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Review

Ex vivo expansion of hematopoietic stem cells by cytokines

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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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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 $Lin^-/Sca\text{-}1^+/c\text{-}kit^+$ 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 $CD34^+/c\text{-}kit^{low}/CD38^{low}$ 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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