LONG-TERM EXPANSION OF TRANSPLANTABLE HUMAN FETAL LIVER HEMATOPOIETIC STEM CELLS

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Supported by grants from La Ligue Suisse contre le Cancer (#228-11-1995), La Recherche Suisse contre le Cancer (KFS 170-9-1995), and La Ligue Vaudoise contre le Cancer

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ABSTRACT

Hematopoietic stem cells (HSC), with their dual ability for self-renewal and multilineage differentiation, constitute an essential component of hematopoietic transplants. Human fetal liver (FL) represents a promising alternative HSC source, and we reported simple culture conditions allowing long-term expansion of FL hematopoietic progenitors. In the present study, the NOD/SCID mouse xenotransplantation assay was used to confirm that human FL is rich in NOD/SCID-repopulating cells (SRC), and to show that these culture conditions repeatedly maintained short and long-term SRC from various FL samples for at least 28 days. Quantitative limited dilution analysis in NOD/SCID mice demonstrated for the first time that a 10 to over a 100 fold net expansion of FL SRC could be achieved after 28 days of culture. The efficiency of this culture system may lead to an increase in the use of FL as a source of HSC for transplantation in adult patients, as previously demonstrated with umbilical cord blood under different culture conditions.

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INTRODUCTION

There is a growing clinical need for large numbers of human hematopoietic stem cells (HSC) for transplantation or gene therapy \(^{1,2}\). In cancer patients where the probability of tumor cell contamination in either leukapheresis or bone marrow (BM) is high, the use of allogeneic cells is preferable. Accumulated evidence suggests that fetal liver (FL) and/or umbilical cord blood (CB) represent alternative and possibly more "universal" sources of "early" HSC possessing a better proliferative potential and also a pre-immune status that may be important in mismatched transplant situations \(^{3-7}\). However, both sources are compromised by the relatively small number of cells available. Therefore, the future clinical potential of FL and CB would be strongly enhanced if methods allowing a reliable HSC expansion, perhaps to a degree as little as 20-100 fold, without a loss of their engraftment ability, could be developed \(^8\). Ex-vivo expansion of CB and of adult NOD/SCID-repopulating cells (SRC, see \(^9\)) has been demonstrated (see for example \(^{10,11}\)), but successful expansion of FL SRC has not been reported so far.

We recently published a simple and reproducible stroma-free liquid culture system allowing long-term (>6 months) expansion of human hematopoietic cells contained within previously frozen crude FL cell suspensions. In these cultures, CD34\(^+\) cells, primitive colony-forming progenitors, and cells possessing a putative stem cell phenotype were not only present, they also underwent a continuous amplification \(^{12}\). However, the maintenance and/or expansion of functional repopulating HSC was not tested. This is critical given the dissociation between stem cell phenotype and in vivo repopulating function observed in hematopoietic cultures \(^{13-15}\). Here, we used the NOD/SCID mouse xenotransplantation assay to quantify FL SRC, and demonstrated
that our culture conditions allowed a 10 to over 100 fold net SRC expansion following 28 days of culture.
STUDY DESIGN

Cryopreserved human FL crude cellular suspensions were prepared from aborted fetuses (gestational weeks 12-17) as described, with the approval of the ethical committee of the Lausanne University Medical Faculty \(^{12,16}\). Total nucleated FL hematopoietic cells were expanded in RPMI-1640 supplemented with 8% human AB plasma, Flt-3 ligand (50 ng/ml), interleukin-6 (10 ng/ml), megakaryocyte growth and development factor (MGDF, 10 ng/ml) and stem cell factor (SCF, 50 ng/ml). Cultures were fed and maintained as described \(^{12}\), except that cells were grown in T25 flasks instead of 24-well plates. For NOD/SCID repopulating assays, 6 to 8 week old NOD/LtSz-scid/scid(NOD/SCID) mice were sublethally irradiated (375 cGy using a Cs-137 source), and cells to be tested (at the doses indicated, in ~600 µl RPMI) were injected into the lateral tail vein 4-24h later. Mice were killed after 6, 8 or 12 weeks, the BM harvested from femora, and human engraftment analyzed by flow cytometry using an anti-human CD45 antibody. In most cases, multilineage engraftment was confirmed using a combination of antibodies as described \(^{17}\). For limited dilution analysis (LDA), a mouse was considered positive (engrafted) when low percentages of engraftment (<0.5% CD45\(^+\) cells) could be unequivocally confirmed by Southern blot hybridization \(^{9,17,18}\). Data from LDA experiments were analyzed by applying Poisson statistics to the single-hit model, and the SRC frequency in each cell source calculated using the L-Calc software (StemCell Technologies, Vancouver, Canada).
RESULTS AND DISCUSSION

