Quantitative Analysis Reveals Expansion of Human Hematopoietic Repopulating Cells After Short-term Ex Vivo Culture

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Summary

Ex vivo culture of human hematopoietic cells is a crucial component of many therapeutic applications. Although current culture conditions have been optimized using quantitative in vitro progenitor assays, knowledge of the conditions that permit maintenance of primitive human repopulating cells is lacking. We report that primitive human cells capable of repopulating nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) mice (SRC) can be maintained and/or modestly increased after culture of CD34$^+$CD38$^-$ cord blood cells in serum-free conditions. Quantitative analysis demonstrated a 4- and 10-fold increase in the number of CD34$^+$CD38$^-$ cells and colony-forming cells, respectively, as well as a 2- to 4-fold increase in SRC after 4 d of culture. However, after 9 d of culture, all SRC were lost, despite further increases in total cells, CFC content, and CD34$^+$ cells. These studies indicate that caution must be exercised in extending the duration of ex vivo cultures used for transplantation, and demonstrate the importance of the SRC assay in the development of culture conditions that support primitive cells.

Ex vivo culture is a crucial component of several clinical applications currently in development including gene therapy, tumor cell purging, and stem/progenitor cell expansion (1, 2). Ideally, each of these therapies requires maintenance or expansion of repopulating cells without induction of differentiation. Clinical transplantation studies have demonstrated that human hematopoietic cells can be cultured ex vivo with or without stroma and still retain the capacity to engraft human recipients, although it is not known if their number is reduced (3–5). Moreover, all of these studies involve autologous transplanting, making it difficult to determine whether long-term repopulation was derived from cultured cells or from surviving endogenous stem cells. Although clinical gene marking studies could address the question of stem cell maintenance in an autologous setting, conclusive evidence that engraftment was derived from a pluripotent stem cell is still lacking (6, 7). It appears that the ex vivo culture conditions for gene transfer may not induce the cycling of repopulating cells (as required for retrovirus-mediated gene transfer; reference 8), or they may induce stem cells to differentiate resulting in the loss of their repopulating capacity (2).

Typically, ex vivo cultures are initiated from mononuclear cells or purified CD34$^+$ cells (1), although in some cases, highly purified CD34$^+$CD38$^-$ and CD34$^+$Thy-1$^+$ cells have been used (9–11). Whether primitive cells are actually expanded during ex vivo culture is controversial because different assays and culture conditions have been used in different studies (10, 12–14). It has recently been demonstrated that highly purified subfractions of CD34$^+$ cells possess the greatest proliferative potential, resulting in a large expansion of colony-forming cells (CFC), while long-term culture initiating cells (LTC-IC) show either a slight reduction (15) or moderate increase (13). Although these in vitro assays provide an essential quantitative assessment of the functional properties of the expanded cells, they do not evaluate repopulation capacity.

Recently, we have identified a novel human hematopoietic cell, termed the SCID repopulating cell (SRC), that is capable of extensive proliferation and multilineage repopulation of the bone marrow of nonobese diabetic (NOD)/SCID mice. Using cell purification and retroviral gene marking, the SRC were found to be biologically distinct from CFC and most LTC-IC (16, 17). The SRC were found exclusively in the CD34$^+$CD38$^-$ cell fraction and, under our conditions, were poorly transduced with retroviral vectors relative to CFC and LTC-IC. A quantitative assay for...
SRC was developed using limiting dilution analysis (18), enabling us to determine that there is 1 SRC in 617 CD34+CD38- cells (17). Using this quantitative assay, we have compared the effect of ex vivo culture on SRC, CFC, mononuclear cells, as well as CD34+CD38- and CD34+CD38+ cell content.

**Materials and Methods**

**Cell Purification.** Samples of cord blood (CB) were obtained from placental and umbilical tissues and the mononuclear cells were collected on Ficoll (Pharmacia, Upsala, Sweden). CD34+CD38- and CD34+CD38+ cells were collected using our standard protocol (17). In brief, CB cells were first enriched for CD34+ cells by negative selection using the StemSep (Stem Cell Technologies Inc., Vancouver, Canada). These cell fractions were further purified on a FACStar Plus each well every other day.

