Brief report

Ex vivo treatment of proliferating human cord blood stem cells with stroma-derived factor–1 enhances their ability to engraft NOD/SCID mice

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Ex vivo proliferation of hematopoietic stem cells (HSCs) is important for cellular and gene therapy but is limited by the observation that HSCs do not engraft as they transit S/G_2/M. Recently identified candidate inhibitors of human HSC cycling are transforming growth factor-β_1 (TGF-β_1) and stroma-derived factor–1 (SDF-1). To determine the ability of these factors to alter the transplantability of human HSCs proliferating in vitro, lin$^-$ cord blood cells were first cultured for 96 hours in serum-free medium containing Flt3 ligand, Steel factor, interleukin-3, interleukin-6, and granulocyte colony-stimulating factor. These cells were then transferred to medium containing Steel factor and thrombopoietin with or without SDF-1 and/or TGF-β_1 for 48 hours. Exposure to SDF-1 but not TGF-β_1 significantly increased (> 2-fold) the recovery of HSCs able to repopulate nonobese diabetic/severe combined immunodeficiency mice. These results suggest new strategies for improving the engraftment activity of HSCs stimulated to proliferate ex vivo. (Blood. 2002;99:3454-3457)

Introduction

Much interest is currently focused on improving in vitro procedures for expanding hematopoietic stem cells (HSCs) that do not compromise either their original developmental or engrafting potential. Growth factor (GF) combinations have now been identified that promote self-renewal divisions in vitro of transplantable human HSCs identified as competitive repopulating units (CRUs) based on their ability to repopulate the marrow of nonobese diabetic-*scid/scid* (NOD/SCID) mice with lymphoid and myeloid progeny.[1-7] We have exploited the precision and selectivity afforded by this assay to show that cord blood (CB) CRUs proliferating in vitro lose their ability to engraft as they progress through the S/G_2/M phases of the cycle,[8] as has also been shown for adult human[9] and murine HSCs[10] detected by similar transplantation assays. Previous approaches to increase the transplantability of proliferating murine and human HSCs have exposed the cells to transforming growth factor-β_1 (TGF-β_1) for 24 hours.[8,11] Although this arrested many of the cells in G_0/G_1, no increases in transplantable HSC activity were seen. We subsequently found that a more prolonged exposure to either TGF-β_1 or the chemokine stroma-derived factor–1 (SDF-1) can block the cycling of long-term culture-initiating cells (LTC-ICs) as well as primitive human colony-forming cell (CFC) progenitors both in vitro and in vivo.[12] We therefore reasoned that such treatments might similarly affect proliferating HSCs and thereby increase their ability to be detected as CRUs. The experiments described here were designed to test this hypothesis. Accordingly, CD34$^+$ cell-enriched suspensions of CB were first cultured in the presence of a GF combination that we have previously shown stimulates all of the CRU to proliferate within 4 days.[13] The cells were then switched to a different GF cocktail to test the effect of added TGF-β_1 and/or SDF-1 on HSC detection. The GF cocktail selected for use in these secondary cultures consisted of Steel factor (SF) plus thrombopoietin (TPO) based on previous studies indicating that these GFs are less mitogenic but still able to maintain the viability and stem cell properties of HSCs.[14-17]

Study design

Short-term expansion cultures

CB was obtained with informed consent, lineage marker–positive (lin$^+$) cells removed using StemSep columns (StemCell Technologies, Vancouver, BC) from previously cryopreserved low-density (< 1.077g/mL) cells pooled from at least 5 donors, and the lin$^-$ cells then cultured at a density of 1×10^5/mL to 2×10^5/mL for 4 days in serum-free medium (SFM) containing a serum substitute (BIT9500, StemCell), 10^{-4} M 2-mercaptoethanol (Sigma Chemicals, St Louis, MO), 40 μg/mL low-density lipoproteins (Sigma), and the following 5 recombinant human GFs: 20 ng/mL interleukin-3 (Novartis, Basel, Switzerland), 20 ng/mL interleukin-6 (Cangene, Mississauga, ON), 20 ng/mL granulocyte colony-stimulating factor (StemCell), 100 ng/mL SF (expressed and purified in the Terry Fox Laboratory), and 100 ng/mL Flt3 ligand (Immunex, Seattle, WA) as previously described.[13] The cells were then harvested, washed twice in SFM, aliquots removed for phenotype and functional measurements (see below), and the remainder resuspended in new SFM and cultured further as indicated (either with the same 5 GFs or with 50 ng/mL human SF and 50 ng/mL human TPO (Genentech, Palo Alto, CA) with or without either 5 ng/mL TGF-β_1 (R&D

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Systems, Minneapolis, MN) or 100 ng/mL SDF-1 (synthesized and purified as previously reported[18]) or both.

