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【Ⅴ】研究成果の刊行物・別刷

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β_1 Integrin/Focal Adhesion Kinase-mediated Signaling Induces Intercellular Adhesion Molecule 1 and Receptor Activator of Nuclear Factor κB Ligand on Osteoblasts and Osteoclast Maturation*

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We have assessed characteristics of primary human osteoblasts, shedding light on signaling mediated by β_1 integrin. β_1 integrins are major receptors for these matrix glycoproteins. 1) Integrins β_1, α_2, α_3, α_4, α_5, α_6, and α_v were highly expressed on primary osteoblasts. 2) Engagement of β_1 integrins on osteoblasts by cross-linking with specific antibody or ligand matrices, such as fibronectin or collagen, augmented expression of intercellular adhesion molecule 1 (ICAM-1) and receptor activator of nuclear factor κB ligand (RANKL) on the surface. 3) Up-regulation of ICAM-1 and RANKL on osteoblasts by β_1 stimulation was completely abrogated by pretreatment with herbimycin A and genistein, tyrosine kinase inhibitors, or transfection of dominant negative truncations of focal adhesion kinase (FAK). 4) Engagement of β_1 integrins on osteoblasts induced tartrate-resistant acid phosphatase-positive multinuclear cell formation in the coculture system of osteoblasts and peripheral monocytes. 5) Up-regulation of tartrate-resistant acid phosphatase-positive multinuclear cell formation by β_1 stimulation was completely abrogated by transfection of dominant negative truncations of FAK. Our results indicate that β_1 integrin-dependent adhesion of osteoblasts to bone matrices induces ICAM-1 and RANKL expression and osteoclast formation via tyrosine kinase, especially FAK. We here propose that β_1 integrin/FAK-mediated signaling on osteoblasts could be involved in ICAM-1- and RANKL-dependent osteoclast maturation.

Bone metabolism in health and disease is based on a self-regulating cellular event. The two major processes of bone remodeling, bone formation and resorption, are closely regulated by intercellular signaling involving soluble factors, systemic hormones, and cellular adhesion (1–5). Osteoblasts play a central role in bone formation by synthesizing multiple bone matrix proteins and by differentiation into osteocytes. However, osteoblasts also regulate osteoclast maturation by producing bone-resorbing cytokines and by direct cell attachment, resulting in bone resorption (6–8). Cell adhesion of osteoblasts and osteoclastic precursors of hematopoietic origin is a prerequisite for osteoclast maturation. Several studies have demonstrated that interaction of receptor activator of nuclear factor κB ligand (RANKL)[1] on osteoblasts and RANK on osteoclast precursors provides an essential signal to osteoclast precursors for their maturation into resorbing cells (9–11). We have previously reported that human osteoblasts express intercellular adhesion molecule (ICAM)-1 and that interaction between ICAM-1, expressed on osteoblasts, and leukocyte function-associated antigen (LFA)-1, expressed on monocytes, is required for osteoclast maturation by RANKL on osteoblasts (12).

Adhesion molecules play a fundamental role in cell-to-cell and cell-to-extracellular matrix (ECM) interactions. However, recent findings have indicated that certain adhesion molecules not only function as glue but also regulate several cellular functions by transducing signaling. We have reported that ICAM-1 on rheumatoid synovial cells induced transcription of interleukin-1β by activation of a nuclear factor, AP-1, and that stimulation of β_1 integrin up-regulated ICAM-1 and Fas, and Fas mediated apoptosis of rheumatoid synovial cells through focal adhesion kinase (FAK) (13, 14). These results prompted us to investigate the adhesion molecules involved in regulating the expression of other adhesion molecules, such as ICAM-1 and RANKL, on human osteoblasts. Cell adhesion to matrices is primarily mediated by integrins, cell surface receptors that comprise an expanding family of transmembrane heterodimers of α and β subunits (15–18). Interaction of integrins with their protein ligands increases tyrosine phosphorylation and triggers the assembly of cytoskeletal proteins, signaling complexes including FAKs, and their substrates into membrane-substratum junctions referred to as focal adhesions (19–23).

Although osteoblasts are always surrounded by and encounter ECMs including type I collagen and fibronectin mainly through β_1 integrin, the relevance of β_1 integrin to the intracellular signaling and functions in osteoblasts remains unclear. It is well established that osteoblast differentiation and maturation are regulated by their interaction with ECMs such as type I collagen (24, 25). However, such an adhesive interaction may act on osteoblasts to modulate bone metabolism, not only bone formation by activating osteoblasts to proliferate and

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[1] The abbreviations used are: RANKL, receptor activator of nuclear factor κB ligand; RANK, receptor activator of nuclear factor κB; ICAM-1, intercellular adhesion molecule 1; FAK, focal adhesion kinase; TRAP, tartrate-resistant acid phosphatase; MNC, multinuclear cell; LFA, leukocyte function-associated antigen; ECM, extracellular matrix; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; Ab, antibody; mAb, monoclonal antibody; VCAM-1, vascular cell adhesion molecule 1; VSV, vesicular stomatitis virus; FAT, focal adhesion targeting domain; FRNK, FAK-related non-kinase; PBS, phosphate-buffered saline; ABC, antibody binding capacity; PI 3-K, phosphoinositide 3-kinase.

synthesize bone matrix protein but also bone resorption by indirectly activating osteoclast function and differentiation mediated through osteoblasts. The aim of the present study was to determine the role of β_1 integrin-mediated signaling in the regulation of cell surface adhesion molecules on osteoblasts. Our results demonstrate that engagement of β_1 integrin by a specific antibody or ligand matrices up-regulated ICAM-1 and RANKL expression on osteoblasts and induced osteoclast formation via tyrosine kinase, especially FAK.

EXPERIMENTAL PROCEDURES

The study protocol was approved by the Human Ethics Review Committee of the University of Occupational and Environmental Health, Japan, and a signed consent form was obtained from each subject prior to taking tissue samples used in the present study.

Purification of Human Osteoblastic Cells—Osteoblast-like cells were purified from metaphyseal trabecular bone in the proximal femur of four osteoarthritis patients during total hip arthroplasty by the established procedures of Russell and colleagues (26, 27). After removing pieces of cortical bone, articular cartilage, and soft connective tissue, the fragments were cut into small pieces and washed extensively. The bone explants were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10% fetal calf serum (FCS, Invitrogen) in 25-cm^2 culture flasks (Falcon, Lincoln Park, NJ) in a humidified 5% CO_2 atmosphere. When cell monolayers were confluent after the 6–8-week culture, the explants were removed, and the cells were replated and incubated, resulting in new cellular outgrowth and eventually a confluent monolayer of cells. At confluence, the cells were trypsinized, passaged at a 1:3 split ratio, and recultured. The medium was changed twice each week, and the cells were used after three to seven passages. The obtained cells showed a flattened polygonal shape with multiple spindle legs and possessed characteristics of osteoblast-like phenotype including osteocalcin, bone sialoprotein, type I collagen, and bone alkaline phosphatase as described previously (27).

Antibodies and Other Reagents—The following monoclonal antibodies (mAbs) were used as purified Igs in preparation of staining and analysis of cell surface or cytoplasmic molecules: control mAb thy1.2 (BD Biosciences), human CD29 (β_1 integrin) mAb MAB13, human CD18 (β_2) mAb TS1/18 (kindly provided by Dr. K. M. Yamada, National Institutes of Health, Bethesda, MD), human CD61 (β_3) mAb, human CD49a (α_1) mAb TS2/7, human CD49b (α_2) mAb, human CD49c (α_3) mAb, human CD49d (α_4) mAb NIH49d-1, human CD49e (α_5) mAb MAB16, human CD49f (α_6) mAb NIH49f-1, human CD51 (α_v) mAb 23C6, human CD54 (ICAM-1) mAb 84H10 (kindly provided by Dr. S. Shaw, National Institutes of Health, Bethesda, MD), anti-human RANKL mAb (Sigma), human CD106 (VCAM-1) mAb 2G7 (kindly provided by Dr. W. Newman, Otsuka America, Rockville, MD), major histocompatibility complex class I mAb W6/32, and anti-glycophorin mAb 10F7 (American Type Culture Collection, Manassas, VA). A human wild-type FAK expression plasmid (VSV-FAK), a human focal adhesion targeting domain (FAT) expression plasmid (VSV-FAT), and a human FAK-related non-kinase (FRNK) expression plasmid (VSV-FRNK) were constructed as described previously (28, 29). Multiple inhibitors of intracytoplasmic signaling including wortmannin (Wako Pure Chemical, Osaka, Japan; a phosphoinositide 3-kinase (PI 3-K) inhibitor), H7 and staurosporine (Seikagaku, Tokyo, Japan; protein kinase C inhibitors), and herbimycin A (Sigma) and genistein (Calbiochem) (tyrosine kinase inhibitors) were applied to each assay system, and all reagents were used at the indicated concentrations. At these concentrations, none of these inhibitors produced cytotoxic effects on synovial cells as confirmed by trypan blue staining.

