original paper

haematologica 2001; 86:837-843
http://www.haematologica.it/2001_08/0837.htm

Differentiation of monoclonal gammopathy of undetermined significance and multiple myeloma using flow cytometric characteristics of plasma cells

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Background and Objectives. The differential diagnosis between multiple myeloma (MM) and monoclonal gammopathy of undetermined significance (MGUS) may be uncertain in some cases; this problem is reflected by discrepancies between different classification systems with an accordance in only 2/3 of cases. We studied whether flow cytometric characteristics of plasma cells (PC) can be used for the differentiation between MGUS and MM.

Design and Methods. Patients were divided into 3 groups: Group A included 13 myeloma patients with a plasma cell infiltration of the bone marrow of 10-30%, serum M-protein ≤ 3,5 g/dL (IgG) or ≤ 2 g/dl (IgA) and without bone lesions in conventional radiography. Group B consisted of 53 patients who fulfilled the Durie and Salmon diagnostic criteria including at least one major criterion, and group C individuals with MGUS (n=17). The ratio of immunophenotypically normal (i.e. $CD19^+/CD56^-$) to all bone marrow plasma cells (BMPC), the number of peripheral blood PC (PBPC), the percentage of BMPC in S-phase and the DNA content of BMPC were analyzed.

Results. All individuals with MGUS and no patient with MM in group A or group B had a ratio of phenotypically normal to all BMPC ≥ 20%. The median of monoclonal PBPC was 0/µL (range 0-2/µL) in MGUS, 1/µL (range 0-30/µL) in MM group A and 2.4/µL (range 0-211/µL) in MM group B. The median percentage of BMPC in S-phase was 1.6% both in MGUS and in group A and 3% in group B. Aneuploidy was found in 12%, 11% and 41% in MGUS, group A and group B, respectively.

Interpretation and Conclusions. The ratio of immunophenotypically normal to all BMPC was the only flow cytometric parameter for the differentiation of MGUS and MM group A (p<0.0005). The other parameters were significantly different between MGUS and MM group B, but not group A.

Key words: DNA analysis, flow cytometry, immunophenotype, monoclonal gammopathy, multiple myeloma.

Monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM) are the most frequent forms of monoclonal gammopathies. MGUS is the most common plasma cell dyscrasia occurring in up to 10% of the population over age 75. For differentiation of MGUS and multiple myeloma, various classification systems exist, based on a combination of clinical criteria as the amount of the bone marrow plasmocytosis, the concentration of the monoclonal globulin or the presence of bone lesions. The most common systems used are those according to Durie & Salmon, Kyle & Greipp, and British Columbia Cancer Agency. With the use of these classification systems, the diagnosis of MM or MGUS is identical in only about 2/3 of cases.[1] Especially in early phases of MM, the differential diagnosis is associated with a certain degree of uncertainty. Therefore we evaluated in this study the usefulness of flow cytometric parameters in the differential diagnosis between MGUS and multiple myeloma.

A combination of monoclonal antibodies allows a precise identification of plasma cells in bone marrow (BM) and peripheral blood (PB). In BM, plasma cells can be identified as $CD38^{++}$/CD138 (B-B4) positive cells.[2-4] Coexpression studies with CD19 and CD56 can distinguish between normal and atypical plasma cells in BM within the same individual. It has previously been shown that plas-

ma cells of normal individuals express a typical phenotype ($CD19^+/CD56^-$), whereas clonal plasma cells are phenotypically aberrant ($CD19^-/CD56^+$, $CD19^-/CD56^-$ or $CD19^+/CD56^+$).[5-8] Recent studies suggested that in BM aspirates of MGUS patients two immunophenotypically different plasma cell populations can be distinguished, one with a normal and the other with an aberrant phenotype.[5,9,10] The detection of circulating plasma cells in PB might also be important for the differentiation of MM and MGUS.[11,12] Whether the percentage of BMPC in S-phase, which is a prognostic parameter in myeloma,[13] or the DNA content of BMPC are different in MM and MGUS, has been controversially discussed.[9,10,14,15] Therefore, in this study we addressed the question whether flow cytometric characteristics of plasma cells can be used for differentiation between MGUS and multiple myeloma cases with a low marrow infiltration, low serum M-protein and without bone lesions.

