FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 43, No. 4, 2005
pp. 197-202

Proliferative senescence in hematopoietic stem cells during *ex-vivo* expansion

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Abstract: The good outcome of hematopoietic stem cell (HSC) transplantation is hampered by low doses of $CD34^+$ cell infusion. Transplanted HSCs undergo a replicative stress that causes accelerated senescence due to rapid telomere shortening. The expansion of human cord blood HSCs is instrumental in obtaining a large number of "good quality" cells, in terms of telomere length and telomerase activity compared to adult HSCs.

Key words: Hematopoietic stem cells - Cell senescence - Telomere shortening - *Ex vivo* expansion - NOD/SCID mice - Transplantation

Introduction

Tissue-specific stem cells, like most somatic stem cells, possess the unique ability of self-renewal and multilineage differentiation. These combined properties are reflected in the ability of hematopoietic stem cells (HSCs) to completely and durably reconstitute hematopoiesis of a myeloablated recipient and maintain it throughout the entire life span [13, 19]. HSC self-renewal is not a perfect process and daughter cells have progressively reduced proliferative capacity, due in part to progressive telomere erosion at each cell division. This, in turn, leads to proliferative senescence that can be observed both *in vivo* and *in vitro*. Telomeres are structures at the end of eukaryotic chromosomes that protect chromosomes from degradation, fusion, and recombination. In mammalian cells, they consist of hexanucleotide (TTAGGG) repeats and several associated protein components. In the absence of compensatory mechanisms, dividing cells undergo gradual telomere erosion. When telomeres reach a critical degree of shortening, cells recognize this as DNA damage and initiate pro-apoptotic programs or enter senescence [10].

The best-characterized compensatory mechanism for maintenance of telomere length is mediated by telomerase, an enzyme that synthesizes terminal telomere repeats [31]. In contrast to normal somatic cells, which are telomerase-negative, hematopoietic stem cells have low levels of telomerase, which can be transiently up-regulated upon cytokine stimulation [30]. $CD34^+$ populations exhibit low telomerase activity, but activity increases significantly in both HSC and progenitor populations as cells progress from G_0 into S phase [6, 7]. In early studies using cytokine combinations that were effective in activating stem cells into the cell cycle, but were less effective in maintaining long-term HSC proliferation, telomerase was up-regulated at early stages of culture but declined rapidly after 3 to 4 weeks [6, 7]. In these studies, 1 to 1.5 kb telomeric DNA was lost over 3 to 4 weeks of culture of $CD34^+$ cells from cord blood (CB), mobilized peripheral blood (MPB), or bone marrow (BM). Thus, telomerase activity in HSCs did not prevent telomere shortening although it may have limited the extent of telomere degradation.

After allogenic and autologous transplants, it has been observed a telomere loss of 1-2 kb (corresponding to 15-40 years of premature aging) due to the extensive cell regeneration after infusion [20, 29]. The kinetics of hemopoietic engraftment impose replicative stress on the HSC, resulting in a pronounced aging effect, which

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Lecture presented at the Third Annual Meeting of the European Stem Cell Therapeutics Excellence Centre, October 6-9, 2005, Cracow, Poland

may be sufficient to accelerate the onset of clonal hematopoietic disorders usually associated with later life [26, 27]. Moreover, short telomeres may limit the remaining replicative capacity of cells, measured in term of colony forming unit (CFU) and long-term culture initiating cells (LTC-IC) efficiency, and may be responsible for subsequent changes and potential deteriorating effects (such as acquisition of secondary myelodysplastic syndromes or acute myeloid leukemia) [26, 27]. Average telomere lengths were 10.4 kbp, 7.4 kbp, and 7.6 kbp in CB, PB, and BM $CD34^+$ cells, respectively [6]. Thus, CB HSCs posses a proliferative advantage of about 3 kbp respect BM and MPB. If the decrease in telomere length results purely from increased replicative demand on engrafted HSC, use of a larger number of repopulating cells with longer telomeres should be beneficial. The use of CB as a source of HSCs for allogeneic transplantation has been limited by the HSC number present in a single harvest, which limits the utility of this source to pediatric use or to adults of low body weight [13, 16]. *Ex vivo* expansion of neonatal HSCs would be of considerable clinical utility and preliminary phase 1 results have been reported [12].

These findings raise the question if the *ex vivo* expansion could be able: (1) to maintain reproductive integrity of the repopulating cells, (2) to induce a not dramatic shortening of telomeres and (3) to produce a larger number of primitive precursors and committed progenitors in order to limit the number of HSC divisions after transplantation.

