Fate of Contaminating (t(14; 18)+ Lymphoma Cells During Ex Vivo Expansion of CD34-Selected Hematopoietic Progenitor Cells

By Lucas Widmer, Gabriella Pichert, Lorenz Michael Jost, and Rolf Arno Stahel

The use of ex vivo expanded CD34-selected hematopoietic progenitor cells (HPCs) for autologous stem cell support or gene therapy is a major area of research and is likely to increase in the future. At present, little is known about the fate of contaminating malignant cells during ex vivo expansion of CD34-selected HPCs. We established a competitive polymerase chain reaction (PCR) titration assay to determine the number of residual lymphoma cells before and after selection and ex vivo expansion of CD34-selected HPCs in patients with t(14;18) translocation carrying non-Hodgkin's lymphoma. Seven bone marrow (BM) and 2 mobilized peripheral blood progenitor cell samples from 8 patients without histologic BM involvement at the time of the harvest were analyzed by competitive PCR titration assay and determined to contain between 10 and 4,000 lymphoma cells/10⁶ mononuclear cells (MNCs). Immunoadsorption enriched CD34+ cells from a mean of 5% (range, 1% to 9%) to a mean of 88% (range, 76% to 94%) of MNCs and resulted in a 1 to 4 log depletion of contaminating tumor cells. Two HPC samples became PCR negative after CD34 selection, whereas 7 samples still contained ≤10 to 200 residual lymphoma cells/10⁶ MNCs. CD34-selected cells were consecutively expanded in suspension culture in the presence of stem cell factor, interleukin-1β (IL-1β), IL-3, and IL-6. The mean increase of cells was 13-fold (range, 4 - to 22-fold) at day 7 and 65-fold (range, 43 - to 110-fold) at day 14 of culture. Expansion resulted predominantly in myelomonocytic differentiation, whereas B-cell antigen-expressing cells became undetectable. Six of the seven PCR-positive CD34-selected samples became PCR-negative for the t(14;18) translocation at day 7 and/or 14 of expansion. One PCR-positive and one PCR-negative CD34-selected sample were PCR-positive after ex vivo expansion, but the number of residual lymphoma cells remained at the limit of detection. We conclude that CD34 selection does not eliminate contaminating lymphoma cells in the majority of t(14;18)+ HPC harvests. However, during ex vivo expansion of CD34-selected HPCs, residual t(14;18)+ lymphoma cells do not proliferate and become undetectable by PCR in the majority of cases.

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MATERIALS AND METHODS

Patients and clinical samples. Clinical characteristics of the 8 patients with t(14;18) carrying non-Hodgkin’s lymphoma (NHL) included in this study are listed in Table 1. Six patients had a low-grade and 2 patients had an intermediate-grade histology according to the International Working Formulation. BM infiltration with lymphoma cells ranges from 5% to 90% at diagnosis. After initial evaluation, all patients received induction or salvage chemotherapy. At the time of HPC harvest, 4 patients had achieved a complete and 4 patients a partial remission. In all patients, morphologic BM involvement was no longer detectable. Informed consent was obtained from each individual and aliquots of BM from 7 patients and mobilized PB from 2 patients were collected. Thirty to 80 mL of BM drawn from the posterior iliac crest under local anesthesia was obtained from patients no. 1 through 4 and 6 through 8. Patients no. 4 and 5 underwent mobilization of PB progenitor cells with 1,500 mg/m² cyclophosphamide intravenously at day 1, with subsequent administration of recombinant human granulocyte colony-stimulating factor (Roche, Basel, Switzerland) at a dose of 10 μg/kg/d from
day 2 until PB progenitor cell collection by leukapheresis with a Fenwal CS 3000 was completed. From patient no. 4, BM was obtained 1 day before leukapheresis of mobilized PB progenitor cells was performed.