Human HSC possessing in vivo repopulating capability can be functionally tested in the SRC assay. We first determined whether FL SRC were maintained after 4 weeks in culture, when a large number of FL hematopoietic cells, potentially sufficient for transplantation into adult patients, could be harvested from a limited supply of unexpanded FL cells. Four different FL specimens were cultured for 28 days, and cohorts of ≥5 mice/sample were transplanted with 20×10^6 expanded viable total nucleated cells (TNC). Both short- and long-term SRC were maintained in culture, as all mice analyzed contained large numbers of CD45+ human hematopoietic cells in their BM, whether tested ~6 or ~12 weeks after transplantation. A representative example of these analyses is shown in Fig. 1A. Overall, engraftment levels were higher at 12 weeks than at 6 weeks, demonstrating that expanded FL samples contained SRC capable of long-term repopulating potential. Additionally, the BM of all these mice contained cells committed to both lymphoid (positive for CD19, CD20) and myeloid lineages (CD14, CD15, CD33), as well as a significant fraction of putative primitive CD34+CD38- hematopoietic progenitors. Interestingly, no significant differences in engraftment levels (total and/or lineage-specific) were observed if expansion was performed in 2 steps, i.e. freezing after 7 days of culture followed by three more weeks of culture, or if expanded cells were stored frozen before injection (data not shown). These results demonstrate that our culture conditions repeatedly maintained short and long-term FL SRC capable of in vivo lympho-myeloid differentiation for at least 28 days.
In order to compare the maintenance and/or expansion of the FL repopulating stem cell compartment with that of other, more widely used HSC sources, we attempted to establish long-term hematopoietic cultures from CB and/or adult sources, under the same conditions we used for FL. With adult BM and/or mobilized peripheral blood, we never succeeded in obtaining long-term cultures that would last more than a few weeks, and after 28 days the number of cells generated was not sufficient for transplantation into NOD/SCID mice. With CB, we were only able to establish long-term cultures with about half of the samples tested, and the maintenance/expansion of SRC in NOD/SCID mice was not tested. However, these experiments cannot be compared with published reports of successful CB and/or adult SRC expansion (see for example 10,11) as culture conditions were different: we used RPMI instead of IMDM as culture medium and expanded TNC instead of purified/enriched CD34^+ cells. We previously showed that optimal long-term expansion of FL hematopoietic cells depended not only on an adequate cytokine cocktail, but was also significantly influenced by the protein source (human plasma vs fetal calf serum), its concentration and the culture medium 12. Thus, our culture conditions may be optimal for expanding FL TNC but not for CB TNC or CD34^+ cells, nor for adult hematopoietic cells.

Using quantitative LDA in NOD/SCID mice 9, we then estimated the frequency of SRC in three different unexpanded FL cell suspensions. Mice were given cell doses ranging from ~6×10^4 to 20×10^6 TNC, and engraftment was analyzed 8 weeks later. A representative example is shown in Fig. 1B. In this FL specimen, the average SRC frequency was found to be 1 SRC in ~700'000 TNC, or ~1 in 50'000 hematopoietic CD45^+ cells as this FL sample contained ~7% CD45^+ cells (Table I). Although not directly comparable, this frequency is much higher than those estimated in CB, adult BM or mobilized peripheral blood cells 9-11, and it correlates well with the high SRC
frequency reported in human fetal blood \cite{20}. As could be expected, larger cell doses usually resulted in higher engraftment. However, the BM of mice transplanted with low cell numbers occasionally contained surprisingly high percentages of human CD45\(^+\) cells \textit{(e.g.} Fig. 1B). This could result from the large variability inherent to the NOD/SCID transplantation system \cite{21}, and/or from the functional heterogeneity of the human HSC compartment, as demonstrated both \textit{in vitro} and \textit{in vivo} \cite{22,23}. Alternatively, this could also be stochastic, these mice having randomly received more SRC. Data from two additional FL samples are summarized in Table I, confirming that human FL is rich in SRC \cite{24,25}.

