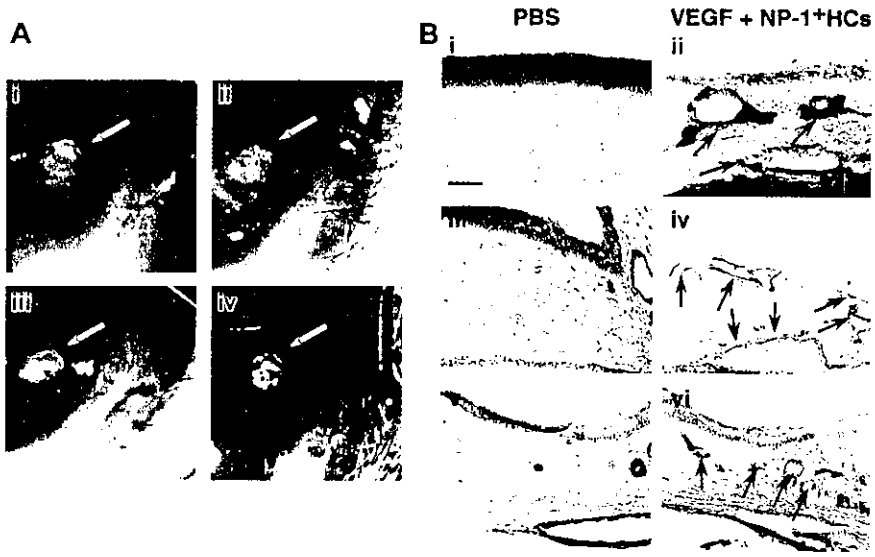

Figure 5. Hematopoietic cells expressing NP-1 induce angiogenesis in corneal neovascularization assay. (A) Gross appearance of neovascularization in the cornea. Pellets containing control buffer (i) or a low-dose VEGF (20 ng/mL) with $CD45^+NP\text{-}1^-$ hematopoietic cells (ii) did not induce corneal neovascularization; on the other hand, pellets containing a low-dose VEGF with $CD45^+NP\text{-}1^+$ hematopoietic cells (iii) or high-dose VEGF (100 ng/mL) (iv) induced corneal neovascularization. (B) Histologic analysis in mouse cornea. Sections were stained with anti–PECAM-1 (i-ii), anti–VEGFR-2 (iii-iv), or anti–VE-cadherin (v-vi) antibody, and positive cells are visualized as dark blue products. Pellets containing a low dose of VEGF and $CD45^+$ cells (ii,iv,vi) induced the blood vessel formation composing with PECAM-1^+ (ii), VEGFR-2^+ (iv), or VE-cadherin$^+$ (vi) ECs into cornea; however, pellets containing PBS alone did not (i,iii,v). Arrows indicate newly formed vessels into cornea. Scale bar indicates 50 μm.

cells (data not shown). Therefore, it seems that membrane-bound NP-1 on hematopoietic cells stimulates VEGFR2 on ECs.

In this study, we used the $B220^+$ B-lymphocyte lineage for the analysis of binding with $VEGF_{165}$ and phosphorylation of VEGFR-2. The reason we selected this lineage is that this lineage expresses NP-1 but does not express other VEGF receptors, which was confirmed by RT-PCR analysis. If we had used a myeloid lineage ($Mac\text{-}1^+$) or erythroid lineage ($Ter119^+$), the abundant endogenous production of VEGF might alter the phosphorylation of VEGFR-2. However, such myeloid and erythroid cells also express NP-1 on their surface (Figure 1A); therefore, they might be able to effectively support vascular development through an autocrine loop in vivo.

The other question that we have to address is the effect of NP-1 on hematopoiesis, because NP-1 expression is observed in mature hematopoietic lineages other than hematopoietic stem cell populations during embryogenesis. The number of hematopoietic cells and maturation of hematopoietic cells in the fetal liver of NP-1 mutant embryos, however, did not differ from those in the wild-type embryos (data not shown). Moreover, we analyzed the role of NP-1 in hematopoiesis using hematopoietic cells from wild-type and NP-1 mutant embryos in an in vitro colony assay (culture colony-forming unit [CFU-c]), and we did not detect a large difference in the number of CFU-c or any difference in lineage commitment (data not shown). Therefore, we concluded that the NP-1 on hematopoietic cells does not alter hematopoiesis, at least during embryogenesis. Recently, it was shown that NP-1 was expressed by human dendritic cells (DCs) and T cells both in vitro and in vivo.[37] The initial contact between DCs and T cells led to NP-1 polarization on T cells and induced proliferation of T cells.

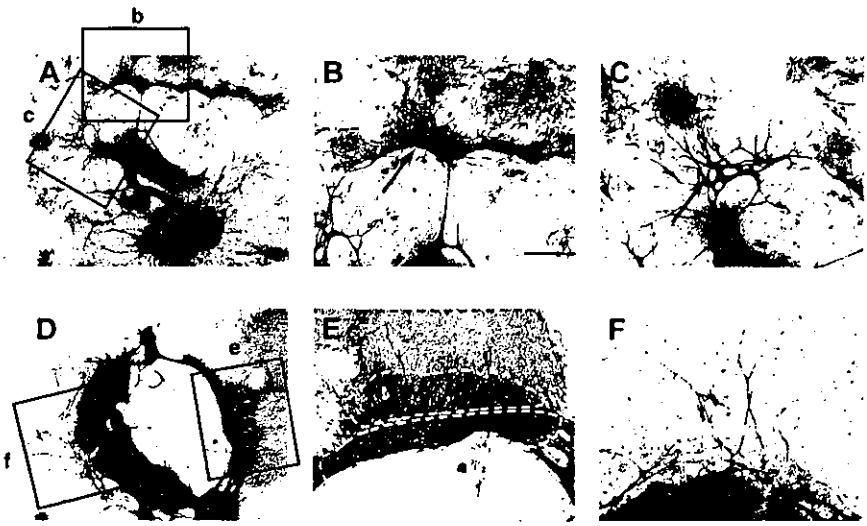

Figure 6. Dimerization of NP-1 is important for inducing endothelial cell growth. ECs developed in P-Sp cultures were stained with anti–PECAM-1 mAb. PECAM-1^+ cells are visualized as dark blue products. Upon the addition of L cells that possessed only the "a" domain (data not shown) or "a" and "b" domains of the NP-1 protein (A-C) to an $NP\text{-}1^{-/-}$ P-Sp culture (2×10^3 cells per well) on the fourth P-Sp culture day, the defective vascularity was not rescued at either the areas that were adherent to the L cells (red dashed line [B]) or the areas that were not adherent to the L cells (C). Small numbers of ECs make cordlike structures, indicated by arrow in panels B and C. Panels B and C are higher magnifications of the areas indicated by the boxes in panel A. On the other hand, upon the addition of L cells that possessed the "a," "b," and "c" domains (D-F) to an $NP\text{-}1^{-/-}$ P-Sp culture (2×10^3 cells per well) on the fourth P-Sp culture day, the defective vascularity was rescued at the areas that were adherent to the L cells (red dashed line [E]); however, it was not rescued at the areas that were not adherent to the L cells (F). Sheetlike structures of ECs (white dashed line) and network formation of ECs (yellow dashed line) are observed (E). Panels E and F are higher magnifications of the areas indicated by the boxes in panel D. Scale bar indicates 400 μm (A,D) and 200 μm (B-C,E-F).

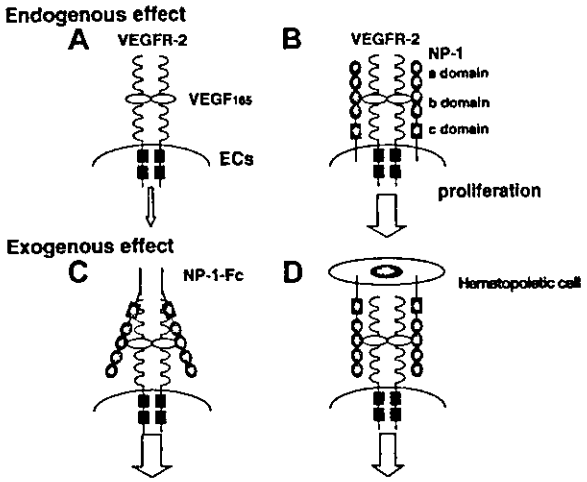

Figure 7. Effect of NP-1 expressed on ECs and non-ECs on the proliferation of ECs. The proliferation of ECs expressing NP-1 together with VEGFR-2 (B) is enhanced compared with that of ECs expressing VEGFR-2 alone (A). Soluble clustered NP-1 effectively enhances the signaling of VEGFR-2 (C). These findings suggest that hematopoietic cells expressing NP-1 also enhance the signaling of VEGFR-2 on ECs (D).

In our experiments, abundant expression of NP-1 was detected in $CD45^+B220^+$ cells (B-cell fraction); on the other hand, T cells ($CD45^+CD4^+/CD8^+$) in fetal liver did not express NP-1 highly. By contrast, in adult bone marrow, abundant expression of NP-1 was detected in T-cell fractions (data not shown). Therefore, correlation between expression and function of NP-1 on T and B cells should be addressed in the future.

It has been reported that the mesenchymal cells surrounding ECs[18] and stromal cells[22] also express NP-1. Moreover, such mesenchymal cells are one of the major sources of VEGF production. This indicates that mesenchymal cells that coexpress VEGF and NP-1 enhance vascular development locally. This hypothesis is supported by 2 analyses reported by Kitsukawa et al[18] and the present results. When NP-1 was overexpressed under the transcriptional control of the actin promoter, excess capillaries and blood vessels were observed in such transgenic mice.[18] Our study also showed that ectopic overexpression of NP-1 on OP9 stromal cells enhanced vascular development in P-Sp culture (data not shown). Taken together, the mesenchymal cells surrounding a blood vessel are one of the key regulators of normal vascular development. However, our discovery that the NP-1 on cells other than ECs has the capacity to induce vascular development came from in vitro findings. Therefore, it is not clear whether this effect of NP-1 is actually involved in normal vascular development. The best way to observe the function of NP-1 on nonendothelial lineages, especially hematopoietic cells, is to construct and analyze the vascularity in mice harboring an *NP-1* gene with a mutation in hematopoietic cells alone using a promoter gene that is specifically expressed in hematopoietic cells. However, such promoter gene is not currently available; therefore, instead of gene targeting, we are currently planning to transplant NP-1 null hematopoietic cells from $NP\text{-}1^{-/-}$ fetal liver into irradiated recipient mice. Using these chimeric mice having $NP\text{-}1^{-/-}$ hematopoietic cells, we will study whether NP-1 on hematopoietic cells contributes to new vessel formation under physiological conditions such as wound healing and ontogeny of tumor.

Recently, double NP-1/NP-2 knock-out mice have been reported to show more severe defects of vascular formation than other NP-1 or NP-1 single knock-outs.[38] Although in this study we did not examine the role of NP-2 in VEGF-induced angiogenesis, a recent report showed that VEGF-induced cellular response was lacking in ECs expressing NP-2 alone.[39] In addition, NP-2 seems to have a minor contribution in embryonic vasculogenesis and angiogenesis, because NP-2 expression is not detected in the heart or capillaries of embryo[40] and NP-2 knock-out mice are viable and fertile.[41] Moreover, in NP-1 knock-out mice, the NP-2 expression was not compensated in hematopoietic cells (data not shown). However, the precise role of NP-2 in angiogenesis is still unknown. Therefore, effects for vascular formation by hematopoietic cells from single NP-1 or NP-2 knock-out mice or double NP-1/NP-2 mutant mice should be compared in the future. Finally, we suggest that induction of the *NP-1* gene in particular locations might be a useful gene therapy in concert with VEGF administration for ischemic diseases.

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Hematopoietic stem cells: generation and manipulation

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Hematopoietic stem cells (HSCs) are at the forefront of both basic stem cell research and clinical applications. Regenerative medicine has recently become a viable form of therapy and can potentially cure several diseases. The generation of blood cells from embryonic stem cells and the manipulation of HSCs continue to provide insights into other stem cell systems. The importance of HSCs as a model of an ideal source for cell therapy is increasing.

