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難治性疾患等克服研究事業
（免疫アレルギー疾患等予防・治療研究事業　移植医療研究分野）

ドナーとレシピエントの双方を改変した、

骨髄非破壊的新規造血幹細胞移植法の開発基盤研究

平成２５年度　総括研究報告書

研究代表者　田代　克久

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目　　次

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総括研究報告書

ドナーとレシピエントの双方を改変した、骨髄非破壊的新規造血幹細胞移植法の開発基盤研究

研究代表者　田代　克久
独立行政法人　医薬基盤研究所
創薬基盤研究部　幹細胞制御プロジェクト　研究員

本研究は、造血幹細胞の移植効率の向上を目指して、遺伝子導入技術を駆使したドナー細胞の機能改変技術の開発、ならびにサイトカインを用いたレシピエント骨髄環境制御法の開発を試みる。本年度は、機能遺伝子を導入したドナー細胞（ヒト CD34 陽性細胞）の in vivo における機能評価を行った。また、サイトカインを投与した免疫不全マウスへのヒト CD34 陽性細胞の移植を行い、以下の結果を得た。

1．抗アポトーシス遺伝子を導入することにより、コントロールと比較し、免疫不全マウスへの移植効率が向上することを明らかにした。
2．G-CSF を投与した免疫不全マウスへ、ドナー細胞を移植したがその移植効率は極めて低く、移植条件の改良が必要であることが示唆された。

研究協力者
川端健二　（独）医薬基盤研究所
平田信恵　（独）医薬基盤研究所
水口裕之　（独）医薬基盤研究所
大阪大学大学院薬学研究科

A．研究目的

造血幹細胞移植の骨髄は、種々の造血障害の根治療法として推進されてきた。しかし、高齢者や合併症をもった患者（レシピエント）へ適応例は少ない。それは、全身放射線照射や大量のアルキル化剤の投与といった移植前処理が、レシピエントに対して大きな負担となっているためである。また、この前処理によりレシピエント骨髄の造血幹細胞ニッチ（niche：本来の居場所）が破壊されていることも推察される。そのため、上記の問題を克服するには、骨髄非破壊的な造血幹細胞移植法の開発が必要不可欠である。一方、骨髄非破壊状態では骨髄に多くの血液細胞が残存しているためドナー造血幹細胞の生着率の低下が懸念される。したがって、造血幹細胞移植の適応拡大には、骨髄非破壊的かつ高生着を可能とする造血幹細胞移植法の開発が重要である。

G-CSF 等のある種のサイトカイン／ケモカインは、骨髄の造血幹細胞を末梢血中へ遊離（動員）する作用を有している。これらの蛋白質の投与により、重篤な骨髄損傷を伴わずに造血幹細胞を骨髄から動員可能であると考えらたため、このような蛋白質はレシピエント骨髄内環境の制御分子として有用であると考えた。そこで本研究では、

ドナー造血幹細胞に機能改変を施すとともに、レシピエント骨髄環境を操作することで、骨髄非破壊的新規造血幹細胞移植法の基盤技術構築を目指すこととした。具体的には、（1）アデノウイルス（Ad）ベクターを用いて種々のサイトカインをマウス全身で発現させることにより、造血幹細胞を効率良く骨髄から動員させる手法、つまり、「ニッチを新たに創り出す方法」を開発するとともに、（2）Ad ベクターを用いた遺伝子導入により機能を増強した造血幹細胞の作製を行う。そして（3）機能増強した造血幹細胞をサイトカインで前処理したマウスへ移植し、キメリズムや生着率を評価する。今年度は主に、以下の研究を実施した。

① ヒトCD34陽性細胞へ抗アポトーシス遺伝子を導入し、in vivo における機能（移植率）を評価した。

② サイトカインを投与した免疫不全マウスへのヒト CD34 陽性細胞の移植を行った。

B. 研究方法

B-1. ヒト CD34 陽性細胞への遺伝子導入

ヒト骨髄由来 CD34 陽性細胞（Lonza 社）は添付のサプリメントを加えた StemPro34 Medium（invitrogen 社）中で解凍した。その後の培養には、サプリメントと L-グルタミン（2 mM、invitrogen 社）、サイトカイン（100 ng/mL human SCF、100 ng/mL human Flt3-ligand、20 ng/mL human IL-3、20 ng/mL human IL-6（全て Peprotech 社））を含む StemPro34 Medium を用いた。96 well ラウンドボトムプレートへ機能遺伝子を発現する Ad ベクターと混合した 1 x 10^4 個のヒト CD34 陽性細胞を播種し、37℃で 90 分培養した。その後、培地を 100uL ずつ加えた。機能増強型抗アポトーシス遺伝子 Bcl-xl-FNK を発現する Ad ベクター、Ad-FNK と Green fluorescent protein（GFP）を発現するコントロール Ad ベクター、Ad-GFP は、昨年度作製したものを用いた。

B-2. ヒト CD34 陽性細胞の移植とキメリズム解析

ヒト CD34 陽性細胞へ Ad-FNK あるいは Ad-GFP を作用させ、2 日間培養した。その後、3Gy の X 線を照射した免疫不全マウス（$Rag2^{-/-}$ $Il2rg^{-/-}$マウス）の尾静脈から、遺伝子導入していない CD34 陽性細胞あるいは Ad ベクターを作用させた CD34 陽性細胞を移入した。4 ヶ月後、免疫不全マウスの末梢血を回収し、ヒト CD45 陽性細胞の割合をフローサイトメーターで解析することにより移植効率を評価した。

B-3. サイトカイン・5FU を併用投与したマウス骨髄細胞のコロニーアッセイ

C57BL/6 マウスの尾静脈内に免疫不全マウスへの尾静脈内に、1 匹あたり 1×10^{10} vector particles（VP）の Ad-GCSF、あるいは Ad-Null を投与した。各 Ad ベクターは昨年度までに作成したものを用いた。そして Ad ベクターを投与して 3 日後に 5-フルオロウラシル（5-FU）あるいは PBS を腹腔投与した。5-FU を投与して 2 日後に各マウスの骨髄細胞を回収し、Methocult M3434 培地（Stem Cell Technologies）を用いてコロニーアッセイを行った。

B-4. 免疫不全マウスへのサイトカイン・5FU の併用投与と CD34 陽性細胞の移植

免疫不全マウスへの尾静脈内に、1 匹あたり 1×10^{10} VP の Ad-GCSF、あるいは Ad-Null を投与した。ベクター投与 3 日後に 5-FU を腹腔投与し、さらに 5-FU を投与して 2 日後に、1 x 10^5 個 CD34 陽性細胞を尾静脈から移植した。

（倫理面への配慮）

本研究は、独立行政法人医薬基盤研究所・組換え DNA 実験委員会および動物実験倫理委員会の承認を得たのちに行った。なお本研究において使用したヒト CD34 陽性細胞はメーカーから購入した細胞である。

