Adhesion molecules are required for development of hematopoietic stem and progenitor cells in the respective hematopoietic microenvironments. We previously showed that development of the erythroid progenitor cells is dependent on their direct adhesion to the stroma cells established from the erythropoietic organs. In this stroma-dependent erythropoiesis, we examined the role of adhesion molecules in erythropoiesis by blocking antibodies. The development of the erythroid cells on stroma cells was inhibited by anti-very late activation antigen-4 (VLA-4 integrin) antibody, but not by antibody against VLA-5, although the erythroid cells express both VLA-4 and VLA-5. Whereas high levels of expression of vascular cell adhesion molecule-1 (VCAM-1) and fibronectin, ligands for VLA-4, were detected in the stroma cells, the adhesion and development of the erythroid progenitor cells were partly inhibited by the blocking antibody against VCAM-1. VLA-5 and fibronectin could mediate adhesion of the erythroid progenitor cells to the stromal cells, but the adhesion itself may not be sufficient for the stroma-supported erythropoiesis. The stromal cells may support erythroid development by the adhesion through a new ligand molecule(s) for VLA-4 in addition to VCAM-1, and such collaborative interaction may provide adequate signaling for the erythroid progenitor cells in the erythropoietic microenvironment.
Diego, CA) and CS-1 peptides of the alternative spliced segment of fibronectin were synthesized by a peptide synthesizer.

**Adherence assay.** MSS31 cells plated onto 24-well plates at $3 \times 10^4$ cells/mL/well were cultured overnight. Before the adherence assay, the cultures were washed twice with serum-free medium. Mouse erythroleukemia (TSA8) cells were cultured in IMDM supplemented 10% FBS. TSA8 cells suspended in serum-free IMDM at $1 \times 10^5$ cells/mL were incubated with 10 μmol/L 2,7'-bis(2-carboxyethyl)carboxyfluorescein-tetraacetoxyethyl ester (BCECF-AM; Wako Pure Chemical Industries, Ltd, Tokyo, Japan) at 37°C for 30 minutes. After washing with IMDM, the fluorescence-labeled TSA8 cells were added to the MSS31 stromal layers at $2 \times 10^4$ cells/0.5 mL/well in the presence or absence of 20 μg/mL antibodies or 50 μg/mL specific peptides and incubated for 30 minutes at 37°C. Nonadherent TSA8 cells were removed by washing with IMDM three times by a multwell-plate mixer. Adherent TSA8 cells were lysed by 1 mL phosphate-buffered saline (PBS) with 1% NP-40, and the fluorescence intensity of the lysates was measured by Fluoroskan (Labosystems, Helsinki, Finland). Percent adherence was evaluated by comparing the intensity of fluorescence with the control experiment.

**Flow cytometry.** MSS31 and MSS62 cells were detached from substrata by 0.02% EDTA-PBS treatment. For immunofluorescence labeling, $10^6$ to $10^7$ cells were incubated with an excess of anti-fibronectin (DAKO Japan Co Ltd, Kyoto, Japan) or antibody M/K-2 (anti–VCAM-1) for 30 minutes on ice and washed three times with PBS. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated antirabbit IgG or antirat IgG, respectively, for 20 minutes on ice. After washing with PBS three times, the cells were resuspended in PBS and analyzed by FACScan (Becton Dickinson) with 488 nm emission. To stain VLA-4 or VLA-5, TSA8 cells were incubated with FITC-conjugated PS/2 (anti–VLA-4) or HMo5-l (anti–VLA-5) for 30 minutes on ice. After washing with PBS three times, the TSA8 cells were analyzed by FACScan.