Stimulation of Osteoblasts by β_1 Integrin Using mAbs and Substrates—After cells were cultured to subconfluence, the medium was changed to DMEM containing 1% FCS on the day before assay. The obtained cells were then incubated with anti-CD29 (β_1 integrin) mAbs such as MAB13 and control mAbs (10 μg/ml) in DMEM without FCS for 30 min at 37 °C. Signal inhibitors were added at the indicated concentration for a 30-min incubation prior to cell stimulation. After washing the cells three times, 1 μg/ml goat anti-rat IgG-Fc was added as the second Ab for β_1 cross-linking. The cells were then incubated in DMEM without FCS at 37 °C for the indicated duration. Purified fibronectin (10 μg/ml), collagen type I (10 μg/ml), and control bovine serum albumin (10 μg/ml, Wako) were applied to 6-well plastic plates in Ca^{2+}/Mg^{2+}-free PBS at 4 °C overnight. Binding sites on plastic were subsequently blocked with Ca^{2+}/Mg^{2+}-free PBS, 2.5% bovine serum albumin for 2–3 h at 37 °C to reduce nonspecific attachment of the cells. Subsequently plates were washed three times with PBS, and the cells were added to each well as described above and were incubated in DMEM without FCS at 37 °C for the indicated duration. After the incubation, the plates were washed twice with PBS and treated with trypsin for 1 min at 37 °C. DMEM containing 10% FCS was added to stop trypsinization. After harvesting from the wells, the obtained cells were washed with PBS and were settled in medium suitable for the following experiments.

Transfection of Plasmids—A human wild-type FAK expression plasmid (VSV-FAK), a FAT expression plasmid (VSV-FAT), and a FRNK expression plasmid (VSV-FRNK) were introduced into osteoblasts using a cationic liposome-mediated transfection method. Plasmids (2.5 μg) dissolved in 100 μl of serum-free medium (OPTI-MEM, Invitrogen) were mixed with 5 μl of Lipofectin reagent (LipofectAMINE 2000™, Invitrogen) in the same volume of OPTI-MEM and incubated for 15 min at room temperature. The plasmids and liposome complex were added to osteoblasts plated in a 6-well culture dish, incubated for 3 h in OPTI-MEM, and then replaced with DMEM containing 10% FCS for 24 h. The expression of VSV-FAK, VSV-FAT, and VSV-FRNK in osteoblasts was confirmed by flow cytometric analysis using anti-VSV Ab after their transfection. High expression of the VSV was observed, and 50–80% of the cells were transfected by intracellular flow cytometric analysis. A marked difference in the transfection efficiency among all three vectors was not observed in osteoblasts, and none of these vectors produced cytotoxic effects on osteoblasts as confirmed by trypan blue staining (data not shown).

Flow Microfluorometry—Staining and flow cytometric analysis of osteoblasts were conducted by standard procedures as described previously (18) using a FACScan (BD Biosciences). In brief, 2×10^5 cells were incubated with negative control mAb thy1.2, integrin β_1 mAb, β_2 mAb, β_3 mAb, α_1 mAb, α_2 mAb, α_3 mAb, α_4 mAb, α_5 mAb, α_6 mAb, α_v mAb, CD54 (ICAM-1) mAb, RANKL mAb, or CD106 (VCAM-1) mAb in FACS medium consisting of Hanks' balanced salt solution (Nissui, Tokyo, Japan), 0.5% human serum albumin (Yoshitomi, Osaka, Japan), and 0.2% NaN_3 (Sigma) for 30 min at 4 °C. After washing the cells three times with FACS medium, they were further incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG Ab, goat anti-rabbit IgG Ab, or rabbit anti-goat IgG Ab for 30 min at 4 °C. The staining of cells with mAbs was detected using FACScan. Quantification of cell surface antigens on single cells was calculated using standard beads (QIFKIT, Dako Japan, Kyoto, Japan) as already described (30, 31). The data were used for the construction of the calibration curve (mean fluorescence intensity (MFI)) against antibody binding capacity (ABC). The cell specimen was analyzed on the FACScan, and ABC was calculated by interpolation on the calibration curve. When the green fluorescence laser detector was set at the 450 level in the FACScan, $ABC = 414.45 \times \exp(0.0092 \times MFI)$ ($R^2 = 0.9999$). Subsequently specific ABC was obtained after corrections for background, apparent ABC of the negative control mAb thy1.2. Specific ABC corresponds to the mean number of accessible antigenic sites per cell, referred to as antigen density and expressed in sites/cell.

Coculture of Osteoblasts and Monocytes and Subsequent Staining of Tartrate-resistant Acid Phosphatase—Osteoblasts were seeded onto 24-well multiwell dishes (1×10^5 cells/well) and cultured to subconfluence. Then osteoblasts, with or without transfection of several plasmids as mentioned above, were stimulated by cross-linking with specific mAb for 6 h. Purified peripheral blood $CD14^+$ monocytes from healthy donors (1×10^6 cells/well) were added to osteoblasts, and they were cocultured for 9 days in DMEM containing 10% heat-inactivated FCS in the presence of 10^{-7} M $1\alpha,25(OH)_2D_3$. Osteoclasts were cytochemically stained for tartrate-resistant acid phosphatase (TRAP, Sigma) as described previously (32). The number of TRAP-positive multinuclear cells (MNCs) that contained more than three nuclei was counted by light microscopy.

Statistical Analysis—Data were expressed as mean ± S.D. of the number of indicated patients. Differences from the control were examined for statistical significance by analysis of variance followed by posthoc Scheffe's F-test. A p value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

β_1 Integrin Was Highly Expressed on Primary Human Osteoblastic Cells—Initially we assessed the expression of various cell surface functional molecules on primary human osteoblastic cells using FACScan. Fig. 1 shows the number of 12 representative molecules, including integrins, ICAM-1, and RANKL, on osteoblasts. Among the screened molecules, β_1 integrin was

