

Clonotypic Myeloma Cells Able to Xenograft Myeloma to Nonobese Diabetic Severe Combined Immunodeficient Mice Copurify with CD34⁺ Hematopoietic Progenitors¹

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ABSTRACT

The identity of the multiple myeloma (MM) precursor(s) is unknown. Our objective was to determine the myelomagenic capabilities of CD34-enriched autografts. Hematopoietic progenitor fractions from fresh or cryopreserved granulocyte-colony-stimulating factor mobilized blood from myeloma patients were obtained by sorting or enrichment, followed by RT-PCR analysis of clonotypic transcripts and/or their ability to transfer myeloma to immunodeficient mice. CD34⁺ enrichment using immunomagnetic methods comparable with those used clinically results in copurification of MM cells able to xenograft nonobese diabetic severe combined immunodeficient mice. Highly purified CD34⁺ progenitors from granulocyte-colony-stimulating factor mobilized blood of myeloma patients include, on average, 31% clonotypic MM cells. CD34⁺ progenitors also include 31% DNA aneuploid cells. For six of six MM patients, enriched progenitors were myelomagenic as measured by engraftment of clonotypic cells and/or the development of lytic bone lesions. Intrasternal injection of enriched progenitor fractions led to clonotypic cells in the femoral bone marrow and bone lesions at distant skeletal locations, confirming dissemination of myelomagenic cells. MM precursors copurify with normal hematopoietic progenitors, emphasizing the need for tumor-free grafts. Autologous MM engraftment is likely to be considerably more efficient than in a xenogeneic host, strongly suggesting that MM autografts contribute to post-transplant relapse. The xenografting myelomagenic component(s) is unlikely to be plasma cells, given the lack of morphologically identified plasma cells among enriched progenitors. Xenografting MM precursors appear to be CD34⁺CD45^{low}, similar to normal progenitors. Precursor function within the MM clone seems to be complex and may involve multiple components of the MM hierarchy.

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INTRODUCTION

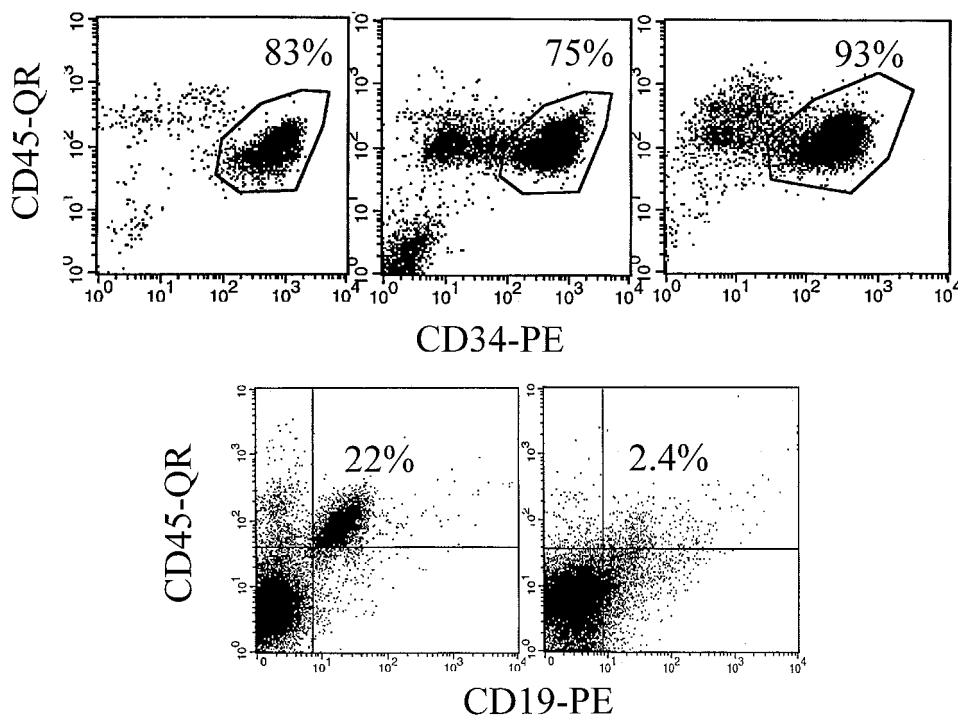
MM³ is a cancer of the B lineage localized to the blood and BM. Although the pathology of MM is mediated by malignant plasma cells located in the BM and secreting post-switch IgG or IgA, the MM clone is a B lineage hierarchy that has been shown to include plasma cells, late stage B cells (1), and pre-switch B cells expressing clonotypic IgM (2–5). MM is characterized by a clonal immunoglobulin gene rearrangement (IgH VDJ), termed clonotypic, that uniquely identifies members of the MM clone, independent of characteristics such as morphology, phenotype or genetic abnormalities. The clonotypic IgH VDJ can be identified in individual cells or populations of cells using PCR or RT-PCR (1, 5–7).