Design and Methods

Patients

Sixty-six patients with MM and 17 patients with MGUS were included in the study. Monoclonal gammopathy was diagnosed using immunofixation of blood and urine. Patients were classified as having MM or MGUS according to the criteria of Durie and Salmon. Stage classification was also done according to Durie and Salmon. In 13 patients (group A) the diagnosis of MM was based on only minor criteria according to Durie and Salmon. These patients had a bone marrow plasma cell infiltration of 10-30%, a low serum M-protein (IgG $\leq$ 3.5g/dL or IgA $\leq$ 2g/dL) and no evidence of osteolytic bone lesions in conventional radiography at the time of flow cytometric analysis. MM was further supported in these patients by bone lesions in MRI scans or signs of progression during a follow-up over 18 months, i.e. appearance of bone lesions in X ray scans, an increase of the M-protein above the mentioned level or an increase of the percentage of bone marrow infiltration >30%. Group B consisted of 53 patients who fulfilled the Durie and Salmon diagnostic criteria including at least one major criterion, and group C individuals with MGUS (n=17). No individual of group C showed signs of progression during the follow up.

Immunophenotypic analysis by flow-cytometry in bone marrow and peripheral blood

BM aspirates from 9 healthy bone marrow donors, 17 patients with MGUS, 13 patients with MM belonging to group A and 53 patients from group B were analyzed. Bone marrow aspirates and peripheral blood samples were collected in EDTA anticoagulant and analyzed within 8 hours. Three-color flow cytometry was performed using the following monoclonal antibodies: CD19 FITC, CD56 FITC, CD56 PE, CD38 PE (Becton Dickinson, San José, CA,USA), CD19 RPE-Cy5, CD45 RPE-Cy5 (DAKO, Glostrup, DK), CD138 FITC (B-B4) (Serotec, Oxford, UK), κ FITC and λ FITC (Becton Dickinson, San José, CA,USA). Erythrocytes were lysed using ammonium chloride lysis Ortho-mune (Ortho-Clinical Diagnostics, Neckargmund, Germany). 50,000 cells of each sample were analyzed on a FACSort flow cytometer (Becton Dickinson) using the Cellquest software. BMPC were identified as $CD38^{++}$ and CD138 (B-B4) positive cells. In peripheral blood, $CD38^{++}/CD45^-$ cells were defined as abnormal plasma cells.[8,16] $CD38^{++}/CD45^+$ phenotype is found in normal individuals, but also some atypic PC express CD45 as shown by some studies.[17,18] In contrast, circulating $CD38^{++}/CD45^-$ cells only occur in myeloma patients.[18] Therefore in our study we focused on the number of $CD38^+/CD45^-$ cells as abnormal PC in peripheral blood.

To confirm the monoclonal origin of the plasma cells, intracellular light chains were analyzed, using the Fix & Perm kit (Caltag Laboratories, San Francisco, CA, USA) according to the manufacturer's recommendation. Monoclonality was defined as κ/λ ratio $\geq$5 or $\leq$ 0.5. The absolute number of circulating plasma cells (/µL) was quantified as 0.01 $\times$ % PC $\times$ white blood cells/µL.

Cell cycle and ploidy analysis

DNA analysis of bone marrow plasma cells was performed to study the percentage of plasma cells in S-phase and their DNA content using a double-staining technique for surface antigens (CD38 FITC, CD138 FITC) and propidium iodide (PI) as described by Vindelov and modified by Orfao.[13,19] Briefly, 100 µL of the BM sample were stained with plasma cell specific surface antibodies (CD38 or CD138). After lysing the erythrocytes using ammonium chloride lysis Ortho-mune, washing and resuspending in 100 µL PBS, 300 µL of solution I (containing 100 mg/L RNAse, 3.4 mM trisodium citrate, 0.1% Igepal, 1.5 mM sperminetetrahydrochloride, 0.5 mM tris(hydroxymethyl)aminomethane dissolved in distilled water) were added. After 10 minutes 300 µl of solution II (containing 208 mg/L propidium iodide, 3.4 mM trisodium citrate, 0.1% Igepal, 1.5 mM sperminetetrahydrochloride, 0.5 mM tris(hydroxymethyl)aminomethane dissolved in dis-

tilled water) were added and incubated for 15 min in the dark. The cells were analyzed immediately after this on a FACSort flow cytometer using the CellFit program. First, 10,000 of all cells were collected, followed by acquisition of 10,000 plasma cells through a lifegate. Plasma cells were identified according to their $CD38^{++}$ or CD138 expression. Both led to comparable results. The percentage of BMPC in S-phase was analyzed in the CellFit program using the RFIT model. The DNA index of BMPC was calculated as G0/G1 peak ($CD38^{++}$ cells)/ G0/G1 peak ($CD38^{-}$ cells). DNA indices ≠ 1.0 were considered as aneuploid.