Since its inception in the 1960s, the hematopoietic stem-cell (HSC) research community has accumulated a tremendous amount of knowledge regarding stem-cell systems, as well as developing a large number of techniques with uses for both basic research and clinical application [1]. HSCs are defined as self-renewing cells with the capacity to differentiate into any of more than eight distinct blood-cell lineages [2,3]. The most stringent way to evaluate the characteristic abilities of HSCs is by transplantation into myeloablated animals. Colony-forming units of the spleen (CFU-S), that is, cells that produce macroscopic hematopoietic-cell colonies in the spleens of lethally irradiated mice, were initially regarded as HSCs. Later, long-term repopulating (LTR) cells, which can reconstitute hematopoiesis over the long-term, were recognized as more immature cells and were thought of as real HSCs. Mouse LTR cells can be purified using a cell sorter and monoclonal antibodies and are the only stem cells that can be purified as a single cell type without contaminating cells.

From the standpoint of hematopoietic development, there exist two types of LTRs, the adult HSC and the embryonic HSC in mouse development [4,5]. Adult HSCs emerge in the AGM (aorta, gonads, mesonephros) region around embryonic day 10 (E10) and possess the ability to reconstitute the hematopoiesis of lethally irradiated adult mice. By contrast, embryonic HSCs develop in the yolk sac at E9. The embryonic HSCs can reconstitute hematopoiesis after the injection to neonatal mice but the cells cannot reconstitute the irradiated adult host. Although these two mouse HSCs are considered distinct cells, differences other than repopulating ability are not yet clear. Also, none of this information is available about human embryonic HSCs. As for adult HSCs, human HSCs are considered as essentially the same as mouse HSCs, although there has been no robust proof. However, it is impossible to examine the repopulating activity of human HSCs experimentally by syngeneic or allogenic transplantation. For *in vivo* analysis of human HSCs, transplantation into immunodeficient NOD (non-obese diabetic) or SCID (severe combined immunodeficiency) mice is well used. It should be mentioned again that the cells detected by transplantation into the NOD or SCID mice might not be precisely equal to human HSCs.

Compared to *in vivo* transplantation, *in vitro* experimental systems are insufficient to establish the self-renewing capacity of HSCs. Although it is difficult to induce the *ex vivo* expansion of HSCs simply by adding cytokines [6], information regarding the molecules that induce the self-renewal of HSCs has been reported recently and is discussed later. In mice, the long-term maintenance of stem cells can be achieved by co-culture of HSCs on a stromal-cell layer [7,8]. Stromal cells can be used not only for maintaining hematopoietic cells but also for inducing hematopoietic development from embryonic stem (ES) cells.

Development of lympho-hematopoietic cells from ES cells

Mouse ES cells are pluripotent cells that can differentiate *in vivo* into any somatic lineage cell or germ cell [9]. Although the *in vitro* differentiation ability of the ES cells is rather limited, their *in vitro* differentiation into several kinds of cell, such as blood cells, cardiomyocytes, endothelial cells, neuronal cells, glial cells, insulin-secreting cells and oocytes, has been reported. In most cases, differentiation from ES cells is achieved by the combination of relatively random differentiation and selection to specific lineages. From this point of view, differentiation 'induction' from ES cells might not be an appropriate semantic word usage. In this Review, however, 'induce' and 'induction' are adopted for convenience and because generally used. One conventional and frequently used method for induction is embryoid body (EB) formation [10]. When ES cells are cultured in suspension without leukemia inhibitory factor (LIF; the cytokine necessary to keep mouse ES cells immature), the cells aggregate, development proceeds to some extent and EBs appear. Inside EBs, various lineages of cells develop and the addition of the appropriate cytokines facilitates hematopoietic development. Not only mouse but also human ES cells give rise to hematopoietic differentiation with EB development in the presence of

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bone morphogenetic protein-4 (BMP-4) and hematopoietic cytokines [11].

Another induction method is the co-culture of ES cells with a stromal cell line. OP9 stroma cells that are deficient in macrophage-colony stimulating factor (M-CSF) induce efficient differentiation; this induction method was named the OP9 system [12,13]. Although lymphoid lineage cells are difficult to induce with the EB method under normal culture conditions [14], the OP9 system efficiently gives rise to pre-B cells with characteristics equivalent to those of bone marrow cells [15]. Simple co-culture cannot give rise to T lymphoid cells but T lymphocytes are generated from the pre-hematopoietic progenitors produced by the OP9 system after the maturation step in fetal thymic organ cultures [16]. It was recently found that the OP9 system also induced mesodermal cells and hematopoietic cells from cynomolgus monkey ES cells. Moreover, the induced cells could be transplanted into fetal sheep where they contributed significantly to hematopoiesis (Y. Hanazono *et al.*, pers. commun.). Mouse stromal cells are also effective for hematopoietic induction from human ES cells [17]. Although normal stroma cells were used in this study, we, and others, have discovered that M-CSF has some deleterious effects on *in vitro* hematopoietic development [12,18,19]. It is likely that hematopoietic differentiation from human ES cells would be induced more efficiently with the OP9 system.

Development of HSCs from ES cells

Lymphopoiesis can be reconstituted by ES-derived hematopoietic cells [14,20,21]. However, neither the EB formation method nor the OP9 system gives rise to transplantable HSCs from mouse ES cells (Figure 1). To improve the hematopoietic activity of the induced hematopoietic cells, several attempts at genetic manipulation have been performed using, for example, transcription factors, such as Lhx-2 and GATA-2, and the oncogenic chimeric gene *bcr-abl*. These genes produced limited improvement in the hematopoietic activities of the ES-induced cells. For example, the overexpression of Lhx-2 in a retrovirus vector gave rise to stem-cell factor (SCF)-dependent hematopoietic progenitor lines that could differentiate into different blood cell types with addition of the appropriate cytokines [22]. The expression of GATA-2 in the tetracycline conditional gene expression system increased the number of hematopoietic progenitor cells by up to tenfold [23]. ES-derived hematopoietic progenitors containing retrovirally introduced *bcr-abl* repopulated irradiated animals, presumably by transforming the embryonic hematopoietic progenitors into adult HSCs [24].

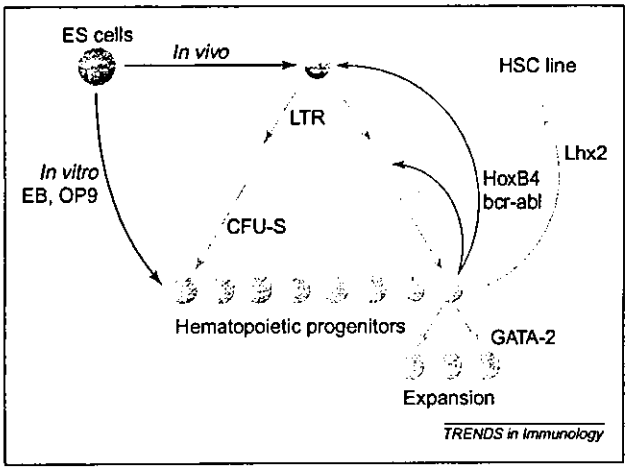

Figure 1. Differentiation induction of hematopoietic stem cells (HSCs) from embryonic stem (ES) cells and its genetic manipulation. ES cells give rise to hematopoietic progenitor cells *in vitro* by embryoid body (EB) formation and co-culture with OP9 stroma cells. However, neither long-term repopulating (LTR) cells nor colony forming units of the spleen (CFU-S) can be induced. Genetic manipulation can alter the hematopoietic activity of the ES-induced hematopoietic progenitors cells.

The most successful HSC production method involves the conditional expression of the *HoxB4* gene [25]. Previous studies suggested that HoxB4 could enhance the potential of hematopoietic cells. The retroviral-mediated overexpression of HoxB4 enhances hematopoietic repopulation, probably through enhancement of self-renewing activity, without inducing leukemia or abnormal differentiation [26–28]. In addition, the constitutive expression of HoxB4 enhanced the formation of immature mixed hematopoietic colonies [29].

The EB method of induction and the OP9 system were combined in the HoxB4 expression experiment [25]. The induction of differentiation was initiated with EB formation. Subsequent differentiation and/or maintenance of hematopoietic cells were achieved by co-culture with OP9. HoxB4 expression transformed ES cell-derived hematopoietic progenitors into HSCs and, surprisingly, the transient expression of HoxB4 for several weeks was sufficient for the conversion. That is, the overexpression of HoxB4 for several weeks in hematopoietic progenitors seemed to reprogram the differentiation program from hematopoietic progenitors to immature transplantable hematopoietic stem cells. The other possibility is that HoxB4 expanded the ES-induced HSCs, which have not been detected yet because of the low number of the cells.

Although transplantations into primary and secondary recipients were successful, it remains unclear whether the ES cell-derived transplantable cells are authentic LTR cells. LTR cells in the bone marrow have the ability to reconstitute the hematopoietic system over the long-term, even when only a single cell is transplanted. However, the percentage of the contribution by ES cell-induced hematopoietic cells to hematopoiesis in the HoxB4 experiment was low despite the transplantation of a relatively large number of cells (10^6). It is intriguing to speculate on the differentiation status of the hematopoietic cells induced by HoxB4; the cells could be HSCs, hematopoietic progenitors, cells intermediate between the two or cells with different characteristics. This experiment also raises the interesting possibility that hematopoietic progenitors might be able to remodel to HSCs during embryogenesis [5,30].

Molecules involved in HSC self-renewal

Transformation from hematopoietic progenitor cells to HSCs is equivalent to granting self-renewing activity to hematopoietic progenitor cells. Although gene-targeting analyses have revealed that many molecules are involved in the development and differentiation of hematopoietic

cells, only a few molecules are directly involved in the self-renewal of HSCs. The primary difficulty lies in defining the self-renewing activity of HSCs. Even if a gene is indispensable for hematopoietic development, it would not be essential for self-renewal [31]. However, a few genes seem to be involved in self-renewal.

Polycomb group (Pcg) proteins, which form DNA-binding protein complexes with gene-suppressing activity, are involved in lympho-hematopoiesis [32]. Mice with null mutations of some *PcG* genes, for example, *rae28*, *Bmi-1* and *Mel-18*, show hypoplasia of lympho-hematopoietic organs [33–35]. Among these genes, *Bmi-1* is a crucial regulator of the self-renewal of adult HSCs [36,37]. *Bmi-1* expression is detected in mouse fetal and bone marrow HSCs, as well as in human normal and leukemic stem cells, and its level of expression declines as differentiation progresses. In *Bmi-1* $^{-/-}$ mice, although the fetal liver contains a normal number of HSCs, the number is markedly reduced after birth and the HSCs can contribute to hematopoiesis only transiently after transplantation. These observations demonstrate that *Bmi-1* is not necessary for development but is crucial for the self-renewal of HSCs.

Because *rae28* also has important roles in the proliferation of HSCs [38], it is conceivable that some common downstream molecules are vital for the self-renewal of HSCs. Both $p16^{INK4a}$ and $p19^{ARF}$, negative regulators of the cell cycle, are strong candidates for such molecules. The levels of $p16^{INK4a}$ and $p19^{ARF}$ were increased in the bone marrow of adult *Bmi-1* $^{-/-}$ mice [36], and defective hematopoiesis in *Bmi-1* $^{-/-}$ mice was corrected by crossing with mutant mouse lines deficient in $p16^{INK4a}$ and $p19^{ARF}$ [39,40]. Other candidates include the homeobox genes because PcG genes suppress previously silenced homeobox genes and some members of the Hox gene family are also targets of Bmi-1. In *Bmi-1* $^{-/-}$ bone marrow cells, HoxB4 expression is not altered but the expression of HoxA9, which functions in determining hematopoietic-cell fate [36], is upregulated.

Another interesting example of HSC maintenance is a G1 checkpoint regulator, the cyclin-dependent kinase inhibitor $p21^{cip1/waf1}$ (p21) [41]. In *p21* $^{-/-}$ mice, the number of HSCs is increased in constitutive hematopoiesis but hematopoietic failure occurs under conditions of stress, such as myeloablative drug administration. HSC self-renewal failure has been observed during the serial transplantation of *p21* $^{-/-}$ cells. These observations suggest that p21 keeps the cell cycle status of HSCs quiescent and is important for the self-renewal of HSCs.

Considering the multitude of biological and ethical challenges facing the use of human ES cells at present, expansion of HSCs represents a more realistic and feasible goal than does *de novo* production of HSCs from ES cells [42]. Based on the rationale of HSC self-renewal, it is reasonable to consider whether manipulation of *Bmi-1* or *p21* could induce expansion of HSCs. Bmi-1, however, is not a good candidate for *ex vivo* expansion because the gene is highly leukemogenic [43]. However, the lentiviral introduction of anti-sense *p21* into human hematopoietic cells enhances the NOD or SCID repopulation capacity to some extent [44].

