REVIEW ARTICLE

Ex Vivo Expansion Of Hematopoietic Cells Today

Zoran Ivanović
Aquitaine-Limousin Branch of French Blood Institute & UMR 5164 CNRS/Ségalen University Bordeaux, France

Correspondence
Professor Zoran Ivanović
Etablissement français du Sang Aquitaine-Limousin et UMR 5164 CNRS/Université Ségalen, Bordeaux, France
E-mail: zoran.ivanovic@efs.sante.fr

ABSTRACT
Ex vivo expansion (amplification) of hematopoietic stem and progenitors cells is a concept aimed to resolve the problem of insufficient number of cells for engraftment and/or to accelerate hematopoietic reconstitution after transplantation. After a long period, during which this approach failed to demonstrate its clinical utility, the first successful clinical trials were achieved. Here, we are explaining this breakthrough, mainly resulting from recent understanding of some fundamental properties of stem cell related to its anaerobic metabolic character.

KEY WORDS
Hematopoietic stem cells, committed progenitors, ex vivo cell expansion, CD34+, stem cell transplantation

(Scr Med 2011;42:92-6)

Submitted: August 15, 2011
Accepted: September 8, 2011

The concept of *ex vivo* expansion of hematopoietic cells for transplantation directly derives from the fundamental knowledge of Experimental Hematology. It enabled us to realize that a critical quantity of different sub-populations of stem and progenitor cells is necessary to get a rapid and sustained hematopoietic reconstitution. These principles, transposed to human cells (originating from bone marrow, peripheral blood, cord blood) inevitably required some fundamentally significant technological innovations (conception of the specific media, recombinant technology of cytokine production, etc.), in order to achieve the first efficient clinical trials (at the moment for cells mobilized in peripheral blood)[1,2]. This goal still remains to be reached for cord blood cells.

Although frequently named "stem cells"[3], the human CD34+ cell population is extremely heterogeneous from a functional point of view. Within an acceptable approximation[4,5], it is considered to be composed of i) committed progenitors (or "Colony Forming Cells – CFC"), representing a relative majority; ii) a low number of short term-repopulating stem cells (usually revealed by the functional *in vitro* and *in vivo* assays as Long-Term Culture Initiating Cells – LTC-IC or cells generating the committed progenitors in the secondary liquid cultures: - pre-CFC); iii) the primitive stem cells exhibiting the capacity of *in vivo* engraftment. Their evidence could be established by transplantation to NOD/SCID (or another immunodeficient strain) mice (Scid-Repopulating Cells - SRC); iv), the most primitive and rare population of stem cells that could be demonstrated by their capacity to maintain the human stem cell potential after being transplanted to the first generation of recipient mice (i.e. on the basis of their capacity to engraft the secondary recipient mice).

Any culture system aimed to expand the CD34+ cells results in the production of precursors and mature cells and, in most cases, in the simultaneous amplification of committed progenitors. The first result relies upon the fact that the differentiation of committed progenitors is enhanced in *ex vivo* cultures, and the second is based upon two simultaneous events: the amplification of committed progenitors by their own divisions and by their production from the stem cells differentiating rapidly in culture and, hence, exhausting themselves.

Most probably, these facts could explain the positive effect of transplanted expanded cells on the shortening of post-transplantation neutropenia. This is the reason why the precursors and committed progenitors should be amplified to the highest possible level. There is however, another opposite demand to an *ex vivo* expansion procedure: to maintain the long-term engraftment capacity, the activity of very primitive stem cells in the expansion product should be preserved or, even better, amplified. In order to reach this goal, enabling the expansion of the whole CD34+ content from one CB unit for transplantation without taking its substantial part as "unmanipulated CB fraction", many research groups studied the different culture conditions (reviewed in: 6) .

The First Clinical Trials

The first clinical trials based on *ex vivo* expanded hematopoietic cells did not demonstrate an acceleration of post-transplantation hematopoietic reconstitution[7-11]. If only the trials based on hematopoietic stem and progenitor cells mobilized into peripheral blood with nowadays knowledge were considered, at least one, but even more reasons could be found for the inefficiency of *ex vivo* expanded grafts. (Reviewed in the reference #6). In most cases, the cytokine combination employed enhances differentiation of stem cells, leading to their exhaustion during expansion culture[12]. In other trials, the fold of expansion was low i.e. the absolute number of cells (whatever functional category considered) obtained after expansion was insufficient. The first clinical trial demonstrating almost complete abrogation of post transplant agranulocytosis was that of Bordeaux group[1]. In this trial, hematopoietic cells, expanded from CD34+ fraction mobilized in peripheral blood of myeloma patients were auto-transplanted The average period of post transplant agranulocytosis, which is about 10 days with non manipulated grafts was completely abrogated or reduced (median of 1.5 days). In the first 14 patients, the amplification of 36, 15, 2.7 fold for total cells chronogenic progenitors, and CD34+ cells respectively, was achieved in a 10 day liquid culture stimulated by SCF, G-CSF, PEG-MGDF. In this first trial, the *ex vivo* amplified cells were transplanted together with a non manipulated fraction of graft. A similar approach was also used for autologous transplantation in the context of breast cancer (11 patients transplanted). This trial also demonstrated a positive effect which, however, was not as spectacular as the first one. Several other trials showed similar yields and results.