Selection and expansion of CD34+ cells from harvested BM or mobilized PB progenitor cells. CD34+ cells from BM or from mobilized PB leukapheresis product were positively selected by immunoadsorption columns (Ceprate LC system; CellPro Inc, Bothell, WA) following the manufacturer’s recommendations. CD34-selected cells were seeded at a concentration of 5 × 10^5/mL in 6-well tissue culture plates (Falcon; Becton Dickinson, Basel, Switzerland) or 25-cm^2 vented culture flasks (Costar, Badhoevedorp, The Netherlands), depending on the amount of cells harvested. Cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM; GIBCO BRL, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS; HyClone Laboratories, Logan, UT) and the following hematopoietic growth factors: 80 ng/mL stem cell factor (SCF; Peprotech, Rocky Hill, NJ) and interleukin-6 (IL-6; 20 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF; 20 ng/mL; provided by Sandoz), and erythropoietin (EPO; 2 U/mL, Cilag, Schaffhausen, Switzerland), as described.

Colonies were scored after incubation for 14 days at 37°C in a humidified atmosphere containing 5% CO2 in a humidified atmosphere containing 5% CO2, representing granulocytes and monocytes that were not removed by the immunoadsorption procedure. To prevent amplification of free DNA released from dead lymphoma cells, cells were incubated with deoxyribonuclease (Boehringer Mannheim, Rotkreuz, Switzerland) in phosphate-buffered saline (PBS) at a final concentration of 3 μg/mL at room temperature for 10 minutes and consecutively washed three times with PBS. The equivalent of 150,000 cells (1 μg of genomic DNA) was analyzed. To ensure that DNA was amplifiable in all samples, PCR was performed in all negative samples using control primers spanning a 506-bp fragment of the bcl-2 gene. With each experiment, DNA from a 10^-3 dilution of RL cells for the MBR and of DHL-16 cells for the mcr (both cell lines were a generous gift from J.G. Gribben, Dana-Farber Cancer Institute, Boston, MA) in normal PB MNCs served as a positive control and PCR reagents without DNA as a negative control. Aliquots of 10 μL of the final reaction product were analyzed by electrophoresis on a 2% agarose gel (Boehringer Mannheim) containing 0.01% ethidium bromide and visualized under UV light.

Comprehensive PCR titration assay. Nested PCR was performed identically, except that 1 μg of competitor DNA was added to 1 μg of sample DNA and the mixed DNA was coamplified. Competitor DNA consisted in genomic DNA extracted from serial dilutions of RL or H2 cells (the H2 cell line was kindly provided by D.C. Roy, Maisonneuve-Rosemont Hospital, Montreal, Quebec, Canada) in MNCs of healthy blood donors, with a 236-bp or 190-bp PCR-amplifiable translocation at the MBR, respectively. Serial dilutions were made in the range of 10^-3 to 10^-6. Samples were analyzed at each order of magnitude. After PCR products were resolved on an agarose gel and visualized under UV light, an image was captured and band intensities were quantified by densitometry using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The log of the competitor dilution added to each reaction was plotted against the log of the ratio of the resultant band intensities. The equivalence point was determined by linear regression analysis and the number of lymphoma cells/10^6 cells in the sample was calculated. To correct for the size difference between competitor and sample bands, the calculated number of lymphoma cells in the sample was multiplied with the size ratio of the competitor and sample PCR products. If the equivalence point was at or below the 10^-3 competitor dilution, the number of lymphoma cells was estimated to be ≤10 in 10^6 cells.

RESULTS

Characterization of CD34-selected and ex vivo expanded HPCs. MNCs from BM or mobilized PB contained a mean of 5% CD34+ cells (range, 1% to 9%). After immunoadsorption, CD34+ cells were enriched to a mean of 88% (range, 76% to 94%) of MNCs, with a mean recovery of CD34+ cells of 42% (range, 26% to 59%). Cell numbers were reduced 68-fold (range, 23- to 175-fold) after CD34 selection. Figure 1 summarizes the results of flow cytometric analysis after selection and during ex vivo expansion of CD34-selected HPCs. CD34-selected cells contained a mean of 11% CD15+ cells (range, 4% to 16%); a mean of 5% CD11b+ cells (range, 4% to 7%); and a mean of 2% CD14+ cells (range, 0% to 4%; Fig 1B through D), representing granulocytes and monocytes that were not removed by the immunoadsorption procedure. Contamination with differentiated B cells was...
Fig 1. Flow cytometric analysis of CD34-selected and ex vivo expanded cells. Flow cytometric analysis of HPCs after CD34 selection and after 7 and 14 days of ex vivo expansion in suspension culture with SCF, IL-1β, IL-3, and IL-6. The percentage of CD34+ (A), CD15+ (B), CD11b+ (C), CD14+ (D), CD34+/CD19+ (E), and CD34-/CD19+ (F) cells at days 0, 7, and 14 is given. N = number of samples analyzed. Mean values are indicated with horizontal bars.

