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REVIEW

Relevance and Clinical Implications of Tumor Cell Mobilization in the Autologous Transplant Setting

John F. DiPersio,[1] Anthony D. Ho,[2] Jessie Hanrahan,[3] Frank J. Hsu,[3] Stefan Fruehauf[4]

Autologous transplantation of peripheral blood (PB) hematopoietic stem cells (HSCs) is a widely used strategy for reconstitution of blood cells following high-dose chemotherapy for hematologic malignancies such as multiple myeloma (MM), non-Hodgkin lymphoma (NHL), and acute myeloid leukemia (AML), among others. Stem cells for transplantation are usually obtained from PB after treatment with chemotherapy with or without cytokine, usually granulocyte-colony stimulating factor (G-CSF), or after treatment with cytokine alone. The use of autologous peripheral blood stem cells (PBSCs) for transplantation is associated with the risk of contamination of the graft with tumor cells; whether this impacts response rates, progression-free survival (PFS), and overall survival (OS) is still debatable. This review summarizes the controversy surrounding tumor cell mobilization (TCM), the complexity of detection of minimal residual diseases, the available diagnostic tools, differences in TCM with available mobilization regimens, and the potential effect of TCM on clinical outcome. Collectively, these data suggest that new treatment paradigms to manage hematologic malignancies, such as MM, NHL, and AML, are needed and should focus on increasing the chemosensitivity of the tumor and eliminating residual disease.

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INTRODUCTION

Tumor cells can be detected in the peripheral blood (PB) of patients with various cancer types and can contaminate autologous stem cell products derived from the bone marrow (BM) or from apheresis [1-3]. Standard cancer treatment such as chemotherapy or radiation might by itself induce significant mobilization of tumor cells into the PB, and has been reported to be associated with metastases [4]. Similarly, common methods employed to mobilize peripheral blood stem cells (PBSCs) such as chemotherapy and the use of agents such as cytokines might also mobilize tumor cells and lead to significant contamination of the apheresis products [4-6].

Given the low number of tumor cells compared with normal cells and the heterogeneity of tumor cells, their detection presents a major challenge. Tumor cell detection is further complicated by the fact that very few tumor-specific cellular markers exist, and many of the available assays are exploratory in nature. Additionally, the sensitivity and specificity of the detection methods vary enormously and are highly dependent upon the methods employed and the source of the cells analyzed (eg, BM sample, apheresis sample, PB sample).

It is possible that tumor cells in the reinfused apheresis product could lead to relapse. In patients with acute myeloid leukemia (AML) or neuroblastoma who relapsed after receiving genetically marked autologous BM cells for autologous stem cell transplantation, the genetic marker (neomycin-resistant gene) could be detected by polymerase chain reaction (PCR) in the relapsed malignant cells, suggesting that contaminating malignant cells in the autologous stem cell product contributed, at least in part, to relapse after transplant [7]. A genetic marking study showed that Ph+ cells present in autologous transplants of patients with chronic myelogenous leukemia can contribute to relapse after transplantation [8]. Nevertheless, these studies do not rule out the likely

From [1]Washington University, St. Louis, Missouri; [2]Department of Internal Medicine V, University of Heidelberg, Heidelberg, Germany; [3]Genzyme Corporation, Cambridge, Massachusetts; and [4]Center for Tumor Diagnostics and Therapy, Paracelsus Klinik, Osnabrück, Germany.

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Correspondence and reprint requests: John F. DiPersio, MD, PhD, Division of Oncology, Siteman Cancer Center, Washington University School of Medicine, 550 S. Euclid Ave., St. Louis, MO 63101 (e-mail: jdipersi@im.wustl.edu).

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possibility that the majority of malignant cells that are detected at the time of relapse after autologous stem cell transplantation are because of the regrowth of residual disease following incomplete eradication of tumor cells by high-dose chemotherapy and radiation therapy [9,10]. This review, with a focus on hematologic malignancies such as multiple myeloma (MM), non-Hodgkin lymphoma (NHL), and AML, provides an overview of the methods used for detection of malignant cells in these entities, and of the levels of tumor cell contamination found following the more common methods of mobilization, including chemotherapy, cytokines, and the newly approved mobilizing agent, plerixafor.

METHODS OF TUMOR CELL DETECTION

Multiple methods have been developed for the detection of tumor cell mobilization, including immunocytochemistry [11-13], cell culture [14], flow cytometry, and PCR (both allele-specific oligonucleotide [ASO] and quantitative real-time assay for tumor-associated markers). The sensitivity of the tumor cell mobilization (TCM) assays can be increased by analyzing multiple samples, increasing the number of cells tested in the sample, and employing sensitive reverse-transcriptase PCR methods (RT-PCR). Flow cytometry is considered a low-sensitivity method capable of detecting 1 tumor cell per 1000 PBMCs [15]. Comparatively, quantitative PCR methods are highly sensitive, detecting as few as 1 tumor cell per million PBMCs. Ultimately, the applicability of each method will be determined by the level of tumor contamination considered to be clinically relevant and the detection limits of each assay.

