Cell Proliferation

Cell Prolif., 2011, **44** (Suppl. 1), 44–47 doi: 10.1111/j.1365-2184.2010.00722.x

Cord blood-circulating endothelial progenitors for treatment of vascular diseases

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Received 14 *June* 2010; *revision accepted* 19 *July* 2010

Abstract

Adult peripheral blood (PB) endothelial progenitor cells (EPC) are produced in the bone marrow and are able to integrate vascular structures in sites of neoangiogenesis. EPCs thus represent a potential therapeutic tool for ischaemic diseases. However, use of autologous EPCs in cell therapy is limited by their rarity in adult PB. Cord blood (CB) contains more EPCs than PB, and they are functional after expansion. They form primary colonies that give rise to secondary colonies, each yielding more than 10^7 cells after few passages. The number of endothelial cells obtained from one unit of CB is compatible with potential clinical application. EPC colonies can be securely produced, expanded and cryopreserved in close culture devices and endothelial cells produced in these conditions are functional as shown in different *in vitro* and *in vivo* assays. As CB EPC-derived endothelial cells would be allogeneic to patients, it would be of interest to prepare them from ready-existing CB banks. We show that not all frozen CB units from a CB bank are able to generate EPC colonies in culture, and when they do so, number of colonies is lower than that obtained with fresh CB units. However, endothelial cells derived from frozen CB have the same phenotypical and functional properties than those derived from fresh CB. This indicates that CB cryopreservation should be improved to preserve integrity of stem cells other than haematopoietic ones. Feasibility of using CB for clinical applications will be validated in porcine models of ischaemia.

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Origin and role of EPC

It is now established that circulating endothelial progenitor cells (EPCs) are mobilized from the bone marrow and are capable of homing to neovascularization sites where they differentiate into endothelial cells and proliferate (1,2), thus participating in the revascularization process. EPCs were identified in the mononuclear cell fraction of peripheral blood, leukopheresis products and in umbilical cord blood (3,4). During the last few years, EPCs have been extensively studied as biomarkers to assess risk of cardiovascular disease in humans. Indeed, lower EPC count predicts severe impaired function in several cardiovascular pathologies such as hypercholesterolaemia (5), hypertension (6,7) and coronary artery disease (8) and in diabetes (9), scleroderma (10–12), aging (7,13), cigarette-smoking changes (7,14,15). Taken together, these studies suggest that the physiological role of EPC consists in participating in maintenance of vascular integrity. Transplantation of EPCs into ischaemic tissues thus has emerged as a promising approach in treatment of peripheral arterial diseases (16–18). In mice, injection of EPCs has led to improved neovascularization in hind limb ischaemia (16–18). *Ex vivo*-expanded EPCs, isolated from peripheral blood mononuclear cells, can also incorporate into foci of myocardial neovascularization (19,20).

Umbilical cord blood as a source of active EPC

Umbilical cord blood (CB) is currently used as a source of haematopoietic progenitor cells for treatment of various haematological malignancies in children and, in the last few years, in adults. CB is also treatment of choice in non-malignant disorders, and in acquired or inherited bone marrow failure syndromes (21).

CB is a main source of EPCs, which can be expanded *ex vivo*. Expression of specific markers has identified them as endothelial cells. When compared to mature endothelial cells, human umbilical vein endothelial cells (HUVECs), endothelial markers were found to be expressed to the same extent except for KDR, which is expressed more in

CB EPCs. They also display higher proliferation potential (4). Functional studies have demonstrated that CB EPCs are more sensitive to angiogenic factors, which confer these cells with greater protection against cell death compared to HUVECs. CB EPCs yield higher number of colonies than adult PB EPC and the colonies can give rise to secondary and tertiary colonies, a property not shared by PB EPC (22), indicating that although these cells have undergone some differentiation steps, they still have properties of immature cells, suggesting greater tissue repair capabilities.

