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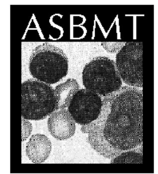

Ex vivo expansion of immature 4-hydroperoxycyclophosphamide–resistant progenitor cells from G-CSF–mobilized peripheral blood

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ABSTRACT

The application of *ex vivo* expansion to cell products pharmacologically purged *in vitro* may provide sufficient numbers of cells for rapid engraftment in a product with reduced tumor burden. To pursue this possibility we evaluated the effect of 4-hydroperoxycyclophosphamide (4-HC) treatment on granulocyte colony-stimulating factor–mobilized peripheral blood stem cells (G-PBSC) and their subsequent expansion potential. A small number of G-PBSC CD34$^+$ cells are resistant to 4-HC and are phenotypically and functionally immature. 4-HC–resistant G-PBSC cells are CD34$^+$ bright, CD38$^{+/-}$, DR^{lo}, CD13^{lo}, CD33$^-$, CD71$^-$, and rhodamine dull. In six experiments, treating G-PBSC with 60 μg/mL of 4-HC at 37°C for 30 minutes reduced the number of colony-forming units (CFUs) per 5000 CD34$^+$ cells by 96.3% (from 1333 ± 137 to 46.5 ± 11). This purging also reduced the frequency of 5-week long-term culture initiating cells (LTC-ICs) from 1/39 (range 1/27 to 1/62) to <1/1680 (range 1/1180 to 1/2420). *Ex vivo* expansion cultures were used to compare the proliferative potential of treated and untreated CD34$^+$ cells. These cells were cultured with either the HS-5 stromal cell line serum-deprived conditioned media supplemented with 10 ng/mL kit ligand (HS-5CM/KL) or a recombinant growth factor mix (GFmix) containing 10 ng/mL each of interleukin (IL)-1, IL-3, IL-6, KL, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, and 3 U/mL of erythropoietin. Culturing untreated CD34$^+$ G-PBSC with 10% HS-5CM/KL increased total nucleated cells by 460-fold after 15 days. Progenitors, which were measured as CFUs, also increased by 47-fold over the same period. More significantly, culturing the 4-HC–treated CD34$^+$ cells with HS-5/KL increased CFUs 98-fold and the nucleated cells increased 4573-fold. The absolute number of CFUs present after expansion of the 4-HC–resistant cells with HS-5CM/KL was threefold higher than that detected before purging and significantly higher than that obtained with GFmix. These data indicate that G-PBSC contain a very immature pool of cells not detectable using the 5-week LTC-IC assay, but have extremely high proliferative potential. Additionally, pharmacological purging of G-PBSC greatly reduces mature cells while retaining an immature population. Also significant is the finding that supernatant from the HS-5 bone marrow stromal cell line plus KL can fully regenerate progenitors from the 4-HC–resistant CD34$^+$ G-PBSC.

KEY WORDS

Purging • Hematopoietic stem cells • CD34

INTRODUCTION

Chemotherapy- or growth factor–mobilized peripheral blood stem cells (G-PBSC) have become the standard of care for autologous treatment of therapy-induced cytopenia. The ability to collect large numbers of stem cells with little duress to patients combined with accelerated engraftment kinetics suggests that the use of stem cell rescue will increase in the future. Mobilization of hematopoietic stem cells (HSCs) also mobilizes tumor cells, which may contribute to relapse [1–3]. To address this issue, investigators have used positive selection of CD34$^+$ cells with and without negative selection to reduce the tumor burden [4–13]. Alternatively, pharmacological agents have been used for

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purging both bone marrow (BM) and peripheral blood autografts [14–24].

Extensive reduction in progenitor cells after purging BM products with 4-hydroperoxycyclophosphamide (4-HC) correlates with the extended duration of cytopenia after transplantation [25]. In theory, this delay in hematopoietic recovery may be reduced by the infusion of large numbers of progenitors, which could be generated *ex vivo*. For the expansion of 4-HC–purged $CD34^{+}33^{-}$ cells, it was initially demonstrated that stroma was an absolute requirement [26]. Subsequently, it was shown that culturing purged cells with a minimum of five recombinant cytokines could also increase the number of nucleated cells. However, to what extent progenitor cells were regenerated was not established.

