Chromosomal stability during *ex vivo* expansion of UCB CD34⁺ cells

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Abstract

Objectives: *Ex vivo* expansion is a feasible strategy, which may overcome limitation of the very low frequency of haematopoietic stem/progenitor cells, in umbilical cord blood (UCB). However, both quality of cells and safety of expanded population are critical issues to be addressed for their clinical application. Hence, in this study, we evaluated genetic stability of UCB-derived CD34⁺ cells during *ex vivo* culture, based on karyotype analysis, as well as its effect on cell proliferation characteristics.

Materials and methods: CD34⁺ cells were isolated from human UCB samples by immunomagnetic separation and were expanded *ex vivo* over a 28-day period. Expansion of total nucleate cells, CD34⁺ cells and CD34⁺ CD38[−] cells was investigated. Karyotype analysis of the expanded cells from six randomly selected UCB samples was performed to evaluate their genetic stability.

Results: Chromosomal abnormality of expanded cells mainly appeared by day 14, but was seldom sustained until day 28. None of the chromosomal abnormal samples displayed neoplastic proliferation, and expanded cells with altered chromosomes did not show obvious transformation phenomena according to soft agar assay.

Conclusions: *Ex vivo* expansion could lead to occurrence of chromosomal abnormality, although here it did not produce excessive proliferative advantage of the expanded cells. Importantly, chromosomal alteration seemed not to be inheritable and unlikely to result in malignant transformation. However, further in-depth evaluation of potential clinical risks of chromosomal abnormality is warranted.

Introduction

Umbilical cord blood (UCB) has been used as a source of transplantable haematopoietic stem/progenitor cells since first used for transplantation into a child with Fanconi anaemia was successfully performed in 1988 (1). Lately, its clinical applications in haematological disorders, immunodeficiency, metabolic disorders and autoimmune diseases have also been reported (2,3). UCB cells have now been considered as an alternative starting population to bone marrow and mobilized peripheral blood haematopoietic stem cell (HSC) for transplantation (4). However, use of UCB as a source of HSCs for allogeneic transplantation is limited to paediatric use as well as for adults of low body weight due to low number of HSCs present in a single harvest. Among the various strategies to overcome limitation of the HSC resource, *ex vivo* expansion of HSCs represents a flexible and convenient technique to be able to manipulate not only haematopoietic, but also the immune component of the graft (5,6), and a preliminary phase I study of *ex vivo* expanded CB-HSCs has been reported (7).

As with all other adult stem cells, HSCs have the ability for self-renewal, differentiation and production of a large number of progeny during *ex vivo* expansion. Cell division is a highly regulated process that leads to cell replication and passage of genetic information from one cell generation to the next (8). Primary mammalian somatic cells can only replicate for a limited number of times *ex vivo*, after which they stop dividing; this is known as replicative senescence. An important factor involved in cell senescence is reduction in mean telomere length as a result of decreased telomerase activity (9,10). However, if cells gain certain genetic alterations, they may bypass senescence and have elevated telomerase activity and undergo malignant transformation (11).

So far, there has been no direct evidence showing that human HSCs, or their progeny, are able to escape cell senescence in a manner similar to immortalized or transformed cells, during *ex vivo* culture. However, for other human stem or progenitor cells, there is an increasing body of evidence showing that they can undergo spontaneous

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transformation to malignant cells (11–13), and transformation seems to be the direct result of spontaneous revealed that human mesenchymal stem cells can undergo spontaneous transformation following long-term *ex vivo* culture (11) and all the transformed cells show high proportions of chromosomal alterations and rearrangements. This suggests that chromosomal abnormality might induce transformation of cultured cells. Moreover, one study of *ex vivo* expansion of UCB-derived endothelial progenitor cells demonstrated high incidence of chromosomal alterations during culture, although the abnormal cells did not show tumourigenicity *in vivo* (12).

