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【Ⅴ】研究成果の刊行物・別刷

Transcriptional regulation of multidrug resistance-1 gene by interleukin-2 in lymphocytes

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P-glycoprotein, encoded by the multidrug resistance (MDR)-1 gene, expels various drugs from cells resulting in drug resistance. However, its functional relevance to lymphocytes and the regulatory mechanism remain unclear. Although MDR-1 is known to be induced by various cytotoxic stimuli, it is poorly understood whether the activation stimuli such as cytokines induce MDR-1 transcription. We investigated the transcriptional regulation of MDR-1 in lymphocytes by activation stimuli, particularly by interleukin (IL)-2. IL-2 induced translocation of YB-1, a specific transcriptional factor for MDR-1, from the cytoplasm into nucleus of lymphocytes in a dose-dependent manner and resulted in the sequential events; transcription of MDR-1, expression of P-glycoprotein on the cell surface, and excretion of the intracellular dexamethasone added *in vitro*. Transfection of YB-1 anti-sense oligonucleotides inhibited P-glycoprotein expression induced by IL-2. Cyclosporin A, a competitive inhibitor of P-glycoprotein, recovered intracellular dexamethasone levels in lymphocytes. We provide the first evidence that IL-2, a representative lymphocyte-activation stimulus, induces YB-1 activation followed by P-glycoprotein expression in lymphocytes. Our findings imply that lymphocytes activation by IL-2 *in vivo*, in the context of the pathogenesis of autoimmune diseases, results in P-glycoprotein-mediated multidrug resistance, and that P-glycoprotein could be an important target for the treatment of refractory autoimmune diseases.

Introduction

Drug resistance is one of the most important issues to be overcome in the treatment of malignancies and chronic diseases, including systemic autoimmune diseases. Among the multiple mechanisms of multidrug resistance, over-expression of P-glycoprotein (P-gp), a 170-kDa product of the multidrug resistance-1 (MDR-1) gene, has emerged as the major molecule involved in multidrug resistance during chemotherapy for various malignancies (Beck *et al.* 1996). P-gp is a member of ATP-binding cassette (ABC) transporter superfamily of genes and functions as an energy-dependent transmembrane efflux pump. Over-expression of P-pg results in reduction of intracellular concentrations of xenobiotics, drugs and poisons, such as vinca alkaloids, anthracyclines (Tsuruo 1983), anti-malarials, colchicines (Ueda *et al.* 1987), cyclosporin (List *et al.* 2002), and glucocorticoids (Bourgeois *et al.* 1993). Thus, P-gp appears to be a double-edged sword, involved both in protecting cells from these drugs and in the development of resistance to them.

Since resistance to chemotherapy induced by P-pg is closely associated with prognosis of human malignancies (Linn *et al.* 1996), recent studies have helped elucidate the association of drug resistance and P-gp expression on malignant cells. P-gp is expressed on various types of tumour cells (Fojo *et al.* 1987), including leukaemic cells (Advani *et al.* 1999), $CD34^+$ haematopoietic stem cells (Chaudhary & Roninson 1991), and epithelial cells in the liver, kidney, pancreas, gut and adrenals (Sugawara *et al.* 1988). On the other hand, treatment resistance is common in patients with not only haematopoietic malignancies, but also systemic autoimmune diseases, such as systemic lupus erythematosus (SLE), which sometimes leads to a poor prognosis of these diseases. However, P-gp expression on immune cells such as T cells and B cells, the functional relevance of P-gp to lymphocytes,

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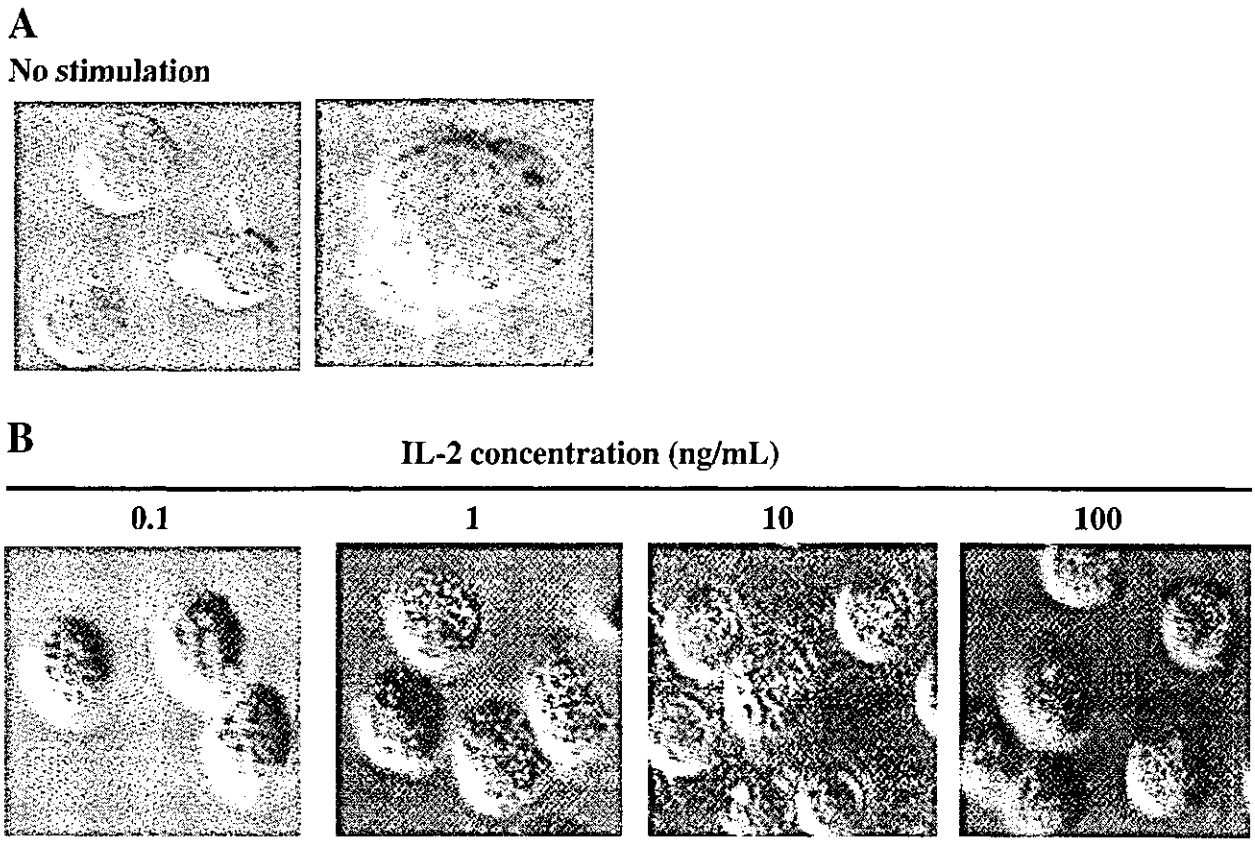

Figure 1 IL-2 induces activation and nuclear translocation of YB-1 in PBMCs. Immunostaining and confocal microscopy analysis of YB-1 in 1×10^5 of PBMCs. (A) YB-1 is expressed in the cytoplasm of all PBMCs without stimulation. (B) In contrast, nuclear translocation of YB-1 was induced in at least 15% of PBMCs incubated with indicated concentration of IL-2 for 20 min at 37 °C. Magnification × 600.

and the regulatory mechanisms for induction of P-gp on these cells remain unclear.