Pharmacological purging can also be used to evaluate the maturation status of hematopoietic cell products. Numerous investigators have compared G-PBSC, enriched $CD34^{+}$ populations, and subpopulations of $CD34^{+}$ cells such as DR^{lo} or $CD38^{lo}$ with analogous populations in BM or steady-state peripheral blood [27–35]. G-PBSC $CD34^{+}$ cells are phenotypically comparable with those detected in steady-state PBSC, but they differ from those in BM. Fewer BM $CD34^{+}$ cells express CD13 and CD33 and a greater number of marrow cells are $CD19^{+}$ and $CD20^{+}$. Although the G-PBSC product phenotypically appears to contain more cells that are committed to the myeloid lineage than do BM products, functional analysis reveals that a comparable percentage of $CD34^{+}$ cells are colony forming units (CFUs) and long-term culture initiating cells (LTC-ICs). Further, relative to the numbers present in steady-state peripheral blood, the mobilized product contains greater numbers of LTC-ICs. Note that the immature $CD34^{+}DR^{-}/38^{-}$ subpopulations in both steady state and G-PBSC contain comparable frequencies of LTC-ICs (0.5–2.0%). However, in G-PBSC the majority of LTC-ICs are within the more mature $CD34^{+}DR^{+}/38^{+}$ populations [35]. A large percentage (90–95%) of the DR^{+} and 38^{+} LTC-ICs are of intermediate maturity and only provide CFUs for up to 5 weeks in culture [35].

By a thorough analysis of the phenotype and the maturation status of 4-HC–resistant cells, we here assess the effects of 4-HC purging on G-PBSC. We demonstrate that committed progenitors, measured as CFUs and 5-week LTC-ICs, are highly sensitive to 4-HC. Further, we demonstrate that the immature 4-HC–resistant cells can undergo extensive proliferation *ex vivo* and generate large numbers of CFUs.

MATERIALS AND METHODS

G-PBSC processing

Normal G-PBSC was obtained by informed consent in accordance with the Fred Hutchinson Cancer Research Center Institutional Review Board guidelines. Red blood cells were removed from apheresis products by hemolysis with 150 mM NH_4CL_2 at 37°C. Nucleated cells were stored frozen in RPMI, 36% fetal bovine serum (FBS), 10% dimethyl sulfoxide (DMSO), 90 U of penicillin, 90 μg/mL of streptomycin sulfate, and 0.36 μg/mL of glutamine. The stored cells were thawed quickly at 37°C and diluted at room temperature with medium-199 plus heparin to a final DMSO concentration below 1%. After an overnight incubation at 4°C, the cells were washed and then treated with 4-HC as described below.

4-HC treatment

G-PBSC was treated according to previously published guidelines for purging BM [36]. Before isolating $CD34^{+}$ cells, the thawed G-PBSC product was divided into two aliquots of 2×10^{7} cells/mL in RPMI plus 20% FBS. The treated sample received 4-HC (Scios Nova, Mountain View, CA) at a final concentration of 60 μg/mL, and both aliquots were incubated at 37°C for 30 minutes. After incubation, the $CD34^{+}$ cells were washed 3 times in 10 vol RPMI/20% FBS to remove residual 4-HC, pelleted, and then resuspended in phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) to stain before sorting.

Isolation of $CD34^{+}$ cells

Treated and untreated cells were labeled with anti-CD34 antibody (HPCA-2 [IgG_1], Becton Dickinson, San Jose, CA) conjugated to fluorescein isothiocyanate (FITC) for 20 minutes on ice, then washed with PBS containing 1% BSA. Cells with medium to high forward light scatter and low side scatter were selected, and $CD34^{+}$ cells were enriched to 60–70% purity using the enrichment mode of a FACStar plus (Becton Dickinson). For purity >98%, the enriched cells were again sorted using the normal-R mode.

Conditioned medium

HS-5 cells were plated at 2×10^{6} cells per 75 cm^2 in RPMI containing 5% FBS. After 24 hours, the serum containing medium was removed and the cultures washed 2 times with Hanks' balanced salt solution (HBSS). The cells were then fed with serum-deprived medium composed of Iscove's modified Dulbeccos medium (IMDM), 1% nutridoma-HU, 2 mmol/L of L-glutamine, 1 mmol/L of sodium pyruvate, 50 U/mL of penicillin, and 50 μg/mL of streptomycin sulfate. Supernatants were harvested after 7 days and culture debris was pelleted by centrifugation at 2000*g* for 10 minutes. Conditioned medium was stored at 4°C, and batches were tested for colony formation activity before concentrated as previously described [36]. Conditioned medium was concentrated fivefold by a reduction in volume using Centriprep 10 concentrators (Amicon, Beverly, MA) with a 10 kd cutoff, according to manufacturer specifications, and is referred to as HS-5 conditioned medium (HS-5CM).

