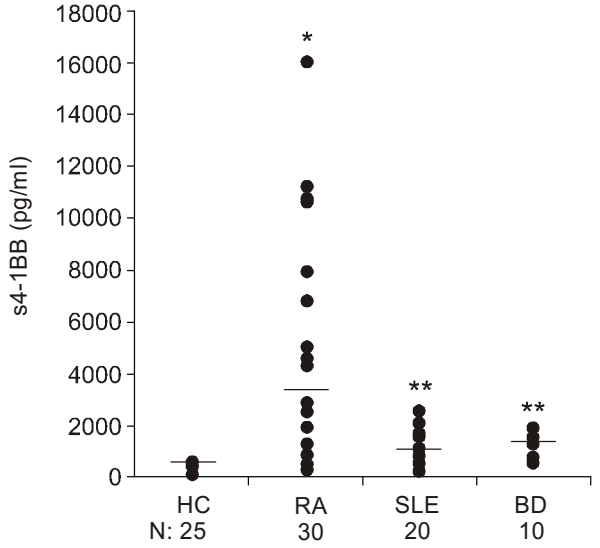

Figure 3. Comparison of the s4-1BB levels in sera of patients with rheumatoid arthritis with those of other autoimmune diseases and healthy control. Serum samples were investigated by ELISA. *$P <$ 0.0001 and **$P < 0.001$ compared with serum levels in healthy controls. Horizontal bars depict means. The data shown are means of triplicates. RA, active rheumatoid arthritis; SLE, systemic lupus erythematosus; BD, Behcet's disease; and HC, healthy control. n. numbers of donors in each group.

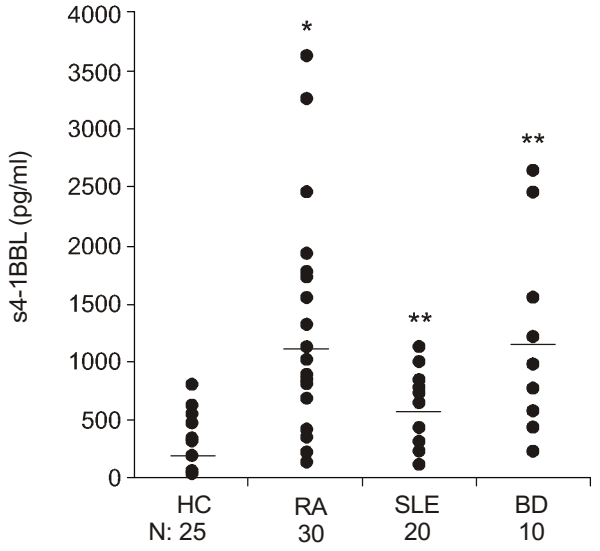

Figure 4. The s4-1BBL profiles in sera of patients with rheumatoid arthritis, other autoimmune diseases and healthy control. Serum samples were investigated by ELISA. *$P < 0.0001$ and **$P < 0.001$ compared with serum levels in healthy control. Horizontal bars depict means. The data shown are means of triplicates. RA, active rheumatoid arthritis; SLE, systemic lupus erythematosus; BD, Behcet's disease; and HC, healthy control. n. numbers of donors in each group

low levels of s4-1BBL (mean±SD; 197±225 pg/ml) (Figure 4) ranging between 36 and 804 pg/ml, with a median of 108 pg/ml. Significantly higher levels

