Cord blood as an alternative source of haemopoietic stem cells

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Introduction

The ultimate value of human umbilical cord blood (CB) for clinical transplantation will mainly depend on whether single donations can reliably engraft adult recipients. Certain inherent advantages of CB over adult marrow donations are potentially attractive. Cord blood collected from healthy full term cord and placenta is likely to be virus free, to have a naive immunophenotype [1], and a low level of mature cytotoxic T-cells [2]. The immunophenotype and functional naivety of CB may allow HLA partially mismatched transplants without inducing life-threatening graft-versus-host-disease (GvHD). In addition, CB may be conveniently banked after collection [3] and in the future banks may be networked world-wide. Cord blood banking could dramatically reduce the 'search to transplant interval' for patients who lack a family donor and at present rely on volunteer marrow donors. In recent years there has been some improvement in the probability of finding HLA matched unrelated marrow donors from volunteer donor registries [4]. However, one prospective study indicated that 400 days after search requests the probability of finding a volunteer marrow donor was only 10% [5]. Furthermore, in a prospective cohort controlled study, survival after unrelated bone marrow transplantation (UD-BMT) was significantly inferior with HLA identical sibling BMT [6].

To date, experience of successful family and unrelated donor CB transplantation has been confined to recipients weighing less than 40 kg [7, 8]. Our main aim has been to evaluate the potential of CB donations for adults who constitute 85% of all marrow allograft recipients. This work has covered two main areas; firstly, optimisation of processing CB donations for banking and secondly, attempts to expand the CD34+ fraction of CB to facilitate engraftment in adult recipients after allogeneic transplantation.

Optimal collection procedures

All donations were collected from full-term normal deliveries with informed consent from the mother. Previously we have reported, in a consecutive series of 132 donations, mean values for volume and nucleated cell count of 117 ml and 15.8 x 10^8, respectively [9]. In our current work on smaller aliquots we have expressed results as for 'standard' 100 ml CB donations. This facilitates yield comparison of different cell types in CB donations with those in adult marrow or peripheral blood progenitor cells.

Volume reduction and red cell depletion of CB donations

The aim of this work was to volume reduce donations by red cell depletion to allow cost effective storage in a CB bank without significant loss from the CD34+ stem cell fraction. Six red cell depletion methods were compared: ficoll and percol density gradient separation, ammonium chloride red cell lysis, and red cell sedimentation with hetastarch, 1% methyl cellulose, or 3% gelatin [10]. Thirteen donations were volume reduced using at least four methods, and recovery of total CD34+ cells and colony forming cells (CFC) were measured (Figures 1A, B). Results were consistently better using 3% gelatin sedimentation giving mean recoveries of 775 nucleated cells, 86% CD34+ cells and 92% CFC (Table 1). We have shown that 3% gelatin sedimentation is a cost effective method which our group will introduce to other participants in the U.K. cord blood banking network for further pre-clinical evaluation and standardisation.

Cryopreservation of CB cells

Cord blood was cryopreserved using up to 1 x 10^8 cells/ml in 10% DMSO with Iscoves modified Dulbecco's medium (IMDM) and fetal calf serum (FCS).
Figure 1. Comparison of the yield of CFC (A) or CD34+ cells (B) from 13 cord blood samples (mean ± SD) following red cell deple-
tion by ficoll, 3% gelatin, red cell lysis or hetastarch. Yields are
corrected for 100 ml of CB representing the average volume for a
clinical donation. All four methods were performed concurrently
on aliquots of each CB tested. * Indicates a statistically significant
difference in yield between gelatin compared with ficoll,
(P < 0.05).

Table 1. Recovery of haemopoietic cells after red blood cell (RBC)
depletion of 100 ml cord blood 'donations' with 3% gelatin.

<table>
<thead>
<tr>
<th></th>
<th>Mean nucleated cells/100 ml ± SD</th>
<th>Mean CFC/100 ml x 10^6 ± SD</th>
<th>Mean CD34+ cells/100 ml x 10^6 ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cord blood</td>
<td>1.7 ± 0.4</td>
<td>3.2 ± 2.2</td>
<td>5.9 ± 3.5</td>
</tr>
<tr>
<td>Post RBC depletion</td>
<td>1.3 ± 0.3</td>
<td>2.8 ± 1.7</td>
<td>4.6 ± 2.6</td>
</tr>
<tr>
<td>Mean cell recovery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>post-RBC depletion</td>
<td>77%</td>
<td>92%</td>
<td>86%</td>
</tr>
</tbody>
</table>

Freezing was at -1 °C per minute to -60 °C, followed
by rapid immersion in liquid nitrogen. Thawed cells
were treated with DNA-ase to prevent cell clumping.
There was approximately 30% reduction in the recov-
ery of CFC using this protocol (data not shown). In
contrast the stem cell compartment, estimated in vitro
by measurement of long-term culture initiating cells
(LTC-IC) in long-term stroma based cultures, appeared
unaffected by cryopreservation [11]. In this study
5 x 10^3/ml fresh or cryopreserved, then thawed,
CD34+ purified cells from seven CB donations were
seeded onto mature irradiated normal bone marrow
stromas. Experiments were paired by seeding fresh and
thawed cells from each CB donor onto stroma from
the same marrow donor. It can be seen from Figure 2 that
there is no difference in the growth characteristics
of fresh and cryopreserved CB LTC-IC.

