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The Endothelial Antigen ESAM Monitors Hematopoietic Stem Cell Status between Quiescence and Self-Renewal

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Whereas most hematopoietic stem cells (HSC) are quiescent in homeostasis, they actively proliferate in response to bone marrow (BM) injury. Signals from the BM microenvironment are thought to promote entry of HSC into the cell cycle. However, it has been cumbersome to assess cycle status of viable HSC and thus explore unique features associated with division. In this study, we show that expression of endothelial cell-selective adhesion molecule (ESAM) can be a powerful indicator of HSC activation. ESAM levels clearly mirrored the shift of HSC between quiescence and activation, and it was prominent in comparison with other HSC-related Ags. ESAMhi HSC were actively dividing, but had surprisingly high long-term reconstituting capacity. Immunohistochemical analyses showed that most ESAMhi HSC were located near vascular endothelium in the BM after 5-fluorouracil treatment. To determine the importance of ESAM in the process of BM recovery, ESAM knockout mice were treated with 5-fluorouracil and their hematopoietic reconstruction was examined. The ESAM deficiency caused severe and prolonged BM suppression, suggesting that ESAM is functionally indispensable for HSC to re-establish homeostatic hematopoiesis. With respect to intracellular regulators, NF-kB and topoisomerase II levels correlated with the ESAM upregulation. Thus, our data demonstrate that the intensity of ESAM expression is useful to trace activated HSC and to understand molecular events involved in stem cell states. The Journal of Immunology, 2012, 189: 000–000.

Hematopoietic stem cells (HSC) are characterized as being extensively self-renewing as well as multipotent. Distinction of HSC from differentiating cells is essential for understanding the essence of “stemness.” Many groups have identified HSC-related Ags, and those markers have made it possible to sort long-term reconstituting HSC (LT-HSC) with high purity. For example, at least one in three lineage (Lin)-c-KithiSca-1CD34-Flk2/Flt3-CD150CD48 fraction cells in adult mouse bone marrow (BM) can be transplanted (1–3). Recent studies using a BrdU-retaining method and/or a histone 2B-GFP transgene have shown that the long-term reconstituting activity of the adult mouse BM is sustained mostly in very quiescent HSC that divide only five to six times during the adult period (4, 5).

However, even the highly purified LT-HSC fraction is heterogeneous with respect to cell cycle status (4, 5).

The cell cycle status and differentiating behavior of HSC are known to fluctuate according to physiological circumstances. During fetal and early postnatal periods, development of the hematopoietic system is essential in supporting the rapid growth of organisms and the explosive expansion of all blood lineages. Indeed, numbers of HSC increase ~40-fold in the fetal liver between embryonic days 12 and 16 (6). Alternatively, upon reaching adulthood, HSC become quiescent and evade exhaustion or mutation to maintain hematopoiesis throughout life (7). Although the quiescent HSC divide at an extremely low rate during homeostasis, they are rapidly activated to proliferate in response to BM injury or by G-CSF stimulation (5). Interestingly, after re-establishment of homeostasis, the activated HSC can return to quiescence. Distinguishing LT-HSC from differentiating progenitors becomes complicated by BM injury because the expression pattern of HSC-related Ags is dramatically influenced (8). Molarcrosstalk between HSC and the BM microenvironment, also known as “HSC niche,” is likely to control the balance of HSC quiescence and activity (9, 10), but precise mechanisms regulating HSC status remain largely unknown. If we could selectively isolate active HSC with a set of surface markers, that should yield significant insights regarding HSC biology and HSC applications for clinical purposes. Furthermore, information about cell surface Ags that mirror HSC states would be invaluable for understanding the relationship between HSC and their niches.

Endothelial cell-selective adhesion molecule (ESAM), which is an Ig superfamily protein, was originally identified as an endothelial specific molecule mediating cell–cell adhesion through homophilic interactions (11, 12). ESAM proteins colocalize with cadherins and catenins in cell–cell junctions of vascular endothelium. ESAM deficiency in endothelial tight junctions disturbs neutrophil extravasation to inflamed tissues by reduction of acti-

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Abbreviations used in this article: BM, bone marrow; E, embryonic day; ESAM, endothelial cell-selective adhesion molecule; 5-FU, 5-fluorouracil; HSC, hematopoietic stem cell; KO, knockout; Lin, lineage; LSK, Lin Sca1/c-Kithi; LT-HSC, long-term reconstituting hematopoietic stem cell; ROS, reactive oxygen species; RU, repopulating unit; SAv, streptavidin; TR, Texas Red; WT, wild-type.