**Knee injections of human cytokines (human stem cell factor, 10 ng/ml of IL-3 (Amgen Biologicals) and IL-6 (Amgen Biologicals), 50 ng/ml of granulocytic CSF (Amgen Biologicals), and 10 ng/ml of IL-3 (Amgen Biologicals) and IL-6 (Amgen Biologicals). Cells were cultured in flat-bottomed suspension wells of 96-well plates (Nunc, Burlington, Canada), incubated for 4 and 9 d at 37°C and 5% CO2, and 50 µl of fresh GF cocktail was added to each well every other day.

**Transplantation of Purified Cells into NOD/SCID Mice.** Cells were transplanted into NOD/LtSz-scid/scid (NOD/SCID) mice according to our standard protocol (16). In all cases, cells were cotransplanted with nonrepopulating CD34-Lin+ cells as accessory cells. The transplanted mice received alternate day intraperitoneal injections of human cytokines (human stem cell factor, 10 µg/ml of human IL-3 and human GM-CSF, 6 µg/ml of human IL-3 and human GM-CSF, provided by Becton Dickinson, San Jose, CA), based on CD34 and CD38 expression using similar sorting gates shown previously (17).

**Clonogenic Progenitor Assays.** Human clonogenic progenitors were assayed under standard conditions (16), except that 10% 5637-conditioned medium was included.

**Liquid Suspension Cultures.** CD34+CD38- and CD34+CD38+ cells were incubated in 50 µl of IMDM supplemented with 1% BSA (Stem Cell Technologies Inc.), 5 µg/ml of human insulin (Humulin R, Lilly, Canada), 100 µg/ml of human transferrin (GIBCO BRL, Burlington, Canada), 10 µg/ml of low density lipoproteins (Sigma Co., St. Louis), 10^-7 M b-mercaptoethanol and growth factors (GF). The GF cocktail was used at final concentrations of 300 ng/ml of stem cell factor (Amgen Biologicals, Thousand Oaks, CA) and Flt-3 (Immunex, Seattle, WA), 50 ng/ml of granulocytic CSF (Amgen Biologicals), and 10 ng/ml of IL-3 (Amgen Biologicals) and IL-6 (Amgen Biologicals). Cells were cultured in flat-bottomed suspension wells of 96-well plates (Nunc, Burlington, Canada), incubated for 4 and 9 d at 37°C and 5% CO2, and 50 µl of fresh GF cocktail was added to each well every other day.

**Transplantation of Purified Cells into NOD/SCID Mice.** Cells were transplanted into NOD/LtSz-scid/scid (NOD/SCID) mice according to our standard protocol (16). In brief, CB cells were first enriched for CD34+ cells by negative selection using the StemSep (Stem Cell Technologies Inc., Vancouver, Canada). These cell fractions were further purified on a FACStar Plus each well every other day.

**Results**

Measurement of the Proliferation and Differentiation of Ex Vivo Cultures Initiated with Human CD34+CD38- and CD34+CD38+ Cells. The proliferative capacity of CD34+CD38- and CD34+CD38+ cells were determined by comparing the number of cells obtained after 4 and 9 d of culture to those originally seeded (Fig. 1). The purified cells were incubated in serum-free media with the addition of growth factors based on a cocktail previously shown to expand LTC-IC 30-50-fold in 10-21 d from an initial inoculum of CD34+CD38- cells (13). The number of cells in the CD34+CD38- wells increased by an average of 4-fold, whereas the CD34+CD38+ wells increased by more than 16-fold by day 4 (Fig. 1). By day 9, the CD34+CD38- wells increased by 8-fold, whereas the CD34+CD38+ wells had expanded by 22-fold compared to day 0.

To determine the maintenance of the CD34+CD38- and CD34+CD38+ phenotypes after 4 and 9 d of culture, individual wells were analyzed by flow cytometry (Fig. 2). The vast majority of cells obtained after 4 d of culture in the CD34+CD38- wells maintained the same phenotype. However, by day 9, no CD34+CD38- cells remained and all of the cells had begun to differentiate, as evidenced by the large number of CD34+CD38+ cells. A similar pattern was seen in CD34+CD38+ wells at day 4 most of the cells retained the same phenotype, whereas at day 9 extensive differentiation had occurred with the majority of cells losing both CD34 and CD38 expression.