Flow cytometry

Expression of CD34 was analyzed after staining aliquots of cells with fluorescein isothiocyanate–conjugated 8G12 (anti-CD34) antibody (kindly provided by P. M. Lansdorp, Terry Fox Laboratory) and of CXCR4 using a phycoerythrin-conjugated anti-CXCR4 antibody (Pharmingen, Baie d'Urfe, Quebec). The cell cycle distribution of $CD34^+$ cells was determined after propidium iodide (PI) (Sigma) staining as previously described.[8]

In vitro progenitor assays

CFC and LTC-IC assays were performed as previously described.[19]

CRU assays

CRU frequencies were determined as described in detail previously.[2] Briefly, 8- to 12-week-old NOD/SCID mice were given a sublethal dose of 350 cGy of total body ^{137}Cs γ-irradiation and were then injected intravenously with test cells plus 10^6 irradiated (15 Gy) normal human bone marrow cells as carriers. After 6 to 8 weeks, the presence of viable (PI^-) human B-lineage ($CD34^-CD19/20^+$) and human myeloid ($CD45/71^+CD15/66b^+$) cells in the marrow of the mice was determined by fluorescence-activated cell sorter (FACS) analysis. A detection limit of 5 human lymphoid ($CD34^-CD19/20^+$) cells and/or 5 human myeloid ($CD45/71^+CD15/66b^+$) cells per 2×10^4 PI^- cells analyzed was used to identify positively engrafted mice. Gates were set to exclude more than 99.99% of nonspecifically stained PI^- cells incubated with irrelevant isotype-matched control antibodies labeled with the corresponding fluorochromes. CRU frequencies (Table 1) were calculated from the data pooled from all experiments using Poisson statistics and the method of maximum likelihood (with L-calc software, StemCell). Engraftment levels varied from 0.2% to 71% human cells according to the number of CRU transplanted, consistent with previous observations.[2]

Data analysis

Mean values (± SEM) were calculated from the data pooled from replicate experiments. Differences between groups were assessed using the Student *t* test.

Results and discussion

A first series of experiments were undertaken to determine the rate at which proliferating $CD34^+$ CB cells would be arrested by TGF-β_1 and/or SDF-1. Proliferating $CD34^+$ cells were isolated by FACS from 4-day primary ("proliferation") cultures originally initiated with lin^- CB cells. These cells were then transferred into new SFM containing SF and TPO with TGF-β_1 and/or SDF-1. As shown in Figure 1A, exposure to TGF-β_1 in the secondary cultures caused a rapid increase (within the first 24 hours) in the proportion of $CD34^+$ cells in G_0/G_1 (from an initial value of 54% ± 2% to > 75%). Maximum levels of G_0/G_1 cells (82% ± 3%) were reached another 24 hours later. In the same cultures, the proportion of $CD34^+$ cells undergoing apoptosis (identified by a DNA content of < 2n) also increased significantly but somewhat more slowly (from 3.6% ± 0.1% to 47% ± 4%; $P < .01$, Figure 1B). In contrast, SDF-1 did not affect either of these parameters ($P > .05$).

