Detection of Germ Cell Tumor Cells in Apheresis Products Using Polymerase Chain Reaction

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ABSTRACT

The contamination of apheresis products with tumor cells was evaluated in patients undergoing autologous peripheral blood stem cell transplantation for germ cell tumors. A blinded, retrospective analysis was performed on 63 apheresis products from 28 patients using the PCR and primers for β human chorionic gonadotropin (β-HCG). Of the 20 patients with β-HCG-secreting tumors, 8 apheresis products from 7 patients were PCR positive. PCR was negative in the 8 patients whose tumors did not secrete β-HCG. Twenty-two apheresis products from patients with lymphoma and breast cancer were negative for β-HCG expression. Evaluating the 20 patients with β-HCG-secreting tumors, 100% of PCR-positive patients had elevated serum β-HCG at the time of apheresis compared to 46.2% of PCR-negative patients (P = 0.04). A positive PCR was also associated with a higher serum β-HCG at diagnosis (P = 0.03). Patients receiving a PCR-positive product had a higher relapse rate (85.7% versus 61.5%) and were more likely to have visceral metastasis (100% versus 61.5%), although the numbers did not reach statistical significance (P = 0.35 and 0.11, respectively). The finding of β-HCG mRNA in apheresis products strongly suggests the presence of circulating tumor cells in a significant number of germ cell patients undergoing autologous transplantation. This assay may be useful in monitoring attempts at tumor cell depletion and in developing improved prognostic models for assessing risk of relapse after transplantation.

INTRODUCTION

Although germ cell neoplasms are rare, they still rank as a leading cause of cancer death in young adult men. Clinical investigations in GCTs at Indiana University and elsewhere during the 1970s and early 1980s saw the development of well-tolerated effective cisplatin-combination chemotherapy for disseminated germ cell cancer (1). Also, initial attempts at developing predictive prognostic models allowed for the development of clinical trials based on risk of failure (2–3). For good-risk patients, trials sought to diminish therapy-related toxicities (4–7). For poor-risk patients, trials have sought treatments that improve therapeutic results. Examples include dose intensity, substitution of ifosfamide for bleomycin, additional drugs, or initial autologous transplantation (8–11).

For those patients who relapse after initial therapy, conventional-dose salvage chemotherapy results in durable complete remissions for only 20–30% of patients (1). Patients who relapse after salvage therapy or are platinum refractory are incurable with conventional-dose chemotherapy. The use of high-dose chemotherapy and autologous BMT has been successful in some of these refractory patients (12–15). Certain subsets of GCTs, in particular those refractory to platinum-based chemotherapy (progression within 4 weeks of recent platinum regimen) or nonseminomatous tumors arising in the mediastinum, only rarely benefit from autologous BMT or other salvage therapies (16, 17).

One potential cause of treatment failure after autologous transplantation for GCTs is the reinfection of cancer cells inadvertently collected at the time of stem cell collection. Contamination of bone marrow products has been shown to contribute to disease relapse after autologous transplantation for childhood acute myeloid leukemia and adult chronic myeloid leukemia using retroviral gene marking (18, 19). Whereas definitive evidence of relapse arising from transplantation of lymphoma cells will require similar marking studies, patients with detectable lymphoma cells in stem cell products have a significantly higher risk of relapse compared to individuals in which no detectable lymphoma cells are present (20). In breast cancer, tumor cell contamination of bone marrow and stem cell products is a common finding (21–23), although the ability of reinfused breast cancer cells to cause disease relapse has not been definitively shown (24).

In this study, we chose to evaluate apheresis products for evidence of GCT contamination. Tumor cell detection used a PCR-based method with primers specific for β-HCG mRNA. Patients with detectable tumor cells were compared to PCR-negative patients with regard to serum β-HCG levels at diag-

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3 The abbreviations used are: GCT, germ cell tumor; BMT, bone marrow transplantation; β-HCG, β human chorionic gonadotropin; AuSCT, autologous stem cell transplantation.
nosis, serum β-HCG at apheresis, sites of disease, and evidence of relapse after transplantation.

MATERIALS AND METHODS

Patient Samples. This study represents a retrospective analysis of patients undergoing AuSCT. Apheresis products were collected after a uniform mobilization protocol using daily s.c. granulocyte colony-stimulating factor (10 μg/kg/day) for 4 days before collection and daily during apheresis. Target cell numbers were ≥6.5 × 10⁸ mononuclear cells/kg for patients with lymphoma and ≥5 × 10⁸ MNC/kg for breast cancer patients. Germ cell patients were scheduled for tandem transplants, and ≥10 × 10⁶ mononuclear cells/kg were collected. For GCT patients, one to four apheresis samples were required to reach the target number of cells. Consent for use of the apheresis product in research studies was obtained at the time of stem cell collection.