MM B cells/preplasma cells persist throughout conventional and high-dose chemotherapy and remain detectable after transplant (1, 5, 8). The persistence of pre-switch MM B cells throughout therapy correlates with reduced survival (5), suggesting that these early stage cells play a highly significant role in disease progression. The source of MM cells mediating after transplant relapse is unknown but is likely to derive from both the cytoreduced host and the autologous G-CSF mobilized blood used for transplant. Mitterer *et al.* (9, 10) showed that monoclonal B cells in mobilized blood correlated with early relapse, and others have shown that MM cells copurify with CD34-enriched populations (9, 11–13). In support of the idea that relapse is mediated, at least in part, by malignant precursor cells in autografts used for transplant, recent work has shown that G-CSF mobilized blood from MM patients in stages of minimal disease is myelomagenic in NOD SCID mice (6), and that on xenotransplant, leukemic B cells can give rise to myeloma (7). Regardless of which cells mediate the transfer of myeloma to NOD SCID mice, our observations clearly establish the ability of mobilized blood cells to transfer disease to a naive host. It seems likely that MM cells able to xenograft human MM would even more efficiently transfer MM to an autologous host. In support of this, MM patients receiving tumor-free syngeneic, identical twin transplants had significantly longer survival than did patients receiving autologous transplants (14, 15).

To evaluate whether stem cell enrichment methods provide effective purging of malignant cells from autografts, and to begin characterization of a MM precursor population(s), enriched hematopoietic CD34⁺ progenitors were enriched using negative selection techniques comparable with those used clinically. Injection of CD34-enriched populations led to lytic bone

³ The abbreviations used are: MM, multiple myeloma; BM, bone marrow; IC, intracardiac; IS, intrasternal; IgH VDJ, immunoglobulin variable diversity joining; G-CSF, granulocyte-colony stimulating factor; NOD SCID, nonobese diabetic severe combined immunodeficient.

Fig. 1 Phenotypic analysis of enriched progenitor fractions used for xenografting. *Top*, representative enriched progenitor fractions from three different donors were stained with CD34-FITC and CD45-PE to determine the degree of enrichment. *Bottom*, unfractionated and enriched progenitor fractions stained with CD19-FITC and CD45-PE, showing the nearly 10-fold depletion of CD19+ cells by the enrichment procedure. Analysis of CD19 staining was not routinely done on enriched fractions because of the limited amount of material recovered after enrichment and the need to preserve the cells for injection into mice. Numerical values represent the proportion of cells within the indicated amorphous gate or quadrant.



lesions and/or PCR-detectable disease, indicating the presence of MM progenitors within the CD34⁺ subset. These experiments show that myeloma precursors in mobilized blood copurify with normal hematopoietic progenitors as measured by their expression of clonotypic transcripts and their ability to transfer human MM to xenografted mice, emphasizing the need for an effective purging strategy before autologous transplant. They also demonstrate that MM precursors can be found among CD34-enriched cells from which CD19-expressing cells have been depleted, suggesting that at least one generative component of the MM hierarchy may lack CD19 and express CD34.

MATERIALS AND METHODS

Patients. After ethics approval and informed consent, aliquots of G-CSF mobilized blood were obtained from 18 patients with MM. For some patients, aliquots of G-CSF mobilized blood autografts were obtained at the time of apheresis and at the time of reinfusion (postcryopreservation). All of the patients involved were in minimal stages of disease at the time of mobilization. For aliquots of cells taken at the time of reinfusion, samples were withdrawn using a sterile syringe and immediately put into 100-fold excess of 37°C RPMI medium plus 5% fetal calf serum to dilute the cryopreservation medium. Aliquots of BM were obtained for six MM patients at diagnosis, at the time of staging or in relapse.