Ex vivo expansion

Expansion cultures were initiated with 1×10^{4} cells/mL in serum-deprived medium (described above) that was supplemented with either 10% HS-5CM/kit ligand (HS-5CM/KL) or GFmix containing 10 ng/mL each of interleukin (IL)-1, IL-3, IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, KL, and 3 U/mL of erythropoietin (Epo). The initial expansions were performed in the absence of FBS, but the expansion of 4-HC–treated cells required the addition of FBS to a final concentration of 10%. Cultures were incubated at 37°C in 5% CO_2 for different time periods and fed at the start of the expansion. For each time point, triplicate wells were analyzed for total viable cell number, CFU content, or LTC-ICs.

Phenotypic analysis

After sorting the treated and untreated cells, the enriched CD34$^+$ products were placed into expansion cultures. The cells were incubated in expansion medium for 48 hours, which provided sufficient time for the 4-HC to kill sensitive cells. After this incubation, the anti-CD34$^+$ antibody was no longer present and thus restaining of cells was required for phenotypic analysis. The cells were then harvested and labeled with either anti-CD34 (HPCA-2, IgG_1), anti-CD38 (Leu-17, IgG_1), anti–HLA-DR (MHC II, IgG_{2a}), anti-CD13 (Leu-M7, IgG_1), anti-CD33 (Leu-M9, IgG_1), anti-CD71 (anti-transferrin receptor, IgG_{2a}), and the following isotype controls: mouse IgG_1-PE, mouse IgG_{2a}-PE, mouse IgG_{2a}-F from Becton Dickinson. The cells were incubated for 20 minutes on ice with the appropriate antibody and then washed with PBS containing 1% BSA. Labeled cells were then analyzed using a FACScan (Becton Dickinson) and data was collected in list mode.

Rhodamine staining

Staining with Rho123 was performed as previously described [37]. Briefly, cells were incubated at 37°C for 45 minutes with 0.1 µg/mL of Rho123. The cells were then washed twice with HBSS and incubated again at 37°C for 15 minutes in HBSS to eliminate excess dye. Cells were washed again and then used for flow cytometry. Staining was stable for at least 3 hours when protected from light.

7-Amino-actinomycin D staining

Before flow cytometric analysis, 15 µL of 7-amino-actinomycin D ([7-AAD] 50 ng/µL) was added to 1.0 mL of cell suspension. The cells were incubated for a minimum of 15 minutes on ice before analysis.

Colony assays

Colony assays were established and scored as previously described [36]. Briefly, cells were cultured in semisolid medium containing 1.2% methylcellulose, 2.5% BSA, 25% FBS (Hyclone, Logan, UT), 100 U of penicillin, 100 µg/mL of streptomycin sulfate, and 0.1 M of β-mercaptoethanol. Colony-stimulating activity was provided by 10% HS-5CM/KL and 3 U/mL of Epo. Hematopoietic cells plus conditioned medium was added to 0.9 mL of the methylcellulose stock. Colony formation was scored at day 14.

LTC-IC limiting dilution analysis

Two- to four-week-old primary LTCs established and maintained according to previously published guidelines [38,39] were irradiated at 2000 cGy with ^{137}Cs, and 25,000 cells were plated into the middle of each of 60 wells of a 96-well plate at least 24 hours before the addition of hematopoietic cells. The stromal cultures were seeded with untreated CD34$^+$ cells at 5, 10, 25, 50, 75, and 100 cells per well (20 wells each) using single-cell deposition on a Becton Dickinson FACStar Plus before expansion, and after demidepletion were fed weekly for 5 weeks. For the first three experiments, the 4-HC–treated CD34$^+$ cells were plated at the same cell densities as those of the untreated cells; for the next three experiments, 25, 50, 100, 200, 400, and 800 cells per well were plated. The CD34$^+$ cells, with and without 4-HC treatment, were expanded for 12 days and then plated at various concentrations, depending on the extent of expansion. LTC-ICs were detected by removal of the adherent layer and culturing in 90 µL of methylcellulose mix plus 10 µL of recombinant GFmix for 2 weeks, at the end of which time colonies were scored. Calculations of absolute numbers of LTC-ICs were derived from their frequency within the expanded population, based on the proportion of negative wells from the limiting dilution analysis using Poisson statistics with maximum likelihood estimation [40].

