Junctional adhesion molecule-A, JAM-A, is a novel cell-surface marker for long-term repopulating hematopoietic stem cells

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Junctional adhesion molecule-A (JAM-A/JAM-1/F11R) is a cell adhesion molecule expressed in epithelial and endothelial cells, and also hematopoietic cells, such as leukocytes, platelets, and erythrocytes. Here, we show that JAM-A is expressed at a high level in the enriched hematopoietic stem cell (HSC) fraction; that is, CD34+/c-Kit+ cells in embryonic day 11.5 (E11.5) aorta-gonado-mesonephros (AGM) and E11.5 fetal liver (FL), as well as c-Kit+/Sca-1+/Lineage− (KSL) cells in E14.5 FL, E18.5FL, and adult bone marrow (BM). Although the percentage of JAM-A+ cells in those tissues decreases during development, the expression in the HSC fraction is maintained throughout life. Colony-forming assays reveal that multilineage colony-forming activity in JAM-A+ cells is higher than that in JAM-A− cells in the enriched HSC fraction in all of those tissues. Transplantation assays show that long-term reconstituting HSC (LTR-HSC) activity is exclusively in the JAM-A+ population and is highly enriched in the JAM-A+ cells sorted directly from whole BM cells by anti-JAM-A antibody alone. Together, these results indicate that JAM-A is expressed on hematopoietic precursors in various hematopoietic tissues and is an excellent marker to isolate LTR-HSCs.

Methods

Mice

C57BL/6(B6-Ly5.2) mice of 8 to 12 weeks of age were purchased from CLEA Japan (Tokyo, Japan). C57BL/6 mice congenic for the Ly5 locus (B6-Ly5.1) of 8 to 12 weeks of age were purchased from Sankyo-Lab Service (Tsukuba, Japan). Mice were kept under specific pathogen-free conditions in our animal facility. The day of appearance of vaginal plug was designated as day 0.5 of gestation. To obtain B6-F1 mice, B6-Ly5.1 male and B6-Ly5.2 female mice were mated. All experiments using mice received approval from the University of Tokyo Administrative Panel for Animal Care.

Isolation of cells and depletion of lineage-positive cells

AGM, FL, and BM cells were prepared as described previously.20-23 CD4, CD8, and B220, Ter119, Mac-1, and Gr-1 were used as lineage markers for BM cells. Since it was shown that Mac-1 is a hematopoietic stem-cell marker at the fetal stage, Mac-1 was excluded from the lineage markers in the FL cells.24 Depletion of lineage marker–positive cells was performed as described previously.25 In brief, FL and BM cells were incubated at 4°C for 30 minutes in a cocktail of HI219.19 (CD4), 53-6.7 (CD8a), RA-3-6B2 (B220), Ly-76 (Ter-119), and RB6-8C5 (Gr-1) antibodies (PharMingen, San Diego, CA). M1/70 (Mac-1) antibody was also added to BM cells.24 Cells (250 × 10^6 cells/mL) were incubated with M-450 sheep anti-rat IgG-conjugated immunomagnetic beads (Dynal, Oslo, Norway) at 4 beads per target cell according to the manufacturer’s recommendations. Beads were removed with a magnetic particle concentrator (MPC-1; Dynal), and unattached cells were collected.
FACS analysis and cell sorting

KSL cells were prepared by sorting Sca1\(^{-}\)c-Kit\(^{+}\) cells from lineage-depleted FL or BM cells as described above. CD34\(^{-}\)c-Kit\(^{+}\) cells in embryonic day 11.5 (E11.5) AGM and E11.5 FL were considered the enriched HSC fraction,\(^{26}\) and lineage depletion was not performed because of the low abundance of lineage-positive cells.