Statistical methods

The results of the immunophenotyping of peripheral blood and bone marrow and the DNA studies of the BMPC were compared between groups, i.e. MM and MGUS, using the Mann-Whitney or the χ^2-test with two-sided *p* values. The Spearman rank test was used to calculate correlations. *p* values <0.05 were considered to be significant. Statistical analyses were performed using the SPSS program.

Results

Immunophenotype of bone marrow plasma cells

Plasma cells strongly positive for CD38 and positive for CD138 (B-B4) could be detected in the bone marrow of normal individuals, myeloma and MGUS patients. In order to distinguish normal from aberrant PC, the expression of the surface antigens CD19 and CD56 was analyzed. Plasma cells in the 9 normal individuals were $CD19^{+}/CD56^{-}$. In myeloma patients, plasma cells had the phenotype $CD19^{-}/CD56^{+}$ in 75% and 80%, $CD19^{-}/CD56^{-}$ in 25% and 20% in group A and group B, respectively. Intracellular light chain staining showed that $CD19^{-}/CD56^{+}$ and $CD19^{-}/CD56^{-}$ cells were monoclonal. In 61% in group A and 72% in group B no normal residual plasma cells could be detected (Figure 1). In contrast, 100% of the MGUS patients had a subpopulation of normal plasma cells. In all MGUS patients two different subpopulations of BMPC were found. The first population showed a normal phenotype ($CD19^{+}/CD56^{-}$) and amounted to 20-95% (median 60%) of all BMPC. Analysis of the cytoplasmatic light chain restriction proved these cells to be polyclonal. A second subpopulation was phenotypically abnormal with $CD19^{-}/CD56^{+}$ in 63% and $CD19^{-}/CD56^{-}$ in 37%. In this study, all individuals with MGUS and no patient with MM had a percentage of ≥ 20% phenotypically normal BMPC to all BMPC. This ratio was highly significant for the differential diagnosis between MGUS and MM, both for group A ($p<0.0005$) and group B ($p<0.0005$).

Peripheral blood plasma cells

In our study, circulating abnormal plasma cells ($CD38^{++}/CD45^{dim}$ or $CD45^{-}$) could be detected in 33% of all MGUS patients and 56% of MM group

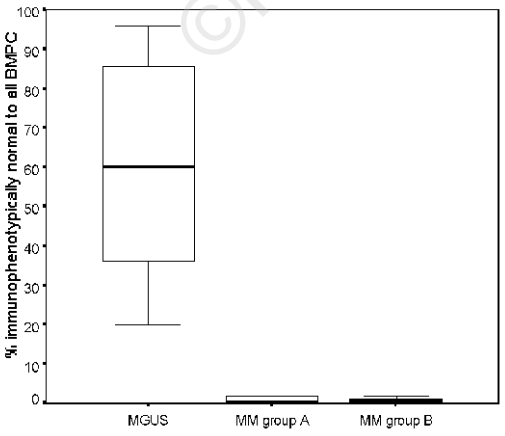

Figure 1.Proportion of immunophenotypically normal bone marrow plasma cells to all bone marrow plasma cells in MGUS and MM.

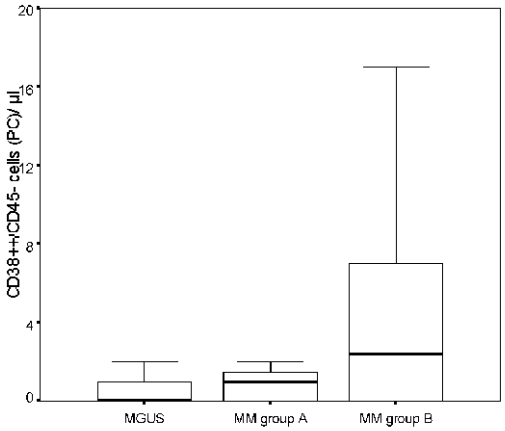

Figure 2.Absolute number of circulating abnormal plasma cells ($CD38^{++}/CD45^{-}$) in the peripheral blood of MGUS and MM patients.

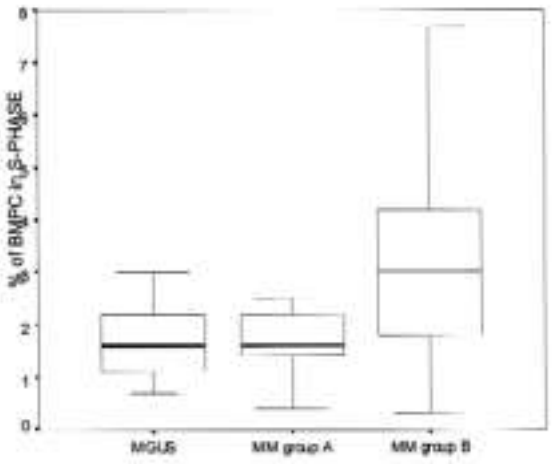

Figure 3. Percentage of BMPC in S-Phase.

