A Critical Role of VLA-4 in Erythropoiesis In Vivo

By Keisuke Hamamura, Hironori Matsuda, Yumiko Takeuchi, Sonoko Habu, Hideo Yagita, and Ko Okumura

Hematopoiesis requires specific interactions with the microenvironments, and VLA-4 has been implicated in these interactions based on in vitro studies. To study the role of VLA-4 in hematopoiesis in vivo, we performed in utero treatment of mice with an anti-VLA-4 monoclonal antibody. Although all hematopoietic cells in fetal liver expressed VLA-4, the treatment specifically induced anemia. It had no effect on the development of nonerythroid lineage cells, including lymphoids and myeloids. In the treated liver almost no erythroblast was detected, whereas the erythroid progenitors, which give rise to erythroid colonies in vitro, were present. These results indicate that VLA-4 plays a critical role in erythropoiesis, while it is not critical in lymphopoiesis in vivo.

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DEVELOPMENT OF hematopoietic cells depends on the interaction with hematopoietic microenvironments that consist of a variety of components, including stromal cells, cytokines, and extracellular matrix (ECM) proteins. Among various ECM, it has been demonstrated that hematopoietic progenitor cells adhered selectively to fibronectin (FN) but not to collagen, laminin, or proteoglycans, indicating a possibility that FN contributes to hematopoiesis. Currenty known major FN receptors are VLA-4 and VLA-5, which belong to the β1 integrin subfamily of adhesion molecules. Integrins not only mediate cellular adhesion, but also transduce signals that regulate cellular responses. Recent studies using in vitro hematopoietic culture systems demonstrated the importance of VLA-4 in B and T lymphopoiesis. In these studies, specific antibodies or peptides interfering with VLA-4-mediated adhesion inhibited lymphoid colony formation in bone marrow culture and stromal cell-dependent thymocyte differentiation. In addition, we recently demonstrated that an anti-VLA-4 monoclonal antibody (MoAb) inhibited stromal cell-dependent erythropoiesis in vitro. These in vitro results raise the question of whether these observations are relevant to hematopoiesis in vivo. To address this issue, we carried out in vivo treatment with a MoAb against mouse VLA-4. A critical contribution of VLA-4 to erythropoiesis in vivo was found.

MATERIALS AND METHODS

Mice. Timed pregnant C57BL/6 mice were purchased from Japan SLC Inc (Shizuoka, Japan). The day of observation of a vaginal plug was designated as day 0 of gestation.

Monoclonal antibodies. The hybridoma cells producing MoAb against α4 subunit of VLA-4 (PS/2.3) and that against mouse VCAM-1 (MK/2) were kind gifts from Dr Kensuke Miyake (Department of Immunology, Saga Medical School, Japan). The MoAbs were purified from ascites by affinity chromatography on protein G-Sepharose column (Pharmacia, Uppsala, Sweden).

Preparation of cells. Thymocytes were released by pressing thymic lobe between two frosted slide glasses, passed through nylon mesh, and suspended in α-modified Eagles medium (α-MEM) (GIBCO, Gaithersburg, MD). Livers were mashed and suspended in α-MEM, passed through nylon mesh, and layered on lymphocyte-separating medium (JIMBO, Gummna, Japan) followed by a centrifugation at 1,200g for 10 minutes at room temperature. Mononuclear cells (MNC) at the interface were collected, washed, and resuspended in α-MEM.

Flow cytometric analysis. Cells, 106 per sample, were incubated at 4°C for 20 minutes with appropriate dilutions of FITC-, PE- or biotin-labeled MoAbs and washed twice with phosphate buffered saline (PBS). When the cells were reacted with biotinylated antibody, they were further incubated with Red613-streptavidin at 4°C for 15 minutes, and washed twice with PBS. Immunofluorescence was analyzed on FACSscan (Becton Dickinson, Mountain View, CA).

MoAbs against mouse cell surface markers included ACK-4 (anti- c-kit), RM4-5 (anti-CD4) (Pharmingen, San Diego, CA), 53-6.7 (anti-CD8), RM2-1 (anti-CD2), RA3-6B2 (anti-B220), M170 (anti-Mac-1), RB6-8C5 (anti-Gr-1), TER119, and PS/2.3 (anti-VLA-4). The Lin cocktail of MoAbs against lineage markers contained RM4-5, 53-6.7, RM2-1, RA3-6B2, M170, RB6-8C5, and TER119. Conjugation of MoAbs with FITC or biotin was performed by standard methods. Optimal concentrations of labeled antibodies were determined by preliminary experiments.