Cell Isolation and PCR. RNA was isolated from 1-ml apheresis samples that had been stored in liquid nitrogen since the day of collection. For analysis, cells were thawed in a 37°C water bath, washed once in 100% fetal bovine serum, and resuspended in Iscove’s modified Dulbecco’s medium with 10% fetal bovine serum. Cells were enumerated, and 1–5 × 10⁶ viable cells were suspended in 1 ml of Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH). RNA was isolated and resuspended in 12 μl of diethyl pyrocarbonate treated water. Ten μl of isolated RNA were added to 90 μl of DNAse I solution (9 parts 10× DNAse I buffer: 1 part 18 units/μl DNase I; Boehringer Mannheim, Indianapolis, IN). After a 15-min incubation at room temperature, the RNA was purified using a High Pure filter tube (Boehringer Mannheim) and resuspended in 20 μl of diethyl pyrocarbonate-treated water. The integrity and quantity of RNA were verified by spectrophotometry and gel electrophoresis. One μg of RNA with 30 pmol of random hexamer primers (Promega, Madison, WI) were heated to 72°C for 2 min and then rapidly quenched on ice. To this mixture, 50 mM Tris-HCl, 30 mM KCl, 8 mM MgCl₂, 1 mM DTT (pH 8.5), 0.5 mM deoxynucleotide triphosphate, 0.75 unit of RNase inhibitor, and 300 units of avian myeloblastosis virus reverse transcriptase were added and incubated at 42°C for 1 h. After cDNA generation, the reverse transcriptase was inactivated by heating to 94°C for 5 min. Primary and secondary PCR included 35 cycles of 1 min of denaturation at 94°C, 1 min of primer annealing at 55°C, and 1.5 min of extension at 72°C after an initial denaturation for 5 min at 94°C. Ten μl of each cDNA product were placed in a sterile 0.5-ml PCR tube in a final volume of 100 μl with the following reaction conditions: 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 2.0 mM MgCl₂; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 500 pmol of each of the two oligonucleotide primers specific for β-HCG (gene products and 2.5 units of Taq polymerase (Perkin-Elmer Corp.)). Two sets of primer pairs specific for the majority of the β-HCG gene cluster (four of six) were designed. The first round primers were 5'-TCGGGTCACGGCCTCCTCT-3' (−351−335) and 5'-'AGGATGCGGGTGGTCCGA-3' (464/480), which amplified 831 bp from cDNA and 1416 bp from genomic DNA (25, 26). The nested PCR was initiated in this study by using a secondary pair of primers, 5'-ACATGGCGATACGAGAG-3' (44/61) and 5'-AGTCGGGATGACTTGGGA-3' (436/453), which gave a 410-bp band in cDNA and a 644-bp band in genomic DNA.

PCR Laboratory Procedures. The laboratory adheres to strict policies designed to decrease the likelihood of false positive results due to contamination by previously amplified DNA. To this end, samples were prepared in a dedicated PCR hood (clean room) and amplified and electrophoresed in a separate products room. Before initiating PCR work in either room, personnel are instructed to always wash hands and don gloves, to wear lab coats that have not come from other areas, and to decontaminate all equipment using bleach or UV irradiation. The PCR hood is decontaminated daily using bleach and UV irradiation before and after each use. Nucleic acids, RNA, and DNA from control or patient samples were stored separately from PCR reagents, and amplified products, which were never removed from the products room, were disposed of once results were obtained.

Statistical Analysis. Fisher’s exact tests were run to compare clinical characteristics (elevated serum β-HCG at apheresis, visceral metastasis, and relapse) and the PCR results among those patients with β-HCG-secreting tumors. The ability of serum β-HCG levels at diagnosis or at the time of apheresis to predict relapse was assessed with logistic regression (27) using relapse as the dependent variable and the log of serum β-HCG as the independent variable. t tests were done to compare serum β-HCG levels for PCR-positive and PCR-negative patients, and a log transformation was performed to correct for unequal variances.

