〈Regular Article〉

Human bone marrow VCAM-1+ macrophages provide a niche for reactive and neoplastic erythropoiesis

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ABSTRACT　　Resident bone marrow macrophages provide a microenvironment for erythropoiesis, forming erythroblastic islands (EBIs) via adhesion molecules. In this study, we examined vascular cell adhesion molecule-1 (VCAM-1) expression in human bone marrow specimens using immunohistochemistry. VCAM-1 was strongly expressed in CD169+ macrophages in EBIs and weakly in sinusoidal vascular endothelial cells. In reactive erythropoiesis, including hemolytic and megaloblastic anemia, the extended cytoplasm of VCAM-1+ CD169+ macrophages circumscribed the erythroid cells. The strong affinity between VCAM-1+ macrophages and erythroid cells was also observed in polycythemia vera (PV), essential thrombocythemia (ET), and chronic myelogenous leukemia (CML). VCAM-1 density was significantly higher in PV than in ET and CML (p < 0.001), and correlated with blood erythrocyte count in all three neoplasms (p < 0.001). In ET, the VCAM-1 density was higher in cases with the JAK2 mutation than with the CALR mutation. In myelodysplastic syndrome with erythroid predominance but unclear EBI formation, punctate VCAM-1+ cytoplasmic processes of macrophages were seen between erythroblasts, similar to those seen between granulocytic precursors in CML, suggesting incomplete contact of VCAM-1+ macrophages with dysplastic erythroid cells. These results suggest that VCAM-1+ macrophages create a niche for reactive and neoplastic erythropoiesis and may be a therapeutic target in PV.

INTRODUCTION

Bone marrow stroma comprises a variety of cell types, including adipocytes, fibroblastic reticular cells, endothelial cells, and resident macrophages[1-4]. These stromal cells provide a microenvironment suitable for hematopoiesis by secreting various cytokines and by attaching to hematopoietic cells via adhesion molecules[5-9]. The

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resident macrophages are located in erythropoietic foci, forming erythroblastic islands (EBIs) in which erythroblasts are physically close to each other but separated by the cytoplasm of the central macrophage. Several studies have suggested that signals from adhesive interactions with the macrophage are important in EBI formation and may contribute to the survival and proliferation of the erythroid precursors[10-14].

Vascular cell adhesion molecule-1 (VCAM-1) is a transmembrane molecule that mediates the adhesion of immune cells to activated endothelial cells and macrophages through very late activation antigen 4 (VLA-4, or $\alpha 4/\beta 1$ integrin) and the integrins $\alpha 4/\beta 7$, $\alpha 9/\beta 1$, and $\alpha D/\beta 2$. VLA4 is a ligand of VCAM-1 and fibronectin, and it has been shown that erythroblasts express it at a high level[8]. The importance of VLA4/VCAM-1 interactions has been demonstrated in the proliferation and survival of erythroid progenitors on mouse fetal spleen stromal cells[10]. In mice, the central macrophage in an EBI expresses not only the macrophage pan-marker CD163[15] and CD169 (known as sialoadhesin or Siglec-1)[16, 17], but also VCAM-1[5, 14]. Following the administration of granulocyte colony-stimulating factor (G-CSF), CD169^{+} VCAM-1^{+} macrophages were observed to decrease in number in bone marrow but not in the spleen; the splenic macrophages were able to sustain extramedullary erythropoiesis *in vivo*[18, 19].

Mice treated by injection with liposome-encapsulated dichloromethylene diphosphonate (Cl2MDP) provide a model for studying the role of macrophages and erythropoiesis. The abrogation of macrophages by these injections has been shown to result in the suppression of mouse splenic erythropoiesis stimulated by phlebotomy[20], and bone marrow erythropoiesis[21]. Other studies involving injections with liposome-encapsulated Cl2MDP have revealed the supporting activity of CD169^{+} VCAM-1^{+} stromal macrophages not only

for reactive erythropoiesis in phenylhydrazine-treated mice, but also for neoplastic erythropoiesis in a JAK2[V617F]-driven mouse model[22, 23].

Much less is known about the morphology, phenotypes, and distribution of bone marrow stromal macrophages and their association with hematopoietic cells, clinicopathological parameters, and gene mutation status in human hematologic diseases. This may be due to the difficulty of analyzing EBIs *in vitro* while retaining their three-dimensional complex structure. Results so far indicate that VCAM-1 is expressed by stromal and endothelial cells and plays an important role in the adhesion of VLA4^{+} hematopoietic stem cells (HSCs) to sinusoidal endothelial cells during their homing into the bone marrow and in the mobilization of HSCs[5, 14, 18]. As yet, there has been no report evaluating the expression of VCAM-1 by bone marrow macrophages in human hematologic diseases.

The aim of this study was to investigate the expression of VCAM-1 in human bone marrow samples and its association with erythropoiesis in myeloproliferative neoplasms. To archive this, we first verified the expression of VCAM-1 in erythropoiesis-associated macrophages by immunostaining formalin-fixed paraffin-embedded sections of spleen from mice treated by phlebotomy and injections of liposomes-encapsulated Cl2MDP. Using the same anti-VCAM-1 antibody, we then immunostained human bone marrow biopsy samples with reactive and neoplastic erythropoiesis, focusing on the expression pattern and density of VCAM-1 in the erythropoiesis-associated macrophages in myeloproliferative neoplasms and myelodysplastic syndrome.

MATERIALS AND METHODS
Primary antibodies

The following commercially available antibodies were used: the anti-VCAM1 rabbit monoclonal

antibody (mAb) (EPR5047, Abcam, Cambridge, UK; 1:100 dilution), which also reacts with mouse, rat, and human antigens; the anti-CD163 mouse mAb (10D6, Leica Biosystems, Newcastle, UK; 1:200 dilution); the anti-CD169 rabbit mAb (SP213, Spring Bioscience, Pleasanton, CA, USA; 1:50 dilution); the anti-CD146 rabbit mAb (EPR3208, Abcam; 1:200 dilution); the anti-CD34 mouse mAb (QBend10, Dako, Tokyo, Japan; 1:200 dilution); the anti-TP53 mouse mAb (DO7, Dako; 1:50 dilution); the rabbit anti-integrin $\alpha 4$ polyclonal antibody (MSD, Darmstadt, Germany; 1:500 dilution); the rabbit anti-myeloperoxidase polyclonal antibody (Dako; 1:500 dilution); the anti-CD71 mouse mAb (H68.4, Santa Cruz Biotechnologies, Santa Cruz, CA, USA; 1:100 dilution); the anti-CD42b mouse mAb (MM2/174, Serotec, Kidlington, UK; 1:200 dilution); and EB-1 mAb (rat anti-mouse erythrocyte band 3 protein mAb), which was produced in our laboratory as described previously[24].