Flow Cytometry

Malignancies such as MM arise from a monoclonal population of B cells, and therefore each tumor cell expresses a unique immunoglobulin (Ig) molecule. In addition, plasma cells from patients with MM generally have an abnormal DNA content (aneuploidy) and an increased RNA content [16-18].

In this detection method, cells are labeled with anti-Ig light chain and/or anti-Ig heavy chain antibodies conjugated to fluorochromes, and the DNA is stained. The cells are then analyzed by flow cytometry, which allows for automated cellular analysis [19]. The results of this method can be easily analyzed, profiling normal cells versus tumor cells. The data from this method are presented as the percentage of Ig light chain cells (kappa and lambda), as a DNA index (ratio of $G_{1/0}$ DNA peaks of tumor and control cells) [20], and/or as an RNA index (ratio of mean RNA content of tumor $G_{1/0}$ cells to control lymphocytes) [18]. Detection of Ig light chain restriction and a DNA index of anything other than 1 is indicative of tumor cells. Other markers such as CD38, in combination with DNA content, can also be used in a similar manner to increase the specificity of the assay [21,22]. Additionally, as mentioned above, MM cells may have increased cytoplasmic immunoglobulin (CIg), which can be detected by flow cytometry [20]. These data are represented as a CIg index (ratio of median CIg fluorescence intensities of aneuploid and diploid G_1 cells from the same patient sample).

Recently, a monoclonal antibody that recognizes a 19-amino acid polypeptide containing a unique C-terminus of the nucleophosmin 1 (NPM1) mutated protein has been generated, which can be used to detect AML with this NPM1 mutation using immunohistochemistry [23].

Flow cytometry is highly reproducible, relatively inexpensive, and a quick throughput method [24]. However, flow cytometry may require large sample volumes [24], and the sensitivity of this assay is limited to approximately 1 tumor cell per 1000 PBMCs [25]. Because the technique of flow cytometry is to count cells labeled with a fluorochrome, for a low-frequency event such as tumor cell contamination, a large number of PBMCs must be counted in order to accurately detect low quantities of tumor cells. Even with modern high-speed machines, there are practical limits to the number of cells that can be analyzed by this method, because of the time required to perform these tests and the quantity of cells available. In addition, the specificity of the assay may be impacted by the reagents used and by their ability to accurately distinguish tumor cells from normal cells [24,26]. Immunophenotyping using multiple antibodies can increase the sensitivity of flow cytometry to $1{:}10^5$ [15], allowing for the detection of rare, contaminating aberrant plasma cells [15,27]. However, this can be technically challenging, as a large quantity of cells to be analyzed is required to achieve accurate results.

PCR Analysis

PCR can be used to detect the presence (or absence) of tumor cells. Tumor cells from a specific cancer type generally have unique DNA sequences, often resulting from gene rearrangements, which are absent in normal cells. The unique DNA sequence can be detected by designing specific PCR primers capable of amplifying the tumor-specific sequences of DNA. PCR has important advantages in that the method requires very little sample and is relatively simple to perform. Disadvantages of the PCR method include its high rate of false positives and the fact that very few gene sequences have been identified that may be broadly applied to a cancer indication (eg, *BCL-2* for certain lymphomas, *Bcr-Abl* for chronic myelogenous leukemia). In many instances, gene rearrangements and mutations are specific to an individual patient's

tumor and are not applicable to other patients with the same type of cancer. In these cases, a patient-specific detection test must be developed for each individual. The detection sensitivity of PCR is 1 tumor cell per 10^5 to 10^7 PBMCs [24].

Patient-specific PCR to detect TCM in multiple myeloma and NHL

In MM and B cell NHL, malignant cells arise from a single B cell clone. During B cell ontogeny, a functional immunoglobulin (Ig) heavy chain gene complex is formed. The formation of the Ig heavy chain results in a unique gene sequence specific to the B cell clone from which it arose [28,29]. Mutational events occur at the junctional splice sites and often within the complementarity determining regions (CDRs). The CDRs represent primary contact points for antigen interaction; these "binding sites" are often subject to significant mutational events following antigen exposure. The CDR3 region of the Ig heavy chain variable gene spans 3 different Ig gene segments: VH-D-JH. Because of the enormous genetic diversity of this region, as well as of the other CDRs, these Ig heavy chain variable gene segments represent true tumor-specific markers. All tumor cells in an individual patient share an identical rearranged Ig gene sequence, resulting from rearrangements of the VH, D, and JH gene segments [28,29]. B cell–specific primers, which are patient specific, can be designed to these gene segments and used to detect tumor cells by sensitive PCR techniques.

Quantitative PCR can be performed to amplify the CDR3 region of the clonally rearranged VDJ segments of the Ig heavy chain gene [30,31] using allele-specific, and therefore patient-specific, oligonucleotides (ASO-PCR) [2,32,33]. ASO-PCR can be used to specifically detect these unique DNA sequences and assess the mobilization of MM tumor cells [34]. The quantitative ASO-PCR method determines the frequency of tumor genes with respect to the total DNA content. This result is then used to calculate the tumor cell frequency compared with total PBMCs. Using the total PBMC concentration, the total tumor cell concentration can be calculated. The detection sensitivity of this method is $1{:}10^4$ cells [24] to $1{:}10^6$ cells [24,34,35].

