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Recent advances in *ex vivo* expansion of human hematopoietic stem cells

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Abstract

Hematopoietic stem cells (HSCs) are a rare cell population present in the bone marrow. They possess self-renewal and multipotent differentiation capacities and play a crucial role in lifelong hematopoiesis and reconstitution of the hematopoietic system after hematopoietic stem cell transplantation (HSCT). HSCT remains the only curative treatment for refractory hematologic disorders. Umbilical cord blood (CB) has several advantages as an alternative donor for HSCT, including HLA flexibility and lack of donor burden. However, CB has limitations in terms of cell dose, restricted donor options, and prolonged time to engraftment. Development of techniques for expanding HSCs *ex vivo*, especially those contained in CB, has become a goal in the field of hematology. Attempts have been made to use various combinations of cytokines for this purpose, but these protocols showed limited expansion rates and did not progress to clinical applications. Recent advances that include the addition of small molecules to cytokines have enabled long-term and stable *ex vivo* expansion of human HSCs. Clinical trials have been conducted with HSCs expanded in CB using these techniques, confirming their efficacy and safety. Furthermore, we recently developed a recombinant cytokine-free, albumin-free culture system for long-term expansion of human HSCs. This approach has the potential to selectively expand human HSCs more effectively than the previous protocols. We herein present an overview of *ex vivo* culture protocols for expanding human HSCs together with the results of clinical trials that utilized these techniques.

Key words human hematopoietic stem cell, *ex vivo*, expansion, polymers, chemicals, clinical trials

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Introduction

Hematopoietic stem cells (HSCs) constitute a rare cell population residing in the bone marrow; they possess self-renewal and multipotent differentiation capacities that sustain lifelong hematopoiesis[1]. HSCs play a pivotal role in the curative treatment of various refractory hematologic disorders through hematopoietic stem cell transplantation (HSCT). HSCs are also present in the umbilical cord blood (CB), which is now commonly used as a source of stem cells[2]. CB offers advantages such as greater tolerance for human leukocyte antigen (HLA) disparities and lack of burden on donors. However, the limited number of HSCs in CB not only restricts the CB units that can be transplanted but also results in delays in engraftment and increases the risk of infection[3]. Therefore, *ex vivo* expansion of umbilical CB-derived HSCs holds significant importance for enhancing the safety of transplantation procedures. This review focuses on recent advancements in *ex vivo* expansion techniques for human HSCs and their application in clinical research, which have sought to address these challenges and promote safer transplantation practices.

Cytokines and Growth Factors

By combining insights into HSC self-renewal *in vivo*, researchers have developed various techniques for *ex vivo* HSC expansion. Central to this process are cytoki-

Table 1. Protocols for *ex vivo* expansion of human hematopoietic stem cells

Compound	Activity	Cell tested	Cytokines	Culture period	Fold expansion	Ref.
SR-1	AHR antagonist	CB $CD34^+$	SCF, TPO, FLT3-L, IL-6	21 days	$CD34^+$ HSPCs: 50-fold SRCs: 17-fold	13
Nicotinamide	Inhibition of NAD^+-dependent enzymes	CB $CD34^+$	SCF, TPO, FLT3-L, IL-6	21 days	$CD34^+$ HSPCs: 80-fold SRCs: 9-fold	15
UM171	Inhibition of LSD1 and HDAC	CB $CD34^+$	SCF, TPO, FLT3-L	12 days	SRCs: 13-fold	16
PCL-PVAc-PEG-based 3a (740Y-P, butyzamide and UM171)	PI3K activator, TPO receptor agonist, Inhibition of LSD1 and HDAC	CB $CD34^+$	None	30 days	$CD34^+$ HSPCs: 55-fold*	21

*Single-cell RNA sequencing analysis after 10 days of culture revealed a higher proportion of cells expressing HSC-specific genes compared to UM171 or SR-1-based medium.