Treatment of mice

Briefly, eight-week-old BALB/c mice weighing 20 − 24 g (SLC, Hamamatsu, Japan) were injected i.v. with 0.4 ml of phosphate-buffered saline liposome suspension or Cl2MDP − liposome suspension[20]. The mice in the phlebotomy group were bled into heparinized capillary tubes from the retro-orbital sinus (0.5ml) 30 min prior to injection. The animals were sacrificed at various times after the treatment. The spleens were excised and fixed with 20% buffered formalin, dehydrated in alcohol, and embedded in paraffin. All procedures in this study were approved by the Kawasaki Medical School Institutional Review Board (number 95-060).

Immunohistochemistry of mouse spleen

Sections of formalin-fixed paraffin embedded tissues 3 μm thick sections were stained with the DISCOVERY XT system (Ventana Medical Systems, Tucson, AZ, USA) using VCAM-1 antibodies. For the identification of erythroid cells with EB-1 mAb, immunohistochemistry was performed with Histofine® Simple Stain Mouse MAX-PO (Nichirei Biosciences Inc., Tokyo, Japan).

Human bone marrow samples

The design of the experiments using human bone marrow samples was approved by the Ethics Review Board of Kawasaki Medical School and Hospital (assigned numbers 1915-1 and 2664). Case materials were obtained from Kawasaki Medical School Hospital (Kurashiki, Japan).

Paraffin-embedded bone marrow samples were obtained from a total of 138 patients: 90 biopsy cases, and 48 aspirate clot cases. The patients had the following diagnoses: hemolytic anemia (n = 10); megaloblastic anemia (n = 6); pure red cell aplasia (n = 3); myelodysplastic syndrome (MDS; 10 in total, 1 MDS-RAEB2, and 9 RDS-RCMD) and acute myeloid leukemia (AML; 8 in total, 7 acute erythroid leukemia and 1 AML with multilineage dysplasia-related changes), of which erythroid precursors comprised ≥50% of the bone marrow nucleated cells and exhibited impaired formation of EBIs, chronic myelogenous leukemia (CML; n = 22); polycythemia vera (PV; n = 20); essential thrombocythemia (ET; n = 26), post-polycythemic myelofibrosis (n = 5), post-ET myelofibrosis (n = 3), primary myelofibrosis with overt fibrotic stage (n = 4); and, as a control, malignant lymphoma with normal hemoglobin values of peripheral blood and no bone marrow involvement (n = 21).

Bone marrow diagnoses were confirmed using smears of peripheral blood cell count (leukocyte count, erythrocyte count, platelet count, hemoglobin value, and hematocrit value), smears of peripheral blood and bone marrow, histology, immunohistochemistry, flow cytometric analysis, chromosome analysis, and gene mutation status according to the current World Health Organization classification[25]. Information on JAK2 and CALR

mutation status was extracted from the chart.

Immunohistochemistry of the bone marrow samples

The bone marrow biopsy tissues were fixed in 10% buffered formalin overnight or in Bouin's solution for 2 h. Formalin-fixed biopsy tissues were decalcified by citrate-buffered formic acid and embedded in paraffin. Bouin-fixed biopsy tissues were embedded in paraffin without decalcification. The bone marrow aspirate clot sections were fixed 10% buffered formalin overnight or in Bouin's solution for 2 h and then embedded in paraffin. The paraffin-embedded sections were cut into 3-μm thick sections and deparaffinized; the sections were then incubated in antigen retrieval CC1 solution for 60 min at 98℃ and were stained using DISCOVERY XT (Ventana) with antibodies against CD163 for macrophages, CD169 for resident macrophages, CD146 for adventitial reticular cells[26], CD34 for endothelial cells and HSCs, myeloperoxidase for granulocytes, CD42b for megakaryocytes, integrin α4 and CD71 for erythroblasts, TP53, and VCAM-1.

Double immunohistochemistry

Double labeling was performed to determine which cell types expressed VCAM-1. Tissue sections were stained for CD163, CD169, CD71, CD34, and CD146, and the signals detected using diaminobenzidine hydrochloride solution and TrueBlue™ Peroxidase Substrate (KPL, Gaithersburg, MD, USA).

Image analysis

A computer-assisted image analysis system was used to quantify VCAM-1 density in the bone marrow specimens[27]. Briefly, bone marrow biopsies and aspirate clots were examined by light microscopy following immunostaining VCAM-1. Four non-overlapping fields per specimen were evaluated at a magnification of ×400 using a fully automated image analysis system (BZ-X Viewer and BZ-X Analyzer; Keyence, Osaka, Japan). The VCAM-1 density was expressed as the stained area with VCAM-1 as a proportion of hematopoietic area (defined as the bone marrow area excluding fat). This allowed us to compare the density of macrophages regardless of the difference in cellularity.

Statistical analysis

The Mann－Whitney U test was used for comparisons of VCAM-1 density between two groups, and the Kruskal－Wallis test was used for comparisons among three groups. Correlations between VCAM-1 density and peripheral blood count were evaluated using Spearman's rank correlation coefficients. P values <0.05 were considered significant. The statistical analyses were performed using EZR (easy R), available on the website of Jichi University (http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/ststmed.html)[28], and StatMate (StatMate Discovery Software, version 5.01, ATMS, Tokyo, Japan).