We and others have reported that transcription of MDR-1 is directly regulated by human Y-box-binding protein-1 (YB-1), a MDR-1 transcription factor, and that activation of YB-1 is induced in response to genotoxic stresses (Ohga *et al.* 1998) such as ultraviolet light (Uchiumi *et al.* 1993a), anti-cancer agents (Kohno *et al.* 1989), serum starvation (Tanimura *et al.* 1992), heat shock (Miyazaki *et al.* 1992) and multiple drugs, including vinca alkaloids and corticosteroids (Chaudhary & Roninson 1993). However, the regulatory mechanisms of YB-1 activation and MDR-1 transcription in lymphocytes remain unclear. Furthermore, although MDR-1 is induced by various genotoxic or cytotoxic stimuli described above, it is poorly understood whether the activation stimuli such as cytokines induce MDR-1 transcription in lymphocytes. The present study was designed to investigate the transcriptional regulation of MDR-1 in lymphocytes, particularly in lymphocytes activated by interleukin (IL)-2.

Results

Activation of lymphocytes induces nuclear translocation of YB-1

We first examined the intracellular distribution of a transcriptional factor YB-1 in PBMCs by immunostaining using anti-YB-1 monoclonal antibody. Using confocal microscopic analysis, we observed that YB-1 was localized in the cytoplasm of PBMCs at basal conditions (Fig. 1A). Then YB-1 was translocated into the nucleus within 20 min after stimulation with IL-2. As shown in Fig. 1B, nuclear accumulation of YB-1 occurred in an IL-2-concentration-dependent manner within the range between 0.1 and 10 ng/mL. These results suggest that IL-2 activate the transcription factor YB-1.

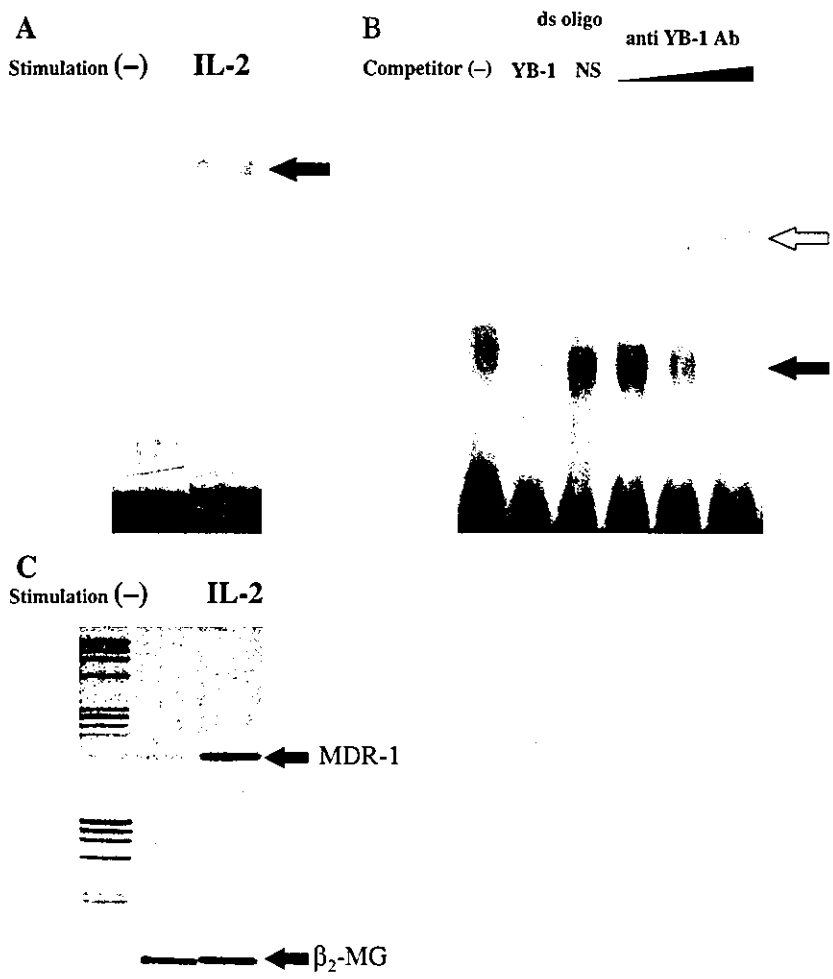

Figure 2 IL-2 activates YB-1 and MDR-1 gene expression in PBMCs. (A) YB-1 DNA binding activity was examined by EMSA. Four hours stimulation with 10 ng/mL of IL-2 induced YB-1 DNA binding activity. (black arrow indicates the complex of YB-1/DNA). (B) The binding of YB-1 (lane 1) was competed with consensus oligonucleotides to YB-1 binding site (lane 2) but not with irrelevant oligos (lane 3). An aliquot of 1–5 μg YB-1-specific antibody (YB-1 Ab) super-shifted the dense band (lanes 4–6). The white arrow indicates supershifted complexes. C, MDR-1 mRNA expression was examined by RT-PCR using total RNA extracted from 1×10^6 of PBMCs incubated with 10 ng/mL of IL-2 for 4 h. Beta 2-microglobulin (β2-MG) transcript was used as an internal standard.

To determine whether the activated YB-1 directly affects MDR-1 gene expression in PBMCs, we investigated DNA binding of YB-1 by electrophoretic mobility shift assay (EMSA). We prepared nuclear extracts from PBMCs at basal conditions and also after stimulation with IL-2, and incubated them with a P^{32}-labelled oligonucleotide containing YB-1 consensus binding sites. We observed dense bands in the mixture of oligonucleotides and nuclear extracts derived from PBMCs stimulated with IL-2. The dense bands markedly reduced in the presence of 25-fold molecular excess of double strand YB-1 oligonucleotides but not by nonspecific oligonucleotides. The presence of anti-YB-1 specific antibody induced a super-shifted band from the basal band of nuclear extracts (Fig. 2A,B). These results imply that activation of PBMCs with IL-2 resulted in a sequence of events; activation of a nuclear factor YB-1, its nuclear translocation and binding of the activated YB-1 to the promoter region of the *MDR-1 gene*.

Activation of lymphocytes induces MDR-1 gene transcription in PBMCs

The nuclear localization of YB-1 is closely associated with MDR-1 gene expression in a human breast cancer cell line (Bargou *et al.* 1997). To test whether activated YB-1 directly affects MDR-1 gene expression in response to IL-2, we examined the expression of MDR-1 mRNA by reverse transcription-polymerase chain reaction (RT-PCR). There was a substantial increase in MDR-1 mRNA relative to β2-microglobulin mRNA in PBMCs activated with IL-2, compared with PBMCs at basal condition (Fig. 2C). This result was consistent with that observed in the mobility shift assay and translocation of YB-1 in immunostaining.