PCL-PVAc-PEG, polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer; CB, cord blood; HSPC, hematopotietic stem/progenitor cell; SRC, SCID repopulating cell; AHR, aryl hydrocarbon receptor; NAD, nicotinamide adenine dinucleotide; HDAC, histone deacetylase; PI3K, phosphatidylinositol-3 kinase; TPO, thrombopoietin; SCF, stem cell factor; FLT3-L, FMS-related tyrosine kinase 3 ligand; IL-6, interleukin-6.

nes and growth factors in widely used combinations, including stem cell factor (SCF), thrombopoietin (TPO), Fms-like tyrosine kinase 3 ligand (FLT3-L), and interleukin-6 (IL-6). SCF, initially identified as a c-Kit (CD117) ligand in mice[4], has been shown to play a pivotal role in HSC survival and expansion[5]. Within the bone marrow niche, SCF expression promotes HSC maintenance and stimulates cell cycle progression via activation of the phosphoinositide 3-kinase (PI3K)/AKT/FOXO pathway[6]. TPO is essential for HSC maintenance and expansion and induces self-renewing cell division. By signaling through the MPL receptor, TPO triggers multiple pathways, including JAK/STAT, MAPK/ERK, and PI3K/AKT[7, 8]. FLT3-L and IL-6, too, contribute to cell amplification via diverse signaling pathways[9, 10]. Despite these advances, cytokine-based culture systems have not achieved long-term stable HSC self-renewal and their full-scale clinical application remains unrealized[11, 12].

Small Molecules

While the expansion of human HSCs using cytokine combinations alone has limitations, recent studies have indicated that the addition of small molecules to these cytokines can enhance expansion efficiency (**Table 1**).

The front-line compound in this field, StemRegenin-1 (SR-1), was identified by screening a library of 100,000 compounds designed to detect molecules that enhance the proliferation of umbilical CB-derived hematopoietic stem and progenitor cells (HSPCs)[13]. SR-1 is a purine derivative and an antagonist of the aryl hydrocarbon receptor (AHR) of human HSPCs. When SR-1 was added to cytokines in a culture of umbilical CB $CD34^+$ cells, the cells expanded 50-fold and SCID-repopulating cells (SRCs) that sustain long-term hematopoiesis in immunodeficient mice were amplified 17-fold. The precise molecular mechanism underlying the action of SR-1 is not fully understood. However, overexpression of the RNA-binding protein Musashi-2 (MSI2) has been suggested to directly suppress the AHR signal and thus expand HSCs *ex vivo*[14].

In 2012, a breakthrough was achieved by combining nicotinamide (NAM), a form of vitamin B-3, with cytokines to enhance the expansion of umbilical CB-derived $CD34^+$ cells. This approach led to 80-fold expansion of these cells within three weeks[15]. Xenotransplantation assays showed that the addition of NAM led to a 9-fold increase in SRCs, enhancing bone marrow homing via the CXCR4-CXCL12 pathway. These grafts allowed for hematopoietic reconstitution using human cells of both myeloid and lymphoid lineages. NAM functions as an inhibitor of NAD^+-dependent enzymes, particularly sirtuin, which play a central role in the proliferation of human HSCs.

In 2014, through the screening of compound libraries, a pyrimidoindole derivative, UM171, was identified to expand human HSCs when used in combination with cytokines in culture[16]. In xenotransplantation assays, a 13-fold increase in SRCs was observed. Recent research has revealed that UM171 activates the CULLIN3-E3 ubiquitin ligase (CRL3) complex, which includes KBTBD4, to ubiquitinate the CoREST complex. As a result, lysine-specific histone demethylase 1 (LSD1) and histone deacetylases (HDACs) within the CoREST complex are inactivated, suggesting that ubiquitination suppresses genes involved in differentiation[17]. Functional and genomic studies have suggested that UM171 acts independent of AHR inhibition, suggesting a synergistic increase in the amplification efficiency of HSCs when used in combination with SR-1.

Polymers and Chemicals

HSC culture has traditionally required the presence of bovine serum albumin (BSA) or fetal bovine serum

(FBS) together with cytokines. Albumin is considered to play a crucial role in HSC culture as a carrier protein or source of amino acids. However, it is difficult to obtain stable results due to significant variations between batches. This instability and uncertainty pose significant obstacles to clinical applications. In 2017, synthetically produced albumin extracted through yeast culture was reported to exhibit functionality equivalent to that of albumin extracted from serum, with reduced batch-to-batch variation and stable outcomes[18]. However, even with synthetic albumin, trace amounts of yeast impurities were unavoidable.

