Brief Definitive Report

Very Late Activation Antigen 4–Vascular Cell Adhesion Molecule 1 Interaction Is Involved in the Formation of Erythroblastic Islands

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Summary

Erythroblastic islands are anatomical units consisting of a central macrophage surrounded by erythroblasts. We studied the adhesion molecules involved in the formation of these structures. Central macrophages of erythroblastic islands isolated from the spleens of phlebotomized mice were clearly stained for vascular cell adhesion molecule 1 (VCAM-1). The surrounding erythroblasts of the erythroblastic islands strongly expressed the $\alpha 4$ integrin of very late activation antigen 4 (VLA-4: $\alpha 4\beta 1$ integrin), the counter receptor of VCAM-1, whereas most reticulocytes and erythrocytes did not. Both monoclonal antibodies (mAbs) against $\alpha 4$ integrin and VCAM-1 disrupted the erythroblastic islands cultured in the presence of erythropoietin. Moreover, adhesion of splenic erythroblasts to tumor necrosis factor α–stimulated mouse splenic endothelial cells, which showed high expression of VCAM-1 but not intercellular adhesion molecule 1, was inhibited by the anti-VCAM-1 and anti-$\alpha 4$ mAbs. These findings suggest that VLA-4–VCAM-1 interaction plays a crucial role in the formation of erythroblastic islands.

Bone marrow resident macrophages (MΦs) establish stroma by extending long cytoplasmic processes and attaching to developing erythroid and myeloid cells (1, 2). These cells are both phenotypically and functionally different from peritoneal MΦs and monocytes (3, 4). In long-term bone marrow culture by which hematopoietic stem cells are maintained, immature myelomonocytic cells are attached to and proliferate on the MΦs defined by the mAb F4/80 (5). The addition of erythropoietin to these cultures alternatively induces erythropoietic activity on the MΦs, which form erythroblastic islands (EI) composed of central MΦs and surrounding erythroblasts (Ebs) (6, 7). These lines of evidence suggest that erythropoietin-responsive erythroid precursors adhere to the MΦs, where they are induced to proliferate and differentiate into erythrocytes by maintaining contact with the central MΦs. To gain insight to the possible function of the MΦs in erythropoiesis, we have considered it a priority to identify specific adhesion molecules involved in EI formation (8).

Recently, Morris et al. (9) characterized the nature of the adhesion using EI isolated from fetal liver and found that the adhesion required divalent cations. Soligo et al. (10) demonstrated that a divalent cation-dependent adhesion molecule, very late activation antigen 4 (VLA-4) ($\alpha 4\beta 1$) integrin, was present on Ebs, and that this molecule was localized at sites of intercellular contact between Ebs and MΦs in human marrow, suggesting that VLA-4 might be involved in the adhesive interaction. The ligand of VLA-4 for cell–cell interaction is vascular cell adhesion molecule 1 (VCAM-1), which was first identified on TNF-α–stimulated human umbilical endothelial cells (11, 12). Using the bone marrow stromal cell culture system, VCAM-1 has been shown to be constitutively expressed in stromal cells, and to be involved in B lymphocyte–stromal cell (13) and hematopoietic stem cell–stromal cell interactions (14). However, morphological precise characterization of VCAM-1–positive cells in hematopoietic tissues has not yet been performed. In the present study, we showed that VCAM-1 was strongly expressed on central MΦs in EI, and that the adhesive interaction between VLA-4 on Ebs and VCAM-1 on the central MΦ plays an important role in the formation of EI.

Materials and Methods

Cells and Antibodies. Bone marrow cells were prepared by flushing the femora of 8-wk-old C3H/HeN mice (Charles River Japan Inc., Tokyo,, Japan) using a syringe with a 26-gauge needle. Splenic Ebs were prepared from the spleens of anemic mice. Briefly, the mice were bled into heparinized capillary tubes from the retroorbital sinus 3 d (0.5 ml) before killing. Mice were killed by cervical dislocation, and their spleens were excised, washed in RPMI 1640 medium (GIBCO BRL, Gaithersburg, MD) containing 0.004% DNase (Sigma Chemical Co., St. Louis, MO), and minced in the medium with scissors. After passing the fragments through a syringe with a 26-gauge needle several times, the suspensions were settled for 5 min and then the small amount of residual undigested white tissue and cellular aggregates were discarded. The suspension was washed in cation-depleted PBS at 80 *g* for 3 min three times and the supernatants containing erythrocytes were discarded. The sedimented Ebs-rich fraction was resuspended in