less than 0.5% in all samples (Fig 1F). However, CD34+/CD19+ B-cell precursors ranged from 32% to 53% of the CD34+ fraction in BM samples, whereas CD34+/CD19+ cells were less than 0.6% of the CD34+ fraction in PB samples, as has been observed by others (Fig 1E).23 Expansion of CD34-selected cells in suspension culture resulted in a 13-fold increase (range, 4- to 22-fold) of cells at day 7 and a 65-fold increase (range, 43- to 110-fold) of cells at day 14 (Table 2). The CD34+ fraction decreased during expansion in suspension culture to a mean of 5% at day 7 (range, 1% to 13%) and 1% at day 14 (range, <0.5% to 4%), respectively, as shown in Fig 1A. The percentage of cells expressing the myelomonocytic markers CD15, CD11b, and CD14 increased to a mean of 52% (range, 43% to 63%), 17% (range, 14% to 25%), and 8% (range, 1% to 14%), respectively, at day 7, and to a mean of 73% (range, 67% to 81%), 54% (range, 46% to 61%), and 14% (range, 3% to 21%), respectively, at day 14 (Fig 1B, C, and D). CD34+/CD19+ B-cell precursors disappeared in culture and no differentiated B cells (CD34+/CD19+) were detectable after 7 and 14 days of expansion, as shown in Fig 1E and F. CFU-GM increased 2.2-fold at day 7 (range, 1- to 5.6-fold) and fourfold (range, 0.9- to 9.3-fold) at day 14, respectively. BFUe/CFUe expanded 3.3-fold at day 7 (range, 0.3-fold to 11-fold). The more mature type (CFUe) predominated after ex vivo expansion. At day 14, BFUe/CFUe were lost. CFU-GEMM (0 to

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Table 2. Fold Increase of Cells After 7 and 14 Days of Ex Vivo Expansion of CD34-Selected Cells in Suspension Culture

Abbreviation: ND, not determined.
EX Vivo Expansion of CD34-Selected HPCs

10 colonies/1,000 CD34-selected cells at day 0; mean, 3) were lost during expansion.

**Accuracy and reproducibility of the competitive PCR titration assay for the t(14;18) translocation.** The sensitivity of the double-amplification PCR assay was determined by amplifying DNA extracted from serial dilutions of RL or H2 cells in normal PB MNCs (Fig 2). The t(14;18) translocation was detected reproducibly in DNA extracted from 10⁻² RL or 10⁻⁵ H2 dilutions. DNA extracted from RL and H2 serial dilutions were used as competitors in a modified PCR titration assay.²⁴-²⁶ To test the accuracy and reproducibility of the assay, DNA from serial dilutions of RL and H2 cells was coamplified, as shown in Fig 2B. Band intensities at the corresponding dilutions were similar (arrowheads), showing that both translocations are amplified with similar efficiency over a range of at least 3 orders of magnitude. The equivalence point of competitor and sample was determined by linear regression analysis, as shown in Fig 3A. In five independent experiments, using the RL cells as a competitor and the H2 cells as a sample and vice versa, sample dilutions of 10⁻⁴, 10⁻³, and 10⁻² were assayed. After correcting for the size difference of the PCR products, the determined sample dilutions were 10⁻².⁰±0.⁴ SD, 10⁻³.¹±0.³ SD, and 10⁻⁴.⁰±0.²⁴ SD, respectively (Fig 3B). Therefore, the determination of the number of t(14;18)+ cells is achievable within less than an order of magnitude. The individual patient bands (between 114 and 238 bp) were similar in size to the competitor bands in 7 of 8 patients. Therefore, amplification efficiencies of the fragments are comparable.²⁷ One PCR product (patient no. 8) was considerably larger (880 bp). The amplification efficiency may therefore be lower, which might lead to an underestimation of the number of lymphoma cells in the sample.