Transplant ex vivo Expanded Cells, Only

These trials provided a solid basis for the analysis related to distinct cell population and quantity to achieve an accelerated blood reconstitution. Given the fact that there were autologous transplantations, the issue of the persistence of primitive stem cells after expansion and their influence on long term hematopoietic reconstitution of the receiver could not be considered. After the analysis of these first trials, a new clinical trial was set up, considering transplantation of only expanded cells. The results concerning acceleration of hematopoietic reconstitution were similar to those of previous studies where both expanded and non manipulated cells were transplanted[2,13]. The analysis of these results showed that the extent of shortening of post-transplant neutropenia was well correlated to the number of nucleated cells and to the dose of clonogenic progenitors transplanted per kg of patient. On the contrary, this correlation was less obvious, and even inexistent, for CD34+ cells. Given that the expansion fold for clonogenic progenitors was more than 13 times higher than the one of CD34+ cells, the final result gave two fold more progenitors than CD34+ cells in term of absolute cell number. That means that expansion culture produced a huge quantity of CD34- progenitors. This trial was interrupted because both clinical grade MGDF and culture medium (Irwin) were not available. In order to restart this trial, we tested several media and thrombopoietin (Tpo) preparations. These trials highlighted Macopharma HP01 medium and Peprotec Tpo molecule. We carried out pre-clinical testing based on these conditions and finally restarted this clinical trial which was successfully achieved, yielding the results very similar to those where the expanded and non-expanded cells were transplanted together[13].

Expansion of Hematopoietic Cells From Cord (placental) Blood

Cord (placental) blood represents a source of stem and progenitor cells for engraftment. These cells are, in general, more primitive with respect to those mobilized into peripheral blood. For example, the CD34+ population of cord blood cells is for 1 to 2 log richer in stem cells capable to engraft the immunodeficient mice (Scid Repopulating Cells-SRC). The cells of cord blood did not respond the same way to the cytokines, and their amplification kinetic *ex vivo* is different from that of the peripheral blood cells. The transplantation of cord blood cells is limited by a low number of cells in one cord blood unit. In addition, given that these cells are more primitive, the time for mature cells production is rather long. This emphasizes an important consequence: a very slow blood reconstitution after transplantation (agranulocytosis period is about one month). Due to this inconvenience, the transplantation of cord blood cells was limited to children and adults of low body weight. This problem is reduced by the practice of simultaneous transplantation of 2 cord blood units. However, even with this approach, the time of post transplant neutropenia is rarely below 2 weeks. So there is an evident interest for *ex vivo* expansion of cord blood cells in order to:

1. Amplify the number of total cells

2. Differentiate several sub-populations of stem cells and progenitors and amplify these populations in order to get a shortage or even abrogation of post-transplant agranulocytosis period.

At the same time the absolute imperative is to:

3. Maintain or even amplify the primitive stem cells in order not to jeopardize the capacity of long term maintenance of hematopoiesis.

This third point would allow to consider the *ex vivo* amplification and consecutive transplantation of the whole cord blood unit without saving a non manipulated part. At the moment, the expansion of hematopoietic cells from cord blood is aimed to the allogenic transplantation, although its use in autologous settings cannot be excluded.

The allogenic hematopoietic transplantation exhibits another dimension with respect to autologous transplanta-

tion: the immune-compatibility aspect. With that respect, the effects of the modification of immunogenesis during *ex vivo* expansion could only be properly evaluated in a clinical trial (transplantation procedure). The CD34+ cells selection, *conditio sine qua non* for successful *ex vivo* expansion, eliminates the immunocompetent cells from the graft. Since the "graft vs. tumor" effect is considered as an important part of therapy mechanism for cord blood transplantation, we considered that it would be of significant relevance to be injected together with *ex vivo* amplified cells, the CD34- fraction cells issued from the same cord blood unit.

The analysis of 4 clinical trials[15-17] concerning transplantation of the *ex vivo* expanded cord blood CD34+ cells however, did not reveal a shortening of the post transplant agranulocytosis period. In our opinion, it is still insufficient, since despite its expansion *ex vivo,* the total number of progenitors and CD34+ cells transplanted per kilo of donor weight, was low. Indeed, a more accurate analysis of these trials revealed at least five reasons that could be responsible for the absence of effects: low extent of expansion; an exhaustion of cell populations capable to induce a rapid short term hematopoietic reconstitution; a time-lag of 8 to 12 days for the injection of expanded cells with respect to non manipulated fraction; only a fraction of cord blood unit was ex vivo expanded.