Immunohistochemical analysis. Fetal livers were fixed in periodate-lysine-parafomaldehyde (PLP) containing 4% paraformaldehyde at 4°C overnight, embedded in OCT (Miles, Elkhardt, IN), and stored at −80°C. Frozen sections were reacted with 5μg/ml primary antibody, PS/2.3 or MK/2, at 4°C overnight. After rinsing with PBS, they were incubated with 2.5 μg/ml biotinylated antirat IgG (Vector, Burlingame, CA) for 60 minutes at room temperature, rinsed, and then reacted with avidin-biotin-peroxidase complex (Vector) for 60 minutes at room temperature. The staining was developed by incubating the specimens in a reaction buffer (0.01% H2O2, 0.3 mg/ml diaminobenzidine in 50 mmol/L Tris-HCl, pH 7.6) for 5 minutes at room temperature. The specimens were washed and then counterstained in 0.1% Mayer's hematoxilin. In controls, primary antibodies were substituted with PBS.

In utero treatment. Pregnant mice were administered 1 mg/d of PS/2.3 or MK/2 intraperitoneally from day 7 of gestation, when hematopoiesis begins in the yolk sac of fetuses, until the day of analysis. Trans-placental delivery of PS/2.3 to the fetuses was checked by flow cytometric analysis for the presence of antibody on fetal liver MNC, as detected by FITC-conjugated antirat IgG (CalTag, San Francisco, CA). The delivery of MK/2 through the placenta was expected based on preliminary experiments in which isotype-matched anti-Fgp-1 MoAb 1M7.8.16 was similarly detectable on fetal thymocytes after administration to pregnant mice. In both of these experiments, normal rat IgG (Sigma, St Louis, MO) or saline were administered as controls.

Histological study. The livers from control or PS/2.3-treated neonates were fixed in phosphate-buffered 10% formalin (pH 6.8), passed through

From the Second Department of Internal Medicine, Tokyo University; the Department of Immunology, Juntendo University School of Medicine, Tokyo; and the Department of Immunology, Tokai University School of Medicine, Isehara-shi, Japan.

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Address reprint requests to Ko Okumura, MD, Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

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Fig 1. Expression of VLA-4 and VCAM-1 in fetal liver. (A) Expression of VLA-4 on fetal liver MNC. Embryonic day 15 fetuses were dissected from timed pregnant mice, and mononuclear cells were isolated. They were stained by FITC-conjugated PS/2.3, PE-conjugated ACK45, and the cocktail of biotinylated-MoAbs against lineage markers (Lin), followed by streptavidin-Red613, and analyzed on FACSscan. ---, autofluorescence; ----, PS/2.3 (anti-VLA-4) staining of total liver MNC; ---, PS/2.3 staining of the electronically gated c-kit+/Lin− cells. Vertical scale represents relative cell number and horizontal scale represents log fluorescence. (B and C) Immunohistochemical staining of embryonic day 15 fetal liver with PS/2.3 or M/K-2. Embryonic day 15 fetal livers were fixed in periodate-lysine-parafomaldehyde (PLP) containing 4% parafomaldehyde. Frozen sections were reacted with a primary antibody, PS/2.3 or M/K-2, followed by biotinylated antirat IgG and avidin-biotin-peroxidase complex. The staining was developed by diaminobenzidine, and the counterstain was done with Mayer’s hematoxilin. Results represent the staining with PS/2.3 (B) and M/K-2 (C).

Fig 3. Histological study of newborn liver treated in utero with PS/2.3. The livers from control (A) or PS/2.3-treated (B) neonates were fixed in phosphate buffered 10% formaline (pH 6.8), embedded in paraffin, and the tissue sections were stained with 0.1% Mayer’s hematoxilin and 0.025% eosin. Arrows indicate erythroblasts, and arrow heads indicate myeloid cells.
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embedded in paraffin, and the tissue sections were stained with 0.1% Mayer's hematoxilin and 0.025% eosin.

