The Clonal Hierachy in Multiple Myeloma

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In this report we evaluated the number and phenotype of blood circulating B-cell subsets at different stages of differentiation in 26 patients with newly diagnosed multiple myeloma (MM). In all patients, plasma cells and/or plasma blasts could be identified by flow cytometry with a mean frequency of 1.20% and 0.07%, respectively. In 76.9% of the patients these cells showed aberrant expression mainly of CD56, CD28 and CD117, none of these markers were found on the earlier B-lymphocytes. Clonal B-cells preceding the plasma blast stage were identified by patient specific IgH RT-PCR on sorted B-cell subsets. The clonal cells included the less differentiated $CD38^{+}/CD19^{+}$ and $CD38^{-}/CD19^{+}$ subsets, illustrating that the clonal cells are part of an ongoing differentiation process. Further, the presence of $CD38^{-}/CD19^{+}$ cells with somatically mutated Cγ transcripts identical to the tumor-specific Cα transcript, shows that the clonal hierarchy in myeloma may include memory B-cells.

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The predominant cell type in multiple myeloma (MM) is the plasma cell displaying a heterogeneous phenotype, including functionally distinct populations (1, 2). The majority of myeloma patients has a $CD38^{++}/CD56^{++}/CD19^{-}$ plasma cell phenotype, in contrast to normal plasma cells which have a $CD38^{++}/CD56^{-}/CD19^{+}$ phenotype (3). Further, myeloma plasma cells may show aberrant expression of a promiscuous array of cell surface markers (4–6). The myeloma plasma cells are frequently identified in the PB and may serve as a prognostic factor (7). Several studies have identified circulating clonal cells recognized by idiotype-specific antibodies (8) or with patient-specific polymerase chain reaction (PCR) methods identifying cells with IgH VDJ rearrangements identical to BM myeloma plasma cells (9, 10). The involvement of earlier stage B-cells with stem cell characteristics (10) and the presence of pre-switch clonal cells (11–13) have been reported, suggesting that the oncogenic event occurs at a differentiation stage preceding the plasma cell. PB-localized clonal cells have been reported to include a drug resistant compartment that may be responsible for both dissemination and relapse in this disease (14, 15). The identification and characterization of clonal cells in the PB of MM patients are of importance for understanding the biology of MM as well as for the introduction of new treatment strategies urgently needed.

In this study we analyzed the PB of myeloma patients for the presence of aberrant or clonal cells in B-cell subsets representing different stages of B-cell differentiation. We discuss the results in relation to the origin of myeloma.

MATERIAL AND METHODS

Patients and samples

A total of 26 PB samples taken at time of diagnosis and one diagnostic BM, were obtained from 26 MM patients after informed consent. The isolation of BM mononuclear cells (BMMNC), peripheral blood mononuclear cells (PBMNC) and RNA was performed as previously described (16).

Flow cytometry

PBMNC and BMMNC were stained as previously described (17) with the monoclonal antibodies CD19 FITC, CD20 FITC, CD23 PE, CD38 APC, CD45 PERCP and CD56 PE (Becton Dickinson Immunocytometry Systems (BDIS), San Jose, CA, USA). CD117 PE and CD28 FITC (Immunotech, Marseille, France). IgM, IgD, IgG, IgA, lamda PE and kappa FITC moabs were obtained from Dakopatts A/S, Glostrup, Denmark. A minimum of 100000 cells was collected using a FACScan (BDIS) in acquisition mode followed by analysis with CELLQuest 1.2 software (BDIS). IgG 1 FITC (BDIS) and IgG 1 PE (BDIS) were used as negative control. Flow-sorting of single cells was performed as previously described (16).

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Generation of allele-specific oligonucleotides (ASO)

To identify and sequence the CDR3 region of the IgH gene, we used a panel of consensus oligonucleotides to amplify the myeloma-specific IgH sequence for each patient, as previously described (16).