Ex vivo expansion of HSCs by genetic manipulation

After bone marrow transplantation, HSC expansion of ~100-fold can be achieved [45]. Although the co-culture of murine HSCs with stroma cells can maintain HSC activity over the long-term, the efficacy of *ex vivo* expansion is rather poor compared to *in vivo* expansion. Assorted signaling molecules and transcription factors have been introduced into HSCs to induce *ex vivo* expansion or immortalization of cells. However, many attempts were unsuccessful because of the poor expansion of HSCs or the undesirable induction of a leukemic phenotype. HoxB4 brought about the best results in HSCs as well as ES-induced hematopoietic cells [28].

The Hox family of transcription factors is reported to be involved in both normal and leukemic hematopoiesis. The functions of several *Hox* genes, especially those genes highly expressed in immature hematopoietic cells (e.g. *HoxA9*, *A10*, *B3* and *B4*), have been studied extensively. The overexpression of each *Hox* gene provides a different effect on lympho-hematopoiesis. For example, HoxB3 impairs lymphoid development but enhances myeloid cells [46]; HoxB4 enhances the *in vivo* repopulating activity of HSCs [26,27]. HoxB4 also induces a 40-fold *ex vivo* expansion of mouse HSCs in cultures containing interleukin-3 (IL-3), IL-6 and SCF [28]. HoxB4 enhances HSC proliferation or the probability of self-renewal, although the gene is not leukemogenic. Overexpression of *HoxC4*, a paralog of *HoxB4*, also induces *ex vivo* expansion of human immature hematopoietic progenitors [47]. These findings suggest the importance of the fourth paralogous group of *Hox* genes for HSC expansion. Although there seem to be no obvious hematological abnormalities in the mice deficient in HoxB4 alone, HSCs and progenitor cells deficient in HoxB3 and HoxB4 displayed impaired activity both *in vitro* and *in vivo* [48]. Interestingly, PBX1, a transcriptional partner of HOX proteins, is dispensable for the HoxB4-induced HSC expansion [49]. It is possible that HoxB4 (and presumably HoxB3 and HoxC4) activates a set of genes that are not involved in HSC self-renewal under physiological conditions. Further evidence, such as enhanced HoxB4 expression by thrombopoietin and the effect of HoxB4 on human HSCs, imply the significance of HoxB4 in HSC activities [50,51].

Two other genes have succeeded in expanding the range of HSCs that could be transplanted without causing leukemia. One is *Lhx2*, a LIM domain-containing transcription factor. HSC cell lines were established from bone marrow HSCs by retroviral introduction of *Lhx2* in the presence of IL-3, IL-6, and SCF. The cells gave rise to mature blood cells in not only primary, but also secondary and tertiary, recipients [52]. Although Lhx-2-targeted mice showed defective hematopoiesis, the abnormality was not due to hematopoietic cells, but was due instead to non-hematopoietic stromal cells [53]. It is likely in this case that overexpression of Lhx-2 activated genes that are irrelevant to the physiological role of Lhx-2.

The other gene that has been used to establish HSC lines is the dominant active form of *Notch* [54]. The Notch pathway regulates cell-fate determination in a wide variety of cell lineages, including neurogenesis, eye development,

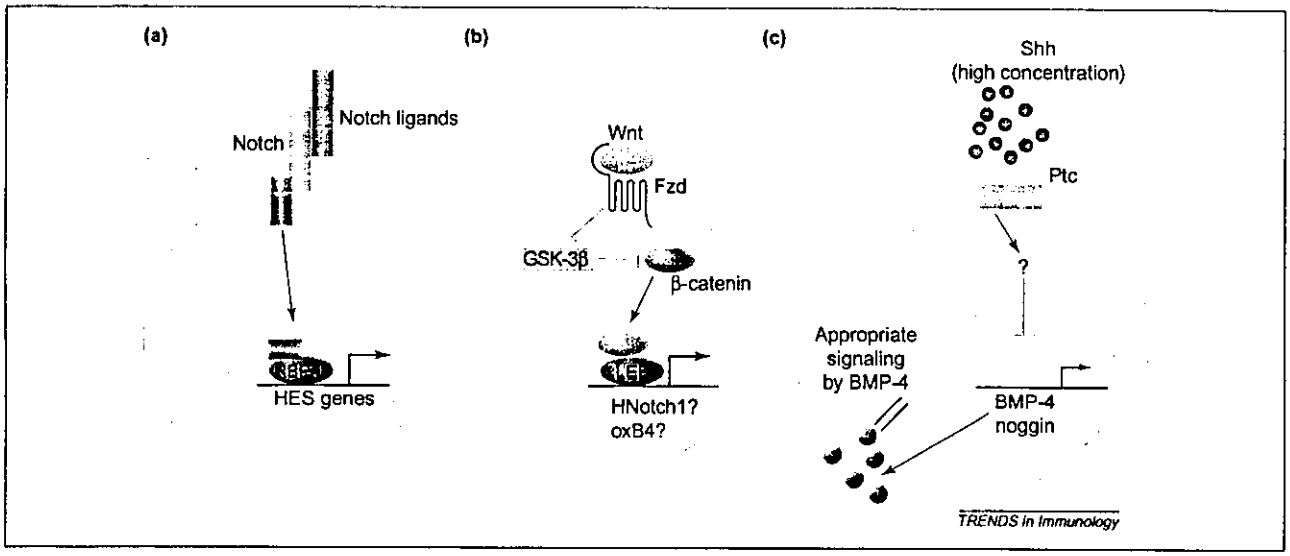

Figure 2. Three important signaling pathways involved in *ex-vivo* expansion of hematopoietic stem or progenitor cells. **(a)** Notch and Notch ligands. Various Notch ligands enhance the self-renewal of hematopoietic cells. Hematopoietic stem cell (HSC) lines can be established by the dominant active form of Notch. **(b)** Wnt. Wnt 3 enhances the self-renewal of HSCs from Bcl-2 transgenic mouse. Similarly, overexpression of β-catenin induces the self-renewal of HSCs. **(c)** Sonic hedgehog (SHH). SHH modifies the expression of bone morphogenetic protein-4 (BMP-4) and its inhibitor noggin through the SHH receptor, patched (Ptc). Self-renewal of hematopoietic cells is achieved by appropriate signaling of BMP-4 produced by the balance of BMP-4 and noggin. Abbreviations: GSK-3, glycogen synthase kinase-3; RBP-J, recombination signal sequence-binding protein-J.

myogenesis and lymphopoiesis [55–57]. The Notch pathway is evolutionally conserved from *Caenorhabditis elegans* to humans and often controls the differentiation program by balancing differentiation and self-renewal through cell–cell interactions. There are four Notch family members, Notch 1 through 4, in mammals. Notch signaling enhances the *in vitro* generation of human and mouse hematopoietic precursors, determines T and B lineage specification, and promotes the expansion of $CD8^+$ T cells [55,56]. Retroviral overexpression of a dominant active form of Notch established immortalized HSCs that were dependent on a cytokine cocktail of SCF, IL-6, IL-11 and Flt3 ligand. The cell line differentiated into both lymphoid and myeloid cells after transplantation. These results show that signaling by Notch ligands could be a regulator of the self-renewal of HSCs.

Ex vivo expansion of HSCs by extracellular signaling

The use of extracellular signaling molecules to enhance self-renewal is ideal for clinical applications because it avoids retroviral gene introduction, which is potentially leukemogenic. Recent studies have implied that some extracellular signaling molecules can induce *ex vivo* expansion of HSCs (Figure 2). The modulation of Notch signaling would have some effects on the enhancement of HSCs. There are six Notch ligands in vertebrates: Delta-like 1 through 4 and Jagged 1 and 2. The effects of the Notch ligands Delta-like 1 and 4 and Jagged 1 have been analyzed [58–63]. In general, Notch ligands enhance the repopulating activity of hematopoietic cells. However, *ex vivo* expansion has not been definitively proven.

Wnt signaling is involved in several events during animal development, including the proliferation of stem cells and the determination of mesodermal-cell fate, both of which are important for the establishment of the hematopoietic system. There are many Wnt molecules in the mouse and at least two members, Wnt3a and Wnt5a, have augmenting effects on hematopoietic cells [64–66]. Although Wnt5a-conditioned medium did not show any effect on hematopoietic cells *in vitro*, the *in vivo* administration of Wnt5a-conditioned medium increased the repopulation of human HSCs in NOD or SCID mice. By contrast, Wnt3a enhanced the *in vitro* self-renewal of mouse HSCs by roughly tenfold in the presence of SCF. Furthermore, the overexpression of β-catenin, one of the central molecules of the Wnt signaling pathway, upregulated the expression of two important molecules in the self-renewal of HSCs, HoxB4 and Notch1. The possible significance of Wnt signaling is exciting but it should be noted that this result was primarily obtained with the HSCs of Bcl-2 transgenic animals, in which apoptosis of HSCs was prevented [64,65]. And the crucial competitive repopulation assay of HSCs expanded with Wnt3a has not been reported. Thus, *ex vivo* expansion of normal HSCs by Wnt3a should be regarded as not yet definitively demonstrated.

The Hedgehog family of proteins is involved in the organization of early mesoderm and embryonic specification of non-hematopoietic tissue. Sonic hedgehog (Shh), one of three mammalian hedgehog proteins, induces the expansion of pluripotent human hematopoietic-repopulating cells in NOD or SCID mice [67]. Noggin, a specific inhibitor of BMP-4, inhibits Shh-induced proliferation, suggesting that Shh acts through downstream BMP signals. Different combinatorial cocktails of cytokines, including SCF, interleukins (IL-1, -3 and -6), Flt3 ligand, thrombopoietin (TPO) and granulocyte-colony stimulating factor (G-CSF), have been used in attempts to expand human HSCs *ex vivo* (these were summarized in a previous comprehensive review [68]). Recently, novel transmembrane protein mKirre, which supports HSCs, has been cloned from OP9 cells with a signal sequence trap

Table 1. Molecules possibly involved in hematopoietic stem cell (HSC) self-renewal

Molecules	Category	Evidence	Refs
Wnt	Soluble factor	Self-renew of Bcl-2 transgenic mouse HSC *in vitro*	[64,65]
		Enhancement of human HSC in SCID (severe combined immunodeficiency)/NOD (non-obese diabetic) mouse	[66]
Shh	Soluble factor	Self-renew of human HSC or progenitors through bone morphogenetic protein (BMP) signaling	[67]
LIF (leukemia inhibitory factor)	Soluble factor	Reduction of HSCs in knockout mouse	[70]
IL-6	Soluble factor	Proliferation of hematopoietic cells in collaboration with the soluble interleukin-6 (IL-6) receptor	[62]
Notch ligands	Trans-membrane protein	Proliferation of hematopoietic progenitors	[56–63]
Mkirre	Trans-membrane protein	Maintenance of HSCs	[69]
gp130	Receptor	Essential for self-renewing division in conjunction with other cytokines	[71,72]
c-mpl	Receptor	Reduction of HSCs in knockout mouse	[73]
Notch	Receptor	Establishment of HSC line by a dominant active form	[52–55]
β-catenin	Cytoplasmic protein	Self-renew of HSCs	[64]
HoxB4	Transcription factor	Self-renew of HSCs	[28]
		'Re-programming' from hematopoietic progenitors to HSCs	[25]
Bmi-1	Chromatin or gene silencing	Lack of HSC self-renewal activity in knockout mouse	[36,37]
rae28	Chromatin or gene silencing	Reduction of HSCs in knockout mouse	[38]
p21	Cell cycle regulator	Failure of HSC maintenance in knockout mouse	[41]

method [69]. Other yet unknown molecules could have crucial roles in hematopoietic-cell maintenance. Molecules that are potentially involved in HSC expansion are listed in Table 1.

Perspective

A limited number of signaling pathways governs the generation of many cell types and patterns in animals. Most cell–cell interactions during embryonic development involve the Hedgehog, Wnt, BMP, Notch, JAK (Janus family kinase)–STAT (signal transducer and activator of transcription), receptor tyrosine kinase (e.g. SCF–c-Kit), and nuclear hormone pathways. In addition, Homeobox genes have major roles in body patterning. From an evolutionary point of view, it is neither surprising nor coincidental that all of these signaling pathways are involved in the maintenance and proliferation of HSCs. Because the majority of important molecules belonging to these signaling pathways seem to have been identified, it is reasonable to search within these signaling systems for the molecules involved in providing the characteristics of HSCs. The combinatorial regulation of known signaling molecules is a crucial next step in these studies. Although it has been suggested that progress in HSC research over the past five years has been slower than that in other stem-cell systems, such as neuronal and mesenchymal stem cells, this is incorrect. Studies of the generation and manipulation of HSCs will continue to provide new insights into many stem-cell systems.

