RAPID COMMUNICATION

The Hematopoietic Stem Cell Antigen, CD34, Is Not Expressed on the Malignant Cells in Multiple Myeloma

By Robert A. Vescio, Charlie H. Hong, Jin Cao, Austin Kim, Gary J. Schiller, Alan K. Lichtenstein, Ronald J. Berenson, and James R. Berenson

Autologous stem cell transplantation has become an important therapy in multiple myeloma (MM). To develop adequate autograft purging methods, it is necessary to determine whether antigens expressed on early hematopoietic progenitors exist on malignant cells. The Ig heavy chain produced by the MM cells shows evidence of prior somatic mutation without intraclonal diversity. As a result, this sequence can be used as a specific marker to detect all members of the malignant clone. The Ig heavy chain sequence expressed by the MM cells was obtained in five patients with advanced disease. Patient specific oligonucleotide primers were designed based on the complementarity determining regions (CDR) of each MM Ig sequence and used to amplify DNA by polymerase chain reaction for the detection of malignant cells. A highly purified collection of CD34+ cells was obtained after passage of the initial bone marrow cells through an immunoadsorption column and fluorescence-activated cell sorting. Despite an assay sensitivity of 1 tumor cell in 2,500 to 44,000 normal cells, none of the CD34+ samples showed product with the myeloma-specific CDR primers. Therefore, positive selection for cells bearing this antigen should yield a tumor-free autograft capable of providing hematopoietic recovery after myeloablative chemotherapy.

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MULTIPLE MYELOMA (MM) is a B-cell malignancy characterized by the accumulation of a clonal population of plasma cells in the bone marrow (BM) that secrete a monoclonal Ig protein. Although the disease is responsive to alkylating agents and corticosteroids, the disease is incurable with standard doses of chemotherapy, resulting in a median survival of 32 months.1,2 In an attempt to improve on these results, high doses of cytotoxic agents have been administered to patients in conjunction with allogeneic or autologous marrow transplantation. Although response rates have been high, patient outcome has been disappointing with both procedures due primarily to toxicity and early relapse.3,4 The early relapse rates in patients after autologous BM transplantation may be caused by the reinfection of contaminating autograft tumor cells in this BM-based malignancy. Consequently, peripheral blood progenitor cell transplantation is being used at many centers to minimize tumor relapse.5,6 However, this technique may not substantially reduce the autograft tumor load because the cell number infused is greater by 10-fold and circulating tumor cells have been detected in the peripheral blood at a concentration as high as 10%.5,6

The positive selection of hematopoietic progenitor cells has been recently shown to reduce autograft breast cancer tumor burden while avoiding exposure of the hematopoietic cells to the cytotoxic agents found in most other purging methods.10 An immunoadsorption column that collects CD34 antigen-expressing cells was used because this antigen is found on the most primitive hematopoietic progenitor cells that are capable of engrafting primates after myeloablative chemotherapy.11,12 A similar CD34+ peripheral blood progenitor cell transplantation in MM would only be advantageous if the antigen was not expressed on the malignant cells. Normally, CD34 antigen expression is gradually lost during cell maturation13-15 but does persist on the earliest B-lymphocyte precursor cells.16 Although the CD34 antigen is not normally found on the surface of plasma cells, the existence of a clonally less differentiated B-lymphocyte or even stem cell precursor has been proposed. Evidence in support of a less differentiated malignant cell include the expression of shared idiotypes by the myeloma cell and IgM producing B lymphocytes,17 the expression of myeloid and megakaryocytic markers on the aneuploid cell population in myeloma,18 and the identification of an IgM-producing cell with the terminal portion of the Ig heavy chain sequence matching that expressed by the myeloma cell.19,20 The purpose of this study was to identify whether any malignant cells in MM express the CD34 antigen by using the heavy chain variable region gene sequence as a marker of clonality.