Single cell RT-PCR on flow-sorted cells

Single cell IgH RT-PCR was performed as previously described (16–18). Briefly, the flow-sorted cells were lysed by heating at 95°C for 5 min, followed by addition of 8 μl DEPC-treated water and 1 μl of oligo(dT) primer (from 50 μM stock). This mixture was heated to 65°C for 10 min and then placed on ice. Aliquots of, 2 μl of 0.1 M DTT, 1 μl of 10 mM dNTP mix, 1 μl RT enzyme (M-MLV, 200 U/μl, GIBCO BRL, Copenhagen, Denmark), and 2 μl of 10× RT buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl, 25 mM $MgCl_2$, and 1 mg/ml BSA) were added to each tube. Synthesis of cDNA was performed at 37°C for 60 min followed by 5 min at 95°C. To the 20 μl RT product, 30 μl of Step 1 mix was added. Step 1 mix consisted of 0.5 μl dNTP mix (10 mM), 0.5 μl Taq polymerase (5 U/μl, GIBCO BRL, Copenhagen, Denmark), 1 μl of each outer primer (IgH PCR = $V_{Hx} - J_H$ or C C, Cμ primers) from 25 μM stock, 22 μl of dH_2O, and 5 μl of 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$). The cDNA was amplified on a Perkin–Elmer 9600 DNA thermal cycler (PE Applied Biosystems, Foster City, CA, USA). Each PCR cycle consisted of 94°C heat denaturation for 0.5 min followed by primer annealing at a temperature optimized for each V_x primer for 0.5 min followed by primer extension at 72°C for 0.5 min. Thirty-five cycles were performed. The first cycle was preceded by a 2 min denaturation step at 95°C, and the last elongation step was prolonged to 10 min. One μl of Step 1 PCR product was added to 24 μl of Step 2 PCR mix. Step 2 PCR mix consisted of 0.5 μl dNTP mix (10 mM), 0.125 μl Taq polymerase (5 U/μl, GIBCO BRL, Copenhagen, Denmark), 0.5 μl of each of the inner primers (IgH PCR = V_{Hx} – ASO) from 25 μM stock, 15 μl of water, 5 μl cresol (60% sucrose/1 mM cresol), and 2.5 μl of 10× PCR buffer. $MgCl_2$ concentration, primer concentration and annealing temperature were optimized for each ASO IgH RT-PCR assay. The second round of PCR was performed as described for the first round and 15 μl of the PCR product was analyzed on a 2% agarose gel. Where sufficient material was available, experiments were performed in duplicate on separate days. The identity of the IgH PCR products was confirmed by direct sequencing of the PCR product.

RESULTS

Frequency and phenotype of B-cell subsets in the PB

The frequency and phenotype of B-cell subsets in the PB were determined by four-color flow cytometry at time of diagnosis in 26 MM patients as described in the Materials and methods section. The frequency of plasma cells ($CD38^{++}/CD45^{-/i}/CD19^{-}$), plasma blasts ($CD38^{++}/CD45^{+}/CD19^{+}$) and B-lymphocytes ($CD38^{-/+}/CD45^{+}/CD19^{+}$) is shown in Table 1. The frequency of aberrant expression of a panel of CD antigens and surface Ig on plasma cells and plasma blasts in MM patients is shown in Table 2.

Table 1

Frequency of B-cell subsets in PB of myeloma patients

Phenotype	Frequency (%)[a]		
	Median[b]	Mean[b]	Range
$CD38^{++}/CD45^{-/i}/CD19^{-}$	0.05	1.20	0–17.71
$CD38^{++}/CD45^{+}/CD19^{+}$	0.04	0.07	0–0.56
$CD38^{-/+}/CD45^{+}/CD19^{+}$	3.85	7.13	1.16–55.04

Flow cytometric analysis of B-cell subsets in blood of myeloma patients. The B-cells are divided into three groups, plasma cells ($CD38^{++}/CD45^{-/i}/CD19^{-}$), plasma blasts ($CD38^{++}/CD45^{+}/CD19^{+}$) and B-lymphocytes ($CD38^{-/+}/CD45^{+}/CD19^{+}$).

[a] The frequency is the percentage of PBMNC.

[b] The median and mean values were calculated from the analysis of PB samples obtained from 26 MM patients at time of the diagnosis.

Plasma cells were observed with a mean frequency of 1.20% of PBMNC, seven patients (26.9%) did not have a level of plasma cells detectable by flow cytometry. Of the 19 patients with detectable plasma cells (73.1%), the plasma cells expressed at least one aberrant marker in 15 of these patients (78.9%).

Plasma blasts were observed with a mean frequency of 0.07% of PBMNC, five patients (19.2%) did not have a level of plasma blasts detectable by flow cytometry. Out of 21 patients with detectable plasma blasts (80.8%), the plasma blasts expressed at least one aberrant marker in 15

Table 2

Aberrant expression of surface markers on circulating myeloma cells

Marker	Frequency (%)[a]
CD56	78.9
CD28	52.6
CD117	26.3
CD23	5.3
CD20	0
sIg	26.3

B-cell subsets in PB of myeloma patients were analyzed by four-color flow cytometry. Of 26 patients analyzed 19 patients had expression of at least one aberrant marker on plasma cells and/or plasma blasts.