RESULTS

Expansion of CD34$^+$ G-PBSC in serum-deprived media

Using serum-free culture conditions previously established with BM cells, the CD34$^+$ cells from G-PBSC were cultured and analyzed for total nucleated cells (TNCs) and CFUs. Figure 1A demonstrates that extensive production of nucleated cells was possible from G-PBSC CD34$^+$ cells in the absence of serum. A significantly greater production of cells after 12 and 15 days was obtained with HS-5CM/KL ($p < 0.001$ for both time points) compared with cultures expanded with recombinant growth factors. Maximum myeloid CFU production was obtained at day 8 with the recombinant factors, whereas HS-5CM/KL maintained production for an additional 4 days, resulting in significant differences in the number of CFU generated ($p < 0.01$) (Fig. 1B).

Immuno-phenotypic analysis

To assess the effect of 4-HC treatment on G-PBSC, we compared the immuno-phenotype, progenitor, and LTC-IC content of the 4-HC–treated CD34$^+$ cells with untreated CD34$^+$ cells. These two populations were evaluated for viability and phenotypic markers 48 hours after either a pulse with 4-HC or mock treatment and initiation of the expansions. Most (98–99%) of the treated CD34$^+$ cells were positive when stained with 7-AAD (not shown), indicating that 4-HC treatment results in extensive cell death. Gating on the 7-AAD negative population revealed that 48 hours after 4-HC treatment the resistant cells differed markedly from untreated cells (Fig. 2). The viable 4-HC–treated cells are CD34 bright, HLA-DR, and CD71^{low}, CD38$^{\pm}$, and rhodamine dull and display low levels of the myeloid markers CD13 and CD33. This phenotypic analysis suggests that a very immature population of cells remains viable after 4-HC purging of peripheral blood CD34$^+$ cells.

Effect of 4-HC treatment on progenitors from normal G-PBSC

Quadruplicate primary colony assays were performed on 2000 4-HC-treated and 200 untreated CD34$^+$ cells from normal donors (n=5). 4-HC treatment significantly reduces the CFU content of the G-PBSC CD34$^+$ cells, from 1333 ± 137 to 46.5 ± 11 per 5000 CD34$^+$ cells ($p < 0.001$). This level of reduction was also observed with 4-HC–treated BM (data not shown) and is consistent with previously published results using BM [26].

Effect of 4-HC treatment on LTC-ICs from G-PBSC CD34$^+$ cells

Before expansion, CD34$^+$ cells were sorted onto preformed irradiated stroma and LTC-IC analysis was per-

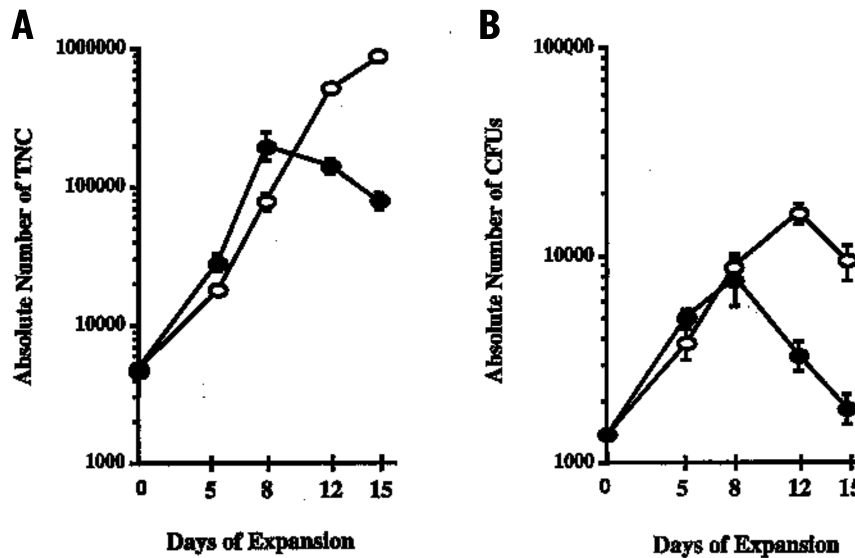

Figure 1. Analysis of total nucleated cell (TNC) production (A) and colony-forming unit (CFU) production (B) from G-PBSC CD34$^+$ cells expanded in serum-free conditions with GFmix or HS-5CM/KL