Sample Preparation. Progenitor cell enrichment used StemSep columns (Stem Cell Technologies Inc., Vancouver, British Columbia) according to the manufacturer's directions. Briefly, 5×10^8 mobilized blood mononuclear cells were incubated on ice for 30 min with StemSep antibody mixture containing anti-CD2, anti-CD3, CD14, CD16, CD19, CD24, CD56,

and CD66b. Next, magnetic colloid was added, and after a 30-min incubation on ice, cells were loaded into the StemSep column. The negatively selected, enriched hematopoietic progenitor fraction was collected and counted. In most cases, aliquots of the enriched progenitor fraction were tested for the expression of CD34 and CD45. These cells were then used to engraft NOD SCID mice. When sufficient cells were available, or with separate populations of enriched progenitors, cells were analyzed for clonotypic transcripts using RT-PCR. For a series of 14 mobilized bloods, the mean number of CD34⁺45^{lo} progenitors in enriched fractions was $70 \pm 6\%$ (mean \pm SE), with a range of 27–94% purity (Fig. 1). This is within the same range reported for clinically used methods of stem cell enrichment (16). Only 1 of the 14 enriched progenitor preparations was found to have less than 50% purity. The clinical use of enrichment methods is based on the assumption that CD34 is not expressed by myeloma plasma cells or earlier precursors of myeloma, and that enrichment of cells bearing this marker will provide strong negative selection against the copurification of tumor cells. The CD34-enriched fractions from StemSep columns were used for xenografting to NOD SCID mice. To obtain progenitor fractions with greater purity, aliquots of mobilized blood were stained with antibody to CD34 and CD45, followed by sorting with an ELITE cell sorter (Coulter, Hialeah, FL) for the CD34⁺45^{lo} subset, as described previously (17). These sorted progenitor fractions were >97% pure as measured by reanalysis of sorted fractions using flow cytometry. Because of the relatively large numbers of cells required for xenografting, analysis of sorted progenitor cells was limited to detection of clonotypic transcripts. For samples from which B cells were purified, mobilized blood cells were stained with antibody to

CD19, followed by sorting of positive cells, as described previously (1).

DNA Content Analysis. BM cells from six MM patients were stained with antibody to CD34 for detection of progenitor cells, CD3 to identify T cells as an internal control for a diploid DNA content, and with a DNA binding dye to quantify DNA content as described previously (18). Files of 50,000 cells were gated for CD34+ cells with low forward and side scatter, and the DNA content was plotted as a histogram. Hyperdiploidy was defined as an amount of staining greater than 1 SD from the coefficient of variation of diploid T cells in the same sample, as described previously (18).

RT-PCR Analysis. RT-PCR and *in situ* RT-PCR were performed as described previously (1, 18, 19). For *in situ* RT-PCR, the number of cells detected as having transcripts for histone was used as the positive control for determining the percentage of cells having clonotypic transcripts, as described previously (1, 18, 19). Samples were scored by an observer blinded to their identity. For each patient, the clonotypic IgH VDJ was determined and validated, and primers for patient-specific CDR2 and CDR3 were designed as described previously (1); all primers were rigorously tested for specificity by analysis of B cells from normal donors and from unrelated myeloma patients.

Xenografting of Enriched Progenitors. Enriched progenitor fractions from six MM patients were injected into irradiated NOD SCID mice via the IC route as described previously (6). Mice were terminated when disease symptoms developed or at defined time points (1 month or 3 months after xenografting). The spleen and femoral BM were removed for RT-PCR analysis, and the carcass was X-rayed to detect lytic bone lesions. Use of femoral BM for molecular analysis may result in an underestimate of the true frequency of engrafted clonotypic MM cells, because the majority of lytic lesions appeared to be in the vertebral column and skull, comparable with patterns in MM patients who usually have few or no lesions in their femurs. All mice that developed visible symptoms also had clonotypic transcripts in spleen and/or BM cells.