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ESAM LEVEL DISTINGUISHES HSC STATUS

Materials and Methods

Mice

Wild-type (WT) C57BL/6, BALB/c, and FVB mice were obtained from CLEA Japan (Shizuoka, Japan). The congenic C57BL/6 strain (C57BL/6SJL; CD45.1 allotype) was purchased from The Jackson Laboratory (Bar Harbor, ME) and used for transplantation experiments. ESAM KO mice were developed by Dr. T. Ishida (Kobe University, Kobe, Japan) as previously reported (18). Mating of heterozygous male and female mice was routinely performed to generate homozygous ESAM KO mice. Three types of PCR primers were used to genotype ESAM KO mice as documented previously (19). All mice used in this article were 8–12 wk old. Animal studies were performed with the approval of the Institutional Review Board of Osaka University.

Abs and reagents

5-Fluouracil (5-FU) was purchased from Kyowa-Hakko Kirin (Tokyo, Japan). Purified anti-Ly6G and Ly6C/Gr1 (RB6-8C5) mAb, PE-conjugated anti-Ly6G (1A8), and CD45.1 (HM46-1) mAb, FITC-conjugated and allophycocyanin-conjugated anti-CD11b/Mac1 (M1/70), Ly6G and Ly6C/Gr1 (RB6-8C5), CD45R/B22 (RA3-6B2), Ter119, CD3e (145-2C11), and CD8a (53-6.7) mAbs, allophycocyanin-conjugated anti-CD117c/Kit (2B8) mAb, PE-Cy7-conjugated anti-Scal (Ly6a/E; D7) mAb, biotinylated anti-Scal (E13-161.7) mAb, PerCP-Cy5.5-conjugated anti-CD45.2 (104) mAb, and Alexa Fluor 647-conjugated anti-CD19 (17A2), Mac1 (M1/70), and Ter119 mAbs, and PE-conjugated and allophycocyanin-conjugated anti-CD45R/B22, FITC-conjugated and allophycocyanin-conjugated anti-CD11b/Mac1, Ly6G and Ly6C/Gr1, PerCP-Cy5.5-anti-CD45R/B22, PE-Cy7-anti-Sca1, and allophycocyanin-anti–c-Kit (2B8) mAbs were purchased from BD Pharmingen (San Diego, CA). Purified anti-CD3 (17A2), Mac1 (M1/70), and Ter119 mAbs, and PE-conjugated anti-CD105/endoglin (M7/18), CD31/PECAM-1 (390), Tie2 (TEK4), and CD135/Fn3 (A2F10) mAbs were purchased from eBioscience (San Diego, CA). PE-conjugated and allophycocyanin-conjugated anti-CD150 (TC15-12F12.2) mAbs and PerCP-Cy5.5-conjugated anti-CD117c/Kit (2B8) mAb were purchased from BioLegend (San Diego, CA). Cy3-conjugated goat anti-rat IgG Ab was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Streptavidin-Alexa Fluor 647 was purchased from Invitrogen (Carlsbad, CA). FITC-conjugated Ki67 (M-19) mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A rat anti-mouse ESAM (1G8) mAb, which was originally developed and supplied by Drs. S. Butz and D. Vestweber (Max Planck Institute, Munster, Germany), was purchased from BioLegend. The Ab was biotinylated in our hands using Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Rockford, IL). Bortezomib (Velcade) was purchased from Janssen (Tokyo, Japan). ICRF-193 was purchased from Funakoshi (Tokyo, Japan). TaqMan FAM dye-labeled MGB probe sets for ESAM and GAPDH were purchased from Applied Biosystems (Foster City, CA).

Flow cytometry

Cells were obtained from adult mouse tissues indicated in each experiment and first incubated with a rat anti-mouse FcRIII/II Ab (2.4G2) to block nonspecific Ab binding via FcR. Then, the cells were stained with the indicated Abs. Lin Abs contain anti-Mac1, Gr1, CD3e, CD45R/B220, and Ter119 Abs for analysis of untreated control mice, and Gr1, CD3e, CD45R/B220, and Ter119 for 5-FU-treated mice, as the level of Mac1 on HSC resurfaces after a 5-FU treatment. A biotinylated anti-ESAM Ab was developed with SAv-PE or SAv-PE-TR. Flow cytometry analyses were performed with FACSVerse or FACSCanto (BD Biosciences). The data analyses were done with FlowJo software (Tree Star, San Carlos, CA).

Cell cycle analyses

Mice were given a single 5-FU injection and analyzed. BrdU was i.p. administered 12 h before analyses. BM cells were stained with biotinylated anti-ESAM, PE-Cy7-anti-Sca1, and allophycocyanin-anti–c-Kit Abs, followed by SAv-PE. The stained cells were then fixed, permeabilized, and incubated with DNase to expose incorporated BrdU by using a BrdU flow kit (BD Pharmingen). Subsequently, the cells were stained with FITC-anti-BrdU Ab for 30 min at room temperature and resuspended with staining medium containing 7- aminoactinomycin D. For another staining set, the cells were incubated with 2 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) and FITC-anti-Ki67 Ab for 30 min at room temperature. Cell cycle statuses were analyzed by FACSVerse (BD Biosciences).