Ex vivo expansion of CB $CD34^+$ cells

The transplantation assay available in sublethally irradiated non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice has been instrumental in defining and characterizing the most primitive cells of the hemopoietic system [14, 23]. The NOD/SCID model system has been shown to support the engraftment and retention of primitive human HSCs with the potential for extensive proliferation and multilineage differentiation [1, 2, 4, 23, 28]. Unlike the majority of LTC-ICs is incapable of repopulation, human long-term NOD/SCID repopulating cells (SRCs) are found exclusively in the $CD34^+CD38^-$ cell fraction [1, 28]. Furthermore, kinetic experiments indicate that engraftment of SRCs is followed by a large expansion of LTC-ICs *in vivo*, suggesting that these are derived from a more primitive cell [2].

Our recent studies [9, 21, 23] have demonstrated the propagation of CB $CD34^+$ cells for months in different culture systems, with extensive expansion of stem cells as measured by Cobbelstone Area-Forming Cell (CAFC) and LTC-IC assay or NOD/SCID engraftment. SRCs in CB can be expanded many-fold for up to 10 weeks of culture in the presence of stem cell factor (SCF), the ligand for c-kit; flt3/flk2 ligand (FL), c-mpl ligand thrombopoietin (TPO), and interleukin 6 (IL-6) and sustain the hematopoietic reconstitution over 3 serial passages in NOD/SCID mice. These systems are critically dependent on provision of TPO, as a recombinant factor added twice a week. The stroma-independent LTC was performed in a continuous manner for 20 weeks or in a fractionated manner stopping it every 4 weeks for the paramagnetic immuno-isolation of $CD34^+$ cells (Fig. 1). The data generated in the stroma-independent expansion-isolation procedure (fractionated cultures) allowed direct evaluation of purified $CD34^+$ cells at monthly intervals. Progenitors (CFCs and granulo-erythro-monocyte-macrophage colony forming units) continued to expand throughout the 20 weeks of study, whereas stem cells expanded through week 16 but then declined sharply by week 20 as measured by *in vitro* LTC-IC and *in vivo* SRC assay. By transplanting limiting dose of $CD34^+$ in mice at different time points we have demonstrated the expansion of stem cell compartment over 16 weeks of culture measured as large increase in SRCs. In contrast to the CB cultures, cytokine-stimulated cultures of $CD34^+$ cells from adult BM or G-CSF MPB did not sustain significant levels of $CD34^+$ production beyond 4 weeks.

This result points to the reduced proliferative potential of adult stem cells versus neonatal stem cells that may be due to a higher probability of differentiation than of self-renewal or to a higher frequency of stem cell apoptosis [9].

Ex vivo CB $CD34^+$ cell telomere shortening and telomerase activity

The ability to maintain HSCs *in vitro* for prolonged periods provides a valuable system for evaluating the role of telomerase in telomere length maintenance and cell expansion. Because previous studies had indicated that progressive telomere shortening would be anticipated with *in vitro* expansion [7, 27] comparable to up to 20 years of normal age-associated telomere loss, we chose to closely monitor telomerase activity and telomere length in the improved long-term CB cultures using the cytokine combinations optimal for SRC expansion. We show that under both these culture conditions, CB $CD34^+$ cells undergo extensive proliferation and self-renewal for 4 to 5 months with sustained elevation of telomerase activity and without concomitant significant telomere shortening. Primitive hematopoietic precursors as detected by CAFC and by LTC-IC assays are maintained for up to 16 to 18 weeks and greatly expand over this time. During this period, repopulating ability is maintained and the SRC number is greatly increased. After this period of telomere stabilization (0-16 weeks), telomeric DNA begins to be lost, coinciding in cytokine-stimulated cultures with a steep decline in LTC-ICs and SRCs by week 20. Most import-