RESULTS AND DISCUSSION

The Reinfused Apheresis Product from MM Patients Includes a High Frequency of Clonotypic B Cells. Previous work has indicated that essentially all mobilized blood collections include clonotypic cells (5). To determine the frequency of clonotypic B cells in mobilized blood immediately before reinfusion to the MM patient, sorted B cells were analyzed for clonotypic transcripts using *in situ* RT-PCR. Positive cells were counted microscopically, and the frequency in the original population calculated. For one patient, aliquots of cells from each of four cryopreservation bags were assessed (Table 1). Approximately 1–5% of WBCs in the apheresis product were clonotypic, and approximately 4×10^7 clonotypic MM B cells were reinfused per liter of apheresis product. Thus, even when plasma cells are undetectable in aphereses, other members of the MM clone are readily detectable. They are likely to be clinically important based on our observation that mobilized blood xenografts transfer human myeloma to NOD SCID mice (6). Gazitt *et al.* (20) have reported that during the last days of

Table 1 The reinfused mobilized blood from a MM patient includes a high frequency of clonotypic B cells

	% B cells in PBSC	% clonotypic ^a	% of WBC that are clonotypic	Absolute no. reinfused/liter of PBSC ^b
1	38%	11%	1.0%	1.6×10^7
2	36%	34%	3.0%	4.8×10^7
3	27%	29%	1.6%	3.2×10^7
4	49%	42%	5.0%	5.6×10^7

^a Mobilized blood cells were stained with anti-CD19, and B cells were sorted, fixed, and analyzed for clonotypic transcripts using *in situ* RT-PCR. The percentage of B cells having clonotypic transcripts is expressed as a percentage of the total number of B cells having histone transcripts, as described previously (1, 18, 19).

^b The absolute number of clonotypic cells per liter of blood was calculated as (the % of WBC \times the number of WBC/liter of PBSC). PBSC, peripheral blood stem cells.

mobilization, mobilized malignant cells express CD19, also indicative of a less mature component of the myeloma clone because plasma cells rarely express CD19. Our microscopic analysis of nine randomly selected apheresis products indicated a plasma cell content of <0.01%, consistent with the fact that the majority of such patients are in minimal stages of disease at the time of mobilization. Despite this lack of plasma cells, mobilized blood cells readily xenografted myeloma to NOD SCID mice (6), indicative of a less mature progenitor cell for the myeloma clone.

Clonotypic B Cells Copurify with Highly Purified CD34⁺45^{lo} Hematopoietic Progenitors. Enrichment of CD34+ stem cells by a variety of methods does not appear to have a detectable effect on regrowth of the tumor clone. Although some observations suggest that clonotypic transcripts are reduced in purified CD34-enriched progenitor fractions (21, 22), patients transplanted with these enriched fractions show no clinical benefit as compared with unfractionated autografts (23–25). Although this may mean that there is no benefit to providing a clean graft, it is equally likely that myeloma stem cells copurify with the normal progenitor cells and contribute to relapse. Our previous work described a subset of CD34+ clonotypic B cells that might be expected to copurify with normal CD34+ progenitors (19). To determine the frequency of clonotypic cells in CD34+45^{lo} progenitors purified by cell sorting, *in situ* RT-PCR with patient-specific primers was used. For five patients, the mean percentage of clonotypic cells copurifying with sorted CD34+45^{lo} progenitors was $31 \pm 15\%$ (Table 2). Clonotypic MM cells detectable in sorted CD34+ populations are unlikely to be plasma cells based on their low-medium scatter profile, their detectable intensity of CD45, which is absent from mature plasma cells, and their expression of CD34, which is also absent from MM plasma cells (18, 26) but shown to be present on subsets of B cells (18, 19).