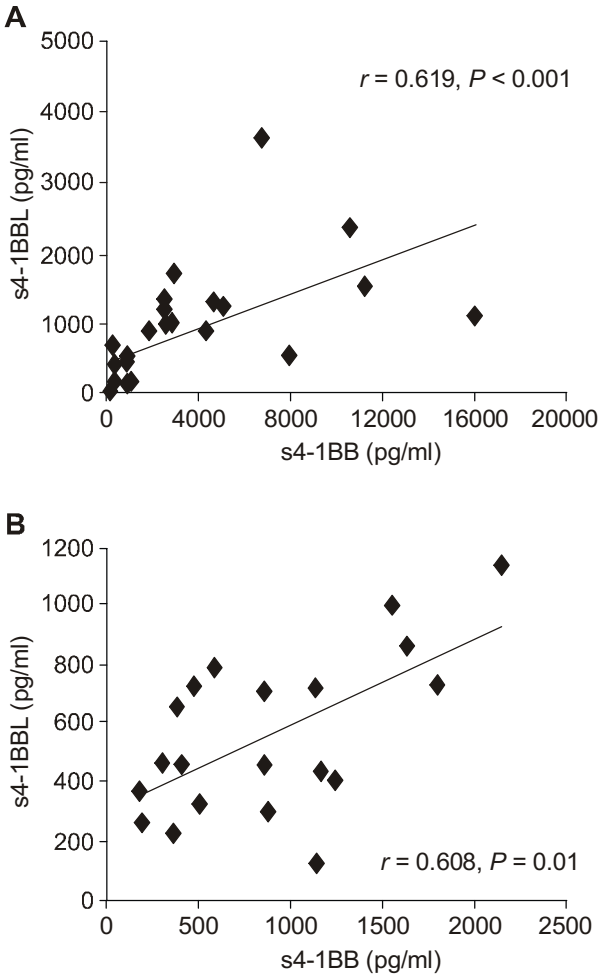

Figure 5. Correlation between s4-1BB and s4-1BBL in sera of the patients with rheumatoid arthritis (A) and systemic lupus erythematosus (B). s4-1BB and s4-1BBL were assessed by ELISA.

were detected in patients with active RA, SLE, and BD. Greatest levels of s4-1BBL were also detected in sera from patients with RA (1,034±916 pg/ml) ranging was between 130 and 3,636 pg/ml, with a median of 882 pg/ml. In SLE patients (598±247 pg/ml), s4-1BBL levels were between 121 and 1135 pg/ml, with a median of 549 pg/ml. In BD patients (1,207±861 pg/ml), they were between 228 and 2,652 pg/ml, with a median of 1,204 pg/ml. The level of s4-1BBL was significantly elevated in sera with patients of RA ($P < 0.0001$), of SLE ($P < 0.001$), and of BD ($P < 0.001$) compared with healthy donors.

The data clearly indicates that levels of s4-1BB and s4-1BBL are greatly increased in sera of patients with autoimmune disorders such as RA, SLE and BD. Serum s4-1BB levels in RA ($r = 0.619$, $P < 0.001$) (Figure 5A) and SLE ($r = 0.608$, $P < 0.01$) (Figure 5B) were strongly correlated with raised serum s4-1BBL values.