We show that when human CB EPC-derived endothelial cells are injected intramuscularly into immunodeficient (NOD/SCID) mice with hind limb ischaemia (resection of the femoral artery), a significant improvement in reperfusion, measured by scanner laser Doppler, has been observed, compared to non-injected animals. Presence of human endothelial cells was observed in vascular structures of treated animals, indicating that the cells truly participated in revascularization.

Indeed, it has been shown that adult PB EPCs form blood vessels that are unstable and regress within 3 weeks, while in contrast, CB EPCs form normal functioning blood vessels that last for more than 4 months. These vessels exhibit normal blood flow, permeability selectivity to macromolecules and induction of leucocyte-endothelial interactions in response to cytokine activation similar to normal vessels (23). CB thus represents a valuable source for production of EPCs to be used in treatment of a wide range of cardiovascular diseases.

As CB EPCs will be allogeneic for patients, it is important to study alloimmunity of these cells. In a study by Suarez *et al.*, phenotype and alloantigenicity of human CB EPC were compared to vessel endothelial cells isolated from HUVECs from the same donor. This study showed that CB EPCs were very similar to HUVECs in expression of proteins relevant to alloimmunity, including MHC molecules, costimulators, adhesion molecules, cytokines, chemokines and IDO, and in their ability to initiate allogeneic CD4 and CD8 memory T-cell responses *in vitro* and *in vivo* (24).

Steps toward cell therapy

Use of existing CB banks

CB banking for allogeneic haematopoietic stem cell transplantation has been established worldwide in the past, with cryopreservation methods allowing maintenance ofviability over years (25). While the impact of freezing on haematopoietic stem cells has been extensively studied (26–30), it has, however, not been clearly shown whether or not these processes maintain viability and functionality of other stem and progenitor cells. This question is of crucial importance as hundreds of thousands of CB units are collected, cryopreserved and stored in anticipation of their potential therapeutic application in regenerative medicine.

As an example of that, in both autologous and allogeneic settings, EPCs should likely be expanded from cryopreserved CB units in CB banks. In our previous studies, we have shown that not all cryopreserved CB units are able to produce EPC colonies in culture, and when they do so, number of colonies yielded from the frozen units was lower than obtained from fresh CB units. However, we have shown that the phenotypical and functional features displayed by EPC-derived endothelial cells were identical when isolated from fresh or frozen units; they express the same endothelial markers (KDR, CD144, CD31, vWF), and they have comparable population doubling capacity, and DILL-LDL uptake capacity (31). Use of cryoCB for producing active EPC-derived colonies is thus possible, provided freezing/thawing conditions of CB units are improved. This improvement may be applied to future collection of CB units, but it will not help the many CB units currently stored worldwide.

Another approach for using these frozen units is to improve initial steps of EPC adherence, colony formation and population growth. Different research directions are currently developed in our laboratory. The first consists of improving matrix composition used for coating culturing surfaces and the second is development of specific media, dedicated to initial steps of EPC adherence and colony formation.

Expansion of EPC for therapeutic purposes

In our hands, one CB can yield from 5 to 30 colonies, this yield depending partly on CB volume. However, CB of similar volumes can yield different numbers of colonies, suggesting that stem cell composition is different between CB units. At early stages of EPC colony formation, endothelial cells with features of progenitor cells remain in the colony, displaying self-renewal capacity and the property of giving rise to secondary colonies (22). Yield of secondary colonies obtained from primary ones varies from 20 to 60, suggesting that primary colonies are different in terms of number of clonogenic endothelial progenitors, reflecting heterogeneity of circulating CB EPC populations in terms of stem cell potential.

Formation of secondary colonies can be used as a powerful expansion process, as one secondary colony can give rise to 10^7 cells after two passages. One unit of cord blood can easily give rise to between 10^8 and 10^9 endothelial cells, which are still growing and functional. This yield can certainly be improved, by increasing numbers of clonogenic endothelial progenitors within primary colonies.