CD34+ Progenitors Include a DNA Aneuploid Subset. In MM, both B and plasma cells have abnormal DNA content as measured by flow cytometry (18, 27, 28). Although DNA aneuploid plasma cells lack CD34, DNA aneuploid cells in blood are CD34+ clonotypic MM B cells (18). As a second measure of abnormalities among CD34+ fractions from myeloma patients, we analyzed the DNA content of CD34+ cells from MM BM because

Table 2 Clonotypic B cells copurify with CD34⁺ hematopoietic progenitor cells

Patient	% of clonotypic cells in CD34 ⁺ 45 ^{lo} fractions ^a
1	17
2	56
3	3
4	<1
5	79
Mean = 31 ± 15%	

^a G-CSF mobilized blood cells were stained with CD34 and CD45, the progenitor fraction sorted as indicated. These fractions were >97% pure on flow cytometric reanalysis. The low scatter profile of the sorted cells precludes their identity as plasma cells. Insufficient cells were available from sorts to allow full phenotypic or morphologic characterization. Sorted cells were fixed and placed on slides for *in situ* RT-PCR and microscopic counting of positive cells. All slides included a positive and a negative control for the procedure itself, as described previously (1, 18, 19). For most experiments, two replicate slides were analyzed; the values shown represent the mean percentage of positive cells. Controls were always done to confirm that patient-specific primers were specific for the appropriate patient and amplified no product from cells of an unrelated patient. As a further positive control, primers to histone were used to verify that the deposited cells had intact transcripts. The person who scored positive and negative cells was blinded to the identity of each slide. The mean value is ±SE.

Table 3 CD34⁺ progenitor cell populations include a DNA aneuploid subset

	% of CD34 ⁺ cells with the indicated DNA content ^a
Diploid	43 ± 6
Hyperdiploid	31 ± 4
Tetraploid	3.5 ± 1
Apoptotic	25 ± 5

^a Cells from six patients with MM were stained with CD34, CD3, and DAPI to determine DNA content. Files were gated as indicated in "Materials and Methods," and the DAPI staining plotted as a histogram. Diploid cells were those having a DNA content identical to that of T cells in the same sample. Hyperdiploid cells were those having a DNA content >1 SD as compared with the T-cell peak. Tetraploid cells were those with a DNA content twice that of diploid cells, and apoptotic cells were those having a DNA content at least 1 SD less than that of T cells. Most apoptotic cells had severely reduced DNA staining. Values are the mean ± SE of six different samples.

these are the cells likely to give rise to mobilized CD34⁺ progenitors in blood. On average, 31% of cells within the CD34⁺ progenitor gate were DNA hyperdiploid (Table 3), a value consistent with the number of clonotypic cells within sorted CD34⁺ fractions (31%; Table 2). This suggests that clonotypic cells may be of malignant origin rather than remnants of the original monoclonal gammopathy that likely gave rise to MM.

CD34-enriched Progenitor Populations Have Clonotypic Transcripts of the Clinical Isotype. Patients who receive CD34-enriched autografts have no survival benefit as compared with those receiving unfractionated autografts (23–25). Enriched progenitors from six MM patients were assessed for clonotypic transcripts, and their isotype expression was characterized using a previously described RT-PCR strategy (Ref. 5; Table 4). Analysis of aggregate populations of CD34-

Table 4 CD34-enriched progenitors have clonotypic transcripts of the post-switch isotype^a

Patient (clinical isotype)	Clonotypic transcripts (CDR2/CDR3 primers)	Clonotypic isotype			
		IgM	IgD	IgG	IgA
11 (IgG)	Yes	–	–	+	–
12 (IgG)	Yes	–	–	+	–
17 (IgG)	Yes	–	–	+	+
18 (IgG)	Yes	–	–	+	+
19 (IgG)	Yes	–	–	+	–
20 (IgA)	Yes	+	+	–	+

^a Purified RNA from each population of CD34-enriched progenitors was subjected to a first and second stage RT-PCR reaction to detect clonotypic transcripts and determine their isotype as previously described (5). Briefly, clonotypic transcripts were detected in a single-stage (25 cycles) RT-PCR with primers annealing to CDR2 and CDR3 regions of the transcript. Isotype was determined in a single-stage RT-PCR using primers to CDR3 and the indicated constant region (25 cycles) or in a nested RT-PCR using first primers to VhLeader and the indicated constant region and a second stage using primers to CDR3 and the indicated constant region. For all samples the clinical isotype indicating the monoclonal Ig for each patient was detectable in a single-stage RT-PCR. For nonclinical isotypes (the nonclinical post-switch isotype and any pre-switch isotypes) product was usually detected only after the second stage nested RT-PCR, suggesting a lower frequency of these transcripts relative to the clinical isotype.