In vitro expansion of CB cells

The main aims of this part of the study were to investi-
gate the probability of improving engraftment after CB
transplants in adults, and to diminish the period of
post-transplant cytopenia by expanding CB CFCs and
stem cells in vitro. Published data shows a clinically sig-
nificant delay in peripheral blood platelet recovery
after allogeneic CB transplantation compared to BMT
[7]. We have investigated the influence of stroma condi-
tion medium (SCM) prepared from standard long-
term culture supernates on in vitro expansion of
CD34+ purified CB cells [12]. CD34+ cells were puri-
fied after mononuclear cell selection using the mini-
MAC® immunomagnetic separation system as previ-
ously reported [11]. Suspension cultures of 5 x 10^3
CD34 cells/ml were incubated in IMDM containing
SCF, IL-6, IL-3, G-CSF and GM-CSF all at 10 ng/ml.
As a result of pilot experiments we elected to expand
CB CD34+ cells over 14 days in vitro. Pre- and post-
exansion total CD34+ cells, CD34+38+ cells, and
granulocyte macrophage colony forming cells (GM-
CFC) were measured by standard methods [11] and
the fold expansion calculated. At weeks five and eight
LTC-IC were quantitated in modified stroma based
long-term cultures by limiting dilution analysis, scoring
cobblestone areas [13]. Three complete experiments in

Figure 2. Comparison of 5 x 10^3/ml fresh or cryopreserved, then
thawed, CD34+ purified cells from seven CB donations seeded onto
mature irradiated normal bone marrow stromas and grown in stan-
dard long-term culture conditions. Experiments were paired by
seeding fresh and thawed cells from each CB donor onto stroma
from the same marrow donor. There is no apparent difference in the
haemopoietic growth characteristics between freshly prepared and
frozen/thawed CD34+ purified CB cells.
the presence, and five experiments in the absence, of SCM were performed. After expansion CD34+38− cell and LTC-IC quantitation was performed on repurified CD34+ cells to allow comparison of similar cell populations pre- and post-expansion. The mean CD34 cell purity was 95 ± 1% pre- and 90 ± 1.7% post-expansion. Fold expansion quoted in the text and in Table 2, measured after re-purification of CD34+ cells, excluded cell losses in the re-purification process. Therefore true expansion of CD34+38− cells and LTC-IC was underestimated. Total expansion of CD34+38− cells and LTC-IC in the non-repurified expanded cell fraction was calculated from the repurified expanded cell population. For the purpose of this calculation it was assumed that CD34+38− cells and LTC-IC were present in the same proportions in in vitro expanded CD34+ cells before and after re-purification. These estimated data are also shown in Table 2. It is emphasised that calculated expansions of CD34+38− cells and LTC-IC as shown in Table 2 need verification by direct measurement of CD34+38− cells and LTC-IC in expanded non-repurified CD34+ CB samples.

There was a trend toward less efficient expansion in the presence of SCM, suggesting the possible presence of inhibitory factors. In the conditions described there was relatively modest expansion of the total CD34+ cells and CFC-GM. More interestingly, approximately two to seven fold expansion of directly measured weeks five and eight LTC-IC and of phenotypically primitive CD34+38− cells was also demonstrated. If the calculated expansions of CD34+38− cells and LTC-IC shown in Table 2 can be verified by direct measurement, these data suggest that the expansion of primitive cells within the CD34+ fraction of CB may be expanded 20–30 fold in simple in vitro conditions suitable for adaptation to clinical transplantation.

Discussion

Our overall aim has been to investigate and optimise CB donations for clinical transplantation. We have emphasised evaluation of CB as a suitable source of allogeneic haemopoietic stem cells for adult patients who currently constitute around 85% of all recipients of allogeneic haemopoietic stem cell transplants. Previously published work on red cell depletion and volume reduction of cord blood cells, as reported in the literature, has been disappointing with unexplained loss of total mononuclear and committed progenitor cells. These data lead to initial reluctance to fractionate CB for clinical banking. More recently, successful transplantation of 3% gelatin sedimented CB has been reported. In contrast to initial studies our data has confirmed that excellent recovery of 70%–90% of total CD34+ cells and CFC from CB donations can be achieved by ficoll density gradient separation, heta-starch and gelatin sedimentation and red cell lysis (Figures 1 A, B). In our hands 3% gelatin sedimentation is the method of choice combining optimal yield of CD34+ cells and CFC (see Table 1) with simplicity and low cost. Growth characteristics of primitive LTC-IC in long-term culture (LTC) following routine cryopreservation by established methods appears to be equivalent to that of fresh cells studied in paired LTC experiments (Figure 2). Further experiments to minimise cell loss during freezing and thawing are required to optimise CB processing for clinical transplantation. If cell losses during manipulations of CB donations can be minimised then it is probable that a single banked CB donation will be adequate to reconstitute adult recipients without in vitro expansion.

