Role of Bone Marrow–Derived Progenitor Cells in Cuff-Induced Vascular Injury in Mice

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Objectives—Arterial injury results in vascular remodeling associated with proliferation and migration of smooth muscle cells (SMCs) and the development of intimal hyperplasia, which is a critical component of restenosis after angioplasty of human coronary arteries and an important feature of atherosclerotic lesions. However, the origin of SMCs and other cells in the development of vascular remodeling is not yet fully understood.

Methods and Results—We utilized a cuff-induced vascular injury model after transplantation of the bone marrow (BM) from green fluorescent protein (GFP)-transgenic mice. We found that macrophages were major cells recruited to the adventitia of the vascular injury lesion along with SMCs and endothelial cells (ECs). While investigating whether those cells are derived from the donor, we found that most of the macrophages were GFP-positive, and some of the SMCs and ECs were also GFP-positive. Administration of the anti–c-*fms* antibody resulted in a marked decrease in macrophages and a relative increase of SMCs, while administration of antibodies against the platelet-derived growth factor receptor-β caused a prominent decrease in SMCs and a relative increase in macrophages.

Conclusions—The current study indicates that BM-derived cells play an important role in vascular injury, and that differentiation of macrophages and SMCs might be dependent on each other. **(*Arterioscler Thromb Vasc Biol.* 2004; 24:477-482.)**

Key Words: macrophage ■ smooth muscle cell ■ endothelial cell ■ vascular injury ■ bone marrow

Arterial injury results in proliferation and migration of smooth muscle cells (SMCs) and the development of intimal hyperplasia, a critical component of restenosis after angioplasty of human coronary arteries and an important feature of atherosclerotic lesions. However, the origin of SMCs, which engage in the development of neointimal thickening during vascular disease, is not yet fully understood. One possibility is that medial SMCs are phenotypically modified and migrate into the intima, where they proliferate and secrete extracellular matrix components.[1] It has also been proposed that adventitial fibroblasts move into the neointima and give rise to cells with smooth-muscle–like properties.[2]

Recently, several groups have reported that cells of recipient origin take part in the formation of neointimal SMCs during the development of transplant vasculopathy.[3–5] These results agree with the notion that adult bone marrow (BM) contains multipotent cells that can develop into various lineages.[6] It has also been shown that endothelial progenitor cells (EPCs) can transdifferentiate into SMCs.[7] Thus, the origin of SMCs in atherosclerotic lesions is a source of controversy, and it is important to understand the contribution of BM-derived cells to neointimal formation in vascular pathology.

In vascular injury or remodeling, it is not clear whether one specific type of multipotent precursor cell can differentiate into endothelial cells (ECs), SMCs, or macrophages, or whether there are different precursor cells for each cell lineage. We have reported that administration of anti–c-*fms* antibody can prevent early atherosclerosis in apolipoprotein E-deficient (apoE$^{-/-}$) mice.[8] We have also shown that administration of antibodies against the platelet-derived growth factor receptor-β (anti–PDGFR-β) can prevent the recruitment of SMCs, but not of macrophages in the atherosclerotic lesions in apoE$^{-/-}$ mice.[9] These results indicate the important role of macrophages in the initiation of the lesion and recruitment of SMCs in hyperlipidemia-induced atherosclerosis. However, it is not known whether the recruitment of macrophages is critical for the migration of SMCs in vascular injury.

Therefore, we have two goals in this study. One is to explore the contribution of BM-derived cells to the development of vascular remodeling. The other is to examine whether blocking the cell differentiation by a specific antibody can affect the lesion formation in vascular injury. For this purpose we have utilized an inflammation-dependent vascular disease

Received November 16, 2003; revision accepted January 5, 2004.

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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

DOI: 10.1161/01.ATV.0000118016.94368.35

model induced by polyethylene cuff placement around the femoral artery after BM transplantation (BMT) from green fluorescent protein (GFP)-transgenic mice.

Methods

Mice

All experimental protocols were performed in accordance with the guidelines of Kyoto University, Japan. GFP-transgenic mice with C57BL/6 background were a generous gift from Dr. M. Okabe[10] (Osaka University, Japan). The mice were kept in a temperature-controlled facility on a 14-hour light/10-hour dark cycle, with free access to food and water. Mice were fed a normal chow diet containing 8.7% (wt/wt) fat and 0.063% (wt/wt) cholesterol (Oriental Yeast, Chiba, Japan) for the entire period of the experiment.

Bone Marrow Transplantation

Femurs of male or female, 8- to 12-week-old GFP-transgenic mice were dissected, and surrounding muscle tissue was removed by microscissors. Bones were then left in Dulbecco's modified Eagle's medium (DMEM). Both ends of the bones were cut with scissors, and the marrow was flushed with DMEM using a syringe with a 21-gauge needle. The marrow clusters were disaggregated by vigorous pipetting. BM cells were washed, resuspended in PBS, and counted. Eight-week-old female C57BL/6 mice were subjected to a lethal dose of total body irradiation (9 Gy) using the Gammacell 40 Exactor Irradiator (Nordion International). Each irradiated recipient received 5×10^5 BM cells extracted from GFP-transgenic mice in 0.5 mL PBS by tail vein injection. Mice used for BMT experiments were housed in sterilized cages and fed sterilized normal chow diet. Drinking water was supplied with 0.1% hydrochloric acid. Four weeks after BMT, the recipient mice were phlebotomized, and the circulating leukocytes were then checked for the expression of GFP by flow cytometry. Cuff placement was performed at least 4 weeks after BMT.

Cuff Placement

Mice were anesthetized with barbiturate complex [propylene glycol 17.9% (v/v), ethanol 8.9% (v/v), sodium 5-ethyl-5-(1-methylbutyl) barbiturate 10.7% (v/v)]. The right femoral artery was dissected from its surroundings. A nonconstrictive polyethylene cuff (PE50, 0.58 mm inner diameter, 0.965 mm outer diameter, 2 mm length; Becton Dickinson) was placed loosely around the right femoral artery.

Antibody Administration

AFS98, a rat monoclonal anti-murine c-*fms* antibody, which inhibits colony formation dependent on macrophage-colony stimulating factor (M-CSF) and cell growth by blocking the binding of M-CSF to its receptor c-*fms*, was previously described as an anti-c-*fms* antibody.[9] APB5, a rat monoclonal anti-murine PDGFR-β antibody, which blocks the PDGFR-β–mediated signaling pathway, was also described.[9] Four C57BL/6 mice in each group were administered 1 mg of AFS98, APB5, or isotype-matched irrelevant rat IgG (γ2A) once a day for 2 weeks after cuff placement.

Tissue Preparation

At euthanization, mice were anesthetized with barbiturate complex. Mouse thorax was opened, and physiological pressure-perfusion-fixation (100 mm Hg) was performed by cardiac puncture with 4% paraformaldehyde in PBS for 10 minutes. After the procedures, bilateral femoral arteries were harvested. The tissue was snap-frozen in OCT compound (Sakura Finetek). Serial cross sections (6 μm thick) were obtained throughout the entire length of the cuffed femoral artery or equivalent portion of the contralateral artery for histological analysis. Rat monoclonal antibody (mAb) BM8, labeled with biotin (BMA Biochemicals AG), was used as a specific marker for mouse macrophages. For macrophage staining, we used the Tyramide Signal Amplification system (NEN Life Science Products) to amplify the weak signal. For SMC staining, we used mouse monoclonal anti-human smooth muscle α-actin (SMA) antibody (clone 1A4), labeled with Cy3 (Sigma). For the staining of smooth muscle myosin heavy chain SM1,[11] we used rat anti-SM1 mAb (clone KM995) (kindly provided by the Kyowa Hakko Kogyo Co, Tokyo, Japan). Sections were secondarily incubated with rhodamine-labeled anti-rat IgG (Chemicon). ECs were identified by immunohistochemical staining with biotin-conjugated rat anti-mouse CD31 antibody (Southern Biotech) and rabbit anti-von Willebrand Factor (vWF) antibody (Sigma). For CD31 staining, the Tyramide Signal Amplification system was employed to augment antigenicity of ECs. For vWF staining, sections were secondarily incubated with rhodamine-labeled anti-rabbit IgG (Chemicon).

Image Analysis and Quantification

Eight equally crossed sections were used from each mouse to quantify the femoral artery lumen, BM-derived cell area, and vascular remodeling lesion size. Sections were evaluated by using Image-Pro Plus (Media Cybernetics). To estimate the effect of anti-c-*fms* or anti-PDGFR-β on vascular remodeling, we calculated the ratio of the number of SMCs or macrophages to the whole vascular remodeling lesion area. The area of the femoral artery lumen, BM-derived cells, and vascular remodeling lesion was calculated and expressed in square micrometers.

Statistical Analysis

Data are expressed as mean±SEM and were analyzed by ANOVA with Abacus Statview software (version 5.0). A value of $P<0.05$ was regarded as significant.

Results

Recruitment of Bone Marrow–Derived Progenitor Cells in Cuff-Induced Vascular Remodeling

To elucidate the involvement of BM-derived cells in cuff-induced vascular remodeling lesions, BM cells from GFP-transgenic mice were transplanted into lethally irradiated C57BL/6 mice before cuff placement. After 4 weeks of BMT, we confirmed the reconstitution of the hematopoietic system by checking the fluorescence of blood leukocytes by flow cytometry. We found that more than 85% of the cells were positive for GFP (data not shown); this finding indicates that most of the leukocytes were derived from the donor BM. One or two weeks after cuff placement, cuffed or sham-operated femoral arteries were examined under fluorescence microscopy. In the cuffed artery, the majority of the cells accumulating in the lesion were GFP-positive (Figure 1A and 1B), suggesting that those cells were derived from the donor BM. In contrast, in the sham-operated artery, GFP-positive cells were hardly detected (Figure 1C). We found that the accumulation of BM-derived cells in the vascular remodeling lesion was significantly increased from 1 week to 2 weeks after cuff placement (Figure 1A, 1B, and 1D). Although we did not find a visible change in intimal thickening after cuff placement, the lumen of the cuffed artery was more restricted than that of the sham-operated artery (Figure 1E).

Macrophages are the Major Component in the Cuff-Induced Vascular Remodeling Lesion

Next, to examine the recruitment of macrophages in the cuffed lesion, we stained the tissue with BM8. We found many cells recruited to the adventitia of the cuffed artery, most of which were positive for BM8 (Figure 2A), indicating the role of monocyte-macrophages in vascular remodeling

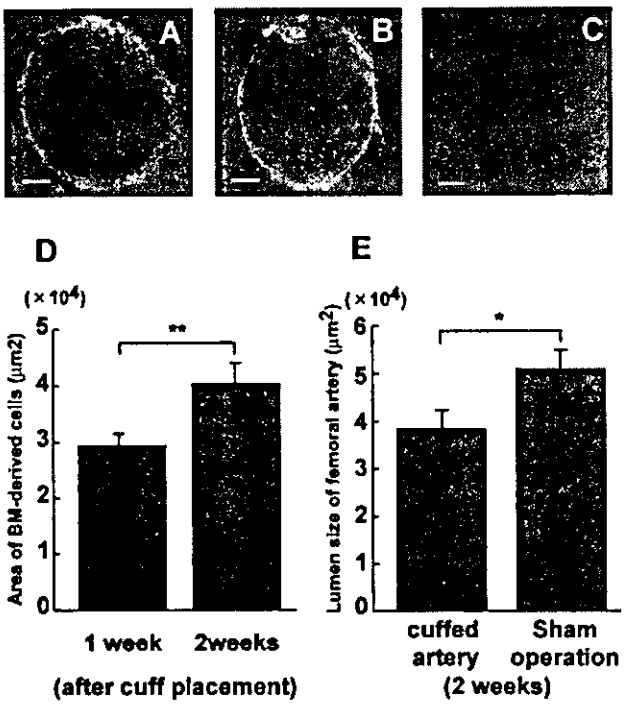

Figure 1. A through C, Representative microscopic photographs of BM-derived GFP-positive cells in C57BL/6 mouse vascular remodeling lesion. Four weeks after BMT, a nonconstrictive polyethylene cuff was placed around the right femoral artery in four mice in each group. The cuffed (A, 1 week after cuff placement; B, 2 weeks after cuff placement) or sham-operated (C) femoral arteries were examined under fluorescence microscopy. D and E, Quantitative analyses of BM-derived cell area (D) and femoral artery lumen area (E) after cuff placement showed a significant difference between 2 groups. Data from 20 slices per mouse artery are shown as mean±SEM. $^{*}P<0.05$, $^{**}P<0.01$. Scale bars: 100 μm

lesions. In the sham-operated femoral artery, we found few BM8-positive cells (Figure 2B).

