Peripheral Blood Stem Cell Tumor Cell Contamination and Survival of Neuroblasto Patients

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Abstract Purpose: Contribution of peripheral blood stem cell (PBSC) contaminating tumor cells to subsequent relapse and overall survival of neuroblasto patients remains controversial. Experimental Design: Neuroblasto cell contamination of 27 PBSC harvests from stage IV neuroblasto patients was assessed by quantitative RT-PCR for both tyrosine hydroxylase (TH) and GD2 synthase (GD2-s). The effect of PBSC contamination on survival was then analyzed. Results: Seven PBSC tested negative for both markers; 19 were positive for GD2-s, 6 for TH, with 5 positive for both. Survival of the 20 patients with positive PBSC did not differ from that of the patients with negative PBSC (log-rank test, \( P = 0.134 \) and 0.218 for event-free survival and overall survival, respectively). By considering the TH and GD2-s results independently, a borderline (\( P = 0.053 \)) negative effect on event-free survival was observed in patients reinfused with GD2-s-positive PBSC. When the status at transplant was taken into account, only the event-free survival of the patients rescued when in complete remission with GD2-s-negative PBSC was better, although not significantly, than that of patients infused with GD2-s-positive PBSC. Conclusions: Our results obtained in a small cohort of homogeneously treated stage IV patients suggest that patient survival is not affected by PBSC contamination with the exception of a borderline negative effect on event-free survival in patients rescued when in complete remission.

Myeloablative chemotherapy followed by autologous peripheral blood stem cell (PBSC) rescue has become standard therapy in high-risk neuroblasto patients. However, PBSC may be contaminated with tumor cells, and few reports on small case series (1–7) have documented a trend to a poorer survival in patients rescued with contaminated PBSC. Rill et al. have also shown that gene-marked neuroblasto cells contaminating PBSC can be detected in case of relapse (8), suggesting their active role in disease progression. However, Corrias et al., by evaluating PBSC contamination by means of immunocytochemistry and qualitative reverse transcription-PCR (RT-PCR), showed the absence of correlation between tumoral contamination of unselected PBSC and patient survival (9).

The use of purified CD34+ cells instead of unselected PBSC was adopted in several pediatric cancer centers (10–14) with the expectation to improve patient survival. Quite surprisingly, Handgretinger et al. reported that survival of neuroblasto patients, rescued with purified CD34+ cells, inversely correlated with the amount of neuroblasto cells contamination detected by immunofluorescence technique. The authors (15) suggested that a small amount of graft contamination might induce a protective antitumor immune response. In conclusion, the contribution of tumor cells to subsequent relapse and to overall and event-free survival remains controversial.

Materials and Methods

Study design. Tyrosine hydroxylase (TH) and disialoganglioside GD2 synthase (GD2-s) are two neuroblasto-specific molecular markers (16, 17). Quantitative RT-PCR for both genes was used to assess neuroblasto contamination of the PBSC collected from stage IV neuroblasto patients. The effect of PBSC contamination on patient survival was then analyzed.

Patients. In the period 1997 to 2001, 27 consecutive International Neuroblasto Staging System (18) stage IV neuroblasto patients had PBSC harvest and myeloablative therapy with unselected autologous PBSC rescue at the Gaslini Institute. They had been enrolled in two consecutive protocols (i.e., AIEOP NB-97 and COJEC pilot study) that adopted the busulfan-melphalan association as conditioning regimen. According to the protocols, PBSC were harvested only in patients responsive to induction chemotherapy. The median age of the patients (16 males and 11 females) at PBSC harvest was 3.2 years. At the time of myeloablative therapy, 12 patients (44%) were in complete remission,
and 15 (56%) were in partial remission (Table 1). Response to treatment was evaluated according to the International criteria for neuroblastoma diagnosis, staging, and response to treatment (18).

The study protocols were approved by the Ethics Committee of the Gaslini Institute, and PBSC were harvested after informed consent in conformity with the Declaration of Helsinki principles. This biological study was initiated 2 years after the last myeloablative therapy.