Cultures were fed at the inception of expansion and at each time point the expansion product was assayed in triplicate for TNCs and quadruplicate for CFUs. The absolute number of TNCs or CFUs are plotted using a logarithmic scale, and each data point represents the mean and standard error of the mean for five separate experiments. ○, *production with HS-5CM/KL;* ●, *production with GFmix.*

formed. Combining the results from six experiments revealed a mean frequency of LTC-ICs in untreated G-PBSC of 1/35.8 ± 2.6 (Table 1). In contrast, the LTC-IC frequency was approximately 1/1666 ± 364 for 4-HC–treated CD34$^+$ cells. This reduction was greater than initially anticipated, preventing detection of this population when a narrow range of cell concentrations was used in the first three experiments. Overall, these data indicate that approximately 96% of the G-PBSC LTC-ICs are sensitive to 4-HC. After 12 days of expansion, the frequency of LTC-ICs in the 4-HC–treated group was too low to detect, suggesting that the extensive expansion of TNCs and CFUs depleted this immature population.

Expansion of G-PBSC

In contrast to results obtained with untreated CD34$^+$ cells, no expansion of 4-HC–treated cells was obtained in the absence of FBS, regardless of whether recombinant cytokines or HS-5CM was added (data not shown). Therefore, parallel expansions of treated and untreated CD34$^+$ cells were performed with the addition of 10% FBS. In cultures with untreated cells, the expansion was rapid through the first 8–12 days of culture (Fig. 3). Adding 10% FBS increased production of nucleated cells fivefold with the GFmix and 2.5-fold with HS-5CM/KL (compare Fig. 1A and Fig. 3). 4-HC–resistant cells constituted only 1–2% of the CD34$^+$ cell population; hence, 4-HC-treated cultures that were initiated with 5000 CD34$^+$ cells contained only 50–100 viable cells. For this reason there was an insufficient number of cells to obtain an accurate cell count until day 8 of culture (Fig. 3, treated arms). The 4-HC–treated cells continued to expand to day 15 with the GFmix and to day 21 with HS-5CM/KL.

Production of progenitors from 4-HC–treated cells

Progenitor production from both treated and untreated CD34$^+$ cells is reported in Figure 4. HS-5CM/KL generated significantly more CFUs than did the GFmix ($p < 0.01$) after 12 days of culture with untreated cells and after 15 days with treated cells ($p < 0.01$). Moreover, under these expansion conditions, only HS-5CM/KL was able to regenerate 2 times more than the initial number of CFUs before 4-HC treatment. In contrast, the GFmix cultures regenerated only 58% of the initial number of CFUs. For both treated and untreated arms, the GFmix had slightly faster production of progenitors through the first 8 days. At this time, the GFmix progenitor production decreased, whereas HS-5CM/KL cultures continued to produce progenitors.

Proliferative index of TNCs and CFUs

By tabulating the mean fold expansion (proliferative index) of 4-HC–treated and untreated cells for the generation of nucleated cells and CFUs, it became apparent that HS-5CM/KL has its greatest effect, in comparison with the GFmix, on the generation of CFUs from the 4-HC–resistant population (Table 2). The CFU production stimulated by HS-5CM/KL from 4-HC-treated cells was 5.16-fold higher than that by the GFmix. In contrast, TNC production was similar under either of the two conditions. Expansions initiated with untreated CD34$^+$ G-PBSC cells generated numbers of TNCs and CFUs approximately 2 times higher with the HS-5CM/KL combination compared with the GFmix.