C. 研究結果

前年度までの研究により、CD34 陽性細胞への遺伝子導入に適した Ad ベクターを決定した。さらに、最適化した Ad ベクターを用いて抗アポトーシス遺伝子（FNK）を導入したヒト CD34 陽性細胞は、コントロール遺伝子（GFP）を導入した細胞と比較し、in vitro において分化能を保持しながら長期間（約 20 日）増殖可能であることを明らかにした。そこで本年度は、抗アポトーシス遺伝子を導入した CD34 陽性細胞を免疫不全マウスへ移植し、その移植効率を評価することとした。遺伝子導入していない CD34 陽性細胞、GFP 遺伝子を導入した CD34 陽性細胞、FNK 遺伝子を導入した CD34 陽性細胞を 3Gy の X 線を照射した免疫不全マウスへ移入し、その 4 ヶ月後に末梢血におけるヒト CD45 陽性細胞の割合を解析した。その結果、遺伝子導入していない CD34 陽性細胞、GFP 遺伝子を導入した CD34 陽性細胞を移植したマウスの CD45 陽性細胞の割合は 5%前後であったのに対し、FNK を導入した細胞の場合には約 15%まで増加していた（図１）。したがって、CD34 陽性細胞への抗アポトーシス遺伝子を導入は、in vivo における機能の増強にも極めて有効であることが明らかとなった。

昨年度までに、サイトカイン GCSF と抗がん剤 5-FU を併用投与がレシピエントの改変法として有効であることを明らかにした。すなわち、G-CSF と 5-FU の併用投与という移植前処理により、放射線非照射条件においても長期骨髄再構築能を有する造血幹細胞が生着することを示した。そこで本年度はまず、この前処理条件下での骨髄中の血液前駆細胞の割合をコロニーアッセイにて解析した（図２）。その結果、Ad-GCSF のみを投与したマウスの骨髄では、コントロールである Ad-Null を投与したマウス骨髄と同程度の血液前駆細胞が含まれていることが明らかとなった。5-FU を投与したマウスでは、いずれの群も血液前駆細胞数は大きく低下していたが、特に、Ad-GCSF と 5-FU を併用投与したマウス骨髄中の血液前駆細胞は激減していることが確認された。したがって、本前処理法は、骨髄改変には有効であることが示唆された。

そこで次にヒト CD34 陽性細胞の移植系においても GCSF と 5-FU の併用投与処置が有効であるか否か検討することとした。まず、免疫不全マウスにおいても GCSF 投与により骨髄動員が誘導されるかどうか検討したところ、末梢組織へ造血幹細胞・血液前駆細胞が動員されることを確認した（データ略）。そこで免疫不全マウスに対して GCSF と 5-FU を投与したところ、C57BL/6 マウスとは異なり、全例が死亡した。一方、Ad-Null と 5-FU を投与したマウスでは死亡は観察されなかった。そのため、5-FU の濃度をこれまでの半量（75 mg/kg）にして再度検討を進めたところ、GCSF と 5-FU 投与マウスにおいても生存していた。そこで本条件で CD34 陽性細胞の移植を実施した。その結果、全ての群において CD45 陽性細胞の割合は 1%以下であり、極めて移植効率は低いものであった。なお、遺伝子導入を行った CD34 陽性細胞についても GCSF と 5-FU 投与したマウスへの移植実験を実施したが、キメリズムは依然として低いものであった（図３）。

D. 考察

本研究では、骨髄非破壊的な新規造血幹細胞移植法の開発を目指し、アデノウイルス（Ad）ベクターを用いて種々のサイトカインをマウス全身で発現させることにより、造血幹細胞を効率良く骨髄から動員させる手法、つまり、「ニッチを新たに創り出す方法」の開発、そして Ad ベクターを用いた遺伝子導入により機能増強型造血幹細胞の作製法の開発を目指している。

昨年度の結果をうけ、本年度は抗アポトーシス遺伝子を CD34 陽性細胞へ導入し、in vivo における機能を評価した。その結果、抗アポトーシス遺伝子を導入した CD34 陽性細胞のキメリズムはコントロール群と比較すると有意に高くなることが明らかとなり、CD34 陽性細胞のアポトーシスの阻害が移植効率の向上に有効であることが示された。ただし、抗アポトーシス遺伝子を導入した場合でも移植効率は 15%程度であり、十分とは言い難い。これまでにヒト細胞の移植系においては種々の免疫不全マウスが用いられており、$Rag2^{-/-}Il2rg^{-/-}$マウスについてもその有用性はみとめられている。しかし、ヒト造血幹細胞移植の実験系においては $NOD/SCID/Il2rg^{-/-}$マウス（NOG マウス）の方が、$Rag2^{-/-}Il2rg^{-/-}$マウスよりも移植効率が高いことが報告されている。したがって、NOG マウスをレシピエントとして用いることでより移植効率が改善されるものと期待される。

$Rag2^{-/-}Il2rg^{-/-}$マウスへ GCSF と 5-FU を併用投与した場合、これまでの条件では全例死亡した。コントロールベクターである Ad-Null と 5-FU を併用投与した場合には生存していたことから、免疫不全マウスと C57BL/6 の 5-FU に対する感受性の違いだけでは説明することができない。細胞増殖が盛んな細胞に対して 5-FU は作用するため、免疫不全マウスの場合、C57BL/6 マウスよりも造血幹細胞動員ならびに増殖・分化が顕著に誘導されており、そのために 5-FU の感受性が増して死亡した可能性もある。5-FU の投与量を半量にすることで死亡は回避可能であったが、その条件では、ヒト CD34 陽性細胞の移植効率は極めて低いものであった。本研究課題で目的としていた手法を確立するには、上述のマウス系統の検討と同時に、GCSF や 5-FU の投与量を厳密に決定する必要があると思われる。

E. 結論

1．ヒト CD34 陽性細胞へ抗アポトーシス遺伝子を導入することで、in vitro だけでなく、in vivo における機能も増強可能であることが明らかとなった。

2．サイトカインを投与した免疫不全マウスへ CD34 陽性細胞の移植については、実験系の更なる改良が必要であることが示唆された。

F. 健康危険情報

該当なし

G. 研究発表

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contributed)