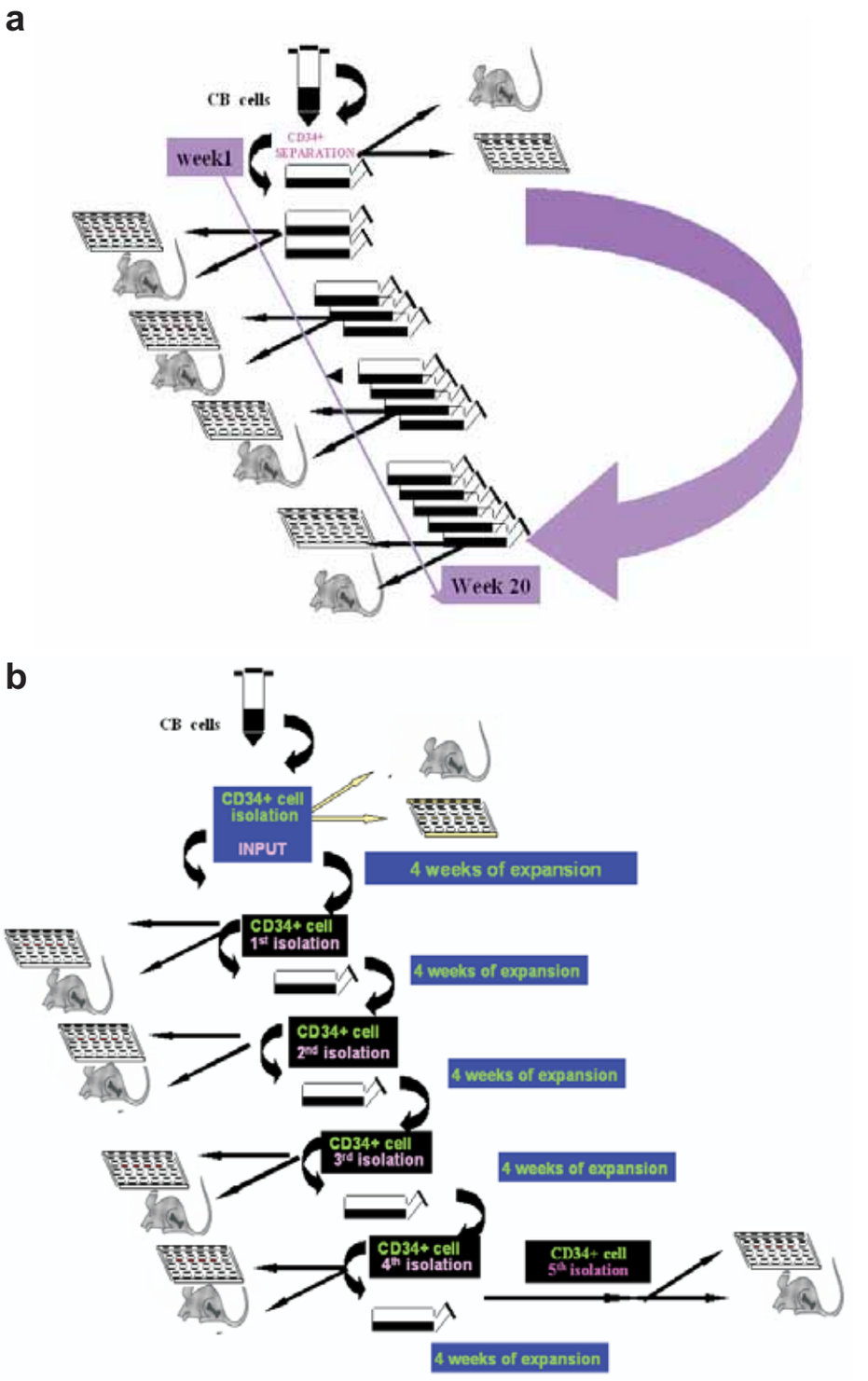

Fig. 1. a. Continuous expansion. Schematic representation of continuous stroma-free LTCs with cytokines. In continuous culture, CB $CD34^+$ cells were plated at 5×10^4/mL in triplicate or quadruplicate tissue culture flasks and biweekly supplied with cytokines until week 20. Every 4 weeks expanded cells was analyzed for LTC-IC and SRC output. **b.** Fractionated expansion. Schematic representation of fractionated stroma-free LTCs with cytokines. In fractionated cultures, CB $CD34^+$ cells were plated at 5×10^4/mL in triplicate or quadruplicate tissue culture flasks. Every 4 weeks all cells were harvested and $CD34^+$ cells were isolated with miniMACS devices, analyzed with LTC-IC and SRC assays, and then recultured.

antly, in these systems this proliferation occurred without significant telomere loss until late stages of culture and correlated with sustained up-regulation of telomerase in the stem/progenitor compartment of $CD34^+$ cells. Because telomerase activity is restricted to the $CD34^+$ cells, the dilution by differentiating cells in continuous cultures underestimated telomerase activity in the stem/progenitor population by a factor of 5- to 10-fold. Adjusting for this dilution by factoring in the CFC content as a measure of these early cells, the stem/progenitor population maintained telomerase activity comparable to levels found in immortal tumor cell lines throughout the culture [7]. Levels were also comparable to those of hTERT-transduced human fibroblast clones that showed stable or elongated telomeres despite extensive proliferation [8, 17].