A and 67% of group B respectively (Figure 2). The median PBPC in the MGUS group was 0/µL (range 0-2/µL), in the MM group A 1/µL (range 0-30/µl) and in group B 2.4/µL (range 0-211/µL). No MGUS patient had PBPC ≥ 3/µL, but 11% of patients in group A and 41% in group B. The number of circulating abnormal plasma cells was significant for the differential diagnosis between MGUS and myeloma group B (p=0.002), but not between MGUS and myeloma patients in group A.

Aneuploidy and S-phase analysis

For ploidy studies, the DNA index was calculated as the ratio between the G0/G1 peak of the plasma cells ($CD38^{++}$, $CD138^{+}$) and of the remaining ($CD38^{-}$/^{dim} or $CD138^{-}$) cells which are considered to be diploid. The mean CV for the G0/G1 peak was 3.8 (median 3.5) within the whole series of patients. Aneuploidy (DNA index <1 or >1) was detected in 12% of MGUS patients and in 11% of MM group A or 42% of MM group B. There was no significant difference between the DNA index of BMPC in MGUS and group A. In the MGUS group, the range of BMPC in S-phase was 0.7-3% (median 1.6%) (Figure 3). The patients of MM group A showed similar results, the range of BMPC in S-phase was 0.4-2.5% (median 1.6). In contrast, in 48% of group B MM cases the percentage of plasma cells in S-phase was > 3% (range 0.3-10%, median 3). The percentage of BMPC in S-phase was significantly different between MGUS and group B MM (p=0.007), but not between MGUS and MM group A.

Differentiation between MM and MGUS

A univariate analysis was performed to evaluate clinical, biological and flow-cytometric parameters for the differential diagnosis between MGUS and MM (Table 1). In MGUS patients, the median bone marrow plasmocytosis was 5%. Normal levels of polyclonal serum immunoglobulins were found in 88% of cases. In contrast, group A patients showed a median plasma cell infiltration of 20%. Using the reference levels according to Durie and Salmon classification (IgM < 500 mg/l, IgA < 1 g/L or IgG < 6 g/L) polyclonal immunoglobulins were reduced in 100% of group A patients. The medium age was

Table 1. Differentiation between multiple myeloma and MGUS.

Parameter	*MGUS (n=17) median±SD*	*MM group A (n=13) median±SD*	*p values**	*MM group B (n=53) median±SD*	*p valus**
% PC in BM	5±2.7	20±7.8	<0.0005	50±25	<0.0005
Hemoglobin (g/dL)	13.4±1.3	12.6± 1.4	0.01	10.4±1.9	<0.0005
CRP (mg/dL)	0.25±0.40	0.42± 0.46	n.s.	0.7±5.0	0.001
β2-microglobulin (mg/L)	1.55±0.77	1.6± 0.9	n.s.	3.3±9.1	<0.0005
Calcium (mmol/L)	2.36±0.13	2.37±0.2	n.s.	2.37±0.35	n.s.
Number of reduced immunoglobuline classes**	0±0.4	1±0.66	<0.0005	2±0.5	<0.0005
Flow cytometric parameters					
PBPC (/µL)	0.0±0.6	1.0± 9.8	n.s.	2.4±42	0.002
Proportion of normal BMPC to all BMPC (%)	60±27	0.0±5.2	<0.0005	0.0±2.3	<0.0005
S-phase of BMPC (%)	1.6±0.7	1.6± 0.6	n.s.	3±2.5	0.007
DNA-index of BMPC	1.0±0.03	1.0±0.10	n.s.	1.0±0.15	0.008

**P-value for differentiation from MGUS. **Number of immunoglobuline classes with a low plasma level according to Durie and Salmon: IgM < 500 mg/l, IgA < 1 g/l or IgG < 6 g/l.*

similar in MM group A and MGUS, 63 years and 57 years, respectively. Serum CRP and β_2-microglobulin levels were not significantly higher in group A compared to MGUS. Concerning the flow cytometric parameters, we found that the proportion of immunophenotypically normal plasma cells to all plasma cells in the bone marrow, the DNA content and percentage of bone marrow plasma cells in S-phase, and the absolute number of circulating clonal plasma cells in blood were significant factors for the differential diagnosis between MGUS and MM group B. If patients in group A were considered, the single flow cytometric parameter which distinguished myeloma from MGUS was the percentage of immunophenotypically normal to all BMPC. This was the most powerful parameter for discrimination between myeloma and MGUS in this study.