RESULTS

PCR Amplification of β-HCG mRNA Using Nested Primers. Using PCR for detection of β-HCG expression is complicated by two factors: (a) β-HCG is a glycoprotein hormone with extensive homology with three other hormones, luteinizing hormone, follicule-stimulating hormone, and thyroïd-stimulating hormone. Differences in the 3’ portion of the β-HCG gene permit the design of specific PCR primers (25, 26), but the relatively small introns within the genomic sequence generate nested PCR products that vary by only 234–236 bp from the mRNA product (Fig. 1); and (b) a second factor to consider is the potential competition for primers between genomic and mRNA sequences. β-HCG is encoded within a multigene cluster composed of six homologous sequences, each containing three exons and two introns (26). Because we anticipate that very few cells in the peripheral blood express β-HCG...
mRNA, whereas all blood cells will contain six copies of the genomic sequence, primer competition may decrease the sensitivity of detecting small numbers of β-HCG-expressing cells. Therefore, we chose to DNase treat isolated RNA before reverse transcriptase treatment to decrease the potential for primer competition. The utility of this approach is shown in Fig. 2. PCR of cDNA from the β-HCG-expressing BeWo cell line generates an extra band of 644 bp corresponding to the genomic DNA band (Fig. 2, Lane 4, Sample w/o DNase I, positive apheresis sample after PCR amplification without DNase I treatment; Lane 5, Sample w/ DNase I, apheresis product shown in Lane 4 treated with DNase I before reverse transcriptase and PCR; Lane 6, BeWo genomic DNA, PCR of genomic DNA from BeWo cell line. B: β2M, PCR for β2-microglobulin as an RNA control.

Fig. 2 Reverse transcription-PCR of apheresis products using nested primers for β-HCG. A, comparison of signals obtained from apheresis samples with and without DNase I treatment. Lane 1, Marker, molecular weight marker; Lane 2, H₂O control, PCR reaction run with H₂O substituting for RNA; Lane 3, BeWo, β-HCG-secreting BeWo cell line RNA; Lane 4, Sample w/o DNase I, positive apheresis sample after PCR amplification without DNase I treatment; Lane 5, Sample w/ DNase I, apheresis product shown in Lane 4 treated with DNase I before reverse transcriptase and PCR; Lane 6, BeWo genomic DNA, PCR of genomic DNA from BeWo cell line. B: β2M, PCR for β2-microglobulin as an RNA control.

Fig. 3 Detection of β-HCG mRNA sensitivity after DNase I treatment. Various concentrations of β-HCG-expressing BeWo cells were mixed with a constant number of nonexpressing K562 cells. RNA was isolated from each mixture, treated with DNase I for 15 min, and then subjected to sequential reverse transcription-PCR using nested primers for β-HCG (β-HCG 2°) or single amplification using β2-microglobulin (β2M) primers. The top row indicates the number of BeWo cells mixed with 10⁶ K562 cells.

At the time of apheresis, elevated serum β-HCG was noted in 100% of PCR-positive patients compared to 46.2% of patients whose PCR was negative (Table 2). This difference was statistically significant when compared by Fisher’s exact test (P = 0.04). As shown in Fig. 4, a positive PCR was associated with a slightly higher serum β-HCG at the time of apheresis compared with PCR-negative values (mean ± SD, 818 ± 1772 versus 117 ± 232, respectively). Using t test analysis and a log transformation to correct for unequal variances, the serum β-HCG at apheresis was slightly higher for PCR-positive patients (P = 0.09). Interestingly, a positive PCR was also associated with a higher serum β-HCG at diagnosis (mean, 169,986 ± 207,712 versus 27,886 ± 77,038, respectively), a difference that was statistically significant (P = 0.03). Of those patients with detectable β-HCG by PCR, 100% had a history of visceral metastasis (pulmonary or hepatic), compared to 61.5% of patients whose PCR was negative (Table 2), but the difference did not reach statistical significance (P = 0.11). A greater number of patients with a positive PCR relapsed after transplantation (85.7 versus 61.5%), but the numbers were not statistically significant (P = 0.35).
Table 2

Clinical characteristics of PCR+ and PCR- patients among those with 3-HCG-secreting tumors (number with characteristic/total number of patients)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total Number of Patients</th>
<th>PCR+</th>
<th>PCR-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sci, B.</td>
<td>100000</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>I., E.</td>
<td>10000</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PCR+</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PCR-</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1

Evaluation of apheresis products for the presence of β-HCG mRNA using nested PCR primers

<table>
<thead>
<tr>
<th>Elevated serum β-HCG*</th>
<th>Sex</th>
<th>No. of patients</th>
<th>No. of apheresis tested</th>
<th>Apheresis with β-HCG by PCR</th>
<th>Patients with β-HCG by PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma</td>
<td>NT</td>
<td>Male</td>
<td>7</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Negative</td>
<td>Female</td>
<td>7</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Germ cell tumor</td>
<td>All patients</td>
<td>Male</td>
<td>28</td>
<td>63</td>
<td>8</td>
</tr>
<tr>
<td>Germ cell tumor</td>
<td>Negative</td>
<td>Male</td>
<td>8</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Germ cell tumor</td>
<td>Positive</td>
<td>Male</td>
<td>20</td>
<td>47</td>
<td>8</td>
</tr>
</tbody>
</table>

* Elevated serum β-HCG refers to patients with elevated β-HCG at any time during their disease. NT, not tested.