In stroma-free cultures of CB $CD34^+$ cells, telomerase levels remained quite high in the first 8 weeks, but then fell to 25% of input levels between 8 and 16 weeks, and to 12% by week 20. In adult $CD34^+$ cell cultures, the telomerase levels of the input cells and of the recovered $CD34^+$ cells at 1 and 3 weeks were comparable but significantly lower than in CB cultures over the first 2 months. In the majority of CB cultures, telomeres remained stable for over 3 to 4 months and only at late stages telomeres begin to shorten. Indeed, in some cultures telomere elongation was noted in the first month. At week 3, in cytokine-stimulated cultures of adult $CD34^+$ cells, telomeres of the $CD34^+$ subset were slightly shorter (-0.1 to -0.3 kb) than input telomere length. The great reduction in $CD34^+$ cell recovery by week 4 precluded telomere measurement of this subset; however, telomere measurement of unseparated culture cells at this time showed a loss of 0.7 kb. This contrasted with the lengthening (+0.9-1.0 kb) seen in CB cultures over the same time period. The telomerase activity level in extracted cell protein does not take into account the role of nuclear localization of the enzyme in determining biologic activity [16].

Nevertheless, stable enzyme up-regulation is the most plausible explanation for telomere stabilization. Given a doubling time of 24 hours, then over 20 weeks the stem cells would have undergone 140 population doublings (PDs). With the average loss of telomeric DNA of 50 to 100 bp/PD seen in telomerase-negative cells, 7 to 17 kb of telomere would have been lost in the absence of telomerase up-regulation, and cultures would have terminated because of proliferative senescence. Even with a more conservative assumption of doubling every 48 hours, the stem cells would have lost telomere DNA equivalent to a lifetime of normal telomere erosion. In this regard, the HSCs in late passage CB cultures would more closely correspond to adult HSCs, with much reduced expansion potential *in vitro*, reduced telomerase levels, and loss of capacity to stabilize telomeres [6, 27]. Thus the 20-week culture may span a developmental stage of transition from fetal/neonatal to adult stem cell.

Does the *in vitro* model predict for the *in vivo* situation?

Analysis of age-related changes in granulocyte telomere length has revealed an average loss of 39 bp/year, but in the first 6 months of life telomeres shorten at a much more rapid rate corresponding to 3 kb/year [20]. This would correspond to 15 to 30 stem cell divisions in the first 6 months of life, followed by less than one stem cell division per year [27]. This would not have been predicted from the *in vitro* data unless the extent of stem cell proliferation in the first 6 months was itself biphasic, with stable telomeres only for the first 3 months followed by rapid loss as HSCs transit to adult type while still actively proliferating. Numerous studies have investigated telomere shortening, particularly following autologous or allogeneic stem cell transplantation [13, 20, 25, 26, 29]. Although significant telomere shortening is reported to occur within the first year after transplantation [25] as yet there is no evidence that this shortening leads to impaired hematopoiesis in the majority of cases [5], but there are reports where late graft failure was associated with significant telomere shortening [26]. Perhaps the most compelling evidence for telomere length relevance in hematopoietic cells is the recent demonstration of a highly significant association between blood leukocyte telomere length and mortality in a group of healthy elderly individuals [3]. Those with the shortest telomeres had poorer survival, attributable in part to higher mortality from heart disease and infectious diseases. The loss in median survival associated with possession of shorter telomeres in any of the age cohorts studied was 4.8 years for women and 4.0 years for men.

The development of an improved *in vitro* CB $CD34^+$ culture system that supports a many thousand-fold amplification of HSCs has provided insight into the role of telomerase up-regulation in maintaining telomere stability in neonatal HSCs. It has also revealed an important distinction between adult and neonatal HSCs with respect to onset of proliferative senescence. The clinical potential of our observations are evident because very extensive expansion of CB HSCs can be obtained over many months without deterioration of HSC quality as measured by NOD/SCID engraftment and stable telomeres. *Ex vivo* expansion can extend the use of CB, not only to all adult recipients, but potentially to multiple recipients, without concern that extensive telomere shortening would compromise the long-term survival of the recipients. Particularly poor results are seen after an unrelated CB transplant when the nucleated cell dose infused is less than 1.5×10^7/kg, or 1.7×10^5 $CD34^+$ cells/kg [11]. CB harvests are generally sufficient for

pediatric engraftment (median age 7 years, 25 kg body weight) and would be approaching the lower limits for optimal engraftment in adults up to 60 kg with engraftment problems likely in larger individuals. When patients receive lower cell doses, risk of graft failure is exceedingly high and time to neutrophil recovery is prolonged. Furthermore, survival in recipients of one or 2 HLA-mismatched CB transplants is improved with higher cell doses [11, 24]. A recent study concluded that raising the nucleated cell dose to approximately 3×10^7/kg may offset the negative effect of one HLA-mismatch [11, 24]. CB *ex vivo* expansion that does not cause proliferative senescence is an attractive option for adult unrelated transplantation, also in the cases with one or two HLA mismatches.

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Received: June 1, 2005
Accepted: June 6, 2005