Flow cytometric evaluation of bone marrow plasma cells using CD19, CD45, CD56, CD38, and CD138 and correlation with bone marrow infiltration ratio in multiple myeloma patients

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ABSTRACT

Objective: To examine the co-expression of CD19, CD45, CD38, CD56, and CD138 molecules in plasma cells of bone marrow (BM) aspirates and their relation with BM infiltration, and treatment in patients with multiple myeloma by flow cytometry.

Methods: Forty BM aspirate samples were assessed from 40 patients at diagnosis and on follow-up at the Medical Oncology Department, Cukurova University, Balcali Hospital, Turkey, between 2002 and 2004. The mean age was 56.83 ± 9.1 and male:female ratio was 2.6. All patients received at least 4 courses of VAD (vincristine, adriamycin, dexamethasone) regimens and 20 of them were also treated with high dose melphalan and peripheral autologous stem cell transplantation. The median follow-up period was 19.1 ± 22.7 months.

Results: Using light microscopy the BM smears stained with hematoxylin and eosin from patients on follow up were classified into one of 3 categories, complete remission (CR) (<5%), partial remission (PR) (>5% and <30%), and extensive infiltration (EI) (>30%). According to infiltration ratio 23 were evaluated CR, 2 were PR and 15 were EI. The mean value of CD19 was 6.01 ± 9.5%, CD56 = 9.9 ± 6.8%, CD138 = 8.6 ± 5.6%, CD45 = 84.2 ± 22.3% and CD38 = 59.5 ± 25.4%. The flow cytometric analyses revealed that only the mean value of CD38 and CD45 expression were significantly high. We correlated infiltration ratio with each parametric and found statistically significant relations. We also correlated independent variables with each other and found a relation between CD38 and CD19 (p=0.005). We also defined the groups whether treated with peripheral autologous transplantation or not and compared the independent variables between them, in which CD138 was statistically significant (p=0.02).

Conclusion: We suggest BM plasma cells expressed mainly by CD38 and CD45 may have a role in generation of BM plasma cells and that CD138 expression may be considered in follow-up for minimal residual disease after autologous transplantation in myeloma patients.

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Plasma cells are characterized by expression of cytoplasmic Ig and surface membrane antigens such as PC-1, PCA-1, BB4, CD38 and CD138.[1] The phenotype of myeloma cells has long been considered the same as that of normal plasma cells. Only recently has the differential expression of surface markers on normal versus malignant plasma cells been described.[2] Normal plasma cells were shown to be $CD56^-/CD19^+$, whereas most myeloma cells were $CD56^+/CD19^-$. Interestingly, both populations are found in monoclonal gammopathy of undetermined significance (MGUS), which

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indicates that a varying percentage of monoclonal and normal plasma cells can be detected concomitantly in this disease,[3] which is a growth factor for plasma cells.

The tendency of myeloma cells to reside in the BM compartment during the main course of disease evolution is mainly attributable to their exclusive repertoire of adhesion molecules.[4] While neoplastic cells retain some of the phenotypic characteristics of normal plasmacytes, such as strong CD38 expression, they are readily distinguished from their physiologic counterparts by CD56 overexpression and absence of CD19 from their surface.[5]

Recent insights into the biological activities of a negative regulator of osteolysis, syndecan-1 (CD138) may introduce novel strategies for the management of MM cases in the future. Although, expression of this heparan sulfate proteoglycan was initially thought to be limited on the surface of myeloma cells, recent studies have demonstrated its presence on normal plasma cells, as well.[6,7] However, neoplastic cells have been shown to actively shed and release high amounts of the molecule, with serum levels of shed syndecan-1 representing a putative prognostic marker in MM, associated with M-protein concentration, tumor mass and short survival.[8,9] Increasing in vitro and in vivo evidence supports a central role of syndecan-1 in MM pathobiology. The addition of intact purified CD138 to human myeloma cell lines leads to a marked induction of apoptosis, down regulation of osteoclastogenesis and concurrent stimulation of osteoblast differentiation. Moreover, following injection in severe combined immune deficient mice, syndecan-1-transfected cells produce significantly less lytic bone lesions than cells expressing low levels of the molecule.[8] Finally, a role in inhibiting tumor invasion has been assigned to the membrane bound form of syndecan-1, possibly through promoting the anchorage of malignant cells to their microenvironment, as well as by suppressing myeloma cell production of extracellular-matrix degrading enzymes.[10] Syndecan-1 (CD138) is expressed by malignant plasma cells from the majority of MM cell lines and patient specimens.[7,11,12] Furthermore, during normal B-cell development, CD138 expression is highly specific for terminally differentiated normal plasma cells as it is absent on highly proliferative normal plasmablasts and all earlier B-cell stages.[12-15]