After staining with a monoclonal antibody according to the manufacturer’s protocol, cells were analyzed by fluorescence-activated cell sorting (FACS) with the FACScalibur system or sorted by the FACS-Vantage system (Becton Dickinson Biosciences, San Jose, CA) for further analysis. E13-161.7 (FITC-Sca-1), RAM34 (FITC-CD34), 2B8 (PE–c-Kit), and 7-ADD antibodies were obtained from BD Biosciences PharMingen (San Diego, CA); 9D1 (APC-CD150) and eBio244F4 (PE-CD48) were from eBioscience (San Diego, CA); HM48-1 (FITC-CD48) was from Abcam (Cambridge, MA); and streptavidin-allophycocyanin (SA-APC) was from Molecular Probes (Eugene, OR). The anti–JAM-A monoclonal antibody was generated by immunization of a rat with E14.5 fetal liver cells. Among the panel of rat monoclonal antibodies, we found one antibody that recognized megakaryocytes.\(^{27}\) We identified the antigen as JAM-A by expression cloning of cDNA, as described previously.\(^{27}\) The specificity of the antibody was confirmed by COS7 cells transfected with JAM-A cDNA by flow cytometry. The monoclonal antibody was biotinylated and was used for flow cytometry.

Colony formation assays

To evaluate hematopoietic potentials, sorted cells were cultured in semi-solid medium (MethoCult GF M3434; StemCell Technologies, Vancouver, BC) according to the manufacturer’s protocols, and colonies were scored after 10 to 14 days.

Histologic analysis

After sorting of JAM-A\(^{+}\) and JAM-A\(^{-}\) cells, cells were collected onto slides by Cytospin at 106g for 3 minutes (Cytospin 3; Thermo Shandon, Pittsburgh, PA). Cells were fixed in methanol for 2 minutes and stained with May-Grunwald Giemsa stains. The percentages of progenitors, mature myeloid (eg, neutrophil and monocyte), lymphoid, and erythroid/megakaryocyte lineage cells were evaluated by examining stained cells under light microscopy.

Transplantation

Test cells (25, 100, 300, 400, and 1000 cells) from a B6-Ly5.1 mouse were mixed with unfractionated adult BM cells (2 \times 10^6) from a B6-F1 mouse and intravenously injected into the retro-orbital plexus of etherized adult recipients (B6-Ly5.2) that had been lethally irradiated at 10 Gy from a \(^{137}\)Cs source. For transplantation assays, 8- to 12-week-old mice were used as test, competitor, and donor. Peripheral blood (PB) samples were collected from the recipients after transplantation. After lysis of erythrocytes, the remaining cells were stained with biotinylated anti-Ly5.2, FITC–anti-Ly5.1, a mixture of PE–anti-Mac-1 and PE–anti-Gr-1, a mixture of PE–anti-CD4 and PE–anti-CD8, or PE–anti-B220, and followed by addition of SA-APC, and were analyzed with FACScalibur. More than 1% of donor cell contribution in recipient PB were considered LTR-HSCs. Donor contribution was calculated as follows: The percentage of donor chimerism = \((100 \times \%\text{Ly-5.1 cells}/\%\text{Ly-5.1 cells} + \%\text{F1 cells})\).

CRU

The limiting dilution assay for competitive repopulating cells (CRUs) has been described previously.\(^{28}\) Cell number ranged from 25 to 300 cells in JAM-A\(^{+}\) KSL cells and from 25 to 400 cells in KSL cells. Hematopoietic repopulation was evaluated by taking PB at 8 weeks after transplantation. After lysis of erythrocytes, donor contribution was calculated. Donor contribution less than 1% was considered negative. The frequency of CRUs was calculated by applying Poisson statistics to the proportion of negative recipients at different dilution with using L-Calc software (StemCell Technologies).

Figures

Figure 1. Expression of JAM-A in AGM, FL, and adult BM. Expression of JAM-A in whole tissues (left) and enriched stem cell fractions (right) in E11.5 AGM (A), E11.5 FL (B), E14.5 FL (C), E18.5 FL (D), and adult (8-12 weeks) BM (E). Representative data from more than 3 independent experiments are shown. Numbers on the graphs are percentages of JAM-A\(^{+}\) cells in whole tissue and on stem cell fractions.

