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MELPHALAN- MOBILIZED BLOOD STEM CELL COMPONENTS CONTAIN MINIMAL CLONOTYPIC MYELOMA CELL CONTAMINATION

SHORT TITLE: Melphalan-mobilized stem cells in myeloma

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Abstract

Optimal methods of stem cell mobilization in multiple myeloma are undefined, and contaminating clonotypic cells could contribute to disease recurrence. A phase 2 trial of intravenous melphalan (60mg/m²) and G-CSF (10ug/kg/d) for mobilization was performed. To enhance reliability, contamination was assessed with two sensitive methods, immunoglobulin light- and heavy-chain variable region patient-specific limiting-dilution PCR. We evaluated 29 stem cell components (SCC) from fifteen patients; for 9 SCC, only V_L PCR was used because of light chain disease or technical problems with V_H primers. For 20 SCC, V_L and V_H PCR results were highly correlated ($r^2 = 0.93$, $p \ll 0.01$) with 35% (7/20) having identical estimates. V_H PCR gave significantly higher estimates for 8, and V_L PCR for 5, SCC, supporting the utility of employing two methods. Estimated clonotypic contamination per SCC was 0.0009% (0-0.1) or 0.5×10^4 clonotypic cells per kg (0-41.2), and contamination correlated with CD34+ cells collected ($r^2 = 0.42$, $p \ll 0.01$). Melphalan-mobilized SCC contain minimal clonotypic contamination.

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Introduction

Multiple myeloma (MM) is an incurable hematologic malignancy. Autologous stem cell transplantation (SCT) prolongs survival compared to conventional chemotherapy (1-3) though patients invariably relapse (3). Autologous SCT followed by reduced intensity allogeneic SCT may improve response rates and survival (4) but, even for such tandem SCT regimens, the contribution to disease relapse of autologous stem cell components (SCC) contaminated by malignant plasma cells will need to be evaluated (5-9).

The optimal mobilization strategy in myeloma might combine anti-myeloma effect with collection of adequate, minimally contaminated stem cell components. Over the past two years we conducted a phase 2 trial employing intravenous melphalan (60mg/m^2) and G-CSF (10ug/kg/d) for stem cell mobilization and reduction of disease. We assessed clonotypic contamination in SCC by limiting-dilution polymerase chain reaction (PCR) assays employing immunoglobulin (Ig) light- and heavy-chain (V_L and V_H) patient-specific primer sets (CDR1/CDR3) (5, 10-13), a strategy that combines a sensitive method and an independent check on estimates of contamination. In this report we describe the technical aspects of the assessment of tumor cell contamination of SCC from patients with myeloma mobilized with melphalan.

Methods

Patients with chemoresponsive multiple myeloma ($> 50\%$ reduction in M protein) were enrolled on this IRB approved protocol after providing written informed consent (14). Bone marrow and stem cell specimens were obtained for molecular studies, and data were recorded, as previously described (15). Stem cell collections began when peripheral blood leukocyte counts exceeded $5000/\text{uL}$.

Clonal Ig V genes: Clonal Ig V_L and V_H genes were identified as previously described, using PCR primers for consensus V_H and C_α or C_γ regions and for V_κ and V_λ subgroups (10-13, 16, 17). Sequences of the *VDJ* (V_H) or *VJ* (V_L) regions were evaluated. V_L and V_H CDR1/CDR3 primers were designed and optimized (12, 17-20). Each primer pair was tested for specificity and sensitivity using buffy-coat DNA from normal donors and DNA from the patient's marrow specimen.

Limiting-dilution PCR: Representative samples were obtained from each SCC without manipulation. To estimate the percent of clonotypic cells, limiting-dilution PCR with patient-specific CDR1/CDR3 primers was performed (10-13, 17-20) with DNA from 2×10^5 cells per SCC serially log-diluted with buffy-coat DNA. Specimens giving all negative results were assessed by RT-PCR, whereas specimens giving positive results underwent further PCR assays, in which five to ten identical PCR reactions were performed simultaneously with DNA at each dilution. The linearity of this approach has been validated (13, 18), and the intra-assay variability was assessed using a control patient specimen with 1% clonotypic cells, repeating the PCR assays for 7 consecutive days. Each 25 μ l PCR reaction contained cellular DNA (diluted as above), PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μ M of each primer and 0.5 U of *Taq* (Invitrogen, Carlsbad, CA). Negative controls with and without buffy-coat DNA were amplified with each assay. PCR conditions were as previously described; annealing temperatures ranged from 50° to 64° C, depending on primer pair (16, 20).