Discussion

The aim of this study was to analyze whether flow cytometric characteristics of plasma cells can be used for differentiation between MGUS and early MM with low marrow infiltration, low M-protein and without bone lesions in conventional radiography. In these cases, the diagnosis is classically made according to Durie & Salmon using a combination of minor criteria only, e.g. bone marrow plasmocytosis more than 10% and normal IgM < 500 mg/L, IgA < 1 g/L or IgG < 6 g/L. This may be uncertain in some cases, since the decision whether plasma cell infiltration is above or below 10 percent may not be clear-cut and the lower reference level for normal immunoglobulins may differ between different laboratories. Moreover, our study showed that a reduction of polyclonal immunoglobulins was predominantly found in MM, but also detected in some MGUS cases. These data are in accordance with the results of other groups that found Ig reduction in MGUS patients.[20,21] In this study other laboratory data, such as β_2-microglobulin or CRP, were not significantly different between MGUS and early multiple myeloma stages. Therefore, additional parameters are needed to distinguish between MGUS and MM in early cases. We analyzed phenotypic and DNA characteristics of plasma cells in order to evaluate whether these parameters can provide additional information for the differential diagnosis.

According to their expression of CD19 and CD56, plasma cells can be divided into two distinct subpopulations, one of them being phenotypically normal and polyclonal and the other one being aberrant and monoclonal. In the marrow of all MGUS patients, both populations could be found. In contrast, in 61% of MM group A no immunophenotypically normal BMPC could be detected by flow cytometry. When normal plasma cells were found in the bone marrow of patients with MM, this population accounted for less than 20% of bone marrow plasma cells in all cases in our study. The ratio of immunophenotypically normal plasma cells to all plasma cells in the bone marrow was the most powerful parameter for the differential diagnosis of MM and MGUS. This result goes in line with findings of other studies.[9,10] and demonstrates that the ratio of immunophenotypically abnormal to all BMPC is the best flow cytometric parameter regarding the differential diagnosis between MGUS and MM. This parameter is highly significant even in early myeloma cases with otherwise uncertain diagnostic criteria (group A), in which the diagnosis of multiple myeloma is supported by abnormal MRI scans or signs of progression during a follow-up over 18 months. Thus immunophenotyping by flow cytometry is a sensitive and specific method to distinguish MGUS and early MM. In contrary, the sensitivity of detection of aneuploid cells in the bone marrow in MM is discussed controversially[22,23] and depends on several parameters like the percentage of diploid cells in the S-Phase, the DNA index of aneuploid cells and the ratio between diploid and aneuploid cells. Particularly if the percentage of aneuploid cells is small and the DNA indices are near to one, a clear separation of both populations is difficult. The reported incidence of aneuploidy found in MM ranges between 23% and 83% in different studies.[24] In our study, we could find DNA aneuploidy in 11% of group A and 42% of group B MM (36 % if all MM cases were considered). There was no significant difference between MGUS and MM group A concerning the DNA index or percentage of BMPC in S-Phase. The percentage of BMPC in S-phase is a known prognostic factor for survival in MM,[13] and in our study the two groups A and B of MM patients revealed a significant difference in this parameter. Nevertheless the percentage of BMPC in S-phase was very similar in MGUS and MM group A.

In summary, our data showed that flow cytometric analyses supply relevant information on plasma cell characteristics which can be used for the differential diagnosis of MM and MGUS. The ratio of immunophenotypically normal plasma cells to all plasma cells in the bone marrow was the most powerful parameter in this context. In this study, all individuals with MGUS and no patient with MM had a ratio of immunophenotypically normal BMPC to all BMPC $\geq$ 20%. This parameter

was the only one that could distinguish between MGUS and MM even in early stages. The number of plasma cells in peripheral blood, the percentage of BMPC in S-phase and the DNA content of the BMPC may offer additional information, but they are of less importance for differentiation.

Contribution and Acknowledgments

All authors contributed to the conception and design of the study. OS was responsible for supervision of the study, analysis and interpretation of data, drafting the article and revising it critically. UH contributed to the collection and analysis of samples, interpretation of data and drafting the article. IZ contributed to the collection and analysis of samples and drafting the article. KP provided critical revision of the article. All authors approved the final version of the paper.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Prof. Jesus F. San Miguel who acted as an Associate Editor. The final decision to accept this paper was taken jointly by Prof. San Miguel and the Editors. Manuscript received May 4, 2001; accepted July 31, 2001.

Potential implications for clinical practice

The percentage of normal to all bone marrow plasma cells as calculated by flow cytometry may allows differential diagnosis between MGUS and early multiple myeloma.

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