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H. 知的財産権の出願・登録状況

1. 特許取得

該当事項なし

2. 実用新案登録

該当事項なし

3. その他

該当事項なし

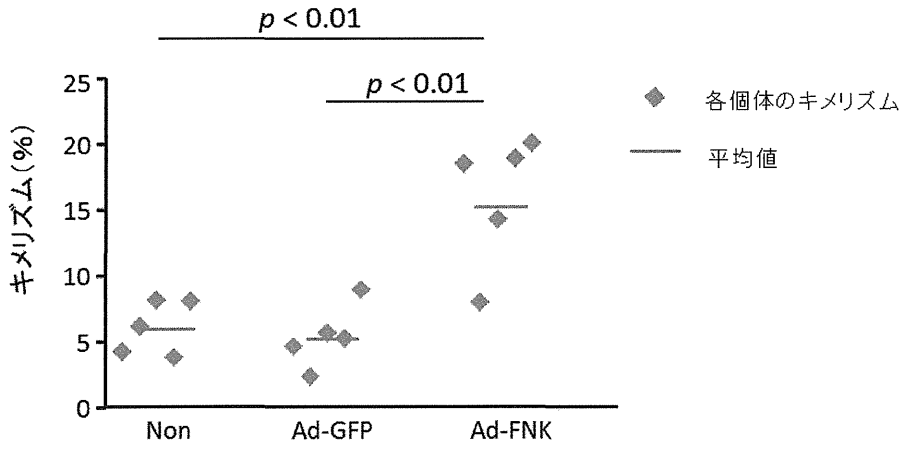

図1 ヒトCD34陽性細胞を移植した免疫不全マウスのキメリズム解析
3GyのX線を照射した免疫不全マウスへ、3,000 vp/cellの濃度でAdベクターを作用させたCD34陽性細胞を移植した。また、コントロールとして、遺伝子導入していないヒトCD34陽性細胞も移植した。移植4ヶ月後に末梢血を回収し、ヒトCD45を発現するドナー細胞の割合をフローサイトメーターで解析した。

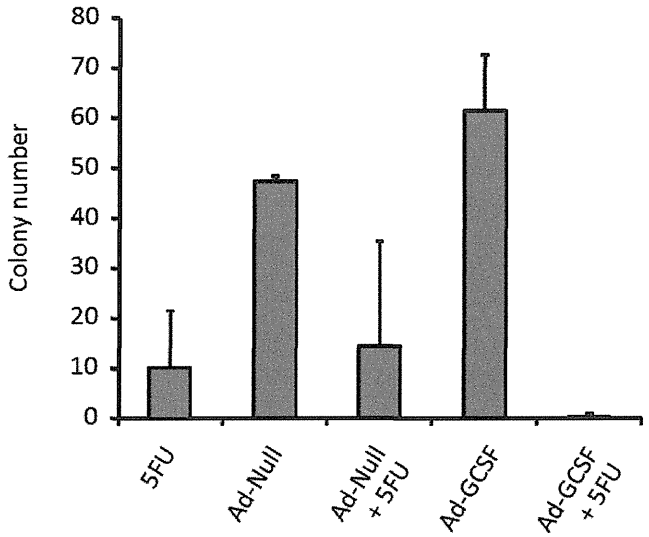

図2 種々の条件下におけるマウス骨髄中の血液前駆細胞数の解析
Adベクター(Ad-NullまたはAd-GCSF)を投与し、その3日後に抗がん剤5-FUを150mg/kgの量で腹腔内へ投与した。その2日後にマウス骨髄細胞を回収し、コロニーアッセイを行った。

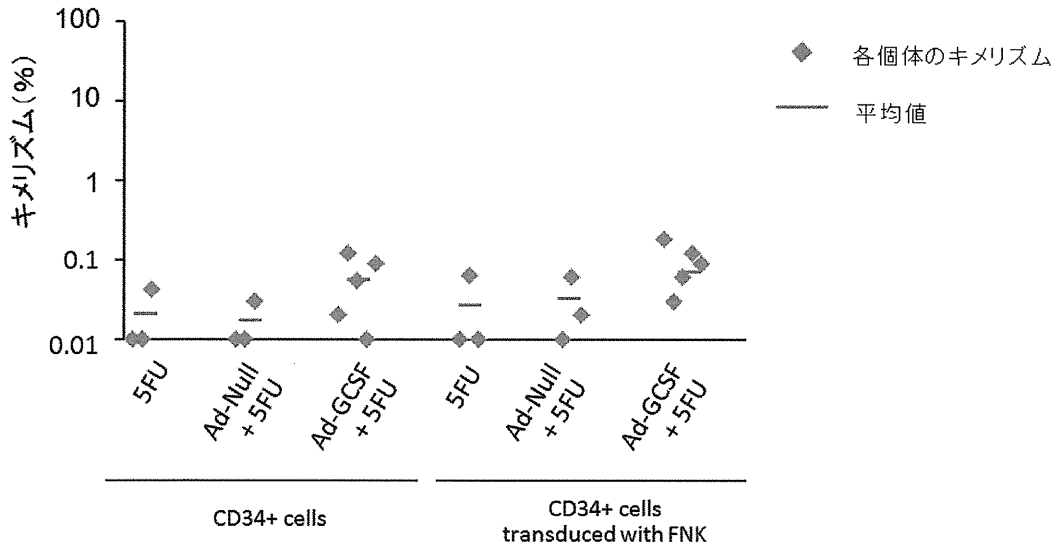

図3 サイトカインにて前処理した免疫不全マウスへのヒトCD34陽性細胞の移植
免疫不全マウスにAd-NullまたはAd-GCSFを投与し、その3日後に75mg/kgの5-FUを腹腔投与した。その2日後に遺伝子導入したCD34陽性細胞あるいは遺伝子導入していないCD34陽性細胞を移植した。移植1ヶ月後に末梢血を回収し、ヒトCD45の発現を指標にキメリズムを評価した。

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tashiro K.*, Nonaka A.*, Hirata N., Yamaguchi T., Mizuguchi H., Kawabata K. (*equally contributed)	Plasma elevation of vascular endothelial growth factor leads to the reduction of mouse hematopoietic and mesenchymal stem/progenitor cells in the bone marrow.	*Stem Cells Dev.*			印刷中

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ORIGINAL RESEARCH REPORT

Plasma Elevation of Vascular Endothelial Growth Factor Leads to the Reduction of Mouse Hematopoietic and Mesenchymal Stem/Progenitor Cells in the Bone Marrow

Katsuhisa Tashiro,[1,*] Aki Nonaka,[1,2,*] Nobue Hirata,[1] Tomoko Yamaguchi,[1] Hiroyuki Mizuguchi,[3–6] and Kenji Kawabata[1,2]

Vascular endothelial growth factor (VEGF) is reported to exhibit potent hematopoietic stem/progenitor cell (HSPC) mobilization activity. However, the detailed mechanisms of HSPC mobilization by VEGF have not been examined. In this study, we investigated the effect of VEGF on bone marrow (BM) cell and the BM environment by intravenous injection of VEGF-expressing adenovirus vector (Ad-VEGF) into mice. A colony assay using peripheral blood cells revealed that plasma elevation of VEGF leads to the mobilization of HSPCs into the circulation. Granulocyte colony-stimulating factor (G-CSF) is known to mobilize HSPCs by decreasing CXC chemokine ligand 12 (CXCL12) levels in the BM. However, we found almost no changes in the CXCL12 levels in the BM after Ad-VEGF injection, suggesting that VEGF can alter the BM microenvironment by different mechanisms from G-CSF. Furthermore, flow cytometric analysis and colony forming unit-fibroblast assay showed a reduction in the number of mesenchymal progenitor cells (MPCs), which have been reported to serve as niche cells to support HSPCs, in the BM of Ad-VEGF-injected mice. Adhesion of donor cells to the recipient BM after transplantation was also impaired in mice injected with Ad-VEGF, suggesting a decrease in the niche cell number. We also observed a dose-dependent chemoattractive effect of VEGF on primary BM stromal cells in vitro. These data suggest that VEGF alters the distribution of MPCs in the BM and can also mobilize MPCs to peripheral tissues. Taken together, our results imply that VEGF-elicited egress of HSPCs would be mediated, in part, by changing the number of MPCs in the BM.