The percent of clonotypic cells per SCC was calculated using a Poisson analysis and MAXLIKE software (gift of F. Cremer and M. Moos) as previously described (13). The amount of contamination was estimated by multiplying the percent clonotypic cells (using the higher of the V_H or the V_L estimate) by the total number of cells collected per kg.

Statistics: All analyses were performed with PRISM (GraphPad, San Diego, CA) and were two-tailed, unless otherwise noted, using a $p < 0.05$ significance level.

Results and Discussion

Optimal methods for stem cell mobilization in myeloma and for assessment of stem cell components (SCC) for contamination have not been defined. We evaluated 29 SCC mobilized with melphalan from fifteen patients, twelve with an IgG or IgA paraprotein and three with light chain disease (Table 1). In all cases the clonal Ig V_L and V_H genes were identified; they were distributed among heavy chain subgroups $V_{H2} = 1$, $V_{H3} = 8$, and $V_{H4} = 3$, and light chain subgroups $V_{\kappa 1} = 7$, $V_{\kappa 4} = 1$, $V_{\lambda 1} = 1$, $V_{\lambda 2} = 3$, and $V_{\lambda 3} = 3$. All genes showed evidence of somatic hypermutation (21, 22).

Twenty-five of 27 primer sets passed specificity and sensitivity testing (15 V_L and 10 V_H). Two V_H primer sets continued to amplify bands similar in size to the target amplicon, despite using buffy-coat DNA from three normal donors as substrate and repeated re-design of primers. (Most target amplicons were ~220bp, ranging from 159 to 255bp). Thus, 10 V_H primer sets passed testing for the 12 patients with IgG or IgA paraproteins. Sensitivity testing showed that all 25 specific primer sets had sensitivities $\leq 0.01\%$. The coefficient of variation (CV) for replicate patient-specific limiting-dilution PCR assays was 24%, acceptable and similar to CVs obtained for radioimmunoassays (23, 24).

Results are available for all 29 SCC. Thirty-five percent of the SCC (7/20) successfully analyzed by both V_L and V_H PCR (n=20) had identical estimates by both methods; in five of the concordant cases the estimate was zero or none detected (although RT-PCR assays for these were positive by both methods). V_L and V_H estimates differed for 65% (13/20) of the SCC; in

these instances, the higher value was employed as the estimate of contamination. V_H PCR was significantly higher for 8 (2.9 versus 0.4×10^4 cells per kg, $p = 0.02$ by one-tailed paired t-test), whereas V_L PCR was higher for 5 SCC (9.2 versus 7.5×10^4 cells per kg, $p = 0.05$). Results for these 20 SCC were highly correlated (Figure 1A; $r^2 = 0.93$, $p \ll 0.01$). For the remaining 9 SCC, only V_L PCR was used because of light chain disease ($n=5$) or technical problems with V_H primer sets ($n=4$).

For the 29 SCC examined, estimated clonotypic contamination was 0.0009% (0-0.1) or 0.5×10^4 cells per kg (0-41.2). Some variability could be explained by the correlation between CD34+ cell mobilization and contamination (Figure 1B). There was no correlation between contamination and stage of disease (data not shown) or timing of mobilization (Figure 1C). In a study of SCC contamination that involved a similar cohort of patients mobilized with cyclophosphamide or ifosfamide-containing regimens and G-CSF, a constant ratio of contaminating clonotypic cells to CD34+ cells collected was also observed using a similar PCR method (18). The investigators reported no difference in CD34+ cells obtained on the first or second days of collection (18), whereas with melphalan mobilization significantly more CD34+ cells were obtained on the second day of collection (Table 1).

In that study the investigators reported a median of 0.0066% contaminating cells per SCC (range, 0-0.71) (18). We performed limiting dilution PCR on 4 SCC mobilized with cyclophosphamide and G-CSF from three chemoresponsive myeloma patients and found a median of 0.038% contaminating cells per SCC (range, 0.029-0.046), using the same technique that demonstrated a median of 0.0009% contamination of melphalan-mobilized SCC. These data suggest that melphalan-mobilized SCC contain minimal clonotypic contamination and that the assessment of contamination is enhanced by use of Ig V_L and V_H patient-specific PCR.