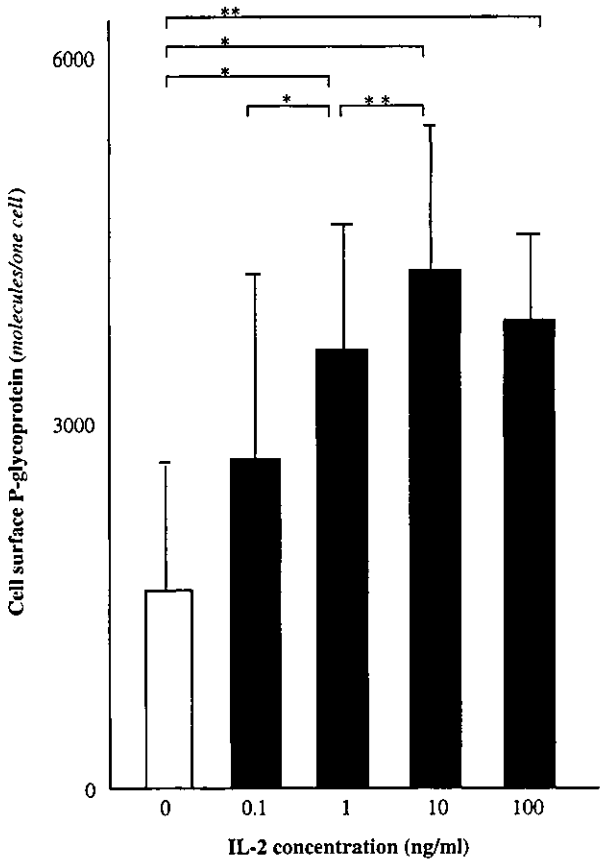

Figure 3 IL-2 induces cell surface P-glycoprotein expression on PBMCs. Flow cytometric analysis showed P-glycoprotein expression on 1×10^6 of PBMCs after 4 h incubation with different concentrations of IL-2. Each value represents the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data represent mean ± SD of five independent experiments. Statistical analysis was performed using the paired *t*-test. $*P < 0.05$, $**P < 0.01$.

Up-regulation of P-glycoprotein on IL-2-activated lymphocytes

Preliminary experiments showed that P-gp expression on PBMCs reached maximum levels within 3 h of incubation with IL-2 and then diminished to basal levels after 24 h of incubation (data not shown). Therefore, we evaluated the expression of P-gp after a 4-h stimulation in the following studies. We observed that expression of P-gp was augmented in a dose-dependent manner up to 10 ng/mL of IL-2 (Fig. 3). Furthermore, to investigate the expression of P-gp on lymphocytes in detail, we next performed two-colour analysis using anti-CD4, -CD8, and -CD19 antibodies and examined P-gp expression on each subset of lymphocytes. Flow cytometric analysis showed that P-gp expression was significantly augmented by IL-2 on $CD4^+$, $CD8^+$ and $CD19^+$ cells (Fig. 4).

Inhibition of P-glycoprotein expression on lymphocytes by YB-1 anti-sense

To determine whether YB-1 is directly coupled with IL-2-induced MDR-1 gene activation, we assessed the P-gp on PBMCs transfected with YB-1 anti-sense expression plasmid (PRC/CMV AS) or control vacant vector and compared the levels of P-gp expression on PBMCs incubated with or without IL-2. In comparison with vector alone, introduction of YB-1 anti-sense significantly reduced the expression of P-glycoprotein on PBMCs. IL-2 stimulation significantly increased the expression of P-gp on control cells, but the inducibility was abolished by transfection of YB-1 anti-sense (Fig. 5).

Inhibition of Pgp-induced excretion of intracellular dexamethasone by cyclosporin A

To investigate the association between expression of P-gp and exclusion of drugs through P-gp, intracellular and extracellular concentration of dexamethasone was determined as described in Experimental procedures. IL-2 simulation resulted in the significant decrease of intracellular dexamethasone on PBMCs during observed periods (3–30 min) as shown in Fig. 6. To confirm the functional involvement of P-gp in the decrease of intracellular dexamethasone, we added cyclosporin A, a competitive inhibitor of P-gp, to IL-2 stimulated PBMCs. Excretion of dexamethasone in PBMCs inhibited by cyclosporin A in a concentration-dependent manner, up to 100 ng/mL of cyclosporin A (Fig. 7).

Discussion

We and others have reported that MDR-1 transcription is directly regulated by a transcription factor YB-1 and that activation of YB-1 is induced in response to genotoxic and/or cytotoxic stresses, such as xenobiotics, drugs, poisons, ultraviolet and environmental stimuli (Uchiumi *et al.* 1993b). However, it is poorly understood whether the activation stimuli such as cytokines activate YB-1 and induce MDR-1 transcription in lymphocytes. We here propose that MDR-1 transcription in lymphocyte is also induced by activation stimuli such as IL-2, a potent stimulus of lymphocytes (Kelly *et al.* 2002; Luxembourg & Cooper 1994), based on the following sequence of events; activation and translocation of YB-1 by IL-2, transcription of MDR-1 by the binding of the activated YB-1, and expression of P-gp on lymphocytes.

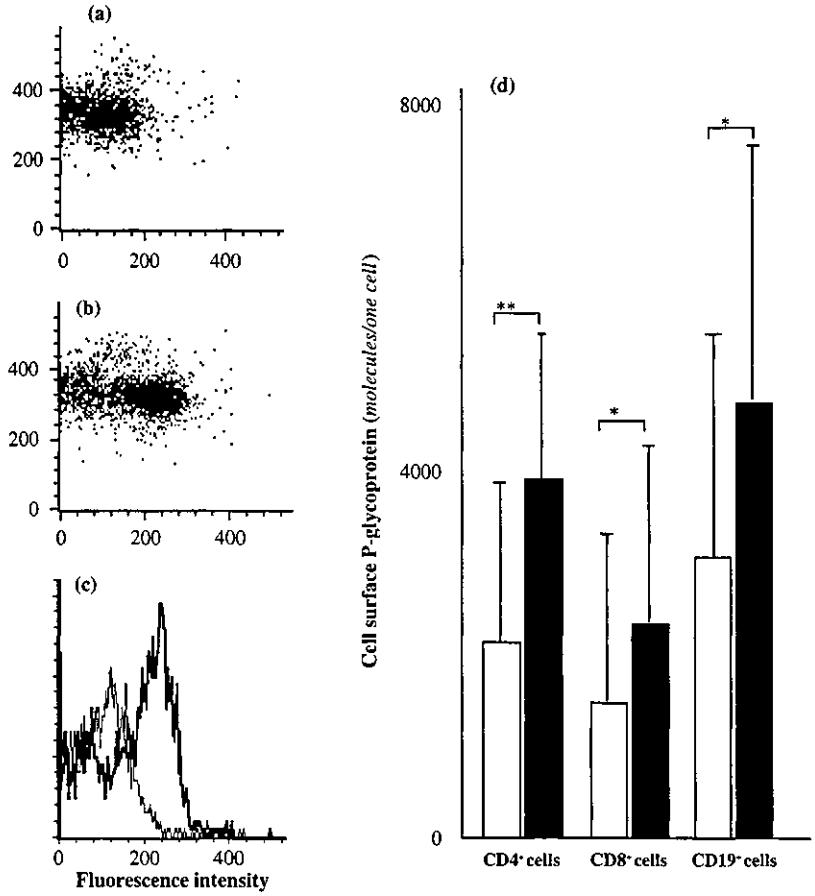

Figure 4 IL-2 induces expression of P-glycoprotein on lymphocytes. Flow cytometric analysis showed P-glycoprotein expression on peripheral $CD4^+$ cells from 1×10^6 of PBMCs (A and grey area in C) increased after 4 h stimulation with 10 ng/mL of IL-2 (B and solid black line in C). Representative experiment from five. P-glycoprotein expression on peripheral $CD4^+$, $CD8^+$ and $CD19^+$ cells from 1×10^6 of PBMCs in five independent donors incubated with (■) or without (□) 10 ng/mL of IL-2 for 4 h (D). Each value represents the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data represent mean ± SD of five independent experiments. Statistical analysis was performed using the paired *t*-test. $*P < 0.05$, $**P < 0.01$.