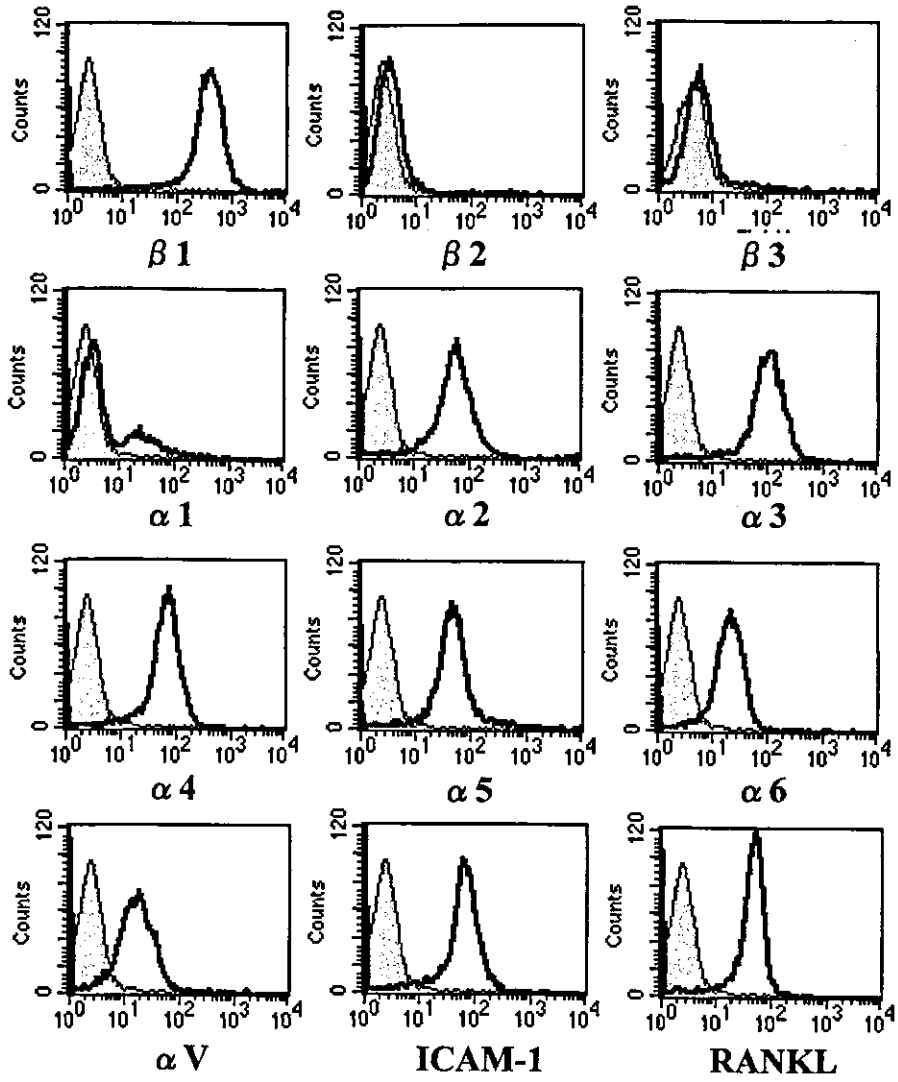

FIG. 1. **β_1 integrin was highly expressed on primary human osteoblasts.** Osteoblasts were stained with CD29 (β_1) mAb MAB13, CD18 (β_2) mAb, CD61 (β_3) mAb, CD49a (α_1) mAb TS2/7, CD49b (α_2) mAb, CD49c (α_3) mAb, CD49d (α_4) mAb NIH49d-1, CD49e (α_5) mAb MAB16, CD49f (α_6) mAb NIH49f-1, CD51 (α_v) mAb 23C6, CD54 (ICAM-1) mAb 84H10, and RANKL mAb. Flow cytometric analyses were performed using FACScan. *Open* histograms represent the number of cells stained with mAb in each logarithmic scale on a fluorescence amplifier. *Shaded* histograms represent profiles of thy1.2 mAb as a negative control. The histogram is a representative result from four different experiments using four different donors.

highly expressed on osteoblasts. Among α subunits of integrins, α_2, α_3, α_4, α_5, α_6, and α_v were expressed on osteoblasts. A histogram implied that the vast majority of osteoblasts expressed β_1. We therefore assumed that β_1 integrin, which is consistently highly expressed on osteoblasts, might play a functional role in primary osteoblasts. ICAM-1 and RANKL were moderately expressed on osteoblasts. Estimation from histograms of multiple donors showed that one-third to two-thirds of osteoblasts expressed ICAM-1 and RANKL without stimulation.

Cross-linking of β_1 Integrin Up-regulated ICAM-1 and RANKL Expression on Osteoblasts—To characterize the function of β_1 integrin on osteoblasts, we assayed the cell surface molecule expression by β_1 cross-linking using a specific mAb and a second cross-linker Ab. Flow cytometry showed that expression of ICAM-1 and RANKL on the surface was markedly augmented by the β_1 cross-linking on osteoblasts. As shown in Fig. 2, ICAM-1 and RANKL were moderately expressed on unstimulated osteoblasts. However, β_1 cross-linking significantly up-regulated ICAM-1 and RANKL expression, whereas cross-linking of major histocompatibility complex class I using their specific mAbs and second cross-linker Ab had no effect. In contrast, cross-linking of β_1 integrin on osteoblasts did not induce VCAM-1 expression (Fig. 2). The results were consistent in osteoblasts derived from four donors. Time course experiments showed that ICAM-1 and RANKL expression reached maximum levels within 3 h after β_1 cross-linking (Fig. 2). These results suggest that β_1 integrin appears to play a pivotal role in ICAM-1 and RANKL up-regulation on osteoblasts.

Engagement of β_1 Integrin by Ligand Matrix Glycoproteins Augmented ICAM-1 and RANKL Expression on Osteoblasts—Fibronectin and type I collagen are major ligands for cell surface β_1 integrin. We next assessed the biological activities of fibronectin and type I collagen on osteoblasts. Expression of both ICAM-1 and RANKL were markedly induced by incubation of these cells on fibronectin- or collagen-coated plastic plates (Fig. 3). In contrast, no change was noted when these cells were incubated on bovine serum albumin-coated plates (Fig. 3). Furthermore ICAM-1 and RANKL expression induced by fibronectin and type I collagen was completely inhibited by pretreatment of cells with anti-β_1 mAb (Fig. 3). These data suggest that fibronectin and type I collagen are possible ligands involved in β_1 integrin-induced ICAM-1 and RANKL expression on osteoblasts.

Involvement of Tyrosine Kinases in β_1 Integrin-mediated Up-regulation of ICAM-1 and RANKL on Osteoblasts—Next, to determine signaling pathways involved in β_1 integrin-induced induction of ICAM-1 and RANKL expression on osteoblasts, we pretreated the cells with or without different concentrations of various inhibitors of intracytoplasmic signaling, and then ICAM-1 and RANKL expression by β_1 cross-linking was determined by FACScan (Fig. 4). Pretreatment of cells with the tyrosine kinase inhibitors herbimycin A or genistein completely

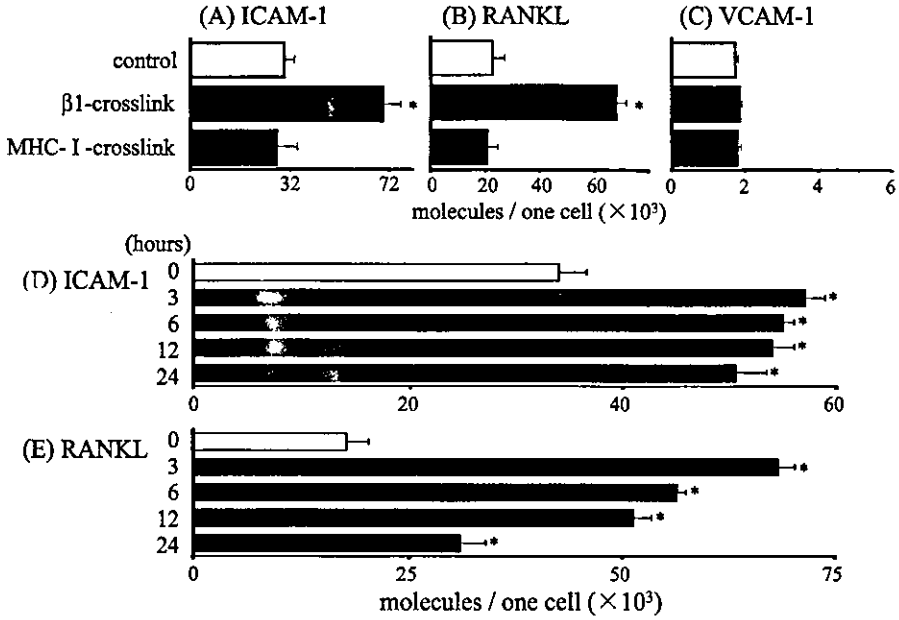

FIG. 2. **Cross-linking of β_1 integrin up-regulated ICAM-1 and RANKL expression on osteoblasts.** Osteoblasts were cross-linked with (*solid bars*) or without (*open bars*) anti-CD29 (β_1) mAb MAB13 and control major histocompatibility complex class I (*MHC-I*) mAb W6/32 at a concentration of 10 mg/ml for 6 h, and then ICAM-1 (*A*), RANKL (*B*), and VCAM-1 (*C*) expressions were analyzed by FACScan. Time course of β_1-triggered ICAM-1 (*D*) and RANKL (*E*) expression on human osteoblasts was also evaluated. Osteoblasts were stimulated with 10 mg/ml anti-CD29 mAb for the indicated duration. Each value represents the number of molecules expressed per one cell calculated using standard QIFKIT beads from four similar experiments as described under "Experimental Procedures." Data are presented as mean ± S.D. *, $p < 0.05$ compared with controls.