To perform this assay, a sample of tumor must be obtained, the Ig VH gene segment must be sequenced, and tumor-specific primers must be designed and tested beforehand. Once the PCR detection assay is established, total genomic DNA is isolated from PB, apheresis product, or other sample. The isolated DNA and the gene-specific primers are then used in a PCR reaction. Quantitative PCR methods can be used to determine the level of contamination within a sample, or in the case of no DNA amplification, to indicate the absence of tumor cells at the level of the sensitivity of the assay. This method is extremely laborious (requiring original patient tumor samples and primers designed for each patient), technically challenging, and expensive. ASO-PCR, however, is highly sensitive, tumor-specific, and requires a small sample.

PCR for chromosomal translocations to detect TCM in NHL

Approximately 80% of patients with NHL with follicular histology have a translocation t(14;18) of the *BCL-2* gene [36]. The presence of this translocation can be exploited through the use of PCR in diagnostic settings [37-39].

Approximately 60% to 70% of breakpoints fall within a well-defined area, known as the major breakpoint region (MBR) of the 3′-untranslated part of the *BCL-2* gene. Using primers that flank the most common consensus regions of the MBR and modified to fluoresce when hybridized with complementary DNA, PCR can be used to detect *BCL-2* gene rearrangements [26]. Methods such as quantitative real-time PCR can be used to detect translocations down to 0.002%, or 1/50,000 cells.

For this assay, total genomic DNA is isolated from PB, apheresis product, or other sample. The isolated DNA and the gene-specific primers are then used in a quantitative PCR reaction; the amplification of the β-actin gene is used as a control. Standard curves are calculated for the *BCL-2* rearrangement and β-actin and are used to quantify DNA within each sample. These curves control for PCR efficiency and detection limits. A typical lower limit of detection for this assay is 0.01 ng of tumor DNA in a 1-μg DNA sample per PCR, which correlates to approximately 2 tumor cells per PCR. If no *BCL-2* amplification is detected in the PCR product, this indicates the absence of the t(14;18) translocation in the MBR of the *BCL-2* gene or that levels are below the limit of detection.

This method is highly sensitive, and commercial primers are available that can be used for all patients. However, rearrangements involving other regions, including the minor breakpoint cluster, are not detectable with this assay. Additionally, given that the translocation is not found in all patients with NHL, this method is not applicable to all patients and could lead to false negatives. Another disadvantage of this method is that the t(14;18) translocation is observed with increasing frequency in normal lymphocytes as a function of age [40].

PCR to Detect TCM in AML

Aberrations in the *NPM1* gene, especially *NPM1-mutA*, represent the most common gene alteration in AML [41,42]. *NPM1* mutations display stability and high prevalence over the course of the disease. Investigators have shown that an ASO-RT-PCR assay

can provide rapid detection of *NPM1-mutA*. In a study that included 52 patients with AML, ASO-RT-PCR detected *NPM1-mutA* in 21 patients, with a sensitivity of 1:100 and, using a seminested method that incorporated a forward primer to enhance sensitivity, 1:100,000 [42]. It should be pointed out, however, that this assay has not been properly validated to detect NPM; consequently, other mutations, even in mutA, may render the assay nonfunctional. The translocation t(8;16)(p11;p13), which is associated with myelomonocytic differentiation, represents another common genetic abnormality in AML cells that may be detected with RT-PCR [43]. Fms-like tyrosine kinase-internal tandem duplication (Flt3-ITD)-positive AML cells can also be detected by PCR, but no clinical correlation has been made associating the presence of contaminating flt3-ITD–positive AML cells with the outcome of autologous stem cell transplantation. This is in contrast to contamination of autologous stem cell products with acute promyelocytic leukemia (APL) cells harboring the *PML-RAR-α* fusion gene, which can be detected by RT-PCR. The presence of contaminating *PML-RAR-α*–positive APL in autologous mobilized PBMC has been associated with decreased leukemia-free survival after autologous stem cell transplantation [44]. No studies have demonstrated that either granulocyte colony-stimulating factor (G-CSF) or chemotherapy plus G-CSF has a differential effect of mobilizing AML cells carrying NPM1, flt3-ITD mutations, or *PML-RAR-α* fusion genes.

PCR to Detect TCM in Neuroblastoma

In a study of 11 patients with neuroblastoma or Ewing's sarcomas, RT-PCR was used to detect neuroblastoma-specific antigens (MoAb14-2Ga, UJ 127.11, 5.1.H11, and Anti-Thy-1) in PBSC and BM harvests [45]. Immediately before harvest, clinical histology revealed no tumor cells in BM. Yet tumor cells were detected by RT-PCR and immunochemistry assays in 9 of 11 (82%) of PBSC harvests and in 8 of 9 (89%) of the BM harvests. Moreover, PBSC mobilization with cyclophosphamide and G-CSF did not affect the incidence or level of contamination in PBSCs.

Other investigators have used RT-PCR to examine the expression of additional markers that are highly expressed in neuroblastoma—tyrosine hydroxylase (TH), neuroendocrine protein gene product 9.5, ElAVL-4, and GD2 synthetase [46]. In this analysis, TH was shown to be the most specific marker for the detection of neuroblastoma.