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Plenary paper

A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow

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To measure the ability of human hematopoietic stem cells (HSCs), the SCID-repopulating cell (SRC) assay has been widely used. Conventionally, human HSCs are transplanted into a nonobese diabetic/severe combined immunodeficient (NOD/SCID) mouse via a tail vein. However, those cells must go through various obstacles until they reach the mouse marrow environment, which could explain the generally low homing efficiency in this system. Thus, the capability of HSCs may not be studied accurately by this intravenous transplantation method. In our attempt to reveal actual SRC potential, ie, self-renewal and multilineage differentiation in recipient bone marrow, we introduced cells into mouse marrow directly (intrabone marrow [iBM]) to minimize the effect of factors that may interfere with the homing of HSCs and compared the results obtained by intravenous and iBM methods. When cord blood $CD34^+CD38^-$ cells were transplanted in NOD/SCID mice by iBM, a 15-fold higher frequency of SRC, 1 in 44 $CD34^+CD38^-$ cells, was achieved compared with 1 in 660 by the intravenous method. Furthermore, the iBM transplant showed high levels of engraftment in the secondary transplantation. Pretreatment of $CD34^+$ cells with antibodies that block either very late antigen 4 (VLA-4) or VLA-5 reduced engraftment partially, whereas blockage of both molecules resulted in complete inhibition of engraftment, which suggests that VLA-4 and VLA-5 are involved in different processes in engraftment or have complementary roles. Our results indicate that the iBM injection strategy is a more sensitive and direct way to measure the capability of human SRCs and is useful to investigate the interaction of HSCs and marrow environment in vivo. (Blood. 2003;101: 2905-2913)

Introduction

Measurement of human hematopoietic stem cell (HSC) activity has been greatly facilitated by the development of xenotransplantation assay. Especially, nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice have proven to be a reliable recipient for detecting human hematopoietic-repopulating cells that differentiate into multilineage mature cells and self-renew in mice.[1-3] Human hematopoietic-repopulating cells identified in this assay, operationally defined as SCID-repopulating cells (SRCs), have been shown to be enriched among an extremely rare $CD34^+CD38^-$ subfraction of lineage-depleted ($Lin^{-/low}$) cells, and the frequency of SRCs was 1 in 617 $Lin^{-/low}CD34^+CD38^-$ cord blood (CB) cells.[4]

This assay, however, quite possibly underestimates human hematopoietic-repopulating cell frequencies. When candidate human stem cells are transferred by intravenous injection, cells travel into the right atrium, ventricle, and lungs in which most of the cells are trapped, then to the systemic circulation and lodge in organs according to organ blood flow; therefore, only a small fraction of injected cells can lodge in bone marrow (BM).[5-8] The marrow seeding efficiency of murine repopulating stem cells was reported around 20%,[9-11] and that of human stem cells in the sublethally irradiated NOD/SCID mouse was even lower than in the syngeneic murine situation. van Hennik et al[12] demonstrated that the seeding efficiencies of human CB $CD34^+$ cells in NOD/SCID mice were 4.4% by week 6 cobblestone area–forming cell (CFAC) assay and 2.3% by flow cytometry analysis. Cashman and Eaves[13] reported that the proportion of total injected human CB competitive repopulating units (CRUs) in the marrow of mice was 7%, as determined by limiting-dilution assays in NOD/SCID mice.

To exclude stem cell homing interference (eg, stem cell trapping in lung and/or liver, transendothelial migration step) and focus on the phases of the stem cell and BM stromal cell interaction, we carried out direct injection of human hematopoietic cells into mouse BM (intra-BM [iBM] injection), as previously reported by Kushida et al,[14] and compared it with the SRC assay conducted by intravenous injection. IBM injection is revealed to be sensitive and adequate means to measure human HSC capability and enabled us to investigate the interaction of HSCs and marrow environment in vivo directly.

Materials and methods

Collection and purification of human CB $CD34^+$ cells

CB samples were obtained from full-term deliveries according to the institutional guidelines approved by Tokai University Committee on Clinical Investigation. Mononuclear cells (MNCs) were isolated by Ficoll-Hypaque (Lymphoprep, 1.077 ± 0.001 g/mL; Nycomed, Oslo, Norway) density gradient centrifugation. Cells were washed and suspended in

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phosphate-buffered saline (PBS) containing 0.1% of human serum albumin (HSA; Sigma, St Louis, MO). $CD34^+$ cell fractions were prepared from Ficoll-separated MNCs using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Sunnyvale, CA) according to the manufacturer's directions. For isolation of $Lin^{-/low}CD34^+CD38^-$ and $CD34^+CD38^+$ cells, $CD34^+$-enriched cells were stained with fluorescein isothiocyanate (FITC)–conjugated antilineage-specific antigens; CD3 (UCHT1), CD41 (P2), glycophorin A (11E4B-7-6) (all Coulter/Immunotech, Marseille Cedex, France), CD4 (SK3), CD7 (4H9), CD14 (MϕP9), CD16 (3G8), CD19 (SJ25C1) CD20 (2H7), CD33 (WM53), and CD56 (NCAM16.2) (all BD Biosciences, San Jose, CA), and phycoerythrin (PE)–conjugated anti-CD38 (HB7; BD Biosciences), phycoerythrin–Texas Red (ECD)–conjugated anti-CD34 (581; Coulter/Immunotech), and allophycocyanin (APC)–conjugated anti-CD45 (J.33; Coulter/Immunotech) monoclonal antibodies (mAbs). Cells were sorted using FACSVantage flow cytometer (BD Biosciences) equipped with HeNe and argon lasers. $CD38^-$ region was defined as below isotype control. The expression of CD34 and CD38 cell surface antigens on a representative $Lin^{-/low}$ population is shown in Figure 1A-B. $Lin^{-/low}CD34^+CD38^-$ cells, which comprise 5% to 8% of total $CD34^+$ population, was isolated with 97% to 99% (n = 16) purity using FACSVantage (Figure 1C).

NOD/SCID mice

Male or female NOD/Shi-*scid* (NOD/SCID) mice were obtained from CLEA JAPAN (Tokyo, Japan) and were maintained until use in the animal facility of Tokai University School of Medicine in microisolator cages. Mice were fed ad libitum with autoclaved food and water. All experiments were approved by the animal care committee of Tokai University.

Intrabone marrow or intravenous injection of human hematopoietic cells

NOD/SCID mice (7-9 weeks old) were irradiated with 300 cGy X-rays and thereafter received acidified water containing 1.1 g/L neomycin sulfate and 131 mg/L polymyxin B sulfate (Sigma). Within a few hours after irradiation, the mice were injected intravenously or by iBM with human CB $Lin^{-/low}CD34^+CD38^-$ cells along with 10^4 irradiated (15 Gy) $CD34^+CD38^+$ cells as carrier cells to support engraftment and expansion of immature $CD34^+CD38^-$ cells.[15,16] IBM injection was carried out as described previously with slight modifications.[14] In brief, a 29-gauge needle was inserted into the joint surface of the right tibia of anesthetized mice, and human hematopoietic cells in 40-μL suspension were injected into the BM cavity. For the punctures and injections, 1-mL insulin syringe with fixed 29-gauge needle (Terumo, Tokyo, Japan) was used. The advantage of this needle is the absence of dead space at the connection between the needle and the syringe, which minimizes the loss of samples.

For in vivo blocking experiments, column-enriched whole $CD34^+$ cells (> 95% purity, n = 10) were preincubated with 20 μg/mL antihuman-CXCR4 (12G5; BD Biosciences), antihuman–VLA-4 (HP2/1; Coulter/Immunotech), or antihuman–VLA-5 (SAM-1; Coulter/Immunotech) mAbs for 30 minutes on ice. After washing, 2×10^5 or 2×10^4 $CD34^+$ cells were injected into mice intravenously or by iBM, respectively. All blocking antibodies used in this study were nontoxic to human $CD34^+$ cells as determined by colony-forming assays and our stromal cell–dependent culture system as described previously (data not shown).[17] For cell surface analysis, column-enriched $CD34^+$ cells were again stained with APC-conjugated antihuman CD34 mAb and with FITC-conjugated antihuman–VLA-4, –VLA-5 mAbs, or PE-conjugated antihuman CXCR4 mAb. Cells labeled with mouse isotype control antibodies were used as negative control. Fluorescence-activated cell sorter (FACS) analysis was performed using FACSCalibur (BD Biosciences).

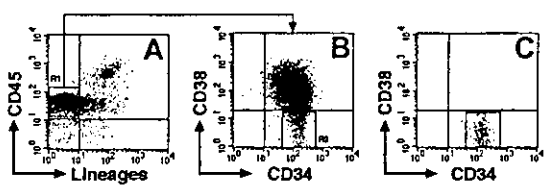

Figure 1. Representative FACS profile of sorted CB $Lin^{-/low}CD34^+CD38^-$ cells. (A) Column-enriched $CD34^+$ cells were stained with anti-CD45 mAb and antilineage-specific mAb cocktail and were gated on lineage marker–negative and/or low expression region (R1). (B) R1 cells were further gated on $CD34^+CD38^-$ region (R2). (C) Re-analysis of isolated $CD34^+CD38^-$ cells from R2 (99.8% purity).

Tracing of intrabone marrow–injected $CD34^+$ cells

Column-enriched CB $CD34^+$ cells (> 95% purity, n = 16), washed once with PBS, were suspended in PKH diluent, and the PKH26 dye (Sigma) at 20 μM was added. Cells were incubated at room temperature for 3 minutes with gentle agitation. Fetal calf serum (FCS; 2 mL) was added to cell suspension to stop the reaction, and then cells were centrifuged and washed twice with alpha-minimal essential media containing 10% FCS. PKH26-stained cells, 10^6 cells per animal, were injected iBM or intravenously into irradiated NOD/SCID mice. Aliquots of cells were reserved for staining control. At 5 minutes and 20 hours after transplantation, BM samples were aspirated from the human cell noninjected side tibia through the knee joint, as previously described.[18,19] Peripheral blood (PB) was aspirated from retroorbital sinus. After washing and hemolysis, samples were suspended in PBS containing propidium-iodide (PI) for flow cytometric analysis using FACSCalibur (BD Biosciences). Dead cells stained with PI were excluded from the analysis.

Flow cytometric analysis

Six to 9 weeks after transplantation, mice were killed, and BM, spleen, and PB were collected for analyzing the presence of human cells by flow cytometry. Human hematopoietic cells were distinguished from mouse cells by the expression of human CD45. BM cells were collected separately from tibia of human cell–injected side and noninjected side and suspended in PBS using a 27-gauge needle. Spleen was teased apart. PB was aspirated from retroorbital sinus. Samples were prepared as single cell suspensions in PBS containing 0.1% HSA and passed through a nylon filter to remove debris. Cells were stained with mAbs to human leukocyte differentiation antigens. FITC-conjugated antihuman CD34 (Coulter/Immunotech), CD14, CD19, CD33, CD56 (all BD Biosciences) mAbs, PE-conjugated antihuman CD38 (BD Biosciences), and APC-conjugated antihuman CD45 mAbs (Coulter/Immunotech) were used. Four-color flow cytometric analysis was conducted using FACSCalibur or FACSVantage (BD Biosciences). Quadrants were set to include at least 97% of the isotype-negative cells. Dead cells stained with PI were excluded from the analysis.

Analysis of human cell engraftment in mice receiving transplants by polymerase chain reaction (PCR)

Genomic DNA was isolated from the BM of mice receiving transplants by standard extraction protocols. DNA samples (100 ng) were subjected to PCR to detect a 1171-bp fragment of human chromosome 17-specific α-satellite using the primers: forward 5′-ACACTCTTTTTGCAGGATCTA-3′ and reverse 5′-AGCAATGTGAAACTCTGGGA-3′ under the following conditions: 94°C for 2 minutes (1 cycle); 94°C for 1 minute, 65°C for 1 minute, 72°C for 2 minutes (30 cycles); and 72°C for 7 minutes (1 cycle). PCR was performed using the RNA PCR kit (TAKARA SHUZO, Tokyo, Japan). PCR products were separated on 1.0% agarose gel and visualized by ethidium bromide staining. The level of human cell engraftment was determined by comparing the characteristic 1171-bp PCR product with that of human/mouse DNA mixture control (detection limit of 0.001% human DNA).