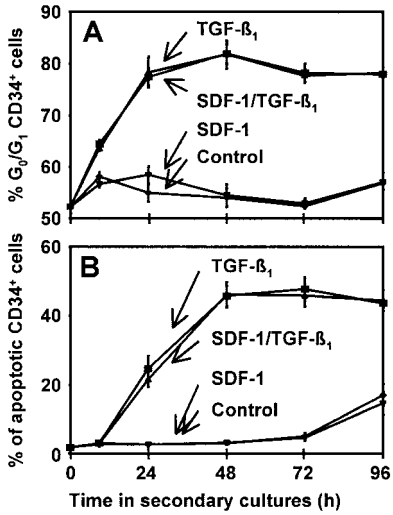

Figure 1. Time course study of the effect of TGF-β_1 and SDF-1 on the cell cycle progression and frequency of apoptotic cells in cultured $CD34^+$ CB cells. Primary cultures of lin^- CB cells were set up in SFM containing 5 GFs and then, after 4 days, $CD34^+$ cells were isolated and transferred to secondary cultures containing SF and TPO (both at 50 ng/mL) either without (control) or with TGF-β_1 (5 ng/mL), or SDF-1 (100 ng/mL), or both. Values shown are the mean ± SEM of measurements made after PI staining of cells from 3 independent experiments. (A) Proportion of $CD34^+$ cells in G_0/G_1. (B) Proportion of apoptotic cells.

Based on these findings, a 48-hour period of exposure to TGF-β_1 (and/or SDF-1) was chosen to evaluate the effects of these agents on the functional activities of various progenitors identified by quantitative in vitro and in vivo assays. Accordingly, a second series of similar experiments were set up with larger numbers of lin^- CB cells. In these, all of the cells (not just the $CD34^+$ subset) harvested from the 4-day primary proliferation cultures were transferred into secondary cultures. Forty-eight hours later, aliquots were removed for FACS analyses of their cell cycle parameters and expression of CD34 and CXCR4 and also for assessment of the

Table 1. Results of CRU assays of cells from secondary cultures of lin^- CB cells

	SF/TPO		SF/TPO + SDF-1		SF/TPO + TGF-β_1		SF/TPO + SDF-1 + TGF-β_1		5 GFs*	
Experiment no.	Cells per mouse, × 10^6	$L^+ + M^+$	Cells per mouse, × 10^6	$L^+ + M^+$	Cells per mouse, × 10^6	$L^+ + M^+$	Cells per mouse, × 10^6	$L^+ + M^+$	Cells per mouse, × 10^6	$L^+ + M^+$
1	1.3	5/6	1.3	6/6	1.3	6/6	1.3	5/6	1.3	6/6
	0.3	3/6	0.3	4/6	0.3	4/6	0.3	4/6	0.3	2/6
2	1.0	5/6	1.0	6/6	1.0	5/6	1.0	5/6	1.0	5/6
	0.4	1/6	0.4	3/6	0.4	1/5	0.4	1/6	0.4	3/6
3	2.0	6/6	2.0	5/5	2.0	6/6	2.0	6/6	2.0	6/6
	0.4	5/6	0.4	3/3	0.4	2/4	0.4	2/6	0.4	2/6
CRUs per 10^6 input cells in second cultures (range defined by SEM)	2.0 (1.6-2.6)		4.6 (3.4-6.2)		2.3 (1.8-3.0)		1.7 (1.3-2.2)		2.0 (1.5-2.5)	

In each of the 3 experiments performed, more than 90% of all the cells from each culture were injected at the doses shown into a minimum of 8 irradiated NOD/SCID mice. L^+ indicates lymphoid engraftment with human cells ($CD34^-CD19/20^+$); M^+, myeloid engraftment with human cells ($CD45/71^+$ $CD15/66b^+$)

*The 5 GFs were Flt3 ligand, SF, interleukin-3, interleukin-6, and granulocyte colony-stimulating factor, as described in "Materials and methods."

number of CFCs and LTC-ICs present. The remainder (> 90% of all the cells harvested from the secondary cultures) were injected into irradiated NOD/SCID mice. Cell cycle analyses revealed similar distributions of the total cell population as shown in Figure 1A for the $CD34^+$ compartment (data not shown). Despite the inhibition of proliferation seen in all cultures exposed to TGF-β_1, the total number of CFCs present increased over the 2 days of incubation in all of the secondary cultures (Figure 2). However, this expansion was generally less in the TGF-β_1–containing cultures and, as expected,[20] the presence of TGF-β_1 selectively suppressed the output of erythroid CFCs ($P < .01$). Yields of LTC-ICs from all cultures were more variable, but neither TGF-β_1 nor SDF-1 had a significant effect on their numbers ($P > .1$, data not shown). Also as expected,[21] exposure to SDF-1 (but not TGF-β_1) down-regulated expression of the SDF-1 receptor (CXCR4), resulting in a decrease of $CXCR4^+$ $CD34^+$ cells from 72% ± 3% to 45% ± 6% ($P = .012$). The results of the NOD/SCID repopulation assays are shown in Table 1. Significantly more CRUs (2.3-fold, $P < .02$) were detectable in the cells harvested from the secondary cultures containing SDF-1 than in those containing SF plus TPO alone or in those maintained in the original 5-GF cocktail used in the primary cultures. In contrast, TGF-β_1 did not affect the number of CRUs detected and even antagonized the enhancing effect of SDF-1.