Tumor Cell Contamination of PB Prior to Mobilization

Tumor cells can be found circulating in the PB of patients with both hematologic malignancies and solid cancers, especially when the cancer is measurable and not in a minimal disease state. One study showed a wide variation prior to treatment (ie, any chemotherapy) in the frequency/number of circulating tumor cells in PB of patients with MM. Among 14 patients with MM, 13 tested positive for the presence of tumor cells by ASO-PCR, with tumor cell frequency varying for 12 patients from 0.001% to 1.0% and 32% for 1 patient [2,3]. In another study of 23 patients with MM, all patients had detectable circulating plasma cells with a range of 0.1% to 1.8% of mononuclear cells (MNC) fraction [47]. Some studies have reported a correlation of tumor burden and stage of disease with the incidence of circulating tumor cells [48-52]; others, however, have reported that the incidence of tumor cells in PB in certain cancers such as MM appeared to be independent of BM tumor burden and stage of disease [3,53].

Tumor Contamination of Stem Cell Products After Mobilization

Tumor cell contamination of PBSC products routinely occurs and has been reported following the use of all common mobilization methods [54]. A wide variation in the frequency of tumor cell levels, ranging from 0.01% to >10%, has been reported in virtually all mobilized PBSC harvests [55]. The kinetics of tumor cell mobilization, the underlying residual tumor burden in the patient, and the mobilization method used are important factors that impact the contamination of the apheresis product. Several groups have reported that higher proportions of hematopoietic progenitor cells are observed early during apheresis (ie, the first 2 days), whereas peak levels of myeloma cells occur in the later stage of apheresis [1,56]. However, other studies have found no variation in the kinetics of tumor cell and $CD34^+$ stem cell mobilization [27,37,57,58].

In addition to G-CSF, several agents have been shown to mobilize PBSC, including pegylated G-CSF (pegfilgrastim), natalizumab, thrombopoietin, and chemokines such as stem cell factor, macrophage inflammatory protein-1α, interleukin-8, and stromal cell-derived factor-1 (SDF-1) analog, among others [59,60]. However, the only Federal Drug Administration–approved agents are G-CSF, granulocyte-macrophage colony stimulating factor (GM-CSF), and the chemokine receptor 4 (CXCR4) antagonist plerixafor.

Chemomobilization

Prior to the development of cytokines for stem cell mobilization, it was noted that there were transient increases in hematopoietic stem cells in PB following the administration of myelosuppressive chemotherapy [61,62]. The earliest protocols for the mobilization of stem cells used chemotherapy alone [63,64], and later protocols used chemotherapy followed by daily

administration of cytokines (G-CSF or GM-CSF) [65-67]. In general, chemotherapeutic agents induce stem cell mobilization by reducing BM hematopoietic cell reserves and forcing stem cell expansion and hematopoiesis [68].

Studies have shown that patients with MM have between 0.1% and 1% clonal B cells in hematopoietic stem cell collections following chemomobilization, resulting in the infusion of 0.5 to 5 $\times$ 10^8 myeloma cells with each transplant [47,55,69,70]. A recent study by Kopp et al. [6] examined the amount of tumor cell contamination in the stem cell product and the resulting outcome following transplantation. The study reported that 27% of stem cell products from 60 patients with MM were contaminated with large quantities of tumor cells (>4.5 $\times$ 10^5 cells/kg) following mobilization with chemotherapy or chemotherapy plus G-CSF, and 73% of stem cell products were contaminated with low levels of tumor cells (≤4.5 $\times$ 10^5 cells/kg). A study by Ho et al. [71] on the presence of contaminating tumor cells in PBSC and its influence on outcomes showed tumor contamination of the PBSC graft in 36% (32/89) of patients with NHL and in 25% (17/69) of patients with MM following chemomobilization using varying regimens (Table 1).

Mobilization with cytokines

The use of cytokines (most commonly, G-CSF and GM-CSF) began in the late 1980s to early 1990s following the report of the mobilizing effects of these agents [66,67]. A later report described long-term hematopoietic reconstitution in patients transplanted with only cytokine-mobilized PBSCs [76]. The most commonly used cytokine, G-CSF, works by granulocyte expansion/activation, protease release, and cleavage of adhesion molecules [68,77].

Tumor contamination of the PB apheresis product has also been noted in patients receiving only cytokines for mobilization. In a retrospective study of 61 patients with NHL, PBSC samples were examined from 26 patients who did not receive a mobilizing regimen and 35 patients who had mobilization of stem cells with cytokines (22 with G-CSF and 12 with GM-CSF) [72]. Among patients in the cytokine-mobilized group, 37% had tumor contamination in the apheresis product, compared with 19% of the nonmobilized group, although this difference was not statistically significant.