Cell isolation

BM cells obtained from femora and tibiae of adult mice were first incubated with purified anti-Lin Abs, followed by goat anti-rat IgG microbeads (Miltenyi Biotec). After Lin–cell depletion, the Lin–enriched cells were stained with FITC-anti-Lin Abs in combination with PE-Cy7-anti-Sca1, allophycocyanin-anti–c-Kit, and biotinylated anti-ESAM Abs. Subsequent to staining with SAv-PE, ESAM+ and ESAM− LSK cells were sorted with FACSAria (BD Biosciences). The purity of the sorted cells was routinely confirmed by using a part of each sorted population to be >97%.

Methylcellulose cultures

C57BL/6 mice were treated with single i.v. 5-FU (150 mg/kg). Five days after treatment, mice were killed and ESAM+ or ESAM− LSK cells of BM were sorted and subjected to methylcellulose colony formation assays. Two hundred cells of each sorted fraction were cultured in IMDM-based methylcellulose medium supplemented with 50 ng/ml recombinant murine stem cell factor, 10 ng/ml recombinant murine IL-3, 10 ng/ml recombinant human IL-6, and 3 U/ml recombinant human erythropoietin (Methocult F 3434; StemCell Technologies, Vancouver, BC, Canada). After 9–10 d, colonies were enumerated and classified as CFU-GM, CFU-M, BFU-E, or CFU-Mix according to shape and color under an inverted microscope.

Competitive repopulation assay

Ly5 congenic mice were used for competitive repopulation assays. Two thousand ESAM+ LSK or ESAM− LSK cells from C57BL/6-Ly5.1 (CD45.1) mice were mixed with 2 × 106 unfractionated adult BM cells obtained from WT C57BL/6-Ly5.2 (CD45.2) mice and then injected into C57BL/6-Ly5.2 mice irradiated at a dose of 8.5 Gy. Peripheral blood analyses were performed at 4-wk intervals after transplantation. Sixteen weeks after transplantation, all recipients were killed and BM cells were collected. BM cells were stained with FITC-anti-Mac1, PE-anti-CD3e, Alexa Fluor 647-anti-Cy7, PerCP-Cy5.5-anti-CD45.2, and PE-Cy7-anti-CD45.1 Abs to analyze the donor-derived chimerism and each lineage differentiation, and they were simultaneously stained with FITC-anti-Lin, PE-anti-CD150, PerCP-Cy5.5-anti-CD45.2, PE-Cy7-anti-Sca1, and allophycocyanin-anti–c-Kit, and biotinylated anti-ESAM Abs, followed by SAv-PE-TR to evaluate ESAM level of LSK fraction. The repopulating unit (RU) was calculated as follows: RU = (% donor-derived cells) × (number of competitor cells/10^7) / (% competitor-derived cells) (20). The total number of RU per BM was obtained by multiplying the number of RU per 2,000 test cells by the number of ESAM+ LSK or ESAM− LSK cells per BM (two femora and two tibiae) divided by 2000. For the second transplantation, two hundred CD45.1+ LSK cells sorted from primary recipient mice were mixed with 1 × 10^5 unfractionated BM cells obtained from Applied Biosystems (Foster City, CA).
from WT C57BL/6-Ly5.2 (CD45.2) mice and were transplanted into irradiated C57BL/6-Ly5.2 mice. Sixteen weeks after transplantation, the contribution of CD45.1 cells to the hematopoietic reconstitution was evaluated.

**Immunohistochemical analyses**

Immunohistochemical staining was performed as described previously (21). In brief, bone samples were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose/PBS. Fixed samples were embedded in OCT medium (Sakura Finetek, Tokyo, Japan) and frozen in cooled hexane. Sections of uncalcified femoral bone were generated via Kawamoto’s film method (Cryofilm transfer kit; Leica Microsystems). The 8-μm-thick cryostat sections were first blocked with 5% FCS/PBS and then stained with mAbs. The following Abs were used for immunostaining: FITC-conjugated mAbs against Gr1, B220, CD3e, CD8, and Ter119; biotinylated mAb against ScA1; and purified mAb against ESAM. For secondary Ab, Cy3-conjugated donkey anti-rat IgG Ab was used. Biotinylated Ab was visualized with SA-Alexa Fluor 647. The nuclei of cells were labeled with DAPI (Dojindo, Kumamoto, Japan). The sections were mounted with PermaFluor (Thermo Fisher Scientific), and confocal microscopic analyses were performed with an LSM 510 META (Carl Zeiss, Oberkochen, Germany). Image analyses were performed using an LSM image browser (Carl Zeiss).

**Luciferase assays**

Promoter sequences of ESAM gene were searched with Genetyx version 9 (Genetex, Tokyo, Japan). Segments of ESAM genes were amplified by PCR and inserted in pGL3 basic vector (Promega, Madison, WI). Luciferase assays were performed using the endothelial bEnd3 cell line. The cells were seeded in 3.5-cm dishes, and 24 h later each pGL3-promoter construct and pRL-CMV encoding the Renilla luciferase gene were transfected into the cells by Lipofectamine. Each culture medium was changed 24 h after transfection. Luciferase assays with use of a luminometer were carried out 48 h after transfection.