This sequence can be used as a marker capable of identifying all of the malignant cells in myeloma for the following reasons. First, the myeloma sequence should be unique because it is derived after the random rearrangement of one of the 100 heavy chain variable (VH) genes with one or more of the 30 diversity (D) and one of the six functional joining (JH) genes. Diversity is further enhanced by the imprecise joining of these regions and the insertion of additional template-independent nucleotides at the VH, D, and JH junction during the pre-B-cell stage of development by the action of terminal deoxynucleotidyl transferase. Furthermore, because a B lymphocyte is exposed to antigen in the lymph node, mutations of the Ig gene sequence occur in an attempt to develop cell progeny that have greater antigen avidity. This
either Cy or CO was hybridized to 1 pg of RNA by heating to 65°C. We and others have determined that the VH sequences of the myeloma cells show evidence of substantial prior somatic mutation. Consequently, the VH sequences of the myeloma cells are very unique and, therefore, useful as a marker for clonality. Second, there is no evidence of intraclonal diversity of the myeloma VH sequence among tumor clones. Finally, the myeloma VH gene sequence does not vary during the course of disease, a process known to occur in two other B-cell malignancies, acute lymphoblastic leukemia (ALL) and follicular non-Hodgkin’s lymphoma (NHL). In this report, a highly purified population of CD34+ cells was collected from five patients with advanced stages of MM. The polymerase chain reaction (PCR) was then used to amplify CD34+ cell DNA using oligonucleotide primers complementary to the most unique areas of the myeloma VH gene sequence to search for malignant cells in a subpopulation expressing this early hematopoietic antigen.

**MATERIALS AND METHODS**

**Patient material.** BM cells were obtained from 5 patients with MM after informed consent was obtained and in accordance with the Human Subjects Review Boards of the Veteran’s Administration West Los Angeles and UCLA Medical Center. The heparinized sample was layered over Histopaque 1077 (Sigma, St Louis, MO) and was collected from five patients with advanced stages of MM. The heparinized sample was layered over Histopaque 1077 (Sigma, St Louis, MO) and was then centrifuged and washed three times in phosphate-buffered saline (PBS) after exposure to cold blood (RBC) lysis buffer (Sigma).

**Myeloma VH gene determination.** Total RNA was extracted using the guanidium isothiocyanate method from 30 × 10^6 BM mononuclear cells. Fifty picomoles of an antisense primer specific for either Cy or Ca was hybridized to 1 μg of RNA by heating to 65°C and then cooling on ice. The Ig RNA was then reverse transcribed by using 200 U of Moloney-murine leukemia virus reverse transcriptase (M-MLV; Gibco, Gaithersburg, MD) in a 30 μL volume of IX First Strand Buffer at 42°C for 1 hour after the addition of IX Taq polymerase buffer. Each PCR reaction was performed by cycling between 95°C for 1.5 minutes, 60°C for 1 minute, and 72°C for 1 minute of denaturation at 94°C, 1.5 minutes of annealing at 50°C, and 1.5 minutes of extension at 72°C except for an initial 5-minute denaturation at 95°C and a final 10-minute extension at 72°C. Thirty-two cycles of PCR amplification were performed because this allowed for detection of the monoclonal VH gene product in most MM patients and yet did not yield a product in normal controls. The polymerase chain reaction (PCR) was then used to amplify CD34+ cell DNA using oligonucleotide primers complementary to the most unique areas of the myeloma VH gene sequence to search for malignant cells in a subpopulation expressing this early hematopoietic antigen.

**Myeloma variable gene sequencing.** The remainder of the PCR product was purified after electrophoresis through a 1.6% low-melt agarose gel and excision of the appropriately sized band. Either GeneClean (Bic 101, La Jolla, CA) or Gelase (Epicenter Technologies, Madison, WI) was used to extract the cDNA from the agarose. The product was then sequenced using T4 polynucleotide kinase (Gibco) and then blunt end ligated into a Smal I cut and dephosphorylated Bluescript II SK− vector (Stratagene) overnight at 12°C using T4 DNA ligase (Gibco) according to the manufacturer’s protocol. One-fifth of the ligation mixture was then used to transform Escherichia coli strain XLI-Blue, which was then plated onto ampicillin-impregnated agar plates. Colonies containing appropriately sized inserts were selected after Magic MiniPrep (Promega, Madison, WI) purification and PeII (Stratagene) plasmid digestion. Dideoxy chain termination sequencing was performed on a minimum of four clones obtained from at least two separate PCR reactions using the Sequenase II kit (US Biochemicals, Cleveland, OH) according to manufacturer’s instructions with 16S α-CATP (Amersham, Arlington Heights, IL).

**Purification of CD34+ BM cells.** BM mononuclear cells were resuspended at a concentration of 100 × 10^6 cells/mL in PBS with 1% bovine serum albumin (BSA; Sigma) and incubated for 25 minutes on ice with a biotinylated 12.8 antibody (murine anti-CD34; Cell-Pro, Bothell, WA). The cells were passed through the CEPRATE LC34-Biotin system (CellPro) per the manufacturer’s protocol and adsorbed cells were collected and cryopreserved in dimethyl sulfoxide (DMSO) freezing media (Sigma) until needed.