BM Cells Can Differentiate into Vascular Smooth Muscle Cells

To examine whether BM-derived cells can differentiate into SMCs in the vascular remodeling lesion, we stained the tissue with Cy3-labeled anti-SMA (clone 1A4) and anti-SM1 (clone

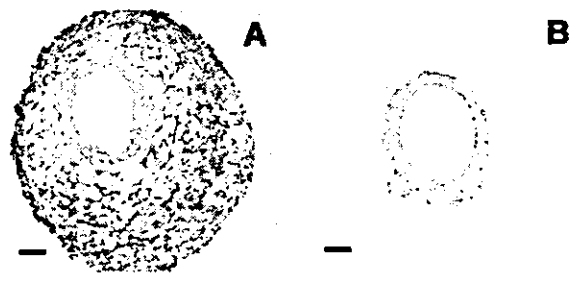

Figure 2. Numerous macrophage-like cells accumulating in the cuff-induced vascular remodeling lesion. After 2 weeks of cuff placement as described in Figure 1, tissues were subjected to immunohistochemistry with biotinylated anti-mouse macrophage antibody BM8. A number of cells were BM8-positive cells in cuffed femoral artery (A), but in the sham operated femoral artery, those cells could hardly be found (B). Scale bars: 100 μm

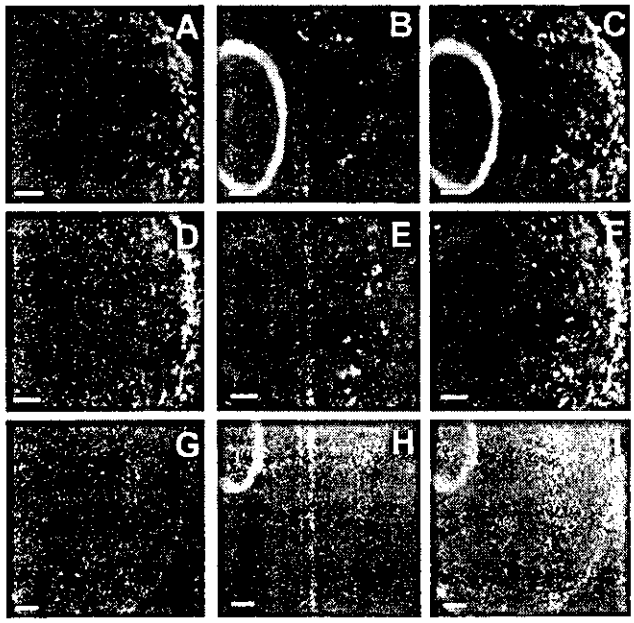

Figure 3. BM-derived SMCs in C57BL/6 mouse vascular remodeling lesion. After the same procedure in Figure 1, tissues were subjected to immunohistochemistry with antibodies to Cy3-labeled SMA (B, H) or SM1 (E). A, D, and G are fluorescent microscopic photographs for GFP. G, H, and I are fluorescent microscopic photographs from femoral artery of 1 week after cuff placement. All the others are samples at 2 weeks after cuff placement. C, F, and I are merged images of GFP and Cy3 signal from A and B, D and E, and G and H, respectively. Scale bars: 100 μm

KM995) antibodies. We found a number of 1A4- and KM995-positive cells in the adventitia of the lesion (Figure 3B and 3E). With the colorization of GFP signals, we observed that some of the 1A4- and KM995-positive cells were also positive for GFP (Figure 3C and 3F), indicating that BM-derived cells can also differentiate to SMCs in the cuff-induced vascular remodeling lesion. However, in the earlier time point at 1 week after cuff placement, we could find few SMCs in vascular remodeling lesion (Figure 3H).

Interference Exists Between Macrophages and Smooth Muscle Cells

To examine whether inhibiting the differentiation to macrophage or SMC by mAb could affect the manner of accumulation and differentiation of BM-derived cells in the vascular remodeling lesion, we administered an antagonistic rat mAb against murine c-*fms* (M-CSF receptor) (clone AFS98) or PDGFR-β (clone APB5) to C57BL/6 female mice which had undergone cuff placement. In comparison with the lesion from mice administered with control IgG (clone γ2A) (Figure 4C), we found that the treatment with AFS98 caused a marked decrease in macrophages in the lesion (Figure 4A and 4G). Interestingly, the density of SMCs was inversely increased (Figure 4D and 4H) in response to this treatment. In contrast, administration of APB5 resulted in a marked increase in macrophages (Figure 4B and 4G) with a concomitant decrease of SMCs (Figure 4E and 4H), suggesting that a certain interaction occurs between macrophages and SMCs during the vascular remodeling process.

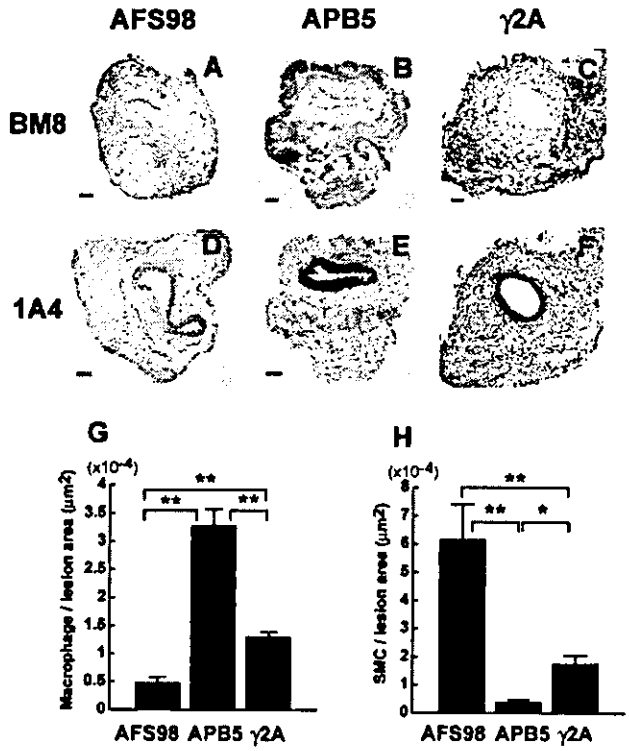

Figure 4. Progenitors of SMC and macrophage have opposite roles in the lesion formation. A total of 12 C57BL/6 mice (8 weeks of age) were injected for 2 weeks with 1 mg of AFS98 (n=4), APB5 (n=4), or γ2A (n=4) every day after cuff placement. Each mouse was euthanized and the femoral artery was subjected to immunohistochemistry with anti-macrophage antibody (A, B, and C) or anti-SMA antibody (D, E, and F). A and D are from mice injected with AFS98, B and E are from mice injected with APB5, and C and F are from mice given γ2A. Ratio of the number of macrophages (G) and SMCs (H) to whole vascular remodeling lesion area had a significant difference in each group. Data from 20 slices per mouse are shown as mean±SEM. *$P<0.05$, **$P<0.01$. Scale bars: 100 μm

To estimate the effects of anti–PDGFR-β or anti–c-*fms* mAb on vascular remodeling, we measured the lumen size of the artery treated with the two kinds of mAb and γ2A. We found no distinct difference in the lumen size of the femoral artery through administration of AFS98, APB5, or γ2A (data not shown).

We also examined whether each antibody administration had any effect on tissue formation after cuff placement. The calculated vascular remodeling lesion area of each mouse treated with AFS98, APB5, and γ2A was $1.18\times10^5\pm5.38\times10^3$ μm², $1.43\times10^5\pm7.27\times10^3$ μm², and $1.82\times10^5\pm1.11\times10^4$ μm², respectively (mean±SEM of 20 slices from each of 4 mice, $P<0.05$ versus γ2A). Less tissue formation was observed in the mice treated with AFS98 and APB5 than in mice treated with γ2A. This result indicates that AFS98 and APB5 administration could inhibit tissue formation after cuff placement. Further, to examine whether APB5 or AFS98 has an effect on BM-derived cell incorporation, we performed cuff placement and administered each antibody to mice that had been subjected to BMT. By measuring BM-derived cells accumulating in the cuff-induced lesion, we found a significant decrease of GFP-positive cells by mAb administration (data not shown), indicating that APB5 and AFS98 also affected the incorporation of BM-derived cells.

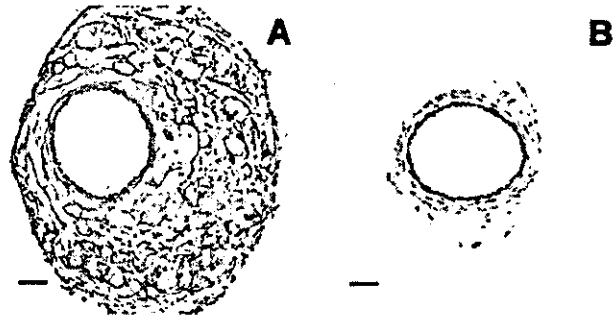

Figure 5. Representative microscopic photographs of BM-derived ECs in cuff-induced vascular remodeling lesion. After the same procedure in Figure 1, tissues were subjected to immunohistochemistry with biotin-conjugated rat anti-mouse CD31. The microscopic photograph of A is from cuffed right femoral artery, and B is from sham-operated left femoral artery. Scale bars: 100 μm

Endothelial Progenitor Cells Are Recruited to the Cuffed Vascular Remodeling Lesion

Because it is not known whether EPCs can contribute to cuff-induced vascular remodeling lesion formation in the injured femoral artery, we performed a series of endothelial staining. We found that the endothelial lining of the intima was clearly stained with anti-CD31 antibody, and that small vessels in the adventitia were also stained. There were also some CD31-positive cells clustered outside the small vessels in the adventitia in the cuffed lesions (Figure 5A), but not in the sham-operated lesions (Figure 5B). Because CD31 can also be expressed on monocyte-macrophages, we stained the tissue with anti-vWF antibody, another EC-specific marker, and compared the expression with GFP-positive cells. As shown in Figure 6E, the endothelial lining of the intima and small vessels in the adventitia were also positive for vWF. Some of the clustered cells in the adventitia were positive for

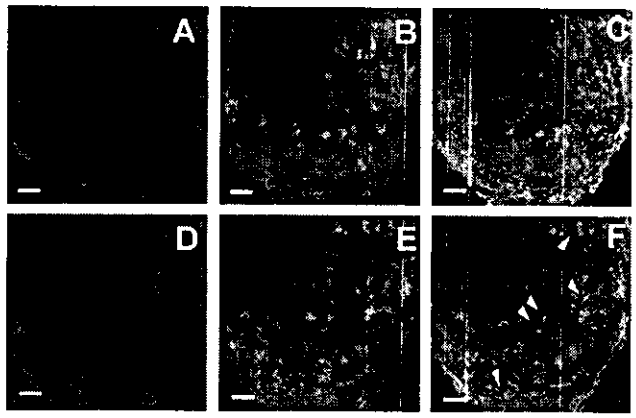

Figure 6. EPCs in cuff-induced vascular remodeling lesions. After the same procedure in Figure 1, tissues were subjected to immunostaining with anti-vWF antibody. A, B, and C are 1 week after cuff placement, and D, E, and F are 2 weeks after cuff placement. A and D indicate GFP signals, and B and E indicate vWF signals. C is a merged image of A and B, and F is a merged image of D and E. Scale bars: 100 μm

vWF and GFP, while the endothelial lining of the intima of the artery and small vessels in the adventitia were only positive for vWF (Figure 6F), indicating the involvement of angiogenesis from vasa vasorum. Notably, as we observed that significantly fewer SMCs could be found 1 week after cuffing (Figure 3B), EPCs could scarcely be found in the vascular remodeling lesion at this earlier phase (Figure 6C).