Colony assay. Semi-solid methyl cellulose culture of hematopoietic cells was performed according to the previously described technique with some modifications. The livers were isolated from control or PS/2.3-treated neonates and were mashed by pressing them between frosted slide glasses. By this manipulation hepatocytes coaggregated, and dissociated hematopoietic cells became a single cell suspension. These cells (2 x 10^5 to 1 x 10^6) were cultured in 1 mL of Iscove's modified Dulbecco's medium (IMDM; Kyokuto, Tokyo, Japan) containing 1.0% nethyl cellulose (Dow Chemical, Midland, MI), 30% fetal calf serum (JRH Biosciences, Lenexa, KS), 1% deionized fraction V bovine serum albumin (BSA; Sigma), and 0.1 mmol/L 2-mercaptoethanol in the presence of 6 U/mL human recombinant erythropoietin (Chugai, Tokyo, Japan), 100 U/mL human recombinant IL-3, and 50 ng/mL human recombinant granulocyte colony-stimulating factor (G-CSF; Chugai). The amounts of colony forming unit-erythroid (CFU-E) were scored as single aggregates of more than eight cells at day 3 of culture. Burst forming unit-erythroid (BFU-E), colony-forming units granulocyte, macrophage, and granulocyte macrophage (CFU-G, CFU-M, CFU-GM), and CFU-Mix were differentially counted at days 7 to 8 as aggregates of more than 40 cells (200 cells for BFU-E) under microscopy. Some colonies were lifted from the cultures using micropipet, cytocentrifuged, and identified by Diff-Quick (Midori-Juji, Osaka, Japan) or benzidine staining.

RESULTS AND DISCUSSION

We first evaluated the VLA-4 expression in fetal liver, which is the main site of early hematopoiesis. Three-color flow cytometric analysis of fetal liver MNC showed that almost all cells strongly expressed VLA-4 (Fig 1A), which implies that VLA-4 can participate in the development of all lineages. The c-kit/"Lin" cells, which are enriched for stem cells, expressed VLA-4 most strongly, suggesting the importance of VLA-4 for these cells. Immunohistochemically, VLA-4 resided on small round cells with a large nucleus/cytoplasm ratio and dense chromatin; these are morphological characteristics of hematopoietic cells (Fig 1B). By contrast, VCAM-1, VLA-4 ligand other than FN, resided on the cells with irregularshape-extending cytoplasmic projections to surrounding hematopoietic cells, which are morphological characteristics of stromal cells (Fig 1C). This implied the possibility that not only FN but also VCAM-1 could act as a VLA-4 ligand in the hematopoietic microenvironment, as suggested by the inhibitory effect of an anti-VCAM-1 MoAb on stromal cell-dependent lymphohematopoiesis in vitro.

In vivo treatment was then performed with an anti-VLA-4 MoAb, PS/2.3, which has been demonstrated to inhibit VLA-4-mediated cell binding to both FN and VCAM-1 and also to efficiently inhibit lymphohematopoiesis in vitro.

Fetuses were treated with PS/2.3 from gestational day 7 to birth by administering the antibody into maternal peritonea. The injected antibody was efficiently transferred to the fetuses as estimated by its deposition on fetal liver MNC. Figure 2 shows that rat IgG was present on MNCs from the PS/2.3-treated fetal livers, but it was absent in controls. Because most liver MNCs from the PS/2.3-treated individuals were positive for rat IgG, we could conclude that the amount of transplacentally delivered PS/2.3 was enough to block VLA-4 molecules on fetal liver MNC. The neonates that had been treated in utero with PS/2.3 looked pale, though they did not show any other morphological abnormality (not shown). In these neonates, a marked anemia was defined by red blood cell counts that were approximately one-fifth of the controls (Table 1). Since the liver is the major site of hematopoiesis in fetuses and neonates, phenotypical analysis was carried out with liver-derived MNC. The number of TER119" cells representing erythroid progenitors was strikingly decreased. In contrast, the number of white cells in the blood and those of Gr-1" or Mac-1" lymphoid cells in the liver were not significantly decreased. CD4^"8^, CD4^"8^, or CD4^"8^ cells in the thymus and B220" cells in the liver were also not significantly affected. These results indicate that the fetal anti-VLA-4 treatment interfered only with erythropoiesis; it did not affect the development of other lineages including granuloid, monocytoid, and lymphoid cells. In addition to the erythroid cells, a significant decrease was also noted for c-kit/"Lin" cells in the liver and CD4^"8^ cells in the thymus. Since these cells are the most immature cells within each organ, VLA-4 may also contribute to stem cell homing from the yolk sac to the liver and homing of T progenitor cells to the thymus.