**Statistical analyses**

Statistical analyses of chimerism status were carried out with Mann–Whitney U tests, and other analyses were conducted with standard Student t tests. Error bars used throughout indicate SD of the mean.

**Results**

**BM injury upregulates ESAM expression on HSC**

We have reported that ESAM is a durable marker for fetal and adult HSC (16). Levels decline in early postnatal life and become high again with age (16). Several Ags, including Mac1 and CD34, are known to be downregulated when fetal HSC switch to quiescent adult ones, but they emerge again on HSC as activation-related changes after myelosuppression (8, 17). We monitored ESAM expression levels in HSC-enriched fraction of adult C57BL/6 BM after a single 5-FU treatment (150 mg/kg) by flow cytometry (Fig. 1A). As shown in a previous report (8), 5-FU injection caused significant downregulation of c-Kit expression on Lin− cells (Supplemental Fig. 1). In the same report, HSC could be enriched in the Lin− Sca1+c-Kitdull fraction after 5-FU treatment (8). Therefore, in our experiments, we applied an enlarged LSK gate covering the c-Kitdull HSC.

We observed remarkable increases in ESAM levels in HSC-enriched fractions from days 2–9 after a 5-FU injection (Fig. 1A). Indeed, the mean fluorescence intensity of ESAM expression on LSK increased by 11-fold in 5 d after a single 5-FU injection, compared with untreated control mice. More than 70% of LSK cells on day 5 expressed high amounts of ESAM (fluorescence intensities >10-fold the maximum level of isotype controls). After reaching peak values around days 5–6, ESAM levels gradually decreased and returned to steady-state levels by day 12.

ESAMhi cells formed a dominant population in the LSK fraction and logarithmically increased from day 3 to 9 in parallel with hematopoietic recovery (Fig. 1B). Quantitative real-time PCR showed gradual increases of ESAM transcripts in the LSK fraction after 5-FU treatment (Fig. 1C), suggesting that something associated with BM injury activates ESAM expression at the gene transcription level. Sublethal total body irradiation caused essentially the same change on the HSC fraction (data not shown). We evaluated ESAM levels of BM HSC fractions in BALB/c and FVB strains other than C57BL/6 mice. Expression of ESAM was also upregulated after 5-FU treatment in the CD150+Lin−c-KitdFlt3− fraction (Fig. 1D). These results suggested that BM injury upregulates ESAM levels on HSC.

**Upregulation of ESAM on HSC after BM injury in comparison with other endothelial markers**

Recent studies have shown that the gate of CD150+CD48− is useful to enrich for LT-HSC activity in various contexts, from aged BM, mobilized splenocytes, or reconstituted mouse BM (1, 22). Therefore, we evaluated whether ESAM expression patterns were shared with those of SLAM family markers on adult C57BL/6 BM LSK cells before and after 5-FU injection. In the homeostatic BM LSK, CD150+CD48− cells were only detectable among the ESAMhi population and the percentage was higher in ESAMhi than ESAMlo (Fig. 2A, left panels). After 5-FU injection, more than half of LSK cells were found in the CD150+ fraction, whose levels of ESAM were clearly upregulated. Additionally, percentages of the population that were CD150+CD48− were also higher in ESAMhi than ESAMlo categories (Fig. 2A, right panels). With respect to mobilized LSK cells, we also detected increases of ESAMhiCD150− cells in spleens and peripheral blood after day 7 from a 5-FU injection (Supplemental Fig. 2).

HSC are known to share various surface Ags with endothelial cells. We next evaluated how ESAM expression correlates with other endothelial-related HSC markers. The ESAMhi LSK population in the homeostatic BM was found in CD34−, Tie2hi, endoglin−, and CD31/PECAM-1hi, which is consistent with the phenotype of adult BM LT-HSC (Fig. 2B). After 5-FU injection, the expression patterns of those markers also changed. Whereas CD34 and Tie2 showed modest increases, endoglin as well as ESAM were clearly upregulated. The CD31/PECAM-1 levels remained unchanged. These results indicate that patterns of several endothelial markers on HSC after BM injury are not homogeneous. Additionally, among the markers, ESAM appeared to be uniquely valuable for monitoring HSC activation.

