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Expression of Adhesion Molecules on Myeloma Cells

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We investigated the expression of adhesion molecules including LFA-1α (CD11a), Mac-1 (CD11b), LFA-1β (CD18), VLA-β_1 (CD29), H-CAM (CD44), VLA-4 (CD49d), VLA-5 (CD49e), ICAM-1 (CD54), N-CAM (CD56), LFA-3 (CD58), VNR-β (CD61), and LECAM-1 (CD62L) on fresh myeloma cells and human myeloma cell lines. By two-color flow cytometric analysis with anti-CD38 antibody, we demonstrated that myeloma cells were located in the strongly CD38-positive ($CD38^{++}$) fractions. Fresh myeloma cells were obtained from 28 patients with multiple myeloma (MM) and 3 patients with plasma cell leukemia (PCL). All myeloma cells expressed VLA-4 on their surface. Most of the myeloma cells also expressed VLA-5, ICAM-1, and LFA-3. H-CAM was strongly expressed in 3 cases of PCL and 2 cases of aggressive myeloma, and moderately expressed in other MMs. N-CAM was expressed in 68% of MMs, but none of the 3 PCLs. LFA-1 was expressed in two cases of aggressive myeloma, but not expressed in other non-aggressive myelomas. Most of the myeloma cells did not express Mac-1, VNR-β, or LECAM-1. These results suggest that VLA-4, VLA-5, ICAM-1, LFA-3, and H-CAM are involved in cellular interaction and migration in MM, and that the expression of N-CAM and LFA-1 varies with disease activity in MM.

Key words: Adhesion molecule — Immunophenotypic analysis — Myeloma cell

Multiple myeloma (MM) is a B-cell malignancy characterized by a monoclonal proliferation of plasma cells usually localized in the bone marrow. Recently, the presence of precursor or premyeloma cells has been reported in the peripheral blood of myeloma patients.[1-3] Caligaris-Cappio *et al.*[4] demonstrated in an *in vitro* study that these precursor cells circulating in peripheral blood selectively bound to marrow stroma, proliferated, and terminally differentiated. In this process, VLA-4-fibronectin interaction is probably required,[5,6] whereas circulating plasma cells are rarely observed in MM, except in the terminal stage of this disease or plasma cell leukemia (PCL). Kalasz *et al.*[7] reported that PCL cells adhered to rat high-endothelial venules (HEV) facilitating their exit into the circulation, while myeloma cells isolated from bone marrow were not able to bind to rat HEV. Tsutani *et al.*[8] reported that leukemic plasma cells from peripheral blood expressed ICAM-1 and not LFA-1, while myeloma cells from a subcutaneous plasmacytoma had the opposite pattern ($ICAM\text{-}1^-$ and $LFA\text{-}1^+$). These observations suggest that adhesion molecules may play a critical role in the biology of this disease.[9,10] However, the reason why plasma cell neoplasms affect bone marrow almost exclusively remains unclear.

Several reports on the expression of H-CAM, VLA-4, ICAM-1, N-CAM, and LFA-3 on myeloma cells and neoplastic plasma cells have been published.[11-18] Furthermore, the identification of adhesion molecules on myeloma cells may be of prognostic value, as is the case with identification of adhesion molecules in B-cell lymphomas.[19-21] Therefore, we investigated the expression of adhesion molecules in 3 myeloma cell lines and myeloma cells from 31 patients with MM including PCL using a panel of monoclonal antibodies. The clinical significance of the results is discussed.

MATERIALS AND METHODS

Patients Fresh myeloma cells were obtained from bone marrow of 27 patients with MM and 1 patient with PCL, from ascites fluid of 1 patient with MM, and from peripheral blood of 2 patients with PCL (Table I): 16 were IgG type, 4 were IgA type, 1 was IgE type, 7 were Bence-Jones type, 2 were biclonal gammopathy, and 1 was non-secretory type; further, 13 were κ and 16 were λ type. We also examined two aggressive myelomas, as defined by Suchman *et al.*[22] The patients were staged according to the clinical staging system proposed by Durie and Salmon[23]: 2 were in stage I, 5 were in stage II, and 24 were in stage III. All samples were collected after informed consent had been obtained.