enriched progenitors indicates that the majority of these cells are post-switch cells (IgG or IgA, depending on the mIg isotype in each patient). Clonotypic IgM or IgD was detected in cells from only one of these patients. For two of the patients, sequencing confirmed that the PCR product encoded a somatically mutated IgH VDJ identical to that of autologous plasma cells (data not shown). Thus, they are likely to be bone fide members of the malignant clone. However the enrichment procedure depletes cells expressing CD19 (Fig. 1), suggesting that the engrafting clonotypic MM cells detectable among CD34-enriched progenitors may not be classical CD19⁺ B cells. To enable sensitive detection of rare plasma cells that might be present, after H&E staining, we screened cytopins from enriched progenitor fractions for cells with plasma cell morphology. No plasma cells were detectable on microscopic examination of cells from five of five preparations of enriched progenitors from either fresh or cryopreserved mobilized blood, giving values of <0.06% of cells with plasma cell morphology.

Enriched CD34⁺ Progenitor Fractions from Mobilized Blood Are Myelomagenic in NOD SCID Mice. To determine their myelomagenic capacity, enriched progenitor fractions of mobilized blood cells were injected into NOD SCID mice and tumor take was measured by the presence of disease symptoms, clonotypic transcripts, and lytic bone lesions. Enriched progenitors from six MM patients were analyzed. These populations are highly enriched in cells having the CD34⁺45^{lo} phenotype (mean = 70%; Fig. 1, *top*) and depleted for cells expressing CD19 (Fig. 1, *bottom*), and lack detectable plasma cell contaminants (<0.06%), as identified morphologically. For four of six patients, the clonotypic IgH VDJ sequence was known. For two patients, enriched progenitors from both freshly harvested and cryopreserved apheresis products were analyzed. For two patients, only freshly harvested cells were analyzed, and for two other patients, only cryopreserved samples were ana-

Table 5 Enriched progenitors engraft clonotypic cells to NOD SCID mice

Patient ^a	No. of enriched progenitors/mouse ^b	No. injected	No. with clonotypic transcripts ^c
11-C	1.5×10^5	6	3/5
12-F	2×10^5	6	2/5
12-C	2.4×10^5	8	1/8
13-F ^d	1×10^5 , 2.5×10^5	7, 3	0/10
14-F	3×10^5	7	ND
15-C	1×10^5	4	ND
16-F	35×10^5	7	1/6
16-C	8.5×10^5 (IC)	7	1/7
16-C	0.8×10^5 (IS)	7	2/7

^a For six patients, mobilized blood, taken fresh (F) at the time of apheresis or after cryopreservation (C), was fractionated to prepare enriched progenitors and injected into NOD SCID mice as indicated in "Materials and Methods."

^b The indicated number of enriched progenitors was injected via the IC route to each mouse. For patient 16-C, a second set of mice was injected via the IS route.

^c Number of mice having clonotypic transcripts/total number tested.

^d Apheresis product was tested on two sequential days of harvest.

lyzed. Of the 62 mice that received injections, 5 became sickly or died, and, at the time of autopsy, brittle bones were detected in 1 mouse. The remainder of mice were terminated at 1–3 months after receiving injections for molecular analysis and X-rays. Most mice that received injections of G-CSF mobilized blood or of enriched progenitor fractions did not exhibit terminal symptoms that were readily apparent when the xenografting cells were predominantly plasma cells (6). This suggests that the xenografting MM cells that copurify with enriched progenitors are unlikely to be plasma cells, consistent with the low levels of plasma cells detected in these fractions (<0.06%). We speculate that earlier members of the MM clone may have more primitive progenitor function, manifesting as delayed myelomagenesis, relative to late stage leukemic plasma cells, which rapidly generate terminal disease (6).