[a] The frequency is the percentage of MM patients displaying the aberrant marker on circulating myeloma cells.

of these patients (71.4%). The proportion of plasma blasts being aberrant was variable and ranged from 7.34% to 100% (mean = 43.07%) of the population.

In the 26 patients analyzed, all had a plasma cell and/or plasma blast population detectable by flow cytometry, and the majority of these patients (76.9%) had circulating myeloma cells with expression of at least one aberrant marker were found. In all patients having both a plasma cell and plasma blasts population evaluable by flow cytometry, aberrant markers found on plasma blast were always present on the plasma cell.

B-lymphocyte levels at time of diagnosis in MM patients were highly variable (mean = 7.13, range 1.16–55.04), but not significantly different from a normal control group (mean = 8.49, range 4.84–14.84, $n = 20$ (unpublished data)), even though the range in $CD19^+$ cell levels was larger in the group of MM patients than in normal donors. The aberrant markers found on plasma cells and plasma blasts were not identified on any of the analyzed B-lymphocyte populations.

Clonal $CD19^+$ cells represent an ongoing differentiating population

To identify clonal $CD19^+$ cells and determine their frequency at different stages of B-cell differentiation, PBMNC from one MM patient were stained with CD19 and CD38 moabs and the $CD19^+/CD38^{++}$, $CD38^+$ and $CD38^-$ subsets were flow-sorted directly to PCR tubes at different numbers, followed by ASO IgH RT-PCR analysis (Fig. 1). The highest frequency of clonal $CD19^+$ cells was found in the late-stage $CD38^{++}$ cells (plasma blasts), containing 18% of clonal cells. The less differentiated $CD38^+$ and $CD38^-$ subsets contained 5% and 4% of $CD19^+$ clonal cells, respectively. The frequency of clonal cells was calculated by limiting dilution statistics (18).

In addition, we performed ASO RT-PCR on the CD38/CD19 subsets discriminating whether the clonal VDJ sequence was linked to $C\gamma$, $C\alpha$ or $C\mu$ (Fig. 2). The isotype of the patient was IgA, and $C\alpha$ transcripts were clearly seen in all subsets. However, in the CD38 negative subset $C\gamma$ transcripts were present.

Thus, at least four populations of clonal cells at different stages of differentiation could be identified in the actual patient based alone on CD38 and CD19. The clonal cells ranged from $CD38^-/CD19^+/C\alpha^+/C\gamma^+$ cells phenotypically similar to a memory B cell, $CD38^+/CD19^+/C\alpha^+$, $CD38^{++}/CD19^+/C\alpha^+$, and to the $CD38^{++}/CD19^-/C\alpha^+$ circulating plasma cell.