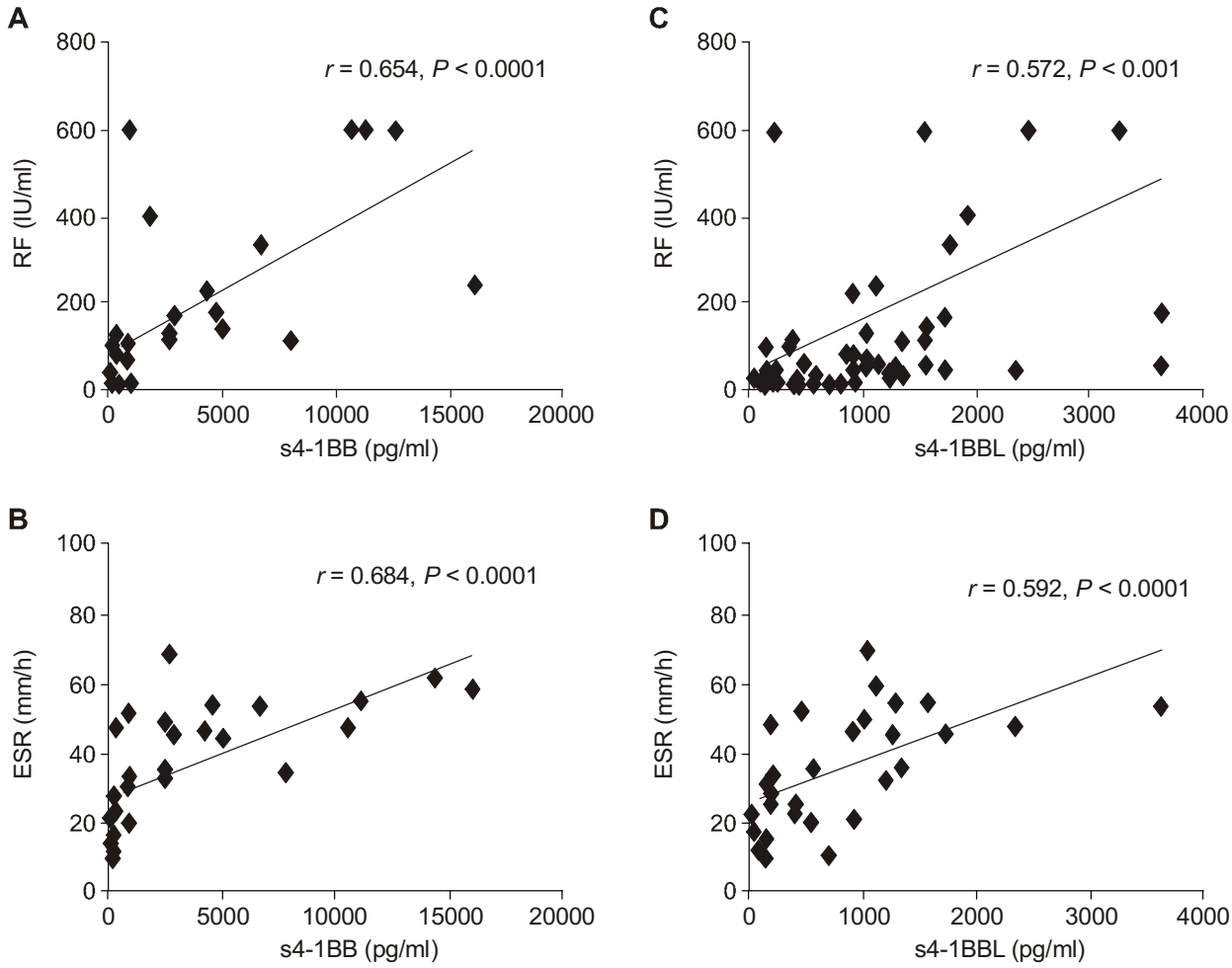

Figure 6. Correlation between s4-1BB (A, B) or s4-1BBL (C, D) in sera of the patients with rheumatoid arthritis and serological parameters. RF; rheumatoid factor (IU/ml) and ESR; erythrocyte sedimentation rate (mm/h).

Correlation between s4-1BB or 4-1BBL and serological RA parameters

We sought to evaluate the relationship between release of s4-1BB and serological parameters of RA such as RF, CRP, WBC and ESR. In the RA patient group, s4-1BB levels were correlated with RF values ($r = 0.654$; $P < 0.0001$, Figure 6A) and ESR values ($r = 0.684$; $P < 0.0001$, Figure 6B). The correlation between serum levels of s4-1BBL and RF or ESR in patients with active RA also reached statistically significant levels, ($r = 0.572$; $P < 0.001$ for RF, Figure 6C) and ($r = 0.592$; $P < 0.001$ for ESR, Figure 6D). Additional two serological parameters, CRP and WBC, were not significantly correlation with s4-1BB and s4-1BBL in the RA patient group (data not shown).