CD56 neural cell adhesion molecule (NCAM) is frequently lost or down-modulated in extramedullary myeloma and peripheral myeloma cells,[16,17] which suggests that loss of this adhesion molecule may act as transmigratory signal, permitting the exit of malignant plasma cells out of the BM. Malignant plasma cells can be detected in the peripheral blood of patients with advanced disease, and were shown to be of adverse prognostic significance.

The rationale of this study was to assess the extent to which CD19, CD45, CD56, CD138 and CD38 correlate with the pattern of BM infiltration ratio, biochemical prognostic factors such as M-protein and lactic dehydrogenase level and their predictive value in 2 different treatment modalities.

Method. ***Patients.*** Forty BM aspirate samples were assessed from 40 patients, mean age 56.8 ± 9.1, male : female ratio 2.6, at diagnosis and follow-up. The patients were defined as stage IIIA, IIIB and smoldering disease according to Durie-Salmon classification. All patients diagnosed as IIIA and IIIB received at least 4 courses of VAD (vincristine, adriamycin, dexamethasone) regimens and 20 of them were treated with high dose melphalan and peripheral autologous stem cell transplantation (PBCT). The median follow-up period was 19.1 ± 22.7 months **(Table 1)**.

Sample preparation. The BM aspirates were obtained from posterior iliac crest. The glass smears were prepared for hematoxylin and eosin staining and 2 milliliters of aspirate were separated for flow cytometric analysis. The proportion of plasma cells and their distribution was assessed on marrow aspirate according to the percentage infiltration by 2 independent hematopathologist using light

Table 1 - Demographic features of patients.

Characteristics	Value
Male/Female	2.6
Mean age	56.8 ± 9.1
Stages	
IIIA	72.4%
IIIB	13.8%
Smoldering	13.8%
Treatment	
Autologous peripheric blood transplantation	37.9%
Chemotherapy	62.1%

Table 2 - The distributions of flow cytometric analysis.

Surface antigen	Minimum %	Maximum %	Mean
CD19	0.2	46.9	6.01 ± 9.5
CD56	0.5	26.6	9.9 ± 6.8
CD138	1.8	21	8.6 ± 5.6
CD38	14	98.3	59.5 ± 25.4
CD45RA	20.2	100	84.2

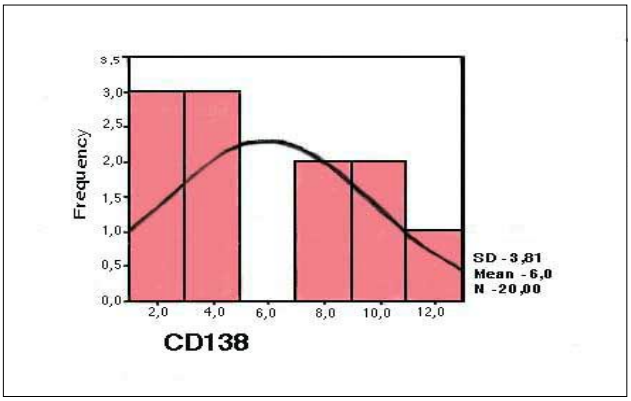

Figure 1 - The histogram of the CD138 for the patients treated with autologous peripheric blood transplantation.

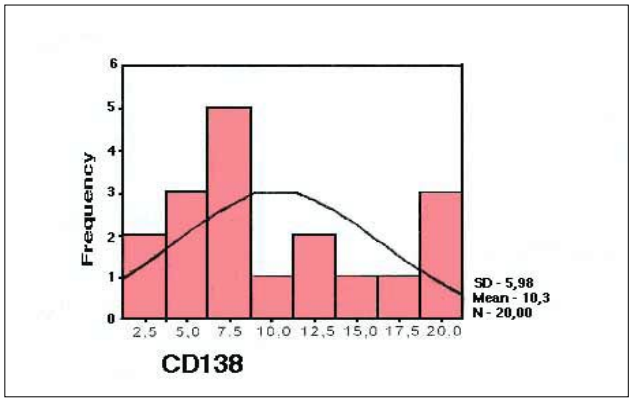

Figure 2 - The histogram of the CD138 for the patients treated with chemotherapy.

microscope. The degree of marrow infiltration was identified by the distribution of plasma cells on an aspiration smear. The smears from patients at presentation were classified into one of 3 categories: complete remission (CR) (<5%), partial remission (PR) (>5% and <30%), and extensive infiltration (EI) (>30%). The percentage >30 was assessed as nonresponder and <5 assessed as complete responder.