Myeloma cell lines Three human myeloma cell lines (U 266, KPMM1, and KPMM2) were used. KPMM1 and KPMM2 were established in our laboratory from pericardial effusion[24] and ascites fluid,[25] respectively, of a patient with MM. These two cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf

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Table I. Characteristics of Patients

	Diagnosis	Isotype	Stage	Disease status	Sample
1.	MM[a)]	IgG/λ	II A	Relapse	AS
2.	MM[a)]	BJ/κ	IIIB	At diagnosis	BM
3.	MM	BJ/λ	IIIB	At diagnosis	BM
4.	MM	IgG/κ	IIIA	Terminal	BM
5.	MM	IgG/κ	I A	Relapse	BM
6.	MM	IgA/λ	II A	Relapse	BM
7.	MM	IgG/λ	IIIA	At diagnosis	BM
8.	MM	IgG/λ	II A	At diagnosis	BM
9.	MM	IgG/λ	IIIB	At diagnosis	BM
10.	MM	IgG/λ	IIIB	At diagnosis	BM
11.	MM	IgG/λ	II A	At diagnosis	BM
12.	MM	BJ/κ	IIIB	At diagnosis	BM
13.	MM	IgG/κ	IIIB	At diagnosis	BM
14.	MM	IgA/λ	IIIA	At diagnosis	BM
15.	MM	IgG/λ	IIIA	Terminal	BM
16.	MM	IgG/κ	IIIA	At diagnosis	BM
17.	MM	IgA/λ	II A	Relapse	BM
18.	MM	BJ/κ	IIIA	At diagnosis	BM
19.	PCL	IgG/κ	IIIA	At diagnosis	BM
20.	MM	Non-secretory	IIIA	At diagnosis	BM
21.	MM	BJ/λ	IIIA	Terminal	BM
22.	MM	IgG/κ, BJ/κ	IIIA	At diagnosis	BM
23.	MM	BJ/λ	IIIB	Relapse	BM
24.	MM	IgG/κ	IIIA	At diagnosis	BM
25.	MM	IgG/λ	IIIA	Terminal	BM
26.	MM	IgG/κ	IIIA	At diagnosis	BM
27.	MM	IgG/κ, BJ/κ	I B	At diagnosis	BM
28.	MM	IgA/λ	IIIA	At diagnosis	BM
29.	PCL	IgG	IIIA	At diagnosis	PB
30.	MM	BJ/λ	IIIB	At diagnosis	BM
31.	PCL	IgE/κ	IIIA	At diagnosis	PB

Abbreviations: MM, multiple myeloma; PCL, plasma cell leukemia; AS, ascites; BM, bone marrow; PB, peripheral blood.
a) Aggressive myeloma.

serum (FCS; Hyclone, Logan, UT) and recombinant human IL-6 (4 ng/ml, Chugai Pharm. Co., Ltd., Tokyo). U266 was obtained from Fujisawa Pharm. Co., Ltd. and maintained in RPMI-1640 medium with 10% FCS.

Isolation of fresh myeloma cells The bone marrow aspirates, peripheral blood, or ascites fluid of MM or PCL patients were collected. The mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation, washed twice, and resuspended in phosphate-buffered saline with 10% FCS. Wright-Giemsa staining of cytocentrifuge preparations of the mononuclear cells was done to determine the percentage and morphology of the myeloma cells according to the criteria of Greipp *et al.*[26]