Already clinical experience has shown that a nucleated cell dose as low as 1.0 × 10^7/kg of recipient body weight can lead to sustained engraftment after allogeneic CB transplantation. In this study the largest recipients reported as successfully engrafted were 35–40 kg, a body weight about half that of a large adult.

Further evidence that single CB donations may be sufficient for larger adult recipients comes from theoretical computer simulations of the kinetics of haematopoesis. These were undertaken to establish the minimum number of haemopoietic 'stem' cells required for sustained engraftment after allogeneic BMT in humans. Simulations suggest that a minimum of 2 × 10^3 'stem' cells would provide sustained engraftment defined by completion of 100 cell cycles without clonal extinction. Our own calculations, based on the frequency of LTC-IC in CD34+ purified CB cells measured by limiting dilution analysis in LTC, indicate that approxi-

Table 2. Comparison of mean (± SEM) fold expansion of CB CD34+ cells with and without addition of stroma conditioned medium (SCM) to suspension cultures containing SCCF, IL-6, IL-3, G-CSF, GM-CSF all at 10 ng/ml.

<table>
<thead>
<tr>
<th>Fold expansion</th>
<th>CD34+ cells</th>
<th>CD34+38−</th>
<th>CFC-GM</th>
<th>Wk 5 LTC-IC</th>
<th>Wk 8 LTC-IC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before repurification</td>
<td>-SCM #5 43 ± 10</td>
<td>16 ± 6*</td>
<td>36 ± 17</td>
<td>25 ± 7*</td>
<td>10 ± 2.0*</td>
</tr>
<tr>
<td>+SCM #3 19 ± 7</td>
<td>5 ± 1.4</td>
<td>11 ± 3.2*</td>
<td>8 ± 3.9*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>After repurification</td>
<td>-SCM #5 8 ± 1.1</td>
<td>2.8 ± 0.8</td>
<td>6 ± 2.7</td>
<td>5 ± 1.1</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>+SCM #3 3 ± 0.6</td>
<td>ND*</td>
<td>0.4 ± 0.1</td>
<td>1.1 ± 0.4</td>
<td>1.2 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

Fold expansion relates to expansion of CD34+ purified CB cells. Measurements of fold expansion of total CD34+ cells and CFC-GM were performed directly prior to repurification of expanded CB cells. Measurement of CD34+38− cells and LTC-IC were performed after repurification of post-expansion CD34+ cells.

* Calculated values; see text for additional explanation.

ND: analysis not done due to insufficient cells.
mately 1 x 10^5 LTC-IC are available in a 100 ml donation of CB (unpublished data). It is probable that this number of LTC-IC are associated with sufficient marrow repopulating units to give sustained engraftment in an adult recipient after allogeneic CB transplantation without in vitro expansion of the CD34^+ cell fraction. There is clear evidence for self renewal and high proliferative potential which places human LTC-IC in the 'stem' cell compartment [17]. However caution is required as there is no direct method to relate the measured number of human LTC-IC to the number of marrow repopulating cells present in a donation.

We have studied expansion of CB CD34^+ cells in vitro with a view to ensuring that sufficient 'stem' cells are available for large adult recipients of allogeneic CB transplants. Table 2 shows that modest expansion of the most primitive cell populations measurable in human CB in vitro, namely CD34^+38^- cells and LTC-IC, can be achieved. Clinically useful expansion of total CD34^+ cells and CFU-GM also occurs which may prevent the documented delay in peripheral blood recovery by early differentiation into mature blood cells after allogeneic CB transplants. Although we have not yet accumulated data to verify our calculations it is likely that expansion of CD34^+38^- cells and LTC-IC is greater than we have directly measured at this stage.

As discussed above, it is becoming apparent that even simple in vitro expansion procedures as described here may not be necessary for allogeneic CB transplantation in larger adults. These data on in vitro expansion of human CB CD34^+ cells may however prove useful for optimising gene incorporation into primitive haemopoietic cells to enhance the level and longevity of gene expression in pre-clinical studies of gene therapy [18]. Familial diseases of lympho-haemoipoiesis such as severe combined immunodeficiency and Fanconi anaemia, and inborn errors of metabolism such as Hurler's syndrome and Gaucher's disease, are suitable disorders for research into gene therapy protocols. Furthermore, others have used similar expansion protocols as a method of purging tumour cells from autologous donations of CD34^+ purified peripheral blood progenitor cells [19].

In conclusion, these studies provide a practical insight into the feasibility of allogeneic CB as an alternative to unrelated and related donor bone marrow transplantation. In the United Kingdom a pilot national CB project has been set up which will be linked with a European CB banking initiative recently funded through the European Commission. Carefully controlled clinical studies of allogeneic CB transplantation in comparison with marrow should be in progress within two to three years in Europe.

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References


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