These results were also confirmed with the reduced expression of P-gp on IL-2-activated lymphocytes when the cells were transfected with YB-1 anti-sense oligonucleotides.

Furthermore, when dexamethasone was added to lymphocytes *in vitro*, excretion of dexamethasone was up-regulated and intracellular dexamethasone was reduced in IL-2-activated lymphocytes, corresponding to higher expression of P-gp. However, the addition of cyclosporin A to the culture, a competitive inhibitor of P-gp (List *et al.* 2002; Fedeli *et al.* 1989; Zacherl *et al.* 1994), inhibited excretion of dexamethasone in a dose-dependent manner, implying that P-gp induced on IL-2-activated lymphocytes play a functional role in the drug-excretion in lymphocytes, which leads to resistance to multiple drugs such as corticosteroids.

The role of MDR-1 and P-gp in tumour cells and haematopoietic malignancies is well known and the regulation of MDR-1 gene by IL-2 varies among cancer cells (Burton *et al.* 1994; Johannessen *et al.* 2000; Stein *et al.* 1996). However, P-gp expression, its functional relevance and the regulatory mechanisms of P-gp in normal or activated lymphocytes still remain unclear. SLE, a representative systemic autoimmune disease, is characterized by activation of T cells and B cells and the presence of activated helper T cells and the Th1/Th2 imbalance are involved in the pathogenesis of SLE (Huang *et al.* 1988; Akahoshi *et al.* 1999). We and others also reported that the numbers of lymphocytes producing cytokines such as IL-2 and serum levels of these cytokines are increased in patients with active SLE (Tanaka *et al.* 1988; Dau *et al.* 1991; Horwitz *et al.* 1994). The increased IL-2 levels in SLE patients usually fall below the threshold, whereas they remain at high levels in patients who respond poorly to treatments and continue to have high disease activity. However, the mechanisms of drug resistance in SLE are largely unclear.

We here propose that the sequential events in IL-2 activated lymphocytes, consisted of YB-1 activation by IL-2, MDR-1 transcription, P-gp expression and excretion of intracellular dexamethasone, could be relevant to poor responsiveness to several immunosuppressants and corticosteroids in SLE patients with a high disease activity. Indeed, we observed that P-gp expression on lymphocytes markedly increased in active SLE patients

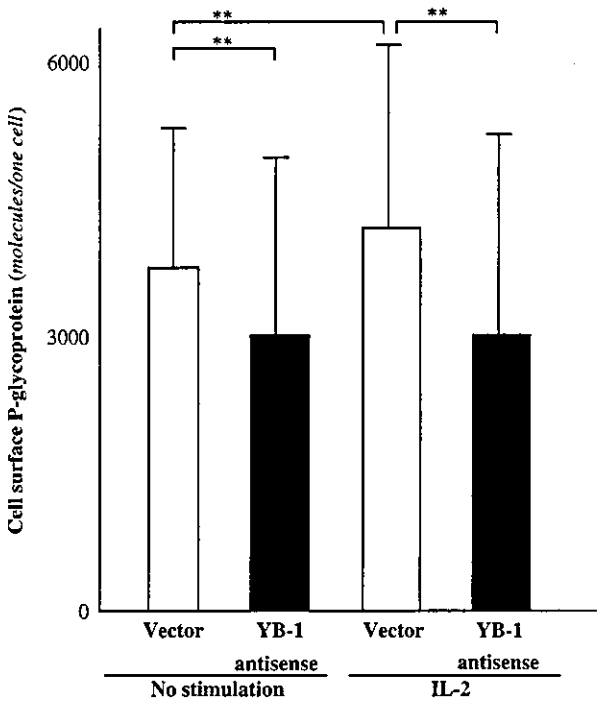

Figure 5 YB-1 anti-sense inhibits IL-2-induced P-glycoprotein expression on PBMCs. Flow cytometric analysis showed P-glycoprotein expression on 2×10^6 of normal PBMCs that were transfected with YB-1 anti-sense constructs (closed bars) or control vacant vector (open bars) and then incubated with or without 10 ng/mL of IL-2. Each value represents the number of molecules expressed per cell, calculated using standard QIFIKIT beads. Data represent mean ± SD of six independent experiments. Statistical analysis was performed using the paired *t*-test. $^{**}P < 0.01$.

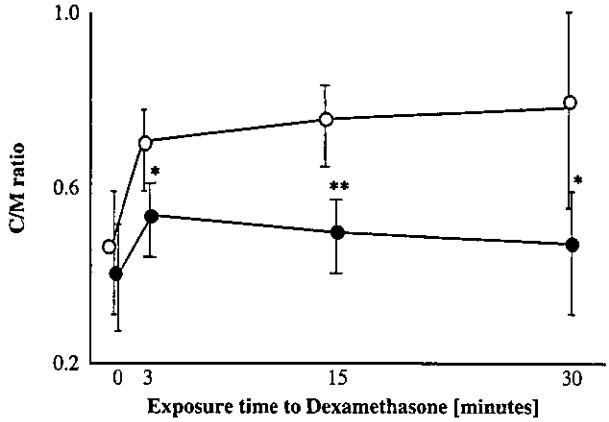

Figure 6 Excretion of intracellular dexamethasone through P-glycoprotein induced by IL-2. Excretion of intracellular dexamethasone was evaluated by C/M ratio, an index of intracellular [6,7-^{3}H(N)]-dexamethasone concentration (C) and extracellular [6,7-^{3}H(N)]-dexamethasone concentration in conditioned medium(M) ratio. Time course of accumulation of [6,7-^{3}H(N)]-dexamethasone in 1×10^6 of PBMCs after incubated with (●) or without (○) 10 ng/mL of IL-2 for 4 h. Each points represent mean ± SD of five independent experiments. Statistical analysis was performed using the non-paired *t*-test. $^*P < 0.05$, $^{**}P < 0.01$.

(data not shown). We further propose that cyclosporin A could be used not only as an inhibitor of NF-AT-dependent IL-2 transcription but also as the competitive inhibitor of P-gp in activated lymphocytes of SLE patients, since dexamethasone concentration in IL-2-activated lymphocytes was recovered by low dose of cyclosporin A. Taken together, the regulation of P-gp on lymphocytes could provide a novel therapeutic strategy in patients with multidrug resistance, including patients in active stages of systemic autoimmune diseases as well as progressive states of leukaemia/lymphoma.

Experimental procedures

Isolation of peripheral blood mononuclear cells from healthy donors

We isolated peripheral blood mononuclear cells (PBMCs) from healthy donors by density gradient centrifugation using Lymphocyte Separation Medium 50494 (Pharmacia Biotech, Uppsala, Sweden) as previously described (Tanaka *et al.* 1997; 1999). We confirmed that purified PBMCs containing more than 90% of lymphocytes (CD4, CD8 or CD20 positive cells) and less than 10% of CD14 positive monocytes by immunostaining.