The effect of therapy in active RA

Serum s4-1BB and s4-1BBL levels in patients with RA were compared at disease onset and upon treatment with immunosuppressive therapy in Figure 7. Six patients with active RA had levels of s4-1BB ranging between 2,784 and 16,069 pg/ml (mean±SD; 6,958 ±5,343) while for s4-1BBL the range was between 1,032 and 2,339 pg/ml (1,392±477). However, at the quiescent stage following immunosuppressive therapy, the levels have markedly decreased; for s4-1BB (Figure 7A) the range was between 388 and 957 pg/ml (672±252) and for s4-1BBL (Figure 7B), it was between 96 and 540 pg/ml (322±191), with $P < 0.05$ and $P < 0.01$, respectively, compared with patients at disease onset. All patients with RA over the duration of study showed improvement and levels of CRP, ESR, and WBC decreased upon immunosuppressive therapy (Table 1).

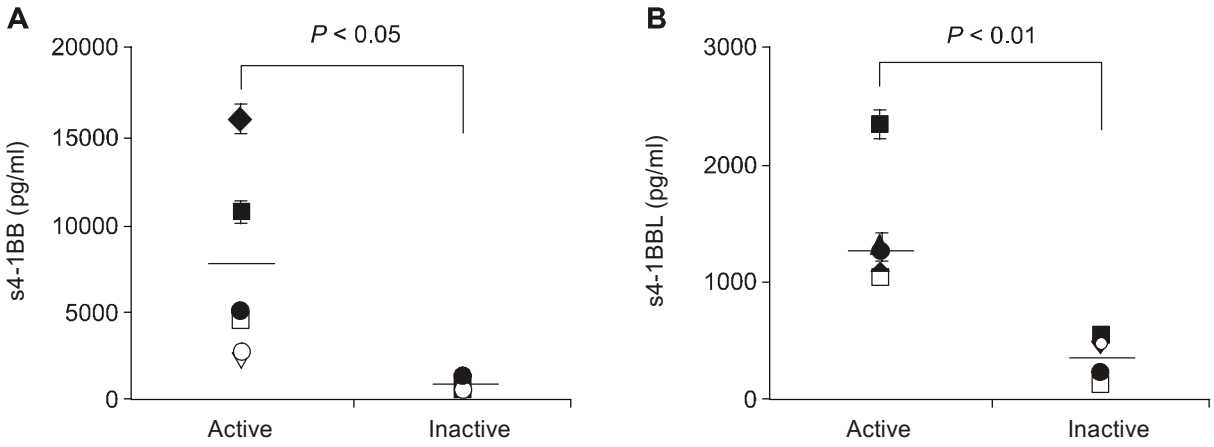

Figure 7. s4-1BB (A) and s4-1BBL (B) levels in sera of six patients with RA in active and inactive stage. s4-1BB and s4-1BBL were assessed by ELISA. $P < 0.01$ and $P < 0.05$ respectively for the comparison of serum levels of s4-1BB and s4-1BBL between active and inactive stage. Donor number 1 (□); donor 2 (■); donor 3 (▲); donor 4 (○); donor 5 (●); and donor 6 (◆).

Table 1. The levels of serological parameters of the patients with rheumatoid arthritis in active and inactive stage

Patient	Age/sex	Active stage			Inactive stage following treatment		
		CRP	ESR	WBC	CRP	ESR	WBC
1.	70/M	0.4	55	15900	0.03	28	4700
2.	42/F	0.32	48	4230	0.05	25	4800
3.	37/F	1.22	33	7170	0.19	12	4790
4.	46/F	14.22	54	7930	0.09	23	8570
5.	49/F	0.18	46	8160	0.08	1	15480
6.	53/F	1.81	59	6090	0.03	10	6730

CRP, C reactive protein (mg/dL); ESR, erythrocyte sedimentation rate (mm/hour); WBC, white blood cell counts (cells/ul); F, female; M, male.