For the four patients for whom the clonotypic IgH VDJ had been derived, spleen and BM cells were analyzed for clonotypic transcripts using RT-PCR with patient-specific CDR2/CCR3 primers. For three of four patients from whom six different preparations of enriched progenitors were separately analyzed, at least one mouse in each xenografted group had detectable clonotypic transcripts (Table 5). Overall, of 56 mice tested, 10 mice (18%) had detectable clonotypic transcripts. This likely represents some degree of homing to and clonal expansion within the murine BM, particularly because two of seven mice that received injections directly into the sternal BM had detectable clonotypic transcripts in the femoral BM (Table 5, row 9). Clonotypic transcripts were found in mice that received injections of freshly harvested or cryopreserved progenitor fractions. This is likely to be an underestimate because only femoral BM was analyzed, which in mice lacks the trabecular bone favored by myeloma cells in human marrow. Mice that received IS injections also had clonotypic cells in femoral BM, indicative of dissemination from the sternal BM to distant BM sites. No EBNA or LMP-1 (EBV virus) was detected, indicating that clonotypic transcripts do not derive from EBV-transformed B

Table 6 Lytic bone lesions in mice xenografted with enriched progenitor cell populations from mobilized blood

Patient	No. of mice with lytic bone lesions/total analyzed ^a
11-C	3/5 (60%)
12-F	3/4 (75%)
12-C	4/6 (66%)
13-F	8/10 (80%)
14-F	6/7 (86%)
15-C	3/3 (100%)
16-F	4/5 (80%)
16-C (IC)	7/7 (100%)
16-C (IS)	6/7 (86%)
Mean = 45/59 mice (76%)	

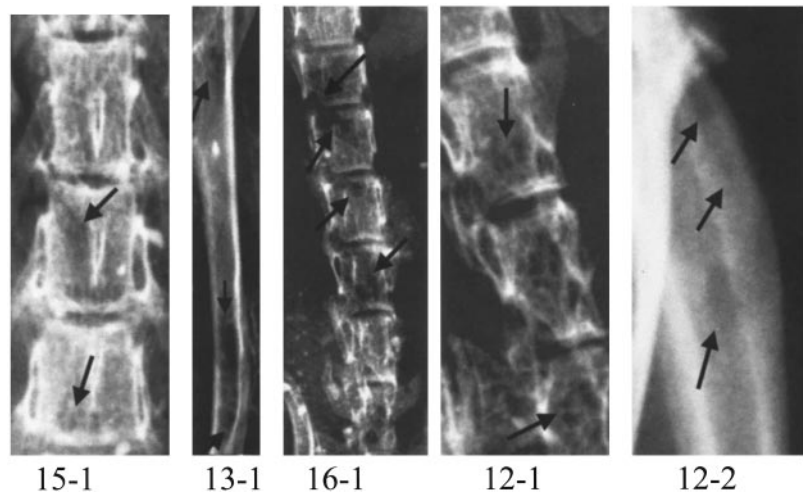
^a Bone lesions were detected after sacrifice by X-ray analysis of frozen carcasses. Abbreviations are as for Table 4.

cells. On the basis of use of a cellular limiting dilution assay (1, 6), for most mice having clonotypic transcripts, the frequency was less than 1/3000 BM or spleen cells. However for one mouse, clonotypic cells were found at a frequency of 1/3000 in BM and 1/100 in spleen. On the basis of the fact that most mice received $1-2 \times 10^5$ enriched cells, the MM precursor(s) being detected here has a minimum frequency of at least $1/10^5$.

Lytic Bone Lesions Are Detectable in the Majority of Mice Xenografted with Enriched Progenitor Fractions of Mobilized Blood. The extent to which human myeloma colonizes murine BM is best measured by X-ray detection of lytic bone lesions. As shown in Table 4, the majority of mice (73%; range, 60–100%) engrafted with enriched progenitors had lytic lesions in skull, spine, pelvis, and/or tibia. Bone lesions were found at distant locations in mice that received IS injections (Table 6, row 9), further confirming the spread of disease originating from the enriched progenitors. Representative examples of bone lesions are shown in Fig. 2. No lesions were detected in irradiated control mice that had not received enriched progenitors.