The study was approved by the human subject research committee of the University of Occupational and Environmental Health, School of Medicine, and informed consent was obtained from all donors who enrolled in the study.

Immunostaining and confocal microscopy analysis

PBMCs were plated in a 12-well culture dish (2×10^5 cells/well) and incubated for 20 min at 37 °C in the presence or absence of 10 ng/mL of recombinant human IL-2 (Becton Dickinson Labware, Mountain View, CA, USA) in RPMI 1640 (Nissui, Tokyo, Japan) containing 5% FCS (Bio-Pro, Karlsruhe, Germany). The cells were then treated with 4% formaldehyde (Sigma Aldrich Japan, Tokyo) in FACS medium for 15 min and then with 0.1% saponin (Sigma Aldrich Japan) in FACS medium. The cells thus obtained were incubated with a specific antibody (Ab) against YB-1 (a binding protein to the Y box and CCAAT box, which is critical for the *cis*-regulatory element that regulates drug-induced MDR-1 gene expression (Ohga *et al.* 1998)) for 30 min at 4 °C. Subsequently, the cells were incubated with FITC-conjugated anti-rabbit IgG Ab at saturating concentrations in FACS medium. We performed confocal analysis of YB-1 using a LSM 410 invert Laser Scan Microscope (Carl Zeiss Microscope Systems, Germany).

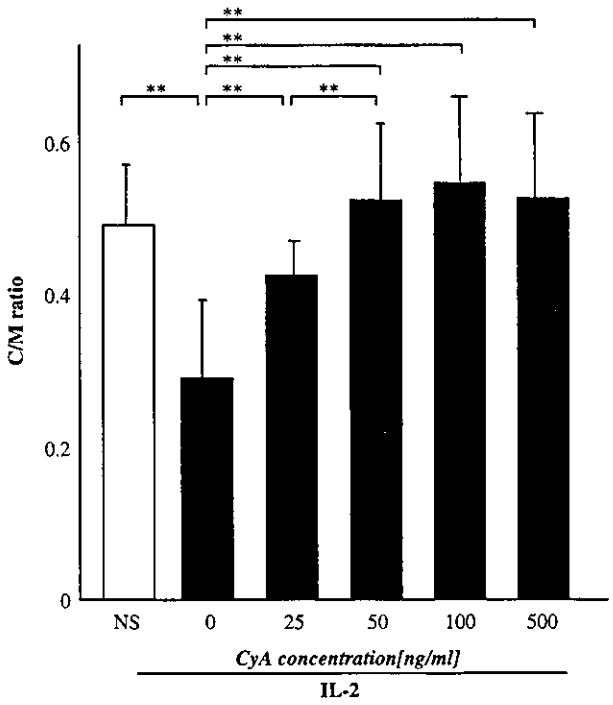

Figure 7 Cyclosporin A inhibits Excretion of intracellular dexamethasone by P-glycoprotein. 1×10^6 of PBMCs were preincubated with (■) or without (□) 10 ng/mL of IL-2 for 4 h. Then 20 min after the addition of [6,7-^{3}H(N)]-dexamethasone, C/M ratio was evaluated in the presence of indicated concentrations of cyclosporin A. Data represent mean ± SD of 10 independent experiments. Statistical analysis was performed using the paired *t*-test. $^{**}P < 0.01$.

Gel shift assay

Nuclear extracts from PBMCs were prepared as previously described (Ohga *et al.* 1998) and then incubated with or without 10 ng/mL of recombinant human IL-2. In the next step, 4 μg of nuclear protein were preincubated for 20 min at room temperature in 15 μL of buffer (10 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM 2-mercaptoethanol, 4% glycerol, and 40 mM NaCl) containing 0.5 μg of poly(dI-dC) (Pharmacia Biotech, Uppsala, Sweden) and a P32-end-labelled double-stranded oligonucleotide containing the YB-1 consensus binding site (5′-GGGCAGTTTTAGCCAGCTCCTCCCTA-3′, 5′-GGGGTAGGGAGGAGCTGGCTAAAACT-3′) as previously described (Dignam *et al.* 1983). The reaction mixtures were electrophoresed on 4% polyacrylamide gels in 0.25 × TAE buffer. For the supershift experiments, nuclear proteins were incubated with a specific antibody (Ab) against YB-1 (a binding protein to the Y box and CCAAT box, which is critical for the *cis*-regulatory element that regulates drug-induced MDR-1 gene expression (Ohga *et al.* 1998) before adding the P^{32}-end-labelled double-stranded oligonucleotide containing the YB-1 consensus binding site (YB-1 oligo). For the cold competition assay, 25-fold molecular excess of double stranded YB-1 oligo or irrelevant double stranded oligonucleotide were preincubated with nuclear extract before the addition of hot probes.

Reverse transcription-polymerase chain reaction

After 4 h of incubation with or without 10 ng/mL of IL-2, total cellular RNA from PBMCs was isolated by a single step isolation procedure with ISOGEN (Wako, Osaka, Japan) and stored purified total RNA at −80 °C. Five hundred ng of total RNA were reverse transcribed at 42 °C for 30 min. Amplification with specific primers for MDR-1 and β2 microglobulin was performed in an iCycler (Bio-Rad, Richmond, CA, USA) for 30 cycles of 45 s at 94 °C for denaturing, 45 s at 55 °C for annealing and 90 s at 72 °C for extension. The primer sequences were as follows: human β2-microglobulin forward 5′-ACCCCCACTGAAAAAGATGA-3′, reverse 5′-ATCTTCAAACCTCCATGATG-3′; human MDR-1 forward 5′-CCCATCATTGCAATAGCAGG-3′, reverse 5′-GTTCAAACTTCTGCTCCTGA-3′. Amplified products were electrophoresed with Marker 4 (Nippon Gene, Tokyo, Japan) on 3% agarose gels.

Flow cytometric analysis

Staining and flow cytometric analysis of PBMCs were conducted by standard procedures as previously described using a FACScan (Becton Dickinson) (Tanaka *et al.* 1997; 1999). Briefly, PBMCs (2×10^5 cells/well) were initially incubated with polyclonal γ-globulin (10 μg/mL, Yoshitomi Pharmaceutical Co.) for the blocking of Fc-receptors and then incubated with MRK-16, a specific monoclonal antibody (mAb) against P-gp (Hamada & Tsuruo 1986), followed by FITC-conjugated anti-mouse IgG Ab (Fujisawa, Osaka, Japan) in FACS medium consisting of phosphate-buffered saline (PBS), 0.5% HSA, and 0.2% NaN_3 (Sigma Aldrich Japan). For the two-colour analysis, we incubated PBMCs with phycoerythrin (PE)-conjugated CD4 mAb, CD8 mAb or CD19 mAb (Fujisawa, Osaka, Japan) after blocking of free anti-mouse IgG-binding sites with irrelevant antibodies. Monoclonal antibodies-two-colour-stained cells were detected by electronic gating based on their CD4, CD8 or CD19 expression using a FACScan. Amplification of mAb-binding was provided by a three-decade logarithmic amplifier. Quantification of the cell surface antigens on one cell was performed using QIFIKIT beads (Dako, Kyoto, Japan) as reported previously (Tanaka *et al.* 1996).

