Ex-Vivo Expanded Umbilical Cord Blood Stem Cells Retain Capacity for Myocardial Regeneration

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**Background:** Umbilical cord blood (UCB) is a source of human hematopoietic precursor cells (HPCs), a stem cell (SC) type that has been used in several trials for myocardial repair. A certain minimal number of cells is required for measurable regeneration and a major challenge of SC-based regenerative therapy constitutes ex-vivo expansion of the primitive cell compartment. The aim of this study was to investigate the ex-vivo expansion potential of UCB-derived HPCs and the ability of these expanded cells to migrate to the site of damage and improve ventricular function in a rodent model of myocardial infarction (MI).

**Methods and Results:** UCB-derived HPCs, defined by coexpression of CD133 and CD34, were expanded using various cytokine combinations. MI was induced by left anterior descending artery ligation in nude rats. Cells were injected intravenously 2 days after infarction. The combination of SC factor, thrombopoietin, flt3-ligand and interleukin-6 was found to be the most effective for inducing proliferation of HPCs. The migratory capacity of expanded HPCs was similar to that of non-expanded HPCs and improvement of ejection fraction was significant in both groups, with a relative increase of >60%.

**Conclusions:** UCB-derived HPCs can be reproducibly expanded ex-vivo and retain their potential to improve cardiac function post-MI. (Circ J 2010; 74: 188–194)

**Key Words:** Myocardial repair; Stem cells; Umbilical cord blood

**S**tem cells (SCs) have been shown to improve function after myocardial infarction (MI). Although all tissues of the human body contain SCs, research is focusing on bone marrow and peripheral blood SCs, because they are easily accessible and available in sufficient numbers. A host of studies on SC biology and hematopoietic precursor cell (HPC) transplantation indicate that umbilical cord blood (UCB) is a suitable source of transplantable human HPCs. Harvesting is a non-invasive collection procedure, concerns “commonly wasted material” only and the cells are better tolerated across the HLA barrier. However, the low amount of obtainable cells represents a major limitation and ex-vivo expansion of HPCs without differentiation has been repeatedly named the holy grail of SC research.

Current evidence suggests the presence of multiple SC subpopulations within human UCB, with both a CD34+ and a CD34− phenotype. CD133 is a surface molecule with unknown functions expressed by more primitive CD34− HPCs and vascular endothelial growth factor receptor-expressing endothelial precursors. The presence of CD133 on a subset of very primitive HPCs, such as CD34+CD133−CD117−lin− cells, points to this marker as an attractive tool to use for isolating human HPCs for both biologic studies and clinical purposes.

CD34-selected cells were successfully engrafted in a fetal sheep transplantation model, and human cells harvested from the chimeric fetal sheep bone marrow engrafted secondary recipients, providing evidence for the long-term repopulating potential of CD133+ cells. Moreover, CD34 is regarded as being involved in neovascularization in ischemic tissue, having been identified on endothelial progenitor cells circulating in the peripheral blood. Accordingly, it has been recently shown that human UCB-derived CD34+ cells directly promote neovascularization in a model of ischemic brain disease and thus facilitate neuronal regeneration.

In the present study, we assessed the self-renewal potential of selected CD133+ cells with respect to their clonogenic capacity and their ability to expand in serum-replete or...
serum-free medium using UCB progenitors in the presence of various cytokine combinations and serum additives under GMP compliant conditions. Further, we examined the in vivo properties (ie, migration of and functional improvement by these cells) in a rat model of acute MI.

**Methods**

**Mononuclear Cell (MNC) Preparation by Ficoll Density Gradients**

UCB was collected from the umbilical cord of healthy full-term deliveries (n=21) after approval by the local ethics committee and written informed consent of the mother was obtained. A total volume of 30 ml UCB/phosphate-buffered saline (PBS; Gibco, Invitrogen Life Technologies, Paisley, UK) 1:1 was layered onto 15 mL of Ficoll-Paque (Pharmacia-Amersham, Piscataway, NJ; ρ=1.077 g/ml) density gradient and centrifuged at 1,160 g for 20 min without brake to deplete red blood cells. The MNC interface was collected, washed twice with PBS, and counted with a Sysmex KX-21 (Sysmex Corporation, Kobe, Japan).