Discussion

In this study, we have clearly shown that BM-derived cells are critically involved in the lesion formation of cuff-induced vascular remodeling in mice. In this setting, BM-derived macrophages, SMCs, and ECs contributed to the lesion formation. However, not all of the SMCs or ECs in the lesion were derived from the BM. Interestingly, when anti–c-*fms* antibody was administered after cuff placement, the recruitment of macrophages was suppressed, but the density of SMCs was increased. On the other hand, administration of anti–PDGFR-β inhibited the recruitment of SMCs in the vascular remodeling lesion, but increased the number of macrophages. These results suggest an interaction between macrophages and SMCs during the lesion formation.

Although previous investigators have shown intimal thickening in the cuff-induced vascular injury model,[12,13] we have not been able to reproduce their results. This may be due to the technique of the cuff placement, because we were able to induce intimal thickening of the cuffed artery when we used apoE$^{-/-}$ mice fed with high-fat diet (data not shown). Indeed, we found a marked inflammatory change in the adventitial region around the cuffed artery. However, little is known about inflammatory responses in the adventitia after vascular injury, and adventitial and perivascular reactions are largely ignored. Recent clinical and experimental data by other investigators suggest that constrictive vascular remodeling is in large part responsible for lumen loss associated with restenosis.[14,15] Scott et al have indicated that the adventitia may be important in the first wave of growth after angioplasty of coronary arteries, with later growth of the lesion occurring in the neointima.[16] Therefore, studying the mechanism of cell recruitment to the adventitia in the vascular remodeling region is important for the understanding of the pathogenesis of restenosis.

Recent studies for transplant atherosclerosis have demonstrated that most of the neointimal α-actin–positive SMCs in recipient coronary arteries or aortas were from host origin,[4,5] suggesting that these SMCs might be at least in part from BM-derived smooth muscle progenitor cells. In this study, we have demonstrated that at least three types of cells, macrophage, SMC, and EC, are recruited from the BM to the adventitia of the cuff-induced vascular injury site. The characteristic feature of those cells is to form a cluster in the lesion. However, we have not determined when and how those cells migrate to the adventitia. Therefore, it is very important to understand the timing and pathway of cell migration in the pathogenesis of vascular injury. Elucidating the involvement of soluble factors in this model, such as chemokines and adhesion molecules, would also be intriguing.

In this study we have shown that administration of anti–c-*fms* antibody inhibited the recruitment of macrophages, and increased the recruitment of SMCs to the vascular injury lesion in wild-type mice. This finding is different from our report on apoE$^{-/-}$ mice, where we showed that the antibody inhibited the recruitment of SMCs as well as macrophages in early atherosclerotic lesion.[8] Thus, in hyperlipidemia-induced atherosclerosis, the recruitment of monocyte-macrophage is prerequisite for the migration of SMCs for the lesion formation; this paradigm was not applied to the current vascular injury model. If the common progenitors for macrophage and SMC exist, our data might indicate that BM-derived cells are playing an important role in vascular injury, but not in hyperlipidemia-induced atherosclerosis. The result with anti–PDGFR-β is also different from our previous observation in apoE$^{-/-}$ mice,[9] in which the antibody to apoE$^{-/-}$ mice failed to affect the density of macrophages in advanced atheromatous lesions. It was also notable that administration of anti–PDGFR-β increased the recruitment of macrophages in this study. Thus, in the vascular injury model, blocking the differentiation of one cell type can increase the recruitment or differentiation of the other cell type. Although we have not determined whether the progenitors of macrophages and SMCs are derived from the same precursor cell, anti–c-*fms* or anti–PDGFR-β might affect the differentiation of common precursor cells.

Schmeisser et al reported that BM-derived macrophages might contribute to neovascularization by in situ transdifferentiation to EC-like cells.[17] We found that in the vascular injury lesion there were many cells positive for CD31, which is an endothelial marker and is also positive for monocyte-lineage. However, vWF-positive cells were much smaller in number in this lesion. Furthermore, most of the cells forming a small vessel were positive for vWF, but negative for GFP, indicating that the source of the ECs forming a small vessel in the adventitia is from vasa vasorum, not from the BM.

Terada et al[18] and Ying et al[19] demonstrated that embryonic stem cells can spontaneously fuse with mononuclear BM cells[18] or brain cells[19] in vitro to form pluripotent tetraploid hybrids. In this study, there are a number of BM-derived cells stimulated after cuff placement in the cuff-induced vascular remodeling lesion. These BM-derived cells play an important role for lesion formation. Those two reports showed that the frequency of cell fusion was very low (2×10^{-6} to 10^{-4}), although it is difficult to directly correlate the in vitro findings of embryonic stem cells to our in vivo study. It is possible that some of the BM-derived cells in our experiments resulted from fusion between BM cells and vascular cells; however, this phenomenon would be an unlikely explanation for the extent of BM involvement seen in this study.

In summary, we have provided evidence that BM-derived cells are playing a critical role in cuff-induced vascular injury in mice. Understanding the interaction among the cells involved in the lesion formation will be important for regulating the accumulation of inflammatory cells in the vascular injury lesion.

Acknowledgments

This study was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (20045982, 12671111, 14571093, and 13307034) (to H.A., M.Y., T.K.); Center of Excellence grants (12CE2006) (to T.K.); a research grant for health sciences from the Japanese Ministry of Health and Welfare (to T.K.); and a grant from the Rotary Yoneyama Memorial Foundation (to Y.X.). T.M. is supported by Establishment of International COE for Integration of Transplantation Therapy and Regenerative Medicine (COE Program of the Ministry of Education, Culture, Sports, Science, and Technology, Japan). We thank Drs Hiroharu Kataoka and Masafumi Morimoto for their advice about cuff placement, and Akiko Kato for her excellent technical assistance.

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Biochimica et Biophysica Acta 1592 (2002) 313–321

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Review

Ex vivo expansion of hematopoietic stem cells by cytokines

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Received 14 November 2001; received in revised form 24 June 2002; accepted 24 June 2002

Keywords: Expansion; Stem cell; Cytokine

1. Introduction

Autologous or allogeneic hematopoietic stem cell (HSC) transplantation has been used successfully in the treatment of hematological diseases, neoplasias, primary immunodeficiency diseases, and metabolic disorders of children and adult patients. Historically, the bone marrow (BM) has represented the main source of HSCs in pediatric and adult individuals. However, in many cases, a suitable donor is hardly found, thus limiting the applicability of this life-saving treatment. This difficulty has led to a search for alternative sources of HSCs for use in human transplantations. Two sources of human HSCs have been identified: cord blood (CB) and peripheral blood (PB). PB is considered to be practical sources of HSCs, especially because methods exist which can mobilize significant numbers of stem/progenitor cells into circulation after administration of G-CSF. However, the poor mobilization happens sometimes, and more importantly, concerns about donor safety have been apprehended because of splenic rupture during G-CSF application [1]. HSCs derived from CB have many advantages for transplantation, but the recipient is restricted to pediatric patients because of its small volume. These inconveniences have been considered to be resolved if ex vivo expansion of HSCs would turn out to reality.

Recently, the identification, cloning, and production of recombinant cytokines together with the identification and purification of HSCs and progenitor cells have greatly increased our understanding of the hematopoietic system. As a result of these fundamental discoveries, many investigators are now attempting ex vivo manipulation of HSCs for potential therapeutic purposes. In general, the objectives of these manipulations are to expand HSCs responsible for long-term hematopoietic repopulation. This review will summarize the present understandings of the roles of cytokines for expansion of human HSCs, together with the development of assay systems to reflect human HSC activity correctly. Moreover, therapeutic applications for ex vivo expanded HSCs will also be discussed [2–4].

2. In vitro and in vivo assessments of human hematopoietic stem/progenitor activity

2.1. In vitro assays

These assays include long-term culture-initiating cell (LTCIC) assay, cobblestone area-forming cell (CAFC) assay, high proliferative potential colony-forming cell (HPP-CFC) assay, and colony-forming unit-blast (CFU-Bl) assay [5,6]. However, even with the extended long-term culture-initiating cell system (ELC-IC) which can detect an immature progenitor population, it turned out to be impossible to reflect human long-term repopulating stem cells [7]. At present, the available in vitro assays do not appear to reflect a true measure of human HSC.

2.2. In vivo assay

In order to enhance the study of human HSC, investigators have directed their attention to the development of small animal models of human hematopoiesis [8]. In mouse, quantitative competitive repopulating assay between phenotypically distinguishable stem cell populations has been developed as a definitive stem cell assay system [9]. In this system, mixtures of BM cells from different donors are injected into irradiated recipient mice, and the relative portion of each donor-origin population is determined as a

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PII: S0167-4889(02)00324-5

ratio. This ratio represents the relative HSC content of each inoculum. The advantages of animal models, particularly small animal models, are obvious. The development, differentiation, and long-term repopulating capacity of human cells, which can only be determined in vivo, can be ascertained in a small animal model without the need for clinical studies. In 1988, the first report of the engraftment of human cells into homozygous severe combined immunodeficiency (SCID) mice was presented. This report included engraftment of human PB mononuclear cells following intraperitoneal injection into unirradiated recipients and transplantation of fetal BM and thymus fragments under the renal capsule. These initial model systems were soon followed by the experiment of human BM engraftment when transferred intravenously into irradiated SCID recipients [10].

Recent advances in mammalian genetics have provided a number of immunodeficient murine models for engraftment and quantitation of human stem cells. One model is mice which are triply homozygous for the beige (*Lyst*^{bg}, *bg*), *nu*, and X-linked immunodeficiency (*Btk*^{xid}, *xid*) loci (*bg-nu-xid*) [11,12]. Additional immunodeficient models include mice deficient in the recombination activating gene-1 (*Rag1*) and *Rag2* genes and mice homozygous for *Prkdc*^{scid} (*scid*) locus [13–15]. During extensive attempts being made to utilize the *bg-nu-xid* mouse, the *Rag1* and *Rag2*-deficient mice, and the *scid* mice as hosts for normal and malignant human hematopoietic cells, the optimal host has been determined to be mice homoxygous for the *scid* mutations. Therefore, small animal models of human HSC engraftment have been attempted mainly based on the use of the *scid* mice (Table 1).

The term SCID-repopulating cell (SRC) has been utilized to describe the reconstitution activity of the human HSCs engrafted in SCID. However, the serious limitation has still precluded the widespread use of SCID mice for detecting human HSC activity. The engraftment levels of human cells are low, representing only 0.5–5% of the total SCID recipient BM population. Systemic approach has been based on the hypothesis that the poor engraftment level of human xenograft cells might be derived from the residual innate immune resistance in host. To evaluate the influence of the residual innate immunity on the engraftment efficiency, an extensive screen of SCID mice backcrossed with mice harboring several impaired innate immunities was evaluated. As a result, the NOD/LtSz-*scid* mouse strain was generated as an efficient recipient to reconstitute human hematopoietic cells [16]. This strain has a variety of immunological abnormalities such as T- and B-cell deficiency, impaired natural killer (NK) cell activity, macrophage dysfunction, and absence of circulating complements. Recently, we also established a similar NOD/Shi-*scid* mice, which showed a similar phenotype with NOD/LtSz-*scid* [17,18]. These mice injected intravenously with human CB CD34$^+$ cells were able to support 5- to 10-fold higher levels of human cell engraftment in their BM than in SCID mice. However, careful examinations of NOD/LtSz-*scid* mice and NOD/Shi-*scid* mice revealed that both mice still preserve a subtle NK activity [19]. NK cell depletion by anti-asialo GM1 antiserum treatment was indispensable in the enhancement of human HSC engraftment in irradiated NOD/Shi-*scid* mice.