Flow cytometry. Six-thousand leukocytes were incubated with 100µl of each pre-tittered antibody per test for 20 minutes at room temperature. After that incubation with antisera at room temperature, in the dark, red blood cells were lysed with distilled water, washed twice, and acquired using a Becton Dickinson FACSort with CellQuest v3[1] software. A minimum of 50000 total cells were analyzed in each test. Plasma cells were identified using a sequential gating strategy, assessing CD19, CD45, CD38, CD56 and CD138 simultaneously, as well as forward and side lightscatter characteristics. Antibodies used for flow cytometry were CD138/syndecan-1 (Lot No: 09, Immunotech) PE, CD56 (NKH-1-RD1, Lot No: 729311, Immunotech), CD38 (OKT10) FITC (Lot No: 28, Immunotech), CD19-B4 (Lytic)-RD1/J5-FITC (Lot No: 732416, Immunotech), CD45RA (4B2) FITC (Lot No: 41, Immunotech). Antibody conjugates were prepared in-house, from hybridoma supernatant and tittered against known antigen positive cell lines unless from a commercial source.

Statistical analysis. The statistic analysis was carried out with SPSS 10.0.

Results. The BM aspirates were studied for flow cytometric analysis of the plasma cells. The mean value of CD19 was 6.01 ± 9.5%, CD56 = 9.9 ± 6.8%, CD138 = 8.6 ± 5.6%, CD45 = 84.2 ± 22.3% and CD38 = 59.5 ± 25.4%. The mean value of CD38 and CD45 expression was significantly higher than other surface antigens (**Table 2**). According to infiltration ratio the 23 were evaluated CR, 2 were PR and 15 were EI.

In MM a nodular infiltration was found in low percentage (<5%) of cases, after defining parameters we analyzed if there were any correlation between BM plasma cell infiltration ratio with each parameters in each treated patient whether treated with autologous peripheric blood transplantation or not. The results revealed that only CD138 (p=0.02) relation was found statistically significant between patients treated with autology transplantation and standard chemotherapy. We correlated variables with each other and found a relation between CD38 and CD19 (p=0.005). We also defined the groups as treated with peripherica autologous transplantation or not and compared the independent variables between them in which CD138 was statistically significant (p=0.02) **Figure 1 & 2**.

Discussion. In this study, we have assessed the flow cytometric analysis of plasma cells in BM of myeloma patients and identified if there were any relation with BM infiltration ratio. Although, in MM a nodular infiltration was found in low percentage (<5%) of cases, we took infiltration ratio as main foundation of the study. We known that FCM analysis of the plasma cells is an acceptable method for detecting myeloma plasma cells and minimal residual disease, but in cases with low infiltration ratio it is difficult. We aim to investigate if FCM analysis of plasma cells in BM of myeloma patients could be identified even in complete remission marrow.

Flow cytometric analysis has not been widely used as a method for residual disease analysis in MM, but has been previously shown to be extremely effective in chronic lymphocytic leukemia. It has been demonstrated that a unique neoplastic plasma cell phenotype is identifiable in more than 98% of

patients, and that the technique can be extremely sensitive.[18-20]

In this study, we found the majority of plasma cells were expressed by CD38 and CD45 surface antigens. Myeloma cells display heterogenous phenotypes with differences in molecules among different patients expressed on the cell surface as well as differences within the same patient at different disease stages.[21] In general, all myeloma cells express high levels of CD38 with immature plasma cell additionally expressing CD45.[22,23] More recently, the authors have shown that the proliferation of normal and reactive plasma cells is associated with a high expression of the CD45. Furthermore, it has shown that these CD45 bright plasma cells are totally depending on interleukin (IL)-6 to survive and to proliferate.[24,25] More recently, Fuji et al,[26] showed that CD45 bright myeloma cells were those, which were more preferentially targeted by IL-6 and that CD45 myeloma cells overlap immature plasmablastic myeloma cells.[27] Altogether, in parallel to our results, these data show that MM results from the expansion of CD45 bright plasmablastic myeloma cells, for these reasons the expressed values of CD38 and CD45 were significantly high. The elevated CD45 expression with elevated CD38 level may be considered as a predictor of early relapsed disease and the role of CD45 expression in plasmagenesis should be evaluated for detecting molecular remission in myeloma patients and its role in plasmagenesis.