**ESAMhi HSC are actively dividing**

Previous studies have proposed that BM HSC in homeostasis are quiescent, a characteristic assumed to protect them from anti-metabolites such as 5-FU (4, 5, 23, 24). However, upon their activation by BM injury, it has been hypothesized that HSC move out of their homeostatic niche to proliferate and differentiate (25). To confirm that the ESAMhi HSC in 5-FU–treated mice are actively proliferating, we conducted cell cycle analyses. Although most ESAMhi and ESAMlo Sca1+c-Kit+ cells after a 5-FU injection left the G0 stage and entered into G1 and S+G2+M, the ESAMhi cells showed even higher percentages entering cell cycle than did the ESAMlo subset (Fig. 3, upper panels). Short-term exposure to BrdU marked more cycling cells in the ESAMhi fraction than in the ESAMlo cohort (Fig. 3, lower panels). CD150+ESAMhi LSK cells also showed higher percentages entering cell cycle than did the CD150+ESAMlo LSK cells (Supplemental Fig. 3A). Based on the fact that total stem cell activity per liver from day 12 to 16 of gestation is higher than that after day 16 (6), we compared ESAM levels in HSC fractions between embryonic day (E)14.5 and E18.5 fetal liver. ESAM levels at E14.5 were 1.9-fold higher than at E18.5 (Supplemental Fig. 3B). These results suggest that expression of ESAM is associated with rapid division before and after birth.
ESAMhi HSC have enhanced repopulating capacity

Next, we performed functional assessments of the ESAMlo and ESAMhi LSK fractions sorted from 5-FU–treated BM. In methylcellulose cultures, both fractions showed high colony-forming activities. However, whereas the ESAMlo fraction mainly contained committed progenitors, primitive multipotent progenitors, CFU-Mix, were significantly enriched in the ESAMhi fraction (Fig. 4). Additionally, those CFU-Mix cells formed high proliferative potential colonies (data not shown).

To analyze long-term reconstitution capacities in vivo, we transplanted 2000 CD45.1+ ESAMlo or ESAM hi LSK cells sorted from 5-FU–treated mice, with $2 \times 10^5$ CD45.2+ competitor BM cells derived from untreated mice, into lethally irradiated CD45.2+ mice (Fig. 5A). Peripheral blood analyses were performed every 4 wk after transplantation, and at any time point, the mice transplanted with ESAM hi LSK cells showed >3-fold higher contributions of CD45.1+ cells to peripheral leukocytes than did the mice transplanted with ESAMlo LSK cells (data not shown). Sixteen weeks after transplantation, all mice were killed and the contribution of donor type cells in BM was evaluated. The mice with ESAMlo LSK cells showed higher chimerism in the myeloid lineage, whereas ESAMhi LSK cells tended to reconstitute the B lineage more than did the myeloid lineage (Fig. 5D). This observation seemed to be consistent with previous reports showing that long-term reconstituting HSC predominantly contributed to the myeloid lineage (26). It is noteworthy that ESAM levels on CD45.1+ donor-derived LSK cells were identical to the homeostatic level 16 wk after transplantation in the BM that was transplanted with CD45.1+ ESAMhi LSK cells (Fig. 5E). The CD45.1+ LSK cells in the primary recipients serially reconstituted hematopoiesis in secondary CD45.2+ recipients (data not shown). These results suggested that LT-HSC are enriched in the ESAM hi fraction of LSK cells after 5-FU injection.

Most ESAMhi HSC are located around perivascular areas in 5-FU–treated BM

Next, immunohistochemical analyses were conducted to locate the ESAMhi HSC in 5-FU–treated BM. Without treatment, ESAM+ HSC were not easily distinguished because the ESAM levels were not high enough for this type of assessment (Fig. 6A, left panels). However, a single 5-FU treatment significantly increased ESAM expression in the HSC-enriched fraction, so that we could discriminate ESAMhi Lin− Sca1+ HSC from areas with background staining (Fig. 6A). The 5-FU treatment remarkably reduced Lin− cells and enlarged sinusoidal vasculature spaces in BM, which favored our ability to locate the activated HSC (Fig. 6A, right panels). Additionally, recipient BM transplanted with ESAMhi LSK cells showed higher chimerism in the myeloid lineage, whereas ESAMlo LSK cells tended to reconstitute the B lineage more than did the myeloid lineage (Fig. 5D). This observation seemed to be consistent with previous reports showing that long-term reconstituting HSC predominantly contributed to the myeloid lineage (26). It is noteworthy that ESAM levels on CD45.1+ donor-derived LSK cells were identical to the homeostatic level 16 wk after transplantation in the BM that was transplanted with CD45.1+ ESAMhi LSK cells (Fig. 5E). The CD45.1+ LSK cells in the primary recipients serially reconstituted hematopoiesis in secondary CD45.2+ recipients (data not shown). These results suggested that LT-HSC are enriched in the ESAM hi fraction of LSK cells after 5-FU injection.