RESULTS

Immunohistochemical distribution of VCAM-1 in the spleens of mice treated with phlebotomy and injections of liposome-encapsulated Cl2MDP

First, we immunostained formalin-fixed paraffin embedded sections of mice treated by phlebotomy and injections of Cl2MDP encapsulated in liposomes using the same anti-VCAM-1 antibody as used for the immunostaining of the human bone marrow specimens. The kinetics of erythropoiesis of splenic red pulp in these mice has been described previously[19-21]. In the normal control mice, VCAM-1 was expressed in cells that extended fine cytoplasmic processes into hematopoietic foci and sinus endothelial cells in the red pulp, and follicular dendritic cells in the white pulp (Fig. 1a). In the spleen of the phlebotomized mouse, large

erythropoietic foci formed and each erythroblast was individually circumscribed by the dendritic cytoplasm of macrophage that were strongly positive for VCAM-1 (Fig. 1b). Erythropoietic foci were identified using EB-1 monoclonal antibody immunostaining. At four days after phlebotomy and the injections of liposome-encapsulated Cl2MDP, VCAM-1^{+} macrophages were not seen in the splenic red pulp of mice, although VCAM-1 was still expressed on reticular and sinus endothelial cells in the red pulp. In this point, no erythropoietic foci were observed (Fig. 1c). The macrophages had almost recovered to their normal level by 10 days

after treatment and strong VCAM-1 expression on the macrophages with cytoplasmic extension was observed in the erythropoietic foci (Fig. 1d). These findings indicate that VCAM-1^{+} cells are stromal macrophages that are closely associated with erythroid precursors.

Immunohistochemistry of normal human bone marrow specimens

CD163^{+} macrophages were uniformly distributed throughout the hematopoietic cords, frequently in association with immature hematopoietic cells and megakaryocytes. CD169 was distributed on
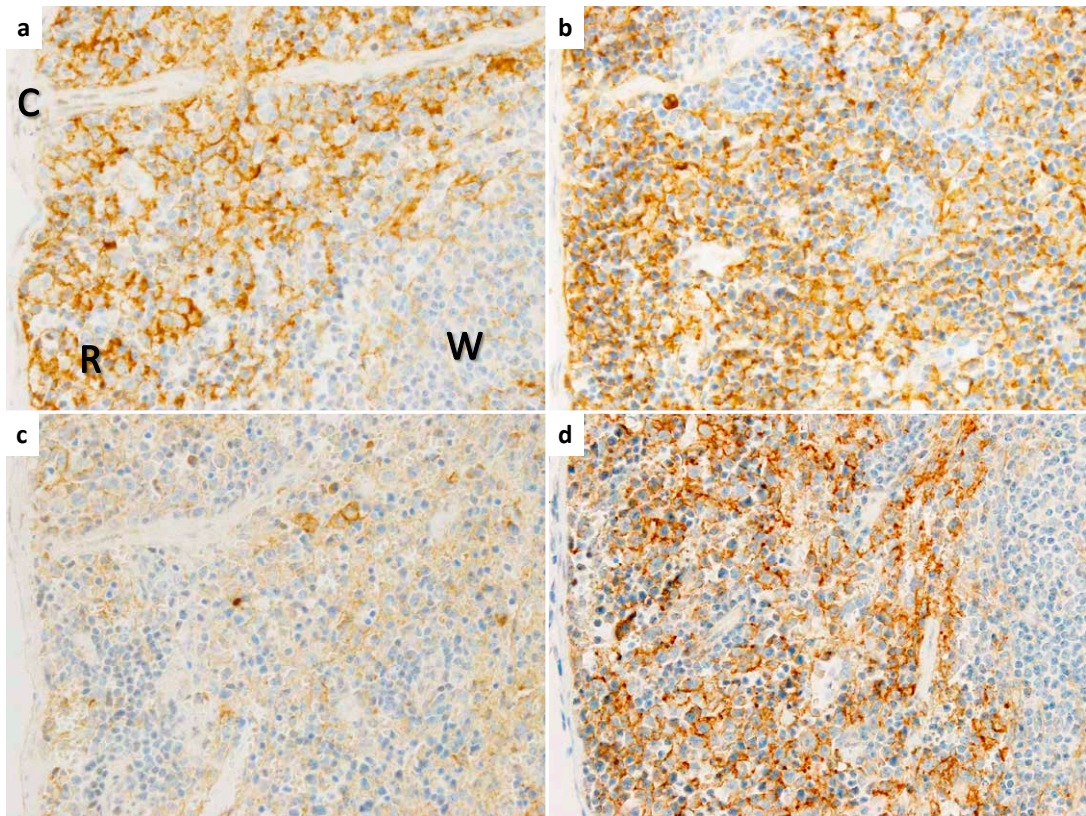