**RESULTS**

**Effect of MoAbs against integrins on the large erythroid colony formation.** In previous studies, we showed that stro-

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**Fig 1.** Effects of anti–VLA-4 antibody on adherence and proliferation of erythroid progenitor cells on stromal cell layer. Fetal liver cells from 13-day gestation were cultured on MSS62 stromal cells in a semisolid medium with (B and D) or without (A and C) 20 μg/mL antibody PS/2 (anti–VLA-4). After 2 days of incubation (A and B), progenitor cells adhered to and proliferated on the stromal cells. In the control experiment (A), both small erythroid (arrow) and large nonerythroid (arrowhead) cells adhered, but in the culture with antibody PS/2 (B), only nonerythroid cells adhered. CFU-E-derived small erythroid colonies were observed as hanging colonies (double arrowheads). Erythroid or nonerythroid (granulo/monocytic progenitor) cells were distinguished by their cell size. After 4 days of incubation (C and D), cultures were directly stained with benzidine. Large erythroid colony formations were suppressed by PS/2 (D). Bar = 100 μm.
nal cells established from the erythropoietic tissues can support large erythroid colony formation. To evaluate the possible function of β1 integrins in erythroid progenitor cells, the inhibitory effect of specific MoAbs to VLA-4 and VLA-5 integrins on the large erythroid colony formation was examined using spleen stromal (MSS31 and 62) cells and 13-day-old fetal liver cells containing erythroid and granulo-monocytic progenitor cells. Formation of the large erythroid colonies after 4 days of culture (Fig 1C) was inhibited by the addition of anti-VLA-4 antibody (Fig 1D). Contrary to its inhibition of the large erythroid colony formation, adhesion and formation of the granulo-monocytic colonies (Fig 1A) were not inhibited by anti-VLA-4 antibody (Fig 1B).

Based on this result, we examined quantitatively the inhibitory effect of MoAbs against two integrins, VLA-4 and VLA-5 (Fig 2). The large erythroid colony formation was reproducibly inhibited by anti-VLA-4 antibody, but not by anti-VLA-5 antibody. The inhibitory activities of anti-VLA-4 antibody were saturated at the concentration of 50 µg/mL, but anti-VLA-5 antibody had no effect even at the concentration of 50 µg/mL (data not shown).

Expression of fibronectin and VCAM-1 in the stromal cells. Inhibitory effect of anti-VLA-4 antibody on the large colony formation supported by the stromal cells strongly suggested the presence of ligand molecules for this integrin in the stromal cells. Because fibronectin and VCAM-1 are known ligands for VLA-4 integrin, the presence of both molecules was analyzed by flow cytometrical detection (Fig 3). Because an excess EDTA treatment of MSS cells did not decrease fluorescence intensities, fibronectin detected in this analysis may be caused by the cell-bound forms. Thus, MSS cells abundantly expressed both ligand molecules.

Involvement of VCAM-1 in the large erythroid colony formation. Functional roles of the ligand molecules for the integrins were examined by the inhibitory activities of anti-VCAM-1 antibody and the synthetic peptides carrying the binding domains of fibronectin to the integrins. Anti-VCAM-1 antibody showed a partial inhibition (30% to 50%) in its concentration that completely blocked lymphopoiesis, whereas anti-VLA-4 antibody almost completely inhibited (80% to 90%) the large erythroid colony formation (Fig 2).

Because fibronectin contains ligand domains for both VLA-4 and VLA-5 integrins, involvement of fibronectin was examined using known CS-1 and RGD sequences recognized by both integrins (Table 1). When these peptides were added to the culture of erythroid progenitor cells on the MSS62 monolayers, dose-dependent inhibition of the large erythroid colony formation was not observed except at highest concentration (80 µg/mL) of CS-1 peptide. This is contrary to its inhibitory effect observed in lymphocyte proliferation in which 50 µg/mL synthetic peptides may be a sufficient amount. A high dose of CS-1 peptide caused growth suppression and morphologic change of MSS cells; thus, the fibronectin CS-1 sequence seems to have a critical role in the maintenance of adhesion and cell proliferation of MSS cells. With such a toxic effect, MSS cells could not provide an adequate microenvironment for erythroid progenitor cells.

