Brief Definitive Report

Evidence for a Bone Marrow B Cell Transcribing Malignant Plasma Cell VDJ Joined to C\(\mu\) Sequence in Immunoglobulin (IgG)- and IgA-secreting Multiple Myelomas

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Summary

Multiple myeloma is a B cell malignancy characterized by the expansion of plasma cells producing monoclonal immunoglobulins (Ig). It has been regarded as a tumor arising at the B, pre-B lymphocyte, or even stem cell level. Precursor cells are presumed to proliferate and differentiate giving rise to the plasma cell clonal expansion. Antigenic features and specific Ig gene rearrangement shared by B lymphocytes and myeloma cells have supported this hypothesis. However, the existence of such a precursor is based upon indirect evidence and is still an open question. During differentiation, B cells rearrange variable (V) regions of Ig heavy chain genes, providing a specific marker of clonality. Using an anchor polymerase chain reaction assay, these rearranged regions from five patients with multiple myeloma were cloned and sequenced. The switch of the Ig constant (C) region was used to define the B cell differentiation stage: V regions are linked to C\(\mu\) genes in pre-B and B lymphocytes (pre-switch B cells), but to C\(\gamma\) or C\(\alpha\) in post-switch B lymphocytes and plasma cells (post-switch B cells). Analysis of bone marrow cells at diagnosis revealed the presence of pre-switch B cells bearing plasma cell V regions still joined to the C\(\mu\) gene. These cells were not identified in peripheral blood, where tumor post-switch B cells were detected. These pre-switch B cells may be regarded as potential myeloma cell precursors.

Materials and Methods

Patients and Nucleic Acid Extraction. Four cases of multiple myeloma were evaluated at diagnosis and one at relapse. Two patients had an IgG (MM-20 and MM-23) and three an IgA (MM-15, MM-67, and MM-125) monoclonal protein. Specimens were obtained during standard diagnostic procedures. Bone marrow plasma cells and lymphocytes were separated on a Ficoll-Hypaque density gradient. DNA was obtained by cell lysis, phenol extraction, and ethanol precipitation. RNA was isolated by the guanidinium isothiocyanate and cesium chloride centrifugation method.

Synthesis of cDNA and Tailing Reaction. Two syntheses of cDNA were performed: (a) total RNA was reverse transcribed using an isotype-specific primer (\(\alpha\) or \(\gamma\)) to have the Ig cDNA; and (b) total...
RNA was reverse transcribed into total cDNA using an oligo dT-15 primer. 20 µg of total RNA was reverse transcribed with 20 pmol of reverse transcription primer (γ: GACCGATGGGCCCTT-GGTGGAGGAC [15], or α: GACCTTGGGCTGCTGGGGA-TGC [16]). A 50-µl reaction was carried out in 10 mM diithothreitol, 1 mM dNTPs (Pharmacia LKB Biotechnology, Uppsala, Sweden), 1× reverse transcriptase buffer (50 mM Tris-HCl, 6 mM MgCl₂, 40 mM KCl), final concentration, adding 20 U of ronucleoside inhibitor (RNasin; Promega, Madison, WI), and 400 U Moloney MuLV reverse transcriptase (Superscript, Gibco BRL, Gaithersburg, MD). The reaction was incubated 1 h at 37°C. The Ig cDNA was then run in 200 µM dATP, 1× terminal deoxynucleotidyl transferase (TdT) buffer, final concentration, adding 30 U of dTdT enzyme (Gibco BRL), and incubated 10 min at 37°C.

**PCR Conditions.** Three reactions were performed. The anchor-PCR assay (Fig. 1) included PCR-1 and -2, whereas PCR-3 was a standard amplification. For PCR-1, 20% of dA-tailed Ig cDNA was amplified in 10% DMSO, 200 µM dNTPs, 1× Taq buffer (50 mM KCl, 10 mM Tris-HCl pH 8, 1 mM MgCl₂, 1% [wt/vol] gelatin), final concentration, adding 2.5 U of TdT enzyme (Gibco BRL), and incubated 10 min at 37°C. The amplified DNA was then run on a 1.5% low melting point agarose and the 500-bp band was cut out, phenol/chloroform-extracted and precipitated in 2 vol of ethanol, and then cloned in a Bluescript SK vector (Stratagene, San Diego, CA) according to standard procedures (14). Restriction enzyme analysis was carried out on plasmid DNAs prepared by the alkaline lysis method (14). Miniprep plasmid DNAs were sequenced with the Sequenase version 2.0 kit (United States Biochemicals Corp., Cleveland, OH) according to the manufacturer's recommendations. Sequence analysis was performed using the PC-GENE programs (IntelliGenetics, Inc., Mountain View, CA).