Studies comparing mobilization methods

An early study in 8 patients with MM compared the tumor load of the apheresis product following mobilization with G-CSF and following mobilization with G-CSF plus cyclophosphamide [34]. This study concluded that mobilization by cyclophosphamide plus G-CSF led to a lower number of malignant cells per $CD34^+$ cells in the apheresis product compared with G-CSF alone. A later study that examined response and survival rates with various treatment regimens in 135 patients with MM reported graft contamination with monoclonal plasma cells following mobilization of stem cells. Of the 102 patients receiving G-CSF alone, 25% had tumor contamination, and of the 33 patients receiving chemomobilization, tumor contamination was noted in 51% [5]. In a randomized study of 47 patients with lymphoma, PCR tumor cell data were available for 22 patients with NHL; 11 patients received mobilization with G-CSF alone, and 10 patients received chemotherapy and G-CSF [73]. In both groups, a high percentage of tumor contamination of the harvested PBSC product was seen (in 92% and 90%, respectively) (Table 1).

Plerixafor plus G-CSF

Recently approved for marketing in the United Sates for use with G-CSF for stem cell mobilization, plerixafor is a selective and reversible antagonist of CXCR4, disrupting its interaction with SDF-1 and thereby releasing hematopoietic stem cells into the circulation.

Plerixafor is not approved for stem cell mobilization in patients with leukemia, owing to a potential for mobilizing leukemic cells. This very property is being explored in investigational trials to determine whether plerixafor can sensitize leukemic cells to chemotherapy and is an area of development [78-82]. The effect of plerixafor plus G-CSF on tumor cell contamination has been investigated in patients with NHL [76,83,84] and those with MM [74,84]. Samples were collected for the evaluation of tumor cell contamination from 11 patients with NHL [75] and from 10 patients [84] and 7 patients [74] with MM who were in plerixafor clinical trials. In the samples of 11 patients with NHL, 5 received G-CSF alone and 6 received plerixafor plus G-CSF. Samples were analyzed by quantitative PCR for *BCL-2* translocation [75]. Of these NHL patients, 10 had undetectable levels of translocated *BCL-2* in the apheresis product, and 1 patient who received G-CSF alone had detectable levels (Table 1).

Tricot et al. [84] analyzed PB samples by flow cytometry in 10 patients with MM. A total of 9 paired samples were available for analysis before and after the first and second dose of plerixafor. None of the PB samples showed >1% light chain–restricted cells, except for the first apheresis product of 1 patient, who had 2% kappa cells with a DNA index of 1 and 1% lambda cells with a DNA index of 1. Plasma cells with aneuploid DNA content were not observed in any of the samples.

In the 7 patients with MM, samples for PBMC were obtained at baseline prior to any therapy, following G-CSF and before plerixafor administration, and

Table 1. Mobilization Methods and Tumor Cell Contamination of Apheresis Products

First Author (Reference)	Mobilization Method	Number of Patients/ Disease Type	Method for Detection of Tumor Cells	Percentage of Patients with Tumor Cells Detected	Level of Contamination
Gazitt [55]	Chemotherapy	10/MM	PCR	100% (10 patients)	<0.4-8400 $\times$ 10^4
Ho [71]	Chemotherapy	89/NHL 69/MM	PCR	36% of collections contaminated with NHL tumor cells 25% of collections contaminated with MM tumor cells	Reported as detectable or not detectable according to the method (method sensitive to detect 1:100,000 cells)
Kopp [6]	Chemotherapy and G-CSF	60/MM	Flow cytometry	27% of collections contaminated with high levels 73% of collections contaminated with low levels	$\leq$4.5 $\times$ 10^5 tumor cells/kg >4.5 $\times$ 10^5 tumor cells/kg
Lemoli [47]	Chemotherapy and G-CSF	23/MM	Flow cytometry	100% (23 patients)	Mean = 0.7% circulating myeloma cells (range: 0.2%-2.7%)
Schiller [69]	Chemotherapy and G-CSF	37 (14 had tumor contamination measured)/MM	PCR	57% (8/14)	0.23%-0.0015%
Demirkazik [72]	Cytokines	26/NHL (not mobilized) 35/NHL (mobilized)	PCR	37% of collections contained tumor contamination in the mobilized group compared with 19% in the nonmobilized group	G-CSF mobilized group: median GM-CFC $\times$ 10^4 kg/liter blood = 0.21 (0.017-5.8) Nonmobilized group: median GM-CFC $\times$ 10^4 kg/L blood = 0.0054 (0.0001300.072)
Anagnostopoulos [5]	G-CSF or chemotherapy	33/MM (G-CSF alone) 102/MM (chemotherapy)	Flow cytometry	25% of collections contaminated with tumor cells (G-CSF alone) 51% of collections contaminated with tumor cells (chemotherapy)	All patients: B-2 microglobulin median = 3.2 (range: 0.2-34.1)
Narayanasami [73]	G-CSF or chemotherapy and G-CSF	47/lymphoma (TCM data reported on 22 patients with NHL)	PCR	11/12 patients (92%) in G-CSF-alone arm 9/10 patients (90%) in chemotherapy + G-CSF arm	Not reported
Cremer [34]	G-CSF or chemotherapy plus and G-CSF	8/MM	PCR	All 8 patients had detectable malignant cells in the apheresis product	0.0006%- 0.256%
Fruehauf [74]	Plerixafor plus G-CSF	7/MM	PCR	3/7 had detectable tumor cells; 4/7 had no detectable tumor cells	Observed fold change from pre-plerixafor to pre-apheresis: 0.11- to 1.08-fold
DiPersio [75]	Plerixafor plus G-CSF	11/NHL (5 G-CSF only; 6 plerixafor plus G-CSF)	PCR	1/5 G-CSF had detectable levels of tumor cells; 6/6 had no detectable levels of tumor cells	Not reported

