Effects of telomerase activity and apoptosis on *ex vivo* expansion of cord blood CD34⁺ cells

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Abstract

Objective: *Ex vivo* expansion of CD34⁺ cells has become critically important in order to obtain sufficient haematopoietic stem cells for clinical application. Among major regulators involved in *ex vivo* expansion, telomerase activity and apoptosis have been revealed to be closely linked to cell cycle progression. However, all exact roles remain to be elucidated. Here, change in telomerase activity and level of apoptosis in cord blood (CB) CD34⁺ cells were evaluated together with specific cell population growth rate during *ex vivo* culture.

Materials and methods: CD34⁺ cells isolated from human CB were expanded *ex vivo* over a 28-day period. Besides monitoring cell proliferation kinetics of the CD34⁺ cells, changes in telomerase activity and apoptotic levels were investigated. Several relevant genes were quantified by qRT-PCR during the culture period.

Results: Significant elevation of telomerase activity had close relationship to activation of CB CD34⁺ cell expansion. Peak apoptotic level was accompanied by a remarkable decline in cell-specific growth rate, and apoptotic level of differentiated CD34⁺ population was significantly higher than that of the CD34⁺ population.

Conclusion: Although telomerase activity was activated during the culture, expansion of CB CD34⁺ cells seemed to be more susceptible to apoptotic suppression when cultured *ex vivo*, which implied that apoptosis may serve as a rate-limiting factor involved in controlling expansion efficiency.

Introduction

Haematopoietic stem cells (HSCs) have the ability to self-renew and differentiate into all haematopoietic lineages, and cord blood (CB) is a promising source of them, for their potential use for treatment of haematological disorders, immuno-deficiencies, metabolic disorders and autoimmune disease (1–3) amongst others. However, inadequate amounts of CB HSCs available from donors is a limiting factor for clinical applications, most cases yielding only sufficient quantities for transplantation into children or adults of low body weight (4,5). In attempts to overcome low cell dose limit and improve transplantation outcomes, *ex vivo* expansion of CB HSCs is necessary to highlight great potential opportunities in their clinical use for different strategies proposed, and results of preliminary phase I trial that have been reported (6,7).

Discovery of telomeres and telomerase has provided a new type of vision in stem cells to understand their regulation of cell proliferation and senescence. Among the factors involved, telomerase has been suggested to play a critical role in compensatory mechanisms for maintenance of telomere length, supposed to be essential for both cell self-renewal and differentiation of HSCs (8). In addition, telomerase activity is also suggested to be directly related to cell-proliferation processes (9). Compelling evidence has revealed that *ex vivo* expansion of CD34⁺ cells, using a combination of cytokines, has induced elevated levels of expression of hyper- and hypophosphorylated retinoblastoma (RB) protein, CDK2, p34^{CDK2} and cyclin A, which are all closely associated with progression of cells into S phase (10–13). To date, several recent studies have illustrated a relationship between telomerase activity and cell-cycle progression (14,15).

Meanwhile, it has been widely agreed that cell proliferation and apoptosis are linked to cell-cycle regulators, and apoptotic stimuli can be related to results of cell proliferation as well as to cell death (16). It has been reported

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that CD34⁺ cells in fresh CB or in mobilized peripheral blood often undergo apoptosis (17,18). Mastino *et al.* found a remarkable difference between apoptotic levels at 24 h and 48 h CD34⁺ cell culture, suggesting that apoptosis may play a crucial role in control of CD34⁺ cell counts in CB expansion (19). Moreover, it was found that apoptotic level of CD34⁺ cells served as an important parameter in functional heterogeneity of cryopreserved CB (20). Consistent with previous data supporting importance of apoptosis in regulating CB CD34⁺ cell count, recent study has also detected dynamic change in apoptotic levels in total expanded nucleate cells, initiated from freshly isolated CB CD34⁺ cells, and apoptotic level with different combinations of growth factors (21). However, the effect of apoptosis on CD34⁺ cell expansion during *ex vivo* culture has seldom been reported.

