bjh short report

Diverse niches within multiple myeloma bone marrow aspirates affect plasma cell enumeration

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Summary

A basic criterion for the diagnosis of multiple myeloma is plasma cell enumeration within the bone marrow (BM). This report showed that flow cytometry under-estimated the number of plasma cells in BM aspirates by an average of 60%, compared with morphological evaluation. The discrepancy was partially because BM smears contain cells associated with the lipid-enriched spicules. In contrast, flow cytometry is performed on the BM fluid, which is depleted of the lipid-adhesive plasma cells. This discrepancy may point to different plasma cell subpopulations associated with diverse niches within the BM.

Keywords: bone marrow, flow cytometry, multiple myeloma, plasma cells.

One of the primary criteria for establishing the diagnosis of multiple myeloma (MM) is the enumeration of plasma cells within bone marrow (BM) samples. As outlined in guidelines for the diagnosis of MM according to the International Myeloma Foundation consensus (Durie *et al*, 2003), the designation of MM requires the presence of at least 10% plasma cells within the BM sample and/or the presence of a biopsy proven plasmacytoma. If no monoclonal protein is detected, then ≥30% monoclonal BM plasma cells and/or a biopsy proven plasmacytoma are required (Durie *et al*, 2003). A significant correlation was found between BM aspirate, trephine biopsy, labelling index and the clinical outcome of MM (Rajkumar *et al*, 2001).

In recent years, flow cytometry has become a fundamental tool in the diagnosis of MM and patient follow-up (Rawstron *et al*, 2002; San Miguel *et al*, 2002; Mateo *et al*, 2003). The cell surface marker pattern of MM cells may correlate with their cytogenetics profile (Mateo *et al*, 2005). Furthermore, flow cytometry can identify possible specific drug targets (e.g. CD20, CD44v6 and CD117), thereby allowing the selection of patient subpopulations who may benefit from targeted therapies (Burton *et al*, 2004; Lin *et al*, 2004; Liebisch *et al*, 2005). A discrepancy between plasma cell enumeration by morphological examination and flow cytometric analysis is known, but adequate scientific documentation, its quantitative extent, and the underlying reasons are yet to be provided.

The present study documented a remarkable discrepancy between morphological evaluation and flow cytometric analysis of MM BM aspirates. This phenomenon appears to be typical of MM, and may point to diverse subpopulations of MM cells associated with different microenvironments in the BM.

Methods

Morphological evaluation of MM clinical samples

Morphological evaluation of MM BM aspirates was performed with 100× magnification fields, with a count of *c.* 500 total nucleated BM cells in each sample. This study was approved by the Tel-Aviv Sourasky Medical Center Institutional Review Board.

Flow cytometric analysis

For staining with directly labelled-antibodies, 50 μl (5×10^5 cells) MM BM samples were incubated with each of the designated monoclonal antibodies (CD38, CD45, CD56,

CD117 and CD138, and immunoglobulin G isotype controls – all from Dako, Glostrup, Denmark), for 30 min at 4°C followed by red cell lysis, and washed with 2 ml of phosphate-buffered saline. From each sample 3×10^4 events were acquired by FACSCalibur (Becton-Dickinson, San Jose, CA, USA).

Results and discussion

Bone marrow aspirates from 41 MM patients at diagnosis, or during an active phase of their disease were analysed simultaneously by morphological evaluation and flow cytometry. Each sample was assessed independently by two qualified laboratory specialists and/or haematologists, in a double-blind manner. Plasma cells were identified by flow cytometry using CD38\CD138 defined regions, and confirmed by aberrant markers, including CD56, CD117 and CD45 (mostly negative). Figure 1A shows an average of *c.* 60% plasma cells within the BM aspirates, as evaluated by morphological enumeration, and *c.* 20% plasma cells, as determined by flow cytometry performed on the same BM aspirates. This discrepancy in plasma cell enumeration was found in MM patients in different stages of the disease, including relapse, irrespective to their response to therapy (data not shown). In 14 of 41 (34%) cases, <10% of plasma cells were observed by flow cytometric analysis (Fig 1B), which was less than the number required for MM definition.

Despite this significant discrepancy, some correlation between these two assessments could be found at the single patient level, although this was not statistically significant ($R^2 = 0{\cdot}5$, correlation coefficient = 0·71, Fig 1B). This indicates that, unlike circulating plasma cells (Nowakowski *et al*, 2005), the plasma cells found in the fluid of the BM sample may reflect the disease burden in solid areas of the BM.

We tested whether the discrepancy between the assessments applied only to MM, or represents a more prevalent trend in other haematopoietic malignancies. When seven BM aspirates of acute myeloid leukaemia patients were analysed in a similar manner to the MM samples, a correlation was found between the morphological and flow cytometric assessments (Fig 2A). These data clearly demonstrated that the discrepancy in MM BM analysis cannot be attributed merely to technical or methodological hindrance, but may stem from some unique characteristics of the malignant plasma cells. The difference between these two essential diagnostic approaches may originate in the biological properties of the malignant plasma cells, which occupy the solid and/or adjacent niches of the BM, including bone surfaces, basement membranes and lipid-enriched spicules.