RPMI 1640 medium containing 0.2 U/ml human recombinant erythropoietin (EPOCH®, Chugai Pharmaceutical Ltd., Tokyo, Japan) and used for the adhesion assay. Peritoneal MΦs were prepared as described previously (2). A mouse splenic endothelial cell line (SPE-1) was established and used for adhesion assay as described previously (15). Hybridoma cells, PS/2 (rat anti-mouse α4 integrin mAb) (16), M/K-1 (rat anti-mouse VCAM-1 mAb) (13), and KM201 (rat anti-mouse CD44 mAb) (17) were obtained through the American Type Culture Collection (Rockville, MD). KAT-1 (rat anti-mouse intercellular adhesion molecule 1 [ICAM-1] mAb) (18) was purchased from Serotec Inc. (Oxford, UK). F10 (rat anti-Forssman glycosphingolipid mAb) was produced as described previously (19). Purified mAbs (PS/2 and M/K-1) were kind gifts from Dr. Kensuke Miyake (Saga Medical School, Saga, Japan).

Flow Cytometry. For the study of VCAM-1, ICAM-1, and α4 expression on SPE-1 cells, the cells were treated with or without 10 ng/ml mouse recombinant TNF-α (Boehringer Mannheim-Yamanouchi, Tokyo, Japan) for 12 h and then detached from culture flasks using 0.1% EDTA. The cells were incubated for 20 min on ice with M/K-1, KAT-1, or PS/2 mAbs. After two washes in PBS containing 1% horse serum, the cells were incubated with PE-labeled anti-mouse goat $F(ab')_2$ IgG (Tago, Inc., Burlingame, CA) on ice for 20 min, washed, and analyzed by FACScan®.

Isolation and Culture of Mouse Splenic EI. EI were isolated and enriched from the spleens of bled mice by collagenase digestion, unit gravity sedimentation, and Percoll density gradient separation as described previously (19, 20). The EI fraction containing 90.1% Ebs and 3.4% MΦs was used for immunocytochemistry and inhibition tests.

Immunocytochemistry. Acetone-fixed cytospin preparations of EI, bone marrow cells, and resident peritoneal MΦs were stained with PS/2, M/K-1, KM201, and KAT-1 as described previously (2).

Inhibition Test on EI Formation by Use of mAbs. After suspending the EI fraction in RPMI 1640 medium containing 0.2 U/ml erythropoietin, 150 μl of the suspension was placed on a 35-mm plastic dish (Falcon, Oxnard, CA) and incubated at 37°C in a CO_2 incubator. 30 min later, the medium was decanted and loosely bound cells were removed by direct flushing with RPMI 1640 medium. This treatment left EI bound on the surface of the dish. The EI were cultivated with RPMI 1640 medium containing 0.2 U/ml human recombinant erythropoietin with antiadhesion molecule mAbs or 0.5 mg/ml of the peptide GPEILDVPST (sequence containing the VLA-4-binding epitope spanning the CS-1 fragment of fibronectin) (Pennisula Laboratories, Inc., Belmont, CA) (12) as inhibitors. 3 h later, the medium was decanted and loosely bound cells were removed by gentle flushing with RPMI 1640 medium. The cultures were fixed in PBS containing 0.3% glutaraldehyde, and the number of cells attached to the MΦs were scored under a phase contrast microscope.

Cell Adhesion Assay. SPE-1 monolayers were prepared by seeding onto 35-mm dishes at 50,000 cells/well 3 d before the adhesion assay. Monolayers were treated with or without 10 ng/ml TNF-α for 12 h. After SPE-1 cells were washed once with RPMI 1640 medium and preincubated for 15 min at 4°C in 1 ml of RPMI medium with or without mAbs (KM201, KAT-1, PS/2, and M/K-1), the mouse Ebs-rich fractions were overlayed on the SPE-1 monolayer at a density of 3×10^6/dish in RPMI 1640 medium containing mAbs and incubated at 37°C for 20 min. Dishes were then rinsed with RPMI 1640 medium three times and fixed in 0.1 mol/liter phosphate buffer containing 2% paraformaldehyde for 30 min. Attached cells were counted under the phase contrast microscope. Inhibition of adhesion was studied using mAbs KM201, PS/2, and M/K-1 each at concentrations of 10 μg/ml. To identify morphology of the cells attached to SPE-1 cells, the dishes were rinsed with cation-depleted PBS, and cells detached from the SPE-1 cells were collected and prepared for Giemsa staining.