To eliminate NK cell activity completely, genetic crosses produced NOD/LtSz mice doubly homozygous for *scid* mutation and the β2 microglobulin null allele (NOD/LtSz-*scid*-β2m$^{-/-}$) [20]. NOD/LtSz-*scid*-β2m$^{-/-}$ mice resulted in the absence of MHC class I expression, loss of NK cell activity, and rapid clearance of human IgG1 in addition to the lacking of mouse mature lymphocytes and serum Ig. According to Kollet's experiments, NOD/LtSz-*scid*-β2m$^{-/-}$ mice are highly efficient recipients for human HSCs, compared with NOD/LtSz-*scid* mice [21].

NOD/LtSz-*scid* mice are highly radiosensitive, have short life spans, and a small number develop functional lymphocytes with age. To overcome these limitations, Shultz et al. have backcrossed the null allele of the *Rag1* gene onto the NOD/LtSz-*scid* strain background (NOD/LtSz-*Rag1*^{null}). Although PB mononuclear cell transplantation has been performed, there were no significant differences in the percentage of CD4 or CD8 cells in PB of engrafted NOD/LtSz-*Rag1*^{null} compared with the PB of engrafted NOD/LtSz-*scid* mice [21]. Now, the engraftment level of human HSCs into this mouse remains to be examined.

In either strain of ontogenetically or genetically modified mice with compromised immunological activity, complete multilineage differentiation including T cells has not been

Table 1
Immunocompromised mice for evaluating human hematopoietic stem cell activity

	T cell	B cell	NK cell	Macrophage	Complement	Engraftment of human HSC	Human T cell
SCID	⇊	⇊				impossible	–
bg-nu-xid	⇊	⇊	↓			impossible	–
NOD/LtSz-*scid*	⇊	⇊	↓	⇊	⇊	possible	–
NOD/Shi-*scid*	⇊	⇊	↓	⇊	⇊	possible	–
NOD/LtSz-*Rag1* null	⇊	⇊	↓	⇊	⇊	possible	–
NOD/LtSz-*scid* /B2m null	⇊	⇊	–	⇊	⇊	good	–

SCID (*Prkdc scid*); *bg-nu-xid* (beige, nude, X-linked immunodeficiency); HSC (hematopoietic stem cell).

reported. Stem cells are defined functionally by the self-renewal and multilineage differentiation. To fulfill these two, complete multilineage differentiation including T cells is required to be demonstrated. Further improvement remains to be performed.

3. Effects of cytokines on human hematopoietic stem/progenitor cells

The optimal choice of cytokines for the ex vivo expansion of human hematopoietic stem/progenitor cells has not yet been determined. During the last two decades, investigators in a number of laboratories used murine transplantation models to characterize the cytokines regulating the human HSCs (Table 2). The positive cytokines may be separated in to two groups: one consisting of stem cell factor (SCF) and flt3/flt2 ligand (FL), and the other consisting of interleukin (IL)-6, IL-11, IL-12, leukemia inhibitory factor (LIF), granulocyte colony-stimulating factor (G-CSF), and thrombopoietin (TPO). Interactions of two cytokines appear necessary to positively regulate the kinetics of stem cells. Surprisingly, IL-3 and IL-1 proved to have profound negative effects on HSCs [22]. In spite of these numerous studies, the definitively continuous expansion of human HSC in ex vivo system has not been achieved yet, resulting in no definitive presentation of efficient engraftment through the ex vivo manipulation procedure. In this review, we are going to follow these progresses historically. Therefore, some contradictions among the presented data might be noticed. Because, earlier studies employed the in vitro assays (LTC-IC, CFAC, $CD34^+/CD38^-$ cells) as the methods for quantitation of human HSCs instead of xenotransplantation assay using NOD/SCID mouse, wrong functions may be ascribed to specific cytokines unless highly enriched target cells are used and serum-free conditions are employed, in order to eliminate any colony-stimulating activity produced by accessory cells or contained in serum [23]. For example, the contribution of IL-3 for maintenance or expansion of human HSCs has continued to be indefinite until recently.

4. SCF

Mice with mutations at either the dominant *W* or *Sl* loci encoding for the c-*kit* and SCF, respectively, display a similar phenotype characterized by a reduction in the number of HSCs with hypoplastic anemia. Although the number of molecules per cell varies extensively according to the cell type, c-*kit* is now known to be expressed by most of the hematopoietic cells [24]. SCF plays a central role in the regulation of early hematopoiesis. In one series of experiments, SCF alone had no effect on HPP-CFCs from $CD34^+/HLA^-DR^-/CD15^-$ human BM cells. However, the addition of SCF to a combination of IL-3 and granulocyte-macrophage colony stimulating factor (GM-CSF) resulted in a 12-fold increase in the number of HPP-CFC-derived colonies. Moreover, SCF addition resulted in an increase in the number of erythroid elements in these colonies. Similar effects were observed on burst-forming unit-erythroid (BFU-E), conoly-forming unit granulocyte-macrophage (CFU-GM), and burst- and colony-forming unit-megakaryocyte (BFU-MK and CFU-MK) [25,26]. SCF has been studied extensively in ex vivo expansion experiments both in stroma-free and stroma-containing cultures. Although it is difficult to draw conclusions on the role of any single cytokine, SCF was frequently represented in the most effective combinations.

5. FL

On its own, FL has minimal activity in agar cultures of murine or human BM or CB enriched CD34+ cells, stimulating only dispersed CFU-GM but not HPP-CFC colonies,

Table 2
Ex vivo generation of primitive hematopoietic progenitor/stem cells from human CD34+ cells

CD34+ source	Cytokines	Test	Fold increase	Reference
BM	SCF, PIXY321	HPP-CFC	5.5	Blood 81:661,1992
PB	SCF, IL-1, IL-3, IL-6, EPO	LTCIC	1.1	Blood 84:2898,1994
PB	IL-1, IL-3, IL-6, SCF, EPO	LTCIC	>1	Blood 84:2829, 1994
CB	IL-1, IL-3, SCF	LTCIC	15–20	Blood Cells 20:468,1994
BM, CB	FL, IL-3, IL-6, SCF	LTCIC	increase	Blood 87:3563,1996
BM	FL, SCF, IL-3	LTCIC	30	J. Exp. Med. 183:2551,1996
CB	SCF, IL-3, IL-6, FL, TPO	LTCIC	0–6	Blood 90:365a,1997
CB	FL, TPO	LTCIC	>200,000	Blood 89:2644,1997
BM	SCF, IL-3, FL, IL-6, G-CSF, NGF	LTCIC	47–68	Proc. Natl. Acad. Sci. U. S. A. 93:1470,1997
CB	SCF, FL, IL-6/sIL-6R	CFU-Mix	increase	Blood 90:4363,1997
CB	FL, TPO, SCF, IL-6	LTCIC	280	Blood 93:3736,1999
CB	SCF, FL, TPO, G-CSF	LTCIC	47	Exp. Hematol. 28:1470,2000
CB	SCF, FL, TPO, G-CSF	E-LTCIC	21	Exp. Hematol. 28:1470,2000
CB	SCF, FL, TPO, IL-6/sIL-6R	CFU-Mix	increase	J. Clin. Invest. 105:1013,2000

mixed colony-forming unit (CFU-Mix), or BFU-E. However, in combination with other cytokines, such as SCF, IL-3, and GM-CSF, FL has addictive or more than addictive effects. These effects remain apparent even on single cells in wells [27]. Several lines of evidence suggest that FL works on the early myeloid progenitors. First, FL receptors are only detectable on $CD34^+$ but not $CD34^-$ human BM cells [28]. Second, in suspension cultures of $CD34^+$ column-separated CB cells, whereas SCF and GM-CSF/IL-3 fusion protein (PIXY321) favor the expansion of the more immature progenitors, the addition of FL enhances the amplification of more immature progenitors [27]. Third, FL was the only cytokine to increase the number of LTC-ICs above the input value after 10 days of liquid cultures of $CD34^+$/$CD38^-$ human BM cells [29]. Fourth, serum levels of FL, but not SCF, are elevated in patients with multilineage BM failure and correlate inversely with the colony-forming abilities of BM progenitors from these patients. The levels do not normalize after correction of a single lineage, such as after the transfusion of red blood cells [30].

In ex vivo expansion experiments, FL was compared to SCF. Neither factor could alone support the proliferation of Lin^-/Sca-1^+/c-*kit*$^+$ cells derived from 5-FU-treated murine BM cells in suspension cultures. Both factors were, however, synergistic with IL-11, enhancing the production of progenitors and of nucleated cells. Longer exposure to FL was required. No engraftment defect after incubation with these cytokines was noted after transplantation into lethally radiated mice except for the cells exposed to SCF and IL-11 for 21 days [31]. In a different set of experiments, FL in combination with TPO resulted in extensive expansion with little differentiation of $CD34^+$ CB cells cultured under stroma-free conditions. In stroma-free cultures of human $CD34^+$ CB cells, FL in combination with TPO induced a several-thousandfold expansion of both $CD34^+$/$CD38^-$ and $CD34^+$/$CD38^+$ populations after 20 weeks of culture. Furthermore, when the expanded cells were stimulated by IL-7, IL-11, and FL, the percentage of $CD2^+$ and $CD19^+$ cells reached 33.8% and 3.7%, respectively, after 14 days, suggesting that T- and B-lymphocyte progenitors were also expanded.

6. TPO

TPO has been shown in several studies to play a pivotal role in the regulation of megakaryopoiesis. In vitro, TPO promotes the proliferation and differentiation of immature megakaryocytes [32]. Its augmenting effects on CFU-MK are synergistic when combined with IL-3 and IL-6 [33]. Compared to those generated in cultures containing IL-3, megakaryocytes produced in the presence of TPO showed increased ploidy. TPO is lineage predominant but not specific [34]. Besides governing platelet production, TPO has a noticeable effect on the myeloid and erythroid lineages. When single adult BM $CD34^+$/$Thy1^+$/Lin^- cells were cultured in the presence of a murine stromal cell line, TPO alone resulted in a plating efficiency of 63% with an average of 531 cells per 1 cell input [35]. Although some culture displayed megakaryocytic differentiation, about 75% of cultures exhibited blast cell outgrowths with myeloid, erythroid, and megakaryocytic potential, suggesting that a hierarchy exists among $CD34^+$/Lin^-/$Thy1^+$ cells and that the least mature cells can be expanded under the influence of TPO while maintaining a multilineage differentiation potential. Similarly, TPO acted synergistically with SCF and IL-3 to support the production of CFU-GM, CFU-E, and CFU-Mix in suspension cultures of $CD34^+$/c-kit^{low}/$CD38^{low}$ human BM cells [36].

7. IL-6/sIL-6R

IL-6 is a member of the IL-6 cytokine family, which also includes IL-11, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), and cardiotropin-1 (CT-1) [37]. These cytokines mediate their biologic action through the common subunit gp130. Binding of IL-6 to IL-6 receptor (IL-6R) induces homodimerarization of gp130, which in turn leads to intracellular signaling events. The gp130 molecule has been shown to play an important role in hematopoiesis, as gp130-deficient mice exhibit a greatly diminished number of hematopoietic progenitors [38]. IL-6 alone does not have distinct biological activity on HSC expansion. IL-6 has been shown to act as a potent cofactor in the expansion of human $CD34^+$ progenitor cells in vitro [39–41]. IL-6 and IL-3 induce synergistically the proliferation of murine pluripotent hematopoietic progenitors in vitro [42]. The combination of IL-6 and IL-3 acts on blast cell colony forming cells to cause them to leave G0 earlier. However, we and others demonstrated that pluripotent HSC express only low levels of Flk2/Flt3 and c-kit while lacking expression of IL-6R [43–45]. Activation of gp130 signaling in cells lacking IL-6R can be achieved through a complex of IL-6 and soluble IL-6R (IL-6/sIL-6R) [46,47]. We will mention later again, direct stimulation of gp130 by IL-6/sIL-6 in the presence of SCF has been shown to induce extensive proliferation of human hematopoietic progenitor cells in vitro [47]. In this system, the high concentration of sIL-6R protein is required. Using a novel approach, a fusion protein consisting of IL-6 and sIL-6R linked by a flexible polypeptide (Hyper-IL-6) was produced and has been shown to be useful at 100- to 1000-fold lower concentrations than unlinked IL-6 and sIL-6R [48]. The combination of Hyper-IL-6 and FL was markedly effective for expansion of PB $CD34^+$ cells [49].