**CD133* Cell Purification**

CD133* cord blood cells were purified from MNC preparations or expansion cultures using magnetic-activated cell sorting columns (MACS CD133 Cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany). Next, 100 μl FcR blocking reagent per 10⁶ total cells was added to inhibit unspecific binding of CD133* MicroBeads to non-targeted cells. Labeling was performed by adding 100 μl CD133* MicroBeads to per 10⁶ total cells. The selected cells were washed and counted.

**Cell Expansion**

A total of 5x10⁶ CD133* cells were cultured in flat-bottomed 6-well plates in 1 ml Iscove’s modified Dulbecco’s medium (Gibco, Invitrogen Life Technologies) supplemented with 10% autologous UCB-derived plasma or 10% peripheral blood plasma and a cocktail of 4 cytokines: SC factor (SCF), thrombopoietin (TPO), flt3-ligand (FL) and 1 of interleukin (IL)-6, IL-10 or IL-3 (each 50 ng/ml) (CellSystems, St Katharinen, Germany). All these factors were chosen because they are early-acting cytokines that are proved to work on SCs according to the scientific literature.¹⁸-²¹

Autologous cord blood plasma was taken from Ficoll gradients and peripheral blood plasma was obtained from 1 healthy donor. After 3 days 50% of the fresh medium was added to each well. On day 7 cultures were harvested and aliquots were taken for flow cytometry. A total of 5x10⁶ cells were suspended in 1 ml medium and cultured for 7 days as described above. The wells were maintained in a fully humidified atmosphere of 5% CO₂/95% relative humidity at 37°C during culture.

**Flow Cytometry for Progenitor Quantification**

We labeled 1x10⁶ cells with a combination of FITC-conjugated anti-CD45 (Anti-CD45, clone 2D1, Becton Dickinson, Oxford, UK), PE-conjugated anti-CD133/2 (Anti-CD133, clone AC141, Miltenyi Biotec) and PerCP-Cy5.5 conjugated anti-CD34 (Anit-HPCA-2, clone 8G12, Becton Dickinson). Replicate samples were stained with FITC/PE/PerCP-conjugated mouse IgG₁ antibodies as an isotype control to ensure specificity. A minimum of 10,000 gated events were analyzed for each sample using 3-color flow cytometry on a FACScan analyzer (Becton-Dickinson, San Jose, CA, USA) according to the CD34+ enumeration protocols developed by the International Society of Hematotherapy and Graft Engineering (ISHAGE). The percentage of CD34+ and/or CD133+ cells in whole or processed UCB before and after culture was calculated as the percentage of CD45+ events with the exclusion of CD45+ events. Expansion efficiency is expressed as the fold-expansion of nucleated cells (NC) and HPCs (CD34+ and/or CD34†).

**Labeling of UCB-Derived HPCs**

Expanded and non-expanded UCB-derived HPCs were cultured with 10 μg/ml lipoprotein labeled with carbocyanine membrane 1,1-dioctadecyl-3,3,3,3-tetramethyl indocarbocyanine perchlorate (Cell-Tracker CM-DiI, Molecular Probes, Leiden, The Netherlands) for subsequent cell tracking. After cell viability assessment, aliquots of 10⁶ UCB-derived HPCs in approximately 300 μl of culture medium were prepared for intravenous injections.

**Model of MI**

MI was induced in 8- to 10-week old male rnu/rnu rats (Harlan Winkelmann, Borchen, Germany) through left anterior descending coronary artery (LAD) ligation. Rats

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*Expanded Stem Cells for Myocardial Repair* (Circulation Journal Vol.74, January 2010)
included in the study weighed 200–300 g. All animals received humane care in compliance with the “Principles of Laboratory Animal Care”, formulated by the National Society for Medical Research, and the “Guide for the Care and Use of Laboratory Animals”, prepared by the Institute of Laboratory Animal Resource and published by the National Institutes of Health.

Cell Injection
On the 2nd day after MI, 10⁶ DiI-labeled expanded UCB-HPCs (n=20), 10⁶ DiI-labeled non-expanded UCB-HPCs (n=20), and saline (n=20) were injected in the tail vein.