Fig. 1 Immunohistochemical expression of VCAM-1 in mouse spleens. (a) Spleen from a non-treated control mouse. VCAM-1^{+} fine cytoplasmic processes could be identified on macrophages and sinus endothelial cells in the red pulp, and follicular dendritic cells in the white pulp. Linear positive staining for VCAM-1 could also be seen under the capsule (C, capsule; W, white pulp; R, red pulp). (b) Mouse spleen, one day after phlebotomy and an injection of liposome-encapsulated Cl2MDP. The individual erythroblasts were circumscribed by the dendritic cytoplasm of the VCAM-1^{+} macrophages. (c) Mouse spleen, four days after phlebotomy and the injection of liposome-encapsulated Cl2MDP. VCAM-1 expression was limited only sinus endothelial and reticular cells. (d) Mouse spleen, 10 days after phlebotomy and the injection of liposome-encapsulated Cl2MDP. VCAM-1 was re-expressed in the macrophage associated with erythroblasts.
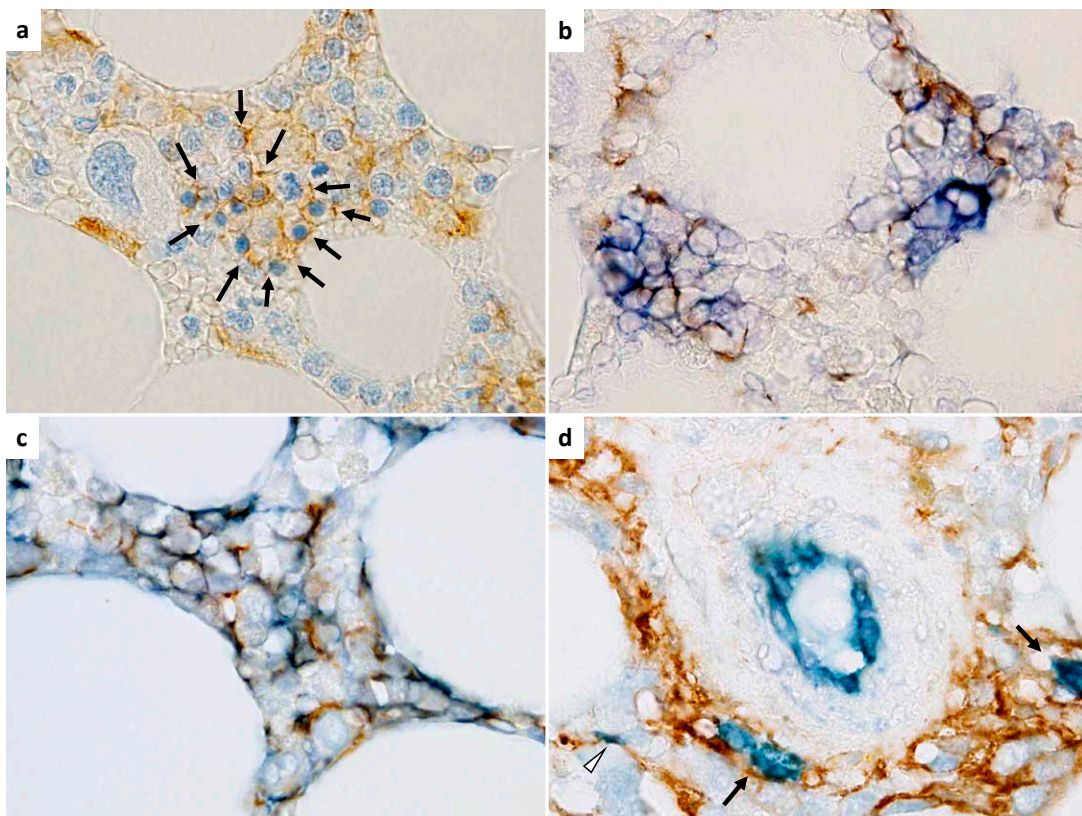
Fig. 2 Immunostaining of the human bone marrow control biopsy specimens. (a) CD169 was expressed in the cytoplasmic processes of resident macrophages. (b) CD169^{+} dendritic extensions of the macrophage (*brown*) appeared to delicately envelope CD71^{+} erythroid cells (*blue*). (c) The VCAM-1^{+} cells (*brown*) were positive for CD169 (*blue*). (d) The arrowheads indicates the sinusoidal endothelial cells that co-expressed VCAM-1 (*brown*) and CD34 (*blue*). The black arrows indicate CD34^{+} blastic cells.

macrophages closely associated with erythroid cells (Fig. 2a), which expressed CD71 (Fig. 2b) and integrin α4. VCAM-1 was strongly expressed on the CD169^{+} cells with dendritic cytoplasmic processes, which circumscribed erythroid cells (Fig. 2c). VCAM-1 was also faintly expressed on CD34^{+} sinusoidal endothelial cells (Fig. 2d), but not on CD146^{+} adventitial reticular cells. Cytoplasmic processes of VCAM-1^{+} macrophages were partly in contact with CD34^{+} blastic cells, in mainly near sinusoid (Fig. 2d).

Reactive erythroid hyperplasia

In the specimens from patients with reactive erythroid hyperplasia, including hemolytic anemia and megaloblastic anemia, the VCAM-1^{+}CD169^{+} macrophages increased in density, extending their cytoplasmic processes between erythroid cells (Fig. 3a). The VCAM-1 density in the reactive erythropoiesis specimens was significantly higher than for the control cases ($p < 0.001$; Fig. 3b). No EBIs were observed in pure red cell aplasia, and VCAM-1 expression was punctuate between the granulocytes (not shown).

Myelodysplastic syndrome and acute myeloid leukemia with ≥ 50% erythroid precursors of nucleated cells

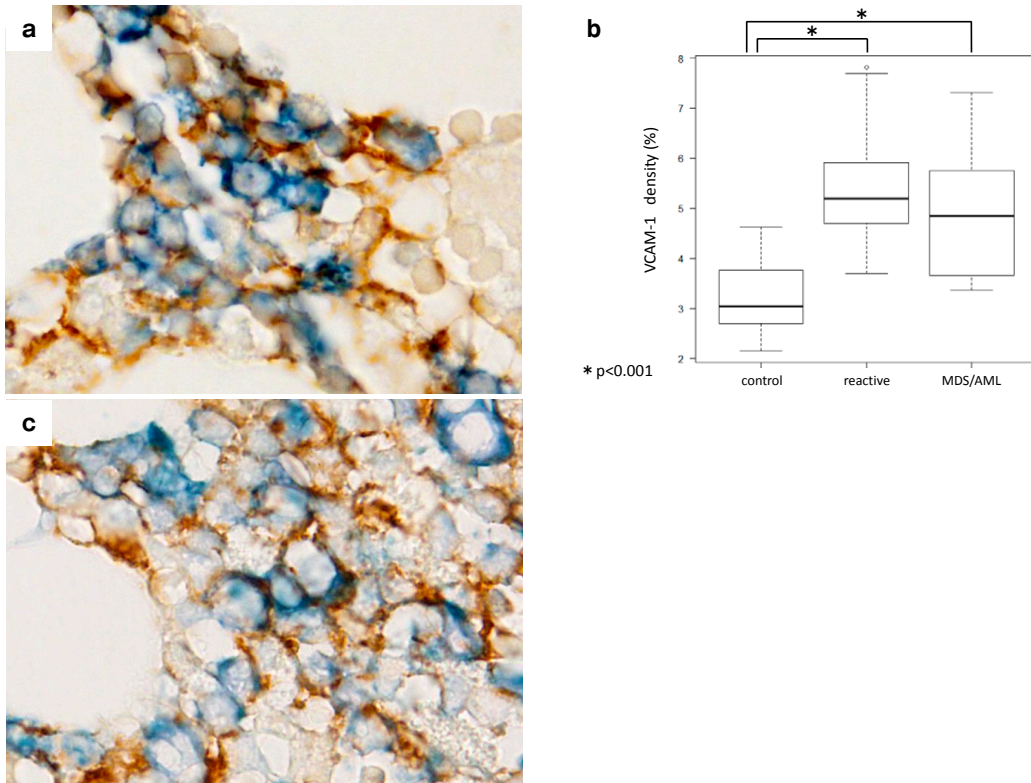Specimens from 10 patients with MDS, seven with acute erythroid leukemia, and one with AML