**Southern Blot and Direct Sequencing Analysis.** 20% of the PCR product was analyzed by agarose gel electrophoresis. The amplified DNA was blotted overnight onto nylon membranes (Stratagene), and hybridized to CDRIII probes end-labeled with γ[32P]ATP (14). The nucleotide sequence of the junction between the VDJ and Cµ genes was determined by direct sequencing of amplified DNA. 5 µl of bone marrow total cDNA was amplified using CDRII and µ-5 (GGGAATTCAAGGAAATCTGGTGCG [17]) primers. µ-5 is located 42 bp from µ-1 in the 3' direction. Amplification conditions were as described above. The CDRIII oligonucleotides (case MM-15 and MM-25) were also used as sequencing primers after end-labeling with γ-[32P]ATP. Sequencing reactions were performed as previously described (18). Reaction products were run on a 6% acrylamide/urea gel, fixed in 10% acetic acid, and then exposed at -70°C for varying periods of time.

**Results**

The anchor-PCR assay is outlined in Fig. 1 (19). Briefly, Ig cDNA was synthesized from bone marrow total RNA using primers specific for plasma cell C region, and then a dATP tail was added by TdT enzyme to the 3' end. The dA-tailed Ig cDNA was amplified twice, using two sets of primers (H-RE2, RE-TTTT, and RE, RE2). Finally, the amplified VDJ were cloned and sequenced. For each patient 10–20 clones were sequenced. The first sequencing gel analysis was per-

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**Table 1. Tumor-specific Primers and Probes**

| Patients | CDRII 5' primer | Distance | CDRIII probe |
|----------|-----------------|----------|--------------|
| MM-15    | TTTCAAGACGCGAGT | 138      | AGACTGAGGGCCAGTTGATC |
| MM-20    | CTACACTGATGGTTACGGAATTC | 126 | GGTTCTCCCCCATACGACTA |
| MM-23    | CCTTATGACATATGATTC | 124 | TTGTTTGGGCGCATATCC |
| MM-67    | ATAGTGGAGGTTGCGATTC | 130 | GGACGGGGAACCTTGATA |
| MM-125   | CGTAGTGGAGGTTGACAAAG | 136 | GGACATAACAGCTGGC |

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Figure 1. Schematic representation of the anchor PCR assay. Bone marrow total RNA was reverse transcribed into Ig cDNA using an isotype-specific primer (CH), and then dA-tailed by TdT enzyme for anchor PCR. The tailed cDNA was amplified twice (PCR1/PCR2) by using two sets of primers (JH-RE2 with RE-TTTT, and RE and RE2). The amplified VDJ were then cloned and sequenced. After sequencing analysis, the CDK were identified and tumor-specific primers and probes were generated.

Figure 2. PCR-based detection of pre-switch B cells. Detection of clonal pre- and post-switch B cells in bone marrow and peripheral blood total cDNA of five myeloma patients. The amplifications were performed using CDRII 5' primer and different C region 3' primers. Amplified DNAs were then hybridized to CDRIII probes. (A) MM-15 patient; (B) MM-20 patient; (C) MM-23 patient; (D) MM-125; and (E) MM-67. Lane 1, bone marrow total cDNA amplified with γ or α primers; lane 2, empty; lane 3, bone marrow total cDNA amplified with μ-1 primer; lane 4, peripheral blood total cDNA amplified with μ-1 primer; lane 5, empty lane; lanes 6 and 7, polyclonal cDNAs; and lane 8, no DNA control. (F) lane a, MM-15 peripheral blood total cDNA amplified with α primer; lane b, MM-20 peripheral blood total cDNA amplified with μ-1 primer; lane c, MM-23 peripheral blood total cDNA amplified with γ primer; lane d, MM-125 peripheral blood total cDNA amplified with μ-1 primer; and lane e, MM-67 peripheral blood total cDNA amplified with α primer.
Figure 3. Demonstration by direct sequencing of the junction of the plasma cell VDJ with the Cµ sequence. (A) Schematic representation of primers used in the amplification (CDRII, µ-5) and sequencing (CDRIII) of the VDJ-Cµ junction. (B) After the amplification of bone marrow total cDNA (case MM-15, and MM-125), direct sequencing analysis showed the joining of the 3' end of plasma cell VDJ (JH6 and JH4) to the first exon (CH1) of Cµ gene. (Arrow) Junction point between JH6, JH4, and CH1. G, A, T, C (dideoxynucleoside triphosphates) represent the order of loading of the sequencing gel.