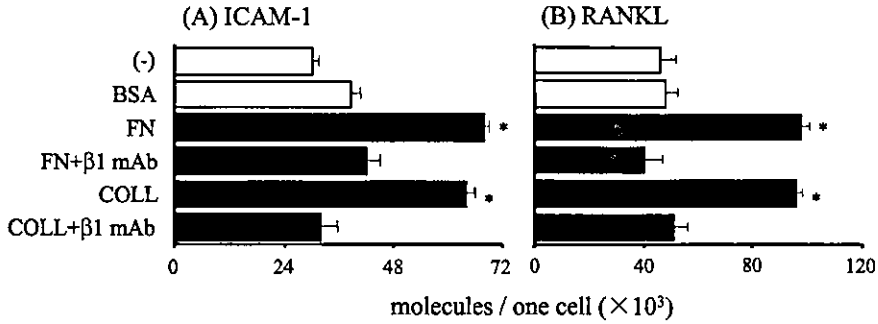

FIG. 3. **Engagement of β_1 integrin by ligand matrix glycoproteins augmented ICAM-1 and RANKL expression on human osteoblasts.** Osteoblasts with or without pretreatment with anti-β_1 integrin blocking mAb were incubated on plastic plates that were precoated with bovine serum albumin (*BSA*), fibronectin (*FN*), or type I collagen (*COLL*) (10 mg/ml) at 37 °C for 6 h. ICAM-1 (*A*) and RANKL (*B*) expression was determined by FACScan. Each value represents the number of molecules expressed per one cell calculated using standard QIFKIT beads from four similar experiments. Data are presented as mean ± S.D. *, $p < 0.05$ compared with controls.

inhibited β_1-induced up-regulation of ICAM-1 and RANKL expression on osteoblasts. In contrast, H7 or staurosporine (protein kinase C inhibitors) and wortmannin (a PI 3-K inhibitor) did not influence β_1 integrin-induced ICAM-1 and RANKL expression. These results suggest that β_1 integrin-induced ICAM-1 and RANKL expression on osteoblasts is mediated mainly through tyrosine kinases, consistent with previous reports from our laboratories and those of others (14, 33, 34).

Involvement of FAK in β_1-mediated Signaling Inducing ICAM-1 and RANKL on Osteoblasts—FAK is known to be an important mediator of the integrin-mediated signaling by adhering to ECM proteins (28). ICAM-1 and RANKL expression was up-regulated by cross-linking of β_1 integrins with a specific mAb on osteoblasts that did or did not express VSV-FAK, a wild-type FAK. However, on the cells expressing VSV-FAT or VSV-FRNK (a dominant negative truncation of FAK), such induction of ICAM-1 and RANKL by stimulation of β_1 was completely reduced to the expression seen on the unstimulated cells (Fig. 5). These results indicate that FAK is involved in signaling via β_1 stimulation, which leads to up-regulation of ICAM-1 and RANKL expression on osteoblasts.

β_1-mediated Signaling Increased TRAP$^+$ MNC Formation in Osteoblasts from Peripheral Monocytes—In a coculture system using osteoblasts with peripheral monocytes, β_1-stimulated osteoblasts that did or did not express VSV-FAK significantly induced the formation of TRAP$^+$ MNCs from the monocytes compared with untreated osteoblasts. However, on the cells expressing VSV-FAT or VSV-FRNK, such induction of TRAP$^+$ MNCs by stimulation of β_1 was markedly inhibited (Fig. 6). These results also indicate that FAK is involved in β_1 stimulation, and this helps commit hematopoietic precursors toward osteoclast development.

DISCUSSION

Integrins are a superfamily of cell surface receptors involved in cell-cell and cell-matrix adhesion. Signals from matrices transduced by integrins play critical roles in regulating gene expression, tissue-specific differentiation, and survival of primary osteoblasts and fibroblasts (6, 35, 36). It has been reported that human osteoblasts express a diverse range of integrins, particularly of the β_1 integrin, including α_1 through α_5 subunits. While our and others' studies show different patterns

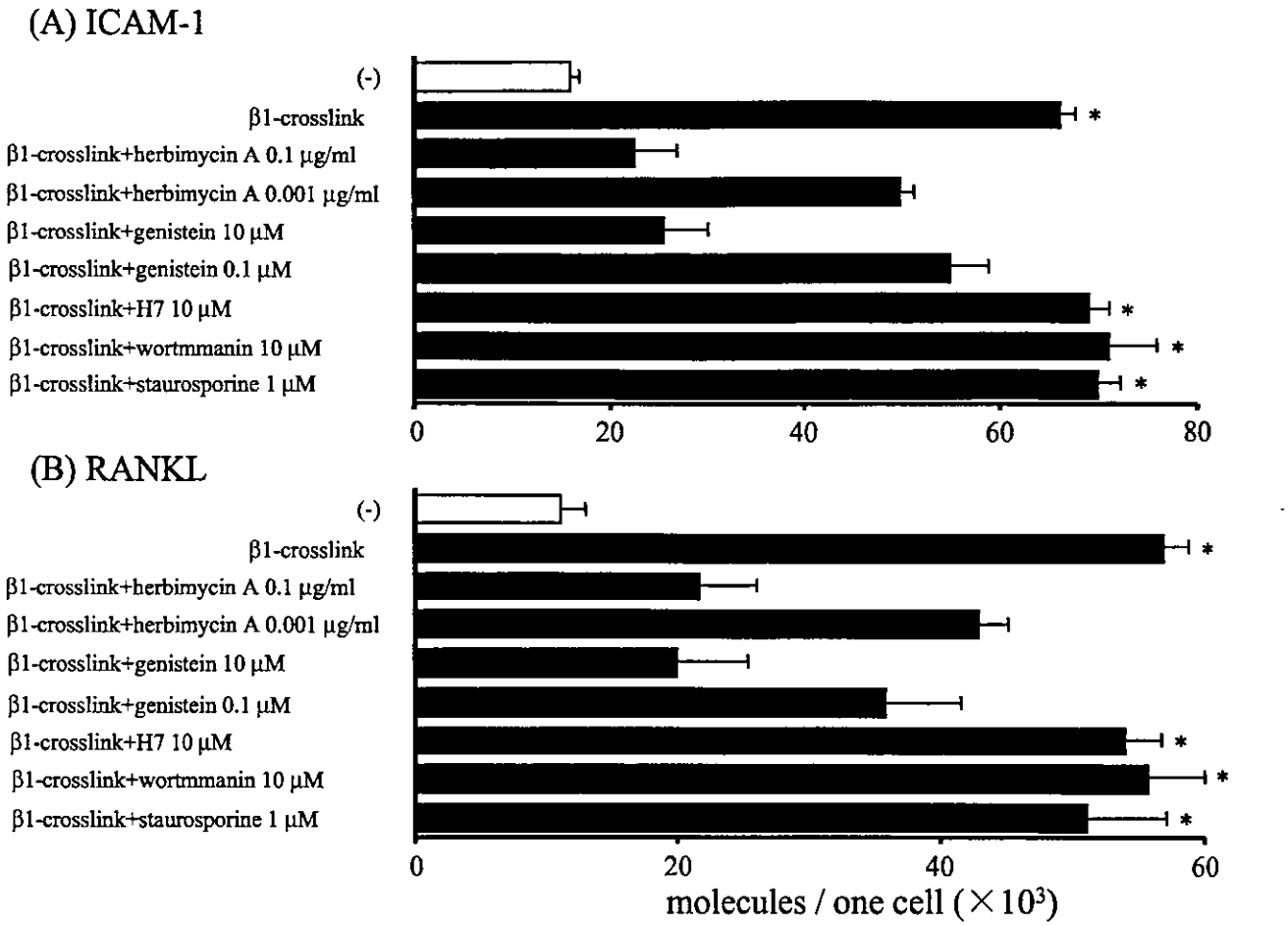

FIG. 4. **Involvement of tyrosine kinases in β_1 integrin-mediated up-regulation of ICAM-1 and RANKL on human osteoblasts.** Osteoblasts were pretreated with or without the indicated concentration of various inhibitors of intracytoplasmic signaling for 30 min. Osteoblasts were then cross-linked with 10 mg/ml anti-β_1 mAb for 6 h. ICAM-1 (*A*) and RANKL (*B*) expression was determined by FACScan. Each value represents the number of molecules expressed per one cell calculated using standard QIFKIT beads from four similar experiments. *, $p < 0.05$ compared with controls.