Fig. 3 (a) Double immunostaining of VCAM-1 and CD71 in the bone marrow of a patient with reactive erythroid hyperplasia (hemolytic anemia). The VCAM-1^{+} macrophages (*brown*) were closely associated with CD71^{+} erythroblasts (*blue*) and formed large erythroblastic islands. (b) Comparison of VCAM-1 expression density in the bone marrow specimens from the control samples (n = 21), patients with reactive erythroid hyperplasia (n = 16, 10 with hemolytic anemia and 6 with megaloblastic anemia) and patients with myelodysplastic syndrome/acute myeloid leukemia with multilineage dysplasia-related changes (n = 18, 10 with myelodysplastic syndrome and 8 with acute myeloid leukemia). The reactive erythroid hyperplasia and myelodysplastic syndrome/acute myeloid leukemia samples showed significantly higher VCAM-1 densities than the control samples (*p < 0.001, Mann−Whitney U test). (c) Double immunostaining of VCAM-1 (*brown*) and CD71 (*blue*) in the bone marrow of a patient with myelodysplastic syndrome, in which erythroid precursors comprised ≥50% of the bone marrow nucleated cells with overexpression of TP53. This revealed the impaired formation of erythroblastic islands and punctate cytoplasmic staining of VCAM-1 between the erythroblasts.

with multilineage dysplasia-related changes, with erythroid precursors comprising ≥50% of bone marrow nucleated cells, were stained for VCAM-1 and CD169. Erythroid cells were markedly increased in these specimens, showing clear megaloblastoid changes, a marked increase in the number of micromegakaryocytes, and a high degree of anemia. Punctate cytoplasmic staining of VCAM-1 was observed between dysplastic erythroblasts. Double immunostaining for CD71 and VCAM-1 revealed that this VCAM-1 staining

pattern represented the impaired formation of EBIs (Fig. 3c). CD169 immunostaining showed almost same pattern. The VCAM-1 density in these MDS cases was significantly higher than for the control cases (p < 0.001, Fig. 3b). However, there was no differences in VCAM-1 density between the reactive erythropoiesis and MDS cases (p = 0.252, Fig. 3b). In 15 cases, more than 20% of the hematopoietic cells were TP53^{+}, indicating the *TP53* mutation[29−33], but there were no significant differences in expression intensity or density of

Reactive erythropoiesis   Myelodysplastic syndrome
                                        (with impaired erythroblastic island formation)
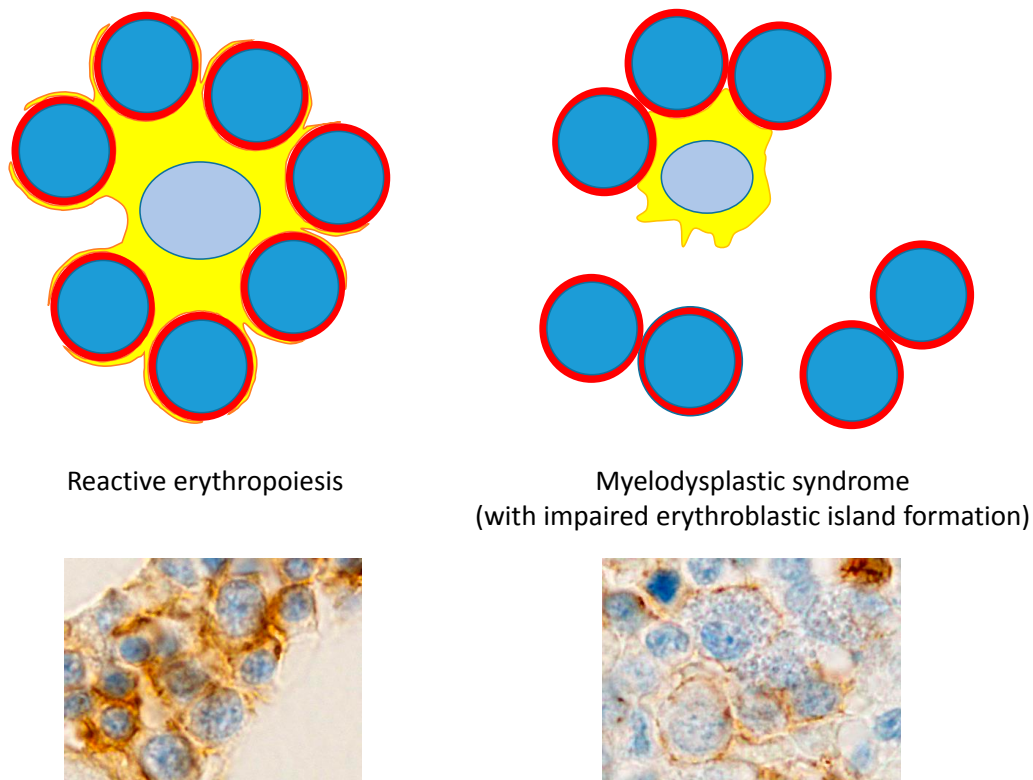

Fig. 4 Schema of erythroblastic islands of reactive erythroid hyperplasia and myelodysplastic syndrome with erythroid predominance with the overexpression of TP53.

VCAM-1 between these cases and those with less than 20% of TP53^{+} hematopoietic cells. Taken together, the findings showed that VCAM-1^{+} macrophages increased in number in MDS but the cytoplasmic processes of VCAM-1^{+} macrophages did not circumscribe the dysplastic erythroid cells. As a result, tight EBIs were not observed. The comparative schema of the immunostaining for VCAM-1 staining in cases with reactive erythropoiesis or MDS are shown in Fig. 4.

Polycythemia vera

Bone marrow samples from 20 patients with PV were examined. All the cases had the JAK2[V617F] mutation and morphologically showed panmyelosis[25]. VCAM-1 was strongly expressed on macrophages with dendritic cytoplasm closely associated with hematopoietic cells, particularly erythroblasts (Fig. 5a). The macrophage surrounded the entire circumference of erythroblasts, as with reactive erythropoiesis, and tight EBIs were observed (Fig. 5b). CD169 immunostaining showed same staining pattern as VCAM-1 immunostaining.