G-CSF indicates granulocyte-colony stimulating factor; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; PCR, polymerase chain reaction; TCM, tumor cell mobilization.

then again following plerixafor and prior to apheresis. These samples were examined for TCM using a highly sensitive and tumor-specific CDR3 PCR test [74]. Consistent with other studies, G-CSF treatment alone resulted in an increase of mobilization of tumor cells over baseline. In contrast, after receiving plerixafor, 3 of 7 patients had slightly increased tumor cells in the PB compared to G-CSF alone, whereas 4 of 7 had decreased tumor cells. For all patients, the change in tumor cell level ranged from 0.11 to 1.08 times the pre-plerixafor level; therefore, there did not appear to be specific mobilization of tumor cells.

In summary, although the total number of patients examined overall was limited, there did not appear to be an increase of tumor cells in the blood or apheresis product following administration of plerixafor greater than that observed or expected with G-CSF. Thus, contamination of an apheresis product following mobilization with plerixafor and G-CSF would be expected to be similar to that occurring with standard G-CSF mobilization in patients with NHL or MM.

Effect of Tumor Cell Contamination on Clinical Outcomes

Early reports examining the presence of tumor contamination in PBSC products and clinical outcome (response and survival) of patients who undergo transplantation with these products have reached different conclusions regarding the clinical implications of this finding [69,85]. A recent study by Ho et al. examined NHL (n = 89) and MM (n = 69) patients undergoing autologous stem cell transplants (Table 2) [71]. The study compared the outcome of patients who underwent transplantation with hematopoietic stem cells (HSCs) free of tumor cells with that of patients who underwent transplantation with contaminated HSCs. The results of this study demonstrated that the contamination of HSCs with tumor cells had no influence on progression-free survival (PFS) or overall survival (OS), even when aggressive or indolent histologies are considered separately. Similarly, another study found that the tumor cell content of PB progenitor cell grafts was not predictive of clinical outcome in patients with diffuse B cell lymphoma and follicular lymphoma [86]. Finally, another study in 61 NHL patients found that lymphoma contamination of transplanted apheresis products had no apparent impact on event-free (EFS) and OS [72].

In contrast, other studies have reported poorer clinical outcomes in patients who underwent transplantation with HSCs contaminated with tumor cells [30,88,91,92,94]. The study by Kopp et al. [6] described a correlation between tumor contamination and outcome in patients with MM receiving chemomobilized stem cell products. The results of this study suggest that the level of tumor cell contamination correlated with a poorer OS. However, the level of tumor cell contamination was high ($>4.5 \times 10^5$ cell/kg). (If one assumes a patient weight of 85 kg and an apheresis cell concentration of approximately 100×10^6 PB mononuclear cells [PBMC] per mL, this correlates to roughly 4 tumor cells per 1000 PBMC.) In this study, lower quantities of tumor had no significant impact on PFS or OS. These findings indicate that the level of tumor cell contamination of the apheresis product needs to be very high in order to affect clinical outcome and that minimal or moderate amounts of contamination may not be clinically significant. Of note, the use of chemotherapy for mobilization did not reduce the subsequent collection of tumor cells or the purported impact on outcome.

In patients with AML, early (after first remission) and late autologous PB transplantations were shown to result in worse outcomes compared with BM transplantation in terms of survival without evidence of relapse or disease progression [95,96]. This difference was attributed to the possible recruitment of tumor cells after growth factor–driven mobilization, and in turn, to higher tumor cell contamination of the PB autographs [95]. There has been a trend away from the use of autologous transplantation for the treatment of AML [97]; thus, the impact of tumor cell contamination of the apheresis product is of diminishing relevance in current clinical practice.

In patients with NHL, such as refractory follicular lymphoma and mantle cell lymphoma, in vivo purging and stem cell mobilization with rituximab and high-dose cytarabine in conjunction with G-CSF have been shown to yield lymphoma-free PB progenitor cells (product of leukapheresis negative for Bcl-2 or Bcl-1) suitable for autologous transplantation [98-100]. Patients who underwent this process, which was preceded by debulking chemotherapy, experienced positive outcomes, with all patients still in complete clinical and molecular remission after a median follow-up of 8.5 months [98]. Rituximab has been incorporated into autologous PB transplantation protocols to minimize disease activity and provide effective in vivo purging [101].