**FIGURE 1.** ESAM expression is upregulated on LSK cells after 5-FU treatment. (A) Flow cytometry analyses were performed with respect to the ESAM expression on murine BM LSK fraction through a single 5-FU (150 mg/kg) treatment. Each panel shows a representative histogram of ESAM level on LSK at days 0 (no treatment control), 1, 3, 5, 9, and 12 after a 5-FU injection. The dashed lines show background levels with an isotype control Ab. The tinted lines show ESAM levels of LSK at the indicated day after a 5-FU injection. The ESAM level of day 0 is added to each panel as an open histogram with a solid line. Upper and lower numbers in each histogram indicate the percentages of ESAM+ and ESAM hi cells, respectively. (B) The numbers of total BM mononuclear cells (MNC), LSK cells, LSK ESAM+ cells, and LSK ESAM hi cells from a pair of femora and tibiae were monitored after a 5-FU injection. Each point summarizes data from five mice. (C) Quantitative real-time PCR analyses for ESAM gene expression of LSK cells after 5-FU treatment were performed. Each bar indicates relative expression level against the expression level of day 0 (ESAM/GAPDH). (D) ESAM expression levels on the BM CD150+Lin− c-Kir−Flt3− fraction of BALB/c and FVB mice were analyzed. The dashed lines and the solid lines show background levels and ESAM levels, respectively, on day 0. The tinted lines show ESAM levels at day 5 after a 5-FU injection (150 mg/kg). Each line of the histogram shows a representative pattern of three mice. *p < 0.05.
panels, Supplemental Fig. 4). Note that some ESAM⁺Sca⁻ cells that were not HSC were also found in these sections (Supplemental Fig. 4F). We confirmed by flow cytometry that some types of progenitors expressed low levels of ESAM after 5-FU treatment. Although megakaryocytes that were conspicuous by their morphology and very high ESAM expression distributed around the vasculature, many ESAM⁺Lin⁻Sca⁺ cells were also found in the same area, and some of them clustered in perivascular areas (Fig. 6A, lower right panel). Indeed, when randomly counted, >80% of the Lin⁻ESAM⁺Sca⁺ cells were localized within 20

FIGURE 2. HSC express other endothelial Ags, but their patterns do not change in a homogeneous way after 5-FU treatment. (A) The expression levels of SLAM family markers (CD48 and CD150) on ESAM⁺, ESAM⁻, and ESAM⁻ fraction of BM LSK cells were analyzed by flow cytometry. (B) The expression of ESAM and endothelial-related Ags (CD34, Tie2, endoglin, and PECAM-1) on BM LSK fraction was analyzed by flow cytometry. (A and B) Left panels show the results of homeostatic state mice (control), and right panels show those of 5-FU (150 mg/kg)–treated mice (day 5). Numbers in each panel indicate the percentages of each fraction. Each panel shows a representative pattern of three mice.

FIGURE 3. ESAM⁺ HSC in 5-FU–treated mice are dividing. C57BL/6 mice were treated with single i.v. 150 mg/kg 5-FU, and cell cycle analyses of Sca⁺c-Kit⁺ cells in BM were performed at day 5 by flow cytometry. Upper panels show Ki67 and Hoechst 33342 staining patterns of ESAM⁺ or ESAM⁻ Sca⁺c-Kit⁻ fraction. Numbers in each panel indicate the percentages of G0, G1, or S/G2/M fraction. Lower panels show the profile of BrdU and 7-aminoactinomycin D (7-AAD) stainings. BrdU was i.p. administered 12 h before analyses. Numbers in each panel indicate the percentages of G0/G1, S, or G2/M fraction. The data represent three independent trials with similar results.

FIGURE 4. Elevated ESAM expression on LSK correlates with CFU activity. C57BL/6 mice treated with single i.v. 150 mg/kg 5-FU were killed 5 d after treatment. ESAM⁺ or ESAM⁻ LSK cells of BM were sorted and subjected to methylcellulose colony formation assays. Each dish contained 200 sorted cells, and colony counts were performed 9 d after culture. The bars indicate the number of CFU-GM, CFU-M, BFU-E, or CFU-Mix per one dish from ESAM⁺ (open bar) or ESAM⁻ (filled bar) LSK cells. The results are shown as means ± SD. The data are shown as one of two independent experiments that gave similar results. *p < 0.05.
From vascular endothelium. Because the distance of 20 μm is approximately three hematopoietic cell diameters, most activated HSC at day 5 of 5-FU treatment were probably adjacent or close to the vascular endothelium. These observations suggested that activated ESAM_{hi} HSC can be intimate with endothelial cells and/or vascular-related cells.

Hematopoietic recovery after BM stress is compromised in ESAM-deficient mice

The results above suggested that ESAM expression levels mirror HSC shifts between quiescence and activation after BM injury. However, it remained unclear whether ESAM plays any role in physiological hematopoietic recovery. To address this issue, we evaluated hematopoietic recovery of ESAM KO mice after 5-FU injection. We did not observe significant phenotypes in peripheral blood of homeostatic ESAM KO mice except for slight anemia. Intriguingly, after injecting 200 mg/kg 5-FU, the KO mice had more severe pancytopenia than did WT mice (Fig. 7A). Whereas leukocyte and platelet counts recovered by day 10, severe anemia was protracted in KO mice (hemoglobin, WT 10.4 ± 1.1 g/dl versus KO 6.0 ± 1.7 g/dl at day 10), and three of nine KO mice died before full hematopoietic recovery (Fig. 7B). With respect to the BM, ESAM deficiency did not affect numbers of total mononuclear, LSK, or Flt3^+ LSK cells at days 0 and 5 after 5-FU. However, all categories were significantly reduced compared with WT mice at day 8 after 5-FU (Fig. 7C). Considering that hematopoietic recovery happened after day 5, when ESAM upregulation on HSC peaked, these results suggested that ESAM is indispensable for normal hematopoietic recovery after BM injury.