8. IL-3

IL-3 has the broadest target specificity of any of the cytokines and the hematopoietic growth factors. The range

of target cells can be summarized as including progenitor cells of every lineage derived from the pluripotent HSCs. Moreover, IL-3 acts on more immature hematopoietic cells. The contribution of IL-3 for the self-renewal of HSCs varies from report to report. IL-3 stimulates the growth in vitro of colonies containing mixtures of myeloid and erythroid cells and stimulates both in vitro and in vivo the division of cells (CFUs) that form splenic colonies in irradiated mice [50]. IL-3 also stimulates the growth of human HSCs with significant capacity for self-renewal [51]. However, according to Peters' report, stimulation with IL-3 may results in a decreased ability of stem cell populations to self-renew as assessed by long-term repopulating capacity [3,52]. We also demonstrated that addition of IL-3 to the cytokine combination of SCF, FL, TPO, and IL-6/sIL-6R abrogated the repopulating ability of the expanded CB $CD34^+$ cells [17].

9. Combination of cytokines for the expansion of human hematopoietic stem/progenitor cells

The optimal choice of hematopoietic growth factors for the ex vivo expansion of human HSCs has not yet been determined. Various combinations of cytokines were examined. One of the most important cytokines is SCF, which was shown to be a survival factor for hematopoietic progenitor cells even in the absence of cell division [53]. Various other cytokines, including IL-1, IL-3, IL-6, G-CSF, GM-CSF, and erythropoietin (EPO), also have been investigated with respect to their ability to expand hematopoietic stem/progenitor cells. Recently, two cytokines (FL and TPO) have been shown to significantly improve the generation of stem/progenitor cells ex vivo. FL strongly potentiates the clonogenic capacity of immature progenitor/stem cells when combined with other growth factors [31,41,54–57]. TPO, in addition to its effects on the megakaryocytic cell lineage, enhances proliferation of stem/progenitor cells [32,58,59]. The representative combinations of cytokines attempted are listed in Table 2. The addition of growth factors (e.g., G-CSF, GM-CSF, and EPO) to these cytokines results in optimal generation of committed progenitor cells.

Recently, we have shown that gp130 is expressed at a low level of most $CD34^+$ cells isolated from human CB, whereas IL-6R is expressed in only 30% to 50% of these cells [47]. The complex of IL-6 and sIL-6R can stimulate the cells which express only gp130 without IL-6R. Clonal cultures of fluorescence-activated cell-sorted $CD34^+IL\text{-}6R^+$ and $CD34^+IL\text{-}6R^-$ cells revealed that most immature progenitors such as multipotent colony-forming units (granulocyte-erythrocyte-macrophage-megakaryocyte colony-forming units [CFU-GEMM] and blast colony-forming units (CFU-Blast)] were included in $CD34^+IL\text{-}6R^-$ cells in methylcellulose culture with a combination of IL-6, sIL-6R, and SCF 45. In a serum-free suspension of $CD34^+IL\text{-}6R^-$ cells in culture supplemented with a combination of IL-6, sIL-6R, FL, and TPO in the presence or absence of SCF, the number of multipotent immature progenitor/stem cells dramatically increased.

We are not sure whether this procedure is also effective for the expansion of progenitor/stem cells derived from BM or PB. Several authors described that the growth factor requirement for the expansion of HSC from different sources is not equivalent. Based on these viewpoints, the optimal combination of cytokines for HSC expansion is required to be evaluated individually.

10. In vivo evaluation of expanded HSCs using NOD/SCID mice

The most challenging question in stem-cell biology is whether the most immature progenitor/stem cells also can be expanded ex vivo. The most reliable method for quantitation of these cells is in vivo assays using xenotransplantation into NOD/SCID mice (SCID-repopulating cell, SRC) [60,61]. To verify whether ex vivo-cultured human $CD34^+$ cell populations might lose or increase true stem-cell properties compared with freshly isolated $CD34^+$ cells, several in vivo models have been investigated. Table 3 reveals the representative experiments for human hematopoietic cells. We demonstrated that the dramatic amplification of human HSCs capable of repopulating in NOD/SCID mice can be obtained in culture system of CB $CD34^+$ cells with SCF, FL, TPO, and IL-6/sIL-6R. The contribution of sIL-6R is indispensable to this efficient expansion. Interestingly, Peters et al. reported that expansion of murine hematopoietic progenitor/stem cell cultures with SCF, IL-3, IL-6, and IL-11 led to an engraftment defect of long-term repopulating cells, although short-term reconstitution capability was maintained [3]. Similar observations have been described by Yonemura et al. [52], indicating that the addition of IL-1 and IL-3 for ex vivo expansion of primitive hematopoietic cells significantly reduces reconsti-

Table 3
In vivo studies with ex vivo-expanded hematopoietic stem/progenitor cells

CD34+ source	Cytokines used for expansion	Long-term reconstitution	References
CB	FL, SCF, IL-3, IL-6, G-CSF	two fold increase in SRC	Proc. Natl. Acad. Sci. 94:9836,1997
CB	FL, SCF, IL-3, IL-6, G-CSF	two- to four fold increase in SRC	J. Exp. Med. 186:619,1997
CB	FL, SCF, TPO, IL-6	increase	Blood 93:3736,1999
CB	FL, SCF, TPO, IL-6/sIL-6R	4.2-fold increase in SRC	J. Clin. Invest. 105:1013,2000
CB	FL, SCF, TPO, IL-3, IL-6, IL-11	increase	Br. J. Haematol. 108:629,2000
CB	FL, SCF, TPO, G-CSF	increase	Exp. Hematol. 28:1470,2000

tution ability compared with cells stimulated without these two factors.

We also observed that the addition of IL-3 to efficient amplification system with SCF, FL, TPO, and IL-6/sIL-6R abrogates the repopulating ability of the expanded cells [17]. However, in contrast, other reports have shown that ex vivo expansion with hematopoietic growth factors—excluding high-dose IL-1 and IL-3—allows for both short- and long-term reconstitution, as well as the ability to transplant these cells serially [62,63]. Based on these data, the use of IL-1 and/or IL-3 has to be evaluated carefully in attempts to expand primitive HSC ex vivo for use in a myeloablative clinical setting.

11. Clinical experience with ex vivo-generated stem/progenitor cells

HSC transplantation has undergone a dramatic change over the past few years both in the number of transplants and in the way that these transplants are being done. There are a variety of clinical applications for culture and/or expansion of immature hematopoietic progenitor/stem cells [64,65]. These include increasing the number of stem cell transplantation cases where a therapeutic dose of transplantable cells cannot be obtained by conventional methods. The first report on the use of ex-vivo-manipulated stem cells to transplant patients came from Brugger et al. [66] and was designed to test the safety of ex vivo manipulation of stem cells. PB progenitor cells were harvested from 10 G-CSF mobilized patients with a variety of solid tumors (lung carcinoma, breast cancer, sarcoma, or undefined tumor) following nonablative chemotherapy. An average of 1×10^7 $CD34^+$ immature hematopoietic cells were cultured for 12 days in media containing autologous plasma, SCF, IL-1β, IL-3, IL-6, and EPO. Each patient received ex vivo-expanded PB progenitor cells following two cycles of induction therapy. Four of the patients also received unmanipulated BM cells to supplement the cultured cells. All of them gained an absolute neutrophil count of >500/mm^3 by day 12 along after receiving a dose of >100,000 CFU-GM/kg body weight. Platelet count also CFU-recovered to >20,000/mm^3 by day 12 and reached to >50,000/mm^3 by day 14. Analyses of the correlation between the time to hematopoietic recovery and the number of transplanted colony-forming cells suggested that a threshold dose of approximately 10^5 CFU-GM/kg was needed for rapid engraftment. Although immature hematopoietic progenitor cells, as defined by LTC-IC, persist under the culture conditions used, the capability of the ex vivo-generated cells for long-term hematopoiesis cannot be determined by this study.

In another clinical study, Alcorn et al. [67] reinfused into patients ex vivo-expanded cells along with unmanipulated cryopreserved PB progenitor cells. The protocol was safe, and hematopoietic recovery was not influenced by the additional transplantation of ex vivo-generated cells. Recently, these investigators reported on four additional patients who received ex vivo-expanded cells only after truly myeloablative chemotherapy [68]. Short-term hematopoietic recovery of neutrophils was observed in three of the four patients. The patient who did not engraft by day 14 had received the lowest dose of CFU-GM. However, at least two of the three patients with short-term reconstitution failed to show long-term engraftment after myeloablative conditioning. These observations indicate that a procedure which allows expansion of committed progenitors but no expansion of more immature hematopoietic cells in fact may not contain sufficient numbers of long-term repopulating stem cells to insure persistent engraftment after myeloablative therapy.

A subsequent report addressed the ability of ex vivo-cultured stem cells to provide durable engraftment in a myeloablative host. Breast cancer patients undergoing high-dose chemotherapy had BM cells harvested for expansion in a perfusion bioreactor just prior to therapy. Cells were used to seed the bioreactor where cultures were grown for 12 days in EPO, PIXY321, and FL. The use of a mononuclear cell population in this bioreactor system was shown to be superior to inoculating with purified $CD34^+/Lin^-$ cells presumably due to the presence of stroma cells in the inoculum [69]. Under these conditions, CFU-GM was expanded 10-fold but LTCIC and $CD34^+/Lin^-$ cells were reduced to half or less of the input amount. Still, all patients showed rapid neutrophil and platelet engraftment (an average of 18 and 23 days) that was sustained for several months of follow-up.

Recently, Stiff et al. [70] reported that the ex vivo expansion of small numbers of BM cells, without prior $CD34^+$ cell selection, in a stroma-based bioreactor with exogenous PIXY-321, FL, and EPO support was able to produce durable hematopoietic reconstitution after myeloablative chemotherapy in 16 patients with advanced breast cancer. McNiece also reported the ex vivo-expanded PB progenitor cells as a source of hematopoietic support to decrease or eliminate the period of neutropenia, with long-term engraftment. $CD34^+$ cells selected from G-CSF mobilized PB progenitor cells from patients with breast cancer were cultured for 10 days in defined medium containing SCF, G-CSF and TPO [71]. The patients who received expanded PB progenitor cells engrafted neutrophils in a median of 8 days. These patients are past the 15-month posttransplantation stage with no evidence of late graft failure.

Several abstracts were reported at the American Society of Hematology meeting in Miami describing studies of ex vivo expansion using the Aastrom cell production system for CB and PBPC. Kurtzberg and colleagues described the expansion of CB products that were transplanted into 21 patients. Each patient received unmanipulated CB cells on day 0 and the expanded cells on day +12. No significant effects on engraftment kinetics were observed in these patients. Stiff and colleagues also reported on expansion of CB cells in the Aastrom system for nine patients. The

median time to neutrophil engraftment was 26 days, with a range of 14 to 36 days. Engraftment of platelets was delayed in these patients. The authors conclude that ex vivo-expanded CB cells may be useful in adults with otherwise incurable hematologic disorders. The Aastrom system has also been used for the expansion of BM cells. Studies have demonstrated that expansion of a small aliquot of BM cells can provide short- and long-term engraftment following myeloablative chemotherapy [72]. Other studies have combined the expanded BM cells with single PB progenitor cell products, and in the reports the patients engraft neutrophils and platelets equivalent to optimal PB progenitor cell products. The evaluation of these reports is required to be properly done.