To analyze whether a net SRC expansion could be achieved, three FL specimens were expanded for 28 days and then analyzed in LDA experiments as above. For example, all mice transplanted with \(\geq1\times10^6\) expanded TNC from FL#841 (Fig. 1C and D), and the majority of mice injected with \(0.5\times10^6\) cells (Fig. 1E), were positively engrafted. Conversely, most mice transplanted with \(0.25\times10^6\) expanded cells were not engrafted (Fig. 1E). The calculated SRC frequency was found to be 1 in \(~365'000\) TNC or CD45\(^+\) cells, as the vast majority (>99\%) of expanded cells in culture at 28 days were CD45-positive \cite{12}. The total cellular expansion (fold increase in viable TNC) for this sample after 28 days of culture was \(~24\)-fold, and the initial percentage of CD45\(^+\) cells was \(~7.0\%\) (Table I). Assuming that only CD45\(^+\) cells in unexpanded FL suspensions contributed to the massive hematopoietic cell amplification observed in culture, this represents a CD45\(^+\) cell expansion of \(~343\)-fold; therefore, the net SRC expansion following 28 days of culture for this FL sample could be estimated to be \(~46\) fold. Data from two additional expanded FL samples are summarized in Table I. Although large differences in SRC frequencies were observed between FL specimens, both before and following culture, a 10-fold or more net SRC amplification was achieved in all cases.
The NOD/SCID transplantation system is extremely useful to test the repopulating potential of human hematopoietic cell populations, whether freshly isolated or after *in vitro* culture. However, it suffers from an important variability, both between but also within similar samples\(^1\), making it difficult to carefully assess the potential differences in engraftment of expanded *vs* unexpanded cells. Despite this variability, overall, no significant difference between the reconstitution capability of expanded *vs* unexpanded FL cells was observed in the present study. In other words, transplantation of equivalent numbers of expanded *vs* unexpanded FL SRC into NOD/SCID mice usually resulted in similar engraftment levels.

Together with our previous study\(^1\), the data presented here demonstrate for the first time that under appropriate conditions, human FL repopulating HSC could be expanded, allowing, in a period of ~4 weeks of culture, the *in vitro* generation of a sufficient number of FL hematopoietic stem, progenitor and mature cells to be used for transplantation in adults. Thus, as for CB, which is increasingly utilized for transplantation (reviewed in \(^8,26\)), FL may become a good alternative clinical source of HSC. Our studies not only support the hypothesis that FL SRC are uniquely different from CB and/or adult sources, in terms of both their *in vitro* expansion and *in vivo* repopulating capabilities\(^4,24,25,27,28\), they also go well beyond, as the first to unambiguously demonstrate that FL HSC can be truly expanded in simple culture conditions, while retaining their lympho-myeloid repopulating potential. Thus, FL SRC may represent one of the best targets for HSC expansion and/or genetic manipulation. Although culture periods longer than one month may not be clinically useful, preliminary experiments suggest that FL SRC could be maintained beyond 28 days in our culture conditions, at least until 6 weeks of culture. For example, when 20 million hematopoietic cells from FL#873 expanded for 42 days were tested, all NOD/SCID mice were positively engrafted 7 weeks later (n=5), with an average engraftment of ~2.2% human.
CD45<sup>+</sup> cells in the recipient BM. Whether the peak of SRC expansion is reached at 4 weeks or whether SRC expansion could still be improved at later time points awaits further LDA analyses. As a dissociation between stem cell phenotype and repopulating function is usually observed in hematopoietic cultures<sup>13-15</sup>, the future challenge will be to characterize expanded human FL HSC, and to compare them to their unexpanded counterpart and/or to HSC from different sources.
ACKNOWLEDGMENTS

We would like to thank Prof. P. Hohlfeld (Department of Gynecology and Obstetrics, University Hospital, Lausanne, Switzerland) and Prof. F. Forestier (Laboratoires Marcel Mérieux, Lyon, France) for providing the FL specimens used in this study, Amgen for their generous supply of MGDF and SCF, Prof. J.E. Dick for his comments on the manuscript, and Prof. R. Mertelsmann for continuous support.
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FIGURE LEGEND

Figure 1. Engraftment of ex-vivo expanded FL hematopoietic cells in NOD/SCID mice.