In the present study, change in telomerase activity and apoptotic level was investigated during CD34⁺ cell expansion culture, and relevant genes were quantified using qRT-PCR. Our findings will contribute to a better understanding of the role of telomerase and apoptosis in regulation of CB CD34⁺ cell expansion.

Materials and methods

Cord blood collection and purification of CD34⁺ cells

Six umbilical CB samples, from full-term deliveries, were harvested in standard 150 mL collection bags (STT, Shanghai, China) and processed within 24 h. All collections were performed after informed consent of the donors and with approval of the local hospital ethics committee. CB mononuclear cells (MNCs) were prepared from around 100 mL CB 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 in phosphate-buffered saline (PBS) and re-suspended in PBS containing 2 mM EDTA and 0.5% human serum albumin. CD34⁺ cells were separated using the MACS cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, CD34⁺ cells were isolated from CB 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 the CB CD34⁺ cells ranged between 93% and 97%, as assessed by flow cytometry.

Stroma-free and serum-dependent culture

Freshly enriched CD34⁺ cells were cultured in 24-well plates in Iscove's modified Dulbecco's medium (Gibco,

Grand Island, NY, USA) supplemented with 20% foetal bovine serum (HyClone, Logan, UT, USA) at 5×10^4 cells/ml density in presence of following growth factors: 50 ng/ml Flt-3, 50 ng/ml TPO, 50 ng/ml SCF (all recombinant human cytokines were purchased from PeproTech EC Ltd, London, UK). Culture was maintained for 28 days. Every 3 days, cells of each well were re-suspended in double their volume, split in two, and replated in new 24-well plates. To analyse cell population growth kinetics, specific growth rate was calculated as:

$$\ln(Nt_2/Nt_1)/(t_2 - t_1)$$

where "Nt" is the number of cells, and "t" is the time point.

Detection of telomerase activity

Telomerase activity was determined using a telomeric repeat amplification protocol (TRAP) assay using TeloTAGGG Telomerase PCR ELISA (Roche Diagnostics, Mannheim, Germany). Briefly, 2×10^5 cells were homogenized in 200 µl ice-cold lysis buffer and incubated for 30 min on ice. After centrifugation at 16 000 g for 20 min at 4 °C, supernatant was collected. Cell extract was incubated with reaction buffer including biotin-labelled P1-TS primer and P2 primer, telomerase substrate and Taq polymerase, for 30 min at 25 °C at final volume of 50 µl. After further incubation at 94 °C for 5 min, the resulting mixture was subjected to PCR for 27 cycles of 30 s at 94 °C, 30 s at 50 °C and 90 s at 72 °C. Amplification products were denatured and mixed with digoxigenin (DIG)-labelled, telomeric repeat-specific detection probe. The mixture was incubated in streptavidin-coated wells of 96-well microtiter plates for 2 h at 37 °C with constant agitation. Amplification product was immobilized using biotin-labelled TS primer, to a streptavidin-coated microtiter plate, and detected with anti-digoxigenin antibody conjugated to horseradish peroxidase. Absorbance values were measured at 450 nm with reference wavelength of 690 nm. Telomerase activity in the sample was calculated as ratio of sample to absorbance value of freshly isolated CB CD34⁺ cells, and represented as relative telomerase activity. A HEK293 cell line provided in the kit served as positive control. Each sample was tested with heat-inactivated control (85 °C for 10 min).

Analysis of apoptosis

For apoptosis, analysis of either CD34⁺ or CD34⁻ cell populations, fresh CB MNCs or expanded nucleate cells were stained with CD34-PE (Becton-Dickinson, San Jose, CA, USA), washed twice in binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl₂ at pH 7.4) and incubated for 15 min in Annexin V-Alexa

Fluor 488 and PI (Molecular Probes, Eugene, OR, USA), for 15 min at RT. Apoptotic cells were analysed by flow cytometry. Proportions of Annexin V⁺ cells among total CD34⁺PI⁻ or CD34⁻PI⁻ cells were measured, while membrane integrity was simultaneously assessed by PI exclusion. Apoptotic level of CD34⁺ cells was calculated as percentage of CD34⁺/Annexin V⁺ population in total viable CD34⁺ cells, and apoptotic level of CD34⁻ cells was calculated as percentage of CD34⁻/Annexin V⁺ population in total viable CD34⁻ cells. Data were analysed using FlowJo V.7.6 software (Treestar, Inc, San Carlos, CA, USA).