Expression of adhesion molecules on myeloma cells For the identification of myeloma cells, monoclonal antibodies against CD38 and plasma cell-associated antigen-1 (PCA-1; Coulter, Hialeah, FL) were used. Myeloma cells were strongly immunoreactive for CD38.[15, 17, 27–31] Therefore, the cell surface expression of adhesion molecules on myeloma cells was determined by two-color flow cytometric analysis using monoclonal antibodies (mAbs) against CD38 and various adhesion molecules, including LFA-1, Mac-1, VLA-4, VLA-5, H-CAM, ICAM-1, N-CAM, LFA-3, VNR, and LECAM-1. The direct immunofluorescence method used has been described previously.[25, 28] Briefly, the isolated mononuclear cells (1×10^5 cells/100 μl) were incubated with fluorescein isothiocyanate (FITC)-conjugated mAbs directed against LFA-1α (CD11a, LFA-1; Dako, Carpinteria, CA), LFA-1β (CD18, Leu1b; Becton Dickinson, San Jose, CA), VLA-β_1 (CD29, IOT29; Immunotech S.A., Marseilles, France), H-CAM (CD44, IOL44; Immunotech S.A.), ICAM-1 (CD54, ICAM; Immunotech S.A.), VNR-β (CD61, GPIIIa; Dako), or LECAM-1 (CD62L, LECAM1; Immunotech S.A.) at 4°C for 30 min. After having been washed twice, the cells were incubated with

phycoerythrin (PE)-conjugated antibody against CD38 (Leu17; Becton Dickinson) at 4°C for 30 min. PE-conjugated mAbs were used against Mac-1 (CD11b, Leu15; Becton Dickinson) and N-CAM (CD56, Leu19; Becton Dickinson) at 4°C for 30 min. The cells were washed twice, then incubated with FITC-conjugated antibody against CD38 (IOB6; Immunotech S.A.) at 4°C for 30 min. An indirect staining method was used for VLA-4, VLA-5, and LFA-3. The cells were incubated first with mouse mAb against VLA-4 (CD49d, HP2/1; Immunotech S.A.), VLA-5 (CD49e, SAM-1; Immunotech S.A.), or LFA-3 (CD58, AICD58; Immunotech S.A.) at 4°C for 30 min. They were washed twice and incubated with PE-labeled $F(ab')_2$ fragments of goat anti-mouse IgG (Coulter) at 4°C for 30 min. They were further washed twice and incubated with FITC-conjugated antibody against CD38 at 4°C for 30 min. Immunofluorescence was analyzed by two-color flow cytometry using an EPICS Profile flow cytometer (Coulter).

RESULTS

Expression of adhesion molecules on fresh myeloma cells

By incubation with antibodies against plasma cell-associated antigens, PCA-1 and CD38, we found that most of the cells located in the $CD38^{++}$ fractions were $PCA\text{-}1^+$, suggesting that $CD38^{++}$ cells were myeloma cells (Fig. 1). A representative staining pattern is shown in Fig. 1 (case 27). More than 90% of the cells in the $CD38^{++}$ fraction were $PCA\text{-}1^+$. The percentages of myeloma cells in the mononuclear cell preparations examined by light microscopy were almost the same as those of $CD38^{++}$ fractions determined by flow cytometry (data not shown). Therefore, we assumed that the percentages of $CD38^{++}$ cells were representative of the percentages of

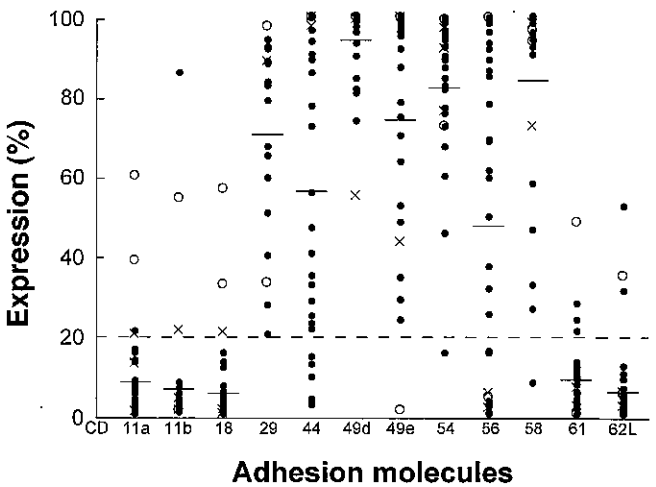

Fig. 2. Expression of adhesion molecules on freshly isolated $CD38^{++}$ myeloma cells. The fresh myeloma cells were obtained from 26 patients with multiple myeloma (●), 2 with aggressive myeloma (○), and 3 with PCL (×). Horizontal lines represent the mean percentage expression of adhesion molecules.