**Nested PCR analysis for t(14;18)+ cells.** Diagnostic lymph node tissue was available from all patients. PCR analysis showed that all lymphomas carried a t(14;18) translocation at the MBR and none at the mcr. All HPC harvests contained residual lymphoma cells with a PCR-amplifiable t(14;18) translocation at the MBR of the same size, as could be expected from the diagnostic lymph node tissue. After CD34 selection, no PCR-amplifiable cells were detectable in aliquots of two harvests. However, one of these harvests was again PCR-positive at days 7 and 14 of ex vivo expansion of CD34-selected cells. In 6 of the 7 PCR-positive CD34-selected harvests, t(14;18)+ cells became undetectable, whereas 1 harvest remained PCR-positive during the 14-day culture period.

**Competitive PCR titration assay before and after selection and ex vivo expansion of CD34-selected cells.** To determine the number of residual malignant cells before and after selection and ex vivo expansion of CD34-selected HPCs, all samples determined to be PCR-positive for the t(14;18) translocation were reanalyzed using the competitive PCR titration assay. Figure 4 shows competitive PCR titration analysis for t(14;18)+ cells in mobilized PB and in BM from patient no. 4, the only patient from which paired samples of BM and mobilized PB progenitor cells were available. A contamination of 3,000 tumor cells/10⁶ MNCs in the PB and of 4,000 tumor cells/10⁶ MNCs in the BM sample was calculated.

The fate of contaminating tumor cells in the PB sample from patient no. 4 before and after selection and ex vivo expansion of CD34-selected cells is shown in Fig 5. PCR shows a tumor-specific band in the PB sample with a size of 140 bp that appears to be slightly less intensive after CD34 selection. After 7 days of expansion in suspension culture, only a very weak band was observed, and after 14 days, no PCR-amplifiable band could be detected (Fig 5A). The number of tumor cells, as determined by competitive PCR titration assay, was 3,000/10⁶ cells in the PB sample, 200/10⁶ cells after CD34 selection cells, and 7/10⁶ cells at day 7 (Fig 5B). Results of the determination of tumor cell contamination before and after selection and expansion of CD34-selected cells of all 8 patients examined are given in Table 3. Contamination with residual lymphoma cells in the unselected HPC harvests ranged between ≤10 and 4,000 tumor cells/10⁶. After CD34 selection, tumor cell contamination ranged from ≤10 to 200 tumor cells/10⁶ cells in 7 of 9 samples. The calculated total tumor cell reduction (relative reduction of tumor cells multiplied with reduction of total cell numbers after CD34 selection) was 1 to 4 logs. After expansion of CD34-selected HPCs, tumor cell contamination in samples from patients no. 1 and 8 remained at ≤10 tumor cells/10⁶ cells. In the other 6 CD34-selected samples that were PCR-positive after selection, the relative numbers of residual lymphoma cells decreased and became undetectable by PCR at day 14 of expansion. No correlation between patient status, tumor contamination in the progenitor harvest, and obtaining PCR-negative progenitors after ex vivo expansion could be made.