Transfection of anti-sense oligonucleotides of YB-1 in PBMCs

YB-1 anti-sense expression plasmid (PRC/CMV AS) was constructed as previously described (Ohga *et al.* 1996). We transfected 2 μg of PRC/CMV AS or control vacant vector into 2×10^6 PBMCs in a six-well culture dish using a cationic liposome-mediated transfection method, with cationic lipid reagents (DMRIE-C, Life Technologies, Rockville, MD, USA) according to the instructions provided by the manufacturer (Itoh *et al.* 1993;

Rodriguez-Viciana *et al.* 1997; Tamada *et al.* 1997). Forty-eight h after transfection, the cells were used for the following experiments.

Dexamethasone accumulation

[^{14}C]n-Butanol(Toho Biochemical, Tokyo, Japan; 1.61 mCi/mmol) diluted with unlabelled butanol (Sigma Aldrich Japan) at a concentration of 0.5 MBq/mL [^{3}H]-dexamethasone (PerkinElmer Life Sciences, Boston, MA. USA; 40.0 Ci/mmol) was dissolved in Dimethyl sulphoxide (DMSO; Nacalai tesque, Tokyo, Japan) before diluting with PBS (final concentration of DMSO was 0.1%). PBMCs incubated with or without 10 ng/mL of IL-2 for 4 h at 37 °C were resuspended in PBS with 7 mM of dextrose for ATP supply, which is dispensable in this assay (Richard & John 1993), at a cell density of 5×10^{6} cells/mL. In the next step, PBMCs incubated with 5.0×10^{-5} M of [^{14}C]n-Butanol and 3.0×10^{-8} M of [^{3}H]-dexamethasone for 0-30 min time range at 37 °C. For competitive studies with cyclosporin A, PBMCs were incubated with 0-100 ng/mL of cyclosporin A (Novartis Pharmaceutical, Japan Co., Tokyo, Japan) for 15 min before incubated with [^{14}C]$_{n}$-Butanol and [^{3}H]-dexamethasone. Cyclosporin A was dissolved in DMSO before diluting with PBS (final concetration of DMSO was 0.03%). After incubation with IL-2 and cyclosporin A, 100 μL of aliquots were layered on 80 μL of the mixture of lauryl bromide and silicone oil (mixture ratio 2 : 1, Nacalai tesque, Tokyo, Japan) in an Eppendorf tube (Assist, Tokyo, Japan). After centrifugation at 10 000 r.p.m. for 2 min, the aliquots were rapidly frozen in liquid nitrogen, the frozen tube was cut between medium-mixture borders. We thereby obtain an upper layer as medium fraction and a lower layer as cell fraction. The obtained cell fractions were melted with soluene-350 and 10 mL of HIONIC-FLUOR (Packard, Meriden, USA) was added. The medium fractions were mixed with 10 mL of mixtures of toluene (Wako, Osaka, Japan), methanol (Wako, Osaka, Japan), ethylene glycol monoethyl ether (Nacalai tesque, Tokyo, Japan) and PERMAFLUOR (Packard, Meriden, USA; mixture ratio 200 : 50 : 50 : 12). Radioactivity of each fraction was counted with scintillation counter. C/M ratio, which is an index of intracellular dexamethasone concentration and extracellular concentration ratio, was computed using the following formula: C/M ratio = [(^{3}H in cell fraction/^{14}C in cell fraction)/(^{3}H in medium fraction/^{14}C in medium fraction)].

Statistical analysis

Student's *t*-test was used to compare data between two groups. One-way ANOVA and Bonferroni correction were used to compare data between three or more groups. Values are expressed as mean ± SD $P < 0.05$ was considered statistically significant.

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企画：竹内　勤+

T細胞内のシグナル伝達分子機構

第17回

β_1インテグリンシグナル伝達とSLE

田中良哉*　中山田真吾*

全身性エリテマトーデス(SLE)の病態形成の過程においては，自己反応性T細胞の活性化とB細胞の活性化が中心的に関与する．自己反応性T細胞のシグナル賦活化には，T細胞レセプターの共刺激分子シグナルが関与するが，代表的な共刺激分子であるCD 28の発現はむしろ顕著に減弱する．われわれは，活動期SLE患者T細胞では，CD 28の低下に反比例して，β_1インテグリン(CD 29)を介するシグナルが量的，質的に亢進し，さらに，β_1-FAKを介する賦活化シグナルの亢進は，CD 28非依存性に自己反応性T細胞の過剰な活性化，ひいては，臓器病変の進展に寄与する可能性を認めた．CD 28の減弱に対するβ_1の相補的役割は，SLEの疾患活動性に密接に関与するとすれば，今後，このような免疫シグナル異常の是正を目的として，新規治療軸の確立が期待される．

はじめに

全身性エリテマトーデス(systemic lupus erythematosus：SLE)は，若年発症と多臓器病変を特徴とする代表的な全身性自己免疫疾患である．その病態形成においては，自己免疫寛容が破綻して活性化された自己反応性T細胞クローンが，B細胞活性化と自己抗体の過剰産生を引き起こし，免疫複合体形成を介した血管炎や多臓器障害を引き起こす[1)-3)]．このような自己免疫の誘導の過程では，免疫シグナルの異常な賦活化，ならびに，免疫抑制性シグナルの機能異常が関与するとされる．

たとえば，自己反応性T細胞の活性化は，抗原提示細胞(antigen-prosenting cells：APC)からT細胞レセプター(T cell receptor：TCR)を介する抗原刺激と共刺激分子からのシグナルの共存でもたらされる(図1)．通常は，共刺激分子を介するシグナルの量的あるいは質的低下により自己反応性T細胞が負の制御を受けることによって自己寛容が維持されている．また，転写因子Foxp3で誘導される制御性T細胞は，APC上の微量の自己抗原を認識し，CTLA-4などの共刺激分子からの負のシグナルを受容し，自己反応性T細胞を抑制する[4)-6)]．自己寛容の破綻，すなわち，自己免疫の誘導は，共刺激分子から伝達される免疫シグナルの異常な賦活化，ならびに，制御性T細胞などで伝達される免疫抑制性シグナルの機能異常によって，誘導される．

〔キーワード〕
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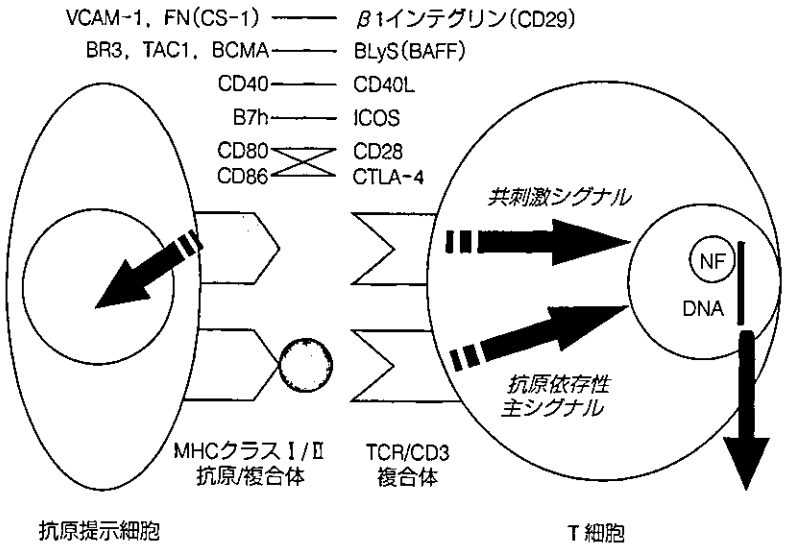