**PCR conditions.** BM mononuclear and CD34+ cell DNA was extracted by phenol–chloroform extraction after proteinase K digestion and quantitated using the DNA DipSticK Kit (Invitrogen, San Diego, CA). Oligonucleotide primers were prepared (Operon, Alameda, CA) complementary to the most unique somatic mutation. One-fifth of the ligation mixture was then used to transform Escherichia coli strain XLI-Blue, which was then plated on ampicillin-impregnated agar plates. Colonies containing appropriately sized inserts were selected after Magic MiniPrep (Promega, Madison, WI) purification and PeII (Stratagene) plasmid digestion. Dideoxy chain termination sequencing was performed on a minimum of four clones obtained from at least two separate PCR reactions using the Sequenase II kit (US Biochemicals, Cleveland, OH) according to manufacturer’s instructions with 16S α-CATP (Amersham, Arlington Heights, IL).

**Patient characteristics.** Each of the five patients had evidence of MM by the criteria proposed by Durie and Salmon. Three of the patients had marrow extracted at the time of diagnosis (Wad#1, Wad#11, and Wad#12), whereas

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Table 1. Comparison of the Myeloma Sequence to the Most Homologous Germline \( V_\mu \) Gene

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>( V_\mu ) Family</th>
<th>Germline Gene</th>
<th>% Overall Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAD#1</td>
<td>V(_1)</td>
<td>DP-5</td>
<td>93.2</td>
</tr>
<tr>
<td>WAD#5</td>
<td>V(_3)</td>
<td>VH26</td>
<td>91.2</td>
</tr>
<tr>
<td>WAD#11</td>
<td>V(_3)</td>
<td>DP-58</td>
<td>84.4</td>
</tr>
<tr>
<td>WAD#12</td>
<td>V(_3)</td>
<td>DP-49</td>
<td>95.2</td>
</tr>
<tr>
<td>WAD#15</td>
<td>V(_4)</td>
<td>DP-65</td>
<td>94.2</td>
</tr>
</tbody>
</table>

The sequence expressed by the myeloma cells in each patient was compared with the most homologous germline \( V_\mu \) gene.\(^{25,26}\) The degree of overall homology with this gene is then noted.

the other two patients had their samples taken at the time of progressive disease (Wad#5 and Wad#15). All of the patients had stage III disease,\(^{28}\) multiple lytic bone lesions, and a large monoclonal Ig protein concentration in their serum (range, 3.7 to 8.0 g/dL). The range of plasmacytosis varied from 8% to 80% in the BM mononuclear fraction used for analysis.

Myeloma \( V_\mu \) gene sequencing. BM RNA was subjected to reverse transcriptase PCR (RT-PCR) with Cy or Ca plus an upstream \( V_\mu \) gene oligonucleotide primer. The \( V_\mu \) genes can be grouped into six gene families by sequence homology; therefore, PCR reactions were performed concurrently for each patient by varying the upstream \( V_\mu \) gene primer to one specific for each of the six \( V_\mu \) families.\(^{28}\) Product was detectable in only one of the six reactions in each case that represented the \( V_\mu \) gene family used by the myeloma clone (Table 1). This PCR product was then sequenced after insertion into a plasmid. At least four plasmid clones were sequenced and all of the sequences were identical in each patient (range, 4 to 5 sequenced). The myeloma heavy chain variable region sequence was then compared with published germline \( V_\mu \), D, and \( J_\mu \) gene sequences to determine the location of the CDRs and amount of somatic mutation that had occurred.\(^{32,33}\) Overall homology to the closest matching germline \( V_\mu \) gene sequence varied from 84.4% to 94.2%, demonstrating the substantial amount of somatic mutation that had occurred in the myeloma clones (Table 1). Patient-specific oligonucleotide primers were then designed complementary to the most unique regions of the CDRs to be used in our PCR assay for clonality (Fig 1 and Table 2).

Sensitivity of the PCR assay to detect tumor. BM mononuclear cell DNA was obtained from each patient and quantitated by the DNA Dipstick kit. The PCR was then used to amplify BM DNA with the patient-specific CDR primers. By serially diluting the BM DNA with placental DNA to maintain a final concentration of 0.1 \( \mu \)g of DNA per reaction, we could determine the sensitivity of our assay to detect clonality in each patient. All sensitivity reactions were performed in triplicate. PCR product was still detectable after dilution of myeloma BM DNA 1,000-fold in three patients and 10,000-fold in two patients (Table 3). After adjusting the results for the percentage of plasma cells in the sample analyzed, the sensitivity of the PCR assay to detect malignant cells ranged from 1:1,250 to 1:22,000 cells per reaction. Because the quantity of cells after CD34\(^{+}\) FACS was limited, only 0.1 \( \mu \)g of DNA (the amount present in approximately 20,000 human diploid cells) was used in each PCR mixture. Thus, the sensitivity to detect malignant cells may have been
higher if a larger quantity of cells were available for PCR analysis.