DISCUSSION

Our data indicated that a small percentage of normal G-PBSC CD34$^+$ cells are resistant to 4-HC. Phenotypic

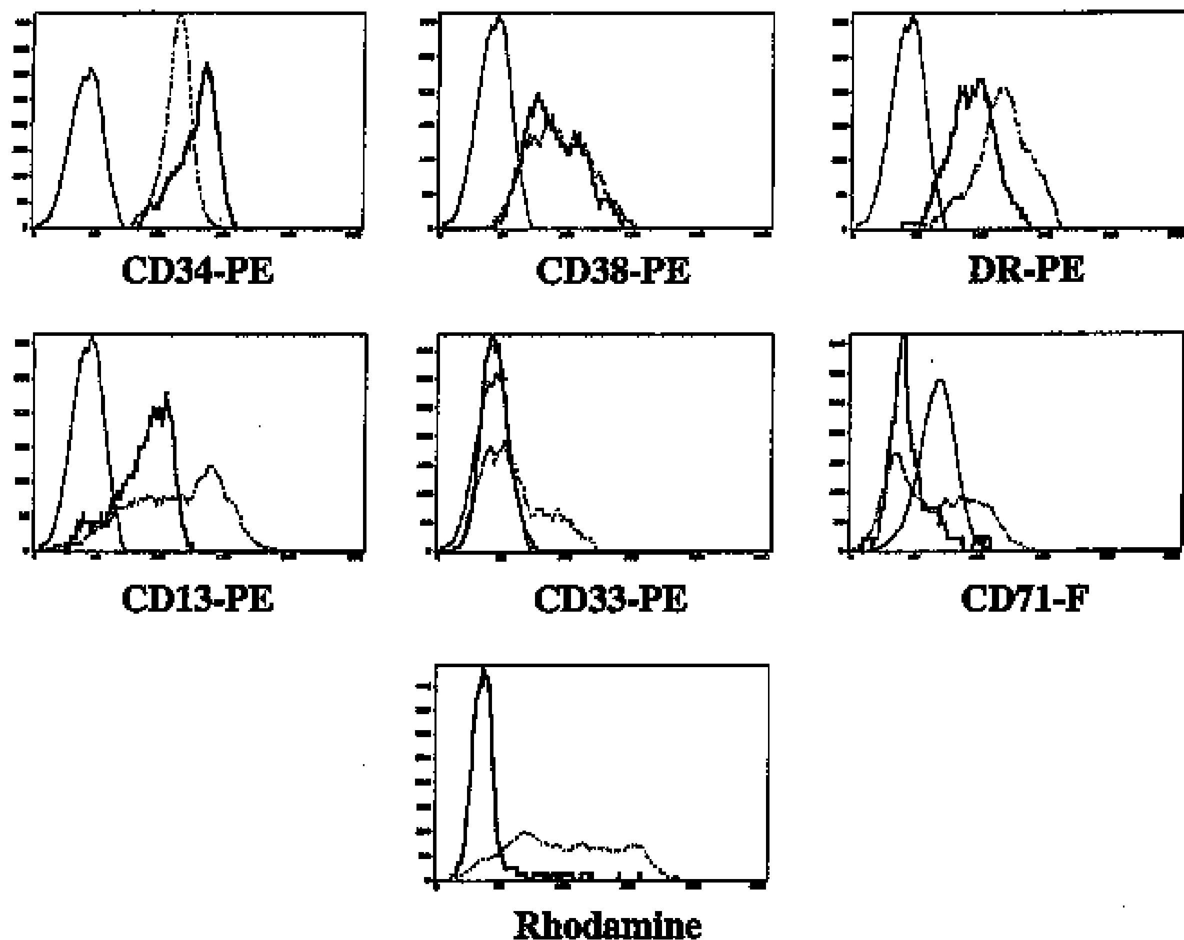

Figure 2. Phenotypic analysis of CD34$^+$ cells from G-PBSC pre– and post–4-HC treatment

Data were collected in list mode and dot plots were generated on pre-gated viable cells as determined by negative staining with 7-AAD. Relevant isotype control IgG_1-PE and IgG_1-FITC were used to detect nonspecific staining. Data are represented as relative fluorescence intensity on the x-axis and relative number on the y-axis. (——) phenotype of 4-HC–treated cells; (·······) untreated cells; (——) isotype control.