Introduction

HEMATOPOIETIC STEM CELLS (HSCs) sustain blood production throughout life. In a steady state, HSCs exist within the bone marrow (BM) and remain largely quiescent and self-renew at a low rate to avoid their exhaustion. By contrast, HSCs can actively proliferate, differentiate into progenitor cells, or egress from the BM into the circulation in some situations, such as tissue damage-induced cell death and increased plasma levels of hematopoietic cytokines, including the granulocyte colony-stimulating factor (G-CSF). These dynamic behaviors of HSCs are controlled by a local specific microenvironment called niches [1–8]. The non-hematopoietic cells, such as endosteal osteoblasts and perivascular mesenchymal progenitor cells (MPCs), are reported to function as niche cells by supplying several HSC maintenance factors, including the CXC chemokine ligand 12 (CXCL12). Indeed, previous studies have shown that decreased levels of CXCL12 in the BM caused hematopoietic stem/progenitor cell (HSPC) mobilization, indicating the pivotal role of CXCL12 signaling in HSPC egress [9,10].

Not only is the vascular endothelial growth factor (VEGF) a well-known factor in angiogenesis, but it also plays an important role in the growth and differentiation of hematopoietic cells. Homozygous or heterozygous deletion of VEGF in mice leads to early embryonic lethality because of impaired vascular angiogenesis and hematopoiesis [11,12]. By conditional deletion of VEGF in hematopoietic cells, but not in stromal cells, Ferrara and colleagues clearly showed that VEGF is required for survival and repopulation

[1]Laboratory of Stem Cell Regulation, National Institute of Biomedical Innovation, Ibaraki, Japan.
Laboratory of [2]Biomedical Innovation and [3]Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Ibaraki, Japan.
[4]iPS Cell-based Research Project on Hepatic Toxicity and Metabolism, Graduate School of Pharmaceutical Sciences, Osaka University, Ibaraki, Japan.
[5]Laboratory of Hepatocyte Regulation, National Institute of Biomedical Innovation, Ibaraki, Japan.
[6]The Center for Advanced Medical Engineering and Informatics, Osaka University, Ibaraki, Japan.
*These two authors contributed equally to this work.

of adult HSCs [13]. Furthermore, VEGF has been shown to be an essential factor for HSC niche formation through endochondral ossification [14]. These observations clearly demonstrate that VEGF exerts physiological actions on hematopoietic systems through both cell-autonomous and -nonautonomous mechanisms.

In addition to the functions described above, VEGF also has a potent HSPC mobilization capacity [15], although the mechanisms of VEGF-induced HSPC mobilization have not been addressed in detail. In the current study, we investigated the effect of VEGF on the BM cell mobilization and BM environment after the intravenous injection of VEGF-expressing adenovirus (Ad) vector (Ad-VEGF) into mice. The results showed that VEGF overexpression in mice could lead to a reduction of not only the HPSC number, but also the MPC number in the BM. We also observed an enhanced chemoattractive activity of BM stromal cells by VEGF. Our data suggest that the plasma elevation of VEGF in mice alters the distribution of MPCs in the BM, and this might cause HSPC egress from the BM.

Materials and Methods

Ad vectors

Ad vectors were constructed by an improved in vitro ligation method [16,17]. The mouse $VEGF_{165}$ cDNA and human G-CSF cDNA were obtained from pBLAST49-mVEGF and pORF9-hGCSFb, respectively (Invivogen). Each cDNA was cloned into a multicloning site of pHMCMV10 [18,19], which contains the cytomegalovirus (CMV) promoter/enhancer and intron A sequence flanked by the I-*Ceu*I and PI-*Sce*I sites, thereby resulting in pHMCMV10-VEGF and pHMCMV10-G-CSF. pHMCMV10-VEGF and pHMCMV10-G-CSF were digested with I-*Ceu*I/PI-*Sce*I and ligated into I-*Ceu*I/PI-*Sce*I-digested pAdHM41-K7 (C) [20], resulting in pAd-VEGF and pAd-G-CSF, respectively. To generate the virus, Ad vector plasmids were digested with *Pac*I and purified by phenol–chloroform extraction and ethanol precipitation. Linearized DNAs were transfected into 293 cells with SuperFect (Qiagen) according to the manufacturer's instructions. The viruses were amplified in 293 cells. Before virus purification, the cell lysates were centrifuged to remove cell debris and were digested for 30 min at 37°C with 200 μg/mL DNase I and 200 μg/mL RNase A in the presence of 10 mM $MgCl_2$. Viruses were purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation. The purified viruses were dialyzed against a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol and were stored at −80°C. The control vector, Ad-Null, is similar in design, except that it contains no transgene in the expression cassette. The biological titers [infectious unit (ifu)] of Ad-VEGF, Ad-G-CSF, and Ad-Null were determined by using an Adeno-X Rapid Titer kit (Clontech).

Administration of Ad vectors in mice

C57BL/6j female mice aged 7–9 weeks were obtained from Nippon SLC, and all animals were maintained under specific pathogen-free conditions. Each Ad vector was intravenously injected into C57BL/6j mice at 1×10^9 ifu through the tail vein. All experiments were conducted according to the institutional ethics guidelines for animal experimentation of the National Institute of Biomedical Innovation.

Cell preparation

Blood and BM were harvested from mice using standard methods on day 5 after injection of Ad vector into mice, and the number of nucleated cells in these tissues was then counted using a Nuclecounter (Chemometec). To collect the nonhematopoietic cells from the femur and tibia, the bone fragments were minced with scissors, and were then incubated at 37°C with a type I collagenase (3 mg/mL; Worthington) in the Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) for 90 min [21]. The cells were filtered with a cell strainer to remove debris and bone fragments, and suspended in a staining buffer [phosphate buffer saline (PBS)/2% FBS]. These cell suspensions were kept on ice for further analysis.

Flow cytometry

The following antibodies (Abs), conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), or PECy7, were used for flow cytometric analysis and cell sorting: biotinylated lineage cocktail [CD3 (145-2C11), B220 (RA3-6B2), Gr-1 (RB6-8C5), CD11b (M1/70), Ter119 (Ter-119)], c-Kit-APC (2B8), Sca-1-PECy7 (D7), Ter119-FITC (Ter-119), CD45-FITC (30-F11), CD11b-FITC (M1/70), Gr-1-PE (RB6-8C5), CD31-FITC (390), CD31-APC (390), CD51-PE (RMV-7), PDGFRa-APC (APA-5), Flt-1-PE (141522), Flk1-PE (Avas12a1), and Alcam-PE. For detection of biotinylated Abs, PerCP-Cy5.5- or FITC-conjugated streptavidin was used. Abs were purchased from e-Bioscience, BD Bioscience, Biolegend, and R&D Systems. Cells were incubated with primary Abs at 4°C for 30 min and washed twice with PBS/2% FBS. After staining, cells were analyzed and isolated by flow cytometry on an LSR II and FACSAria flow cytometer, respectively, using FACSDiva software (BD Bioscience).