Analysis of the integration site of lentivirally marked $CD34^+$ cells

Transduction of enhanced green fluorescent protein (EGFP) encoded gene to $CD34^+$ cells by recombinant lentivirus infection was carried out as described previously.[20] Briefly, CB $CD34^+$ cells were prestimulated by incubating in StemPro-34 medium (Invitrogen, Carlsbad, CA) containing cytokines at 37°C in 5% CO_2 for 24 hours. Recombinant human thrombopoietin (TPO; 50 ng/mL; kindly donated from Kirin Brewery, Tokyo,

Japan), stem cell factor (SCF; 50 ng/mL; donated from Kirin Brewery), and Flk-2/Flt-3 ligand (FL; 50 ng/mL; R&D Systems, Minneapolis, MN) were used. Prestimulated CD34$^+$ cells were cultured for 12 hours under the same conditions in the presence of highly concentrated lentiviral supernatant at an MOI (multiplicity of infection) of 500. Lentiviral-infected CD34$^+$ cells were transplanted into irradiated NOD/SCID mice by iBM injection. Genomic DNA was extracted from the injected side tibia, noninjected side tibia, and spleen of mice receiving transplants at 8 weeks after transplantation. DNA (10 μg) was digested with *Eco*RI, which recognizes a unique site in viral genome, and was electrophoresed on 0.7% agarose gel. After transferring to nylon membranes, DNA was hybridized with ^{32}P-labeled random-primed EGFP probe.

Secondary transplantation

BM cells were obtained from tibiae and femurs of highly engrafted primary mice that received iBM transplants (23%-87% chimerism of human CD45$^+$ cells, n = 10) at 6 weeks after transplantation, and cells were injected intravenously or by iBM into irradiated secondary NOD/SCID recipients. Six weeks after transplantation, presence of transplanted human cells in recipient tibiae was analyzed by flow cytometry, as was described for primary recipients.

Statistical analysis

Engraftment was determined positive when more than 0.01% human CD45$^+$ cells in FACS or 0.01% human satellite PCR products in PCR were detected. For limiting dilution analysis, results of mice that received transplants scored positive by both methods were used. The data from several limiting dilution experiments were combined and used for analysis. The frequency of SRCs in the test BM sample was calculated by applying Poisson statistics to the proportion of negative recipients at different dilutions using L-Calc software (StemCell Technologies, Vancouver, BC, Canada). Data are represented as mean ± SD. The 2-sided P value was determined, testing the null hypothesis that the 2 population medians are equal. $P < .05$ was considered significant.

Results

Tracking intravenous or iBM transplanted human primitive hematopoietic cells

We first examined the distribution of infused human primitive hematopoietic cells. PKH26-stained CB CD34$^+$ cells (10^6 cells per mouse) were transplanted to irradiated NOD/SCID mice by direct injection into a BM cavity (iBM) or conventional tail vein infusion (intravenous). PB from retroorbital sinus and BM cells from noninjected side tibia were aspirated from each animal at 5 minutes and 20 hours after transplantation. The donor cells were detected by PKH26 fluorescence using FACS analysis (Figure 2A). As expected, PKH26$^+$ donor cells appeared in the blood stream of intravenously injected animals at 5 minutes (0.6% ± 0.39%) and, albeit fewer, at 20 hours after transplantation (0.047% ± 0.025%). However, in the BM, intravenously injected donor cells could be detected only at 20 hours after transplantation (0.078% ± 0.016%) (Figure 2B). Because one tibia contains approximately 5% of the total BM cellularity of a mouse,[21] a calculated seeding efficiency of intravenously injected PKH26$^+$ donor cells at 20 hours after transplantation is 1.56% ± 0.32% and is consistent with a previous report by van Hennik et al[12] However, in mice receiving iBM transplants, very few donor cells were present in the blood stream at both time points analyzed: 5 minutes (0.008% ± 0.008%), 20 hours (0.002% ± 0.004%). In addition, none, at 5 minutes, or very few, 0.006% ± 0.005% at 20 hours, of donor cells were detected in the noninjected side tibia (Figure 2B). The levels of iBM-transplanted PKH26$^+$ cells in PB and left (noninjected side) BM were significantly lower than those of intravenously transplanted cells on either time points ($P < .01$). The results confirmed that there was little, if any, leakage of the injected cells when iBM strategy was used.

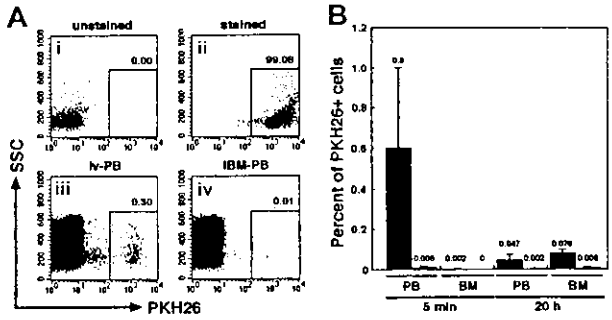

Figure 2. Tracing of intravenously or iBM-injected PKH26-labeled CB CD34$^+$ cells. (A) Representative FACS analysis of PKH26-labeled cells. Column-enriched CB CD34$^+$ cells (i) were stained with PKH26 (ii) and were transplanted into irradiated NOD/SCID mice by intravenous (iii) or iBM (iv) injection. Five minutes later, PKH26$^+$ cells circulating into peripheral blood stream were identified by flow cytometry. The number at each panel represents the percentage of PKH26 bright cells detected. (B) Comparison of the percentage of PKH26$^+$ cells detected in PB and BM from intravenous (filled bars; ■) or iBM (open bars; □) injected NOD/SCID mice at 5 minutes and 20 hours after transplantation. BM cells were aspirated from left (noninjected side) tibia. Fifty thousand events were acquired to calculate the proportion of PKH26$^+$ cells. Data shown are the mean ± SD values of 3 independent experiments (n = 5). The number above each bar represents the mean percentage of PKH26$^+$ cells.

IBM injection of CB Lin$^{-/low}$CD34$^+$CD38$^-$ cells resulted in the higher frequency of engrafting human hematopoietic cells in NOD/SCID mice

No or very little leakage of injected cells in iBM method may result in more efficient engraftment than intravenous transplantation. To compare the frequency of CB Lin$^{-/low}$CD34$^+$CD38$^-$ cells capable of engrafting NOD/SCID mice, cells were transplanted by iBM or intravenous injection for limiting dilution assay. In the human-specific PCR-based method, mice were scored positive for engraftment when more than 0.01% human DNA was detected in the murine BM (detection limit of human DNA is 0.001%) (Figure 3C). In FACS analysis, more than 0.01% of human CD45$^+$ dots was considered positive. Statistical analysis was performed using data from 84 recipients receiving transplants of pooled CB from multiple donors (Figure 3; Table 1), and the frequency of human cells capable of engraftment was calculated as described.[4,22] As determined by FACS and human DNA measurements, the frequency of CB Lin$^{-/low}$CD34$^+$CD38$^-$ cells capable of engrafting NOD/SCID mice was 1 in 44 by iBM injection (95% confidence intervals, 1 in 27 to 1 in 70). In contrast, the frequency by intravenous injection (95% confidence intervals, 1 in 289 to 1 in 1510) was 1 in 660 Lin$^{-/low}$CD34$^+$CD38$^-$ cells, which is consistent with the previous report by Bhatia et al.[4] By using the iBM transplantation method, more than 15-fold higher frequency of human SRCs could be detected in NOD/SCID mice.

Higher levels of human hematopoietic cell engraftment by iBM injection

The level of hematopoietic repopulation is a critical parameter in stem cell transplantation. Therefore, we compared the engraftment levels of mice that received transplants in both injection strategies using the same number of Lin$^{-/low}$CD34$^+$CD38$^-$ cells. Transplantation of CB Lin$^{-/low}$CD34$^+$CD38$^-$ cells into NOD/SCID mice by

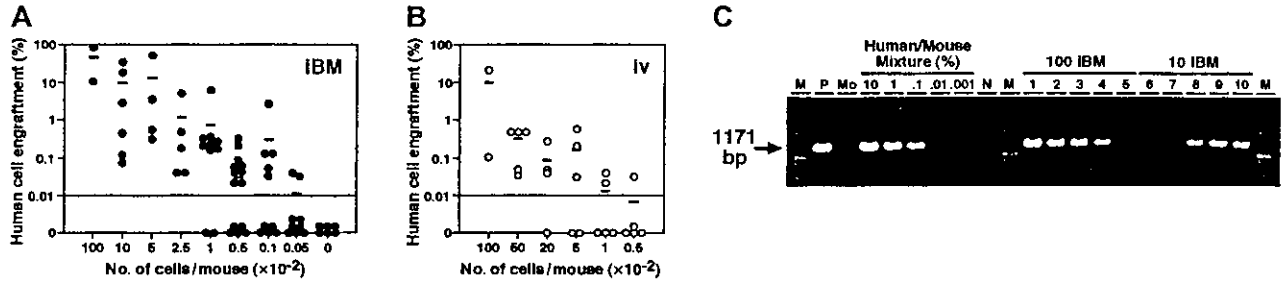

Figure 3. Summary of human cell engraftment levels in the BM of NOD/SCID mice. (A-B) NOD/SCID mice were given transplants of CB Lin$^{-/low}$CD34$^+$CD38$^-$ cell fractions by iBM (●) (A) or intravenous (○) (B). Eight weeks after transplantation, BM cells obtained from NOD/SCID mice were analyzed by FACS and PCR. Numbers indicate the dose of Lin $^{-/low}$CD34$^+$CD38$^-$ cells transplanted. Number "0" means mice were given transplants of 10^4 irradiated CD34$^+$CD38$^+$ carrier cells alone. Each dot represents 1 mouse, and bars indicate the average levels of engraftment. The horizontal lines indicate threshold of positive engraftment. (C) Representative PCR analysis of individual NOD/SCID mice given transplants of CB Lin$^{-/low}$CD34$^+$CD38$^-$ cells by iBM (lanes 1-5 indicate 100 cells injected, lanes 6-10 indicate 10 cells injected). DNA was extracted from the BM cells 8 weeks after transplantations and amplified using a human chromosome 17 α-satellite specific primer. Representative PCR analysis of 5 independent experiments is shown. M, size marker; P, 100% human DNA; Mo, 100% mouse DNA; N, distilled water (DW).

iBM injection achieved higher levels of engraftment than by intravenous injection in NOD/SCID mice (Figure 3; injected cell number, 500 cells, $P < .04$; 100 cells, $P < .03$; 50 cells, $P < .05$). Furthermore, the engraftment level of mice injected with 1000 cells by iBM was significantly higher than those injected intravenously with 2000 or 5000 cells (2000 cells, $P < .02$; 5000 cells, $P < .05$).