Functional Assessment by Transthoracic Echocardiography
Transthoracic echocardiography was performed before and 2 days after MI, and 2 weeks and 4 weeks after cell injection with a Hewlett Packard SONOS 5500 Echocardiography system (Hewlett Packard, Andover, MA, USA), using a commercially available high-frequency linear-array transducer system.

Tissue Preparation for Morphologic Studies
Rats were killed humanely, hearts were harvested, and fibrous tissues removed. After intracardiac blood was rinsed away, the hearts were divided into 3 parts of equal thickness representing the base, middle, and apex of the heart. Each was snap-frozen in liquid nitrogen after being embedded in optimal cutting temperature compound (Tissue-Tec OCT Compound, Miles Inc. Elkhart, IN, USA). From each part, 5 μm slides were prepared using a cryostat. Transplanted cells were detected by fluorescence microscopy. In addition, standard hematoxylin-eosin staining was performed to permit morphologic assessment.

Statistical Analysis
Data were analyzed retrospectively using SPSS 15.0 for windows (Chicago, IL, USA). Continuous variables are shown as mean with standard deviation. For comparison of control animals and animal treated with expanded or non-expanded UCB-HPCs we used the Mann-Whitney U-test. P values <0.05 were regarded as statistically significant.

Results
Effect of Different Interleukins on CD133+ Expansion
CD133+ cells were isolated to an average purity of 93.0±3.8%, with a viability of 97–99%. In order to maximize CD133+ cell expansion, we tested a standard cytokine cocktail containing SCF, TPO and FL in combination with IL-3, IL-6 or IL-10. Autologous cord blood plasma was used as an additional culture component. The expansion of NCs grown in SCF/TPO/FL medium spiked with IL-6 (A6) was
Expanded Stem Cells for Myocardial Repair

Expansion Efficiency of Different Progenitor Subtypes During Cultivation

The evaluation of the expansion of HPCs under various culture conditions was based on the starting population of >95% of the progenitor type CD133+/CD34+. The majority of HPCs remained double positive CD133+/CD34+ (55–70%) throughout the expansion period. However, during the first 7 days of cultivation 2 additional distinct single-positive subpopulations emerged, namely CD133+/CD34− and CD133−/CD34+. After 14 days the 22-fold expanded SC population consisted of CD133+/CD34+ (63.7±4.4%), CD133+/CD34− (22.4±2.5%), and CD133−/CD34+ (13.9±5.3%). Interestingly, this distribution pattern did not change over the cultivation-period. A complete summary of the results of cell expansion is given in Table 1 and Figure 1.

Survival and Migration of Transplanted Expanded and Non-Expanded CD133+ Cells to Ischemic Myocardium

Intravenous injection of both expanded and non-expanded, Dil+ human UCB-HPCs resulted in a similar degree of infarction in the infarcted area at 2 days after induction. Non-ischemic myocardium was not infiltrated by either expanded or non-expanded HPCs (Figure 2). This finding indicates that UCB-derived HPCs not only survive after transplantation but also migrate to the infarct area.

Improvement of Myocardial Function

We measured the left ventricular ejection fraction (LVEF) as a marker of left ventricular function by means of transthoracic echocardiography (Figures 3a–c). LVEF significantly decreased from 70% to 36±5.8% after induction of MI in all animals, with no statistical difference between groups. Akinetic areas were detectable in the anterior wall and apex. In control animals, LVEF decreased further to 32±4.5% at 2 weeks after injection of saline. Treatment with expanded cells induced a significant improvement of LVEF to 61±5.9% at 2 weeks after LAD ligation (expanded cell treatment vs control: P<0.001). Application of non-expanded cells also proved to ameliorate LVEF significantly to 64±4.1% (non-expanded

Figure 3. M-mode echocardiography of rat hearts. (a) M-mode of rat heart prior to intervention. (b) M-mode after induction of myocardial infarction. (c) M-mode 4 weeks after cell therapy with improved ejection fraction.
cell treatment vs control: P<0.001). The difference in LVEF between animals treated with expanded and non expanded cells did not reach statistical significance 2 weeks after LAD ligation (P=0.114). At 4 weeks after induction of MI, the mean LVEF of control animals was still 33±3.8%. Animals treated with expanded cells (LVEF 62±5.5%) and with non-expanded cells (LVEF 64±3.7%) also showed significantly improved left ventricular function after 4 weeks (expanded cells vs control: P<0.001; non-expanded cells vs control: P<0.001). The difference between hearts treated with non-expanded cells and those treated with expanded cells remained non-significant after 4 weeks (P=0.382) (Figure 4).