CD34+ cell purification. BM mononuclear cells from the same sample used to determine assay sensitivity were obtained from each patient, exposed to a biotinylated anti-CD34 antibody (12.8),31 and passed through the CEPRATE LC34-BIOTIN immunoadsorption column (CellPro, Bothell, WA). When the adsorbed cells were assayed for the CD34 antigen by a second antibody that binds to a different epitope (HPCA-2PE), the percentage of CD34+ cells after this initial purification varied from 50% to 92% (Fig 2). The adsorbed cell yield ranged from 0.5% to 1.5% of the initial BM sample. The adsorbed cell CD34+ purity and yield from these myeloma patients is similar to that obtained from normal BM specimens studied in our lab (data not shown) and by others36 and compares well with the expected percentage of CD34+ cells in normal BM, which is 0.5% to 2.5%.37 An aliquot of this initial population of cells was stored for PCR analysis. A second aliquot of cells was further enriched by sorting the cells through a FACSStar Plus after labeling CD34+ cells with the HPCA-2PE antibody. The positively staining cells were collected and, when reanalyzed, the purity of CD34+ cells was greater than 99.99% (Fig 3). When these cells were placed onto a slide and Wright stained, they had the typical appearance of a hematopoietic precursor cell.

Assay for clonality in the CD34+ population. As a control experiment, the PCR was used to amplify 0.1 µg of DNA extracted from the purified CD34+ cell population with primers specific for the β-actin gene (present as a single copy gene in all cells). A similar PCR reaction was performed using 0.1 µg of placental DNA and the patient’s BM DNA. All reactions were performed concurrently, and when the PCR products were run on an agarose gel, the intensity of the PCR product was identical. This implies that the DNA was adequately quantitated in each sample and was of sufficient quality to allow successful PCR amplification.

CD34+ cell DNA obtained after the initial immunoadsorption step was then amplified with the patient-specific oligonucleotide primers, and PCR product remained detectable in the four patients analyzed. When the PCR product was quantified and compared with a concurrently run control of serially diluted unseparated BM DNA, a 1.5 to 2.5 log reduction in myeloma contamination was noted for each patient (log reduction: WAD#1, 1.5; WAD#5, 2.0; WAD#12, 2.5; and WAD#15, 1.5).

Using the same PCR conditions with the more purified aliquot of FACS CD34+ DNA, no detectable product was obtained for any patient (Fig 4). All reactions using CD34+ immunoadsorbed or FACS DNA were performed in duplicate and, concurrent with amplification of BM DNA, serially diluted with placental DNA to maintain an equivalent amount of total DNA (0.1 µg/reaction). There was never any PCR product detectable when placental DNA alone was amplified with the patient-specific primers. In addition, there was no detectable PCR product after amplification of a different patient’s BM DNA under the same conditions depicting the specificity of the patient CDR primers used. Because our assay was capable of detecting 1 tumor cell in 1,250 to 22,000 normal cells and because these PCR reactions were performed in duplicate, we should have been able to detect 1 tumor cell in 2,500 to 44,000 BM cells (Table 3). We specifically chose patients with advanced and untreated disease to maximize our chance for detecting a CD34+ malignant cell. Our inability to do so strongly suggests that the myeloma cell population does not harbor a CD34 antigen expressing cell component.

DISCUSSION

The identity of the myeloma precursor cell remains controversial because of conflicting data but its characterization remains crucial if methods of autograft purging are to be accurately assessed. The existence of shared idiotypes be-

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>% PCs in BM Sample Analyzed</th>
<th>Sensitivity of PCR</th>
<th>Sensitivity to Detect Tumor</th>
<th>PCR Product Detectable in CD34+ Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAD#1</td>
<td>80</td>
<td>1:1,000</td>
<td>1:2,500</td>
<td>No</td>
</tr>
<tr>
<td>WAD#5</td>
<td>15</td>
<td>1:1,000</td>
<td>1:13,000</td>
<td>No</td>
</tr>
<tr>
<td>WAD#11</td>
<td>45</td>
<td>1:10,000</td>
<td>1:44,000</td>
<td>No</td>
</tr>
<tr>
<td>WAD#12</td>
<td>60</td>
<td>1:1,000</td>
<td>1:33,000</td>
<td>No</td>
</tr>
<tr>
<td>WAD#15</td>
<td>8</td>
<td>1:1,000</td>
<td>1:26,000</td>
<td>No</td>
</tr>
</tbody>
</table>