In a phase 3 trial of SCT for myeloma, cyclophosphamide-mobilized SCC purged by CD34-selection were not superior to unpurged components (8). However, gene-marking studies have demonstrated that infused tumor cells in SCC can contribute to relapse (25). Myeloma patients mobilized with melphalan on this trial had minimally contaminated SCC collected, and 40% of them experienced a further 50% disease reduction (14). All fifteen patients we report had adequate SCC collected, underwent autologous stem cell transplantation with a median of 6.8 million melphalan-mobilized CD34+ cells per kg (range, 3.1-10.7), and had routine and full hematologic recoveries (see Table 1). We believe these results support the further assessment of melphalan for stem cell mobilization given its anti-myeloma effect. Such studies can determine whether this mobilization strategy has a clinical impact on progression-free and overall survival.

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Table 1. Data on 15 patients mobilized with melphalan (60mg/m²) and G-CSF

Age	58 (33-73)
Gender	8 men, 7 women
Myeloma protein	
IgG	$\kappa = 3, \lambda = 3$
IgA	$\kappa = 3, \lambda = 3$
Light chain only	$\kappa = 1, \lambda = 1, \text{Non-secretory } [\kappa] = 1$
Stage of disease	
II	7
III	7
Plasma cell leukemia	1
Days from melphalan to first collection	19 (13-22)
Number of leukaphereses	2 (1-5)
Total CD34+ cells collected x 10 ⁶ per kg	14.1 (3.5-40.0)
CD34+ cells x 10 ⁶ per kg infused for SCT	6.8 (3.1-10.7)
Days from SCT to ANC > 500	9 (8-11)
Days from SCT to platelets > 20,000/uL untransfused	12 (10-16)
CD34+ cells in 13 paired (day 1, day 2) collections	
Day 1	%
	1.6 +/- 1.4
	# (x 10 ⁶ per kg)
	3.2 +/- 2.9
Day 2	%
	1.9 +/- 1.3
	# (x 10 ⁶ per kg)
	6.8 +/- 5.6
Day 1 vs Day 2 (paired t-test)	
	%
	p = 0.11
	#
	p < 0.01
Clonotypic cells* in 13 paired (day 1, day 2) collections	
Day 1	%
	0.009 +/- 0.013
	# (x 10 ⁴ per kg)
	1.9 +/- 2.7
Day 2	%
	0.016 +/- 0.033
	# (x 10 ⁴ per kg)
	4.0 +/- 7.7
Day 1 vs Day 2 (paired t-test)	
	%
	p = 0.44
	#
	p = 0.37
Clonotypic cells* (n = 29 collections) (median (range))	
	%
	0.0009 (0-0.1)
	# (x 10 ⁴ per kg)
	0.5 (0-41.2)

*All 15 patients had stem cell collections on Day 1. Thirteen had collections on Day 2, and one had a 3rd collection on Day 3, giving a total of 29 collections. CD34 and clonotypic

contamination data for the 13 paired collections (means \pm SD), and clonotypic data for all 29 collections, are shown. To compute estimates of contamination, the higher percentage obtained with V_L or V_H PCR was used.

Figure 1. Estimates of clonotypic contamination in stem cell components collected after melphalan and G-CSF mobilization in 15 patients are shown as a function of different factors.

In (A), estimates of clonotypic contamination in 20 stem cell components (SCC) are shown as a function of the type of PCR assay used. The results with light- and heavy-chain (V_L and V_H) patient-specific PCR were correlated ($r^2 = 0.93$, $p \ll 0.01$), and 35% (7/20) of the time were equal. Means were $V_L = 2.7$ and $V_H = 3.3 \times 10^4$ clonotypic cells per kg per SCC.

In (B), estimates of clonotypic contamination in 29 SCC are shown as a function of CD34+ cells per kg in each SCC, calculated by flow cytometry. By simple linear regression, the line of best fit is $y = (0.707)x - 0.669$ with 95% confidence intervals as shown ($r^2 = 0.42$, $F = 19.1$, $p \ll 0.01$). There is a relatively constant ratio of CD34+ and clonotypic cells, as previously suggested by Moos and colleagues (18). In (C), clonotypic contamination is shown in relationship to the timing of mobilization. Medians for days 12-18 and 19-23 were 0.5 and 0.35×10^4 clonotypic cells per kg. Differences due to timing of mobilization were not significant (Mann-Wilcoxon, $p = 0.68$).