Discussion

In the current study we evaluated the feasibility of ex-vivo expansion of human UCB-derived HPCs and their potential to improve myocardial function after acute ischemic damage. Although an approach to expanding HPCs has been described,7 this is the first study to introduce the concept of expansion of human UCB-derived HPCs with subsequent successful injection of these cells for the treatment of MI. Ex-vivo expansion of HPCs is a very promising approach with different clinical applications, ranging from rescue after myelo-ablative therapy to tissue regeneration. Although the first attempts to isolate and culture HPCs ex-vivo included CD34+ cells obtained from peripheral blood,17 large-scale isolation of human UCB could represent a primary step to gaining access to a SC pool that is useful for therapeutic hematopoietic transplantation.22,23 SC expansion for clinical purposes, and research on tissue-regenerating therapies. Human UCB as a source of hematopoietic SCs has several advantages because it contains up to 10-fold higher amounts of endothelial precursor cells as non-mobilized adult peripheral blood,24 and is characterized by a higher proliferative capacity.25 In the present study human UCB-derived HPCs were a readily available cell source with a high proliferation potential for regeneration of ischemically damaged rat myocardium and for that purpose athymic nude rats were used to avoid a xenograft reaction by the rats against human cells. The most likely clinical scenario involves the autologous use of these cells, whereby they are stored at birth for repair of possible ischemic damage later in life. Alternatively they could be stored in cell banks for allogeneic transplantation after HLA matching and myelo-ablative therapy.

In most studies, early-acting cytokines (SCF, TPO, FL) have been used to expand CD34+ cells, isolated using magnetic beads.26,27 To overcome the limitations of a low-cell dose in UCB, novel approaches to ex-vivo expansion of HPC are being intensively explored. Astori et al describe a novel protocol that enables short-term expansion of CD34+ cells for optimized production of early pluripotent hematopoietic SCs and committed cells from various lineages, and their strategy might represent a significant improvement for clinical application.18

The application of ex-vivo SC expansion in cell-based therapy requires a standardized and well-controlled GMP-compliant methodology. The use of 6-well plates and bovine serum culture protocols allows adequate expansion levels with a high degree of reproducibility. However, the risk of contamination and transmission of animal viral diseases may jeopardize cell-based therapies and this strategy can therefore not be applied in the clinical setting.

This report documents the establishment of an expansion protocol for CD133+ cells isolated from UCB under GMP-compliant conditions. Clinical application of ex-vivo generated cells requires the biological integrity of the cells to be guaranteed. Because cytokines and early-acting hematopoietic growth factors may induce both proliferation and differentiation, HPC expansion would result in a mixed population of uncommitted and already committed cells.

We studied the effect of various cytokine combinations, thus minimizing the effects of unpredictable influence of growth factors on the biological integrity of HPC in 6-well cultures. A triple cocktail of cytokines (TPO, FL, SCF) in combination with IL-6, IL-3 or IL-1019–21 was used, supple-
mented with autologous cord blood plasma. Maximum expansion of NC as well as HPCs (AC133+ cells and/or CD34+ cells) was obtained with the 4-cytokine combination of TPO, FL, SCF and IL-6. To assess whether the source of protein does have an impact on expansion, the same cytokine combination was tested with either purified human serum albumin or plasma extracted from adult peripheral blood. Peripheral blood was less effective in promoting expansion of NC and HPCs compared with Panserin 406 or autologous cord blood plasma. Despite the fact that the latter resulted in an almost 100-fold expansion of NC after 14 days of culture, human serum albumin allowed reproducible expansion rates.

We additionally examined the possibility of transferring the culture dish expansion protocol to a GMP-compliant version using the same culture protocol in closed culture bags. The use of high-concentration human serum albumin, as suggested by other research groups, allowed closed-culture bag-expansion similar to that observed in the 6-well plate. Interestingly, the fold-expansion of NCs increased constantly during the cultivation period of 14 days, whereas the HPC-expansion rate was reduced after day 7. This fact may be attributed to limited oxygen supply (gas exchange), despite the gas permeability of the culture bags.