Results and Discussion

Expression of Adhesion Molecules in EI. EIs, a form of close association of MΦs with Ebs, have been observed in humans and other mammals (6, 8). Crocker and Gordon (1) reported that central MΦs in EI are positive for F4/80 antigen and are different from fibroblastic stromal cells. Recent interest has been focused on the adhesion molecules involved in the formation of these structures (2, 3, 8). Thus, we first examined the expression of some adhesion molecules (VCAM-1, ICAM-1, CD44, and α4 integrin) in EI isolated from anemic mouse spleen by collagenase digestion. The central MΦs in these structures were positive for VCAM-1, ICAM-1, and CD44 (Fig. 1, *B–D*), and some were weakly positive for α4 integrin. Surrounding Ebs in the EI were clearly positive for α4 and CD44 (Fig. 1, *A* and *D*), whereas erythrocytes and reticulocytes were negative for α4. The EI from mouse bone marrow showed the same staining reactions with these mAbs (data not shown). In contrast to central MΦs of EI, no resident peritoneal MΦs were stained with anti-VCAM-1.

Inhibitory Effects of mAbs against α4 Integrin and VCAM-1 on EI Formation in vitro. To examine the possible function of VCAM-1 in the formation of EI, we used the short-term culture system of splenic EI that we reported previously (20). Splenic EI cultured in the presence of erythropoietin remained intact for a short period. Characteristically, Ebs showed gradiental arrangement on the surface of central MΦs; more differentiated Ebs and enucleated cells were seen at the periphery. In the absence of erythropoietin, erythropoietic activity completely disappeared within 24 h, and granulocytic cells were found in some isolated EI.

3 h after the addition of anti-VCAM-1 (M/K-1) or anti-α4 integrin (PS/2) mAbs to the EI cultures, Ebs became ar-

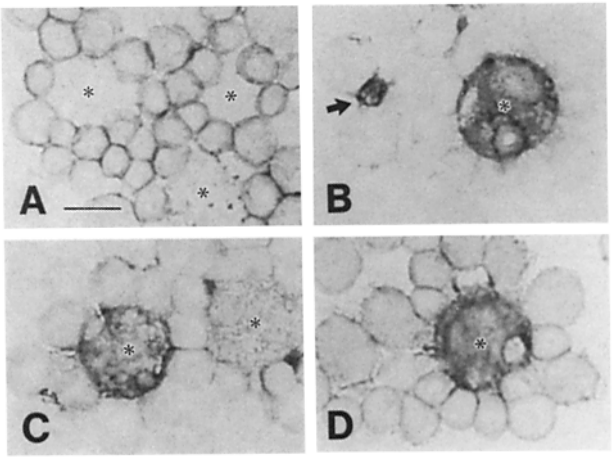

Figure 1. Immunocytochemical localization of α4 integrin (*A*), VCAM-1 (*B*), ICAM-1 (*C*), and CD44 (*D*) in EI isolated from mouse spleen. (*) Central MΦs. (*Arrow*) A cytoplasmic process of the central MΦ. Bar, 13 μm.

ranged loosely on the MΦs and showed no gradiental arrangement. Gentle flushing of the medium using Pasteur pipettes easily destroyed the EI structure, although some EI were resistant to the treatment (Fig. 2, *B* and *C*). On the other hand, anti-CD44 (KM201), anti-ICAM-1 (KAT-1), or anti-Forssman glycosphingolipid (F10) mAbs had hardly any effect on the EI structure (Fig. 2, *D–F*). Quantified effects of the various mAbs on EI in these cultures are shown in Table 1. These results support the possibility that Ebs are capable of attaching to the central MΦs via VLA-4–VCAM-1 interaction.

VLA-4 also functions by binding to the CS-1 domain of fibronectin (12). Therefore, we tested CS-1 peptide in the EI culture system, but this peptide had no effects on EI formation (data not shown). This is consistent with the previously reported observation that fibronectin, RGD-containing peptides, and specific antisera against the fibronectin receptor, had no effects on EI formation in vitro (9).