The ability of serum β-HCG to predict relapse, irrespective of PCR results, was analyzed with logistic regression using relapse as the dependent variable and log of β-HCG at diagnosis or log of β-HCG at BMT as the independent variable. In both cases, the odds ratio was not significant (P = 0.14 and 0.069, respectively).

DISCUSSION

In this study, GCT patients undergoing autologous transplantation were found to have circulating tumor cells at the time of apheresis, as measured by PCR for β-HCG mRNA. The PCR assay seemed specific for β-HCG-secreting tumors, and no detectable β-HCG mRNA was noted in apheresis samples from patients with breast cancer, lymphoma, and GCTs that did not secrete β-HCG. Positive detection of β-HCG mRNA was associated with poor-risk factors at diagnosis, specifically a markedly elevated serum β-HCG. All seven patients with detectable β-HCG had a history of pulmonary or liver metastasis, and six of the seven patients relapsed after transplantation.

The high cure rate of GCTs challenges us to intensify treatment for patients destined to fail standard therapies without increasing treatment-related toxicity for those patients destined to be cured. A variety of prognostic factors have been identified, and classification systems have successfully used clinical and serologic parameters to identify those patients at high risk of treatment failure (1, 2, 28). Although these systems are accurate at predicting those patients who are destined to do well (4–7), a relatively large number of curable patients are included in high-risk groups. The next level of prognostication will likely come from combinations of currently available and newly developed laboratory-based predictors of outcome. PCR has been used to identify cancer cells in apheresis products of patients with hematologic malignancies and breast cancer (20, 23). It has also been used to predict disease relapse after allogeneic BMT for patients with chronic myeloid leukemia and acute lymphoid leukemia (29–31). Interestingly, detecting minimal residual disease does not necessarily predict disease relapse after standard-dose chemotherapy or BMT, but a quantitative increase in PCR-detectable disease has correlated with disease recurrence (29, 32, 33).

In this study, we chose to evaluate patients for the presence of β-HCG mRNA in peripheral blood cells as a marker of circulating tumor cells. Using PCR, β-HCG mRNA was identified in eight apheresis products from seven patients. Data to support our hypothesis that detection of β-HCG mRNA in peripheral blood represents circulating GCT cells include: (a) no
detectable β-HCG mRNA in 22 apheresis products from breast and lymphoma patients; (b) no detectable β-HCG mRNA in 16 apheresis products from GCT patients whose tumors did not secrete β-HCG; (c) 100% of PCR-positive patients had elevated serologic β-HCG levels at the time of apheresis; and (d) all seven PCR-positive patients had a history of metastatic disease to the lung or liver, indicating prior hematogenous spread of tumor cells.

The precise role of β-HCG mRNA PCR in determining prognosis after AuSCT requires further evaluation. Our finding that six of seven patients with a positive PCR relapsed suggests that this may be more accurate than serum β-HCG at the time of transplantation in predicting outcome. Because serum β-HCG at diagnosis, a known poor prognostic factor, was associated with a positive PCR, a multivariate analysis on a larger group of patients will be required to determine if PCR will serve as an independent variable in predicting response to autologous transplantation. Of note, the one patient with a positive PCR who has not relapsed received three cycles of oral VP-16 (50 mg/m² daily for 21 days of a 28-day cycle) after transplantation (34). The utility of using PCR to identify candidates for posttransplantation chemotherapy deserves further study.

The ability to detect circulating GCT cells may have other applications in GCT management. PCR performed at diagnosis may identify patients with advanced disease destined to fail standard chemotherapy. PCR may also identify patients currently treated with surgery alone who are destined to relapse and may benefit from adjuvant chemotherapy. In the setting of autologous transplantation, PCR may identify those patients who could benefit from stem cell manipulations such as tumor cell purging or CD34 selection.

The finding of β-HCG mRNA in apheresis products strongly suggests the presence of circulating tumor cells in germ cell patients undergoing autologous transplantation. Whether these tumor cells have the capacity to contribute to disease relapse remains to be determined. Nevertheless, PCR technology may prove a useful prognostic tool in predicting response to transplantation and identifying those patients requiring additional therapy after AuSCT.

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