Essential thrombocythemia

An examination of the 26 specimens from patients with ET showed VCAM-1 was mainly expressed on the macrophages in EBIs (Fig. 5c). In addition, VCAM-1 expression was observed around megakaryocytes (not shown). Image analysis revealed significantly greater VCAM-1 expression in ET with the JAK2 mutation than in ET with the CALR mutation (p = 0.188; Fig. 6a). However, no significant correlations were found between the

VCAM-1 expression level and leukocyte count, platelet count, or erythrocyte count.

Chronic myelogenous leukemia

All 22 cases of CML showed granulocytic hyperplasia. The dendritic immunostaining of
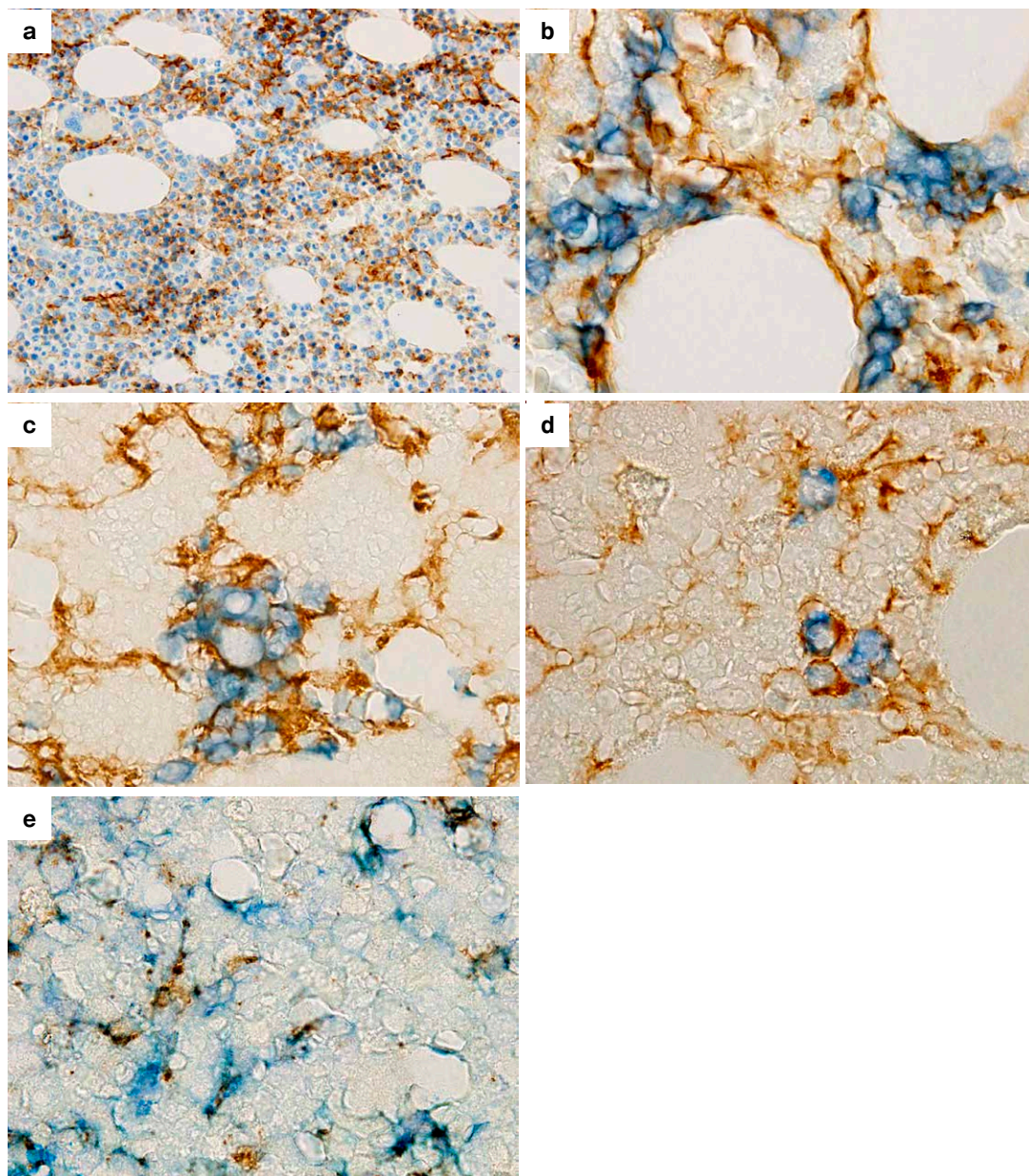

Fig. 5 Immunostaining of VCAM-1 (a) and double immunostaining of VCAM-1 (*brown*) and CD71 (*blue*) (b-e) in bone marrow specimens from the patients with myeloproliferative neoplasms. (a,b) In polycythemia vera, the VCAM-1^{+} macrophages were closely associated with CD71^{+} erythroblasts and formed erythroblastic islands. (c) In essential thrombocythemia, VCAM-1^{+} macrophages were also closely associated with CD71^{+} erythroblasts and formed erythroblastic islands (JAK2-positive case). (d) In chronic myelogenous leukemia (CML), the erythroblastic islands were smaller and less fewer in number. (e) In a CML patient, VCAM-1 (*brown*) was expressed on fine cytoplasmic extensions of macrophages at sites other than erythroblastic islands.

VCAM-1 was mainly observed on macrophages in EBIs (Fig. 5d); a punctate staining pattern was seen sparsely around granulocytic cells. Double immunostaining of VCAM-1 and CD163 or CD169 confirmed that the cells with the punctate expression of VCAM-1 around granulocytes were macrophages and not reticular cells (Fig. 5e).

Comparison of the myeloproliferative neoplasms

Next, we compared VCAM-1 density between the PV, ET, and CML specimens. Image analysis revealed that VCAM-1 density was significantly higher for PV than for ET and CML (chi-squared = 35.2, df = 2, p < 0.001, Fig. 6b). A significant correlation was observed between VCAM-1 density and peripheral blood erythrocyte count (correlation coefficient = 0.554, p < 0.001, Fig. 6c), but not between VCAM-1 density and the leukocyte or platelet count.