Quantitative RT-PCR (qRT-PCR)

RNA from freshly isolated and immunomagnetic (MiniMACS; Miltenyi Biotech, Bergisch-Gerbach, Germany) purified expanded CD34⁺ cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Expressions of survivin and hTERT were assessed by quantitative real-time reverse-transcription and polymerase chain reaction (qRT-PCR), using ABI 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All qRT-PCR reactions were performed in duplicate in 25-μL volume. Fold changes relative to target genes normalized to GAPDH and relative expression of fresh controls were calculated for each sample using the 2^{-CT} method, where

$$-C_T = (C_{T,Target} - C_{T,GAPDH})_{Expandedsample} - (C_{T,Target} - C_{T,GAPDH})_{Freshsample}$$

Specific primers for survivin, hTERT and Gapdh were designed as shown in Table 1.

Statistics

Experimental results from different experiments were reported as mean ± standard deviation of the mean (SD). Significance analysis was performed using two-tailed paired Student's *t*-test and *P*-value ≤ 0.05 was considered to be significant; *represents *P*-value ≤ 0.05.

Table 1. Primers used in this study

Gene	Primer sequence (5'-3')	Size(bp)
Survivin	Sense: TCAAGGACCACCGCATCTC	55
	Antisense: GCGCAGCCTCCAAGAA	
hTERT	Sense: CCATCCCCAGGACAGGCTCA	80
	Antisense: GGCATACCGACGCACGCAGT	
Gapdh	Sense: ACCCACTCCTCCACCTTT	93
	Antisense: GTAGCCAAATTCGTGTGCATA	

Results

Ex vivo expansion and growth kinetic analysis of CB CD34⁺ cells

CB CD34⁺ cells were isolated and cultured in SCF+FL+TPO for 28 days; fresh medium with growth factors was replenished every 3 days. Proportions of CD34⁺ cells were assayed by flow cytometry and expanded total nucleate cells at days 4, 7, 14, 21, and 28, and fold expansion of CD34⁺ cells was calculated. As shown in Fig. 1a, continuous increase in total CD34⁺ cells was observed up to 21 days culture with approximately 20-fold maximum expansion. To detect changes in cell growth kinetics, cell-specific growth rate was also calculated. Based on results, despite comparable values between days 4 and 7, specific growth rate declined strikingly after 14 days culture when compared to day 7 (*P* < 0.05); this was followed by continuous reduction thereafter until achieving zero expansion by day 28 (Fig. 1b).

Telomerase activity and hTERT mRNA expression of ex vivo cultured CD34⁺ cells

Considering the important role of telomerase activity in cell proliferation and senescence as reported previously (9), we first investigated dynamic changes in telomerase

Figure 1. *Ex vivo* expansion of CB CD34⁺ cells and analysis of growth kinetics (a). Absolute expansion fold of total CD34⁺ cells at indicated time points during *ex vivo* culture. (b) Specific growth rate of CD34⁺ cells was also calculated at indicated time points. Data are mean ± SD from five independent experiments. *Indicates statistically significant difference between indicated time points (*P* < 0.05).