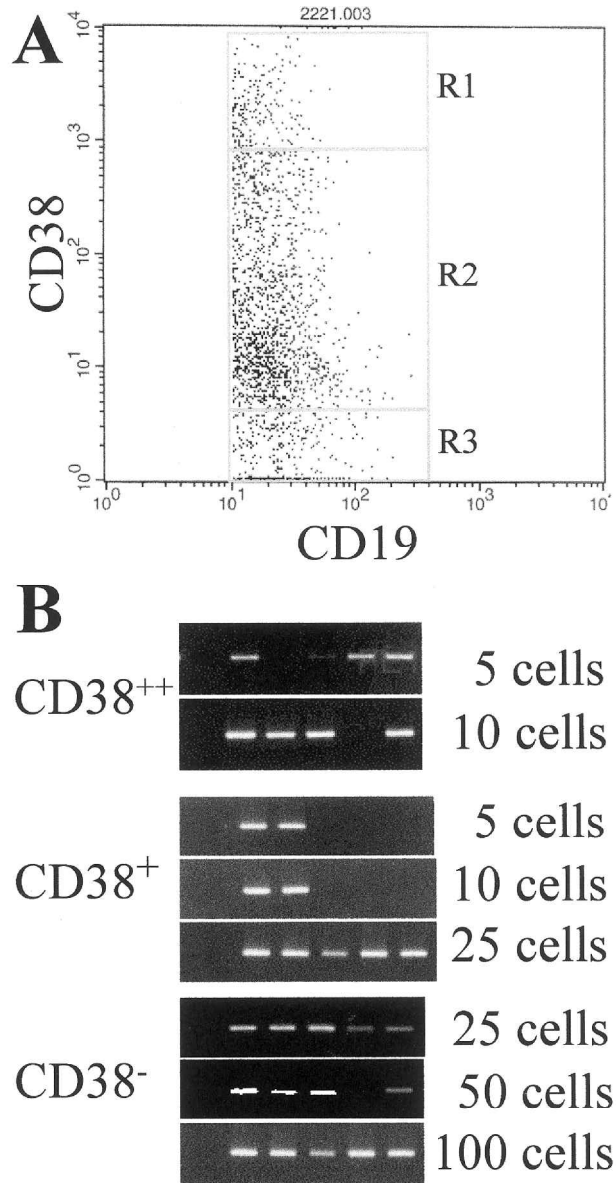

Fig. 1. The frequency of clonal $CD19^+$ cells at different stages of B-cell differentiation. (A) Flow cytometric analysis of PBMNC showing CD19 vs. CD38 expression. The rectangles R1-R3 identifies the sort gates used for the CD19 positive $CD38^{++}$, $CD38^+$ and $CD38^-$ subsets, respectively. (B) ASO IgH RT-PCR performed on the sorted subsets. From left to right: negative control, five wells with the specified number of cells. The frequency of clonal cells in the $CD19^+$ subsets were calculated to 18% ($CD38^{++}$), 5% ($CD38^+$) and 4% ($CD38^-$) as described in the Materials and methods section.

Clonal $CD19^+$ cells in the BM

The presence of $CD19^+$ clonal cells in the PB of MM patients has been reported by several groups (19–22). To investigate whether a similar fraction of $CD19^+$ cells in the BM was clonal we isolated BMMNC from one patient and flow-sorted the $CD19^+$ cells to PCR tubes followed by ASO IgH RT-PCR (Fig. 3). The BMMNC contained 0.86% $CD19^+$ cells, 8.37% of these cells were clonal. In comparison, the PBMNC obtained from this patient contained 1.47% $CD19^+$ cells out of which 6.10% were clonal cells (published data).

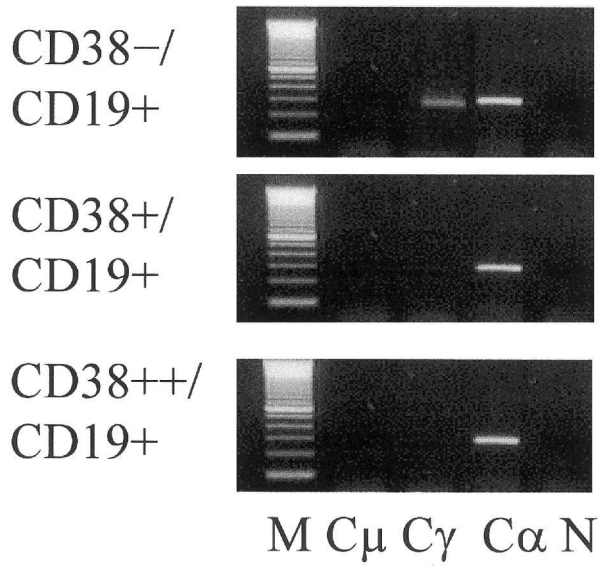

Fig. 2. ASO RT-PCR discriminating whether the clonal VDJ sequence were linked to Cγ, Cα or Cμ, were performed on the CD38/CD19 subsets defined by the R1–R3 sort gates (Fig. 1). The isotype of the illustrated patient was IgA, and Cα transcripts is clearly seen in all subsets. However, in the CD38 negative subset Cγ transcripts were present. M, molecular size marker; N, negative control.

DISCUSSION

We have investigated plasma cells, plasma blasts and B-lymphocytes for the expression of surface markers CD20, CD23, CD28, CD56, CD117 and for surface immunoglobulin (sIg), known to be abnormally expressed on mature myeloma plasma cells (3–6). When speculating on the origin of myeloma cells based on surface antigen expression, several precautions need to be taken. Normal plasma cells show expression of cell surface antigens known to be expressed in both lymphoid and myeloid-specific differentiation (23). Thus, the presence of early stage lymphoid markers or myeloid markers on myeloma cells may not be linked to the oncogenesis of MM. The panel of markers used in this study (see Tables 1 and 2) facilitates the identification of the myeloma plasma cell. The identification of aberrant plasma blasts was based on the expression of CD28 in combination with CD117, CD28 in combination with CD56 over-expression or on CD56 over-expression alone. These markers may be regarded as 'tumor specific antigens' for plasma cells and blasts (3, 5, 6). Although normal plasma cells may express CD56 (6), myeloma cells are distinguished by their high expression of this marker. Thus, based on the flow cytometric analysis we may conclude that the PB contains plasma cells phenotypically similar to the BM localized myeloma plasma cells. In addition, a population of $CD19^-$ plasma blasts was present in PB that displayed the same aberrant phenotype as the plasma cells. The flow cytometric analysis of B-lymphocytes did not identify cells with an aberrant phenotype.