We then histologically examined the liver, thymus, and spleen of the PS/2.3-treated neonates as well as major nonhematopoietic organs including the heart, lung, kidney, and intestine. In the control liver (Fig 3A), many clusters of small round cells with condensed nucleus and scant cytoplasm, characteristic of erythroblasts (arrow), were seen. In contrast, these cells were almost completely absent in the PS/2.3-treated liver, while other cells of myeloid morphology (arrow head) were maintained (Fig 3B). The PS/2.3-treated thymus...
Table 1. Effect of In Utero Treatment With PS/2.3 on Hematopoiesis

<table>
<thead>
<tr>
<th>Source of Cells</th>
<th>Cell Numbers</th>
<th>Control</th>
<th>PS/2.3-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood* (x10^6 cells/µL)</td>
<td>Red cell</td>
<td>55.9 ± 7.2</td>
<td>11.0 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>White cell</td>
<td>0.125 ± 0.096</td>
<td>0.119 ± 0.036</td>
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<tr>
<td>Liver MNC (x10^6 cells/organ)</td>
<td>TER119+</td>
<td>14.3 ± 2.04</td>
<td>0.79 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>Mac-1+</td>
<td>7.96 ± 1.09</td>
<td>8.23 ± 1.17</td>
</tr>
<tr>
<td></td>
<td>Gr-1+</td>
<td>6.98 ± 0.80</td>
<td>7.92 ± 1.06</td>
</tr>
<tr>
<td></td>
<td>B220+</td>
<td>17.6 ± 0.07</td>
<td>12.5 ± 1.44</td>
</tr>
<tr>
<td></td>
<td>c-kit+/Lin-</td>
<td>1.14 ± 0.08</td>
<td>0.378 ± 0.087</td>
</tr>
<tr>
<td>Thymocyte (x10^6 cells/organ)</td>
<td>CD4-8+</td>
<td>25.5 ± 11.1</td>
<td>15.8 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>CD4-8+</td>
<td>85.9 ± 22.3</td>
<td>91.0 ± 21.0</td>
</tr>
<tr>
<td></td>
<td>CD4-8-</td>
<td>2.00 ± 0.72</td>
<td>1.73 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>CD4-8-</td>
<td>10.2 ± 5.9</td>
<td>9.41 ± 5.51</td>
</tr>
</tbody>
</table>

Neonates that had been treated in utero with PS/2.3 were analyzed on the day of birth. Two microliters of the blood was aspirated from the right atrium, diluted appropriately with PBS or Türk’s solution, and the numbers of red and white blood cells were counted using Neubauer’s hemocytometer. Thromocytes and splenocytes were released from each organ by pressing between two frosted slide glasses; they were suspended in α-MEM. Thromocytes and splenocytes, and liver MNC were stained with labeled antibodies against the indicated cell surface markers and analyzed on FACScan. Data indicate means ± SD of three or four (*) different individuals.

and spleen did not show any histological abnormality nor any other nonhematopoietic organs (not shown).

Next, we evaluated the colony forming capacity of the PS/2.3-treated liver to determine the stage at which the erythropoiesis was affected by the anti–VLA-4 treatment. Table 2 represents the mean colony numbers per control or treated neonatal liver. Erythroid colonies (BFU-E and CFU-E) from the PS/2.3-treated liver were reduced to 40% of the normal liver, but this reduction was much less prominent than the almost complete absence of erythroblasts in the treated liver. A similar extent of reduction in the migration of stem and progenitor cells of all lineages. This notion is consistent with the recent observations by other investigators that VLA-4 appeared to be involved in the lodging of CFU in murine spleens and that peripheralization of multiple hematopoietic progenitors was induced by anti-VLA-4 antibody administration in primates. However, the normalized development of myeloid and lymphoid cells after the anti-VLA-4 treatment (Table 1) suggests that some other VLA-4-independent pathways can compensate for the lymphomyelopoiesis but not the erythropoiesis.