図 1. 抗原提示細胞による T 細胞の活性化機構
自己反応性 T 細胞の活性化は，抗原提示細胞から TCR を介する抗原刺激と共刺激分子からのシグナルの共存でもたらされる．

このような免疫寛容の破綻によってもたらされる自己反応性 T 細胞の活性化には，共刺激分子の発現や機能の異常が関与するが，SLE の病態形成過程においては不詳な点が多く，また，モデル動物の結果だけでは説明できない現象も決して少なくない．本稿では，SLE 患者 T 細胞の賦活化の機構と共刺激分子としての β_1インテグリンを介したシグナル異常との観点から概説する．

1．β_1インテグリンで誘導される多彩な細胞機能

インテグリンは，細胞膜表面に発現し，細胞と細胞，あるいは，細胞外基質との接着を媒介する主要な糖蛋白質である．インテグリンの構造と機能はきわめて多岐にわたり，生体内の基本的機能全般において中心的役割を担っている．インテグリンは，分子量 90～180 kDa の α 鎖と 90～110 kDa の β 鎖が非共有結合する膜貫通型糖蛋白質で，β 鎖の違いにより β_1や β_2サブファミリーなどに分類され，α 鎖とのペアにより $\alpha_4\beta_1$(VLA-4)，$\alpha_L\beta_2$(LFA-1)などの接着分子名が決定する．

β_1インテグリン(CD 29)は，赤血球以外の細胞膜表面にユビキタスに発現する分子である．β_1インテグリンは，フィブロネクチンやコラーゲンなどの細胞外基質，および，血管内皮細胞や樹状細胞に発現する VCAM-1 との接着に関与し，生体の発生・分化，常態維持などの基本的生体機能の全般において中心的役割を担う．たとえば，β_1のノックアウト(KO)マウスは，初期発生が障害され胎生 5 日で致死的となる．また，インテグリンは，PMA やケモカイン刺激で伝達される細胞内シグナルによって活性化されて，立体構造の変化や多量体化によってアフィニティーやアビディティーが高まり，基質との十分な接着活性を得る(inside-out シグナル)．

β_1インテグリンは，単に細胞接着のみならず，基質との接着によって細胞内にチロシンリン酸化や低分子量 G 蛋白質などの活性化シグナルを伝達し，細胞増殖やサイトカイン産生などの細胞の活性化をもたらす．すなわち，インテグリンは，細胞外情報を細胞内へ伝達して(outside-in シグナル)，炎症などの病態形成，癌細胞増殖・転移など

に広く役割を担う[7)~9)]．また，T細胞，とくに，メモリーT細胞にβ_1は強く発現し，T細胞の共刺激分子として作用し，T細胞増殖，IL-2産生を誘導する[10)]．実際，健常人T細胞において，CD3抗体架橋刺激によるTCRの刺激，または，β_1抗体架橋によるβ_1の刺激を加えても何ら変化がないが，両刺激の共存によって，IL-2産生や細胞増殖が誘導される．また，CD40LやCD69は，無刺激の健常人T細胞では発現しないが，CD3とβ_1の刺激の共存により6時間以内に発現が誘導される．すなわち，β_1は，共刺激分子としてT細胞の活性化においても重要な機能分子と考えられる．逆に，インテグリンなどの接着分子が伝達するシグナルを阻害することによって，より効率的で特異的な疾患の制御が試みられる．

2．SLEにおけるT細胞の共刺激分子

SLEのモデル動物の発症においても，TCRと共刺激分子を介するシグナルの活性化，ならびに，制御性T細胞による調節異常の結果，自己反応性T細胞クローンの増幅がもたらされる．

共刺激分子としては，CD40Lに代表されるTNFファミリー分子群の関与が注目される．SLE自然発症モデルマウスでは，CD40LをKOすると発症遅延，二本鎖DNA抗体産生抑制，糸球体腎炎改善が観察され，また，CD40L抗体投与により蛋白尿が改善され，顕著な延命効果をもたらす[11)~13)]．同様にTNFファミリーに属するBAFF (B lymphocyte stimulator：BLyS)とそのレセプターTACI, BR3, BCMAとの相互作用も，SLEの病態形成を担う[14)]．*BLyS*遺伝子導入マウスでは，B細胞数が増加してSLE様の病態を形成し，SLEモデルマウスでは，可溶性BLySの添加，*TACI-Ig*キメラや*BR3-Ig*キメラの遺伝子導入により，生存期間が著明に延長する[15)]．さらに，SLE患者のT細胞ではBLySの発現が増強し，血清BLyS値は，疾患活動性やds-DNA抗体価と相関する．実際，SLE患者へのBLyS抗体投与によるds-DNA抗体価の改善が報告される[16)]．

CD28も，CD80とCD86をリガンドとする代表的な共刺激分子である．同様にCD80とCD86のリガンドであるCTLA-4は，自己反応性T細胞に負のシグナルを伝達し，免疫寛容を誘導する．関節リウマチ(rheumatoid arthritis：RA)に対しては，CD28を介する共刺激拮抗的阻害を目的としたCTLA-4-Igキメラの治験がなされ，第II相臨床試験でRA症例の60%以上にACR 20が得られている[17)]．しかし，健常人にくらべSLE患者のCD4陽性T細胞およびCD8陽性T細胞では，CD28発現の著しい減少，ないし，消失が報告され，正常T細胞と異なる刺激伝達系の存在が示唆されている[18)19)]．実際に，SLE症例に対してCTLA-4-Igキメラを使用しても，有効性はほとんど報告されていない．

われわれも，健常人および無治療の活動期SLE患者から採取した末梢血T細胞の細胞表面機能分子の発現を検討し，SLE患者T細胞は，無刺激下でCD40LやCD69などの細胞表面抗原を高発現し，活性化が示された(図2)．これに対して，SLE患者T細胞，とくに$CD4^+CD45RO^+$(メモリー)T細胞で，CD28発現が健常人にくらべ著明に低下した．また，CD28の発現低下は，活動期の症例やループス腎炎などの臓器病変を有する症例でとくに顕著であった．CD28陰性T細胞は，マイトーゲンなどで刺激しても活性化，増殖できないとされ[20)]，SLEのT細胞の活性化過程においては，共刺激分子としてのCD28の役割は軽度で，相補する分子が存在し，正常T細胞と異なる刺激伝達系の存在が示唆された．

3．SLE患者T細胞活性化におけるβ_1インテグリンシグナル伝達の役割

特記すべきこととして，SLE患者T細胞では，β_1インテグリン(CD29)の発現が健常人T細胞とくらべ2~3倍に増強し，その発現量は疾患活動性に比例した(図2)．β_1の発現は，CD4陽性メモ