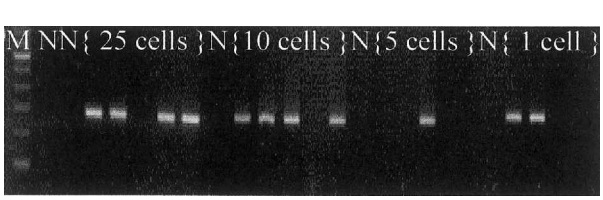

Fig. 3. To determine the percentage of clonal cells in the BM localized $CD19^+$ population, these cells were sorted in numbers of 1, 5, 10 and 25 cells, five wells at each cell number and ASO RT-PCR analysis was performed. M, molecular weight size markers (100 basepair ladder); N, negative control. The frequency of clonal cells in the $CD19^+$ population were calculated as described in the Materials and methods section to 8.37%.

The frequency of $CD19^+$ cells determined at time of diagnosis in this study was not significantly different from normal donors. These results are in accordance with the findings of others (19, 24, 25). However, the findings contradict the reported presence of a generally high level of $CD19^+$ cells (20, 26).

To investigate whether cells belonging to the myeloma clone were present in B-cells preceding the plasma blast stage, we combined the flow cytometric analysis with ASO IgH RT-PCR in a MM patient. The high frequency of clonal cells found in the plasma blast population confirmed that the aberrant plasma blast identified by flow cytometry was indeed part of the myeloma clone. In addition, clonal cells were also identified in the less differentiated $CD38^+/CD19^+$ and $CD38^-/CD19^+$ subsets. Further, the presence of cells expressing Cγ and Cα transcripts with somatically mutated VDJ sequences identical to the tumor-specific Cα transcript, identified in the myeloma plasma cells, were restricted to the $CD38^-/CD19^+$ subset. These findings are in line with the accumulating evidence that the myeloma cells are part of an ongoing differentiation process, responsible for the heterogeneity of clonal cell-types involved.

In one MM patient we showed that the BM contained a frequency of clonal $CD19^+$ cells similar to that observed in PB. Clonal $CD38^-/CD45^+$ cells expressing Cμ, Cγ and Cα transcripts have also been identified in the BM (12). These findings may suggest that the less differentiated clonal cells are truly circulating cells and not a 'spill-over' of the clonal compartment in the BM.

Myeloma cells show stable VDJ joining sequences, thus myeloma cells lack the recombinase activities found in the early stages of B-cell differentiation. This is in accordance with the absence of clonal cells in the $CD34^+/CD19^+$ compartment (16, 27, 28). Although clonally related B-cells, including cells with a clonotypic VDJ sequence linked to the Cμ constant region of the IgH gene, have been found (11–13), myeloma cells show a very high frequency of somatic mutations in the complementary determined regions (29–32) and an absence of intra-clonal diversity (29, 30, 33). These findings support the idea that myeloma

cells are derived from a germinal center (GC) or post-GC B-cell that is no longer exposed to antigen and selection. The presence of clonal $CD38^-/CD19^+$ B-lymphocytes with clonal VDJ linked to multiple constant regions, all characteristics of a memory B-cell, suggest that the first oncogenic event occurs in a post-GC B-cell, no longer exposed to antigen and selection. Whether the less differentiated clonal B-lymphocytes are part of the malignant clone or are non-malignant clonal relatives (MGUS state) that gave rise to myeloma, is at present unknown. The oncogenesis of MM may be regarded as a multistep process (34) and it may be speculated that non-malignant clonal relatives exist that acquires an early oncogenic event, e.g. translocation of an oncogene into the IgH locus.

Variable levels of clonal cells in the CD19 compartment have been reported (19–21), and in general no correlation has been observed between disease status and the level of $CD19^+$ clonal cells. Although clonal $CD19^+$ cells may contain the drug-resistant compartment in myeloma (15), no proof of their role in the disease process has been provided. It is of major importance for future studies on myeloma cell biology to clarify the role of the clonal B-lymphocytes in the disease process and to identify the true nature of these cells.

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