NF-κB and topoisomerase II are likely important for ESAM upregulation in HSC

Next, we searched the promoter sequence of the ESAM gene to find molecular mechanisms possibly involved in ESAM upregulation.
An NF-κB binding sequence at 363 bp upstream of the ESAM exon 1 drew our attention because it was well known to be an antiapoptotic factor after cell injury (27). We also found upregulation of NF-κB in the BM LSK cells after 5-FU treatment (data not shown). To examine the possible involvement of NF-κB, we administered bortezomib, a proteasome-inhibiting drug whose main action is inhibition of NF-κB, to 5-FU–treated mice (Fig. 8B). We found that bortezomib partially cancelled the ESAM upregulation caused by 5-FU injection (mean fluorescence intensity, 4790 ± 497 with 5-FU alone, 4090 ± 1050 with 5-FU and bortezomib) (Fig. 8C). Then, we conducted luciferase reporter assays to confirm the importance of the NF-κB binding sequence for ESAM transcription (Fig. 8E). We used endothelial bEnd3 cells that constantly express high levels of ESAM. The luciferase reporter activity remarkably decreased from construct A to B (Fig. 8F).

**FIGURE 6.** ESAMhi HSC at day 5 after 5-FU injection are mainly localized around vascular areas. (A) BM sections from C57BL/6 mice were stained with Abs against ESAM (red), Sca1 (blue), and Lin (Gr1, B220, CD3ε, CD8, and Ter119) sets (green). The nuclei of the cells are labeled with DAPI. The left panels are representative images of a BM section of untreated mouse, and the right panels are from the mouse 5 d after a 5-FU treatment (150 mg/kg). The frames in the upper panels are zoomed in to the lower panels. In the lower panels, ESAMhi large cells are megakaryocytes (arrows), ESAMhiLin"Sca1" cells are indicated by arrowheads. (B) The distances from each ESAM"Lin"Sca1" cell to the vascular endothelium (VE) and to the endosteum (ES) were measured, and their distribution frequencies were evaluated for 5-FU–treated BM. Open bars show the frequencies for 50 ESAMhiLin"Sca1" cells each in four separate specimens. As controls, random 100 DAPI+ cells each in the same specimens were evaluated (filled bars). The cells were classified into four categories: close to VE (within 20 μm) but not close to ES (>20 μm); close to ES but not close to VE; close to both ES and VE; not close to either ES or VE. *p < 0.05.

**FIGURE 7.** ESAM is required for normal hematopoietic recovery after BM injury. (A) Peripheral blood was examined every 5 d after single 200 mg/kg 5-FU treatment to WT or ESAM KO mice (n = 5 in each). All mice were 8-wk-old males. WBC, RBC, hemoglobin (Hb), and platelet (Plt) counts were compared between WT and ESAM KO mice at each time point. The results are shown as means ± SD. (B) Kaplan–Meier survival curves are shown regarding WT or ESAM KO mice treated with single 200 mg/kg 5-FU (n = 9 in each). (C) Total mononuclear cells (MNC), LSK cells, and Flt3-LSK cells in BM of WT or ESAM KO mice were evaluated at day 0 (homeostatic state), day 5, and day 8 after 150 mg/kg 5-FU treatment. The results are shown as means ± SD of five mice. *p < 0.05, **p < 0.01.
sharply decreased from construct C to D probably because DNA sequences between the two are necessary for binding of the basic transcription complex. Immediately downstream of that site, we found a consensus sequence for cleavage by topoisomerase II (28). Because topoisomerase II was also known to correlate with HSC activation, we tested ICRF-193, a topoisomerase II-specific inhibitor, in 5-FU–treated mice. Similar to bortezomib, ICRF-193 partially inhibited the ESAM upregulation (mean fluorescence intensity, 3260 ± 665 with 5-FU and ICRF-193) (Fig. 8C). Interestingly, ESAM upregulation was markedly abrogated when bortezomib and ICRF-193 were administered simultaneously (mean fluorescence intensity, 1980 ± 392 with 5-FU, bortezomib, and ICRF-193) (Fig. 8C). The mice treated with bortezomib and ICRF-193 showed significant reduction of LSK cells compared with control mice at day 5, although the mice treated with a single inhibitory drug did not respond in that way (Fig. 8D). These results indicated that NF-κB and topoisomerase II synergistically regulate ESAM expression on HSC after BM injury.