Multilineage differentiation and distribution of human hematopoietic cells in NOD/SCID mice that received transplants by iBM injection

Multilineage differentiation of iBM-injected SRCs was observed in the injected side BM of NOD/SCID mice that received transplants, which included lymphoid CD45$^+$CD19$^+$ B cells, CD45$^+$CD56$^+$ natural killer (NK) cells (Figure 4Aiii,v), and myeloid CD45$^+$CD14$^+$, CD45$^+$CD33$^+$ cells (Figure 4Aii,iv). In addition, presence of primitive Lin$^{-/low}$CD34$^+$CD38$^-$ cells were evident in the marrow of these mice (Figure 4Avi,vii). To examine the distribution of reconstituted human hematopoietic cells in iBM or intravenously injected NOD/SCID mice, BM cells were collected separately from injected (right leg) and noninjected (left leg) side of tibia. Lymphoid, myeloid, and stem/progenitor cell lineages are defined as CD19$^+$/CD45$^+$ cells, CD33$^+$/CD45$^+$ cells, and CD34$^+$/CD45$^+$ cells, respectively. As shown in Table 2, human hematopoietic cells with the ability of lymphomyeloid differentiation were detected not only in the injected side tibia but also in the noninjected side tibia, spleen, and PB in all animals analyzed (Figure 4B; Tables 2-3). Multilineage differentiation was also detected in mice with low chimerism of human cell (Table 2, mouse iBM-injected mice 1, 8, 9). In the spleen, lymphoid cells were 85.64% ± 6.31% (iBM) and 90.65% ± 5.91% (intravenous), and myeloid cells were 1.85% ± 1.13% (iBM) and 2.064% ± 1.59% (intravenous). In the PB, lymphoid cells were 71.19% ± 6.23% (iBM) and 74.84% ± 14.51% (intravenous), and myeloid cells were 4.16% ± 2.63% (iBM) and 8.21% ± 4.22% (intravenous) (data not shown). Although 10 times more CD34$^+$ cells were transplanted by intravenous injection (2×10^5 cells), the levels of engraftment were the same as the cases of iBM injection (2×10^4 cells) (Table 2; iBM injected right tibia, 45.51% ± 26.96% versus intravenously injected BM, 27.98% ± 13.56% [mean percentage

Table 1. Comparison of the frequency of SRCs by limiting dilution assay

Route	No. cells injected	No. engrafted mice	No. transplantations
iBM	10 000	2	2
	1 000	6	6
	500	4	4
	250	5	5
	100	8	10
	50	7	12
	10	5	10
	5	2	9
Intravenous	10 000	2	2
	5 000	5	5
	2 000	3	4
	500	3	5
	100	2	5
	50	1	5

NOD/SCID mice (n = 84) were transplanted with various numbers of Lin$^{-/low}$CD34$^+$CD38$^-$ CB cells by iBM or intravenous injection. Engraftment in murine BM was analyzed at 8 weeks. Mice were considered to be engrafted when they were determined positive by both FACS and PCR analysis. The frequency of engrafting cells in CB Lin$^{-/low}$CD34$^+$CD38$^-$ cells transplanted by iBM and intravenously was found to be 1 in 44 Lin$^{-/low}$CD34$^+$CD38$^-$ cells (95% confidence limits, 1 in 27 to 1 in 70) and 1 in 660 Lin$^{-/low}$CD34$^+$CD38$^-$ cells (95% confidence limits, 1 in 289 to 1 in 1510), respectively. SRC frequencies were calculated using Poisson statistics and the method of maximum likelihood with the assistance of the L-Calc software (StemCell Technologies).

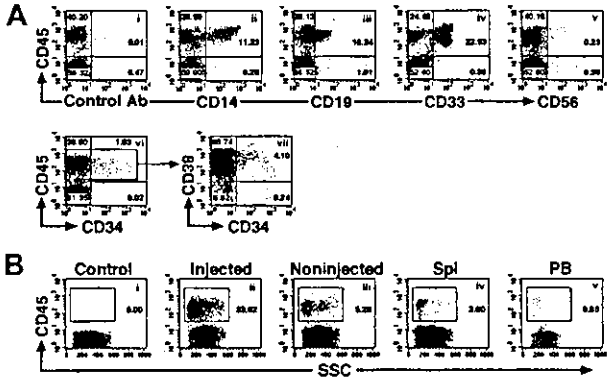

Figure 4. Representative FACS profile of human multilineage engraftment in a NOD/SCID recipient given transplants of Lin$^{-/low}$CD34$^+$CD38$^-$ cells by iBM. (A) At 8 weeks after transplantation of 500 Lin$^{-/low}$CD34$^+$CD38$^-$ human CB cells, BM cells were removed from iBM-injected side tibia of a NOD/SCID mouse and analyzed for the presence of human CD45$^+$ cells (i). Human lineage–specific mAbs were used to detect lymphoid CD45$^+$CD19$^+$ (iii), CD45$^+$CD56$^+$ (v), myeloid CD45$^+$CD14$^+$ and CD45$^+$CD33$^+$ (ii,iv), and immature CD34$^+$CD38$^{-/low}$ (vi,vii) progenitor cells in the marrow of engrafted NOD/SCID mice. (B) Distribution of human hematopoietic cells in iBM-injected NOD/SCID mice. Eight weeks after transplantation, BM cells (ii,iii), spleen cells (iv), and PB (v) of NOD/SCID mice were stained with antihuman CD45 mAbs and analyzed by flow cytometry. BM cells were collected separately from injected (ii) and noninjected (iii) tibiae. BM cells that were injected with irradiated CD34$^+$CD38$^+$ (used as carrier cells in these experiments) alone were used as negative control (i). The relative frequencies of each population are indicated. Representative FACS analysis of 5 independent experiments is shown.

Table 2. Distribution of human hematopoietic cells in iBM or intravenously injected NOD/SCID mice

	Percentage of human CD45$^+$ cells				Lymphoid		Myeloid		Stem/progenitor	
Mouse	Right	Left	Spl	PB	Right	Left	Right	Left	Right	Left
iBM										
1	44.21	0.60	2.46	ND	67.13	63.26	6.96	18.68	28.94	13.98
2	15.83	12.00	0.85	ND	82.82	82.79	3.64	9.54	19.62	6.09
3	34.85	77.90	34.65	ND	69.43	59.60	4.39	5.57	20.66	29.92
4	55.73	50.43	31.92	ND	62.21	68.69	7.22	8.96	19.86	22.83
5	17.57	4.76	2.42	ND	69.35	70.87	8.62	16.00	26.59	22.68
6	77.81	84.74	41.83	14.48	65.16	84.50	31.78	8.53	13.29	22.75
7	96.07	34.23	34.15	11.67	67.21	63.01	14.64	13.89	24.83	21.56
8	28.40	1.84	4.33	0.97	57.12	62.66	12.41	10.76	12.84	10.89
9	39.11	1.87	4.04	2.05	70.45	68.73	4.71	5.96	22.37	28.04
Mean (± SD)	45.51 (26.96)	29.82 (33.79)	17.41 (17.52)	7.29 (6.79)	67.88 (6.99)	69.35 (8.88)	10.49 (8.79)	10.88 (4.47)	21.00 (5.48)	19.86 (7.91)
Intravenous										
1	49.10	52.46	39.18	8.19	74.02	75.04	14.40	11.50	12.71	19.36
2	32.29	21.77	3.64	1.97	86.51	85.86	4.08	5.07	6.00	9.03
3	42.37	39.48	18.35	5.52	69.54	71.22	6.99	9.32	11.59	14.02
4	22.69	16.63	9.20	2.01	86.32	73.07	10.38	12.67	19.31	21.79
5	28.16	22.76	21.16	4.85	80.36	79.76	13.65	15.07	16.88	14.22
6	19.67	22.54	7.55	1.36	68.41	65.59	7.98	11.65	19.28	16.71
7	12.90	8.92	6.50	1.74	62.05	95.64	30.65	3.18	11.18	9.29
Mean (± SD)	29.6 (12.77)	26.37 (14.72)	15.08 (12.41)	3.66 (2.58)	75.32 (9.40)	78.03 (10.08)	12.59 (8.76)	9.78 (4.26)	13.85 (4.89)	14.92 (4.79)

BM cells, spleen cells, and PB of NOD/SCID mice 6 to 9 weeks after iBM or intravenous transplantation of column-enriched but unsorted whole CD34$^+$ cell populations (20 000 cell for iBM and 200 000 cells for intravenous) were stained with antihuman CD45 mAbs and analyzed. BM cells were collected separately from injected (right leg) and noninjected (left leg) side tibia. Lymphoid, myeloid, and stem/progenitor cell lineages are defined as CD19$^+$/CD45$^+$ cells, CD33$^+$/CD45$^+$ cells, and CD34$^+$/CD45$^+$ cells, respectively, and each number indicates the percentage of human CD45$^+$ cells expressing respective surface marker. The proportion of each lineage was calculated from 50 000 to 100 000 events acquired using CELLQuest software.

ND indicates not determined.

of sum of 2 legs], $P = .14$). Human hematopoietic cells were also detected in noninjected side tibia, spleen, and PB of Lin$^{-/low}$-CD34$^+$CD38$^-$ cells transferred to NOD/SCID mice (Table 3). As few as 5 Lin$^{-/low}$CD34$^+$CD38$^-$ cells successfully engrafted in the noninjected side tibia (data not shown). These results suggested that the engrafted human hematopoietic cells in the injected side BM migrate to a noninjected BM by blood flow circulation and differentiate into multilineage cells even when the human hematopoietic cell chimerism showed low level.

Clonal analysis of engrafted human hematopoietic cells transplanted by iBM injection

Relatively high frequencies of human hematopoietic cells found in noninjected BM might suggest that cells entering the blood stream because of the pressure applied in iBM procedure or release after engraftment in injected side tibia. Although we found little evidence of leakage (Figure 2), we proceeded in examining the clonalities of cells present in different hematopoietic organs. Retroviral gene marking provides the ideal tool for studies of clonal analysis because they randomly and permanently integrate into the genome of the host cell. Thus, each genomic integration site is a distinct clonal marker that can be used to trace the progeny of individual stem cells after transplantation.[19,23-25] Therefore, we transduced the *EGFP* gene to CB CD34$^+$ cells by lentiviral infection and transplanted them into NOD/SCID mice by iBM injection. Eight weeks after transplantation, BM and spleen were isolated and analyzed by flow cytometry. BM cells were isolated separately from tibiae of injected side and noninjected side. High frequencies of EGFP$^+$ cells were demonstrated in the engrafted mice (61.16% ± 23.99%, n = 4) (Figure 5A). As shown in Figure 5B, common clones were evident among the injected side BM and other hematopoietic organs. The results suggest that HSCs, directly injected into BM, engraft in the marrow environment and migrate to other hematopoietic organs by mobilization through a systemic circulation.

Table 3. Distribution of human hematopoietic cells (CD34$^+$CD38$^-$) in iBM-injected NOD/SCID mice

	Percentage of human CD45$^+$ cells			
No. cells	Injected tibia	Noninjected tibia	Spleen	PB
10 000	86.13	57.91	64.68	27.86
1 000	33.62	5.28	3.80	0.85
500	47.94	1.28	2.44	ND
250	5.00	0.10	0.10	0.04
100	5.58	0.12	0.35	0.02

BM cells, spleen cells, and PB of NOD/SCID mice 8 weeks after transplantation of CB Lin$^{-/low}$CD34$^+$CD38$^-$ cells by iBM were stained with antihuman CD45 mAbs and analyzed. BM cells were collected separately from tibiae of injected and noninjected side.

ND indicates not determined.

Higher engraftment in secondary NOD/SCID mice that received transplants by iBM injection

Self-renewal of hematopoietic stem cells can be assessed by serial transplantation in SRC assay. A theory behind this assay is that stem cells which give rise to multilineage hematopoiesis in primary recipients are also capable of repeating this process in recipients of secondary transplants. The proportion of human cells in the secondary mice, however, is usually more than 10 times lower than in the primary mice. Consistent with previous studies, secondary mice that received intravenous transplants showed low levels of engraftment (Figure 6A). In contrast, engraftment levels in secondary recipients were significantly higher when they received transplants by iBM (Figure 6B; the reduction rate in chimerism from first to second recipients is 0.031 ± 0.018 in intravenous versus 0.546 ± 0.268 in iBM; $P < .01$). Thus, human cells recovered

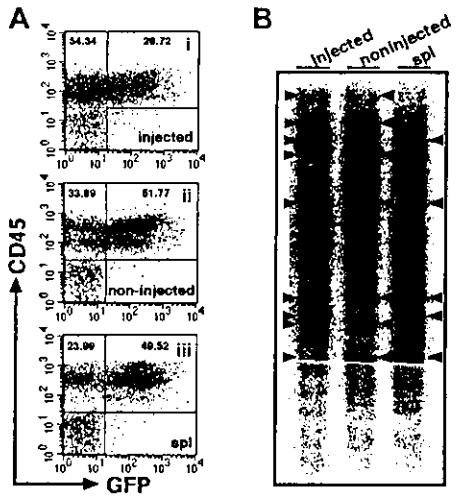

Figure 5. Clonal analysis of iBM-injected SRCs. (A) The proportion of EGFP+ human cells was analyzed at 8 weeks after iBM transplantation. Samples were obtained from injected side tibia (i), noninjected side tibia (ii), and spleen (iii). Cells were stained with antihuman CD45 mAb and analyzed by flow cytometry. Representative FACS profiles are shown from 4 independent experiments. The relative frequencies of each population are indicated. (B) Southern blot analysis for lentivirus integration sites. Genomic DNA extracted from samples indicated earlier was digested with *Eco*RI, which recognizes a unique site in lentivirus vector, and was hybridized with an EGFP probe. Each band represents a unique lentiviral integration site, and it corresponds to each clone. Arrowheads indicate common integration sites. Representative Southern blot analysis of 2 independent experiments is shown.

from BM of primary NOD/SCID mice that received iBM transplants possessed sufficient ability for consecutive multilineage engraftment in secondary recipients (Figure 6C), suggesting that human HSCs transplanted by iBM injection can self-renew in murine BM.