**DISCUSSION**

This report shows that, although the majority of CD34-selected HPCs from patients with advanced t(14;18)+ NHL still contain malignant cells, residual lymphoma cells do not proliferate under the conditions used to expand CD34-selected HPCs in suspension culture. The fate of minimal residual disease during ex vivo expansion of CD34-selected HPCs is of interest in many respects. Residual lymphoma cells may contribute to relapse after transfusion of autologous HPCs after high-dose chemotherapy.⁷ Therefore, growth of contaminating malignant cells during ex vivo expansion of CD34-selected HPCs may preclude the use of expanded progenitors as autologous support after myeloablative therapy for NHL. Furthermore, gene therapy is currently a major research focus in hematologic malignancies. Cycling residual tumor cells may inadvertently be transduced concomitantly with HPCs in gene therapy protocols aiming at transferring drug resistance genes, such as dihydrofolate reductase,²⁸ or MDR1,²⁹ to HPCs. We determined the number of residual t(14;18)+ lymphoma cells in HPC harvests of patients with NHL before and after selection and ex vivo expansion of CD34-selected cells using a competitive PCR titration assay. The availability of two cell lines with different t(14;18) MBR breakpoints yielding PCR products of similar but distinguishable sizes obviated the need to construct a synthetic template and allowed us to assess the reproducibility and accuracy of the assay.
Fig 2. Competitive PCR titration assay. (A) Agarose gel electrophoresis of PCR-amplified DNA isolated from serial dilutions of RL and H2 cells in normal MNCs. The dilution factor is given at the top of the figure. (B) Competitive PCR titration assay with DNA from serial dilutions of RL and H2 cells. DNA was mixed and competitively amplified. The dilution of RL cells and H2 cells are given at the top and at the bottom of the figure, respectively. The position of the PCR product from RL and H2 cells is indicated on the left of the figure. The arrowheads indicate equivalent dilutions of RL and H2 cells.
EX Vivo Expansion of CD34-Selected HPCs

First, we evaluated the effectiveness of CD34 selection in reducing the load of contaminating tumor cells in the HPC harvests. Our results confirm previous reports showing that all patients with a t(14;18)+ lymphoma still have PCR-detectable lymphoma cells in their BM at the time of the HPC harvest. However, the residual tumor load in the HPC harvests was variable and ranged from 500 to 4,000 tumor cells/10⁶ MNCs. In 1 patient, paired samples of mobilized PB and BM were available. The number of tumor cells/10⁶ MNCs was similar in the mobilized PB and in the BM. This finding is in contrast to studies in breast cancer patients, in whom mobilized PB harvests were found to contain less tumor cells than the corresponding BM.

Although CD34 selection of HPCs reduced the number of residual tumor cells by 1 to 4 logs, contaminating lymphoma cells were not eliminated in 7 of 9 HPC harvests. Gorin et al. reported 8 of 9 t(14;18)+ BM harvests from patients with NHL to become PCR-negative after CD34 selection. The sensitivity of tumor cell assessment reported by Gorin et al. was 1 malignant cell in 10⁷ normal cells. We were able to reproducibly detect 1 lymphoma cell in 10⁶ cells, which may explain the discrepancy of our results and those of and Gorin et al.

Although the CD34 antigen is not expressed on mature lymphoma cells, t(14;18)+ cells were recently detected in the CD34+/CD19+ B-cell precursor and in the CD34-/CD19+ mature lymphocyte fraction from BM. The involvement of the IgH locus in the t(14;18) translocation suggests that the translocation takes place at the time of the D-JH rearrangement that occurs early in B-cell ontogeny at the CD34+/CD19+ B-cell precursor stage in the BM. Therefore, t(14;18)+ cells may be coselected with CD34+/CD19+ B-cell precursors. In the BM, the percentage of CD34+/CD19+ B-cell precursors in the CD34+ fraction is much higher than in the PB (32% to 53% vs < 0.6% in our study). It is not known whether t(14;18)+ cells are also present in this small PB CD34+/CD19+ B-cell precursor fraction or only in the mature PB CD34+/CD19+ lymphocyte population. Our findings that t(14;18)+ cell contamination was similar in the mobilized PB as in the BM as discussed above suggest that the majority of tumor cells are among the CD34+/CD19+ mature B-cell population.

Secondly, we monitored the fate of contaminating t(14;18)+ translocation carrying lymphoma cells during ex vivo expansion of CD34-selected HPCs. In 5 of the 6 harvests with PCR-detectable cells after CD34 selection, PCR-positive cells were no longer detectable at days 7 and/or 14 of ex vivo expansion. One harvest remained PCR-positive after 14 days of expansion, but the number of tumor cells decreased to the limit of detection. One of the PCR-negative CD34-selected harvests became PCR-positive at days 7 and 14 of ex vivo expansion. In the positive samples from this...
particular harvest, the number of tumor cells was at the limit of detection. Quantification of tumor cells is therefore inaccurate and no conclusions concerning tumor cell growth can be drawn.