Statistics

Data are presented as the means plus or minus SD, unless otherwise stated. Statistical significance was assessed by means of a Student t test. A P value less than .05 was considered statistically significant.

Results

Expression of JAM-A in hematopoietic tissues

Using flow cytometry, we examined the expression of JAM-A in hematopoietic tissues during development and found that it was expressed in all of the hematopoietic tissues we examined. JAM-A was highly expressed in AGM and FL; however, the percentage of JAM-A\(^{+}\) cells was reduced dramatically in adult BM (Figure 1A-E). JAM-A was expressed not only on hematopoietic cells but also on nonhematopoietic cells in AGM and FL (eg, endothelial cells in AGM and hepatocytes in FL). Hematopoietic cells are a major cell population in BM, and the percentage of JAM-A\(^{+}\) cells is not significantly influenced by nonhematopoietic cells in BM. Furthermore, immature hematopoietic cells with JAM-A decreased along with development.

We then examined the expression of JAM-A in the enriched stem-cell fractions. It is known that HSCs are highly enriched in CD34\(^{-}\)c-Kit\(^{+}\) cells in early embryonic hematopoietic tissues, such as E11.5 AGM and FL,\(^{28}\) whereas HSCs are in KSL cells from FL after E14.5 and adult BM. Most of the CD34\(^{-}\)c-Kit\(^{+}\) cells in E11.5 AGM and E11.5 FL expressed JAM-A (Figure 1A,B), and most of the KSL cells in the E14.5 FL, E18.5 FL, and adult BM cells were also JAM-A\(^{+}\) (Figure 1C-E). These results indicate that a majority of the enriched stem-cell fractions express JAM-A.

CD34\(^{-}\)c-Kit\(^{+}\) and KSL cells are enriched in JAM-A\(^{+}\) cells

Next, we compared the abundance of HSCs in JAM-A\(^{+}\) and JAM-A\(^{-}\) cells in the AGM and FL. JAM-A\(^{+}\) and JAM-A\(^{-}\) cells were isolated from E11.5 AGM, E11.5 FL, E14.5 FL, and E18.5 FL cells using anti–JAM-A antibody, and the HSC fraction in each population was compared. CD34\(^{-}\)c-Kit\(^{+}\) cells were significantly
enriched in the JAM-A⁺ population of E11.5 AGM and FL as well as KSL cells in E14.5 FL and E18.5 FL (Figure 2A-D). These findings suggest that HSCs are highly enriched in JAM-A⁺ cells in all of those hematopoietic tissues.

Most of KSL cells in BM are JAM-A⁺

Next, we compared the fraction of KSL cells in JAM-A⁺ and JAM-A⁻ cells from BM. Compared with the fetal stage, KSL cells in the JAM-A⁻ cell population were dramatically reduced in the BM, regardless of age (ie, from a 1-week-old to a 36- to 40-week-old mouse; Figure 3A-C). Moreover, even without depletion of lineage marker-positive cells, the percentage of JAM-A⁺ in the c-Kit⁺Sca-1⁺ population of BM was much higher than JAM-A⁻ c-Kit⁺Sca-1⁺ cells (ie, 29.99% ± 8.72% vs 0.15% ± 0.08%; P < .001; Figure 3D). While it was shown that JAM-A is expressed in some leukocytes, platelets, and erythrocytes, JAM-A⁺ cells in adult BM were only 2.1%. Thus, JAM-A⁺ cells were enriched in KSL cells, suggesting that JAM-A is an excellent marker for HSCs.