activity over cells' time in culture. Telomerase activity of freshly isolated and cultured CD34⁺ cells at the different time points were measured by telomeric amplification protocol (TRAP) assay (Fig. 2a). Results indicated that freshly isolated CD34⁺ cells exhibited moderate telomerase activity, $55 \pm 15\%$ of that in the representative telomerase-positive cell line (HEK293). During the subsequent 4 days culture, telomerase activity of CD34⁺ cells was significantly higher than that of fresh CD34⁺ cells ($P < 0.05$); no significant change was observed from day 4 through to day 21. However, the relatively high level of telomerase activity did not persist throughout the whole culture period, its notable reduction was observed at day 28 compared to day 7 ($P < 0.05$) (Fig. 2a). Net absorbance values of all heat-treated negative controls were less than 0.1% of the HEK293 cell line (data not shown). Additionally, real-time qRT-PCR was also performed from cultured CD34⁺ cells at indicated time points (Fig. 2b), and expression of *hTERT* mRNA exhibited a similar change in trend to that of telomerase activity, indicating a direct relationship between telomerase activity and *hTERT* mRNA expression.

Figure 2. Telomerase activity associated with *hTERT* mRNA expression in *ex vivo*-cultured CD34⁺ cells. (a) Telomerase activity of CD34⁺ cells analysed using TRAP assay at indicated time points during culture. (b) Time course of *hTERT* mRNA expression in CD34⁺ cells by qRT-PCR assay. Results represent mean $\pm$ SD from four independent experiments. *Indicates statistically significant differences (Student's *t*-test) between indicated time points ($P < 0.05$).

Apoptotic level of CD34⁺ population associated with *survivin* mRNA expression

To determine apoptotic level of cultured CD34⁺ cells, cultures were monitored over time using Annexin V/CD34/PI staining, as mentioned in the Materials and methods section. As shown in Fig. 3a, the data revealed that $9.4 \pm 2.8\%$ input CD34⁺ cells were apoptotic (Annexin V⁺/PI⁻). After subsequent 4 days culture, addition of growth factors induced significant decline in proportion of CD34⁺ apoptotic population to its lowest level (1.4%); this was followed by gradual increase thereafter, and apoptotic level at day 7 was found to be significantly higher than that on day 4 (3.4% versus 1.4%, $P < 0.05$). After further 7 days culture, when peak apoptotic level was achieved with a proportion of around 4-fold higher than that of day 4 (6.1%), it declined slightly (Fig. 3b).

To determine whether changes in level of apoptosis were associated with inhibitor of apoptosis (IAP)-related molecular regulation, *survivin* mRNA expression of CD34⁺ cells at the indicated time points was examined by qRT-PCR (Fig. 3c). Results showed that significantly upregulated and downregulated *survivin* mRNA expressions at days 4 and 14 were consistent with lowest and highest levels of apoptosis, respectively. Considering potential inhibitory effects of differentiated CD34⁻ cells co-cultured on expansion of CD34⁺ cells, apoptotic levels between CD34⁺ and CD34⁻ populations at indicated time points were compared, and a remarkably higher proportion of apoptotic CD34⁻ population cells was observed compared to the CD34⁺ population between days 7 and 28 (Fig. 3d). This indicated that CD34⁻ cells might serve as an important factor contributing the apoptotic process in CD34⁺ cells.

Discussion

Suppression and activation regulators, or their effects, on expansion capacity of HSCs have drawn much attention since *ex vivo* expansion has proved to be a promising strategy to overcome limits to cell number in a sample. It is well known that telomerase and apoptosis are associated with expansion of CB CD34⁺ cells *in vitro* (9,22), although complex molecular mechanisms underlying regulation of cell proliferation remain to be further elucidated.

To study internal relationships between telomerase activity, apoptotic level and specific population growth rate during CD34⁺ cell proliferation *ex vivo*, we monitored alterations in these parameters over a 28 day expansion period. Consistent with previous reports (11), significant increase in telomerase activity was observed

by TRAP assay after addition of growth factors, sustaining a relatively stable level from days 4 to 21, without significant decline. On the other hand, hTERT expres-

sion was related to cell proliferative status and S phase of the cell cycle (23). It has been demonstrated that hTERT is recruited to telomeres from sub-nuclear loci during S phase, and forms functional telomerase to synthesize telomeres, serving as rate-limiting factor for telomerase activation (24). By qRT-PCR assay, direct correlation between change in hTERT mRNA expression and telomerase activity was revealed, which is in agreement with previous reports (14). Even though theoretically there is close relationship between telomerase activity and cell proliferation, it was found that the remarkable reduction in CD34⁺ cell-specific growth rate at day 14 (compared to day 7) was not consistent with the relatively stable telomerase activity or hTERT expression level at that time.