Wilkinson et al. conducted screening for chemically synthesized substances that could be substituted for albumin. By adding SCF and TPO to a medium containing polyvinyl alcohol (PVA), they determined that it was possible to amplify mouse HSCs *ex vivo* without the addition of albumin[19]. Surprisingly, after one month of culture under these conditions, the number of functional HSCs increased 236- to 899-fold. PVA not only acted as a suitable substitute for albumin but also achieved even greater amplification of HSCs. There are various types of PVA with different molecular weights and degrees of hydrolysis. Sudo et al. reported that PVA with a lower degree of hydrolysis efficiently amplifies both mouse and human HSCs, while the molecular weight has no impact on the *ex vivo* expansion of HSCs[20]. PVA can be synthesized stably and inexpensively in large quantities, offering advantages in terms of cost compared with the use of animal-derived components or synthetic proteins in cultures. However, the efficiency of human HSC expansion remains limited under the same culture conditions.

To investigate the differences between murine and human HSPCs, we analyzed the phosphorylation status of major signaling pathways in cultured HSPCs[21]. The results demonstrated a significant decrease in the PI3K/AKT pathway in humans. To activate the PI3K/AKT pathway, we added the PI3K activator 740Y-P and obtained improved expansion rates of human HSPCs on day 7. In addition, we found that SCF could be substituted with 740Y-P, and TPO with the TPO receptor agonist butyzamide[22, 23]. We confirmed that a medium containing PVA, 740Y-P, and butyzamide (2a medium) allowed for a 7-day culture of human HSPCs without the need for cytokines. However, using this approach, HSPCs began to differentiate into megakaryocytes after 14 days. To achieve long-term stable culture, we explored compounds that could prevent the differentiation of HSCs. We discovered that by adding the pyrimidoindole derivative UM171 to the 2a medium (3a medium), human HSCs could be cultured stably for up to 30 days. We also confirmed that the capacity for hematopoietic reconstruction was maintained by transplanting cells cultured in 3a medium into immunodeficient mice after irradiation.

To further improve expansion efficiency, we conducted screening for polymers and determined that the polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer (PCL-PVAc-PEG)[24, 25] possesses superior cell proliferation potential compared to PVA. When 740Y-P, butyzamide, and UM171 were added to culture medium containing PCL-PVAc-PEG instead of PVA, the proliferation capacity of human HSPCs was further enhanced. Over 30 days, the total cell count increased 75-fold and $CD34^+$ cells expanded 55-fold. Transplanting these cells after a 30-day culture into immunodeficient mice confirmed their engraftment. Moreover, robust human CD45 chimerism was observed in secondary xenotransplantation recipients.

To investigate the characteristics of cells cultured in the PCL-PVAc-PEG-based 3a medium, we conducted single-cell RNA sequencing analysis. This analysis revealed that a high proportion of the cells expressed genes specific to HSCs. Notably, this proportion was higher than those reported in recent clinical trials involving UM171 or SR-1 for human HSC culture. These findings suggest that the PCL-PVAc-PEG-based 3a medium achieves selective expansion of HSCs. This medium holds some advantages, as it does not contain recombinant proteins and is chemically defined, resulting in improved batch-to-batch consistency, reduction in reagent costs, and acceleration of clinical applications.

Clinical Applications

Clinical trials using *ex vivo*-expanded CB have already shown promising results (**Table 2**). Among these approaches, NAM is pioneering. In a Phase I trial, double CB transplantation was performed on 11 patients with hematologic malignancies after myeloablative conditioning[26]. This procedure involved simultaneous transplantation of NAM-expanded CB, NiCord (Gamida Cell, Israel), and unmanipulated umbilical CB. NiCord is a product obtained by culturing $CD133^+$ cells from umbilical CB in a medium containing NAM for 21 days and then adding unmanipulated T cells purified from the $CD133^-$ fraction. After three-week culture, $CD34^+$ cells expanded by a median of 72-fold. No severe adverse events related to NiCord infusion were observed. The median time to neutrophil engraftment in the NiCord transplantation group was significantly shorter than that in the historical control group (13 vs 25 days). Complete or partial engraftment of neutrophils and T cells derived from NiCord was observed in eight patients, and the engraftment of NiCord remained stable in all patients throughout the median follow-up period of 21 months.