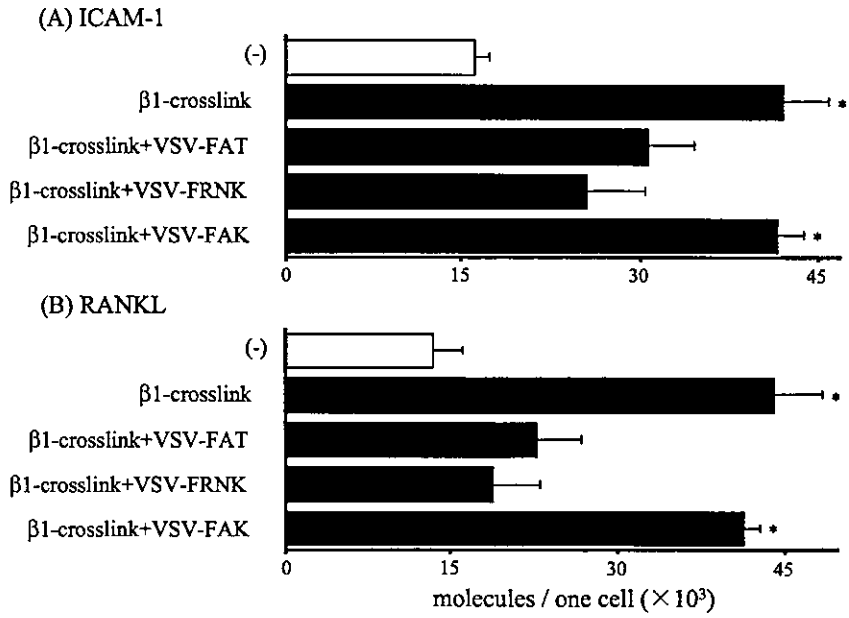

FIG. 5. **Involvement of FAK in β_1-mediated signaling inducing ICAM-1 and RANKL on human osteoblasts.** Osteoblasts transfected with or without control vectors encoding VSV-FAK, VSV-FAT, or VSV-FRNK were cross-linked with 10 mg/ml anti-β_1 mAb for 6 h and analyzed for expression of ICAM-1 (*A*) and RANKL (*B*) using FACScan. Each value represents the number of molecules expressed per one cell calculated using standard QIFKIT beads from four similar experiments. *, $p < 0.05$ compared with controls.

of integrin expression, this may reflect the heterogeneity of osteoblast-like populations and the different stages of osteoblasts from fetal or adult bone (37–40). Although osteoblasts express α_v integrins, they differ from osteoclasts in that β_1 integrins appear to have the major functional role, which has been underscored by *in vivo* data (41). β_1 integrins are major adhesion receptors mediating interactions between osteoblasts and ECMs found in bone, such as collagen, fibronectin, osteopontin, thrombospondin, and vitronectin. The $\alpha_2\beta_1$ integrins bind to type I collagen, which is the dominant bone matrix

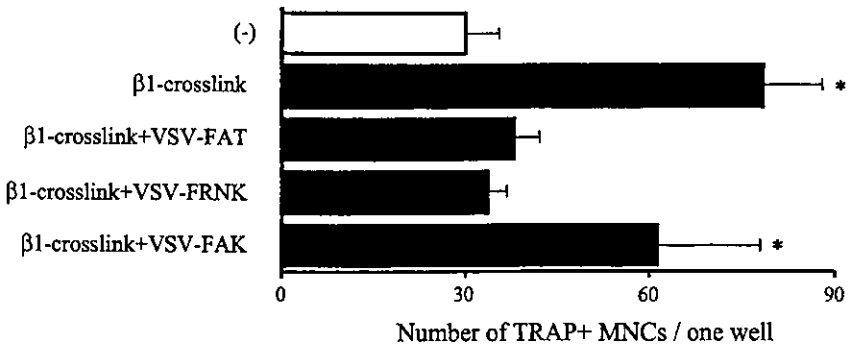

FIG. 6. **β_1-mediated signaling increased TRAP$^+$ MNC formation in osteoblasts from peripheral monocytes.** In a coculture system using osteoblasts with peripheral monocytes, β_1-stimulated osteoblasts transfected with or without control vectors encoding VSV-FAK, VSV-FAT, or VSV-FRNK were evaluated for the formation of TRAP$^+$ MNCs from the monocytes. The data are representative of four similar experiments. Numbers ± S.D. represent the number of TRAP$^+$ MNCs in one well. *, $p < 0.05$ compared with controls.

protein, and several studies have reported that $\alpha_2\beta_1$ integrin-mediated binding to ECM regulates osteoblastic differentiation (24, 25). The $\alpha_5\beta_1$ integrin, the selective fibronectin receptor, is important in both the development and maintenance of bone. Globus *et al.* (42) have demonstrated that $\alpha_5\beta_1$ ligand binding is necessary for cell survival and that receptor blockade leads to cell apoptosis in mature osteoblasts. Thus, the accumulating evidence indicates that β_1 integrin-mediated adhesion to bone matrix induces proliferation, differentiation, and bone matrix synthesis of osteoblasts and that β_1 integrin-mediated signaling on osteoblasts could be involved in bone formation. However, based on the results of the present study, we propose a new concept that stimulation of the adhesion molecule β_1 integrin plays a pivotal role in the regulation of ICAM-1 and RANKL expression and osteoclastogenesis. We deduced this from the following novel findings. 1) Engagement of β_1 integrins on purified human osteoblasts by cross-linking using a specific antibody as well as ligand matrices markedly augmented surface ICAM-1 and RANKL expression. 2) Up-regulation of both ICAM-1 and RANKL by β_1 integrin stimulation was mediated by the tyrosine kinase signaling pathway, especially involving FAK. 3) Engagement of β_1 integrins on osteoblasts induced TRAP-positive MNC formation in the coculture system of osteoblasts and peripheral monocytes. 4) Up-regulation of TRAP$^+$ MNC formation by β_1 stimulation was completely abrogated by transfection of dominant negative truncations of FAK. Therefore, we further propose that β_1 integrin-dependent adhesion to bone matrix proteins in osteoblasts can transduce signaling to induce ICAM-1 and RANKL expression through tyrosine kinase involving FAK- and RANKL-induced osteoclast formation.

As RANKL, a member of the tumor necrosis factor family expressed on the cell surface membrane of COS cells and osteoblasts/stromal cells, induces osteoclast formation from its precursor through cognate interaction between osteoblasts and osteoclast precursors, it is thereby required for RANKL-induced osteoclastogenesis (9–11). Thus, higher affinity adhesion between osteoblasts and osteoclast precursors is emerging as a prerequisite for interaction of membrane-bound RANKL to be efficiently presented to its receptor, RANK. However, the binding affinity between tumor necrosis factor family molecules and tumor necrosis factor receptor family molecules, including CD40/CD40L, CD30/CD30L, Fas/FasL, and RANKL/RANK binding, is not sufficient to support static or firm cell adhesion (43). Furthermore we reported that anti-RANKL antibody did not inhibit the adhesion of osteoblasts to osteoclast precursors, whereas anti-LFA-1 antibody completely blocked the adhesion in a human cell culture system (12). Thus, based on our *in vitro* study, it can be assumed that up-regulation of ICAM-1 and RANKL expression on osteoblasts by β_1 integrin-mediated signaling could affect cellular adhesion between osteoblasts and osteoclast precursors through the ICAM-1/LFA-1 and RANKL/RANK pathways and lead to differentiation of osteoclast progenitors to osteoclasts *in vivo*.