Our findings show that CD34+ HPCs have a growth advantage over residual t(14;18)+ lymphoma cells. Recent reports on contaminating breast cancer and myeloma cells in suspension culture of HPCs showed similar findings. Whether residual lymphoma cells were diluted below the limit of detection by PCR in the rapidly expanding myelomonocytic cell population or whether they died during ex vivo expansion cannot be decisively answered by our results. The fact that t(14;18) translocation carrying cells remained detectable by PCR at both time points analyzed during ex vivo expansion in 2 HPC harvests suggests that residual lymphoma cells may remain viable in some cases. It is unlikely that DNA from dead cells was picked up by PCR, because all samples were treated with deoxyribonuclease before DNA extraction.

Ex vivo expansion of CD34-selected cells resulted in myelomonocytic differentiation of CD34+ HPCs as described by Haylock et al., without concomitant expansion and differentiation of B-lymphoid progenitors. Our results on nucleated cell expansion and decrease of the percentage of CD34+ cells during culture are similar to those Brugger et al. observed in their clinical study using ex vivo expanded cells as a hematopoietic support after high-dose chemotherapy. CFU expansion values were comparable with the values observed by Williams et al using PIXY321 in their culture system, but lower than in the study of Brugger et al. The fact that we and Williams et al did not include EPO in the cultures may account for this difference.

It is still controversial as to whether the potential for long-term engraftment is maintained after ex vivo expansion of HPCs. Ultimately, the quality of ex vivo expanded cells can only be judged by in vivo studies. A long-term engraftment defect after ex vivo expansion of mouse progenitor cells has been observed by Peters et al. Engraftment failure after retransfusion of ex vivo expanded cells in patients with multiple myeloma treated with chemotherapy and total body irradiation has been reported. Hematopoietic reconstitution using ex vivo expanded cells after high-dose chemotherapy was rapid in the study of Brugger et al. However, because the chemotherapy conditioning regimen used by Brugger et al may not be myeloabiative, the study may not prove long-term reconstitution capability of ex vivo expanded cells. Further clinical trials and gene marking studies are required to clarify this issue.

In conclusion, we showed that CD34 selection of HPCs is insufficient for obtaining a PCR-negative autograft in the

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**Fig 4.** Quantification of t(14;18)+ cells in mobilized PB and BM. Competitive PCR titration assay for t(14;18)-carrying cells in mobilized PB and BM from patient no. 4. The position of the PCR product from patient cells and RL cells is indicated on the left of the figure. The RL dilution is indicated at the top of the figure.
EX VIVO EXPANSION OF CD34-SELECTED HPCS

Fig 5. Quantification of t(14;18)- cells before and after CD34 selection and ex vivo expansion. PCR (A) and competitive PCR titration assay (B) for t(14;18)-carrying cells in mobilized PB after selection and after 7- and 14-day ex vivo expansion of CD34-selected cells (patient no. 4). The position of the PCR product from patient cells and RL cells is indicated on the left of the figure. The RL dilution is indicated at the top of the figure.

majority of patients with t(14;18) lymphoma. Because there is evidence that retransfusion of HPCs contaminated with t(14;18) lymphoma cells contribute to relapse after high-dose chemotherapy, additional purging may be required even after CD34 selection. The safety and efficacy of the combination of CD34 selection with immunomagnetic anti-B-cell purging is currently being evaluated in a phase I-II trial at our institution. We provide evidence that contaminating t(14;18) lymphoma cells do not proliferate during ex vivo expansion of HPCs. Because only dividing cells are transduced with retroviral vectors, this finding has important implications for further clinical studies on retroviral transduction of ex vivo cultured HPCs with a chemoresistance gene.

Table 3. Number of Contaminating t(14;18)- Cells/10⁶ Cells Before and After Selection and at Days 7 and 14 of Ex Vivo Expansion of CD34-Selected Cells Determined by Competitive PCR Titration Assay

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Abbreviation: ND, not determined.

ACKNOWLEDGMENT

We thank Heidi Ems for excellent technical assistance, Prof H.P. Honegger for BM samples, E. Niederer for flow cytometry analysis, C. Weber for secretarial assistance, Dr F. Bofferding and Dr D. Schmitter for helpful discussions, and Dr D. Vasella and Sandoz (Basel, Switzerland) for providing cytokines.

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