Complexity of telomerase regulation lead us to investigate the role of apoptosis, which is usually inevitable once the process is initiated with a loss of membrane phospholipid asymmetry (25). The important effect of apoptosis on CD34⁺ cell count control and quality heterogeneity has been reported in fresh and cryopreserved CB (19,20). Our data demonstrated that the remarkable decline in specific cell population growth rate by day 14 was accompanied by the highest level of apoptosis; this suggested that apoptosis also played a critical role in controlling expansion capacity of our *ex vivo* CD34⁺ cells. Moreover, comparison of changes in apoptotic level and telomerase activity showed that reduction in apoptotic level in early stages of culture was concomitant with elevation of telomerase activity, which revealed a contrary relationship between them. However, no association between them was confirmed over following culture periods, as they seemed to vary independently. Survivin, one of the few inhibitors of apoptosis, that blocks apoptosis upstream of caspases -3 and -7 activation (26,27), has also been reported to play an important role in proliferation and survival of normal haematopoietic cells (28–30). Our data showed that significant elevation and decline in survivin mRNA

Figure 3. Apoptosis and survivin mRNA expression of CD34⁺ and CD34⁻ populations during *ex vivo* culture. (a and b) Total nucleate cells from culture were stained with AnnexinV- Alexa Fluor 488, CD34-PE and PI. PI⁻ population was first gated, percentage of annexin V⁺ cells among CD34⁺PI⁻ and CD34⁻PI⁻ populations was calculated respectively, at indicated time points. Data from representative flow cytometry analysis of input cells (a) and time course of apoptotic level of CD34⁺ population during culture (b) are shown. (c) qRT-PCR analysis of survivin mRNA expression at indicated times. (d) Comparison of apoptotic levels of CD34⁺ and CD34⁻ populations. Results represent mean $\pm$ SD of five independent experiments. *Indicates statistically significant differences between indicated time points (a and b) or between CD34⁺ and CD34⁻ populations (c) (**P* < 0.05).

expression of the CD34⁺ cells at days 4 and 14, were concomitant with lowest and highest levels of apoptosis, respectively, indicating the importance of survivin in regulating survival advantage of these cells. However, at other time points, changes in levels of apoptosis seemed not completely consistent with regulation of survivin, suggesting complexity of molecular mechanisms underlying apoptosis regulation *in vitro*. Additionally, it is generally accepted that differentiated mature CD34⁺ cells co-cultured had inhibitory effects on CD34⁺ cell proliferation (8). Our results showed that CD34⁺ populations had exhibited significantly higher apoptotic levels than CD34⁺ populations since day 7, and showed a similarly changed tendency over the culture period, implying the possibility that differentiated CD34⁺ cells, more prone to undergo apoptosis, may potentially play an important role in contributing to apoptosis of CD34⁺ cells *via* intercellular interactions.

It is well agreed that telomerase activity is tightly associated with cell-cycle status, and that the cell cycle and apoptosis are coupled, implying correlation between telomerase activity, apoptotic level and cell proliferation. Although it is difficult to elucidate direct relationships between telomerase activity, apoptotic level and expansion capacity of CB CD34⁺ cells throughout culture periods, due to complexity of *ex vivo* culture conditions, our results revealed the critical role of apoptosis in controlling expansion efficiency of CB CD34⁺ cells *in vitro*, suggesting that cell proliferation may be much more susceptible to suppression of apoptosis rather than to telomerase activation. These findings should be helpful in understanding behaviour regulation of *ex vivo*-cultured CB CD34⁺ cell population expansion.

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