Tumor cell contamination has driven the development of methods to reduce, or purge, the quantity of tumor cells within the stem cell product. These "purging" methods have included the direct removal of tumor cells by antibodies plus complement, by magnetic bead separation, exposure of the stem cell product to chemotherapy in vitro, or the selection and use of purified stem cells. Because malignant cells from patients with MM and NHL generally do not express CD34, positive selection of $CD34^+$ cells has been used to separate stem cells from tumor cells. Studies have demonstrated that this method markedly reduces tumor contamination by several logs (median 2 to 3 logs) [69,102-106]. However, despite large reductions

Table 2. Effect of Tumor Cell Contamination of Apheresis Products on Clinical Outcomes

First Author (Reference)	Mobilization Method	Number of Patients/Disease Type	Conclusions
Blystad [86]	Chemotherapy plus G-CSF	43/NHL	The level of contamination of the PBPC/$CD34^+$ cells ranged from 0% to 8.3%. No relationship could be shown between the total number of tumor cells infused and relapse. Patients receiving PCR-positive or PCR-negative PBPC grafts had similar progression-free survival ($P = .49$).
Bourhis [87]	Chemotherapy and G-CSF	111/MM	Despite significant tumor cell reduction, $CD34^+$ selection does not reduce RR and increases the risk of severe posttransplant infections. There was also no difference in RR between patients in either arm who received grafts with detectable tumor cells and those receiving grafts with no detectable tumor cells. This suggests that reinfused tumor cells may not be the main cause of relapse after autologous transplant in myeloma.
Brown [88]	Chemotherapy or chemotherapy and G-CSF	103/NHL	The only predictors of decreased progression-free survival proved to be histologic BM involvement at time of harvest (HR, 2.27, 95% CI, 1.3-3.9, $P < .004$) and PCR detectable disease in the BM product after purging (HR, 4.18, 95% CI, 1.99-8.8, $P = .0002$). No significant predictors of overall survival were identified.
Ho [71]	Chemotherapy	89/NHL 69/MM	Patients with MM or NHL with contaminating tumor cells in autologous PBSCs do not have worsened overall survival or progression-free survival. Tumor cells detected by sensitive molecular methods in PBSC collections may be distinct from cells contaminating marrow and appear to have limited utility in identifying patients with MM and B-cell NHL who would benefit from purging strategies.
Kopp [6]	Chemotherapy and G-CSF	60/MM	Patients with $>4.5 \times 10^5$ plasma cells/kg contaminating the PBSC graft received after high-dose chemotherapy have a significantly reduced overall survival. Whether high contamination of grafts with plasma cells might reflect residual in vivo tumor mass prior to stem cell transplantation and a generally more aggressive behavior of malignant myeloma cells in these patients, or whether reinfused plasma cells contribute to an unfavorable course of disease remains to be determined.
Kornnacker [89]	G-CSF or chemotherapy and G-CSF	241/NHL	Patients with disease recurrence within 1 year after transplant and those who had received autoSCT as second-line treatment had significantly reduced survival by multivariate analysis, whereas FLIPI score, age, remission status at autoSCT, high-dose regimen, and ex vivo purging had no impact.
Lopez-Perez [30]	G-CSF alone (12 patients) and chemotherapy plus G-CSF (11 patients)	23/MM	Patients with clonally free products were more likely to obtain complete response following transplant (64% versus 17%, $P = .02$) and a longer progression-free survival (40 months in patients transplanted with polyclonal products versus 20 months with monoclonal products, $P = .03$). These results were consistent when the overall survival was considered, because it was better in those patients with negative apheresis than in those with positive (83% versus 36% at 5 years from diagnosis, $P = .01$). These findings indicate that the presence of clonality rearranged V, D, JH segments is related to the response and outcome in MM transplanted patients.
Schiller [69]	Chemotherapy plus G-CSF	37/MM	PBPCs are an effective form of purified hematopoietic support achieving substantial reduction in myeloma cell contamination. At infused cell doses of greater than 2×10^6 CD34′ cells/kg, this product provides safe, rapid, and sustained hematologic recovery in patients receiving myeloablative chemotherapy. Whether this form of purified stem cell support will produce improved progression-free survival in patients with MM will require further trials.
Stewart [90]	Chemotherapy and G-CSF	190/MM	This phase 3 trial demonstrated that although CD34 selection significantly reduces myeloma cell contamination in PBPC collections, no improvement in disease-free or overall survival was achieved.
Vogel [91]	Chemotherapy and G-CSF	74/MM	Patients with graft contamination of $>4.5 \times 10^5$ plasma cells kg^{-1} had a high risk of early disease progression after high-dose chemotherapy.
Vose [92]	G-CSF alone	93/NHL	Patients with aggressive NHL receiving HSC transplantation randomized to PBSC transplantation versus BM harvest demonstrated improved neutrophil engraftment and platelet and RBC transfusion independence. The complete response rate and event-free survival were not statistically different by randomization arm. Patients whose harvests were positive for minimal residual disease by molecular analysis had poorer event-free survival.
Williams [93]	Not reported	224/NHL	Time to hematologic engraftment, response to autologous BM transplantation, and number of procedure-related deaths were similar in purged and unpurged patients. The overall survival rate was 54% at 5 years in purged patients and 48.3% in unpurged patients ($P = .1813$). The progression-free survival rate was 44.3% and 44.6%, respectively ($P = .1961$). Patterns of relapse, including bone marrow relapse, were similar in both groups.

BM indicates bone marrow; CI, confidence interval; FLIPI, Follicular Lymphoma International Prognostic Index; HR, hazard ratio; HSC, hematopoietic stem cells, MM, multiple myeloma; NHL, non-Hodgkin lymphoma; PBPC, peripheral blood progenitor cell; PCR, polymerase chain reaction; RR, response rate; PBSC, peripheral blood stem cell.

of tumor burden, long-term follow-up has not demonstrated that purging leads to clinical benefit [89].