The sensitivity of the PCR assay to detect tumor was determined by multiplying the percentage of plasma cells (PCs) in the BM mononuclear cell specimen by the maximal fold dilution of BM DNA with placental DNA that yielded reproducibly visualizable PCR product. This sensitivity was then doubled as all CD34+ PCR reactions were performed in duplicate.
between myeloma cells and IgM-expressing pre-B lymphocytes has been heralded as evidence for clonality in this less-differentiated cell population. However, the specificity of anti-idiotypic antibodies may not be enough to prove clonality because we have identified specific antibodies that are occasionally shared by patients expressing different \( V_H \) genes. Later studies of surface antigen expression on myeloma cells showed the presence of myeloid antigens on some of the malignant cells, implying derivation of the disease from an undifferentiated progenitor cell. However, the aberrant expression of these antigens on terminally differentiated myeloma cells could also explain this observation.
This aberrant expression was then confirmed when a purified population of plasma cells from normal patients were studied by Terstappen et al. After collecting normal plasma cells by their light scattering properties and high expression of the CD38 antigen, a small proportion of these cells were detected that coexpressed the myeloid antigens CD33 and CD13. Consequently, the presence of these antigens on a malignant cell cannot be cited to prove the existence of a malignant less-differentiated B-cell or stem cell component.

We have chosen to use the unique Ig heavy chain variable region sequence of the myeloma cells as the marker of clonality in myeloma. To maximize specificity, the PCR was used with oligonucleotide primers designed for each patient to two unique areas of the heavy chain variable region sequence. We were able to collect an extremely pure population of CD34 cells by using two methods of purification (immunoadsorption and FACS), and both antibodies used to identify these cells recognize a different CD34 epitope, permitting the collection of a CD34 fraction of greater than 99.99% purity. Because the CD34 antigen is expressed on the majority of pluripotent stem cells, our inability to detect the malignancy in a highly purified population of these cells makes the existence of a stem cell precursor unlikely. In addition, aberrant expression of the CD34 antigen onto the malignant plasma cells did not occur in these patients.

Because Ig heavy chain gene rearrangement does not occur until the pre-B-cell stage of differentiation and because this assay will only detect cells that have undergone this process, one could postulate that a malignant CD34 cell that has yet to undergo Ig gene rearrangement could exist and still give rise to a malignant plasma cell after further differentiation and escape detection by this assay. However, after analyzing the Ig gene sequences of the myeloma cells, this scenario seems unlikely. We have sequenced the VH genes in 40 patients with myeloma and compared these sequences with known germline VH genes. A high frequency of somatic mutation was detected (an average of 8.5% of the nucleotides were changed when compared with germline). In addition, there was evidence of prior antigenic selection in many cases, as noted by the high incidence of replacement versus silent mutations in the CDRs (the areas most responsible for determining antigen avidity). These findings imply that the myeloma cell developed from a cell previously exposed to antigen and selected for by its antigen-binding capability. If an undifferentiated cell in MM were to exist, to account for these findings of somatic mutation and prior antigenic selection pressure the plasma cell progeny should have an extremely diverse Ig gene sequence. However, after sequencing multiple clones in each patient, we found no evidence of intraclonal diversity in the 40 patients studied.

This finding is in concordance with the findings of a previous study by Bakkus et al. When the myeloma VH gene was sequenced from BM obtained at different time points in a patient’s disease, no additional VH sequence changes were noted. These results further attest to the stability of the myeloma VH gene sequence over time.

The initial passage of BM cells through the immunoadsorption column resulted in an average 2 log reduction in myeloma cell contamination. This result is not surprising because the percentage of CD34 cells is increased approximately 2 logs by this procedure. The actual efficiency of this process in eliminating myeloma cells as applied clinically in autologous transplantation is much greater, however, because the total number of cells reinfused after CD34 selec-
tion is also reduced by an additional 2 to 3 logs. Because the collection of CD34+ hematopoietic cells can engraft humans after myeloablative chemotherapy, the selection of these cells should allow an efficient and simple means of purging autografts in MM. A multi-institutional trial using CD34+ peripheral blood progenitor cell transplantation as treatment for myeloma has been enrolling patients for more than 1 year, and we are currently quantifying the reduction of tumor contamination in these CD34-selected peripheral stem cell autografts.

REFERENCES

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