In order to determine whether the inhibitory effect of anti-VLA-4 on erythropoiesis was exerted by its interference with VLA-4/FN or VLA-4/VCAM-1 interaction, we performed the in utero treatment with an anti-VCAM-1 MoAb (M/K-2), which has been demonstrated to inhibit in vitro lymphopoiesis as efficiently as PS/2.3, according to the same protocol as PS/2.3. The M/K-2 treatment did not cause anemia (red blood cells, 5.31 ± 0.62 and 5.59 ± 0.72 × 10^6 cells/µL, mean ± SD, in the treated or control neonates, respectively), but leukocytosis in the blood was notable (white blood cells, 2.39 ± 1.31 and 1.25 ± 0.99 × 10^6 cells/µL, mean ± SD, in the treated or control neonates, respectively). This effect was quite different from that of PS/2.3 (Table 1), which suggests that VCAM-1 does not play a critical role as the VLA-4 ligand for supporting erythropoiesis in vivo, although it is abundantly expressed in the hematopoietic microenvironment (Fig 1C). VCAM-1 may, rather, contribute to the lodging as indicated by leukocytosis after the anti–VCAM-1 treatment. Although the presence of other VLA-4 ligands has not been excluded, FN would act as the critical VLA-4 ligand for supporting erythropoiesis in vivo, as previously suggested by the FN requirement for in vitro differentiation of murine erythroleukemia cells into reticulocytes, which was associated with erythocyte-specific protein induction.

It has been demonstrated that VLA-4–mediated signaling led to cytokine gene expression and autocrine growth in mature T lymphocytes and eosinophils. Interestingly, VLA-4 can transmit a unique signal distinct from that of other members of the integrin family. It remains to be determined how VLA-4–mediated adhesion and signaling participate in the erythropoiesis in collaboration with c-kit/stem cell factor and erythropoietin receptor/erythropoietin systems, which are also required for erythropoiesis.

Table 2. Colony Forming Capacity of Neonatal Liver Treated In Utero With PS/2.3

<table>
<thead>
<tr>
<th>Colony</th>
<th>Control</th>
<th>PS/2.3-Treated</th>
<th>(% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BFU-E</td>
<td>9.69 ± 3.94</td>
<td>3.88 ± 1.48</td>
<td>(40.0)</td>
</tr>
<tr>
<td>CFU-E</td>
<td>276 ± 36</td>
<td>113 ± 23</td>
<td>(40.9)</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>6.59 ± 4.73</td>
<td>3.68 ± 2.05</td>
<td>(55.8)</td>
</tr>
<tr>
<td>CFU-G</td>
<td>6.66 ± 4.32</td>
<td>4.19 ± 1.60</td>
<td>(62.9)</td>
</tr>
<tr>
<td>CFU-M</td>
<td>33.1 ± 7.17</td>
<td>29.0 ± 4.0</td>
<td>(87.6)</td>
</tr>
<tr>
<td>CFU-Mix</td>
<td>3.54 ± 1.41</td>
<td>0.990 ± 0.018</td>
<td>(28.0)</td>
</tr>
</tbody>
</table>

Semisolid methyl cellulose cultures, of the hematopoietic cells derived from new born livers from control or in utero PS/2.3-treated individuals were obtained. Livers were mashed by pressing between frosted slide glasses, and dissociated hematopoietic cells (2 × 10^6 to 1 × 10^7) were cultured in 1 mL of IMDM containing 1.0% methyl cellulose, 30% fetal calf serum, 1% deionized fraction V BSA, and 0.1 mmol/L 2-mercaptoethanol in the presence of 6 U/mL human recombinant erythropoietin, 100 U/mL mouse recombinant IL-3, and 50 ng/mL human-recombinant G-CSF. The numbers of CFU-E were scored as cell aggregates of more than eight cells at day 3 of culture. BFU-E, CFU-G, CFU-M, CFU-GM, and CFU-Mix were differentially counted at days 7 to 8 as aggregates of more than 40 cells (200 cells for BFU-E) under microscopy. Data indicate means ± SD of three different individuals.
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REFERENCES

A critical role of VLA-4 in erythropoiesis in vivo

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