Adhesion of Erythroblasts to TNF-α–stimulated SECs. To confirm the involvement of VLA-4–VCAM-1 interaction in EI formation, we used another adhesion assay system to test adhesion of Ebs to the SPE-1 established from mouse primary splenic culture and characterized by typical endothelial cell morphology distinct from those of fibroblasts, smooth muscle cells, and MΦs. These cells take up acetylated low density lipoprotein, react with antibodies against an endothelial cell–specific antigen (Meca 99) and against basement membrane proteins, and are stained by fluorescein-labeled *Griffonia simplicifolia* lectin known to stain mouse endothelial cells (15). Upon examination of the expression of VCAM-1, ICAM-1, and α4 integrin on nontreated and TNF-α–stimulated SPE-1 cells by flow cytometry, it was found that TNF-α stimulation greatly induced only VCAM-1 expression on SPE-1 cells.

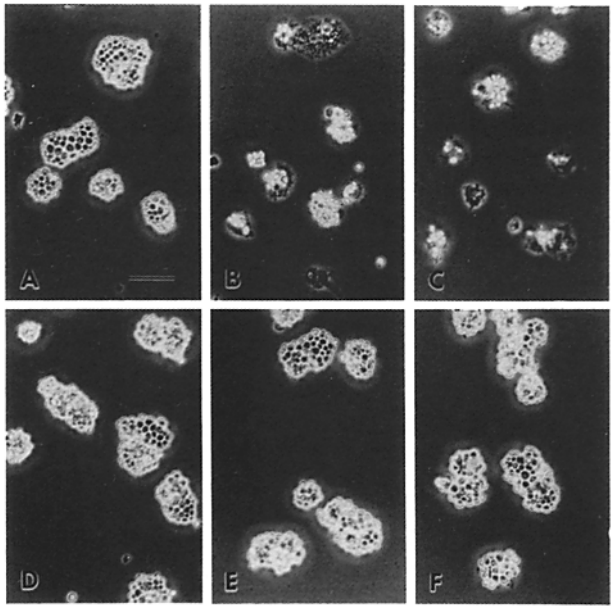

Figure 2. Effects of mAbs on maintenance of EI in culture in the presence of erythropoietin. (*A*) Control; (*B*) anti-α4 integrin; (*C*) anti-VCAM-1; (*D*) anti-ICAM-1; (*E*) anti-CD 44; and (*F*) anti-Forssman glycosphingolipid. Bar, 48 μm.

Table 1. *Quantitative Effects of mAbs on EI Cultured in the Presence of Erythropoietin*

mAb (Specificity)	Dose	Number of Ebs/MΦ
	μg	
No Ab		23 ± 9*
M/K-1 (VCAM-1)	5	6 ± 6‡
M/K-1	10	5 ± 6‡
PS/2 (α4 integrin)	5	10 ± 7‡
PS/2	10	7 ± 6‡
M/K-1 + PS/2	5 + 5	5 ± 5‡
KAT-1 (ICAM-1)	10	22 ± 9
KM201 (CD44)	5	23 ± 10
F10 (Forssman GSL)	10	20 ± 8

EI from anemic mouse spleen were placed on 35-mm dishes and incubated in RPMI 1640 containing 0.2 U/ml erythropoietin and mAbs. 3 h later, the dishes were gently flushed of the culture medium, fixed in 0.3% glutaraldehyde, and observed under the phase contrast microscope.
* Data are shown as the mean ± SD (n = 50) of the total number of Ebs attached to an underlying MΦ.
‡ p <0.01 compared with the control (no Ab) by Student's t-test.

Neither nonstimulated nor TNF-α–stimulated SPE-1 cells were stained for ICAM-1 (Fig. 3). In the adhesion assays of Ebs obtained from phlebotomized mouse spleen, the Ebs adhered strongly to TNF-α–stimulated SPE-1 cells but not to