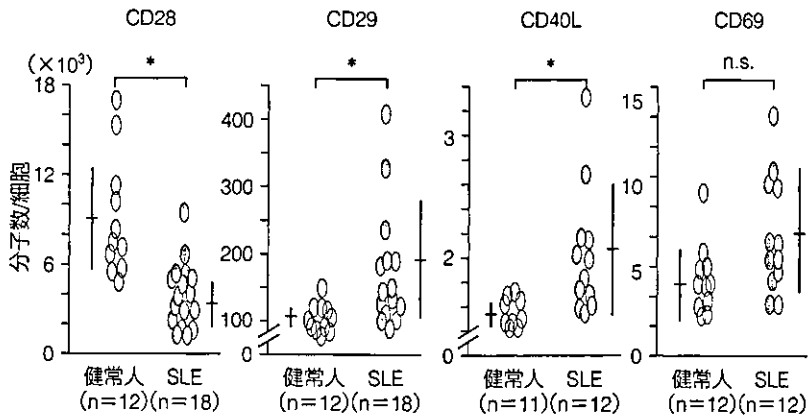

図 2. 健常人および SLE 患者 T 細胞上の細胞表面機能分子の発現
SLE 患者末梢血の T 細胞では，CD 28 の発現低下，CD 29(β_1)および CD 40 L の発現増強がみられた．
*$p<0.05$

リーT 細胞でとくに強く，活性型 β_1 の発現量も増加していた．さらに，SLE の T 細胞の β_1 の発現量，発現率はおのおの，CD 28 のそれらと反比例した．他施設からも，SLE 患者 T 細胞の β_1 発現と血清 ds-DNA 抗体価との相関，SLE 患者 CD 8 陽性細胞の β_1 発現増強と血清 IgG との相関，血管炎を伴う SLE における T 細胞 VLA-4($\alpha_4\beta_1$)の発現増強が報告されている[21)~23)]．

以上は，SLE の病態，疾患活動性の鍵を握る T 細胞の活性化と β_1 インテグリン発現の関連性を示唆するものである．そこで，SLE の T 細胞の活性化における β_1 の機能的な役割を検討した．その結果，健常人 T 細胞の活性化には，CD 3 と β_1 の共刺激が必須であるのに対して，SLE の T 細胞では，β_1 単独刺激でも細胞増殖が誘導された．さらに，SLE 患者 T 細胞上の CD 40 L や CD 69 の発現も，β_1 単独刺激で誘導され，SLE 患者 T 細胞における β_1 を介する刺激は，TCR 刺激を不要とする強力なシグナルを伝達する可能性が示唆された．

β_1 インテグリンを介する接着部の細胞質内にはチロシンリン酸化された蛋白質が豊富に集積し，また，細胞骨格成分が重合して接着斑を形成する．β_1 の細胞内シグナル伝達では，非レセプター型チロシンキナーゼである focal adhesion kinase (FAK)が中心的に関与し，リガンドが結合しリン酸化された FAK は下流分子と結合し，Src 型キナーゼの接着斑への移動と活性化，Ras などの低分子 G 蛋白質を介して MAPK，PI-3 K などの経路を活性化し，細胞増殖やサイトカイン産生などのさまざまな細胞機能を効率よく誘導する(図 3)[24)]．

健常人由来の T 細胞に，*FAK* の野生型遺伝子，または，優勢抑制型遺伝子(*FAT* や *FRNK*)を遺伝子導入すると，CD 3 と β_1 インテグリン架橋刺激の共存で誘導される T 細胞の増殖は抑制された．また，これらの刺激で誘導された CD 40 L や CD 69 の発現も，優勢抑制型遺伝子の導入により完全に阻害された．さらに，SLE 患者 T 細胞の無刺激下や β_1 架橋単独刺激下で誘導された細胞増殖や CD 40 L の発現も，チロシンキナーゼ阻害薬による前処理，または，*FAK* の優勢抑制型変異遺伝子を導入することで阻害された．以上，SLE 患者 T 細胞の活性化に伴う細胞増殖や CD 40 L の発現には，FAK などのチロシンキナーゼを介するシグナル伝達の関与が示唆された．

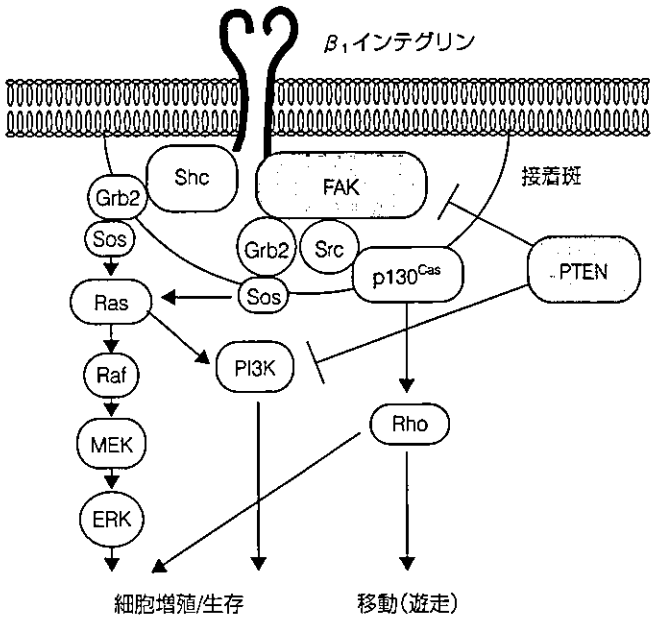

図 3. β_1インテグリンで誘導される細胞内シグナル(Parsons JT, 2003[24]より改変引用)
リガンドが結合しリン酸化されたFAKは下流分子と結合し，Src型キナーゼの接着斑への移動と活性化，Rasなどの低分子G蛋白質を介してMAPK，PI-3Kなどの経路を活性化し，さまざまな細胞機能を誘導する．

4．SLE病態形成におけるβ_1インテグリンの関与とその制御

SLE患者におけるT細胞のβ_1インテグリンの発現や活性化は，ループス腎炎などの臓器病変を有する症例で顕著であった．ループス腎炎組織では，T細胞，単球やメサンギウム細胞から産生されたインターフェロン(IFN)-γなどにより血管内皮細胞上のVCAM-1の発現が増強し，β_1強陽性T細胞が効率よく浸潤し，炎症病態を展開するものと考えられる[25]．実際，MRL/lprなどのSLEモデルマウスでは，ループス腎炎発症早期の糸球体組織においてFAKのチロシンリン酸化が著明に亢進する[26]．同様に，SLEの主要臓器病変である中枢神経ループスでは，微小血管傷害が病態に寄与する．これらのモデルマウスに対して，抗α_4インテグリン抗体または抗VCAM-1抗体を投与すると，中枢神経系（central nervous system：CNS)病変での血管内皮細胞への炎症細胞の接着・ローリングが著明に阻害されることから，VLA-4/VCAM-1シグナル系のCNS病変進展への関与が示唆される[27]．

以上の結果より，活動期SLE患者T細胞では，代表的な共刺激分子であるCD 28の発現が，特徴的に低下しているが，CD 28の発現低下に反比例して，β_1インテグリン(CD 29)を介するシグナルが量的，および，質的に亢進している．さらに，β_1-FAKを介する賦活化シグナルの亢進は，CD 28非依存性に作用し，自己反応性T細胞の過剰な活性化，臓器病変の進展に寄与する可能性が示唆される(図4)．CD 28の減弱に対するβ_1の相補的シグナルは，CD 40 Lなどの他の共刺激分子の発現や細胞の病態組織への効率的な浸潤による炎症の遷延をもたらし，自己反応性T細胞の過剰活性化を介してSLEの疾患活動性に密接に関与するとすれば，今後，このような免疫シグナル異