**Quantitative RT-PCR analysis.** One milliliter of the PBSC harvest was processed for total RNA extraction by means of the RNeasy blood mini kit (Qiagen, Cologne, Germany) and then stored at −80 °C until quantitative RT-PCR was done. All RNA samples were simultaneously thawed, and after measuring the RNA concentration (Nanodrop spectrophotometer, Labtech International, Ringmer, United Kingdom), 1 μg of total RNA was reverse-transcribed in a volume of 50 μL. Then, 5 μL of cDNA, equivalent to 100 ng, in triplicates were amplified in a volume of 25 μL in a 7700 SDS (Applied Biosystems, Foster City, CA) with primers for β2-microglobulin (Applied Biosystems), together with a standard curve of total RNA (100 ng to 10 pg) extracted from the neuroblastoma cell line IMR-32. Afterwards, 5 μL of cDNA in triplicate were separately amplified for 40 cycles with GD2-s-specific (19) and TH-specific (20) primers and probes.

Absolute quantification of TH and GD2-s transcripts was done by using standard curves run simultaneously and consisting of logarithmic dilutions of TH calibrator (20) and of GD2-s plasmid, ranging from 10^5 to 10 copies per 5 μL. The number of transcripts in the positive PBSC was then calculated from the TH and GD2-s standard curves and normalized to 1 ng of total RNA through the β2-microglobulin standard curve.

Relative quantification of PBSC contamination was carried out by the comparative threshold method (2^ΔΔCt, ref. 21) using β2-microglobulin as endogenous reference gene and the IMR-32 neuroblastoma cell line as the exogenous reference sample. To evaluate the total number of transcripts infused in each patient, the total number of cells was multiplied by the amount of transcripts found in 10 pg total RNA, the latter being the average amount of total RNA extracted from a single cell (16).

To evaluate the relative amount of neuroblastoma cell infused in each patient, the total number of cells was multiplied by the 2^ΔΔCt value (21) of the corresponding PBSC.

**Statistical analysis.** Survival analysis was done according to the Kaplan-Meier method and truncated at 5 years after myeloablative therapy due to the small number of patients at risk after that period. Patients were further stratified according to TH and/or GD2-s status of their PBSC and the log-rank test was used to compare survival. P < 0.05 was considered statistically significant. The statistical software Stata (release 7.0, Stata Corp. 2001, College Station, TX) was used for all the analyses.

### Results

**Expression of TH and GD2-s mRNA in PBSC.** Seven (26%) of the 27 PBSC samples analyzed by quantitative RT-PCR for TH and GD2-s were negative for both markers (Table 1), and 20 (74%) were positive for at least one marker (19 for GD2-s and 6 for TH, with five positive for both markers). Twenty-one PBSC

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<th>Table 1. Clinical features of the 27 patients and summary of quantitative RT-PCR results from the autologous PBSC</th>
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Abbreviations: PR, partial remission; CR, complete remission; BM, bone marrow; PT, primary tumor; B, bone.

*Status at transplant as assessed by the International Neuroblastoma Response Criteria.

<sup>c</sup>Number of infused TH– or GD2-s–positive cells × 10⁶, calculated by using IMR-32 cells as reference.

<sup>e</sup>Number of transcripts normalized per ng of total RNA.

<sup>f</sup>Months from transplant and relapse.

<sup>g</sup>Months from transplant (+, censored).
were negative, and six were not assessable by anti-GD2 immunocytochemistry. In the positive samples, the number of TH transcripts ranged between 1 and 281 per ng total RNA and that of GD2-s between 1 and 23 per ng total RNA (Table 1; Fig. 1A and E, respectively). Distribution of the results according to the clinical status at transplant is reported in each plot of Fig. 1. The relative TH and GD2-s PBSC contamination (21), with respect to the reference IMR-32 cell line, ranged between 0.06% and 24% for TH-positive PBSC and between 0.7% and 16% for GD2-s positive PBSC (Fig. 1B and F, respectively).