May-Giemsa staining of JAM-A⁺ and JAM-A⁻ cells

JAM-A⁺ and JAM-A⁻ cells were sorted from the whole adult BM with anti–JAM-A antibody and were stained with May-Giemsa solution. Stained cells were classified into 4 phenotypically distinct groups: progenitors (more immature than myelocytes), mature myeloid, lymphoid, and erythroid/megakaryocyte lineage cells. Although both JAM-A⁺ and JAM-A⁻ cells were heterogeneous, the progenitor population in JAM-A⁺ cells was larger than that in JAM-A⁻ cells (27.75% ± 0.25% vs 1.50% ± 1.25%, respectively).
To evaluate the LTR-HSC potential of JAM-A⁺ cells, we examined LTR-HSC activity by transplantation of sorted cells into irradiated mice. JAM-A⁺ KSL, JAM-A⁻ KSL, KSL, and JAM-A⁻ cells were isolated from adult B6-Ly5.1 mice and were transplanted together with 2 × 10⁵ unfractionated B6-F1 BM cells into the B6-Ly5.2 recipients. Donor contribution in PB was evaluated by flow cytometry 20 weeks after transplantation. High levels of donor contribution (>1%) were found in 7 of the 9 and 4 of the 4 mice that received 100 and 300 JAM-A⁺ KSL, respectively (Table 1). In contrast, none of the mice receiving a transplantation of 1000 JAM-A⁻ KSL cells showed donor contribution (>1%) in PB (Table 1). Donor contribution was detected in 5 of the 6 mice receiving 300 KSL cells as a positive control (Table 1). Furthermore, donor contribution was detected in 4 of the 7 mice that received a transplantation of 100 JAM-A⁻ cells that were sorted from whole BM by JAM-A expression alone (Table 1). Consistent with the results of CFU assays, these results indicate that LTR-HSC activity in KSL cells is present in JAM-A⁺ cells but not in JAM-A⁻ cells, and that LTR-HSCs in BM are highly enriched in JAM-A⁺ cells.

**CRU frequency of JAM-A⁺ KSL and KSL cells**

To further examine the effectiveness of JAM-A antibody, we performed limiting dilution assays to determine the CRUs of JAM-A⁺ KSL cells and KSL cells. The frequency of CRUs was estimated by assessing donor contribution in PB of recipient mice 8 weeks after transplantation. The frequency of CRUs in the fraction of JAM-A⁺ KSL and KSL was 1 in 64 (95% confidence interval for mean, 1 in 45 to 1 in 92, n = 16) and 1 in 109 (95% confidence interval for mean, 1 in 77 to 1 in 154, n = 18), respectively (Table 2). Importantly, selection of JAM-A⁺ cells from KSL cells resulted in the increase of HSC frequency more than 1.7-fold. This finding is consistent with colony assay data, showing that more primitive KSL cells, including HSCs, can be enriched using anti-JAM-A antibody (Figure 5 and Table 2).

![Graph showing colony-forming activity of JAM-A⁺ and JAM-A⁻ cells in BM](https://example.com/graph.png)

**Figure 5. Colony-forming activity of JAM-A⁺ and JAM-A⁻ cells.** Colony-forming activity of sorted cells from fetal and adult tissues was examined. (A) CFU-Mix activity of 800 JAM-A⁺ cells versus 800 JAM-A⁻ cells in CD34⁺ c-Kit⁺ cells in E11.5 FL and KSL cells in E14.5, E16.5, and E18.5 FL. (B) CFU-mix activity of 100 JAM-A⁻ cells without lineage depletion versus 100 KSL cells (i). CFU-mix activity of 100 JAM-A⁺ cells versus 100 JAM-A⁻ cells in KSL cells in 8- to 12-week-old BM (ii). Results from more than 3 independent experiments are shown. Error bars indicate SEM; *P < .05, **P < .005, ***P < .0001, #P > .2.