Enzyme linked immunosorbent assay

Blood samples were collected through the inferior vena cava on day 5 after Ad vector injection, and transferred to polypropylene tubes containing heparin. Plasma was harvested by centrifugation. The BM supernatant was obtained by flushing a femur with 500 μL of PBS, followed by centrifugation at 500*g* for 5 min. The levels of VEGF and CXCL12 in the plasma and BM supernatant were measured using a commercial ELISA kit (R&D Systems) following the manufacturer's instructions.

RT-polymerase chain reaction analysis

CD45-negative($^-$) $Ter119^-$ nonhematopoietic cells in the BM were sorted from mice injected with Ad-VEGF or Ad-Null, and total RNA was then extracted using ISOGEN (Nippon Gene). cDNA was synthesized from DNase I-treated total RNA with a Superscript VILO cDNA synthesis ◀AU3

AU3 ▶ kit (Invitrogen), and quantitative real-time RT-polymerase chain reaction was performed using the Fast SYBR Green Master Mix with an ABI StepOne Plus system (Applied Biosystems). Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase. The sequences of the primers T1 ▶ used in this study are listed in Table 1.

Colony assay

BM cells (2×10^4 cells) and peripheral blood cells (2×10^5 cells) were plated in the Methocult M3434 medium (StemCell Technologies, Inc.). Cultures were plated in duplicate and placed in a humidified chamber with 5% CO_2 at 37°C for 10 days. The number of individual colonies was counted by microscopy. The colony number was normalized to the total number of the nucleated cells.

Colony forming unit-fibroblast assay

BM-derived $CD45^-Ter119^-$ cells were added to the MesenCult MSC Basal Medium, including supplements (Stem Cell Technologies, Inc.), and then plated on a six-well plate at 1×10^5 cells per well. Cells were cultured for 14 days and stromal cell colonies (fibroblast-like colonies: >50 cells) derived from colony forming unit-fibroblasts (CFU-Fs) were stained with the Giemsa solution (Wako) after fixation with methanol. The colony number was counted by microscopy.

Cell migration assay

BM-derived stromal cells, including MPCs, were tested for migration toward VEGF using 8-μm pore-sized cell culture inserts (BD Falcon). Stromal cells (1×10^5 cells) resuspended in 200 μL of DMEM/2% FBS were added to the upper chamber, and 750 μL of DMEM/2% FBS containing recombinant mouse VEGF (10 or 100 ng/mL; Peprotech) was added to the bottom chamber. After 6 h of incubation at 37°C, the upper side of the filters was carefully washed with PBS, and cells remaining on the upper face of the filters were removed with a cotton wool swab. The filters were fixed with 100% methanol and stained with the Giemsa solution. Cells migrating into the lower compartment were counted manually in three random microscopic fields (×200).

Homing assay

Mice were administrated with Ad-Null or Ad-VEGF at 1×10^9 ifu. Five days later, BM cells (1×10^7 cells) derived from green fluorescent protein (GFP)-expressing transgenic mice [22] were intravenously transplanted into Ad-Null- or Ad-VEGF-injected mice. At 16 h after BM transplantation, the percentage of GFP-expressing donor cells in the BM was determined by flow cytometry.

Results

Effect of systemic VEGF overexpression on the distribution of myeloid cells and HSPCs in mice

To evaluate the effect of VEGF on the mobilization of hematopoietic cells, we generated a VEGF-expressing Ad vector, Ad-VEGF, because plasma VEGF levels were rapidly decreased with a $t_{1/2}$ of ~25 min after intravenous injection of recombinant VEGF [23]. Single intravenous injection of Ad-VEGF (1×10^9 ifu) into mice led to a significant elevation of VEGF levels in plasma on day 5 compared with Ad-Null-injected mice (control mice) (Fig. ◀F1 1a). On the other hand, unexpectedly, BM VEGF levels in the Ad-VEGF-injected mice were almost equivalent to those in the Ad-Null-injected mice (Fig. 1b). There were no signs of toxicity in mice treated with Ad-VEGF and Ad-Null at this dose (1×10^9 ifu). To investigate whether the hematopoietic cells could be mobilized from the BM into the circulation after injection of Ad-VEGF, we examined the number of total nucleated cells, myeloid cells ($Gr\text{-}1^+CD11b^+$ cells), and HSPCs [$c\text{-}Kit^+Sca\text{-}1^+Lineage^-$ ◀AU4 (KSL) cells or CFU-GEMM/CFU-Mix] in the peripheral blood. Compared with Ad-Null-injected mice, Ad-VEGF-injected mice showed an increased number of total nucleated cells and myeloid cells in the peripheral blood (Fig. 1c, d). We found that the number of multipotent hematopoietic progenitor cells, CFU-GEMM/CFU-Mix, in the blood of Ad-VEGF-injected mice was four times as great as that of Ad-Null-injected mice (Fig. 1e). Importantly, in Ad-VEGF-injected mice, the number of KSL cells in the blood was also increased (Fig. 1f). These results indicate that hematopoietic cells, including immature hematopoietic cells with colony-forming potentials, would be mobilized from the BM following systemic Ad-VEGF administration.

An increased number of mobilized cells in VEGF-treated mice were reported previously [15], but little is known about the effect of VEGF on BM cells during the mobilization period. Thus, we next investigated the number of total BM cells, myeloid cells, and HSPCs. In contrast to the peripheral blood, the number of total hematopoietic cells, myeloid cells, and CFU-Mix was significantly decreased (Fig. 2a–c). ◀F2 It is of note that the VEGF overexpression in mice resulted in the reduction in both the frequency and the absolute

TABLE 1. ◀AU5

Gene name	*(5′) Sense primers (3′)*	*(5′) Antisense primers (3′)*
Gapdh	TTCACCACCATGGAGAAGAAGGC	GGCATGGACTGTGGTCATGA
Cdh2	CAAGAGCTTGTCAGAATCAGG	CATTTGGATCATCCGCATC
Vcam-1	GACCTGTTCCAGCGAGGGTCTA	CTTCCATCCTCATAGCAATTAAGGTG
Angpt1	CTCGTCAGACATTCATCATCCAG	CACCTTCTTTTAGTGCAAAGGCT
Thpo	GGCCATGCTTCTTGCAGTG	AGTCGGCTGTGAAGGAGGT

Gapdh, glyceraldehyde 3-phosphate dehydrogenase; *cdh2*, N-cadherin; *Vcam-1*, vascular cell adhesion molecule-1; *Angpt1*, angiopoietin-1; *Thpo*, thrombopoietin.