Table 2. Clinical trials of hematopoietic stem cell transplantation using *ex vivo* expanded graft

Compound	Product name	Phase	Cord blood unit	Fold expansion of CD34⁺ cells (median)	Median days to neutrophil engraftment		Ref.
					Study group	Control group	
Nicotinamide	Nicord	Phase I	Double, one manipulated	72	13	25	26
Nicotinamide	Nicord	Phase I/II	Single	33	11.5	21	27
Nicotinamide	Omidubicel	Phase III	Single	130	10	20	28
SR-1	HSC835	Phase I/II	Double, one manipulated	330	15	24	29
UM171		Phase I/II	Double, one manipulated (4 pts) Single (23 pts)	28	18	NA	30

NA, not applicable.

Next, a phase I/II clinical trial involving the transplantation of NiCord alone was conducted after myeloablative conditioning in 36 patients with hematologic malignancies[27]. The culture with NAM resulted in a 33-fold expansion of $CD34^+$ cells, and the median time to neutrophil engraftment after NiCord transplantation was significantly shorter than that in the historical control group (11.5 vs 21 days). For the patients who achieved platelet recovery, the median time to platelet recovery was 34 days for the NiCord group and 46 days for the historical control group, indicating significant improvement in the NiCord group. One patient experienced primary graft failure and two patients experienced secondary graft failure. There was no significant difference in the occurrence of grade III-IV acute graft-versus-host disease (GVHD) or chronic GVHD between the two groups. Moreover, no significant difference was observed in the 2-year disease-free survival (DFS) or overall survival (OS) after transplantation.

Subsequently, a phase III randomized controlled trial was conducted[28]. Eligible patients were aged 12 to 65 years with high-risk hematologic malignancies, were candidates for myeloablative allogeneic HSCT, and lacked immediately available matched sibling or unrelated donors. A total of 125 patients were enrolled, of whom 62 were assigned to the omidubicel (formerly NiCord) group and 63 to the conventional umbilical CB transplantation group. There were no significant differences in the patient characteristics. $CD133^+$ fractions were cultured for 21 ± 2 days with NAM, and transplanted along with $CD133^-$ cell fractions, including T cells. After culture, $CD34^+$ cells were expanded approximately 130-fold (range, 32-233 fold), resulting in a $CD34^+$ cell count of 9.0 (range, 2.1-47.6) $\times$ 10^6 cells/kg in the omidubicel group and 0.3 (range, 0.1-1) $\times$ 10^6 cells/kg in the control group. The median time to neutrophil engraftment was significantly shorter in the omidubicel group (12 vs 22 days). Among the patients receiving omidubicel, higher total $CD34^+$ cell counts and $CD34^+$ cell doses (per weight) correlated with a shorter time to neutrophil engraftment. In addition, platelet engraftment rates up to day 42 were significantly higher in the omidubicel group (55% vs 35%). The cumulative incidence of bacterial or invasive fungal infections was significantly lower in the omidubicel group (37% vs 57%) and the number of days spent outside the hospital within 100 days post-transplantation was significantly higher in the omidubicel group (61 vs 48 days). There were no significant differences in the cumulative incidence of grade III-IV acute GVHD or chronic GVHD between the groups. However, despite the lack of statistical significance, favorable trends were observed in terms of the cumulative incidence of non-relapse mortality (NRM), DFS, and OS in the omidubicel group. Based on these results, on April 17, 2023, the U.S. Food and Drug Administration (FDA) approved omidubicel-onlv (marketed as Omisirge by Gamida Cell Ltd.) for adult and pediatric patients (12 years and older) with hematologic malignancies scheduled for umbilical CB transplantation after myeloablative conditioning; the aim is to reduce neutrophil recovery time and infection rates. This represents a significant step in the *ex vivo* expansion technology of human umbilical CB.

In 2016, the results of a phase I/II trial involving expanded CB transplantation using SR-1 were reported[29]. In this clinical trial, a product called HSC835 was used, which involved culturing $CD34^+$ cells from CB in a culture medium containing SR-1 for 15 days and then adding cryopreserved $CD34^-$ fraction cells. Double CB transplantation was performed by transplanting HSC835 and another unmanipulated CB unit. Seventeen patients who underwent myeloablative conditioning received this product. The $CD34^+$ cells expanded approximately 330-fold throughout the culture period. All patients achieved engraftment. The median time to neutrophil engraftment was significantly shorter in the HSC835 group than in the historical control group (15 vs 24 days), and the median time to platelet engraftment, too, was significantly shorter (49 vs 89 days). Peripheral blood chimerism analysis revealed that the engrafted cells of the six individuals were entirely derived from HSC835, while those of another six individuals were entirely derived from the unmanipulated umbilical CB unit. Those of

the remaining five patients exhibited a unique chimerism pattern in which myeloid cells originated from HSC835, while T cells originated from the unmanipulated umbilical CB unit. Although no significant differences were observed in terms of the incidence of acute GVHD, NRM, or OS compared to the historical control group, hospitalization duration was significantly shorter in the HSC835 group (30 vs 46 days).