(A) Maintenance of both short and long-term SRC in culture. Three FL samples were expanded for 28 days, and 20×10^6 viable nucleated cells were injected into individual NOD/SCID mice. Engraftment was analyzed ~6 weeks (short-term) or ~12 weeks (long-term) post-transplantation (p.t.), and is expressed as the percentage of CD45-positive human cells in the mouse BM. (B) Summary of the levels of human cell engraftment in the BM of NOD/SCID mice transplanted with various doses of FL#841 cells, unexpanded (upper panel) or expanded for 28 days (lower panel). The percentage of human cells in the individual recipients was determined 8 weeks post transplantation by flow cytometry analysis of BM cells expressing human CD45. The dotted lines separate engrafted from non-engrafted animals, as confirmed by Southern blot analysis. More detailed analyses of the engraftment of expanded cells from this FL specimen are shown below. (C) Flow cytometry analysis of a highly engrafted representative mouse transplanted with 5×10^6 expanded FL#841 cells. About 56% of the cells in this mouse BM stained positively for the human pan-leukocyte marker CD45 (upper left panel, gate R1, and upper right panel, region M1); these human cells, once backgated to an FSC/SSC plot, mostly fell within a typical viable (but heterogeneous) cell population (lower left panel). The middle and bottom right panels show a CD45 staining of a control (not transplanted) NOD/SCID mouse BM, and an isotypic control (mouse IgG1) of a positively engrafted NOD/SCID mouse, respectively. Both panels show only a few rare events falling in gate R1. (D). Representative examples of positive engraftment of NOD/SCID mice transplanted with 1 to 10×10^6 expanded FL#841 cells as indicated. Human cells
appear in gate R1, and their percentages (of total BM cells) are shown in the respective panels. 

(E) Southern blot analysis of human cell engraftment in the BM of individual NOD/SCID mice transplanted with the indicated doses (0.25 to 10×10^6 TNC) of FL#841 cells following 28 days of expansion. 4 µg of Eco RI-digested DNA were loaded in each lane, and the blot was hybridized to a human chromosome 17-specific α-satellite probe as described ⁹. Positive engraftment is indicated by + signs above the respective lanes. Human/mouse (H/M) DNA controls are given as percentage of human DNA. M lanes were loaded with DNA molecular weight markers.
Table I: Limited dilution analysis of human FL engraftment in NOD/SCID mice

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SRC frequency: 1 in 704,503 nucleated cells
95% confidence limits: 1 in 370,751 to 1 in 1,338,698
(=7.0% CD45, or 1 SRC in 49,000 CD45-positive cells)

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SRC frequency: 1 in 299,293 nucleated cells
95% confidence limits: 1 in 249,522 to 1 in 712,1047
(=8.7% CD45, or 1 SRC in 250,000 CD45-positive cells)

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SRC frequency: 1 in 366,045 nucleated cells
95% confidence limits: 1 in 180,551 to 1 in 742,065
(=11.8% CD45, or 1 SRC in 43,000 CD45-positive cells)

**NOD/SCID mice were transplanted with serial dilutions of total nucleated cell from three different human FL samples before or following 28 days of culture.** Murine BM was analyzed 8 weeks post-transplantation by flow cytometry and confirmed by Southern blot analysis. Mice were scored as engrafted if a specific band was detectable by Southern blot analysis, as shown in Fig. 1E (limit of detection, ~0.1% human cells). Poisson statistics were applied to the single-hit model, and the frequency of SRC calculated with the maximum likelihood estimator. **
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