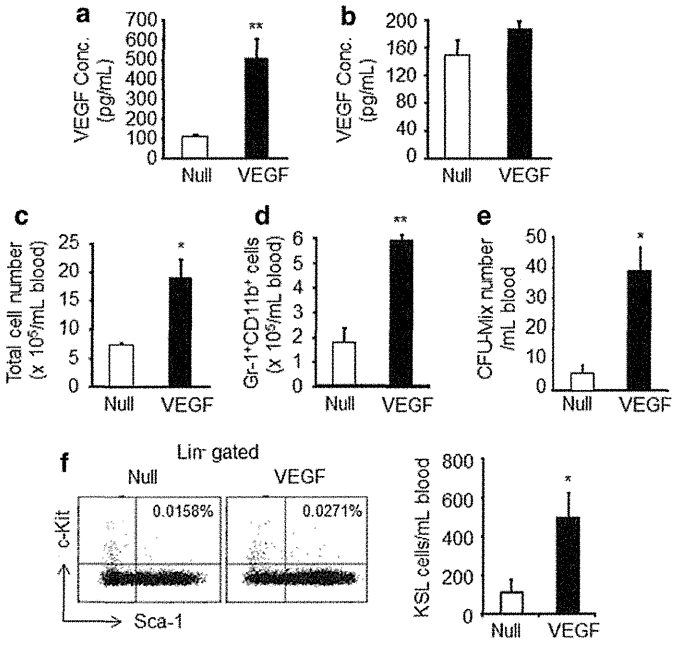

FIG. 1. Effect of vascular endothelial growth factor (VEGF) on the number of myeloid cells and hematopoietic stem/progenitor cells (HSPCs) in peripheral blood. **(a, b)** Mice were intravenously injected with adenovirus (Ad)-Null (Null) or Ad-VEGF (VEGF). Five days later, the concentration of plasma **(a)** and bone marrow (BM) **(b)** VEGF levels was determined by enzyme linked immunosorbent assay (ELISA). Data are expressed as mean ± standard deviation (SD) ($n=4$). **(c)** The number of total PBMCs was counted on day 5 after administration of each Ad vector. **(d)** The percentage of Gr-1^+CD$11b^+$ myeloid cells was determined by flow cytometric analysis, and the absolute number was then normalized to the total PBMC number. Data are expressed as mean ± SD ($n=4$). **(e)** The number of colony forming unit (CFU)-Mix/CFU-GEMM, a multipotent hematopoietic progenitor cells, in PBMCs was determined by a standard colony assay. The colony number was normalized to the total PBMC number. **(f)** A representative analysis of the c-Kit$^+$Sca-1^+Lineage$^-$(KSL) subset in the blood is shown (*left*). The proportion of cKit$^+$Sca-1^+ cells in the lineage-negative (Lin$^-$) population is indicated in the dot blot. The number of KSL cells in the blood was normalized to the total cell number (*right*). Data are expressed as mean ± SD ($n=4$). *$P<0.05$, **$P<0.01$ as compared with Null.

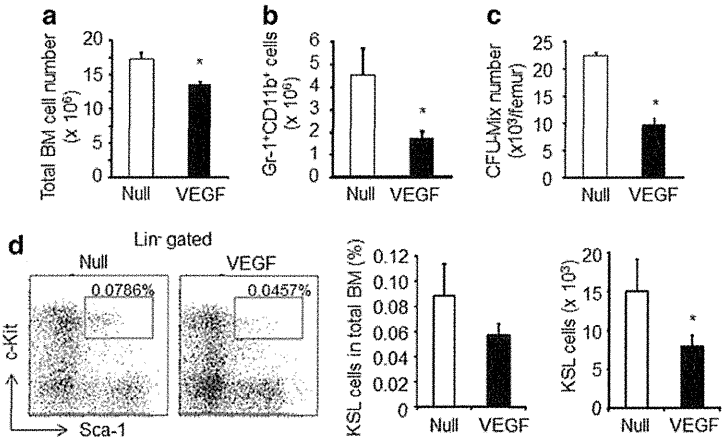

FIG. 2. Plasma elevation of VEGF leads to a decrease in the myeloid cells and HSPCs in the BM. **(a)** The number of total BM cells was counted on day 5 after Ad-Null or Ad-VEGF injection. **(b)** The number of Gr-1^+CD$11b^+$ cells in the BM was determined by flow cytometric analysis. **(c)** The number of CFU-Mix/CFU-GEMM in the BM was determined by a colony assay. The colony number was normalized to the total BM cell number. **(d)** A representative analysis of the KSL subset in the BM after administration of Ad-Null or Ad-VEGF is shown (*left*). The proportion of KSL cells in the total BM is indicated in the dot blot. Frequencies (*middle*) and absolute numbers (*right*) of KSL cells in the BM were calculated. Data are expressed as mean ± SD ($n=5$). *$P<0.05$ as compared with Null.

number of KSL cells in BM (Fig. 2d). Thus, these data suggest that VEGF exerts a physiological effect on the various types of cells within the BM.

Unchanged level of CXCL12 after VEGF overexpression

To examine the mechanisms of BM cell mobilization by VEGF treatment, we analyzed the expression levels of genes associated with HSC maintenance in the BM [*N-cadherin* (*cdh2*), *vascular cell adhesion molecule-1* (*Vcam-1*), *angiopoietin-1* (*Angpt1*), and *thrombopoietin* (*Thpo*)]. The expression levels of these genes in BM nonhematopoietic cells were modestly downregulated after Ad-VEGF injection (Fig. 3a). We next measured the CXCL12 levels in Ad-VEGF-injected mice. Chemokine CXCL12 is an indispensable factor for the maintenance and retention of HSPCs in the BM [5,24]. Previous studies showed that the BM CXCL12 levels were reduced by the injection of mobilization-inducing factors, such as G-CSF and stem cell factor (SCF) [10,25]. We also found that the CXCL12 levels were markedly reduced in the BM, but not the plasma, of Ad-G-CSF-injected mice (Fig. 3b). However, there was almost no difference in the BM CXCL12 levels between Ad-VEGF-injected mice and Ad-Null-injected mice (Fig. 3b). Therefore, these data indicate that VEGF would alter the BM microenvironment, probably by a different mechanism from other mobilization factors.

Reduction of MPCs in the BM after Ad-VEGF injection

Recent studies have demonstrated that MPCs play a pivotal role in HSPC maintenance in the BM [4,6–8]. Therefore, we examined the disposition of MPCs in the BM after Ad-VEGF administration. Flow cytometric analysis of the enzymatically dissociated BM cells revealed that Ad-VEGF overexpression led to a significant reduction of $CD45^-Ter119^-CD31^-Alcam^-Sca\text{-}1^+$ cells, which are reported to be MPCs [21,26] (Fig. 4a). In addition, the percentage of other MPC populations, such as $CD45^-Ter119^-PDGFR\alpha^+Sca\text{-}1^+$ cells [27] and $CD45^-Lineage^-CD31^-CD51^+Sca\text{-}1^+$ cells [28], in the BM of Ad-VEGF-injected mice was also lower than those of Ad-Null-injected mice (Fig. 4b, c). These data clearly showed the decreased number of phenotypically identified MPCs in the BM after injection of Ad-VEGF.