Discussion

4-1BB has been found on activated T cells and NK cells (Melero *et al*., 1998). 4-1BBL on several kinds of APCs (Pollok *et al*., 1994; Zhou *et al*., 1995; Futagawa *et al*., 2002) such as activated B cells, monocytes, activated macrophages, splenic dendritic cells; it can also be induced on T lymphocytes. A two-way transduction of signals seems to operate in the 4-1BB receptor-ligand system. Cross-linking of 4-1BB activates T-cells leading to cytokine release. On the other hand, cross-linking of its ligand by 4-1BB induces apoptosis of activated T lymphocytes (Michel *et al*., 1999). Reverse signaling following cross-linking of 4-1BBL in lymphocytes inhibits proliferation and induces apoptosis (Langstein *et al*., 1998; Ebta *et al*., 2001). In addition, 4-1BB also causes apoptosis of B-cells by direct cell contact (Kienzle and Kempis, 2000).

4-1BB is normally expressed when T cells receive signals through TCR. Therefore, anti-CD3 mAb (OKT3) is one of the better 4-1BB inducer in T cells. 4-1BBL can be induced by stimulating B cells, monocytes and dendritic cells by PHA. We hypothesized that both 4-1BB and 4-1BBL were expressed on higher percentage of PBMC in RA patients. Therefore we compared their expression patterns between healthy control and RA patients before and after respective stimuli. Although 4-1BBL showed higher expression before and after PHA stimulus as expected, 4-1BB on T cells was unexpectedly resistant to induction in RA patients. This may indicate that higher percentage of T cells is in anergic state in RA patients.

The level of 4-1BB was lower on activating PBMC from RA patients than that of healthy controls (Figure 1). In contrast constitutive expression of 4-1BBL on PBMC from RA patients was higher than that on PBMC from healthy donors. Since reverse signaling through 4-1BBL in T cells induces apoptosis (Langstein *et al*., 1998), the high expression of 4-1BBL therefore

is likely to play an important role in disease progress and regulating the outcome of cell interactions in autoimmune disease such as RA. 4-1BB was expressed on both activated $CD4^+$ and $CD8^+$ T-cell subpopulations by OKT3 stimulation (Figure 2). On the other hand, 4-1BBL was expressed on small populations of $CD19^+$ B-cells and $CD14^+$ monocytes.

In RA, large number of memory T cells was detected in the synovium (Toshihiro *et al*., 2000; Maldonado *et al*., 2003). Such enhanced migration of the memory cells to sites of inflammation may play a role in the pathogenesis of RA (Maldonado *et al*., 2003). 4-1BB was expressed upon activation in the memory T cell populations in humans (Garni-Wagner *et al*., 1996). In the future studies, 4-1BB expression in the nave and the memory T cells from both synovial fluid and peripheral blood will be determined in different phases of RA. These studies may provide a clue to the role of 4-1BB and 4-1BBL in the pathogenesis of RA.

Several receptor and their ligands belonging to the TNF family are released in soluble form following cleavage from the cell surface (Metkar *et al*., 2000). These may affect cell-cell interactions and responses to receptor-ligand interactions or provoke more distal responses to cellular activation. In the case of soluble Fas (sFas), the presence of an excess of sFas may interfere with the trimerization of the Fas/CD95 receptor by its ligand leading to apoptosis (Schneider *et al*., 1988; Seo *et al*., 2002). s4-1BB is released by activated lymphocytes and is generated by proteolytic cleavage (Michel and Schwarz, 2000). s4-1BBL is also released constitutively from leukocytes by differential splicing of the membrane-bound molecule and more is released following activation, and this is prevented by the addition of a metalloproteinase inhibitor (Hulmut *et al*., 2001). We analyzed s4-1BB (Figure 3) and s4-1BBL (Figure 4) levels in serum samples from patients with RA and other disorders as well as healthy donors and found that, while only very low levels of s4-1BB and s4-1BBL were present in sera from healthy control group, elevated levels were detectable in the sera of patient group. RA was particularly severe in releasing s4-1BB and s4-1BBL. We found that both s4-1BB and s4-1BBL were detected at high level in sera of patients of another autoimmune disease, SLE and an inflammatory disorder of unknown etiology, BD. The significance of these findings is currently unknown. This finding confirms and extends results from earlier studies demonstrating that patients with rheumatic disease (Michel *et al*., 1998), multiple sclerosis (Sharief, 2002) and a variety of subclasses of acute myeloid leukemia (Salih *et al*., 2001) or hematological malignancies (Hulmut *et al*., 2001) have elevated levels of s4-1BB or s4-1BBL.