Engraftment of CD34+ cells by iBM injection is dependent on CXCR4, VLA-4, and VLA-5

Stem cell engraftment involves multistep processes, including activation of specific adhesion molecules. A number of studies have

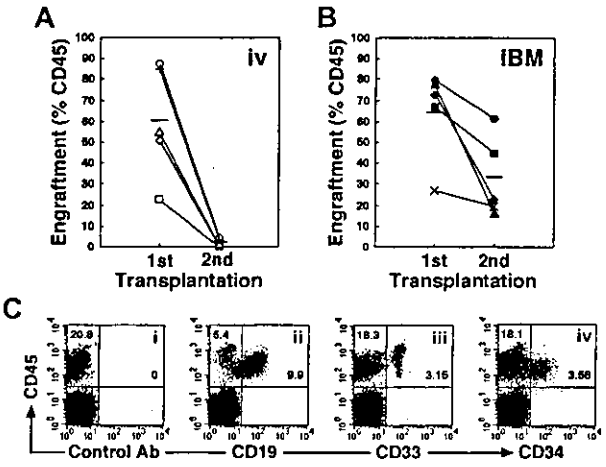

Figure 6. Secondary transplantation. (A-B) Whole human BM cells obtained from each primary recipient mouse given an iBM transplant (n = 10) were transplanted to a secondary recipient mouse by intravenous injection (n = 5) (A) or iBM injection (n = 5) (B). Secondary recipient mouse BM cells were analyzed for the expressions of human CD45 at 6 weeks after transplantation. Each symbol represents 1 mouse, and bars indicate the average engraftment level in 3 independent experiments. (C) Representative FACS analysis of a NOD/SCID mouse that received a secondary transplant. Human CD45+ cells in BM were analyzed for the expression of CD19 (ii), CD33 (iii), and CD34 (iv). The relative frequencies of each population are indicated.

reported crucial roles of VLA-4, VLA-5, and other molecules in interaction of HSCs and microenvironment during homing and engraftment processes (reviewed in Prosper and Verfaillie[26] and in Lapidot[27]). Up-regulation of these molecules through SDF-1–CXCR4 (stromal cell–derived factor 1 and CXC chemokine receptor 4) interaction is reported to increase homing and engraftment of primitive SRCs.[27-29] Primitive human CB CD34+ cells express a chemokine receptor CXCR4 and major β1 integrin VLA-4 and VLA-5 (Figure 7A). The iBM transplantation offers the opportunity to investigate the interaction between stem cells and BM stromal cells in vivo by eliminating the processes before entering marrow environment. To determine the in vivo role of CXCR4, VLA-4, and VLA-5 during the engraftment process of human SRCs, enriched CB CD34+ cells were pretreated with antibodies against the cell surface molecules mentioned and then transplanted. When CB CD34+ cells were transplanted intravenously, neutralizing antibodies to CXCR4, VLA-4, and VLA-5 completely blocked BM engraftment as described previously (Figure 7, indicated as open circles, $P < .01$),[27-29] whereas neutralizing antibodies to either VLA-4 or VLA-5 caused only partial inhibition of engraftment in iBM transplants (Figure 7, indicated as filled circles, $P < .05$). However, when CD34+ cells were pretreated with anti-CXCR4 or with both anti–VLA-4 and anti–VLA-5 antibodies, human SRCs engraftment was blocked completely (Figure 7, indicated as filled circles, $P < .01$).

Discussion

Primitive human hematopoietic cells can be assayed on the basis of their ability to repopulate immune-deficient NOD/SCID mice and have been termed SCID-repopulating cells (SRCs).[1-3] By using this

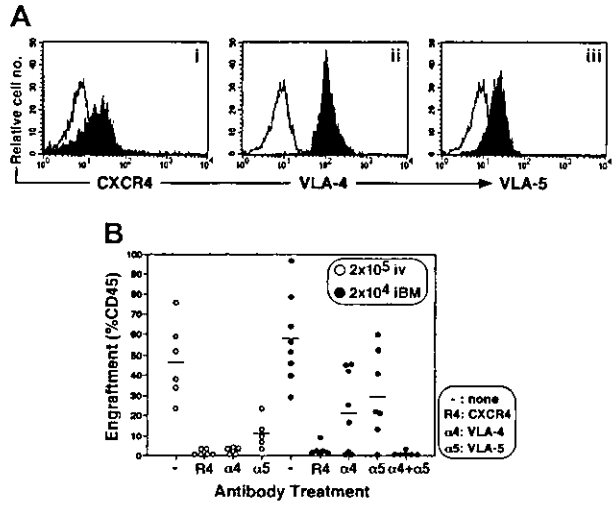

Figure 7. Effect of antibodies to CXCR4 and β1 integrins on engraftment of cord blood CD34+ cells in NOD/SCID mice BM. (A) Expression of CXCR4 (i), VLA-4 (ii), and VLA-5 (iii) on enriched CD34+ cells. Expression levels of CXCR4, VLA-4, and VLA-5 on gated CD34+ population are shown. A representative FACS analysis of 3 independent experiments is shown. The white histogram indicates negative control staining with isotype control antibody; the black histogram indicates CXCR4 (i), VLA-4 (ii), and VLA-5 (iii). (B) Percentage of engraftment in murine BM by CB CD34+ cells pretreated with antibodies to either CXCR4 (R4), VLA-4 (α4), VLA-5 (α5), or VLA-4 + VLA-5 (α4 + α5) was quantified at 6 weeks after transplantation by immunostaining with antihuman CD45 mAb. Open circles (○) represent the mouse that received a transplant of 2×10^5 of CD34+ cells intravenously. Filled circles (●) represent the mouse that received a transplant of 2×10^4 CD34+ cells by iBM. Each circle represents 1 mouse, and bars indicate the average of engraftment. Results were combined from 3 independent experiments.

human-to-mouse xenogeneic transplantation model, studies reported substantially low recoveries of SRCs.[12,13] These results imply that homing of primitive hematopoietic cells to the BM is nonselective and/or an inefficient process. A number of factors, such as entrapment in liver and/or lung, molecular incompatibility between human integrins and its ligands expressed on mouse, would interfere with homing and engraftment of HSCs. Therefore, it has been speculated that if one could eliminate such factors, SRC frequency should become markedly higher than that reported previously.[4,22,30] Here we report that by introducing cells directly into the marrow environment with the use of the iBM method, the frequency of SRCs became more than 15-fold higher (1 in 44 $Lin^{-/low}CD34^+CD38^-$ cells; 95% confidence intervals, 1 in 27 to 1 in 70) than intravenous injection (1 in 660 $Lin^{-/low}CD34^+CD38^-$ cells; 95% confidence intervals, 1 in 289 to 1 in 1510) (Figure 3; Table 1). This finding is compatible with the previous speculation that the SRC frequency could become 10 to 20 times higher, assuming there is no interference. Furthermore, multilineage reconstitution (Figure 4; Table 2) and self-renewal (Figure 6) were demonstrated by human SRCs injected by iBM method. One might argue that iBM injection created a hematoma in the injected site and reflected survival of human cells. However, the results clearly demonstrated maintenance of hematopoiesis as was seen in intravenous injection of human HSCs. Detection of no human cells in mice given transplants of 10^4 irradiated $CD34^+CD38^+$ carrier cells alone also eliminated the former possibility (Figure 3). Furthermore, a mouse given a transplant of 5 human $CD34^+CD38^-$cells showed 0.04% of chimerism in the injected tibia (Figure 3). On the basis of the number of cells recovered from the tibia (2×10^6), a calculated value of human cells was approximately 800, which could represent a 160-fold proliferation of injected cells.

In addition, human hematopoietic cells with the ability of multilineage differentiation were detected in the noninjected side tibia, spleen, and PB (Figure 4; Tables 2-3). In our iBM strategy, there was little, if any, leakage of the injected cells into the peripheral circulation (Figure 2). In much the same way, recently Zhong et al,[31] using the mouse-to-mouse intrafemur injection method, demonstrated that the donor murine cell in PB was undetectable at early points. At the late time points, transplanted donor cells in the noninjected femur were detected as the same level to the injected femur. In our experiments, however, engraftment in the noninjected BM tends to be low (Tables 2-3). The discrepancy may come from the lowering ability of human HSCs to home to the noninjected BM in xenoenvironment. Importantly, we confirmed the existence of common clones in the injected BM and other hematopoietic organs using a retroviral gene marking (Figure 5). We speculate that the human cells introduced directly into the murine marrow environment proliferate, migrate through a physiologic circulatory system, and engraft in other hematopoietic spaces. The level of chimerism may depend on the ability of proliferation and survival in each engrafted clone.[19] Independent clones were also present in the injected side and the noninjected side of legs (Figure 5). There may be 2 possibilities: leakage of HSCs during iBM injection and behavioral differences in SRC clones engrafted in the injected BM. The results of our tracing experiments (Figure 2) strongly support the later possibility. Also, Wright et al[6] demonstrated a dynamic circulation of HSCs. HSCs rapidly and constitutively migrate from BM to blood stream, and this circulation plays a physiologic role in the functional reengraftment of another place of BM. Using this iBM method, we might shed light on the mechanisms of human HSCs homing (noninjected side BM) and engraftment (injected side BM).

Self-renewal is a key characteristic of primitive stem cells and distinguishes them from short-lived progenitor cells. However, assessment of self-renewal in SRC assay requires transplantation into secondary recipients and has been difficult because of the lack of a sensitive and reliable method. Treatment of marrow cells derived from primary recipient mice with interleukin 6 (IL-6) and SCF up-regulated the surface expression of CXCR4 and resulted in higher engraftment levels than those of untreated cells when cells were transplanted in secondary recipients.[28,29] Thus, the expression of CXCR4 appears essential for homing, and therefore engraftment, of human cells. In other words, reduced expression of CXCR4 and other homing-related molecules in primary-engrafted SRCs may be a reason for the diminished level of human cells in a secondary recipient. The iBM strategy we used in this study can disregard the effect of homing factors, and we successfully demonstrated high levels of engraftment and multilineage differentiation of HSCs in secondary recipient (Figure 6).

Homing and lodgement of transplanted HSCs to recipient BM are critical steps in engraftment and initiation of marrow reconstitution. In the first phase "homing," transplanted cells must home to vascular sites and need to penetrate the basal lamina that is composed of extracellular matrix (ECM) proteins. In the second phase "lodgement," HSCs must stay in the appropriate niches of microenvironment where these cells survive, proliferate, and differentiate to reconstitute hematopoiesis, that is, "engraftment." Human HSC engraftment in NOD/SCID mice depends on the expression of the chemokine SDF-1 and its receptor CXCR4.[28,29,32] The SDF-1 activates the integrins lymphocyte function antigen 1 (LFA-1), VLA-4, and VLA-5 on human $CD34^+$ cells. HSCs polarize and migrate through the ECM toward local gradients of SDF-1, which are produced by specialized stromal cells, and orient themselves through the different elements of the BM microenvironment and settle in the stem cell niches.[27,29] Although previous studies showed the inhibition of engraftment in Ab blocking experiments,[28,29,33,34] whether each molecule contributes to either the homing or lodgement step or both has not been clarified, because this system could not differentiate the homing and lodgement. Our iBM strategy is useful to evaluate lodgement as it bypasses the homing step.

In line with the previous studies by Peled et al,[28,29] intravenous injection of human $CD34^+$ cells pretreated with anti-CXCR4, –VLA-4, and –VLA-5 mAbs resulted in complete inhibition of engraftment in NOD/SCID mice (Figure 7). However, the same researchers demonstrated that pretreatment with anti-integrin mAbs reduced homing, but not engraftment, of human cells into BM by approximately 30% to 50%.[32] From these results, we hypothesize that VLA-4 and VLA-5, expressed on HSCs, are involved in part in both homing and lodgement processes. With the use of the iBM strategy, blocking of either VLA-4 or VLA-5 affected engraftment only partially, whereas neutralization of CXCR4 or VLA-4 and VLA-5 together completely inhibited human HSC engraftment (Figure 7). Therefore, engraftment requires interaction between integrins expressed on BM microenvironment and each VLA-4 and VLA-5 expressed on human HSCs. A number of in vitro studies suggested the importance of integrin-mediated signaling pathways for localization of HSC in BM microenvironment[35-39] as well as survival and proliferation of human HSCs.[40-43] To our knowledge, the results of our iBM strategy are the first to demonstrate directly the important in vivo roles of integrins and chemokine receptor for not only homing but also lodgement of HSCs in BM microenvironments. Further studies will determine the molecular mechanisms concerning the HSCs lodgement by our iBM strategy. We are in the

process of examining the molecules participating only in HSC lodgement.