The combination of points 1, 2 and 4 gave as a result, an insufficient number of cells belonging to the populations critical for hematopoietic reconstitution.

After several years of experimentation at basic and preclinical level of our and other groups[18,19], and taking into consideration the results from our above mentioned clinical trials (autologous transplantation)[1,2,14], we developed a very efficient protocol for *ex vivo* expansion of hematopoietic cells starting from CD34+ cells of cord blood.

This could have been achieved today, since the advancements in the media quality and the cytokines selections allowed it. As a matter of fact, these major advancements are related to the compensation of the negative effects of a culture hyperoxygenation (atmospheric 20% O2) i.e. to the cultures that better represent the physiologic conditions of hematopoiesis[20-22]. The same principle, considered through the evolutionary prism (as explained by "Oxygen Stem Cell Paradigm") is targeting the regulation of ancestral genes involved in the basic cellular functions (simple proliferation and survival of cells), considered to be the factors of "stemness" or of "self renewal"[13]. Most of these genes are proved either to be activated by a low O2 concentration or to have a sequence called "Hypoxia Responsing Elements" (HRE). In that context, it should be noted that the latest approaches in *ex vivo* expansion research try to exploit these features[24-27].

Our *ex vivo* expansion procedures are based on the principles that the association of a medium with a powerful system of antioxidants with MGDF (Tpo) (stabilizing HIF1a transcripts[28] mimics the physiological low O2 environment of hematopoiesis, whereas the other cytokines (SCF, G-CSF, Flt3 ligand) in relatively high doses provoke a "regenerating bone marrow-like" effect (Reviewed in: 6).

As an example, here is presented the development of a clinical-grade procedure for the *ex vivo* expansion of cord blood CD34+ cells isolated from previously frozen cord blood units with or without volume reduction, allowing the amplification of total cells by factor ~350 and of committed progenitors by factor ~130 (mean values) without significantly impairing the activity of primitive stem cells. The last point was explored using NOG/SCID model both in usual transplantation model as well as in a model of serial transplantation (i.e. the only way to detect the activity of primitive stem cells today)[29]. That was important since we aimed to amplify *ex vivo* the CD34+ cells from a whole cord blood unit without saving its substantial fraction as a non manipulated background. This issue was also critical to obtain an agreement from the State Sanitary Authority enabling us to avoid a split in 2 parts of one cord blood unit in the clinical expansion set up. Our two-steps clinical grade serum-free culture system [SCF, FLT3-L, MGDF, (100 ng/m each), G-CSF (10 ng/ml)], initially upgraded[30] on the basis of the experimental data of Douay's group[31] in one-step cultures with the medium IRWIN (no longer commercialized)[30], was subsequently improved with serum-free medium Macopharma HP01 an Tpo instead of MGDF[32]. In the design of clinical protocol, the intention was to ensure an immunologic power of the graft. Since it was not possible, with the amplified fraction only, the protocol proposed to inject – together with the expansion product – a CD34-negative fraction that was refrozen after selection of CD34+ cells and thawed before transplantation. It was necessary to demonstrate that this procedure does not impair the immunocompetent cells (T and B cells), although it does destroy an important fraction of granulocytes (cells out of our interest).

Epilogue

Based on these data with some minor modifications a clinical grade procedure was set up. This ongoing clinical trial led by Professor Noel Milpied started in 2010. Seven patients were transplanted so far, with very promising results. For example, the first patient transplanted more than one year ago exhibited peripheral blood reconstitution only one week after transplantation and still has a hundred percent donor chimerism.

No potential conflict of interest relevant to this article was reported.

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Ex vivo ekspanzija hematopoetskih ćelija

Zoran Ivanović

APSTRAKT

Ekspanzija (umnožavanje) hematopoetskih matičnih ćelija i progenitora *ex vivo* je koncipirana da bi se rešio problem nedovoljnog broja ćelija za transplantaciju i ubrzala posttransplantacijska hematopoetska rekonstitucija. Posle dugog perioda, tokom koga ovaj pristup nije davao zadovoljavajuće rezultate, pojavili su se prve uspešne kliničke studije. Članak opisuje u čemu se sastoji ovaj kvalitativni pomak koji je većim delom zasnovan na razumevanju nekih elementarnih svojstava matične ćelije kao što je njen anaerobni metabolički karakter.

KLJUČNE RIJEČI

Matične ćelije hematopoeze, opredeljeni progenitori, umnožavanje ćelija ex vivo, transplantacija matičnih ćelija