Discussion

The involvement of pre-B and/or B lymphocytes in multiple myeloma has been mainly deduced from the observation that they share some of the plasma cell antigenic features (1-5, 20) and that myeloma-specific IgH gene rearrangements take place in PBMC (7, 8). However, some technical aspects have prevented such involvement from obtaining universal acceptance. For instance, myeloma protein may nonspecifically bind to Fc receptors of PBL, which may then erroneously be recognized as neoplastic by anti-idiotypic antibodies (21). In addition, the specificity of these antibodies has been shown to be limited, since they react with more than one myeloma protein and also recognize several normal B cell clones (22, 23). Finally, the presence in the peripheral blood of myeloma-specific IgH gene rearrangements has been ascribed to plasma cells (8, 9). For these reasons, therefore, the B lymphocyte origin for multiple myeloma has thus been disputed, and it has been regarded as a tumor originating at plasma cell level.

We devised an experimental strategy based upon the molecular analysis of IgH genes. The CDRs provided a specific marker of clonality, whereas the presence of Cµ gene allowed the distinction between pre- and post-switch B cells (24). The hypothesis that a malignant plasma cell derives from a pre-switch B cell implies the existence of a B cell in which the tumor VDJ is still joined to Cµ gene. We showed that the VDJ sequences identified by our assay represent the malignant plasma cell VDJ, and then we looked for a clonal pre-switch B cell population. Analysis of bone marrow RNAs demonstrated the existence of such a population. Conversely, these pre-switch B cells were not found in the peripheral blood, where tumor post-switch B cells were detected. It should

reamplified using CDRII and µ-5 primers. The use of µ-5 allowed the amplification of a longer fragment of Cµ first exon. PCR products were then sequenced and the junction of tumor VDJ to the Cµ gene was definitively demonstrated (Fig. 3). 80 bp of the Cµ gene was read and showed a germline configuration.

Our analysis was also extended to the peripheral blood total cDNA samples. The amplifications performed with CDRII and µ-1 primers did not detect the presence of clonal pre-switch B cells. Conversely, PCR reactions performed with CDRII and α or γ 3' primers revealed the presence of tumor post-switch B cells (Fig. 2 F).
be pointed out that pre-switch B cells in the bloodstream could be too few to be detected by our assay. An IgH transcript in which the VDJ is still attached to Cμ sequences can be attributed to pre-B and/or B lymphocytes, even though memory B lymphocytes might be included. It has been reported that memory B lymphocytes may coexpress Cμ and Cγ or Cε genes (25, 26), i.e., they can synthesize Ig molecules with different isotype linked to the same idiotype. The switch mechanism postulated for this finding is the alternative splicing of a large Ig RNA transcript, and not the switch recombination characterized by Cμ gene deletion (for a review see reference 27).

The bone marrow B cells carrying the tumor VDJ, and still expressing Cμ genes (pre-switch B cells), may be regarded as plasma cell precursors. It should be pointed out, however, that our data do not provide a direct demonstration of malignancy, and do not exclude the existence of even more immature precursors. The presence of peripheral blood cells in which the tumor VDJ sequences are linked to Cα or Cγ genes (post-switch B cells) leads us to speculate that these cells may be responsible for the dissemination of the disease throughout the axial skeleton. The contamination of tumor cells in peripheral blood is lower than bone marrow, and the apparent absence of pre-switch B cells in the peripheral blood indicates that it could be an alternative source of normal stem cells for autograft procedures.

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