Myelofibrosis

In specimens from the five cases of post-polycythemic myelofibrosis, CD163^{+} macrophages exhibited spindle morphology in fibrotic areas, as previously observed using immunohistochemistry with the anti-CD68 mAb (PG-M1) (Fig. 7a)[27].
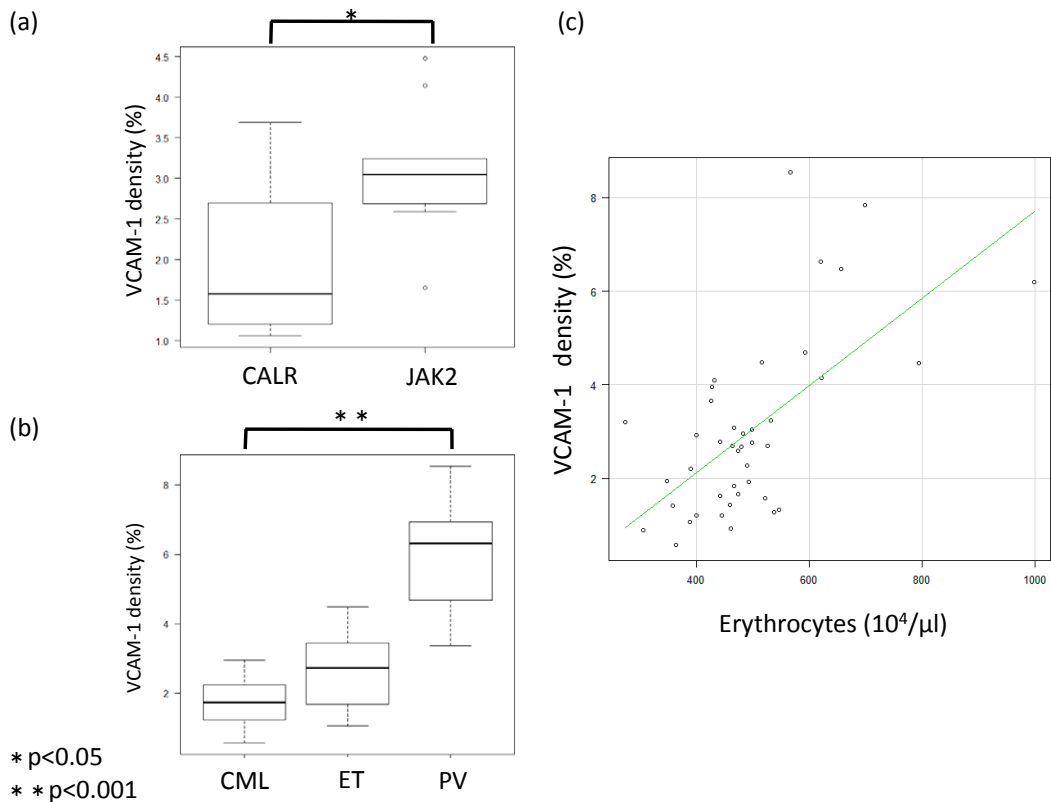

Fig. 6 (a) The VCAM-1 expression density in the bone marrow biopsy specimens of essential thrombocythemia (ET) patients with the JAK2 mutation (n = 9) was significantly higher than that for patients with the CALR mutation (n = 9) (*p < 0.05, Mann−Whitney U test). (b) The VCAM-1 expression density in the bone marrow biopsy specimens of patients with polycythemia vera (PV; n = 20) was higher than that for patients with ET (n = 26) or chronic myelogenous leukemia (CML; n = 22) (**chi-squared = 35.2, df = 2, p < 0.001, Kruskal−Wallis test). (c) The VCAM-1 expression density in the bone marrow specimens of patients with myeloproliferative disorders (PV, ET, and CML) correlated significantly with the erythrocyte count (correlation coefficient = 0.554, p < 0.001, Spearman's correlation test).
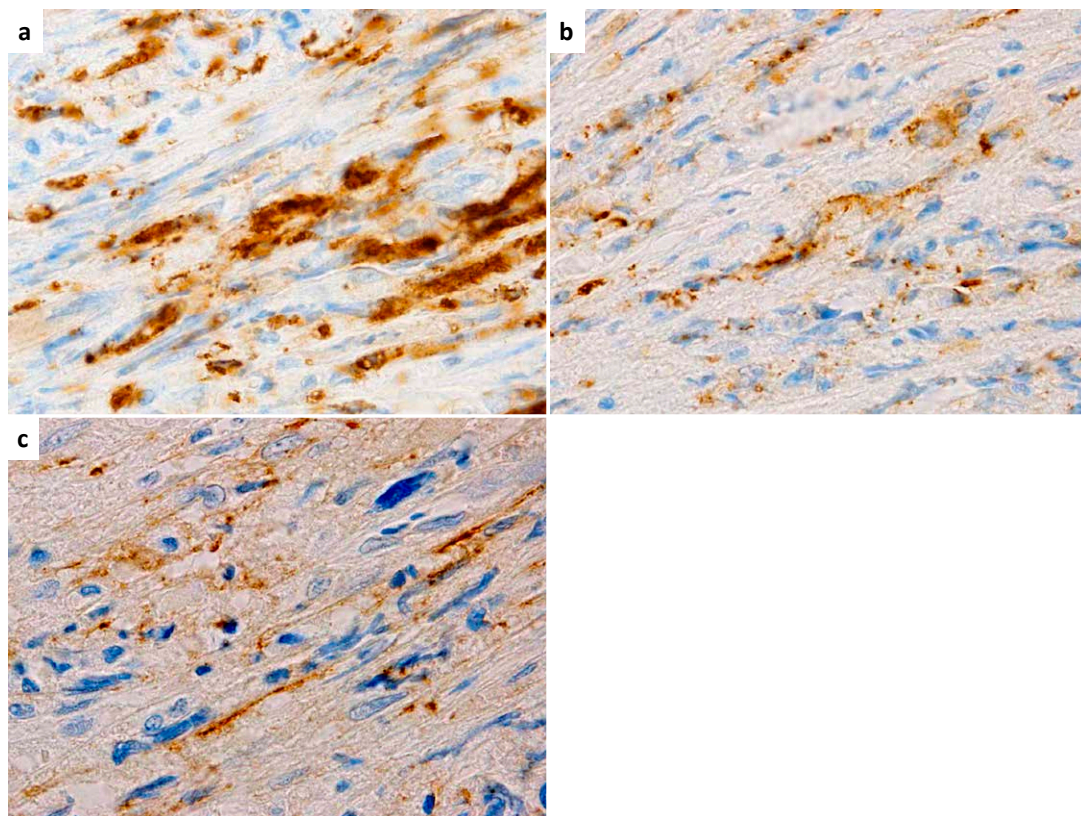
Fig. 7 Immunostaining of the bone marrow of patients with post-polycythemia vera myelofibrosis. (a) CD163^{+} macrophages elongated cytoplasmic processes along the array of collagen fibers (b,c). The CD169^{+} (b) and VCAM-1^{+} (c) cells showed a similar staining pattern.