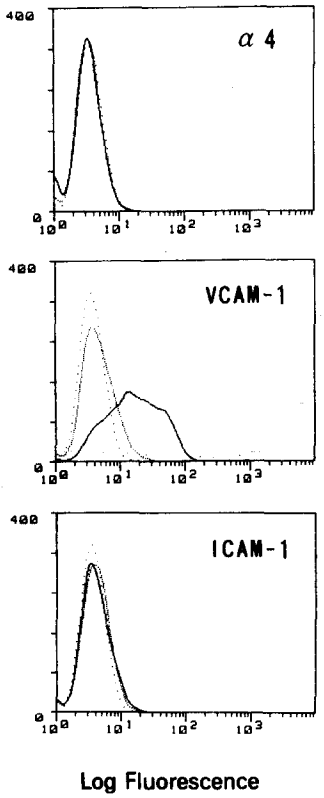

Figure 3. Flow cytometric analysis of the expression of α4 integrin (*top*), VCAM-1 (*middle*), and ICAM-1 (*bottom*) in nontreated (------) and TNF-α–stimulated (———) SPE-1 cells. Control staining (· · · · · ·) was performed without primary Ab. 10^4 cells in each group were analyzed by FACScan®. Histograms plotted as cell number vs log fluorescence intensity. Some unstimulated cells were positive for VCAM-1, and TNF-α stimulation significantly increased VCAM-1 expression.

Table 2. *Effects of mAbs on Adhesion of Bone Marrow Cells and Splenic Ebs to SPE-1 Cells*

Treatment			
TNF-α	Ab (10 μg/ml)	Bone marrow cells	Splenic Ebs
–	–	9 ± 2*	17 ± 7
–	M/K-1 (VCAM-1)	8 ± 4	ND
+	–	73 ± 36	164 ± 33
+	M/K-1	17 ± 4‡	12 ± 5‡
+	PS-2 (α4 integrin)	7 ± 4‡	4 ± 2‡
+	KAT-1 (ICAM-1)	76 ± 17	165 ± 37

* Each number represents mean ± SD (n = 4) of total number of cells attached to 20 SPE-1 cells in different incubation dishes. Bone marrow cells and splenic Ebs prepared as described in Materials and Methods were incubated at 37°C with unstimulated or 12-h TNF-α-stimulated SPE-1 cells in 35-mm dishes. Adhesion of Ebs to SPE-1 cells was quantified by counting adherent cells under the phase contrast microscope at 20 SPE-1 cells per incubation dish.

‡ p <0.01 compared with the control (TNF-α + no Ab) by Student's t-test.

nonstimulated SPE-1 cells. This adhesion was completely inhibited by anti-VCAM-1 and anti-α4 mAbs but not by anti-CD44, or anti-ICAM-1 mAbs, as shown in Table 2.

α4 integrin has been shown to be distributed on a variety of types of hematopoietic cells including hematopoietic stem cells, lymphocytes, Ebs, monocytes, and immature granulocytes (10, 14, 16). We confirmed this by morphological study of bone marrow cells attached to TNF-α-stimulated SPE-1 cells that showed strong expression of VCAM-1 but not ICAM-1 (Table 2). These findings suggested the possibility that several types of hematopoietic cell islands might be formed via VLA-4–VCAM-1 interaction. In vivo, however, complete rosettelike structures were observed only in Ebs-MΦs adhesion (namely EI), but not in myelomonocytic cell-MΦs or lymphocyte-MΦs adhesions. This suggests that not only VLA-4–VCAM-1 interaction but also some unknown adhesion molecules or unknown factors might be involved in the formation of EI in vivo.

The present results raise intriguing questions about the biological significance of the VLA-4–VCAM-1 interaction of EI. In this regard, the following possible functions of EI have been speculated (3, 8): (*a*) The Ebs-MΦ contact may serve to allow rapid expansion of Ebs; (*b*) The contact may promote Ebs enucleation in the same fashion as the Ebs-fibronectin interaction (21); and (*c*) The Ebs-MΦ adhesion may regulate the release of reticulocytes from hematopoietic tissues. In addition, considering the recent finding that VLA-4–VCAM-1 interaction in follicular dendritic cell–B cell clusters serve to inhibit apoptosis of germinal center B cells (22), it may also be possible that the central MΦ may play a supportive role in erythropoiesis by inhibiting apoptosis of Ebs through the VLA-4–VCAM-1 interaction.

In conclusion, the present results indicate that attention should be paid to the resident MΦs as well as stromal cells to understand the in vivo mechanisms underlying hematopoiesis.

This work was supported by grant 5-602 from Kawasaki Medical School.

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Received for publication 27 June 1994 and in revised form 26 September 1994.

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