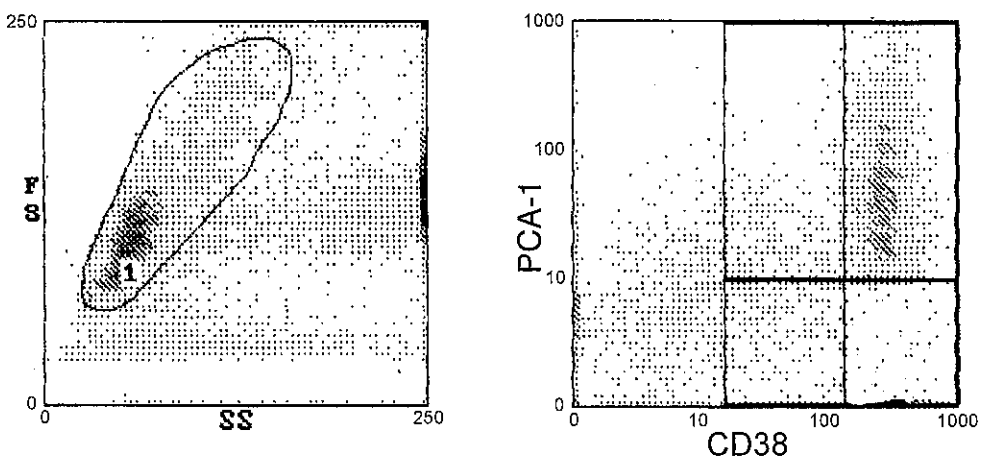

Fig. 1. Two-color flow cytometric analysis of myeloma cells (case 27). The isolated mononuclear cells were incubated with mouse monoclonal antibodies (mAbs) against PCA-1 and PE-conjugated $F(ab')_2$ fragments of goat anti-mouse IgG, and then with FITC-conjugated antibody against CD38. The horizontal axis shows FITC staining, and the vertical axis shows PE staining (log scale). The scatter cytogram on the left illustrates the gate of this phenotypic study. Forward scattering (FS, vertical axis) and side scattering (SS, horizontal axis) profiles of bone marrow mononuclear cells in a representative case of myeloma (case 27). A circle represents an area analyzed. Myeloma cells are located in the $CD38^{++}$ and $PCA\text{-}1^+$ fraction. More than 90% of $CD38^{++}$ fractions in case 27 are $PCA\text{-}1^+$.

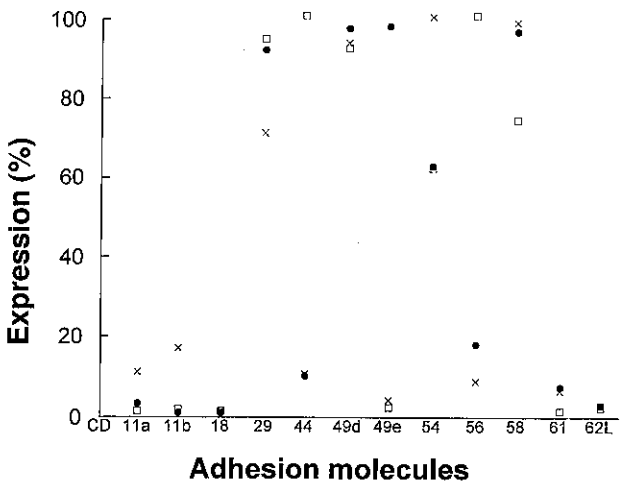

Fig. 3. Expression of adhesion molecules on myeloma cell lines; U266 (●), KPMM1 (×), and KPMM2 (□).