There are 3 ways that SDF-1 could have elicited the increases in CRU numbers seen. One mechanism could be the stimulation of a faster rate of CRU proliferation (faster cell cycle transit time). However, given the evidence that SDF-1, like TGF-β_1, inhibits the proliferation of primitive hematopoietic cells, including LTC-ICs,[12,22,23] such a possibility seems unlikely. A second mechanism would be the induced dedifferentiation of cells that had already progressed to a later progenitor stage prior to their exposure to SDF-1. Although such a possibility cannot be formally ruled out, the increased numbers of CFCs seen do not favor this explanation; nor was there any evidence of an increase in LTC-ICs, which are generally viewed as closely related to CRUs. A third way that increased numbers of CRUs could have been produced is by recruitment from a previously undetectable, but developmentally equivalent, cell population. Such a situation is known to exist because proliferating HSCs transit $S/G_2/M$,[8,10] and the recent evidence of a cytostatic effect of SDF-1 on closely related progenitor types[12] is consistent with a proliferation blockade of HSCs.[12,22,23] Moreover, the 2-fold higher number of CRUs detected when the cells were exposed to SDF-1 is exactly what would be anticipated from an arrest in G_1 of those that would otherwise have entered $S/G_2/M$. Alternatively, SDF-1 may exert its effects on the transplantability of HSCs independent of effects on HSC cycling. However, incubation of freshly isolated quiescent CB HSCs with SDF-1 has been found to decrease the ability of these HSCs to engraft NOD/SCID mice.[21] An SDF-1–mediated arrest of cycling HSCs thus seems a more plausible explanation for the enhancing effect observed here. The effectiveness of SDF-1 in the absence of stroma also suggests that a direct action on HSCs was obtained. Similar results have been recently reported for preactivated primate HSCs maintained for 4 days in secondary cultures containing SF and retronectin (but no other GFs or chemokines).[24]

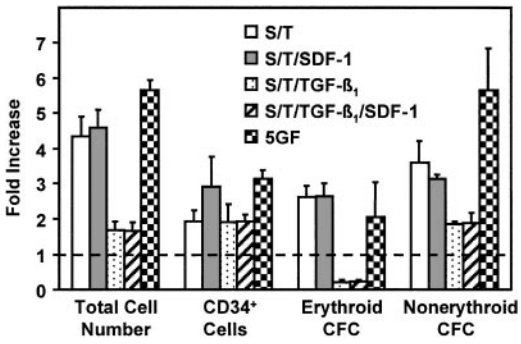

Figure 2. Effect of TGF-β_1 and SDF-1 on the generation of various types of cells in secondary 48-hour cultures of lin^- CB cells. Lin^- CB cells were first cultured for 4 days in SFM with 5 GFs and then transferred to secondary cultures for an additional 48 hours under the same 4 conditions described in Figure 1 (plus one group kept under the same conditions as in the primary cultures). The fold increase (mean ± SEM, n = 3) of each of the indicated cell types is shown relative to the corresponding input value (at the beginning of the secondary culture). S indicates SF; T, TPO.

At first glance, our finding that TGF-β_1 did not have a similar effect on the number of CRUs detected seems contradictory. However, given the high frequency of apoptotic cells seen in the $CD34^+$ compartment when these cells were exposed to TGF-β_1, such an effect may have offset any increase in CRU numbers. In line with this interpretation was the finding that a net increase in CRUs detected was also not obtained when the cells were cultured in TGF-β_1 as well as SDF-1. However, regardless of the mechanism(s) ultimately found to explain the findings described here, they suggest an interesting new approach to enhancing HSC yields in a variety of cellular and gene therapy strategies that involve stimulating HSCs to proliferate ex vivo prior to their transplantation in vivo.

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