Our study indicates that iBM transplantation is a method that can accurately evaluate the innate ability of human HSCs. By using this highly sensitive method, more primitive human hematopoietic stem cells, including cells that have lower capability for homing, such as Lin^-CD34^- cells,[44,45] can be identified at a single cell level as previously demonstrated in the murine experiment.[46] The iBM method is also suitable to analyze BM cells and mobilized PB cells that exhibit low engraftment capability compared with CB in the SRC assay.[12,22] As shown by Kushida et al,[14,47] the iBM strategy makes it possible to coadministrate hematopoietic and hematopoietic-supporting mesenchymal stromal cells into recipient BM, which in turn may facilitate the human hematopoietic cell engraftment in murine BM. These studies would further our understandings of the mechanisms of homing and engraftment of human HSCs and lead to possible applications in clinical transplantation medicine.

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Development of both human connective tissue-type and mucosal-type mast cells in mice from hematopoietic stem cells with identical distribution pattern to human body

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The transplantation of primitive human cells into sublethally irradiated immunedeficient mice is the well-established in vivo system for the investigation of human hematopoietic stem cell function. Although mast cells are the progeny of hematopoietic stem cells, human mast cell development in mice that underwent human hematopoietic stem cell transplantation has not been reported. Here we report on human mast cell development after xenotransplantation of human hematopoietic stem cells into nonobese diabetic severe combined immunodeficient (NOD/SCID)/γ_c^{null} (NOG) mice with severe combined immunodeficiency and interleukin 2 (IL-2) receptor γ-chain allelic mutation. Supported by the murine environment, human mast cell clusters developed in mouse dermis, but they required more time than other forms of human cell reconstitution. In lung and gastric tract, mucosal-type mast cells containing tryptase but lacking chymase located on gastric mucosa and in alveoli, whereas connective tissue-type mast cells containing both tryptase and chymase located on gastric submucosa and around major airways, as in the human body. Mast cell development was also observed in lymph nodes, spleen, and peritoneal cavity but not in the peripheral blood. Xenotransplantation of human hematopoietic stem cells into NOG mice can be expected to result in a highly effective model for the investigation of human mast cell development and function in vivo. (Blood. 2004;103: 860-867)

Introduction

Mast cells are recognized as the principal cells which initiate immunoglobulin E (IgE)–dependent immediate hypersensitivity and also as the cells which contribute to innate immunity and tissue remodeling.[1,2] There are 2 phenotypically distinct mast cell subpopulations in rodents: connective tissue-type mast cells (CTMCs) and mucosal-type mast cells (MMCs). These populations differ in location, cell size, staining characteristics, ultrastructure, mediator content, and T-cell dependency.[3] Proliferation of rodent MMCs is dependent on T-cell–derived cytokines,[3,4] whereas that of CTMCs is supported by stem cell factor (SCF). In humans, mast cells are distinguished on the basis of their protease composition,[5,6] MC_{TC} contains tryptase and chymase in its granules and is predominant in skin and intestinal submucosa, like CTMCs in rodents. MC_T also contains tryptase, but lacks chymase, and is predominant in the alveolar wall and gastric mucosa, similar to MMCs in rodents. Human mast cells were reported to develop only under the influence of SCF, but T-cell–derived interleukin 3 (IL-3) has little affect on their differentiation.[7] Recently, human intestinal mast cells were reported to respond to IL-3 by enhancing their growth,[8] but SCF is still an indispensable factor for human mast cells. Mast cells are the progenies of hematopoietic stem cells (HSCs).[9,10] In mice, the progenitor cells capable of becoming mast cells leave the bone marrow and enter the circulation but complete their differentiation into mast cells only after arriving in peripheral tissues such as lung, bowel, and skin.[10,11] Unfortunately, the developmental mechanism of human mast cells remains far less clear, possibly because the lack of an appropriate in vivo assay system.

The transplantation of primitive human cells into immunedeficient C.B-17-Prkdc^{scid} *(scid)*[12,13] and into NOD/LtSz-*scid* or NOD/Shi-*scid* (nonobese diabetic severe combined immunodeficient [NOD/SCID]) mice[14,15] is thought to constitute an appropriate functional in vivo system for human HSCs. However, it has been suggested that residual natural killer (NK) cell activity in NOD/SCID mice might interfere with engraftment.[16,17] Recently, we developed NOD/SCID/γ_c^{null} (NOG) mice by backcrossing IL-2 receptor γ-chain deficient (γ_c^{null}) mice to NOD/Shi-*scid* mice.[18] Compared with NOD/SCID mice treated with anti-NK cell antibody[17] and NOD/SCID/β_2 microglobulin^{null} mice,[19,20] both of which were established for reducing residual NK cell activity, the newly developed NOG mice were superior in terms of efficiencies of human HSC engraftment, because they lack NK cell activity and show reduced interferon γ production from dendritic cells.[18] In addition, human CD3$^+$ T cells can be generated and matured from human HSCs in NOG but not in other mice.[21,22] These results encouraged us to check human mast cell development in NOG

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mice, even though there are no reports of mast cell development after human HSC transplantation into mice.

This is, therefore, the first report of human mast cell development in mice after transplantation of human HSCs, with NOG mice as recipients. Moreover, development of human mast cells in NOG mice was supported by the murine environment, and, depending on their protease compositions, the distribution of human mast cells was similar to that in the human body.

Materials and methods

Mice, human cell preparation, and xenotransplantation

NOG mice were established at the Central Institute of Experimental Animals (Kawasaki, Japan) by backcrossing γc^{null} mice to NOD/Shi-*scid* mice, as reported previously.[18] The mice were shipped to the animal facility of Kyoto University (Kyoto, Japan) and kept under specific pathogen-free conditions in accordance with the facility's guideline.

Human cord blood was collected from healthy full-term deliveries after obtaining informed consent. Mononuclear cells were isolated on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) after phagocyte depletion with silica (ImmunoBiological Laboratories, Gunma, Japan).[23] $CD34^+$ cell fractions were further isolated by using AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). After the enrichment, assessment of their purity by flow cytometry showed that approximately 95% of the cells were $CD34^+$ cells. In the experiments using lineage-depleted cells ($lin^-/CD34^+$ cells), cord blood mononuclear cells were treated with StemSep (Stem Cell Technologies, Vancouver, Canada), followed by $CD34^+$ selection.

Xenotransplantation of purified human cells into NOG mice was also described previously.[18,21] Mice were irradiated at 8 to 12 weeks of age with 240 cGy. Enriched $CD34^+$ cells (50 000) were injected intravenously through the tail vein. After the transplantation, mice were given sterile water containing prophylactic neomycin sulfate (Invitrogen, Carlsbad, CA). The experimental protocol was approved by the Human Studies Internal Review Board at Kyoto University (no. 322).

Flow cytometry

Human cell development in NOG mice was periodically monitored with a flow cytometer (FACS Calibur; BD Cytometry, San Diego, CA) with fluorescein isothiocyanate (FITC)–conjugated antihuman CD45 monoclonal antibody (mAb) and allo-phycocyanin (APC)–conjugated antimouse CD45 mAb (BD Pharmingen, San Diego, CA), as previously reported.[18,21] The lineage analysis was performed with APC-conjugated antihuman CD45; phycoerythrin (PE)–conjugated anti-CD3, anti-CD33 (BD Pharmingen), and anti-CD203c mAb which recognized both human mast cells and basophils[24,25]; PC5-conjugated anti-CD19, anti-CD56, and anti-Kit (CD117) mAb (Immunotech, Marseille, France); and biotin-conjugated anti-CD123 (IL-3 receptor α-chain) and streptavidin-FITC (BD Pharmingen).

Murine mast cell determination

Tissue samples were frozen in O.C.T. Tissue-Tek compound (Miles Labs, Elkhart, IN) or fixed in 10% buffered formalin or Carnoy solution (60% ethanol, 30% chloroform, and 10% acetic acid). Those sections were stained with acidic toluidine blue. Carnoy fixed preparations were used for safranin-O and Alcian blue staining.

To collect mast cells from the peritoneal cavity, 5 mL prewarmed Hanks balanced salt solution containing 1% fetal calf serum was injected into the mouse peritoneal cavity. The abdomen was gently massaged for 1 minute, after which the peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was collected with a pipette. One part of the collected cell suspension was used for direct counting of living cells, and the remaining cells were used for staining with toluidine blue or with safranin-O and Alcian blue on the cytospin preparations and for cytometry. On the cytospin preparation, a proportion of the positively stained cells among the 200 nucleated cells was determined.

Immunochemistry

To detect human mast cells, acetone-fixed frozen sections were blocked with donkey serum before incubation with antihuman CD45 mAb (Nichirei, Tokyo, Japan) and then incubated with Cy3-conjugated 2nd Ab (Jackson, West Grove, PA), FITC-conjugated avidin bound to mast cells,[26,27] and Hoechst 33342 (Molecular Probes, Eugene, OR). Specificity of avidin binding to mast cells was confirmed with both human and mouse tissue preparations from skin, lung, and gastric stomach.

We used acetone-fixed frozen sections for chymase, because the routinely used Carnoy solution reduces the number of $chymase^+$ cells.[28] Antihuman chymase mAb (Chemicon, Temecula, CA) labeled slides were stained with alkaline phosphatase (AP)–conjugated 2nd Ab (Vector, Burlingame, CA). For tryptase, formalin-fixed paraffin-embedded sections and methanol-fixed cytospin preparations were incubated with antitryptase mAb (Chemicon) and with AP-conjugated 2nd Ab. The color was developed with naphthol AS-BI/new fuchsin. In some experiments, biotin-conjugated antichymase mAb-labeled cells were incubated with horseradish peroxidase–conjugated streptavidin, and the color was developed with 3-amino-9-ethylcabbazole (Vector). The cells were sequentially labeled with AP-conjugated antitryptase mAb, and the color was developed with fast blue substrate (Vector). We used healthy parts of skin obtained after mastectomy as positive controls.

For SCF distribution in NOG mouse skin, we sequentially incubated acetone-fixed frozen sections with antimouse SCF polyclonal Ab (R&D systems, Minneapolis, MN) and Cy3-conjugated 2nd Ab (Jackson) and observed with a confocal laser microscopy (Olympus).

RNA purification and RT-PCR (reverse transcription–polymerase chain reaction)

Cellular total RNA was isolated with the phenol/guanidine isothiocyanate method using a Trizol reagent (Invitrogen) and reverse-transcribed to complementary (cDNA) with oligo dT primers and SuperScript Synthesis System (Invitrogen). Reaction mixtures were amplified with 0.2 U Taq polymerase (Sigma) using 25 cycles for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and 40 cycles for others under the following conditions: denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. Oligonucleotide primers for SCF which recognized both human and mouse SCF, 5′-TCTTCAGCTGCTCCTATTT-3′ and 5′-ACTGCTACTGCTGTCATTC-3′; human tryptase, 5′-GGAAAACCACATTTGTGACG-3′ and 5′-ATTCACCTTGCACACAGGG-3′; and human chymase, 5′-AAGGAGAAAGCCAGCCTGACC-3′ and 5′-TCCGACCGTCCATAGGATACG-3′ were synthesized.

To evaluate the species origin of SCF, PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA) and digested for 1 hour with restriction enzymes, *Xma*I and *Nsi*I. Mouse keratinocyte Pam212 and human keratinocyte DJM-1 were positive controls.

Human mast cell culture in vitro

Human cord blood $CD34^+$ cells were cultured in AIM-V medium (Invitrogen) with either human SCF (Amgen, Thousand Oaks, CA) or murine SCF (Kirin Brewery, Gunma, Japan) at concentration of 10 ng/mL (suboptimal) or 100 ng/mL (optimal), as described previously but with a minor modification.[29-31] During the first week, 50 ng/mL human IL-6 (Kirin Brewery) was also added. Flow cytometry at the constant flow rate was used to assess viable cell number with propidium iodide and mast cell percentage with anti-Kit mAb.

Statistical analysis

Data are presented as the mean ± SD values. Statistical significance was determined with the Student *t* test, and $P < .01$ was considered significant.