Although, we found a good correlation between CD38 and CD19 expression (p=0.005), the phenotypic heterogeneity of myeloma cells in fact describes the differentiation process that is an important part of disease development. The identity of the myeloma stem cell still remains an enigma. Generally, CD19 is a B-cell surface antigen; therefore, the probability of contamination of myeloma cells is high during flow cytometric analysis. The expression of CD19 on malignant plasma cells has been found by some authors[28,29] as we found, but not by others.[17,27,28] The CD19 expressed myeloma cells are typically the less differentiated $CD38^+$, $CD45^+$ plasma cell population;[28] thus, gating strategy is needed to be optimized to exclude contaminating events and it depends on how the gates are set. However, B-cells expressing CD19 can be induced to mature with the help of stromal cells into monocytic plasma cells, suggesting that this cellular compartment may comprise of myeloma cell progenitors.[30]

Neural cell adhesion molecule CD56 is one of the few adhesion molecules that are aberrantly expressed on malignant plasma cells. Although its role in neural development is well described,[31] its activity in myeloma is less clear. In this study, CD56 levels were not correlated with plasma cell infiltration and other surface immunophenotyping. Unlike other prognostic factors, that are surrogate markers of disease bulk, such as ß-2 microglobulin. In parallel to this argument, the results of this study demonstrate that CD56 is a stable feature of the neoplastic plasma cells. It is therefore of great interest to study how CD56 affects the rate of change of BM disease in sequential samples. The lack of correlation with infiltration ratio may be due to the fact that so few patients have been studied, and that CD56 expression has been assessed as positive or negative, rather than as a continuous variable. Further studies are therefore required to determine whether CD56 is an independent prognostic factor. Luque et al[32] remark that the blood plasma cells had a dimmer expression of CD56 than did marrow plasma cells. Others[33-35] have shown that cells from patients with plasma cell leukemia typically have a lower frequency of expression of CD56 or do not express CD56 in the marrow or blood. Recently, it has been demonstrated that lower CD56 expression on marrow plasma cells indicates a higher likelihood of plasma cells in the blood.[18,36] At this time, it appears that the main difference between circulating and marrow plasma cells is a loss of CD56 expression on the blood plasma cells. In addition, Sonneveld et al[29] suggested that samples from patients with MGUS were $CD56^-$; however, others have found that these samples are usually $CD56^+$, indicating that CD56 expression cannot be used to differentiate MGUS from active myeloma.[3]

It has been suggested that neoplastic plasma cell levels might remain stable over time in some patients after transplantation.[37] However, in this study, neoplastic plasma cell levels were shown to have significant relation between BM infiltration with CD138 expression in transplanted patients (p=0.002). Bone marrow plasma cells are best defined by high levels of CD38 and significant CD138 expression. Although, the number of patients treated in our study with PBCT was low, we found after comparing the independent variables between 2 groups in which CD138 was statistically significant. As our results in transplanted patients showed that to monitor myeloma cells, it is possible to discriminate them from their normal counterparts by evaluating CD138 expression for detection of minimal residual disease. However, Luque et al[17] studied the adhesion receptors in blood and marrow $CD138^{++}$ cells and found no difference in the adhesion receptor profile. Witzig et al[32] found CD138 expression on blood and marrow plasma cells to be similar in 36 patients in whom blood and marrow were simultaneously sampled; therefore, loss of CD138 cannot explain how plasma cells escape the marrow compartment.[32,33]

In conclusion, the most definitive and most reliable markers of plasma cells are CD38, CD45

and CD138, but neither are specific for plasma cells nor differentiate benign from malignant plasma cells. Especially in autologous transplanted myeloma patients, CD38, CD138 may be used to detect residual or disease monitoring during conventional therapies. The FCM is not sufficient for detecting minimal residual disease and another method such as real time-polymerase chain reaction (RT-PCR) should be used together with FCM. Additionally, we never forget that most of the adhesion receptors that have been found on malignant plasma cells are also present on normal plasma cells.

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