Recent evidence from the study of human leukaemia has revealed that normal HSC and their neoplastic clone shared similar molecular mechanisms regulating proliferation, suggesting that normal HSCs can be transformed (14,15). As *ex vivo* expansion of UCB–HSCs is promising, to address the issue of limited HSC quantity, it is essential to fully understand the biological properties of expanded UCB cells to ascertain their clinical safety. Here, we have evaluated chromosomal stability of cells expanded from human UCB-CD34⁺ cells, by assessing their karyotype and proliferative characteristics during a 28-day culture period. Our findings provide important evidence concerning chromosomal instability of *ex vivo* expanded UCB cells, and show that occurrence of chromosomal abnormality does not favour development of neoplastic proliferation *in vitro*.

Materials and methods

UCB collection and purification of CD34⁺ cells

Thirty UCB samples from full-term deliveries were harvested in 100-ml collection bags (STT, Shanghai, China) and processed within 24 h. All collections were performed with informed consent of participants and with approval by our local hospital ethics committee. UCB mononuclear cells (MNCs) were prepared from UCB by density gradient separation using lymphocyte separation medium (Shisheng, Shanghai, China). Cells were centrifuged at 400 g for 30 min at 25 °C. MNCs at the interface were washed with phosphate-buffered saline (PBS) and resuspended in PBS containing 2 mM EDTA and 0.5% human serum albumin. CD34⁺ cells were separated using MACS cell isolation kit (Miltenyi Biotech, Gladbach, Germany) according to the manufacturer's instructions. Briefly, CD34⁺ cells were isolated from UCB-MNC fractions by incubation with FcR blocking reagent and CD34 microbeads for 30 min at 4 °C, followed by sequential passages through MiniMACS column. Purity of UCB-CD34⁺ cells was between 93% and 97%, as assessed by flow cytometry.

Cell culture

Freshly enriched CD34⁺ cells were cultured in 24-well plates in IMDM (Gibco, Grand Island, New York, USA) supplemented with 20% foetal bovine serum (HyClone, Logan, UT, USA) at 5×10^4 /ml density, in presence of the following growth factors: 50 ng/ml Flt-3, 50 ng/ml TPO and 50 ng/ml SCF (all recombinant human cytokines were purchased from PeproTech EC Ltd, London, UK). Cultures were maintained for 28 days. Each well was split in two every 3 days. No antibiotics were supplemented during the whole period. Cultures were analysed on days 7, 14, 21 and 28.

Flow cytometry analysis of surface antigen expression

Expanded cells at different time points were analysed on FACS Calibur flow cytometry (Becton-Dickinson, San Jose, CA, USA). Cells were stained with phycoerythrin (PE)-conjugated anti-human CD34 and fluorescein isothiocyanate (FITC)-conjugated anti-human CD38 (Becton-Dickinson). A replicate sample was stained with FITC-mouse IgG1 and PE-mouse IgG1 as an isotype control to ensure specificity. Data were analysed using FlowJo V.7.6 software (Treestar Inc, San Carlos, CA, USA).

Karyotype analysis

Metaphase chromosome spreads were prepared from cultures at the indicated time points. Briefly, 0.1 µg/ml colcemid (Sigma, St. Louis, USA) was added directly to cultures and incubated for 2 h at 37 °C. Cells were harvested, washed with PBS and incubated in 0.075 mol/l KCl at 37 °C for 15 min. Cells were fixed in methanol/acetic acid (3:1), G-banded and analysed. Images of individual metaphase spreads were captured and karyotyped. In the region of 20 cells from each sample were examined.

Telomerase activity assay

Telomerase activity was determined by telomeric repeat amplification protocol (TRAP) assay using a TeloTAGGG Telomerase PCR ELISA Kit (Roche Diagnostics, Mannheim, Germany). Briefly, 2×10^5 cells were lysed in 200 µl lysis buffer, and 1 µg of supernatant was incubated with PCR reaction buffer, in a final volume of 50 µl. After PCR amplification, products were denatured and hybridized with digoxigenin (DIG)-labelled telomeric repeat-specific detection probe, and detected by ELISA. Absorbance values were measured at 450 nm with reference wavelength of 690 nm. Relative telomerase activity was calculated and translated in fold of absorbance value of

HEK293 cell line, provided in the kit. Each sample was tested with heat-inactivated negative control (85 °C for 10 min).