CD169^{+} macrophages were reduced in number and were often associated with residual erythropoietic foci (Fig. 7b), but the macrophages exhibited spindle morphology in fibrotic areas, as seen with the CD163 immunostaining. VCAM-1 was also expressed on the spindle cells, not only on macrophages and endothelial cells, but also on fibroblastic stromal cells (Fig. 7c). The same tendency was seen in specimens from three cases of post-ET myelofibrosis and four cases of primary myelofibrosis.

DISCUSSION

The result of this study clearly showed, using the same antibody for VCAM-1 as used in the immunohistochemical study of the mice injected with liposome-encapsulated Cl2MDP, that the main human bone marrow VCAM-1^{+} cells are a subpopulation of CD163^{+} macrophages and that they express CD169. We also confirmed the association between VCAM-1^{+} macrophages and erythroblasts in reactive erythropoiesis induced by phlebotomy in mice, and hemolytic and megaloblastic anemia in humans. In addition, image analysis confirmed an increase in VCAM-1 density in reactive erythropoiesis in human bone marrow.

So-called tumor-associated macrophages have been shown to form a tumor microenvironment and affect tumor progression and metastasis[34, 35]; however, there have been few studies of bone marrow macrophages in hematopoietic neoplasms. We found that VCAM-1^{+} macrophages tended

to be more closely associated not only with reactive erythropoiesis but also with neoplastic erythropoiesis, than with granulopoiesis. The macrophages were mainly associated with erythroid cells, with their cytoplasmic circumscribing each erythroid cells. VCAM-1 density was higher in specimens from cases of PV than from cases of ET and CML. Furthermore, VCAM-1 expression density showed a strong correlation with peripheral blood cell count in PV, ET, and CML. These observations suggest that the erythropoiesis in PV remained under the control of VCAM-1^+ macrophages.

The following can be inferred about the role of macrophages in PV from previous studies of mice. First, VCAM-1^+ macrophages could help the proliferation of erythroblasts, and/or inhibit their apoptosis. Second, VCAM-1^+ macrophages efficiently perform phagocytosis of the pyrenocytes (nuclei surrounded by plasma membranes) excluded from erythroblasts[36]. One possible mechanism underlying the significant increase in VCAM-1^+ macrophages in erythropoietin-dependent reactive erythropoiesis and JAK2-mutated PV and ET is that JAK2/STAT5 signaling was activated simultaneously in both erythroblasts and macrophages. The regulatory mechanism for VCAM-1 expression in the macrophages that provide a niche for erythropoiesis warrants investigation.

A comparison of the cases with JAK2 and CALR mutations in ET showed a significantly higher density of VCAM-1^+ macrophages in those with the JAK2. VCAM-1^+CD169$^+$ macrophages were associated not only with erythropoietin-dependent reactive erythropoiesis, but also with neoplastic erythropoiesis caused by the mutation, suggesting that the kinetics of the VCAM-1^+CD169$^+$ macrophage subpopulation was regulated by some unknown factor involved in both reactive and neoplastic erythropoiesis.

A recent study suggested that the loss of bone marrow VCAM-1 expression in myelofibrosis caused a bone marrow homing defect of CD34$^+$ HSCs, although the cell type that expressed VCAM-1 was unclear[37]. In the present immunohistochemical study of myelofibrosis, VCAM-1^+ macrophages changed to a spindle morphology aligning to fiber's arrangement, regardless of whether the myelofibrosis was post-PV, post-ET, or primary. This morphological change may be associated with insufficient functioning of the macrophages and could result in peripheral erythroblastosis, probably through the loss of the tight adhesive interaction between macrophages and erythroblasts.

It has previously been shown that the number of CD68$^+$ bone marrow macrophages increased in MDS[33, 38, 39]. This increase may contribute to the elimination of apoptotic cells. In the present study, VCAM-1^+ macrophages were present in MDS with a dysplastic erythroid predominance, but each dysplastic erythroid cell was not well enveloped by the cytoplasm of the VCAM-1^+ macrophages. Another recent study reported that the impaired formation of EBIs of dysplastic erythroblasts have reduced expression of $\alpha 4$ integrin on dysplastic erythroblasts[40]. As a result, there may be no tight EBI formation, as is shown schematically in Fig. 4. It has also been shown that impaired formation of EBIs is associated with erythroid feature and poor prognosis in a significant proportion of patients with myelodysplastic syndromes[41]. Insufficient envelopment of erythroblasts by VCAM-1^+ macrophages may not only lead to a decrease in anti-apoptotic signaling by adhesion molecules, including VLA4/VCAM-1, but may also facilitate erythroblast−erythroblast interaction and induce apoptosis by Fas−Fas ligand signaling, because high expression of Fas and Fas ligands was observed in MDS[42, 43].

In summary, the present results showed that CD169$^+$VCAM-1^+ macrophages were closely associated with reactive and neoplastic erythropoiesis

in human diseases. The macrophages may promote late erythroid maturation, particularly in PV, which is characterized by extremely high erythrocytosis and is associated with the constitutively active JAK2[V617F] mutation. Thus, modulation of the macrophages by targeting VCAM-1 may be a new strategy to treat PV.

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DISCLOSURE STATEMENT

The authors declare no financial or commercial conflict of interest.

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