Fig 2. Effect of antibodies and peptides on the large erythroid colony formation. Fetal liver erythroid progenitor cells from 13-day gestation were cultured on MSS62 (A) and MSS31 (B) stromal cells in semisolid medium with 0.1 U/mL Epo. Specific antibodies and peptide were added at 20 µg/mL. Large erythroid colony formation was performed using antibody PS/2 (anti-VLA-4), antibody HMu5-1 (anti-VLA-5), antibody M/K2 (anti-VCAM-1), synthetic peptides CS-1 (VLA-4 binding domain), RGD peptides (VLA-5 binding domain), and PBS (control). After 4 days of incubation, cultures were directly stained with benzidine. In the control experiments, 54.5 ± 4.4 large erythroid colonies were formed from 1 x 10⁶ fetal liver cells. Values are the average of 4 wells of 24-well plates from two independent experiments (A) and two wells from a single experiment (B). All antibodies and peptides were dissolved with PBS, and an adequate volume of PBS was added as a control experiment.
ROLES FOR VLA-4 IN ERYTHROPOIESIS

Fig 3. Expression of VCAM-1 and fibronectin in MSS31 and MSS62. MSS31 (A and B) and MSS62 (C and D) cells were incubated with antibody M/K-2 (anti-VCAM-1) (A and C) or antifibronectin rabbit serum (B and D) as first antibody, then with FITC-labeled antirat IgG antibody or FITC-labeled antirabbit IgG antibody as second antibody respectively (--), and with second antibody or irrelevant antibodies alone (---). Fluorescence intensity was assayed by flow cytometry. An excess EDTA treatment did not cause a decrease of fluorescence intensity.

Similarly, RGD peptide had no inhibitory effect. When CS-1 peptide was added with either anti-VCAM-1 antibody or anti-VLA-5 antibody, no additive effect was observed. Thus, the CS-1 sequence of fibronectin may not act as a major ligand for VLA-4 integrin in the erythropoietic microenvironment.

Anti-VLA-4 antibody blocks binding of erythroid cells to the stromal cells. Because the above studies showed that VLA-4 is involved in the stimulation of proliferation of erythroid progenitor cells, we asked whether anti-VLA-4 antibody blocks the binding of the erythroid cells to the stromal cells. We used the erythroleukemic cells (TSA8) for binding assay. TSA8 cells have characteristics of the erythroid progenitor cells corresponding to colony-forming units-erythroid (CFU-E) and differentiate into Epo-responsive progenitor cells by treatment with dimethylsulfoxide (DMSO), and large colonies formed from the erythroid progenitors at a stage close to the CFU-E. Flow cytometric analysis (Fig 4) demonstrated that both integrins were ex-

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Table 1. Effects of Peptides on the Large Erythroid Colony Formation

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<thead>
<tr>
<th>Peptides</th>
<th>No. of Large Erythroid Colonies on MSS62 Cells</th>
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<tbody>
<tr>
<td></td>
<td>Experiment No. 1</td>
</tr>
<tr>
<td>Control (PBS)</td>
<td>39 ± 9.9</td>
</tr>
<tr>
<td>RGD</td>
<td>20</td>
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<tr>
<td></td>
<td>40</td>
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<td></td>
<td>80</td>
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<td>CS-1</td>
<td>20</td>
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<td>80</td>
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Fetal liver erythroid progenitor cells from 13-day gestation were cultured on MSS62 stromal cells in a semisolid medium with 0.1 U/mL Epo. Specific peptides were added at various concentrations. After 4 days of incubation, cultures were directly stained with benzidine. Varying numbers of colonies were derived from 1 × 10⁵ fetal liver cells. Values are the average of two wells of 24-well plates. In the case of CS-1 peptide at the concentration of 80 µg/mL, MSS62 cells were affected in their growth and adherence to a substratum. Both peptides were dissolved with PBS, and an adequate volume of PBS was added to the control experiment.

Abbreviation: ND, not determined.