analysis of the 4-HC–resistant population suggested that these cells were immature, a factor consistent with their extensive proliferation upon stimulation with cytokines. We confirmed that the frequency of 5-week LTC-ICs in CD34$^+$ G-PBSC (1/39) was comparable with that of CD34$^+$ cells from BM (1/40). The G-PBSC–derived LTC-ICs were extremely sensitive to 4-HC treatment as were the myeloid progenitor cells measured as CFUs. These data were consistent with previous observations that G-PBSC CD34$^+$ cells contain a large number of LTC-ICs that may be of an intermediate maturity status [35]. It was observed that a large percentage of 5-week LTC-ICs were in the more CD38$^+$ or DR$^+$ subpopulations of G-PBSC CD34$^+$ cells [35]. Hence, the observed reduction of 5-week LTC-ICs was further substantiated by lower levels of HLA-DR expression on the resistant population. In contrast, the lack of CD38 expression did not correlate with maturation status, or at least with 4-HC resistance in this study. This factor was consistent with the inability to separate committed progenitors in peripheral blood CD34$^+$ cells from less mature LTC-ICs using this marker [41].

Previous work established that cultures initiated with immature CD34$^+$38$^-$ marrow cells produced large numbers

Table 1. *LTC-IC content in CD34$^+$ cells from G-PBSC pre– and post–4-HC treatment*

	Pre–4-HC treatment		Post–4-HC treatment		
Experiment	Frequency[a]	Absolute number[b]	Frequency[a]	Absolute number[b]	Percent reduction
1	1/27	185	ND	ND	ND
2	1/34	143	ND	ND	ND
3	1/43	116	ND	ND	ND
4	1/32	156	1/1428	3.5	97.8
5	1/35	143	1/1190	4.2	97.1
6	1/43	116	1/2380	2.1	98.2

[a]*LTC-IC frequency was determined by limiting dilution analysis.*
[b]*Absolute number of LTC-ICs was calculated by multiplying the frequency times the total number of cells.*
ND, no LTC-ICs detected after 4-HC treatment using the standard range of cell concentrations for LTC-IC analysis (see Materials and Methods).

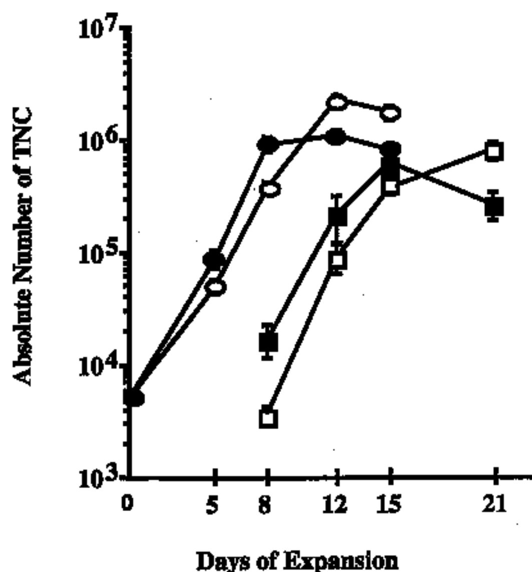

Figure 3. Nucleated cell production from 4-HC–treated and untreated cells

Analysis of total nucleated cell (TNC) production from untreated and 4-HC–treated G-PBSC CD34$^+$ cells expanded in 10% fetal bovine serum (FBS) with GFmix or HS-5CM plus 10 ng/mL KL (HS-5CM/KL). Cultures were fed once at the inception of expansion and each time the expansion product was assayed in triplicate. The absolute number of TNCs is plotted using a logarithmic scale, and each data point represents the mean and standard error of the mean for five separate experiments. Circles represent untreated cells and squares represent the 4-HC–treated cells. HS-5CM/KL cultures are indicated by open symbols and GFmix cultures by closed symbols.