Discussion

Although adult stem cells divide infrequently, they have high proliferative capacity. Emerging evidence suggests that both quiescent and active stem cells simultaneously exist in different but consecutive niches under normal steady-state conditions (9). It is also well known that the quiescent stem cells proliferate after wounding or transplantation (5, 29). Accurate identification of stem cells according to their proliferative states is essential to understand the biological nature of “stemness” and to develop tissue-regeneration therapies. We now report that ESAM, a new marker for HSC, represents a powerful tool to monitor the transition of HSC between quiescence and activation after BM injury. Furthermore, ESAM is required for normal recovery from marrow ablation.
Treatment of mice with 5-FU enriches primitive HSC by eliminating most proliferating progenitors while sparing non-cycling quiescent HSC. Additionally, 5-FU has been widely used to stimulate quiescent adult HSC to proliferate (8, 30–33). In the present study, we exploited this method to evaluate whether ESAM levels would change, and we observed dramatic upregulation. Previous studies showed that expression levels of many adult HSC markers change during hematopoietic recovery after 5-FU–mediated myeloablation. Whereas expression of c-Kit and N-cadherin decrease, levels of Mac1, CD34, and AA4 that are markers for fetal HSC revive (8, 31, 33). CD150 and Sca1 are also known to increase on activated HSC, as confirmed in our study (see Fig. 2A, Supplemental Fig. 1). However, we stress that the degree of change in ESAM greatly exceeds that of other markers. The ESAMhi LSK fraction includes more LT-HSC defined by SLAM markers than does the ESAMlo subset (Fig. 2A, right panels). However, it is controversial whether HSC can only be found in the CD150− fraction (34, 35). For that reason, we did not use SLAM family markers to purify HSC when analyzing functions of ESAM.

Our cell cycle analyses showed that virtually all ESAMhiSca1+c-Kit+ cells after 5-FU treatment exit the G0 phase (Fig. 3). Alternatively, long-term repopulating HSC can be enriched in the ESAMhi fraction (Fig. 5B, 5C). These results suggest that the ESAMhi LSK fraction includes cycling, long-term multipotent HSC. Our data are in accordance with a previous report by Haug et al. (31) showing that the N-cadherinhi HSC in 5-FU–treated BM have high cell cycle entry rates and, at the same time, robust long-term reconstituting potential. These features do not match those of adult HSC under steady-state, but rather are reminiscent of fetal HSC. It is interesting that activated adult HSC after BM injury resemble fetal HSC regarding not only cell cycle status but also surface markers.

Although it was reported that the LSK Thy1hi Fik2/Fit3+ cell fraction in mobilized BM contains authentic HSC less frequently than those in untreated BM (36), our present data have demonstrated that HSC of 5-FU–treated BM can effectively reconstitute long-term hematopoiesis. One interpretation for the discrepancy is that because the HSC change their phenotype by activation, our sorting method, which depended on high expression of ESAM, might have enriched the authentic HSC more efficiently than did the conventional sorting gate. Another possibility is that active ESAM might have enriched the authentic HSC more efficiently than did the conventional sorting method, which depended on high expression of ESAM, vascular endothelial cells (Fig. 6), HSC might receive necessary signals directly or indirectly via interaction with ESAM. In fact, ESAM deficiency resulted in insufficient Rho signaling in endothelial cells, which potentially regulates the stabilization of endothelial tight junctions (13). Rho is also expressed in hematopoietic progenitors and involved in their polarity and mobility (45). Further studies are necessary to learn precisely how ESAM controls HSC function during BM recovery.

It is very important to know how ESAM expression is regulated in HSC. NF-kB is known to be activated by BM stress and induces cyclin D1, a key regulator of the G1 checkpoint (46). Indeed, we observed upregulation of NF-kB in HSC after 5-FU treatment (data not shown). Topoisomerase II is required for the G2-phase transition in mammalian cells (47). NF-kB was implicated as a key ESAM transcription factor by luciferase assay (Fig. 8F). In addition to NF-kB, we focused on topoisomerase II, because this enzyme is required for regulated transcription (48). Although independent administration of inhibitors for each factor showed only subtle effects on ESAM levels, the drugs synergistically but independently abrogated the ESAM upregulation in 5-FU–treated BM (Fig. 8C). Decreased ESAM expression with combined bortezomib and ICRF-193 treatment had a suppressive effect on the number of LSK cells, although treatment of a single inhibitory drug did not have significant effects (Fig. 8D). Given that ESAM KO mice did not show significant cytopenia at 5-FU day 5 compared with WT mice (Fig. 7C), bortezomib and ICRF-193 may suppress cell cycle-related pathways other than ESAM. Recently, reactive oxygen species (ROS) are attracting attention because levels in HSC influence their cell cycle status, self-renewal ability, and differentiation potential (49, 50). Accumulation of ROS is known to activate NF-kB signaling (51). Additionally, several studies have suggested that accumulation of ROS is likely involved in HSC aging (52–54). We reported that whereas ESAM levels on the LSK fraction decrease after the neonatal period, they increase again with aging (16).

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fold in aged mouse BM (55–57). At present, we are studying 10 ESAM LEVEL DISTINGUISHES HSC STATUS

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Disclosures

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References