**Table 1. JAM-A marks LTR-HSCs in BM**

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Cell no.</th>
<th>Engrafted mice</th>
<th>Total</th>
<th>Myeloid cells</th>
<th>T cells</th>
<th>B cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>KSL</td>
<td>300</td>
<td>5/6</td>
<td>16.7 ± 10.6</td>
<td>15.6 ± 17.2</td>
<td>32.1 ± 19.8</td>
<td>28.0 ± 17.0</td>
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<tr>
<td>KSL JAM-A⁺</td>
<td>100</td>
<td>0/4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KSL JAM-A⁻</td>
<td>300</td>
<td>0/6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KSL JAM-A⁺</td>
<td>1000</td>
<td>0/6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KSL JAM-A⁻</td>
<td>100</td>
<td>7/9</td>
<td>32.4 ± 26.4</td>
<td>29.7 ± 25.7</td>
<td>38.7 ± 25.3</td>
<td>24.6 ± 29.2</td>
</tr>
<tr>
<td>KSL JAM-A⁺</td>
<td>300</td>
<td>4/4</td>
<td>77.4 ± 29.6</td>
<td>82.3 ± 20.8</td>
<td>73.8 ± 31.5</td>
<td>83.4 ± 17.1</td>
</tr>
<tr>
<td>JAM-A⁺</td>
<td>100</td>
<td>4/7</td>
<td>15.4 ± 24.4</td>
<td>13.8 ± 21.9</td>
<td>10.4 ± 13.6</td>
<td>14.5 ± 24.4</td>
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</table>

Results of competitive hematopoietic stem-cell transplantation assay. Percentages of donor-derived cells of recipient mice were analyzed after 20 weeks of transplantation. Cell number indicates the test cells (B6-Ly5.1) that were transplanted with 2 × 10⁵ competitive adult BM cells (B6-F1).
Table 2. CRU of JAM-A+/KSL and KSL

<table>
<thead>
<tr>
<th>Cell dose injected</th>
<th>No. negative</th>
<th>No. analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAM-A+/KSL</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td>CRU frequency</td>
<td>1 in 64 (95% confidence interval: 1 in 45 to 1 in 92)</td>
<td></td>
</tr>
<tr>
<td>KSL</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>0</td>
</tr>
<tr>
<td>CRU frequency</td>
<td>1 in 109 (95% confidence interval: 1 in 77 to 1 in 154)</td>
<td></td>
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</tbody>
</table>

To determine the frequency of CRUs, recipient mice were analyzed at 8 weeks after transplantation. Negative mice were defined as animals with less than 1% of donor cell contribution in recipient PB. The frequency of CRUs was calculated by applying Poisson statistics with L-calc software.

Discussion

JAM proteins are expressed in various tissues in the human and murine body and are important for tight junction assembly, leukocyte transmigration, platelet activation, angiogenesis, and virus binding. It was reported recently that two of the JAM family members, JAM-B and JAM-C, are expressed in HSCs. Sakaguchi et al. showed that JAM-B is highly expressed in undifferentiated embryonic stem cells (ESCs), neural stem cells (NSCs), and HSCs, although it is not required for the maintenance of HSCs. JAM-A is expressed in a variety of hematopoietic cells, however, the expression of JAM-A in HSCs had not been known. In this paper, we show that JAM-A is highly expressed in fetal hematopoietic tissues, AGM, and FL. Immunohistochemical staining revealed that JAM-A is detected in vascular networks with PECAM-1 from E9.5 to E12.5. Consistently, we also found that JAM-A is an endothelial marker, because almost all PECAM-1+ and Flk-1+ cells in E11.5 AGM were JAM-A+ (>99%, data not shown). Because of the increase of cells that do not express JAM-A during development, apparent JAM-A expression in whole fetal hematopoietic tissues decreases accordingly. However, the enriched HSC fraction maintains JAM-A expression during development.

In adults, JAM-A has been shown to be expressed in various hematopoietic cells in human PB. We also found that although as much as 25% of mouse PB mononuclear cells expressed JAM-A (data not shown), only about 2% of BM cells expressed JAM-A (Figure 1E). The KSL cells in BM showed high-level expression of JAM-A (Figure 1E), and Sca-1+/c-Kit+ cells were more abundant in the JAM-A+ cells than JAM-A- cells in BM, regardless of age (Figure 3A-C). Compared with the fetal stage, the HSC fraction in JAM-A+ BM cells was much higher than that in JAM-A- cells throughout life. As only 2% of BM cells expressed JAM-A, the abundance of JAM-A+ cells in the enriched HSC fraction was quite high. In fact, c-Kit+Sca-1+ cells were enriched approximately 200-fold from whole BM cells by anti–JAM-A antibody alone; that is, the c-Kit+Sca-1+ cells in whole BM were only 0.15% (Figure 3D) and in JAM-A+ cells were 30% (Figure 3D). CFU-mix activity in the KSL population was almost exclusively in JAM-A+ cells from FL to BM (Figure 5Bii). Furthermore, CFU-mix activity of cells sorted by anti–JAM-A antibody alone was similar to that of KSL cells (Figure 5B), whereas CFU-GM and CFU-MegE were not different between JAM-A+ KSL and JAM-A- KSL cells (data not shown). Morphologic observation also supports that JAM-A+ cells contain more immature cells (Figure 4A).