In 2020, the results of a phase I/II trial utilizing UM 171 were reported[30]. This clinical trial targeted patients with hematologic malignancies who lacked a suitably matched HLA donor. The trial involved transplantation of umbilical CB-derived $CD34^+$ cells cultured for 7 days in a medium containing UM171, along with a $CD34^-$ fraction containing lymphocytes. Initially, double umbilical CB transplantation involving UM171-cultured CB along with unmanipulated CB was performed in four patients. Subsequently, UM171-cultured CB alone was transplanted into 22 patients. The median time to neutrophil and platelet engraftment was 18 and 42 days, respectively. No cases of graft failure were observed. The median time to recovery from febrile neutropenia was significantly shorter than that in the historical control group. The incidence of grade III-IV acute GVHD was 10%, and there were no cases of moderate or severe chronic GVHD. Infection-related mortality was absent, the 1-year NRM rate was 5%, and the 1-year OS rate was 90%.

The trial aimed to determine the minimum umbilical CB cell dose required for achieving engraftment. The expansion process revealed that a pre-culture $CD34^+$ cell count of 0.52×10^5/kg was sufficient. Based on these results, patients weighing 70 kg who were previously eligible for only 5% of the available umbilical CB units based on conventional criteria could now be eligible for 47% of the units based on the criteria established in this clinical trial. The authors concluded that the UM171 single umbilical CB protocol offers the clinical benefits of faster engraftment and reduced early infection complications, while also allowing for the use of HLA-matched umbilical CB units with lower total cell counts through an expanded approach.

Recent advancements in the culture protocols of human HSCs have not only enabled the substantial expansion of human HSCs but have also led to promising results in clinical trials. Clinical evaluations of *ex vivo* umbilical CB expansion have confirmed the safety and rapid recovery of neutrophils and platelets, which are associated with favorable early clinical outcomes post-transplantation. However, long-term clinical benefits have not yet been directly demonstrated in comparative clinical trials.

Regarding immune reconstitution, the median number of infused T cells was 2-3 times lower in UM171-expanded CB than in unmanipulated CB, even though the quantity and phenotype of T cells after transplantation were similar between the two groups[31]. T cell receptor sequencing analysis indicated that the patients who received UM171-expanded CB displayed increased T cell diversity and rapid virus-specific T cell responses at 12 months post-transplantation, leading to a significant reduction in the occurrence of severe infection. Furthermore, in the case of omidubicel, immune reconstitution was comparable to that achieved using unmanipulated CB[32].

To evaluate the impact of expanded CB on prognosis, a retrospective comparison was conducted between the results of clinical trials utilizing UM171-expanded CB and outcomes using non-manipulated CB and matched unrelated donor (MUD) transplantation[33]. The results revealed that the UM171 group exhibited lower NRM compared to the CB control group, along with improved 2-year graft-versus-host disease-free relapse-free survival (GRFS) and 1-year OS. Furthermore, compared to the MUD-peripheral blood stem cell (PBSC) group, the UM171 group experienced fewer cases of grade III-IV acute GVHD and chronic GVHD. These results suggest that recipients of UM171-expanded CB may benefit from reduced NRM and improved GRFS. In addition, a meta-analysis of clinical trials utilizing expanded CB indicated a lower risk of death at the study endpoint for patients who underwent *ex vivo* expansion[34].

Conclusion

Collectively, progress in *ex vivo* expansion techniques for HSCs, coupled with accumulating clinical evidence, makes expanded CB transplantation a feasible option in clinical practice. Further mature results are eagerly anticipated.

Author Contributions

M.S. conceptualized, wrote, and edited the manuscript.

Conflicts of Interest

M.S. is co-founder and shareholder in Celaid Therapeutics. Disclosure form provided by the author is available on the website.

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