Next, to investigate whether functional MPCs in the BM were reduced in Ad-VEGF-injected mice, we performed a CFU-F assay and homing assay. Consistent with the above data, we observed decreased CFU-F numbers in the BM in Ad-VEGF-injected mice (Fig. 4d). For homing studies, Ad-Null- or Ad-VEGF-injected mice were used as the recipient mice. Donor BM cells derived from GFP transgenic mice were intravenously injected into nonirradiated recipient mice, and the frequency of GFP-expressing cells in the recipient BM was then estimated by flow cytometry. The results showed that the homing activity of GFP-expressing cells was partially inhibited in Ad-VEGF-treated recipient mice (Fig. 4e). Thus, the decreased homing efficiency of donor HSPCs in Ad-VEGF-injected mice suggests the decreased number of niche cells in the BM. Taken together, our findings indicate that overexpression of VEGF in mice leads to a reduction of phenotypic and functional MPCs in the BM.

VEGF stimulates the migration of MPCs

We next examined the mechanisms of the reduction of MPCs in the BM after VEGF overexpression. In vitro-expanded primary mouse BM stromal cells (mBMSCs), including MPCs, showed slight expression of Flt-1 (VEGFR1), but not Flk-1 (VEGFR2), on the cellular surface (Fig. 5a). We speculated that MPCs might egress from the BM in response to the plasma level of VEGF, because there was almost no change in the BM VEGF levels in Ad-VEGF-injected mice (Fig. 1b). We performed an in vitro migration assay and found a dose-dependent chemoattractive effect of VEGF on mBMSCs (Fig. 5b), suggesting the possibility that a decreased number of BM MPCs in Ad-VEGF-injected mice would result from the mobilization of MPCs to the peripheral tissue in response to an elevation of plasma VEGF.

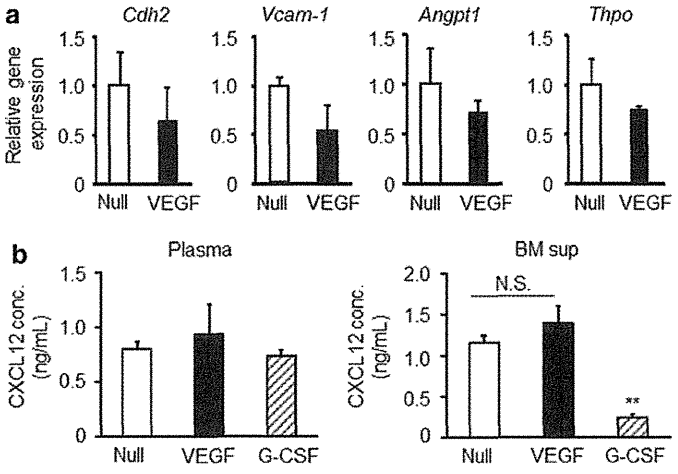

FIG. 3. Expression of HSPC maintenance factor genes after Ad-VEGF administration. **(a)** Expression levels of cadherin2 (Cdh2), vascular cell adhesion molecule-1 (Vcam-1), angiopoietin-1 (Angpt1), and thrombopoietin (Thpo) in nonhematopoietic cells ($CD45^-Ter119^-$ cells) were measured by quantitative polymerase chain reaction analysis. Data are expressed as mean ± SD ($n=3$). **(b)** The plasma and BM supernatants of mice injected with Ad-Null, Ad-VEGF, or Ad-GCSF were collected. The levels of CXC chemokine ligand 12 (CXCL12) in the plasma (*left*) and the BM supernatant (*right*) were measured by ELISA. $^{**}P<0.01$ as compared with Null. N.S. stands for not significant.

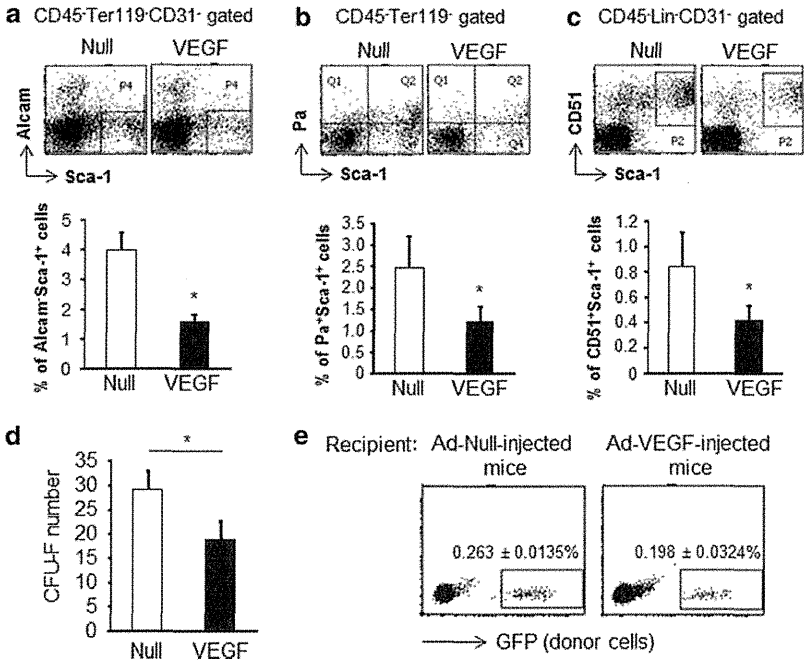

FIG. 4. The number of mesenchymal progenitor cells (MPCs) in the BM is decreased following Ad-VEGF injection. (**a–c**) After BM stromal cells were collected from bone by treatment with collagenase, the proportion of MPC populations [$CD45^-Ter119^-CD31^-Alcam^-Sca\text{-}1^+$ MPCs (**a**), $CD45^-Ter119^-$ $PDGFRa^+$ (Pa^+) $Sca\text{-}1^+$ MPCs (**b**), or $Lin^-CD45^-CD31^-CD51^+Sca\text{-}1^+$ MPCs (**c**)] in the BM was determined by flow cytometry. Data are expressed as mean ± SD ($n=5$). (**d**) A colony-forming unit-fibroblast (CFU-F) assay was performed using $CD45^-Ter119^-$ BM cells. The number of CFU-Fs was counted using a microscope after staining with the Giemsa solution. Data are expressed as mean ± SD ($n=3$). (**e**) Homing assay. After injection of Ad-Null or Ad-VEGF into mice, green fluorescent protein (GFP) transgenic mice-derived BM cells (donor cells) were transplanted into Ad vector-administrated mice. The percentage of donor cells (GFP-expressing cells) in the BM of Ad-Null- or Ad-VEGF-injected mice was analyzed by flow cytometry at 16 h after BM transplantation. The percentage of donor cells in the BM is indicated in the dot blot. Data are expressed as mean ± SD ($n=5$). $^*P<0.05$ as compared with Null.