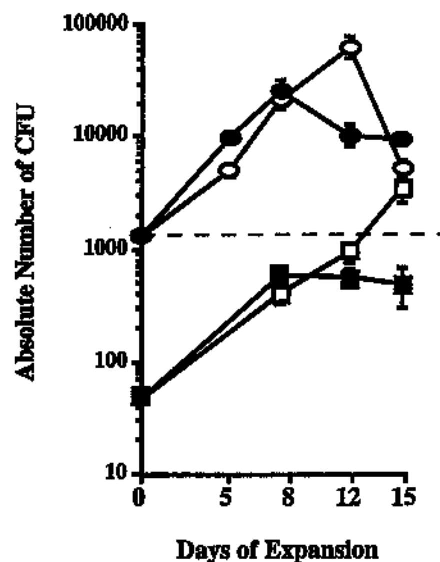

Figure 4. Progenitor production from 4-HC–treated and untreated cells

Analysis of CFU production from untreated and 4-HC–treated G-PBSC CD34$^+$ cells expanded in 10% FBS with GFmix or HS-5CM/KL. Cultures were fed once at the inception of expansion and each time CFUs were assayed in quadruplicate. The absolute number of CFUs is plotted using a logarithmic scale and each data point represents the mean and standard error of the mean for five separate experiments. (---) starting number of CFUs in 5000 CD34$^+$ cells (99% purity) without 4-HC treatment. Circles represent untreated cells and squares represent the 4-HC–treated cells. HS-5CM/KL cultures are indicated by open symbols and GFmix cultures by closed symbols.

of nucleated cells and committed progenitors in serum-free medium [42]. Hence, the dependence on serum for expansion of the 4-HC–treated cells was initially surprising. One possible explanation is that 4-HC damages the immature cells and the FBS helps to facilitate their repair. Another is that the 4-HC–resistant cells are less mature than the CD34$^+$38$^-$ BM population, and thus require additional support for expansion. Alternatively, 4-HC–resistant peripheral blood cells may be equivalent to BM CD34$^+$38$^-$ cells with respect to maturity, and hence the serum requirement is associated with unknown differences between these populations. For example, circulating CD34$^+$ cells are quiescent in contrast, i.e., 30–40% of their marrow counterparts are in S-phase [43].

The first published report on *ex vivo* expansion of 4-HC–treated BM cells indicated that stroma was an absolute requirement for proliferation [26]. With the availability of additional cytokines it became apparent that, by combining a large number of factors, similar results could be obtained in the absence of stroma. In this study, we demonstrated that the HS-5 stromal supernatant was superior to a recombinant growth factor mix for recovery of committed progenitors. This was consistent with the observation that HS-5CM/KL consistently generates more progenitors in comparison with recombinant factors [42]. It is not clear whether this activity was associated with a novel factor, an ideal concentration and combination of cytokines, or an appropriate glycosylation of known factors.

Ideally, for clinical application, exclusion of FBS is warranted because of the potential for adventitious viral exposure and the inherent variability of animal products. We found that incorporating 2% autologous plasma in the serum-free expansion cultures could increase the number of myeloid progenitors twofold from untreated normal donor cells and from untreated patient cells (unpublished observation, B.A.R.). The use of autologous plasma and the HS-5CM have received initial FDA approval for a phase I clinical trial of untreated G-PBSC. However, what effect autologous plasma has on the expansion of 4-HC–resistant cells is unknown and further investigation into its use and the optimization of *ex vivo* expansion techniques should be pursued.

Purging of autologous products could be beneficial by extending remission duration or survival after transplantation. *In vitro* pharmacological purging of BM with 4-HC typically results in delayed hematopoietic recovery [25]. Clinical trials have established that the delay in hematopoietic recovery correlates with the extent of reduction in committed progenitors [25]. To spare normal cells and yet provide tumor reduction, the cell product was pretreated with the cytoprotectant amifostine before 4-HC purging [44]. This product engrafted significantly faster than standard 4-HC–treated cells. However, the effect on relapse or survival has yet to be determined. An alternative approach would follow *in vitro* purging with *ex vivo* expansion of at least part of the product. In the work presented here, treated

Table 2. *Effects of culture conditions and 4-HC treatment on CFU and nucleated cell output*

	Culture condition and treatment	Fold increase in CFU[a]	Fold increase in TNC[a]
HS-5CM/KL	–4-HC	47 X[b]	460 X
	+4-HC	98 X[c]	4573 X
GFmix	–4-HC	19 X[d]	200 X
	+4-HC	19 X[c]	3549 X

[a]*Fold increase was calculated by dividing the total number of cells or CFU produced by the number present before initiating the culture. Maximal productions, which occurred at different times depending on culture conditions and treatment, are compared.*
[b]*Day 12.*
[c]*Day 15.*
[d]*Day 8.*

cells were taken from normal healthy donors. The effect of 4-HC purging on the expansion potential of cells from patients that have a history of cancer and previous chemotherapy is unknown. Currently, however, our studies using cells from normal donors indicate that HS-5CM/KL seems optimal for this purpose.

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