Existing commercial endothelial cell culture media act mainly on endothelial cell proliferation. These media are probably not fully adapted to the first steps of EPC-derived colony formation, because they may rapidly consume the pool of endothelial progenitors still present inside EPC-derived colonies, limiting their capacity to generate secondary colonies. Media rather favouring self-renewal of such progenitors would increase the number of secondary colonies that can be produced. We are currently developing serum-free media with this specific self-renewal capacity.

Another important issue is security of the expansion process. We have validated culture of EPC in closed culture devices, from initial colony formation step to final freezing step. We used 25 cm^2 closed culture systems (Clinicell) developed by Mabio (Tourcoing, France), on which different experimental tools (connecting devices, sterile bags) developed by MacoPharma (Tourcoing, France) can be connected to the culture device for controlling medium changes, cell dissociation and passages. We have shown that CB mononuclear cells could yield primary and secondary colonies in these devices with a yield comparable to that obtained in open cultures. EPC primary colonies grown in one initial culture device can be split into two devices to generate secondary colonies, then into 16 devices at passage 2, yielding more than 2×10^7 endothelial cells. We have also shown that cells can be frozen directly in these close culture devices, yielding more than 90% living cells after thawing. Thawed cells are directly available for further experiments without manipulation other than replacing freezing medium with culture medium. This close culture system is thus convenient for future use of CB EPC-derived endothelial cells for therapeutic purposes.

Validation in pre-clinical models

Many studies have shown that EPC-derived endothelial cells are active in improving revascularization of ischaemic tissues.They also show that CB-derived EPC are far more efficient than those derived from adult peripheral blood in actively forming stable vascular structures inside these ischaemic tissues (22, 23). However, the vast majority of these *in vivo* experiments were performed in immunodeficient mice, which are not necessarily predictive of what would happen in patients. First, mouse vascular system is very different from that of humans, and natural healing from ischaemia is much higher in these animals, and second, as the mice were immunodeficient, information on inflammation and reaction of the immune system is lacking. Pig vascular biology appears to be relatively more similar to that of humans, and for this reason, this animal is considered as a predictive model for cardiovascular diseases. However, as the pigs used are immune competent, human cord blood cells cannot be studied in these animals, and techniques concerning EPC purification and expansion have to be adapted to this animal model. Several studies have already described purification of pig peripheral blood EPC, and have shown their efficacy in ischaemic diseases (32–34).

By transposing our human CB EPC technology and by adapting published methods, we are currently producing newborn pig EPC that are under characterization for determining their phenotypic and functional properties compared to those from humans. We will then compare efficacy of EPC in ischaemic models performed in pigs from the same litter or in non-genetically related animals. This study will provide clues concerning efficacy of newborn pig EPC and on limits of tolerance of grafted cells in allogeneic conditions.

Conclusions

Because of their tropism to sites of neoangiogenesis and their strong angiogenic capacity, EPCs represent a potential therapeutic tool for peripheral artery diseases. However, use of autologous EPCs in cell therapy is limited by rarity of these cells in adult peripheral blood. In addition, EPCs of patients with peripheral artery diseases may be functionally altered, these alterations being directly or indirectly responsible for their vascular disease. CB contains more EPCs than peripheral blood, which are functional and can be expanded in culture. However, these endothelial cells express markers of alloimmunity, and would be rejected by the patient in allogeneic conditions. If efficacy of cord blood EPC is demonstrated in predictive pre-clinical models, it will be then possible to use frozen CB units from existing cord blood banks. However, our studies show that freezing conditions used for CB cryopreservation are not fully adapted to EPC recovery after thawing. Functional EPC are obtained from these frozen CB units, but in lower numbers compared to fresh CB. Freezing conditions and/or improvement in colony formation process are thus required for making possible the use of these existing CB banks.

A further important issue is to expand the cells in conditions compatible with clinical use. We are currently working on this topic using close culture systems and serum-free media adapted to different phases of the culture, initial step of colony formation and population growth, expansion and freezing.

Once efficacy and safety of EPC-derived endothelial cells are validated in ischaemic models performed in large animals, clinical trials will be ready to start in selected cohorts of patients.

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