As shown in Fig. 1C and G, the total number of TH and GD2 transcripts infused in the patients varied between 0 and 4,890 × 10⁶ (median = 0 × 10⁶) and between 0 and 816 × 10⁶ (median = 52 × 10⁶), respectively. The total amount of tumor cells infused in the patients, calculated by taking IMR-32 neuroblastoma cells as reference, varied between 0 and 575 × 10⁶ (median = 0 × 10⁶) and between 0 and 865 × 10⁶ (median = 47 × 10⁶), when either TH (Fig. 1D) or GD2-s (Fig. 1H) was considered. For the sake of simplicity, only the total number of hemopoietic cells and the number of TH-positive and GD2-s positive cells infused in the patients, calculated by taking IMR-32 cells as reference, were reported in Table 1. The number of CD34+ cells infused with the PBSC ranged between 2.1 × 10⁶/kg and 22 × 10⁶/kg (median = 4.3 × 10⁶/kg).

Event-free and overall survival analysis. Within 5 years after myeloablative therapy (range = 4.2-58.5 months, median = 14.3 months), 15 children (56%) relapsed, and 13 (48%) eventually died because of tumor progression (Table 1). The 5-year event-free survival of the entire cohort was 36.9% (SE = 0.11), and the overall survival was 48.0% (SE = 0.10). Only one relapsed patient died after the 5-year follow-up, and this event did not contribute to survival analysis.

Four (80%) of the five patients rescued with a TH-positive/GD2-s-positive PBSC, and 9 (64%) of the 14 with a TH-negative/GD2-s-negative PBSC relapsed. The only patient (no. 10; Table 1) with a TH-positive/GD2-s-negative PBSC never relapsed and is still alive 72.3 months after myeloablative therapy. Survival of the six patients with TH-positive PBSC was not different from that of the 21 rescued with TH-negative PBSC (Fig. 2A and B; log-rank test, P = 0.857 for event-free survival and P = 0.992 for overall survival). When survival analysis was based on GD2-s results, no difference was found in overall survival (Fig. 2D; log-rank test P = 0.110) between patients rescued with GD2-s-negative (19 patients) or GD2-s-positive PBSC (8 patients), whereas a borderline negative effect on event-free survival of GD2-s-positive PBSC was found.
(Fig. 2C; log-rank test, $P = 0.053$). When the TH and GD2-s results were pooled, the survival of the 20 patients with positive PBSC was not different from that of the seven patients with negative PBSC (Kaplan-Meier plot not shown; log-rank test, $P = 0.134$ and $P = 0.218$ for event-free survival and overall survival, respectively). Similar results were obtained when survival analysis was done by considering the relative contamination, evaluated by means of the $2^{-\Delta\Delta Ct}$ method (21), or by considering the total number of transcripts or the total number of tumor cells infused in each patient (data not shown).

Finally, survival analysis was also done taking into account the clinical status at transplant, as assessed by the International Neuroblastoma Response Criteria (18). Among the 15 patients transplanted while in partial remission and rescued with contaminated or clean PBSC, no difference was found in both event-free survival and overall survival when TH and/or GD2-s results were considered (Kaplan-Meier plots not shown; log-rank test, $P = 0.543$ and $P = 0.533$ for GD2-s and $P = 0.787$ and $P = 0.653$ for TH, respectively). Similarly, among the 12 children rescued while in complete remission, no difference was found in overall survival when the TH or the GD2-s status of their PBSC was considered (Kaplan-Meier plot not shown; log-rank test, $P = 0.575$ and $P = 0.112$, respectively).

Conversely, the patients rescued while in complete remission with GD2-s-negative PBSC had a borderline better event-free survival than those receiving GD2-s-positive ones (Kaplan-Meier plots not shown; log-rank test, $P = 0.061$). No difference was found in event-free survival of patients rescued while in complete remission by considering the TH status of PBSC (Kaplan-Meier plots not shown; log-rank test, $P = 0.852$).