Discussion

Recent studies have clearly reported that the HSPC numbers in the BM are significantly decreased by conditional deletion of MPCs, including nestin-expressing stromal cells [4] and CXCL12-abundant reticular cells [5]. It is of note that deletion of MPCs led to the increased number of HSPCs in the spleen, demonstrating the mobilization of HSPCs from BM to peripheral tissues [4]. Therefore, maintenance and retention of HSPCs in the BM would

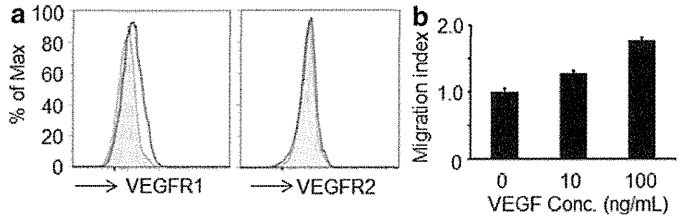

FIG. 5. VEGF enhances the migration capacity of MPCs. BM-derived stromal cells were collected and propagated in a tissue culture dish. (**a**) Expression levels of VEGF receptors, VEGFR1 (*left*) and VEGFR2 (*right*), in the cells was determined by flow cytometry. Staining profiles of specific mAb (dotted lines) and an isotype-matched control mAb (gray area) are shown. (**b**) BM stromal cells were exposed to various doses of recombinant VEGF. Cells that had migrated toward the VEGF (lower chamber) by passing through a membrane filter were counted by microscopy after staining with the Giemsa solution. Data are expressed as mean ± SD ($n=3$).

considerably be dependent on the number of MPCs [4,5]. In the present study, we examined the effect of VEGF on the disposition of BM HSPCs and MPCs in mice. Our main finding was that VEGF overexpression in mice resulted in a reduction of not only HSPCs but also MPCs in the BM. We also found that VEGF could promote the migration of mBMSCs in vitro. The data described here suggest that, as in the case of HSPCs, MPCs would also be mobilized to the peripheral tissues in response to an elevation of plasma VEGF levels, and a reduced number of BM MPCs by VEGF would lead to HSPC egress from the BM, because MPCs would function as niche cells in the BM.

It is well known that BM CXCL12 levels are down-regulated following G-CSF administration and thereby induce an egress of HSPCs [25,29]. Christopher et al. previously showed the reduced BM CXCL12 levels after administration of other mobilization factors, such as SCF and Flt3-ligand [10]. In addition to their mobilization-inducing effects, these factors also impact the number of stem and progenitor cells in the BM. For instance, it has been reported that the number of HSPCs and MPCs in the BM was significantly increased after G-CSF administration [30,31]. Unlike in the case of G-CSF and other mobilization factors, however, VEGF had almost no effect on BM CXCL12 levels (Fig. 3b). Furthermore, systemic VEGF expression resulted in a significant reduction in the number of HSPCs (KSL cells and CFU-Mix) in the BM (Fig. 2). The number of MPCs in the BM was also reduced in Ad-VEGF-injected mice (Fig. 4). Therefore, these data strongly indicate that VEGF would induce HSPC mobilization by altering the BM environment through different mechanisms from G-CSF. Notably, a recent study showed that HSPCs could be mobilized from the BM into the circulation by administration of a prostagrandin E_2 (PGE_2) inhibitor, and this effect was independent of CXCL12-CXCR4 signaling [32]. A nucleotide sugar, uridine diphosphate (UDP)-glucose, has also been shown to mobilize subsets of HSPCs functionally distinct from those mobilized by G-CSF, suggesting that UDP-glucose-induced HSPC mobilization would be mediated, at least in part, by different mechanisms from G-CSF [33]. Thus, it would be of interest to examine whether VEGF could influence the levels of BM PGE_2 and/or plasma UDP glucose.

The expression levels of HSC maintenance genes (*Cdh2*, *Vcam-1*, *Angpt1*, and *Thpo*) in BM nonhematopoietic cells were decreased in Ad-VEGF-injected mice (Fig. 3a). This would be due to the reduction in the number of MPCs in the BM after Ad-VEGF injection (Fig. 4). However, we have no idea why BM CXCL12 levels were not changed in Ad-VEGF-injected mice, because MPCs abundantly produce CXCL12 [7,8]. A detailed investigation would be required to clarify the regulation of CXCL12 expression in niche cells, including MPCs, endosteal osteoblasts, and endothelial cells.

We observed enhanced in vitro migration activities of mBMSCs by VEGF, suggesting the possibility that MPCs in the BM would be mobilized to the peripheral tissue in response to the plasma VEGF concentration. However, at present, we did not detect the CFU-F in the blood in Ad-VEGF-injected mice (data not shown). MPCs are known to be rare cells even in the BM, representing ~1 in 10,000–100,000 total nucleated cells [34], and it is therefore possible that the frequency of MPCs in the blood was too low to detect under our experimental conditions. Alternatively, it is also possible that VEGF overexpression in mice might lead to the homing of MPCs to organs, such as the liver, because transgene expression in the liver was extremely high following systemic Ad vector injection [35]. Therefore, it might be necessary to investigate whether or not the frequency and the number of MPCs are changed in tissues or organs other than the peripheral blood.

Recently, Liu et al. showed that MPCs could be mobilized to the peripheral tissue when rats were exposed to hypoxic conditions, and this hypoxia-induced MPC mobilization was caused by the elevation of plasma CXCL12 levels and BM VEGF levels [36]. Under our conditions, however, plasma CXCL12 levels and the BM VEGF levels in Ad-VEGF-injected mice were almost equal to those in Ad-Null-injected mice (Figs. 1b and 3b), suggesting that the mechanisms of decreased number of BM MPCs in Ad-VEGF-injected mice would be different from those of hypoxia-induced MPC mobilization.

Consistent with previous reports [15], we confirmed the HSPC mobilization from BM into the circulation by VEGF overexpression using an Ad vector system (Fig. 1). On the other hand, a previous report was that administration of a recombinant VEGF protein into mice failed to induce the HSPC mobilization [37]. In our Ad vector systems, plasma VEGF levels were maintained at 400–600 ng/mL on day 3–5 after single intravenous injection. Although we do not know the VEGF levels in the plasma under their experimental protocols, plasma VEGF levels might not be sufficient for HSPC egress from the BM, because exogenous VEGF levels in the plasma were rapidly decreased after administration of a recombinant VEGF protein [23]. Therefore, this difference would be partly due to the difference in the plasma VEGF levels, and we concluded that an Ad vector system would be an appropriate one to estimate the in vivo physiological action of VEGF.

In summary, our results showed that plasma VEGF levels could regulate the distribution of BM HSPCs and MPCs, probably by a mechanism distinct from that of other mobilization factors, and we suggest that a reduction in the number of MPCs in the BM would be one of the mechanisms involved in VEGF-induced HSPC mobilization. Although further investigation of the BM environment will be needed to uncover the VEGF-mediated HSPC mobilization, our findings obtained in this study provide a novel insight into the mechanisms of HSPC mobilization and would be helpful in the development of new clinical mobilizing agents.

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Author Disclosure Statement

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