Soft agar colony formation assay

For analysis of transformation *in vitro*, growth medium (10% FBS-IMDM) containing 0.5% agar was introduced into plate wells as base layer, then test cells (or positive control) were resuspended in growth medium containing 0.3% agar and plated on to the base layer. Following 2 weeks incubation in a humidified incubator at 37 °C, colonies were evaluated using an inverted microscope at 100× magnification.

Results

Cell expansion during *ex vivo* culture

In this study, 30 UCB samples were obtained from healthy donors. Freshly isolated and *ex vivo*-expanded cells were analysed by flow cytometry for expression of CD34 and CD38 surface antigens, at different time intervals. After 28 days, combination of SCF/FL/TPO growth factors was capable of expanding absolute number of nucleate cells (TNC), CD34⁺ cells and CD34⁺ CD38[−] cells with maximum folds of 1069.5, 151.8 and 21.8 (Fig. 1a–c) respectively.

Effects of *ex vivo* expansion on CB cell chromosomal stability

To examine chromosomal stability of the UCB cells expanded *ex vivo*, we randomly selected six samples of expanded cells and performed karyotype analysis at indicated time points. Results of G-band staining for each sample are shown in Fig. 2 and summarized in Table 1. Only two samples (CB3 and CB6) retained stable and normal karyotypes throughout the *ex vivo* expansion. Cells of the other four samples had random chromosomal abnormalities during culture, including numerical and structural changes. Of the four chromosomally malformed samples, CB4 showed three different types of defect, while CB1, CB2 and CB5 exhibited only one type of chromosomal fault. Karyotype analysis was also performed on all CB samples before culture, and no chromosomal alterations had been found (data not shown).

Moreover, the chromosomal abnormalities mainly occurred at 14 days after culture (Table 1). Two samples (CB4 and CB5) showed chromosomal aberrations at day 7. The karyotype of CB4 showed that 10% of metaphase chromosome spreads had 46, XX, t(2:8) karyotype but 10% had 45, XX-19 karyotype at day 7, a further abnor-

Figure 1. Cell expansion during culture. Expansion fold of (a) TNC, (b) CD34⁺ cells and (c) CD34⁺ CD38[−] cells was monitored after different time intervals during culture. Data are mean ± SD from 30 samples.

mal karyotype 45, XX-8 was observed at day 14. However, only 10% of metaphase cells kept an abnormal karyotype (45, XX-19) to the end of day 28. For CB5, in the region of 5% of metaphases lost an X chromosome by day 7, but no abnormal metaphases were observed thereafter. The other two samples (CB1 and CB2) showed chromosomal abnormalities at day 14. For CB1, 10% of metaphase cells had 45, XX-7, 6q+ karyotype at day 14, and proportion of abnormality decreased to 5% by day 21. CB 2 had tetraploidy of chromosome 13 (46, XY+D) with incidence of 13% at day 14, and proportion of polyploidy decreased to 5% by day 28. These data demonstrate that chromosomal alteration might indeed occur in CB cells during *ex vivo* expansion.

Proliferative characteristics of CB cells with normal or abnormal chromosomes

Proliferation potential of the six CB samples with normal or abnormal chromosomes was evaluated during *ex vivo*

Figure 2. Karyotype analysis of expanded UCB cells. Metaphase analysis on six randomly selected samples of expanded UCB cells, every 7 days over 28 days culture. Only two of six samples (CB3 and CB6) maintained normal karyotype. Representative G-banding staining for each chromosomally normal or abnormal sample is shown.

culture. All samples showed limited replication lifespan. CB4 and CB5, which exhibited chromosomal abnormalities by day 7, expanded 28.5- and 36.7-fold after 7 days

of culture, respectively, while normal samples CB3 and CB6 expanded 45.3- and 42.7-fold, respectively. In addition, CB1 and CB2, which exhibited chromosomal abnor-