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Fig 4. Expression of VLA-4 and VLA-5 in mouse erythroleukemia (TSA8) cells. TSA-8 cells were incubated with FITC-labeled antibody PS/2 (anti-VLA-4) or HM5-1 (anti-VLA-5) or the second antibodies (---), and fluorescence intensity was assayed by flow cytometry.
pressed in TSA8 cells. VLA-4 is more abundant than VLA-5 and this pattern of expression is essentially similar to that observed in the early erythroid progenitor cells. The binding assay was performed in the presence or absence of anti-VLA-4, anti-VLA-5, anti-VCAM-1, CS-1, and RGD peptides (Fig 5). Inhibitory activities of these agents on the binding assay were essentially the same as observed in the large erythroid colony formation assay (Fig 2). Anti-VLA-4 antibody dramatically inhibited the binding of TSA8 cells to MSS31 cells, but anti-VLA-5 blocked their binding only 15% to 20%. Anti-VCAM-1 antibody partially inhibited the binding, whereas the binding was not inhibited by either CS-1 or RGD peptide. We then examined the effect of these antibodies on the adhesion of the fetal liver erythroid progenitor cells (CFU-E) to the stroma cells by measuring the CFU-E colony formation (Table 2). The conventional CFU-E colonies were formed in the semisolid culture for 2 days on the stromal cells as previously reported and these stroma-independent CFU-E colonies were not affected by the addition of antibodies and peptides (Table 2, left column). After the fetal liver progenitor cells adhered to MSS62 cells for 2 hours, the nonadherent cells were recovered and the CFU-E colonies were formed. Approximately half of the CFU-E colony-forming cells were adhered to MSS62 cells. Anti-VLA-4 most strongly inhibited the adhesion of the CFU-E colony-forming cells, whereas anti-VLA5 and anti-VCAM-1 inhibited adhesion weakly. These results were consistent with those of TSA8 cells. However, RGD peptide strongly inhibited their adhesion, whereas CS-1 peptide did not, although both peptides did not inhibit the large erythroid colony formation on the stromal cells.

**DISCUSSION**

Hematopoietic stem cells and their progenitor cells can proliferate and differentiate in a microenvironment of bone marrow, spleen, and fetal liver. We previously established stromal cell lines from fetal liver (FLS cells), which is a major erythropoietic organ, and from newborn spleen (MSS cells), in which the stem cells are predominantly committed to erythroid development. As expected, these stromal cells supported erythropoiesis in vitro. The large erythroid colonies were formed from 13-day-old fetal liver erythroid progenitor cells in a semisolid medium in the presence of Epo under direct cell contact with the stromal cell layers. We have shown that production of SCF, a ligand for c-kit, is essential for the stroma-supported erythropoiesis, but another factor(s) is expected to be required for the direct cell-to-cell adhesion, because many stromal cell lines producing SCF abundantly did not support erythropoiesis.

The integrin family may be the most probable candidate(s) for such an adhesion molecule(s) required for erythropoiesis, because the importance of their adhesive interactions in lymphopoiesis has been well studied. In the present study, we examined functional roles of the β1 integrin subfamily, VLA-4 and VLA-5, because both integrins are expressed in erythroid progenitors of bone marrows and fetal livers and fibronectin was shown to provide adhesive ligands for the erythroid cells. We found that murine erythroleukemic cells (TSA8), which can be induced toward Epo-re-
sponsive erythroid progenitor cells\textsuperscript{29,30} corresponding to CFU-E, abundantly expressed VLA-4 and VLA-5 (Fig 4). However, the quantitative binding assay indicated that VLA-4 is mostly involved in the binding of TSAS cells to MSS stromal cell layers. The adhesion of the fetal liver CFU-E colony-forming cells to the stromal cells showed essentially similar result with that of TSAS cells (Table 2), and the large colony formation of the fetal liver erythroid progenitor cells on the stromal cell layers was inhibited by anti-VLA-4, but not by anti-VLA-5, antibody. These results clearly indicate that VLA-4, but not VLA-5, is essential for a rapid expansion of the erythroid progenitor cells in the erythroidic microenvironments such as fetal liver and spleen. This is consistent with the report by Simmons et al\textsuperscript{31} that human erythroid progenitors (BFU-E) bind to stromal cells via VLA-4.