Bone marrow direct smears are considered to have the most representative distribution of cell types, because its preparation involves the least manipulation of the sample. BM smears are prepared from lipid-enriched spicules, which serve as a solid sanctuary for plasma cells. In contrast, flow cytometric analysis merely utilises cells from the BM fluid. When we isolated and disrupted spicules from MM BM samples, by repeated passages through a 21 g needle, there was a nearly 40% increase in the plasma cell percentage, compared with the fluid of the same BM samples (Fig 2B). Hence, the average ratio between morphological evaluation and flow cytometry performed directly on spicule-derived cells was reduced to 2·4. This partial release of cells from the spicules was encouraging but not complete. Previous studies indicated that the first millilitre drawn in a BM aspirate represents pure BM sampling (Batinic *et al*, 1990). Most BM aspirates contain at least 2–3 ml and, hence, BM resident cells are diluted with peripheral blood. However, this dilution should not have a major impact on the BM flow cytometric analysis because of the high WBC counts (average of $50{\cdot}4 \pm 22{\cdot}7 \times 10^9$ cells/l in five different BM samples assessed) within BM aspirates, compared with relat-

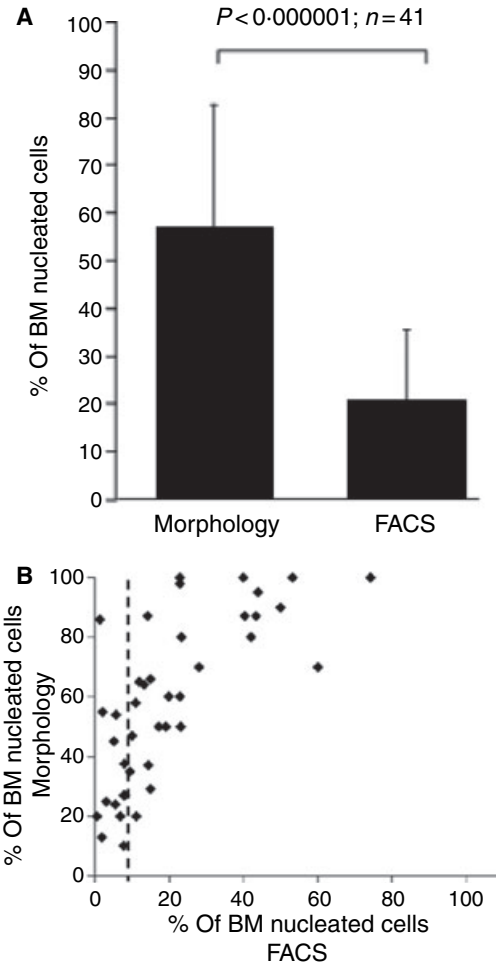

Fig 1. Plasma cell enumeration in bone marrow aspirates of multiple myeloma (MM) patients. Bone marrow (BM) aspirates from 41 MM patients at diagnosis, or during an active phase of their disease were routinely analysed simultaneously by morphological evaluation and flow cytometry as described in the *Methods* section. (A) The average and standard deviation (±SD) of the percentage of plasma cells in the total nucleated BM cells as evaluated by the two methodologies. Differences between the methodologies were statistically significant ($P < 0{\cdot}000001$, *t*-test). (B) Individual presentations of the morphological evaluation *versus* flow cytometric analysis of each of the individual BM aspirates. The dashed line indicates the 10% plasma cell percentage required for MM diagnosis.

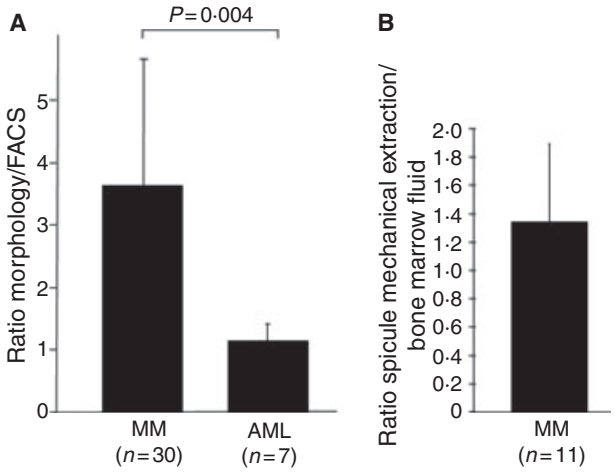

Fig 2. (A) Comparison of disease infiltration in multiple myeloma (MM) and acute myeloid leukaemia (AML) bone marrow (BM) aspirates. Thirty MM and seven AML BM aspirates were analysed independently by morphological evaluation and flow cytometry as described in the *Methods* section. The average disease burden, as evaluated by morphological enumeration was 74·6 ± 20·4% and 71·4 ± 23% for the MM and AML samples, respectively, (the difference was not statistically significant). The average ratio ± SD of morphological evaluation and flow cytometric analysis are shown. (B) Flow cytometric plasma cell enumeration in lipid-enriched spicules *versus* BM fluid of MM patients. Lipid-enriched spicules were isolated from the upper phase of BM aspirates of 11 MM patients. The spicules were suspended in 500 μl of phosphate-buffered saline, and then subjected to repeated passages through 21 g needle, until homogeneous fluid was obtained. Spicule-associated plasma cells were then enumerated by flow cytometric analysis, and compared with flow cytometric analysis performed on the BM fluid from the same aspirates. The ratio ± SD between these measurements are shown.

ively low WBC counts (average of $7{\cdot}7 \pm 3{\cdot}7 \times 10^9$ cells/l) in the peripheral blood of the same individual.

This brief report documented a major discrepancy between two routine laboratory tools utilised for the diagnosis and research of MM: BM morphological evaluation, and flow cytometry. We propose that this discrepancy is partially attributable to two microenvironments occupied by MM cells in the BM sample, the lipid spicules and the fluid phase.

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