With regard to immunomagnetic isolation of CD133+, we observed a high percentage of cell purity, which corresponds to past experience. Although all cultures were performed with a starting population of CD133+/CD34+ cells independent of the culture conditions, a balance between 3 different proportions of SC subpopulations, CD133+/CD34+, CD133+/CD34− and CD133+/CD34−, was established after expansion. The CD133+/CD34− fraction has been described by other groups as part of the CD34+ SC fraction (23% of the total number of CD34+ cells in cord blood) with reduced clonogenic potential. Relatively little is known about the CD133+/CD34− sub-population (25% of all HPC), which cannot be detected in unmanipulated cord blood. Recently, Handgretinger et al described the appearance of CD133+/CD34− adherent cells after ex-vivo culture of purified CD133+ cells with the cytokines FL and IL-6. After incubation of these cells with SCF, non-adherent cells of the type CD133+/CD34− with a high NOD/SCID engraftment potential re-appeared, indicating the plasticity of HPCs.

The development of 3 subpopulations originating from the CD133+/CD34+ starting population supports other studies highlighting the enormous plasticity of HPCs. To demonstrate their potential for clinical application, we tested the number of clonogenic cells in the isolated CD133+ fraction. Only 40.9% total colony-forming units (CFU) were recovered from the total CFU of unseparated MNCs after immunomagnetic separation, suggesting clonogenic potential resides within the CD133− cell fraction and is lost during the isolation procedure. Nevertheless, we were able to demonstrate that isolated progenitor cells would not lose their potential during ex-vivo cultivation. Throughout the culture period of 14 days CFU production matched the increase in HPC, regardless of the expansion efficacy of the individual culture medium. This finding can be explained by the fact that CD133+ cells possess a higher renewal capacity and a lower apoptosis rate as compared with CD133− cell populations.

In a rodent model of ischemic myocardial injury we clearly demonstrated that ex-vivo expanded and non-expanded UCB-HPCs are preferentially attracted to and retained in the ischemic tissue, but not in the remote or intact myocardium, after systemic application via the tail vein, apparently attracted by specific receptors or ligands that the injured tissue expresses to facilitate trafficking, adhesion, and infiltration of SCs to the site of injury. We tested a mix of subpopulations of expanded UCB-HPCs that had been previously characterized (CD133+/CD34+, CD133+/CD34− and CD133+/CD34−). UCB-HPCs can be successfully labeled with Dil without affecting cell viability, and this technique is useful for tracking distribution by fluorescence microscopy.

Kocher et al reported that infusion of human bone marrow progenitor cells enhances neoangiogenesis and subsequently results in decreased apoptosis of hypertrophied myocytes in the peri-infarct region, long-term salvage and survival of viable myocardium, reduction in collagen deposition and sustained improvement in cardiac function after MI. By 48 h after injection, examination of the heart identified donor cells in the ischemic zone only. A number of signaling factors responsible for selective migration of bone marrow and blood derived SCs to sites of ischemia have been described. Most likely the same factors play a role in directing native and expanded UCB-HPCs to damaged tissues. We showed that myocardial function increases within 2 weeks after injection of the cells, reflecting a mechanism of regeneration of the infarcted heart. The present study was not designed to confirm or refute the ability of the transfused cells to differentiate into vascular cells after colonization in the ischemic myocardium. Rather, our findings suggest that UCB-HPCs are preferentially attracted to and retained in the ischemic tissue, colonize there, and might contribute to healing of the heart.

Conclusion

Taken together, our findings suggest that GMP-compliant protocols and culture techniques enable highly reproducible expansion of CD133+ isolated progenitor cells from UCB during a short-term culture period, and that UCB-HPCs are able to colonize infarcted myocardium when transfused after MI. Furthermore, both expanded and non-expanded SCs lead to a significant improvement in cardiac function. This approach is relevant for myocardial repair and site-specific therapeutic gene targeting. Indeed, the high plasticity of hematopoietic SCs in ex-vivo culture might even make costly and time-consuming cell purification procedures obsolete. Therefore UCB might provide a valuable source for regenerating ischemic myocardium in the clinical setting.

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