To determine the effect of in vitro culture on the clonogenic progenitors present in the CD34+CD38- and CD34+CD38+ and CD19, conjugated to PE, and CD45 was conjugated to peridinin chlorophyll protein. For each mouse analyzed, an aliquot of cells was also stained with mouse IgG conjugated to FITC, PE, and peridinin chlorophyll as isotype controls.

**Figure 1.** Increase in cell number after in vitro culture of CD34+CD38- and CD34+CD38+ cells. Purified cells were counted and seeded (25–2,000) in wells containing serum free media (day 0). Cells were harvested from individual wells after 4 and 9 d, counted, and the mean fold increase in absolute cell number of CD34+CD38- (solid bar) and CD34+CD38+ (shaded bar) was calculated.
CD38\(^{+}\) cell fractions, plating assays were performed at day 0, 4, and 9 (Table 1). Initially, both the CD34\(^{+}\)CD38\(^{-}\) and CD34\(^{+}\)CD38\(^{+}\) cells had a high plating efficiency (PE): 1 in 3.3 and 1 in 3.4 cells gave rise to CFC, respectively. After 4 d of culture, the PE of the CD34\(^{+}\)CD38\(^{-}\) wells increased to 1 in 1.1, whereas the CD34\(^{+}\)CD38\(^{+}\) had decreased slightly to 1 in 4.6. After 9 d, the PE of the CD34\(^{+}\)CD38\(^{-}\) wells had decreased to 1 in 4.5 cells, whereas the CD34\(^{+}\)CD38\(^{+}\) wells had decreased even further to 1 in 12.2. Overall, much higher numbers of progenitors were recovered from CD34\(^{+}\)CD38\(^{-}\) wells than the CD34\(^{+}\)CD38\(^{+}\) wells. Moreover, the total number of clonogenic progenitors continued to increase between 4 and 9 d CD34\(^{+}\)CD38\(^{-}\) wells, whereas the fold increase was less in the CD34\(^{+}\)CD38\(^{+}\) wells by day 9.

Quantitative Analysis of SRC After Ex Vivo Culture. We have demonstrated through limiting dilution analysis that SRC are found exclusively in the CD34\(^{+}\)CD38\(^{-}\) fraction at a frequency of 1 in 617 cells (17). In the present study, we used the quantitative SRC assay to measure the number of SRC present in the wells at days 0, 4, and 9 of ex vivo culture. At day 0, wells were seeded with 500–1,000 CD34\(^{+}\)CD38\(^{-}\) or CD34\(^{+}\)CD38\(^{+}\) cells. The contents of each well at day 4 and 9 were either transplanted into one NOD/SCID mouse or divided into three or eight equally sized aliquots before injection into NOD/SCID mice. Human cell engraftment was determined 6–8 wk later to determine whether an SRC had been present in the well. The results of one representative experiment are shown in Fig. 3A. In this experiment, five wells were initially seeded with 500 CD34\(^{+}\)CD38\(^{-}\) cells. The total cell number increased fourfold to \(~2,200\) cells/well after 4 d in culture. The mouse transplanted with the entire contents of one well was engrafted with human cells. Surprisingly, two out of three mice transplanted with...
730 expanded cells and two out of eight mice transplanted with only 275 expanded cells were also engrafted (Fig. 3A). None of the mice transplanted with cells that had been expanded for 9 d were engrafted, despite the injection of 16-fold more cells compared to the day-4 expanded cultures (4,500 versus 275, respectively). Fig. 3B summarizes the level of engraftment in 81 NOD/SCID mice transplanted with expanded CD34⁺CD38⁻ cells, at different doses, from 10 CB samples cultured for 4 d. The frequency of SRC in the ex vivo–cultured cell populations was calculated from this data to be 1 SRC in 668 expanded cells (range 1 in 427 to 1 in 1,043). This frequency is similar to that in CD34⁺CD38⁻ cells before expansion (1 in 617; reference 17). Since the absolute number of cells increased fourfold after 4-d, and there was no significant change in the frequency of SRC, these results indicate a fourfold increase in the absolute number of repopulating cells.