During bone remodeling processes, adhesion-dependent interaction among osteoblasts and osteoclasts causes an imbalance in bone metabolism by favoring bone resorption through the expression of RANKL, ICAM-1, and other factors involved in cellular interaction. Although several studies have reported that β_1 integrin-mediated adhesion to bone matrix induces proliferation, differentiation, and bone matrix synthesis of osteoblasts, our novel findings suggest that β_1 integrin/FAK-mediated signaling on osteoblasts could be involved in ICAM-1- and RANKL-dependent osteoclast maturation. Thus, it can be assumed that β_1 integrin-mediated signaling on osteoblasts could be involved in high turnover on bone metabolism through two paradoxical features of bone formation and bone resorption. However, it is as yet unclear how the same signaling pathway controls such diverse cellular events. After ligation of β_1 integrins with surrounding ECMs, the integrins are found in focal adhesion plaques where various cytoskeletal proteins accumulate. Engagement of β_1 integrins leads to initiation of intracellular signal transduction through accumulated cytoskeletal signaling kinases, resulting in the activation, differentiation, development, and mobility of various cell types (15, 16). Several studies have established that among various cytoskeletal proteins, FAK, a cytoplasmic protein-tyrosine kinase that localizes focal adhesions, is an important mediator of integrin-mediated signaling and that the initial events triggered by the stimulation of β_1 integrin are tyrosine phosphorylation and activation of FAK. In the present study, we observed that β_1 integrin-mediated induction of ICAM-1 and RANKL expression was completely inhibited when the cells were pretreated with tyrosine kinase inhibitors. Furthermore β_1 integrin-induced up-regulation of ICAM-1 and RANKL on osteoblasts expressing FRNK or FAT (dominant negative truncations of FAK) was completely inhibited. These findings suggest that β_1 integrin-induced up-regulation of both ICAM-1 and RANKL expression was brought about by the signaling pathway of tyrosine kinases, specifically involving FAK. Phosphorylated FAK activates several transduction molecules including Src and Grb2, which may cause the activation of mitogen-activated protein kinase or PI 3-K via Ras (22). Recent evidence indicates that small guanine nucleotide-binding regulatory proteins (G-proteins) control signaling pathways critical for diverse cellular functions. Among several small G-proteins, Ras proteins are molecular switches that act as a "hub," which radiates multiple signaling pathways critical for diverse cellular functions, including Raf-1/mitogen-activated protein kinase and PI 3-K (30, 44, 45). We have reported the relevance of H-Ras and its downstream effectors to functions of osteoblasts and proposed that H-Ras signals, especially those followed by the Raf-1/mitogen-activated protein kinase pathway, but not PI 3-K, induce cell cycle arrest and subsequent apoptosis via Fas up-regulation and Bcl-2 down-regulation (46). Although further evidence is required, there is a possibility that such diverse cellular regulation by the β_1 integrin-bone matrix interaction may be mediated by these G-protein signaling cascades and that FAK is

the immediate transducer of β_1 integrin-mediated signaling. In conclusion, our results suggest a novel mechanism of β_1 integrin-bone matrix cross-talk and a pivotal role in osteoclastogenesis. Further studies will be required to understand β_1 integrin function in bone metabolism.

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連載講座

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Foxp 3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〕
CD 29
β_1インテグリン
Focal adhesion kinase
T細胞
全身性エリテマトーデス

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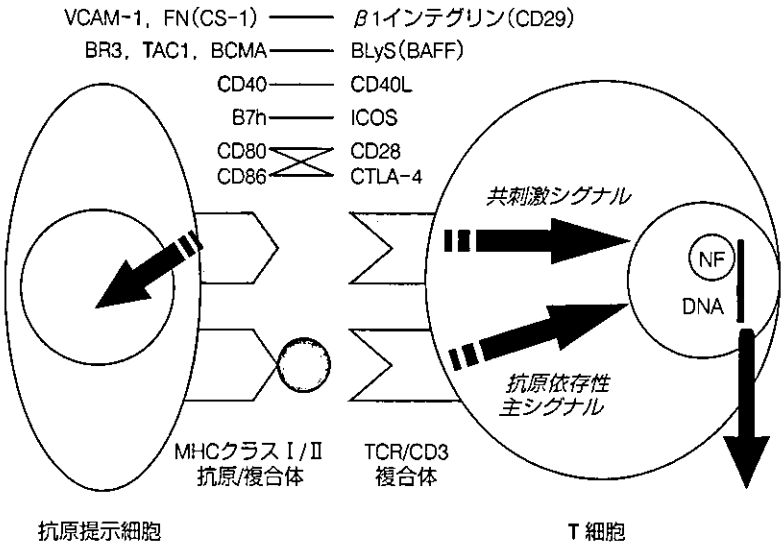

図 1. 抗原提示細胞による T 細胞の活性化機構
自己反応性 T 細胞の活性化は，抗原提示細胞から TCR を介する抗原刺激と共刺激分子からのシグナルの共存でもたらされる．

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，CD 3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，CD 40 LやCD 69は，無刺激の健常人T細胞では発現しないが，CD 3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，CD 40 L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，CD 40 L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，CD 40 L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, BR 3, 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)]．

CD 28も，CD 80とCD 86をリガンドとする代表的な共刺激分子である．同様にCD 80とCD 86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，CD 28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CD 4陽性T細胞およびCD 8陽性T細胞では，CD 28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CD 40 LやCD 69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$CD\,4^+CD\,45\,RO^+$(メモリー)T細胞で，CD 28発現が健常人にくらべ著明に低下した．また，CD 28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．CD 28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CD 28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(CD 29)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，CD 4陽性メモ

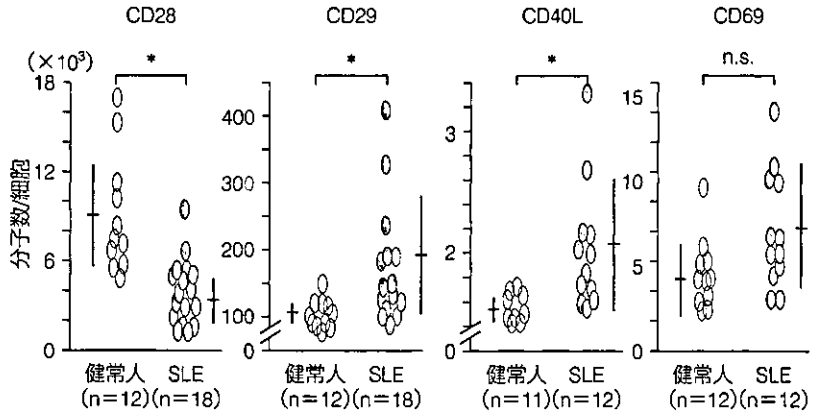

図 2. 健常人および SLE 患者 T 細胞上の細胞表面機能分子の発現
SLE 患者末梢血の T 細胞では，CD 28 の発現低下，CD 29(β_1)および CD 40 L の発現増強がみられた．
*$p<0.05$

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．

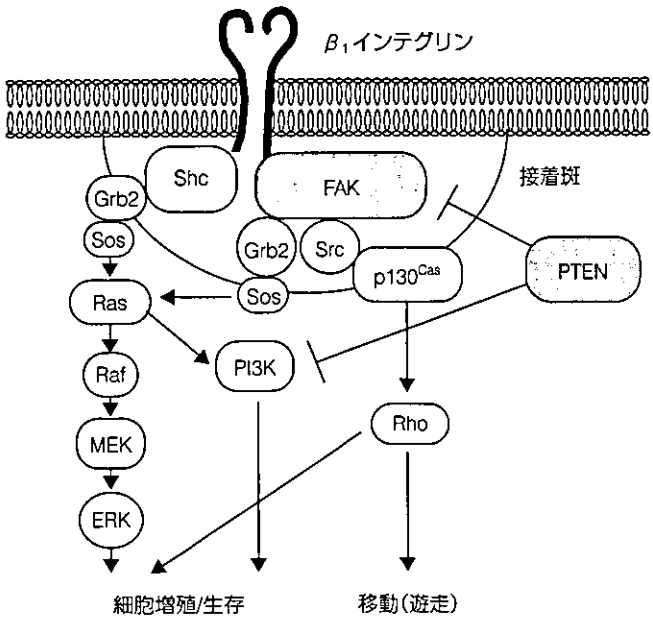

図 3. β_1インテグリンで誘導される細胞内シグナル(Parsons JT, 2003[24]より改変引用)
リガンドが結合しリン酸化されたFAKは下流分子と結合し，Src型キナーゼの接着斑への移動と活性化，Rasなどの低分子G蛋白質を介してMAPK，PI-3Kなどの経路を活性化し，さまざまな細胞機能を誘導する．

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

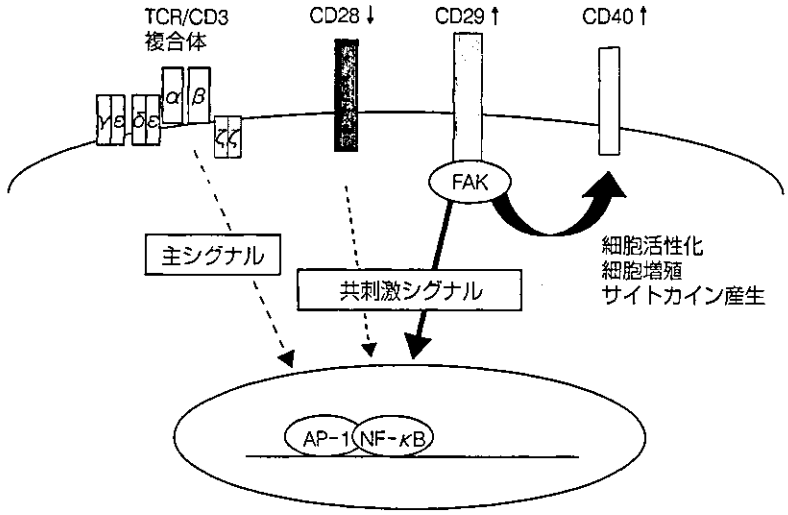

図 4. SLE 患者 T 細胞活性化における CD 28 の減弱に対する β_1インテグリンの相補的役割
活動期 SLE 患者 T 細胞では，CD 28 の低下に反比例して，β_1インテグリン(CD 29)を介するシグナルが亢進し，さらに，β_1インテグリン-FAK を介する賦活化シグナルの亢進は，CD 28 非依存性に作用し，自己反応性 T 細胞の過剰な活性化に寄与する．