**Discussion**

This study, although based on a small sample size (27 patients), suggests that the presence of tumor cells in autologous unselected PBSC infused in first-line responder stage IV neuroblastoma patients may not have an effect on survival. At variance with previous reports (1–7, 9), in this study, neuroblasts were detected using two sensitive quantitative RT-PCR assays (16, 17). Moreover, a homogeneous patient population together with the clinical status at transplant was considered.

In our study, we did not document any effect of the TH and/or GD2-s status of PBSC on probability of event-free survival.
and overall survival, regardless of the clinical status at transplant, the only exception being a better, but not significant, event-free survival of the patients rescued with GD2-s-negative PBSC while in complete remission. Given the small size of the cohort, cautiousness in interpreting the results is needed until future, multicenter study, such as the one within the E-SIOP-NB High-Risk protocol, will provide more data.

Our study confirmed a previous report (9) on a larger but heterogeneous cohort of neuroblastoma patients, showing no correlation between survival and PBSC contamination, evaluated by means of anti-GD2 immunocytochemistry and qualitative RT-PCR for TH and pgp9.5 genes. Here, because all the PBSC tested negative by immunocytochemistry, we were unable to compare the somehow high number of TH- and GD2-s-positive cells with a precise number of neuroblasts. However, it is conceivable that the use of a single cell line as reference may account for error in estimating the number of tumor cells. In fact, the number of TH and GD2-s transcripts per ng of total RNA found by us in a panel of 11 neuroblastoma cell lines ranged between 0 and 485 and between 28 and 726, respectively, supporting the possibility that PBSC-contaminating tumor cells expressed higher number of transcripts than the IMR-32 cells (18 and 28 transcripts per ng RNA of TH and GD2-s, respectively).

The results presented here do not confirm those by Handgretinger et al. (15) who showed a favorable effect of the infusion of tumor cell–contaminated CD34+ cells on stage IV patients survival. In that study (15), contaminant neuroblasts were detected by anti-GD2 immunofluorescence, and it was suggested that systemic administration of low number of tumor cells during hematopoietic reconstitution induced an effective anti-neuroblastoma immune response. Discrepancy between our and Handgretinger's results (15) may be due to cross antigen presentation of certain CD34 epitopes found in primary neuroblastoma cells and cell lines (22, 23). However, our and Handgretinger's (15) studies can not be easily compared because different techniques to detect neuroblasts have been used. In this respect, it is of note that RT-PCR analysis has been reported (9, 16, 17) to perform better than immunofluorescence at detecting neuroblasts in peripheral blood and PBSC samples.

Several previous studies based on smaller and heterogeneous cohorts (1–7) had shown borderline negative effect of PBSC contamination on patients' survival, as reported here when the GD2-s results were considered. However, by taking into account the clinical status at transplant, this effect was confirmed only for patients rescued while in complete remission. Thus, the present results do not support the need for a generalized use of purging procedures, also in view of the increased risk of EBV-related lymphoproliferative disorders in CD34+-transplanted patients (24).

TH and GD2-s quantitative RT-PCR assays have already been used to detect neuroblastoma cell contamination (19, 20, 25–28); however, this is the first study in which the two assays were done simultaneously on PBSC samples. The concordance between the TH and GD2-s results was limited because 14 of 19 GD2-s-positive PBSC were TH negative. Such discrepancy may be due to the higher specificity of the TH assay or to its lower sensitivity (16, 17). Recently, Swerts et al. (29) reported that GD2-s was less sensitive than TH, but they used different primers and probes, studied bone marrow samples, and considered results obtained by immunocytochemistry as golden standard. However, in accordance with previous reports (9, 16, 17), immunocytochemistry did not provide relevant information regarding PBSC contamination in this study. Further quality-controlled multicenter studies will be necessary to identify the best gene marker for detection of neuroblasts in patients' samples.

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References

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