Both integrins share the same $\beta 1$ subunit, but differ in $\alpha$ subunits. VLA-4 recognizes fibronectin by CS-1 peptide domain, whereas VLA-4 recognizes fibronectin by RGD peptide domain. Anti-$\alpha$-VLA-5 antibody was reported to block attachment of both CFU-E and proerythroblasts to fibronectin,\textsuperscript{17} and it partially blocked the adhesion of the progenitor cells to the stroma cells (Table 2); thus, VLA-5 integrin expressed in the erythroid progenitor cells may be functional. We found that adhesion of the CFU-E colony-forming cells was inhibited by RGD peptide (Table 2), whereas it did not affect the large erythroid colony formation (Table 1). In addition, we have tried to culture the fetal liver erythroid progenitor cells on bovine native fibronectin-coated dishes for the simple reconstitution of the stromal cell functions, but did not observe formation of the large erythroid colonies (data not shown). Thus, VLA-5 and fibronectin could mediate adhesion of the erythroid progenitor cells to the stromal cells, but the adhesion itself may not be sufficient for the stroma-supported erythropoiesis. Our results demonstrated that binding of the erythroid progenitor cells to the stromal cells may be separated from their successive rapid proliferation in the erythroidic tissues. It was reported recently that another integrin, $\alpha 4\beta 7$, is involved in binding to both fibronectin and VCAM-1.\textsuperscript{32} We could not observe the expression of $\alpha 4\beta 7$ on TSAS and fetal liver progenitor cells ($c-kit$-positive/lineage-negative) by flow cytometry (Yagita, unpublished observation). It seems probable that $\alpha 4\beta 7$ is not involved in erythropoiesis.

Whereas fibronectin is the only known ligand for VLA-5, VLA-4 has more redundant ligands, such as VCAM-1, which is involved in adhesion of B cells to germinal centers\textsuperscript{33} and in adhesion of leukocytes to activated endothelium.\textsuperscript{34,35} Partial inhibition of binding of TSAS cells (Fig 5) as well as CFU-E colony-forming cells (Table 2) and the large erythroid colony formation on the stromal cells (Fig 2) by anti-VCAM-1 antibody suggests that VCAM-1 is not the sole ligand of this integrin in the stromal cells; there may be another ligand molecule(s) on these cells. VLA-4 may recognize VCAM-1 and another ligand(s) in similar affinity and may cause binding of the erythroid progenitor cells to the stromal cells and stimulation of their proliferation. Both ligands may be required for stroma-supported erythropoiesis.

At present, it is not known how VLA-4 integrin stimulates proliferation of the progenitor cells bound to the stromal cells. Collaborative interactions of integrins with other integrins\textsuperscript{36,38} and other molecules than integrins\textsuperscript{37,38} were shown to be required for proliferative responses. $c$-kit was shown to be responsible for the rapid expansion of the erythroid cells mediated by the interaction with SCF expressed in the stromal cells.\textsuperscript{24} The extent of the inhibition of the large erythroid colony formation by anti-VLA-4 is higher than that by anti-$c$-kit antibodies, and no additive inhibition was observed by the simultaneous addition of the two antibodies (data not shown). This suggests the collaborative interaction between VLA-4 and $c$-kit on the proliferation signals as well as on the cell adhesion, because $c$-kit acts as an adhesion molecule of the mast cell-stromal cell contact.\textsuperscript{39} Rosen et al\textsuperscript{40} reported that VLA-4-VCAM-1 interactions have a role in secondary myocyte formation in culture. Their work and our present work suggest the importance of integrins not only in cell adhesion but also in cell differentiation.

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Roles for integrin very late activation antigen-4 in stroma-dependent erythropoiesis

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