常の是正を目的として，新規治療軸の確立が期待される．実際，インテグリンの拮抗薬として開発されたヒト化抗 α_4抗体 natalizumab が中等度から重度のクローン病，および再発性多発性硬化症に対して臨床試験がなされ，すぐれた臨床効果と忍容性が認められ，米国食品医薬品局(Food and Drug Administration：FDA)に承認申請中である[28)29)]．

おわりに

現在，免疫シグナル異常の是正を目的とした病態特異的な新規治療軸の確立が期待されるが，SLE では，CD 40/CD 40 L，B 7 h/ICOS などを介する共刺激分子シグナルが標的分子として注目され，CD 40 L 抗体，BlyS 抗体などの臨床試験が開始されている[7)16)30)]．今回，SLE の T 細胞では，代表的な共刺激分子である CD 28 の減弱に反して，β_1インテグリン(CD 29)と CD 40 L が増強することを見出した．その機構として，β_1-FAK を介する賦活化シグナルの量的，および，質的な亢進は，従来の T 細胞活性化の経路をバイパスした CD 28 非依存性の共刺激として作用し，CD 40 L などの発現を誘導して自己反応性 T 細胞の過剰な活性化をもたらし，ループス腎炎などの臓器病変の進展を引き起こす可能性が示唆された．癌の化学療法の分野では，チロシンキナーゼや FAK の恒常的活性化を認める白血病や肺癌，乳癌に対して，その阻害薬が分子標的治療薬として試験が先行し，ことに，癌抑制遺伝子 *PTEN* が FAK の活性化を抑制する負の制御機構が注目される[31)]．一方，リンパ球特異的に PTEN を欠損させると，SLE 様の自己免疫疾患の発症，腫瘍化が引き起こされる[32)]．したがって，SLE においても，β_1インテグリンや FAK に対する阻害薬，さらに，PTEN による制御も魅力的な治療の手法となりうる．

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Expression Cloning of a Human cDNA Restoring Sphingomyelin Synthesis and Cell Growth in Sphingomyelin Synthase-defective Lymphoid Cells*

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Sphingomyelin (SM) synthase has been assumed to be involved in both cell death and survival by regulating pro-apoptotic mediator ceramide and pro-survival mediator diacylglycerol. However, its precise functions are ambiguous due to the lack of molecular cloning of SM synthase gene(s). We isolated WR19L/Fas-SM(−) mouse lymphoid cells, which show a defect of SM at the plasma membrane due to the lack of SM synthase activity and resistance to cell death induced by an SM-directed cytolytic protein lysenin. WR19L/Fas-SM(−) cells were also highly susceptible to methyl-β-cyclodextrin (MβCD) as compared with the WR19L/Fas-SM(+) cells, which are capable of SM synthesis. By expression cloning method using WR19L/Fas-SM(−) cells and MβCD-based selection, we have succeeded in cloning of a human cDNA responsible for SM synthase activity. The cDNA encodes a peptide of 413 amino acids named SMS1 (putative molecular mass, 48.6 kDa), which contains a sterile α motif domain near the N-terminal region and four predicted transmembrane domains. WR19L/Fas-SM(−) cells expressing SMS1 cDNA (WR19L/Fas-SMS1) restored the resistance against MβCD, the accumulation of SM at the plasma membrane, and SM synthesis by transferring phosphocholine from phosphatidylcholine to ceramide. Furthermore, WR19L/Fas-SMS1 cells, as well as WR19L/Fas-SM(−) cells supplemented with exogenous SM, restored cell growth ability in serum-free conditions, where the growth of WR19L/Fas-SM(−) cells was severely inhibited. The results suggest that SMS1 is responsible for SM synthase activity in mammalian cells and plays a critical role in cell growth of mouse lymphoid cells.

Diverse kinds of phospho- and glycerolipids such as diacylglycerol (DAG),[1] inositol phosphatides, and phosphatidic acid are recognized as bioactive molecules in cell growth and survival (1, 2). Sphingolipid ceramide has recently emerged as a signal mediator of cell functions including apoptosis, differentiation, and secretion (3). Various stresses such as ultraviolet, irradiation, heat shock, hypoxia, and biological factors such as tumor necrosis factor-α, interferon-γ, and Fas antibody require ceramide generation to execute apoptosis, suggesting the implications of SM as a source of ceramide generation in the induction of cell death (4, 5). It was reported that SM dose-dependently inhibits both deoxycholate-induced apoptosis and subsequent hyper-proliferation in colon epithelial cells (6) and decreases the number of aberrant crypts of colon (7), suggesting the implications of SM in cell death and growth.

SM is produced by SM synthase, which is thought to be the only enzyme to synthesize SM in mammalian cells (8). The enzyme catalyzes the reaction in which phosphocholine moiety is transferred from phosphatidylcholine (PC) to ceramide. Thus, the activation of SM synthase subsequently increases the levels of DAG and decreases ceramide at the same time (8). DAG is an important signaling molecule for cell growth through protein kinase C activation (9–12) and acts competitively against ceramide-induced apoptosis (4, 13). It has been reported that after thioacetamide-induced injury, the SM/PC ratio significantly increased in microsomal fraction from liver, suggesting the involvement of SM synthase in tissue recovery (14). In cerebellar astrocytes, the level of ceramide is rapidly down-regulated by basic fibroblast growth factor via activating SM synthase (15). In SV40-transformed lung fibroblasts, SM synthase regulates the levels of ceramide and DAG in an opposite direction (16). We recently reported that SM synthase was activated to inhibit ceramide generation in IL-2-induced proliferation of natural killer cells,[2] whereas the activity in nucleus was inhibited with ceramide generation in Fas-induced T cell apoptosis (17). We also showed its *in vivo* implication that the level of ceramide was decreased via activation of SM synthase in chemotherapy-resistant blast cells obtained from refractory leukemia patients than in chemotherapy-sensitive leukemic blasts (18). Thus, SM synthase is assumed to play an important role in cell death and survival, *in vitro* as well as *in vivo*.

We previously proposed the "SM cycle," a pathway that consisted of SM synthase and sphingomyelinase as a novel biological system to regulate the cellular level of ceramide for cell death and differentiation (19). In contrast to the studies of the acid and neutral sphingomyelinases in cell death (20, 21), the biological implication of SM synthase has not been elucidated due to the lack of molecular cloning of its responsible gene(s).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB154421.

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[1] The abbreviations used are: DAG, diacylglycerol; SM, sphingomyelin; PC, phosphatidylcholine; MβCD, methyl-β-cyclodextrin; SAM, sterile α motif; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; FBS, fetal bovine serum; FACS, fluorescence-activated cell sorter; MBP, maltose-binding protein; NBD, 12-(*N*-methyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)).

[2] Y. Taguchi, T. Kondo, M. Watanabe, Y. Kozutumi, and T. Okazaki, submitted for publication.

We recently found mouse lymphoid cell variants designated WR19L/Fas-SM(−), which are defective of SM synthesis and susceptible to methyl-β-cyclodextrin (MβCD)-induced cell death (30). By an expression cloning method using WR19L/Fas-SM(−) cells and MβCD-based cell selection, we isolated a human cDNA responsible for SM synthase activity. The cDNA clone encodes a peptide of 413 amino acids, named SMS1, which contains a sterile α motif (SAM) domain and four putative transmembrane domains. SMS1 was identical to the peptide that was recently identified as a human SM synthase by Huitema *et al.* (24). In serum-free condition, where the cell growth of WR19L/Fas-SM(−) was inhibited, the cells expressing SMS1 cDNA (WR19L/Fas-SMS1) restored the growth ability and accumulation of SM at the surface of the plasma membrane. The restoration of cell growth was also observed when WR19L/Fas-SM(−) cells were maintained in the serum-free medium supplemented with exogenous SM. Here, we show the critical role of SM synthesized through SM synthase in mammalian cell growth, and the localization, active site and biological function of SMS1 are also discussed.