Two phase 3 randomized trials compared patients with MM who received autografts purged of tumor cells ($CD34^+$-selected PBSC) with those with unselected PBSCs [87,90]. Both studies used a highly sensitive, tumor-specific PCR technique to identify the Ig heavy chain sequence of the myeloma clone and determine the level of tumor cell contamination. Both studies achieved a large reduction in tumor burden (approximately 1 to 6 log tumor load reduction), but no significant differences were observed between the 2 groups in terms of response, EFS/PFS, or OS.

Given these findings, the authors of each of these phase 3 studies speculated that reinfused myeloma cells may not be a significant cause of relapse, but that failure to eradicate the disease within the patient with high-dose chemotherapy may be a stronger contributor to relapse. Findings showing allogeneic (and therefore tumor-free) BM transplants for myeloma following which patients had a high risk of relapse approaching 50% [107] likewise suggest that improved disease eradication within the patient may be a more important goal than purging tumor cells from the reinfused product. Similar clinical outcomes and results have been reported for patients with NHL who received tumor-purged products in a case-matched comparison study using the European Group for Blood and Marrow Transplantation (EBMT) registry [93].

Mechanism of tumor cell mobilization and metastatic potential

Tumor cells appear to increase, or are "mobilized," into the PB following treatment with standard chemotherapy and radiation [4]. In preclinical models, Biswas et al. [4] demonstrated that when established tumors are treated with chemotherapy or radiation, an increase in circulating tumor cells could be measured, and that this increase in tumor cells results in increased lung metastases. The observed tumor cell mobilization and metastatic effect was blocked by neutralizing antibodies to transforming growth factor-beta (TGF-β). TGF-β is known to be a critical inducer of epithelial mesenchymal transition (EMT), which causes a morphologic and physiologic change in the tumor cell, leading to increased migration and metastases. Interestingly, in studies in which putative breast cancer stem cells have been identified, TGF-β has been demonstrated to have similar effects on the induction of EMT in these cells [108]. Therefore, tumor cell mobilization appears to occur following standard cancer treatments, and although other factors may be involved, this effect appears to be primarily driven by the prometastatic effect of TGF-β.

The tumor microenvironment is now recognized as a key factor in the growth of tumor cells. This symbiotic relationship includes the production of cytokines such as TGF-β and vascular endothelial growth factor (VEGF) by the tumor that stimulate the production of stroma, blood vessels, and other factors that provide the tumor with a supportive microenvironment in which to grow. Stromal cells produce and express SDF-1, which engage tumor cell CXCR4 receptors and induce antiapoptotic signaling. Several groups have demonstrated that blockade of CXCR4 with plerixafor or similar agents can sensitize tumor cells to chemotherapy, increase their effectiveness, and lead to prolonged survival of animals [109-113]. These data are the basis for ongoing clinical studies investigating the use of plerixafor in combination with chemotherapy or biological treatments for AML and chronic lymphocytic leukemia. Therefore, even though tumor cells might migrate/mobilize under certain conditions such as CXCR4 inhibition, they may readily undergo apoptosis, and their metastatic potential may be diminished.

CONCLUSION

The detection of tumor cells continues to remain extremely difficult, given the low number of tumor cells compared with normal cells and the large variety of potential tumor types. The sensitivity and specificity of the various tumor cell detection methods varies and is highly dependent on the method used and the source of the cells analyzed, making it difficult to compare results across studies published in the literature. Despite these limitations, it has long been recognized that tumor cell contamination occurs during the mobilization and collection of stem cells from patients with MM and lymphoma following G-CSF or chemomobilization. Tumor cell contamination following plerixafor administration appears to be similar or less than that following G-CSF mobilization alone when the same number of $CD34^+$ stem cells are collected.

Of critical importance is the relevance of tumor cell mobilization on clinical outcome. It is possible that tumor cells in the reinfused apheresis product could be responsible for relapse. However, it is more likely that relapse is because of the regrowth of residual cancer in the patient following incomplete eradication of tumor cells by high-dose chemotherapy and radiation therapy. The latter scenario is demonstrated by the significant relapse rate in cancer patients receiving "tumor-free" allogeneic stem cells from healthy donors and the fact that in autologous transplants, the most common sites of relapse are prior sites of disease [92]. In addition, phase 2 and large, randomized phase 3 studies have demonstrated that even a large reduction in the number of tumor cells in the apheresis product had no impact on clinical outcome as measured by disease-free survival or OS. In studies in which tumor cell contamination was associated with

poorer OS, the correlation was only significant when the level of tumor contamination was very high ($>4.5 \times 10^5$ cells/kg), as detected by flow cytometry. Finally, the reinfusion with tumor cells during autologous HSC transplantation in MM and NHL patients appears to have no influence on PFS or OS, even when aggressive or indolent histologies are considered separately. Collectively, these data suggest that improved treatments to control and eliminate residual tumor are needed and are critical for improving outcomes with stem cell transplantation. New therapies for hematologic malignancies, such as MM, NHL, and AML, should focus on increasing the chemosensitivity of the tumor and eliminating residual disease.

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