12. Future prospect

A great deal of experiences over the past 10 years in terms of the ability to isolate and culture primitive hematopoietic stem and progenitor cells has been attempted. However, nobody has yet reached a stage to use ex vivo-expanded cells routinely for hematopoietic replacement therapy [65]. Issues related to the identification of human HSC still remain to be dissolved to characterize the expanded population that keeps durable hematopoietic reconstitution. The advantage of animal models of transplantation has allowed us to create reliable criteria for evaluating the stem cell content of a population. However, these models require several months to provide an answer. Growth factors and other soluble factors of stem cell proliferation have provided great insight into the biology of stem cells, but we do not yet understand how to organize these molecules in a physiologically relevant manner. Up to now, regrettably, no clinical state has been identified in which transplantation of expanded cells was clearly advantageous over unmanipulated cells. Moreover, successful expansion of HSCs is not to be expected from heavily pretreated patients. To be widely accepted for clinical use, procedures for ex vivo stem cell expansion are required to meet the requirements for Good Manufacturing Practices (GMPs).

Although many issues to be settled remain now, the tremendous progress has been in progress in this field. The perpetual discoveries in molecular biology and developmental biology help this process cooperatively. The establishment of the procedure for clinical application of ex vivo-expanded HSCs should pave the way to the new era for intractable disorders now and build up hope for these patients.

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Complete reconstitution of human lymphocytes from cord blood CD34$^+$ cells using the NOD/SCID/γ_c^{null} mice model

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Establishment of an assay capable of generating all classes of human lymphocytes from hematopoietic stem cells (HSCs) will provide new insight into the mechanism of human lymphopoiesis. We report ontogenic, functional, and histologic examination results of reconstituted human lymphocytes in NOD/SCID/γ_c^{null} mice after the transplantation of human cord blood (CB) CD34$^+$ cells. After transplantation, human B, natural killer (NK), and T cells were invariably identified in these mice, even though no human tissues were cotransplanted. Immature B cells resided mainly in bone marrow (BM), whereas mature B cells with surface immunoglobulins were preferentially found in spleen. NK cells were identified in BM and spleen. T cells were observed in various lymphoid organs, but serial examinations after transplantation confirmed human T lymphopoiesis occurring in the thymus. These human lymphocytes were also functionally competent. Human immunoglobulin M (IgM), IgA, and IgG were detected in the sera of these mice. T cells showed a diverse repertoire of T-cell–receptor Vβ (TCR Vβ) chains, proliferated in response to phytohemagglutinin, and were cytotoxic against cell lines. NK activity was demonstrated using the K562 cell line. Immunohistochemical analysis revealed that human lymphocytes formed organized structures in spleen and thymus that were analogous to those seen in humans. In the thymus, CD4 and CD8 double-positive T cells were predominant and coexpressed CD1a and Ki-67, thereby supporting the notion that T lymphopoiesis was taking place. NOD/SCID/γ_c^{null} mice provide a unique model to investigate human lymphopoiesis without the cotransplantation of human tissues. (Blood. 2003;102:873-880)

Introduction

Reconstitution of functional human lymphocytes in experimental animals has been intensely explored because this approach could provide a valuable means of assessing human immunity or of preclinical testing of vaccines, pathogens, and new therapeutic strategies. In 1988, the first reports on engraftment of human hematopoietic cells in homozygous severe combined immunodeficiency disease (*scid*) mice were published.[1,2] McCune et al[1] reported that the simultaneous transplantation of fetal liver hematopoietic cells, fetal thymus, and fetal lymph node resulted in the differentiation of mature human T and B cells (SCID-hu mice model). Mosier et al[2] reported that the transplantation of human peripheral blood (PB) mononuclear cells into *scid* mice resulted in the successful transfer of a functional human immune system (hu-PBL-SCID mice model). These initial models were soon followed by experimental human bone marrow (BM) transplantation into *scid* mice models,[3-9] with modification—the cotransplantation of other human tissues, such as bone, or treating recipient mice with a combination of human cytokines. These modified models showed the multilineage differentiation of human hematopoietic cells to varying degrees. In some experimental settings, SCID-hu mice generated all classes of human immunoglobulins, thereby indicating the presence of human lymphocytes that interacted in response to environmental antigens and that induced B cells to undergo immunoglobulin switching. Although the use of these systems is feasible, they require various kinds of fetal human tissues or human cytokines. Moreover, the procedure is laborious, and engraftment of human hematopoietic cells is highly variable.

To overcome these problems, systematic approaches have been explored. One major approach involves suppressing the innate immunity of *scid* mice, based on the hypothesis that an efficient engraftment level can be achieved by eliminating residual innate immunity in the host. The NOD/SCID mouse strain was found to be an efficient recipient for the reconstitution of human hematopoietic cells.[10-13] However, complete multilineage differentiation, including T cells, has not been achieved using this strain. To suppress residual natural killer (NK) activity in recipient mice, the β2 microglobulin-null ($\beta2m^{-/-}$) allele was backcrossed onto the NOD/LtSz-*scid* background, and a new strain of NOD/SCID-$\beta2m^{null}$ mice was established. In these mice there was an efficient engraftment for human hematopoietic stem cells (HSCs),[14,15] but human T cells were not detected. To improve engraftment efficiency, we established a strain of NOD/Shi-*scid* mice that have a defective common cytokine receptor, γ_c (NOD/SCID/γ_c^{null}).[16] Mutation in the common cytokine receptor γ chain led to a life-threatening, X-linked, severe combined immunodeficiency disease (XSCID) in humans, characterized by an extremely low

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Submitted September 9, 2002; accepted March 13, 2003. Prepublished online as *Blood* First Edition Paper, April 10, 2003; DOI 10.1182/blood-2002-09-2755.

Supported by the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan and by a Grant-in-Aid (13GS0009) for Creative Scientific Research, from the Ministry of Education, Science, Technology, Sports and Culture of Japan.

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number of T and NK cells.[17,18] We observed that NOD/SCID/γ_c^{null} mice had no lymphocytes, no NK activity, and impaired dendritic cell function, thought to lead to high engraftment efficiency and full-lineage differentiation, including those of T cells.[16] Following reports of the generation of functional T cells in these mice,[19] we describe here the results of a comprehensive analysis of human T, B, and NK cells generated in NOD/SCID/γ_c^{null} mice, with regard to developmental process and functional maturation. Human T cells developed in the thymi of these mice, moved to the periphery, and functionally matured to produce cytokines and to have cytotoxicity. Human B cells matured to produce not only human IgM but also IgG and IgA. As in T cells, NK cells matured and had cytotoxicity against K562 cells. Histologic examinations revealed the formation of organized structures of lymphoid organs. This new mouse model is expected to pave the way toward the reconstitution of a human immune system within the body of a laboratory animal.

Materials and methods

Mice

NOD/Shi-*scid*, NOD/SCIDβ2m^{null}, and NOD/SCID/γ_c^{null} mice were used in this study. The NOD/Shi-*scid* strain was established, as reported.[20] NOD/SCID-β2m^{null} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). NOD/SCID/γ_c^{null} mice were developed at the Central Institute of Experimental Animals (Kawasaki, Japan) by crossing NOD/Shi-*scid* mice with C57B6/J-IL-2Rγ^{null} mice. All mice were bred as a homozygous line, shipped to the animal facility of Kyoto University (Kyoto, Japan), and kept under specific pathogen-free conditions in accordance with the guidelines of the facility.

Purification of cell populations

Human CB was collected during normal full-term deliveries, after obtaining informed consent. Mononuclear cells (MNCs) were separated by Ficoll-Hypaque density gradient centrifugation after the depletion of phagocytes with Silica (Immuno Biological Laboratories, Fujioka, Japan). CD34$^+$ cell fractions were isolated using AutoMACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Purity was evaluated using flow cytometry. By using Silica and AutoMACS with the most sensitive "Possel d2" protocol, no less than 95% (typically 98%) of the positively selected cells were CD34$^+$. In experiments using lineage-depleted CD34$^+$ cells (Lin$^-$ CD34$^+$), CB MNCs were depleted of lineage-positive cells using StemSep (Stem Cell Technologies, Vancouver, BC, Canada) followed by CD34$^+$ selection with AutoMACS. StemSep contains a cocktail of monoclonal antibodies of antihuman CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, and antihuman glycophorin A. After combined cell selection with StemSep and AutoMACS, almost all the lineage-positive cell fraction was removed. Depletion of CD56$^+$ cells was carried out with AutoMACS and CD56 microbeads (Miltenyi) according to manufacturer's protocol. By choosing the "deplete 025" protocol, almost all CD56$^+$ cells were successfully eliminated.

Transplantation of CB CD34$^+$ cells into mice

Xenotransplantation of purified CB CD34$^+$ cells was performed using a modification of a previously reported method.[11,12] Briefly, 8- to 12-week-old NOD/Shi-*scid*, NOD/SCID-β2m^{null}, and NOD/SCID/γ_c^{null} mice received 240 cGy radiation. The indicated dose of CB CD34$^+$ cells was injected through the tail vein. Only NOD/Shi-*scid* mice were treated with 400 μL phosphate-buffered saline (PBS) containing 20 μL antiasialo GM1 antiserum (Wako, Osaka, Japan) shortly before cell transplantation and every 11th day thereafter. After transplantation, mice were prophylactically provided sterile water with added neomycin sulfate (Gibco BRL, Grand Island, NY).

Flow cytometric analysis of mice with transplanted human cells

PB was taken from the retro-orbital venous plexus at the indicated times after transplantation. Blood was collected through heparinized calibrated pipettes and transferred to EDTA (ethylenediaminetetraacetic acid)–2 Na containing CAPIJECT (Terumo Medical, Somerset, NJ). Lineage analysis of human hematopoietic cells was made using flow cytometry (FACScalibur; BD PharMingen, San Diego, CA) according to the manufacturer's protocol. Mice were killed by cervical dislocation more than 5 months after cell transplantation. BM, spleens, and thymi were analyzed using flow cytometry. Antibodies used for flow cytometric analysis were antihuman CD45-fluorescein isothiocyanate (CD45-FITC), CD3-FITC, TdT-FITC, CD34-phycoerythrin (CD34-PE), CD10-PE, CD3-PE, T-cell receptor (TCR) γδ-PE, CD158a-PE, KIR p70-PE, antihuman surface IgD-PE, IgM-PE, IgG-PE, IgA-PE, CD8-phycoerythrin 5-succinimidylester (PC5), CD19-PC5, TCR αβ-PC5, and antimouse CD45-allophycocyanin (CD45-APC). Antibodies conjugated with FITC, PE, or APC were purchased from BD PharMingen except anti–CD158a-PE and anti–KIR p70-PE. Antibodies conjugated with PC5, anti-CD158a-PE, and anti-KIR p70-PE were purchased from Immunotech (Marseilles, France). The Vβ repertoire of TCR was analyzed using IOTest Beta Mark TCRVβ Repertoire kits (Immunotech).

Reverse transcription–polymerase chain reaction

RNA was isolated from the spleen and BM of NOD/SCID/γ_c^{null} mice with or without transplanted CD34$^+$ cell using TRIzol Reagent (Invitrogen, Carlsbad, CA). Total RNA (1 μg) was taken from each sample and subjected to reverse transcription using SuperScript (Invitrogen). Using one-twentieth synthesized cDNA, PCR amplification was carried out with Takara LA Taq (Takara Bio, Ohtsu, Japan). Samples were denatured at 94°C for 5 minutes, then amplified by rounds consisting of 94°C for 30 seconds (denaturing), 60°C for 30 seconds (annealing), and 72°C for 30 seconds (extension) for 31 cycles using primer sets as follows: interleukin-2 (IL-2), 5′-CTTCAGTGTCTAGAAGAAGAACTCAA-3′ and 5′-GGTAAAC-CATTTTAGAGCCCC-3′; IL-15, 5′-CTGACTCTCAGTTCAGTTT-TACTCT-3′ and 5′-TCTAAGCAGCAGAGTGATGTTTG-3′; human hypoxanthine phosphoribosyltransferase (HPRT) 5′-AATTATGGACAG-GACTGAACGTC-3′ and 5′-CGTGGGGTCCTTTTCACCAGCAAG-3′; mouse HPRT 5′-GCTGGTGAAAAGGACCTCT-3′ and 5′-CACAGGAC-TAGAACACCTGC-3′. PCR products were separated on 2.0% agarose gel, stained with ethidium bromide, and photographed.