Table 1. Karyotype analysis of expanded CB cells

UCB no.	Days in culture	Numerical normal (%)	Structural normal (%)	Karyotype (%)
1	7	100	100	46,XX(100)
	14	90	90	46,XX(90) 45,XX-7,6q+(10)
	21	95	95	46,XX(95) 45,XX-7,6q+(5)
	28	ND	ND	ND
2	7	100	100	46,XY(100)
	14	87	100	46,XY(87) 46,XY+D(13)
	21	88	100	46,XY(88) 46,XY+D(12)
	28	95	100	46,XY(95) 46,XY+D(5)
3	7	100	100	46,XX(100)
	14	100	100	46,XX(100)
	21	100	100	46,XX(100)
	28	100	100	46,XX(100)
4	7	90	90	46,XX(80) 46,XX,t(2:8)(10) 45,XX-19(10)
	14	80	85	46,XX(65) 46,XX,t(2:8)(15) 45,XX-19(10) 45,XX-8(10)
	21	88	92	46,XX(80) 46,XX,t(2:8)(8) 45,XX-19(12)
	28	90	100	46,XX(90) 45,XX-19(10)
5	7	95	100	46,XX(95) 45,X(5)
	14	100	100	46,XX(100)
	21	100	100	46,XX(100)
	28	100	100	46,XX(100)
6	7	100	100	46,XX(100)
	14	100	100	46,XX(100)
	21	100	100	46,XX(100)
	28	100	100	46,XX(100)

Metaphase analysis performed on six different samples of expanded CB cells during 28-day culture period. In the region of 20 metaphases per sample were analysed by typical G-banding staining. ND, not determined.

malities by day 14, expanded 153.2- and 125.5-fold after 14 days of culture, respectively, while normal samples expanded 170.7- and 186.7-fold respectively. By the end of 28 days culture, all chromosomally abnormal samples (CB1, CB2, CB4 and CB5) showed comparable or even lower expansion capacity versus normal samples (CB3 and CB6) (Fig. 3a). Further investigation of changes in specific growth rate revealed that chromosomally abnormal samples did not show higher specific growth rates than normal samples at any time point analysed, and all decreased gradually during culture (Fig. 3b). These data indicate that the chromosomally altered cells had no proliferative advantage over their normal counterparts. Moreover, over 85% of further CB samples showed expansion capacity between or close to the highest and

Figure 3. Proliferative characteristics of UCB samples. (a) Comparison of expansion capability between four samples with chromosomal abnormalities, and two normal samples. No proliferative advantage was observed in samples with chromosomal alterations. (b) Comparison of specific growth rate. All samples had progressive decrease in specific growth rate during culture, and no obvious difference was observed between chromosomally normal and abnormal samples.

lowest levels of the above-mentioned six samples, at the indicated time points (date not shown), implying that these randomly selected CB samples were adequately representative for this study.

Effects of ex vivo expansion on in vitro transformation potential

Expanded cells at different time points from the above six CB samples were also tested for their ability to proliferate in soft agar without supplementation of growth factors, indicative of neoplastic transformation. For CB 4, 1–2 small colonies were observed at day 21 per 5×10^4 cells seeded (Fig. 4a). However, expanded cells from all other UCB samples were not able to grow in soft agar at any time point during culture (Fig. 4b). In contrast, more and larger colonies were observed in leukaemic cell line K562, which served as positive control (Fig. 4c). These results suggested that *ex vivo* expansion of UCB cells did not usually result in neoplastic transformation.

Effects of ex vivo expansion on telomerase activity

Considering the important role of telomerase activity involved in cell neoplastic transformation, telomerase activity of the selected UCB samples was also investigated over time, using the TRAP assay. As shown in

Figure 4. *In vitro* transformation analysis. (a) CB4 formed a colony at day 21. (b) No colony formation was observed for other CB samples. (c) K562 cell line was used as positive control.

Fig. 5, none of the chromosomally abnormal samples exhibited activation or sustenance of advantage of telomerase activity compared to their normal counterparts.

Figure 5. Telomerase activity of expanded UCB cells. Telomerase activity of the six UCB samples selected was individually determined by TRAP assay during culture; none of the chromosomally abnormal samples displayed excessive activation of telomerase compared to their normal counterparts, throughout the whole culture. All samples had progressive decrease in telomerase activity over time.