Although the identity of the MM precursor cell detected here cannot be definitively established, it is unlikely to be a classical plasma cell. BM plasma cells lack CD34 (18, 26) and have a scatter profile that is excluded from populations of sorted CD34⁺ progenitors. In addition, CD34-enriched fractions lack cells with characteristic plasma cell morphology. The myelomagenic cell in CD34-enriched populations is also unlikely to be a classical B cell because CD19 antibodies are included in the antibody mixture used for depletion, and the enriched cells have considerably reduced staining for CD19 (Fig. 1, bottom). Nevertheless, the involved cells are likely to be postgerminal center, memory B cells, given that clonotypic transcripts detected in sorted or enriched progenitor populations appear to be predominantly post-switch cells IgG or IgA cells with a somatically mutated IgH VDH clonotypic sequence identical to that of autologous plasma cells. The relationship between the probably post-switch B cells detected here as being myelomagenic, and the pre-switch B cells previously shown to correlate with reduced survival (5), is unclear. It is possible that pre-switch transcripts detected after transplant derive from drug-resistant B cells that persist in the cytoreduced host. However, it is equally likely that pre-switch clonotypic transcripts are less frequent

Fig. 2 Lytic bone lesions in xenografted NOD SCID mice. Representative X-ray images are shown of bone lesions in mice engrafted with CD34-enriched progenitor cells. Each *panel* represents bone lesions in a different mouse from groups engrafted with the patient cells indicated on the figure. From *left to right*, the bones shown are vertebral column, pelvis, vertebral column, vertebral column, and tibia. *Arrows* indicate lesions.



and/or more labile than the transcripts encoding the clinical isotype. If so, their absence from CD34-enriched progenitors could be an artifact of the purification procedure, during which pre-switch clonotypic transcripts may be reduced to undetectable levels even though pre-switch cells themselves remain among the enriched progenitor cells. Preliminary work supports this latter explanation. However, for one patient for whom CD34⁺ progenitors were sorted from BM immediately before transplant, clonotypic transcripts appeared to be exclusively of the IgM isotype⁴, indicating that pre-switch clonotypic B cells do persist in the host.

These experiments indicate that cyclophosphamide/G-CSF treatment comobilizes myeloma progenitors into the blood together with normal hematopoietic progenitors. Highly purified CD34⁺ progenitors include a subpopulation of cells expressing clonotypic transcripts. CD34⁺ progenitors from MM patients also include a DNA aneuploid subset, indicative of malignant properties. Enrichment of CD34⁺ progenitors, using a method comparable with those used for preparing CD34 autografts clinically (16), results in a population that includes malignant MM cells able to xenograft human myeloma to murine BM, as detected by engraftment of clonotypic cells and lytic bone lesions. Consistent with the finding that these types of enriched progenitor fractions do include clonotypic cells (16), it is plausible to speculate that these cells may engraft and undergo clonal expansion with even greater efficiency in an autologous host after transplant. Using a functional readout, this work confirms that enrichment of CD34⁺ progenitors does not purge cancer cells from autografts, and that posttransplant relapse is very likely to arise from both the host and the autograft. This work underscores the need for a tumor-free graft in myeloma and reemphasizes the urgency in developing efficient purging strategies for use before autologous transplant.

This work also shows that MM precursor function can be detected in cell populations largely depleted of B cells and

having few or no detectable plasma cells. The apparent lack of B cell markers may reflect adaptive differentiation of putative MM precursors to a hematopoietic stem cell-like phenotype amenable to BM homing and extensive clonal expansion (the “wolves in sheep’s clothing” model). Unlike mice xenografted with leukemic plasma cells (5, 6), symptoms for mice that received injections of enriched progenitor fractions were less pronounced, and clonotypic transcripts were less frequent. This may reflect different degrees of differentiation for MM precursors among leukemic plasma cells and those among earlier stage B cells, because end stage plasma cells have higher levels of Ig/Ig transcripts that are more readily detected. Within the 1–3-month window evaluated here, MM precursors present among CD34-enriched progenitors may not complete differentiation to plasma cells, resulting in MM cells with lower transcript numbers. This work suggests that precursor activity in MM may be complex and may involve multiple components of the MM hierarchy, each with differing degrees of generative potential. Enriched progenitor fractions, comparable with those used for transplant of “purified” stem cells to patients, maintain the ability to transfer human myeloma to immunodeficient mice, confirming that myeloma progenitors copurify with CD34⁺ hematopoietic progenitors. To our knowledge, this is the first demonstration that myeloma cells within enriched progenitor populations have the ability to transfer disease to a recipient host, in this case a mouse. This work strongly supports the speculation that for myeloma patients, relapse after transplant is due, at least in part, to malignant cells within the graft.

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