Functional analysis of human lymphocytes generated in NOD/SCID/γ_c^{null} mice

Anti–CD3-dependent cytotoxic T lymphocyte (CTL) activity was tested using a calcine release assay, with minor modification.[21] Briefly, human MNCs in the spleen of a mouse that underwent cell transplantation were separated by Ficoll-Hypaque density gradient centrifugation. MNCs were cultured at 1 × 10^6/mL in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) with phytohemagglutinin (PHA; 1 μg/mL) and human IL-2 (50 IU/mL) for 48 hours. After taking the supernatant to analyze human cytokine production, culture was continued without PHA for 8 additional days. Expanded lymphocytes were suspended in Hanks balanced salt solution supplemented with 5% FBS. Target cells labeled with calcine-AM (Molecular Probes, Eugene, OR) were mixed with T blasts at the indicated effector-target (E/T) ratios in the presence of anti-CD3 monoclonal antibody (mAb). Cells with isotype-control mAb or without mAb were also prepared as negative controls. After 3 hours of incubation, the release of calcine into the supernatant was measured with an automated fluorescence scanner (Wallac 1420ARVOsx; Perkin Elmer, Fremont, CA). NK cell activity was measured in a similar way. In analyzing NK cell activity, expanded lymphocytes were mixed with calcine-labeled K562 cells without antibodies. The CD56$^+$ NK cell population comprised less than 10% expanded lymphocytes. To measure the total release of calcine, 100 μL lysis buffer (50 mM sodium-borate, 0.1% Triton X-100, pH 9.0) was added to the wells with only labeled target cells after aliquots of supernatant had been taken to measure spontaneous release. Percentage cytotoxicity was determined using the following equation: (F CTL assay − F spontaneous

release)/(*F* total lysis − *F* spontaneous release) × 100 = % cytotoxicity, where *F* represents fluorescence. All assays were performed in quadruplicate, and PB MNCs of a healthy adult donor served as a positive control. Human cytokine analysis was made using the Cytometric Bead Array Kit for human cytokines according to the manufacturer's protocol (BD PharMingen). Intracellular cytokine staining was performed as follows: PHA-stimulated splenocytes were further stimulated with 10 ng/mL phorbol ester (PMA; Sigma, St Louis, MO) and 1 μg/mL Ca^{2+} ionophore (ionomycin; Sigma) for 6 hours. Brefeldin A (Sigma) was added at final concentration of 10 μg/mL during the last 2 hours. Cells were washed with PBS with 2% FCS and stained with antihuman CD56-PC5 (Immunotech). Then the cells were washed with PBS with 2% FCS, fixed, and permeabilized with Cytofix/Cytoperm (BD PharMingen) for 20 minutes. After washing with Perm/Wash (BD PharMingen) twice, the cells were incubated with FITC- or PE-conjugated isotype control mAb (BD PharMingen), anti–IFN-γ (BD PharMingen), anti–IL-4 (BD PharMingen), and anti–tumor necrosis factor-α (anti–TNF-α) (Immunotech) for 30 minutes at 4°C. After washing with Perm/Wash, the cells were analyzed by flow cytometry.

Immunoglobulin analysis

Human IgA, IgM, and IgG in plasma of NOD/SCID/γ_c^{null} or NOD/SCID-$\beta 2m^{null}$ mice were measured using human immunoglobulin assay kits (Bethyl, Montgomery, TX). Cross-reactivity against mouse IgG, IgA, and IgM checked with mouse serum standard (Bethyl) were 0.1% for IgM and less than 0.01% for IgG and IgM.

Immunohistochemical and immunofluorescence analysis

Frozen blocks were cut at 4 to 6 μm, and, after air-drying, sections were fixed in cold acetone. After blocking with 0.5% casein and 5% goat serum, the following primary antibodies were incubated for 1 hour at room temperature: mouse antihuman CD3 (UCHT-1), mouse antihuman CD20-EPOS (2L6), mouse antihuman CD1a (BL6), and mouse antihuman Ki-67 (MIB-1). An appropriate secondary antibody was incubated for 30 minutes, and either DAB or VECTOR Blue (Vector Laboratories, Burlingame, CA) was used for visualization. For confocal microscopy, each tissue was stained sequentially with mouse antihuman CD4 (NU-$T_{H/I}$) followed by a Cy3-labeled F(ab')$_2$ fragment of donkey antimouse IgG and with biotinylated mouse antihuman CD8 (HIT8α) followed by streptavidin Alexa 488.

Statistical analysis

Data are presented as the mean ± SEM. Statistical significance was determined using the Mann-Whitney *U* test. $P < .05$.

Results

Efficient engraftment and full lineage lymphoid differentiation from CB CD34$^+$ cells in NOD/SCID/γ_c^{null} mice

To evaluate the efficiency of engraftment of human hematopoietic cells in NOD/SCID/γ_c^{null} mice, 1×10^4 or 5×10^4 CB CD34$^+$ cells were transplanted into NOD/SCID/γ_c^{null} mice, and 1×10^5 CB CD34$^+$ cells were transplanted into NOD/Shi-*scid* (NOD/SCID) mice; the engrafted human hematopoietic cells were then analyzed using flow cytometry. Three months after transplantation, the percentage of human CD45$^+$ cells in the peripheral blood was much higher in NOD/SCID/γ_c^{null} mice, even though the transplanted cell dose was only one half or one tenth that for NOD/SCID mice (Figure 1A). Four months after transplantation of 1×10^5 CB CD34$^+$ cells, the percentage of human CD45$^+$ cells in the BM of NOD/SCID/γ_c^{null} mice was much higher than that in NOD/SCID (72.6% ± 6.3% vs 10.6% ± 4.4%; n = 8; $P < .01$).[16]

Analysis of BM and spleen revealed that the NOD/SCID/γ_c^{null} mice carried all classes of human lymphocytes—human CD3$^+$ T cells, CD3$^-$CD56$^+$ NK cells, and CD19$^+$ B cells. CD5$^+$ B cells were considered to constitute an ontogenically unique subpopulation (Figure 1B). Expression of killer inhibitory receptor (KIR) antigens, such as CD158a and KIR p70, was also identified on a population of CD56$^+$ NK cells (Figure 1C). Human B cells and NK cells were detected in NOD/SCID mice, but the percentage of these cells was low and human CD3$^+$ T cells were never identified.

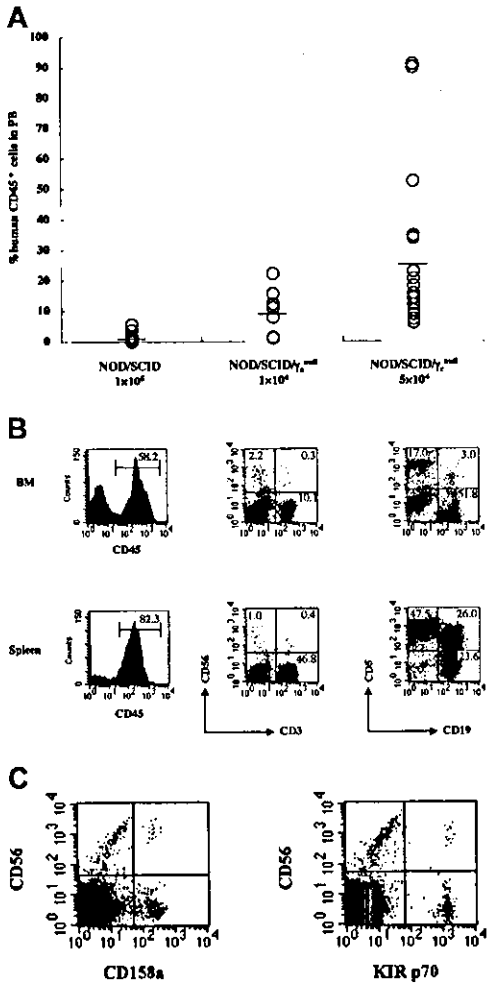

Figure 1. Efficient engraftment and complete reconstitution of human lymphocytes in NOD/SCID/γ_c^{null} mice that underwent transplantation with CB CD34$^+$ cells. 1×10^4 (n = 7) or 5×10^4 (n = 17) CB CD34$^+$ cells were transplanted into NOD/SCID/γ_c^{null} mice, and 1×10^5 (n = 15) CB CD34$^+$ cells were transplanted into NOD/SCID mice. (A) Percentages of human CD45$^+$ cells in PB of mice were analyzed 3 months after transplantation using flow cytometry. Percentages of human CD45$^+$ cells were calculated as: % human CD45$^+$ = human CD45$^+$ cells/(human CD45$^+$ cells + mouse CD45$^+$ cells) × 100. Results of 7 independent experiments are shown. (B) Representative FACS analysis of human lymphocytes in BM and spleen of NOD/SCID/γ_c^{null} mice that underwent transplantation with CB CD34$^+$ cells. (C) Representative FACS analysis of KIR antigens is shown. CD158a or KIR p70 were detected on 7.7% or 3.8% of CD56$^+$ cells, respectively.

Flow cytometric analysis of human T cells in NOD/SCID/γ_c^{null} mice after transplantation of CB CD34$^+$ cells

Next, we focused on human T cells in the NOD/SCID/γ_c^{null} mice. After the transplantation of 4×10^4 CB CD34$^+$ cells into NOD/SCID/γ_c^{null}, NOD/SCID-$\beta 2m^{null}$, and NOD/SCID mice, human CD3$^+$ T cells in PB were measured sequentially using flow cytometry. Human T cells were identified only in NOD/SCID/γ_c^{null} mice, and the percentage of T cells was gradually increased with time (Figure 2A).

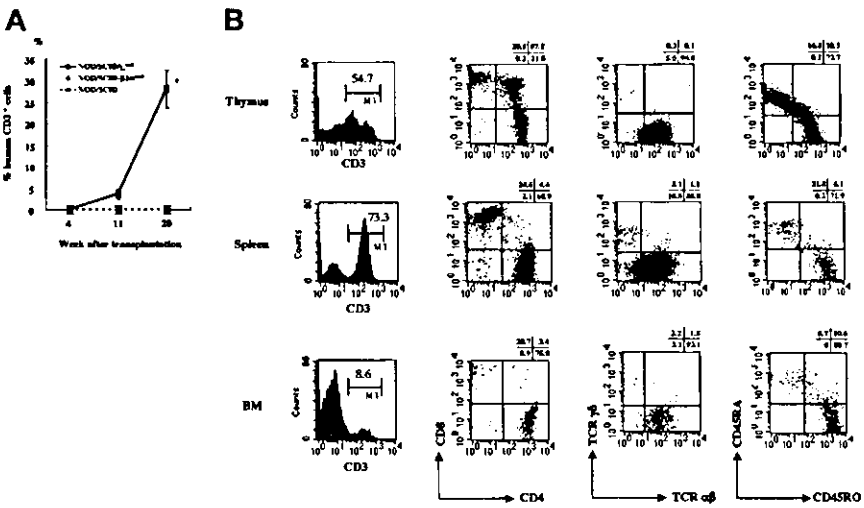

Figure 2. Human CD3⁺ cells in PB of NOD/SCID/γ_c^{null}, NOD/SCID-β2m^{null}, and NOD/SCID mice that underwent transplantation with CB CD34⁺ cells. (A) Percentage of human CD3⁺ cells among human CD45⁺ cells in PB was analyzed sequentially after the transplantation of 4×10^4 CB CD34⁺ cells. Results of 2 independent experiments (n = 3 each) are shown. Error bars represent SDs. (B) Surface phenotypes of human CD3⁺ T cells in thymus, spleen, and BM of NOD/SCID/γ_c^{null} mice were evaluated 5 months after the transplantation of 2 to 4×10^4 CB CD34⁺ cells. Representative data from 5 independent analyses of similar results are shown.