Serum levels of s4-1BB and s4-1BBL were also correlated with disease severity in RA patients (Figure 5). The levels of s4-1BB and s4-1BBL did not strictly correlate with surface expression levels of 4-1BB or 4-1BBL (data not shown), indicating that the soluble forms of the receptor are not merely produced by shedding of the membrane-bound receptors. This is in line with generation of s4-1BB or s4-1BBL from distinct, differentially spliced mRNAs or proteolytic cleavage from the cell surface. The inverse relationship between s4-1BB and cell surface 4-1BB suggests that the increased level of s4-1BB is associated with a switch from membrane-bound to s4-1BB. They may affect cell-to-cell interactions and responses to ligand, or provoke more specific responses to activation.

Because 4-1BB can be involved in the induction of programmed cell death (Michel and Schwarz, 2000), it is tempting to speculate that s4-1BB may regulate apoptosis of activated lymphocytes in a manner similar to other soluble members of the TNF receptor family, such as sFas, which exhibits apoptosis-blocking properties through antagonizing the activity of the membrane-bound receptor (Cheng *et al*., 1994; Cascino *et al*., 1996). Indeed, high circulating levels of s4-1BB have been suggested to suppress immune responses in inflammatory diseases by providing a mechanism to escape immune surveillance, partly through the modulation of apoptosis (Michel *et al*., 1998; 1999). The high level of release of s4-1BBL in patients may permit the cells to escape local immune surveillance by limiting costimulation of the host lymphocytes (Hulmut *et al*., 2001), and by reducing apoptosis signals through 4-1BBL back into the tumor cell (Kashii *et al*., 1999). Thus, *in vivo* release from cells, and/or dispersion to distal site, may be responsible for some of the pathophysiological effects of the disease. In addition, the high level of release of s4-1BB or s4-1BBL from the cell surface may be mediated by different cleavage mechanism in the different patient groups, and is likely to regulate interactions mediated by 4-1BB- 4-1BBL *in vivo*.

Although s4-1BB and s4-1BBL levels were significantly different in the discrete patient groups, we did find a direct relationship between s4-1BB/s4-1BBL and the disease markers such as RF and ESR (Figure 6). In addition, the elevated s4-1BB and s4-1BBL levels characteristic of RA onset rapidly decreased when the patients respond to the treatment as well as serological parameters (Figure 7). Although additional studies are needed, it suggests that s4-1BB and s4-1BBL levels may serve as a diagnostic tool in assessing disease activity in RA. The importance of serum s4-1BB and s4-1BBL release in this autoimmune disease is further illustrated by the positive correlation with clinical severity. As the clinical severity of RA does not necessarily correlate with other disease parameters like CRP and WBC longitudinal

studies would be desirable including serial monitoring of s4-1BB and s4-1BBL to assess their effect in modulating the clinical course of RA. The correlations we have observed are clearly relevant to the underlying immune activation, but further studies are required of the interactions between the soluble forms and other immune regulators in the serum. In conclusion, we have detected high levels of s4-1BB and s4-1BBL in patients with clinically active RA. In view of the emerging role of s4-1BB or s4- 1BBL in modulating lymphocytes functions, our results suggest a role in regulating lymphocyte function in RA. The effect of s4-1BB and s4-1BBL on cell-cell interactions and in the pathological disorders requires further elucidation.

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