Expanded SRC Give Rise to Multilineage Differentiation. To determine whether expanded SRC possessed the same in vivo proliferative and differentiative capacity as uncultured SRC, BM samples of engrafted mice were analyzed by multiparameter flow cytometry. A representative analysis of an NOD/SCID mouse transplanted with 1,200 expanded CD34⁺CD38⁻ CB cells after 4 d of ex vivo culture is shown (Fig. 4).

Figure 3. Quantitative analysis of SRC after ex vivo culture. (A) Representative Southern blot analysis of individual NOD/SCID mice transplanted with expanded cells from replicate wells at days 4 and 9. Lane 1 represents one mouse transplanted with the contents of one well containing 2,200 expanded cells, lanes 2–4 represent three individual mice transplanted with 730 expanded cells recovered from one well, lanes 5–12 represent eight mice transplanted with 275 expanded cells recovered from one well, all at day 4. At day 9, two mice were transplanted with 4,500 and 1,250 expanded cells (lanes 13–14 and 15–16, respectively). DNA was extracted from the murine BM 8 wk after transplant and hybridized with a human chromosome 17–specific α-satellite probe. (B) Summary of the level of human cell engraftment in the BM of 81 mice transplanted with expanded CD34⁺CD38⁻ cells at 4 d from 10 CB samples.
in the numbers of CFC and CD34 ture concomitant with increases in the total cellularity, and that the number of SRC increased fourfold after 4 d in cul-
verted during ex vivo serum-free culture using a man repopulating cells can be maintained and even mod-
ifying factors that govern their developmental program (21). This identify and characterize human repopulating cells and the
Discussion
The recently developed SRC assay provides a method to identify and characterize human repopulating cells and the factors that govern their developmental program (21). This report provides the first demonstration that primitive human repopulating cells can be maintained and even modestly expanded during ex vivo serum-free culture using a cocktail of cytokines. By quantitative analysis, we found that the number of SRC increased fourfold after 4 d in culture concomitant with increases in the total cellularity, and in the numbers of CFC and CD34\(^+\)CD38\(^-\) cells. Despite even greater increases in the total number of cells, CD34\(^+\) cells, and CFC, no SRC were detected when cells were cultured for 9 d. Others have shown continued increases in the number of LTC-IC for up to 21 d of culture (13). This dissociation between SRC and in vitro progenitors is consistent with our earlier results from gene marking and cell purification experiments indicating that SRC are distinct from and more primitive than CFC and most LTC-IC assayed in 5-wk stromal cultures (16, 17). Thus, the SRC assay will play an important role in identifying factors that cause SRC to proliferate without inducing differentiation and loss of repopulating activity and in the design of methods to transduce primitive human cells for stem cell gene therapy.

All prior studies aimed at developing clinical applications have used surrogate assays to optimize the conditions for ex vivo cultures including quantification of the number of CD34\(^+\) cells, CFC, or LTC-IC (12, 13, 22). Most conditions result in marked expansion of CFC and CD34\(^+\) cells (1, 12, 23), with smaller increases in the number of more primitive cells such as LTC-IC and extended LTC-IC (13, 24). Some studies have shown a decline in LTC-IC during 10 d of culture, probably because assay methods or culture conditions differ (15). Our results indicate that total cellularity, CFC content, and the number of CD34\(^+\) cells do not correlate with the repopulating potential of cultured cells. Moreover, the more mature CD34\(^+\)CD38\(^-\) population, which has been previously shown to be devoid of repopulating cells (17), had a higher proliferative capacity with respect to both total cellularity and CFC number compared to cultures initiated with CD34\(^+\)CD38\(^-\) cells after 4 and 9 d of in vitro culture. Although we have shown that only a fraction of CD34\(^+\)CD38\(^-\) cells have SRC activity, this combination of cell-surface markers seemed to provide good correlation with repopulating potential. The number of CD34\(^+\)CD38\(^-\) cells increased by four- to fivefold during the first 4 d of culture, whereas all the cells had differentiated by 9 d, consistent with the loss of SRC. We conclude that the SRC assay will play an important role in the development of clinical methods for ex vivo expansion.

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