EXPERIMENTAL PROCEDURES

Materials—Lysenin, MβCD, and ceramide from bovine brain were purchased from Sigma; PC from egg yolk, SM from bovine brain, and a cell viability assay kit with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) were from Nacalai tesque (Kyoto, Japan); GP2–293 packaging cell, pLIB retroviral expression vector, and human HeLa cDNA retroviral expression library were from Clontech; D-erythro-C6-NBD-ceramide and C6-NBD-sphingomyelin were from Matreya (Pleasant Gap, PA); L-[U-^{14}C]serine, cytidine 5′-diphospho [methyl-^{14}C]choline, L-3-phosphatidyl [N-methyl-^{14}C]choline, 1,2-dipalmitoyl, and [N-methyl-^{14}C]sphingomyelin were from Amersham Biosciences.

Cell Culture—WR19L/Fas cells were kindly gifted from Dr. Yonehara (Institute for Virus Research, Kyoto University). The SM-defective WR19L/Fas-SM(−) cells and the SM-containing WR19L/Fas-SM(+) cells were isolated from the original WR19L/Fas cells by a dilution cloning method. The cells were routinely maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, and 75 μg/ml kanamycin in 5% CO_2 and 100% humidity at 37 °C. For culture in serum-free medium, the cells were washed, reseeded at 1×10^5 cells/ml, and incubated in the RPMI 1640 medium with 5 μg/ml human insulin and bovine holo transferrin in the presence or absence of 50 μM SM in 5% CO_2 at 37 °C. After 48 h incubation, the cell numbers were counted with dye exclusion method using 0.25% trypan blue (Nakalai tesque, Kyoto, Japan).

Cell Labeling—The cells were reseeded at 5×10^5 cells/ml in the RPMI 1640 medium with 2% FBS and L-[^{14}C]serine (specific activity; 155 mCi/mmol) and incubated at 37 °C in 5% CO_2 for 36 h. The labeled cells were incubated at 37 °C in 5% CO_2 for 2 h. The cell lipids were extracted by the method of Bligh and Dyer (19), applied on a silica Gel 60 TLC plate (Merck), and developed with solvent containing methyl acetate/propanol/chloroform/methanol/0.25% KCl (25:25:25:10:9). The radioactive spots were visualized and quantified by using a BAS 2000 Image Analyzer (Fuji Film).

FACS Analyses—The cells were incubated with 500 ng/ml lysenin in the presence of 20 μg/ml propidium iodide (Molecular Probes) at room temperature for 15 min and analyzed with FACS Calibur (BD Biosciences). For detection of SM localized at the plasma membrane, the cells were stained on ice for 30 min with non-toxic lysenin fused to maltose-binding protein (MBP-lysenin) (25), kindly provided by Dr. T. Kobayashi (The Institute of Physical and Chemical Research (RIKEN), Japan). The cells were washed with ice-cold phosphate-buffered saline supplemented with 1% FCS and 0.1% NaN_3 and incubated with rabbit anti-MBP antiserum (New England BioLabs, Beverly, MA) on ice for 30 min. After being washed again, the cells were incubated for 30 min with phycoerythrin-conjugated anti-rabbit IgG (Sigma) and subjected to fluorescence-activating cell sorter (FACS) analysis using FACS Calibur. The data analysis was performed by Cell Quest software (BD Biosciences).

Confocal Microscopy—For visualization of SM localized at the plasma membrane, the cells settled onto slides coated with poly-L-lysine were fixed in 4% formaldehyde and stained with lysenine-MBP at 4 °C for 45 min followed with anti-MBP. After being stained with a phycoerythrin-conjugated anti-rabbit IgG monoclonal antibody, the cells were examined using confocal microscopy using a Zeiss LSM 310 laser scan confocal microscope (Carl Zeiss, Oberkochen, Germany).

Expression Cloning of SMS1 cDNA—The expression cloning method performed in this study was based on the study of Hanada *et al.* (26). Pantropic retroviral particles containing the G glycoprotein of vesicular stomatitis virus (VSV-G) were prepared using a human HeLa cDNA retroviral expression library kit and GP2–293 packaging cells (Clontech). After infection for 24 h, the WR19L/Fas-SM(−) cells were cultured in the RPMI 1640 medium containing 2% FBS overnight. After being washed with serum-free RPMI 1640 medium, the cells were incubated in 1.5 mM MβCD in RPMI 1640 medium for 5 min at 37 °C, replenished with the normal culture medium to a final concentration of FBS at 5%, and then cultured at 37 °C for 60 h. The cells were reseeded, cultured in the RPMI 1640 medium containing 2% FBS overnight, and subjected again to the treatment with appropriate concentrations of MβCD. After a total of two cycles of 1.5 mM MβCD treatment followed by two cycles of 3 mM and two subsequent cycles of 5 mM, an MβCD-resistant variant of WR19L/SM(−) was isolated by a limiting dilution.

By genomic PCR using primers specific to the pLIB expression vector (5′ and 3′ pLIB Primer, Clontech), the 2.0-kb cDNA integrated in the genome of the MβCD-resistant cell was amplified and cloned into pGEM-T Easy vector (Promega, Madison, WI). After sequencing and computer analysis, the cDNA was subcloned into the pLIB expression vector and transfected into the WR19L/Fas-SM(−) cells via the VSV-G retroviral particles. A resultant cell was isolated by a limiting dilution method, which was designated WR19L/Fas-SMS1 cells, and subjected to various assays. Integration of the cDNA into the genome of WR19L/Fas-SMS1 cells was confirmed with PCR.

Assay for Sphingomyelin Synthase Activity—The cells were homogenized in an ice-cold buffer containing 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 2.5 μg/ml leupeptin. The lysates containing 500 μg of cell protein were added to a reaction solution containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 20 μM C6-NBD-ceramide, 120 μM PC and incubated at 37 °C for 30 min. The lipids were extracted by the method of Bligh and Dyer (19), applied on the TLC plates, and developed with solvent containing chloroform/methanol/12 mM $MgCl_2$ in H_2O (65:25:4). The fluorescent lipids were visualized by FluorImager SI system (Amersham Biosciences). For the assay for transferase activity, 20 μM ceramide and 120 μM [N-methyl-^{14}C]PC (specific activity; 57 mCi/mmol) or [methyl-^{14}C]CDP-choline (specific activity; 54 mCi/mmol) were used in the reaction solution instead of the NBD-ceramide and PC. The radioactive spots were visualized using the BAS 2000 system.

Assay for Viability and Growth Rate of Cells Exposed to MβCD and Lysenin—For the assay using MβCD, 1×10^6 of the cells were washed and resuspended in 1 ml of the serum-free RPMI 1640 medium, treated with appropriate concentrations of MβCD, and incubated in 5% CO_2 at 37 °C for 5 min. After the addition of 1 ml of the normal culture medium, the cells were further incubated for 12 h. The viability of the cells was measured using a cell viability kit with WST-8 (Nakalai tesque). For the assay using lysenin, 7×10^6 of the cells were washed and resuspended in 1 ml of prewarmed phosphate-buffered saline, treated with the appropriate concentrations of lysenin and incubated in 5% CO_2 at 37 °C for 1 h. After the addition of FBS, the cell number was counted with the 0.25% trypan blue dye exclusion method.

RESULTS AND DISCUSSION

Mouse Lymphoid Cells Defective of Sphingomyelin Synthase Activity—During investigation of the sphingolipid metabolism in mouse lymphoid cells named WR19L/Fas, which overexpress the human Fas antigen, the variant clones altering SM synthase activity (from 150 to nearly 0 pmol/mg protein/h) have been isolated. One of the variants (clone 6) severely diminished the SM synthase activity (Fig. 1*A*). Conversion of C6-NBD-ceramide to C6-NBD-SM in the cell lysate of the clone 6, named WR19L/Fas-SM(−), was not detected on a TLC plate, in contrast to the clone 2 showing the highest SM synthase activity, named WR19L/Fas-SM(+) (Fig. 1*B*). This finding was supported by the fact that WR19L/Fas-SM(−) cells did not synthesize [^{14}C]serine-labeled SM (Fig. 1*C*).

Lysenin is reported as an SM-direct cytolysin purified from the earthworm (27), for which binding to SM causes poring of the plasma membrane and subsequent cell death (22, 23, 25).