These results also indicate that most of the immature progenitors in FL and BM are highly enriched in JAM-A+ cells. Finally, transplantation of JAM-A+ cells demonstrates their LTR-HSC activity. Most of the mice received only 100 JAM-A+ KSL cells showed long-term engraftment (Table 1). More interestingly, donor contribution was detected in 4 of the 7 mice transplanted with only 100 JAM-A+ cells that were sorted directly from whole BM by JAM-A expression alone (Table 1). Since the ratio of JAM-A+ cells to JAM-A- cells in the KSL fraction was about 6.5:3.5 (Figure 1E), 100 JAM-A+ KSL cells are equivalent to 285 total KSL cells. Consistently, most of the mice (5 of 6) receiving 300 KSL cells were engrafted for the long term. In contrast to JAM-A+ cells, 1000 JAM-A- KSL cells, which are equivalent to more than 2850 total KSL cells, failed to engraft for the long term. Taking these results and consideration together, HSCs in the KSL population are exclusively in JAM-A+ cells, and LTR-HSCs in BM can be highly enriched in JAM-A+ cells. The frequency of CRUs in the fraction of JAM-A+ KSL and KSL was 1 in 64 and in 1 in 109, respectively (Table 2). Importantly, after selection of JAM-A expression, the frequency of CRUs increased approximately 1.7-fold. These results indicate that JAM-A is a useful marker for further purification of KSL cells. It was previously shown that CD34+ KSL cells are a highly enriched HSC population, and we found that about 24% of CD34+ KSL cells expressed JAM-A (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article), suggesting that JAM-A+ CD34+ KSL may be the more purified HSCs. Yilmaz et al. showed that SLAM family receptors CD150 and CD48 are expressed in HSCs and that CD150+CD48+ CD244- KSL cells in BM are the most purified stem cells (1 in 2.1). They also showed that 21% of CD150+CD48+ CD244- cells engrafted for the long term in recipient mice. We found that about 25% of CD150+CD48- BM cells were JAM-A+CD244-. Therefore, selection of JAM-A+ cells from CD34- KSL or CD150+CD48-CD244- may enhance the purity of HSCs.

Several lines of evidence suggest that JAM-A interacts with leukocyte via LFA-1 and αvβ3 in transendothelial migration of leukocytes. Leukocytic LFA-1 is a receptor for JAM-A and is involved in transendothelial migration of leukocytes. JAM-A is known to play a role in platelet activation and aggregation. JAM-A-/- polymorphonuclear leukocytes (PMNs) were shown to adhere more strongly and transmigrate less efficiently through endothelial monolayers, suggesting that the absence of JAM-A impairs PMN movement. JAM-A–deficient mice also revealed that JAM-A expression prevents spontaneous and random motility. By contrast, a role for JAM-A in hematopoiesis has not been elucidated. Considering the role of JAM-A in the adhesion of blood cells to endothelial cells, an interesting possibility may be that JAM-A is involved in the interaction between HSCs and their niche. In conclusion, the results in this paper show that JAM-A is an excellent marker for HSCs in BM and can be used to prepare a highly enriched HSC population by using anti–JAM-A antibody.
References


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Junctional adhesion molecule-A, JAM-A, is a novel cell-surface marker for long-term repopulating hematopoietic stem cells

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