The surface phenotype of human T cells in the thymus, spleen, and BM was analyzed 5 months after transplantation using flow cytometry (Figure 2B). The thymi were always highly atrophic at this time, and approximately 2 to 5×10^5 human nucleated cells were identified.

Two subpopulations of CD3⁺ T cells, CD3^{high} and CD3^{low}, were identified in the thymus, but almost all the T cells in the spleen and BM had the CD3^{high} phenotype. Simultaneous staining of CD4 and CD8 revealed that CD4⁺CD8⁺ double-positive (DP) T cells were predominant in the thymus, whereas CD4⁺ or CD8⁺ single-positive (SP) T cells were predominant in the spleen and BM. Analysis of the TCR revealed that almost all T cells had αβ TCR in the thymus but that T cells with γδ TCR were also detected in the spleen and BM. Considerable numbers of naive T cells of the CD3⁺CD45RA⁺ phenotype were identified in all organs analyzed. NOD/SCID/γ_c^{null} mice with transplanted CB CD34⁺ cells showed little clinical evidence of graft-versus-host disease (GVHD), whereas transplantation of PB CD3⁺ cells evoked GVHD responses even shortly after the procedure (data not shown). Transplantation of CB CD3⁺CD45RA⁺ cells did not result in the maintenance of human T cells for more than 3 months (data not shown). These results are highly suggestive of the de novo generation of human T cells from HSCs in these mice.

Development of human T cells in the thymi of NOD/SCID/γ_c^{null} mice

To confirm the de novo generation of human T cells from HSCs and to clarify the site(s) responsible, we transplanted further purified lineage-depleted (Lin⁻) CB CD34⁺ cells into NOD/SCID/γ_c^{null} mice. The purity of transplanted Lin⁻ CD34⁺ was greater than 98%, and contamination of CD3⁺ cells was not identified by flow cytometry (Figure 3A). After the transplantation of 5×10^4 Lin⁻ CD34⁺ cells, various organs were analyzed through flow cytometry (Figure 3B-C).

After the transplantation of CB Lin⁻ CD34⁺ or CB CD34⁺ cells, human T cells became detectable in PB at approximately the 8th week. Therefore, we conducted our analysis shortly before (6th week after transplantation) and after (9th week after transplantation) the emergence of human T cells. At the 6th week, T cells were identified in the thymus, yet spleen, BM, and liver had no T cells. T cells in the thymus consisted mainly of DP T cells with barely detectable levels of double-negative (DN) T cells. At the 9th week, the thymus was further engrafted with DP T cells, and the spleen began to show a small number of T cells with the SP phenotype. These results clearly indicated that de novo generation of human T cells occurred in the thymus of NOD/SCID/γ_c^{null} mice, followed by seeding of these cells in peripheral organs such as the spleen.

Human B-cell development and immunoglobulin production in NOD/SCID/γ_c^{null} mice

B-cell–specific markers of human hematopoietic cells in the BM and spleen of NOD/SCID/γ_c^{null} mice were evaluated using flow cytometry (Figure 4A). In the BM, phenotypically immature B cells were predominant. A large number of human CD19⁺ B cells simultaneously expressed human CD34 or CD10. Terminal deoxytransferase (TdT)⁺ B cells were also identified in BM and corresponded to premature pro-B cells. Human surface immunoglobulin expression showed immature B cells (IgM⁺) to be the major population. On the other hand, most human B cells in the spleen were mature. The expression of human CD34 or TdT was hardly detectable, and human surface IgM⁺ and IgD⁺ B cells were more abundant than in the BM. To determine whether these B cells were functionally mature, we measured plasma human immunoglobulin levels using enzyme-linked immunosorbent assay (ELISA) (Figure 4B).

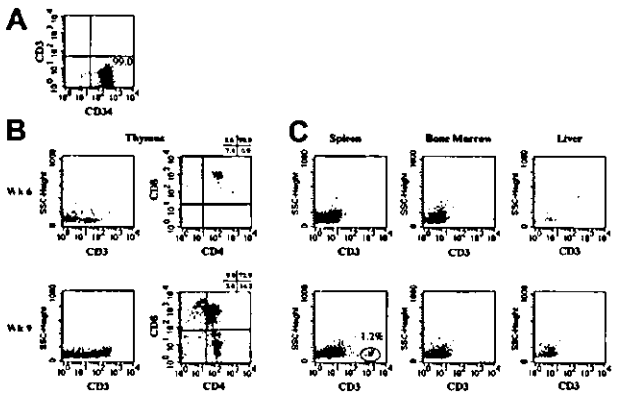

Figure 3. Human T cells in various organs of NOD/SCID/γ_c^{null} mice that underwent transplantation with CB Lin⁻ CD34⁺ cells. Human CD3⁺ cells in thymus, spleen, bone marrow, and liver were analyzed using flow cytometry at 6 and 9 weeks after the transplantation of 5×10^4 CB Lin⁻ CD34⁺ cells. (A) The purity of Lin⁻ CD34⁺ cells was evaluated using flow cytometry. (B) Expressions of human CD3, CD4, and CD8 on human CD45⁺ cells in thymus are shown. (C) Expressions of human CD3 on human CD45⁺ cells in spleen, bone marrow, and liver are shown.

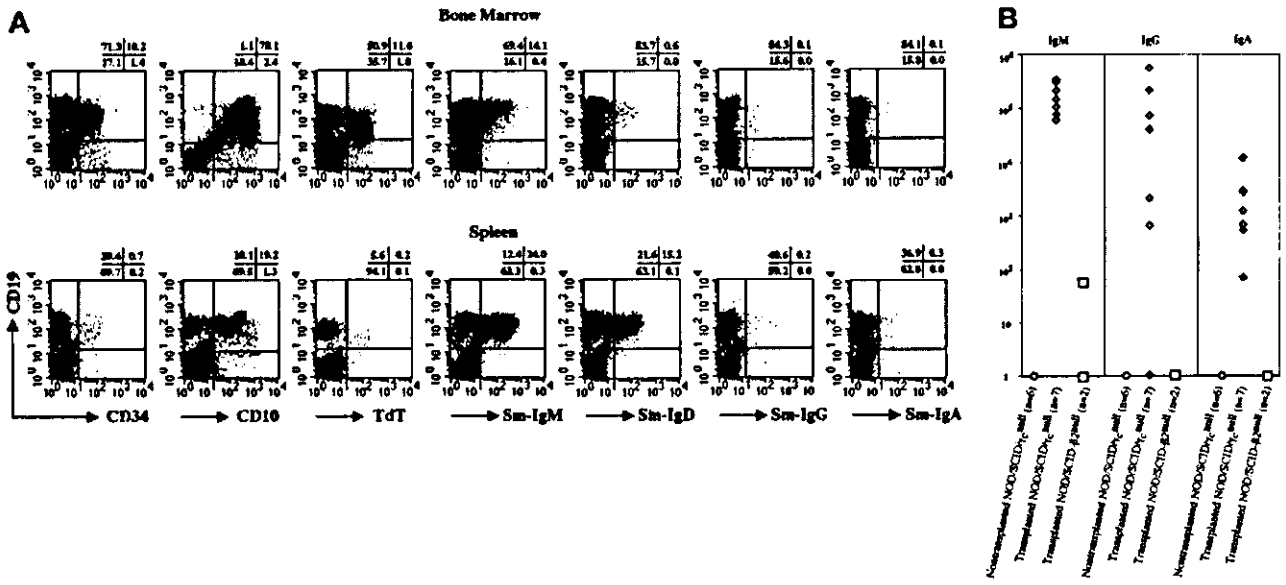

Figure 4. B-cell development in NOD/SCID/γ_c^{null} mice that underwent transplantation with CB CD34$^+$ cells. (A) Surface expressions of CD34, CD10, CD19, IgM, IgD, IgG, and IgA and intracellular expression of TdT were examined after setting a gate on the human CD45$^+$ population. (B) Human IgM, IgG, and IgA concentrations in plasma of NOD/SCID/γ_c^{null} and NOD/SCID-$\beta2m^{null}$ mice that underwent transplantation with 4 × 10^4 CB CD34$^+$ cells were measured by ELISA.

All NOD/SCID/γ_c^{null} mice that underwent CB CD34$^+$ cell transplantation produced not only human IgM but also IgG and IgA. For comparative purposes, NOD/SCID-$\beta2m^{null}$ mice, which were reported to be better recipients for human HSCs,[14] were also evaluated for the production of human immunoglobulin after transplantation with 4 × 10^4 CB CD34$^+$ cells. Only one NOD/SCID-$\beta2m^{null}$ mouse had very low levels of IgM, and other types of immunoglobulin were not detected. Thus, the differentiation of B cells occurred mainly in the BM of NOD/SCID/γ_c^{null} mice then functionally maturated to produce human immunoglobulin with isotype switching from IgM to IgG and IgA.

Diverse Vβ repertoire of human T cells undergoing differentiation in NOD/SCID/γ_c^{null} mice

To determine whether the differentiation of human T cells in NOD/SCID/γ_c^{null} mice proceeded in a coordinated manner, recombination diversity of TCR was analyzed using flow cytometry. Five to 6 months after the transplantation of CB CD34$^+$ cells, the TCR repertoire of human T cells in the spleen of NOD/SCID/γ_c^{null} mice was quantified using a panel of 24 different antibodies specific to each Vβ (Figure 5).

Surprisingly, a diverse T-cell repertoire was confirmed in each experiment. These results imply that in the NOD/SCID/γ_c^{null} mice with transplanted CB CD34$^+$ cells, a tightly regulated program of sequential TCR gene expression occurred that altered the phenotypes of developing cells and finally promoted the generation of a diverse Vβ repertoire.

Functional maturation of human T cells and NK cells differentiated in NOD/SCID/γ_c^{null} mice

We further analyzed the functions of human T cells and NK cells developing in NOD/SCID/γ_c^{null} mice. Human mononuclear cells in spleens of mice that underwent transplantation were cultured in the presence of PHA and human IL-2 for 2 days, after which the supernatant was taken to assess human cytokine production. Cultures were continued without PHA for 8 additional days to yield enough cells to evaluate cytotoxicity. Human T cells in NOD/SCID/γ_c^{null} mice responded to the stimulation of PHA and showed characteristics of T-cell blasts. The number of cells expanded more than 10-fold at day 10 (Figure 6A). These data suggest that the signals of PHA were transduced through the TCR of these T cells and resulted in cell expansion. Human interferon-γ (IFN-γ), TNF-α, IL-10, IL-5, and, though barely detectable, IL-4 was also identified in the supernatant of PHA-stimulated T cells (Figure 6B). To investigate the cytokine production of NK cells, we performed intracellular cytokine staining. Considerable populations of CD56$^+$ NK cells produced IFN-γ or TNF-α, and IL-4 production was limited (Figure 6C). Concerning NK cell development, it has been

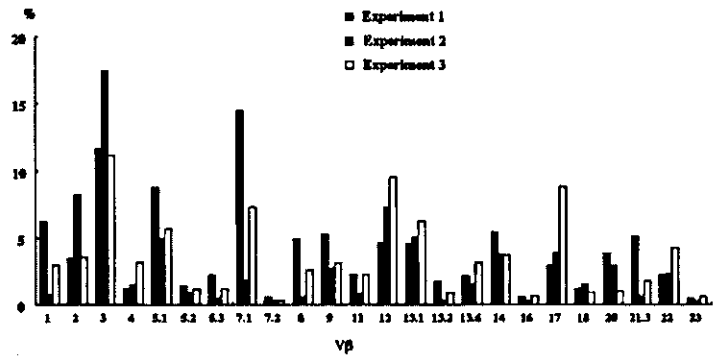

Figure 5. TCR Vβ repertoire analysis of human T cells in spleen. Four to 6 months after the transplantation of 2 × 10^4 to 5 × 10^4 CB CD34$^+$ cells, spleen cells were taken and the TCR Vβ repertoire was analyzed by flow cytometry using a panel of 24 different antibodies. The results of 3 independent experiments are shown.