CB3, which had a normal karyotype and the maximal expansion capacity, also had highest telomerase activity over most of the time period; while CB4, which had an abnormal karyotype and minimal expansion capacity, had the lowest telomerase activity throughout the culture, indicating that occurrence of chromosomal abnormality did not disturb the direct correlation between telomerase activity and cell proliferative capacity. Moreover, neither the normal samples nor the abnormal samples of the chromosomes could sustain stable telomerase activity throughout the study, and progressive decline of telomerase activity was observed in all 6 samples after day 7, which suggested that these chromosomal alterations did not induce abnormal activation of telomerase function.

Discussion

The first discovery of spontaneous transformation of human adult stem cells not only supported the hypothesis of cancer stem-cell origin but also indicated the importance of biosafety studies on adult stem cells or their progeny before their clinical transplantation (11). Although numerous investigations have demonstrated that *ex vivo* expansion of UCB-HSCs is a feasible strategy to overcome HSC cell resource limitation and holds potential for clinical application, clinical safety of expanded cells remains to be confirmed.

Genetic instability in human tumours usually involves chromosomal alterations (16), and spontaneous transformation of somatic cells seems to be the direct result of spontaneous genetic alterations that are accumulated during cell culture (11). To examine effects of *ex vivo* expansion on chromosomal stability of expanded UCB cells, we isolated CD34⁺ cells from UCB of 30 healthy donors and cultured them for 28 days. Absolute quantity of total nucleate cells, CD34⁺ cells and CD34⁺ CD38⁻ cells (believed to be tightly correlated with transplantation outcomes), was expanded to 1069.5-, 151.8-, and 21.8-fold

respectively. For clinical application, serum-containing culture has been regarded unsuitable for HSCs *ex vivo* expansion due to its potential safety risks. However, to reveal potential effect of culture duration on cell chromosomal stability, we considered that this ill-defined nature was preferable for the particular purpose of this study, over its serum-free counterpart, as genetic alterations are more prone to accumulate in this culture condition. In the present study, six CB samples cultured in serum-containing medium were randomly selected for karyotype analysis, and we assumed that they could represent all 30 CB samples cultured in the same conditions, based on their similar proliferative characteristics, and the assumed potential relationship between cell proliferative characteristics and chromosomal stability. Overall, it was found that four of these 6 samples developed random chromosomal abnormalities during culture, and alterations mainly occurred by day 14. Moreover, proportions of chromosomally abnormal cells neither remained constant nor increased over time in all four abnormal samples. Instead, it gradually reduced or even disappeared at later time points. Furthermore, none of these chromosomally abnormal samples had any proliferative advantage over their normal counterparts. Results from soft agar assay indicated that only one abnormal sample (CB4) was able to grow in semisolid agar. Thus, chromosomal abnormalities did not seem to be directly correlated with neoplastic transformation.

In addition in many cases, development of polyploidy is associated with malignancy and poor prognosis for patients (17,18), and it has been demonstrated that cells need to accumulate specific genomic changes, with tetraploidization, before full transformation occurs (19). In this study, although CB2 had exhibited tetraploid chromosome 13 during culture, random chromosome loss was more frequent as was observed in CB1, CB4 and CB5.

One possible mechanism of chromosome instability is loss of sufficient telomerase activity, which is responsible for compensating for telomere shortening at chromosome ends (20). Moreover, the process of transformation is always associated with elevated telomerase activity (11,21,22). The potential target gene encoding telomerase is located on the short arm of chromosome 5. However, in the present study, we did not find any abnormalities in chromosome 5 among all UCB samples tested. Hence, chromosomal alterations described herein did not seem to be supportive of neoplastic transformation, although the possibility that certain subtle molecular changes affecting oncogenes might have occurred in expanded cells, could not be completely ruled out. In addition, excessive upregulation of telomerase activity was not observed in expanded cells as detected by TRAP assay during the whole culture period.

Even though high incidence of chromosomal abnormality was not associated with neoplastic proliferation during this 28-day culture in the present study, biosafety when applying expanded cells to *in vivo* transplantation remains uncertain. Furthermore, the possibility that extended culture may induce certain chromosomal abnormalities, which would cause potential risk of neoplastic transformation, cannot be ruled out. Nevertheless, considering the potential clinical risk in *ex vivo* expansion of UCB-HSCs, more detailed studies on biosafety of expanded HSCs and their progeny are required.

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