myeloma cells in the samples, and we proceeded with double-labeling flow cytometry. The expression of adhesion molecules in all the samples is summarized in Fig. 2. All myeloma cells expressed VLA-4 on their surface. Almost all myeloma cells expressed VLA-β_1, ICAM-1, and LFA-3. H-CAM was strongly expressed in 3 cases of PCL and 2 cases of aggressive myeloma, and moderately expressed in other MMs. N-CAM was expressed in 19 of 28 MMs analyzed and none of the 3 PCLs. LFA-1 (CD11a/CD18) was expressed in 2 cases of aggressive myeloma and weakly expressed in 1 case of PCL, but not expressed in the non-aggressive myelomas. VLA-5 was expressed in most of both morphologically immature and mature myeloma cells (data not shown). Most myeloma cells did not express Mac-1, VNR-β, or LECAM-1. There was no correlation between the expression of adhesion molecules and the isotype or the clinical stage.

Expression of adhesion molecules on myeloma cell lines

There are some variations in the expression of adhesion molecules among myeloma cell lines (Fig. 3). U266 expressed VLA-5 and KPMM2 expressed H-CAM and N-CAM. None of the 3 myeloma cell lines expressed LFA-1, Mac-1, VNR-β, or LECAM-1, like fresh myeloma cells.

DISCUSSION

In the present report, we investigated the expression of surface antigens on myeloma cells in bone marrow, peripheral blood, or ascites by two-color analysis using anti-CD38 antibodies as previously reported.[30] Recently, several reports of adhesion molecules expressed on myeloma cells have been published.[11–18, 32] These studies demonstrated that myeloma cells express H-CAM, VLA-4, ICAM-1, N-CAM, and LFA-3, which is consistent with our findings.

Among these adhesion molecules, VLA-4 is the principal integrin in myeloma.[33] VLA-4-fibronectin interactions have been reported to play an important role in the migration and homing of tumor cells and to facilitate cell-to-cell interactions in MM, e.g., migration and differentiation of B cells[5, 6] and localization of tumor cells to the marrow, triggering IL-6 secretion by marrow stroma cells.[9, 10] In the present study, H-CAM was strongly expressed in 3 cases of PCL and 2 cases of aggressive myeloma, and moderately expressed in other MMs. Recently, Okada *et al.*[34] reported that a combination of mAbs against H-CAM and VLA-4 significantly inhibited the adherence of cultured myeloma cells to stroma cells and that binding of cultured myeloma cells to macrophages was partially blocked with an antibody against ICAM-1. This finding, together with ours, suggests that H-CAM-hyaluronan interactions may play a critical role in migration of PCL cells and aggressive myeloma cells, which is important for plasma cell proliferation.

N-CAM is expressed in about two-thirds of myelomas, but not on non-malignant plasma cells,[11–14] which agrees with our observations. We did not detect this adhesion molecule in 3 cases of PCL. In myeloma cell lines, only one of three lines was N-CAM$^+$. van Riet *et al.*[13] reported that N-CAM was absent from the circulating plasma cells of one patient with PCL. Barker *et al.*[14] reported that two cases of PCL and six cell lines had either weak or no expression of N-CAM in comparison to myeloma cells in the bone marrow. Recently, Pellat-Deceunynck *et al.*[17] reported that extramedullary spreading was associated with a dramatically reduced expression of N-CAM. These observations, together with ours, suggest that N-CAM may play a critical role in migration of PCL cells to extramedullary sites.

LFA-1 was expressed in two aggressive myelomas and weakly expressed in 1 case of PCL, but not expressed in non-aggressive myelomas. This observation is consistent with the findings reported by Ahsmann *et al.*[32] They demonstrated that LFA-1$^+$ plasma cells were scarce in myeloma patients in a nonactive phase of their disease, whereas plasma cells in some patients with active disease and all patients with fulminant disease expressed LFA-1. They also reported that the expression of LFA-1 on plasma cells correlated well with the labeling index of the tumors in the individual patients. Recently, Vacca *et al.*[18] reported that both angiogenesis and high proportions of plasma cells expressing LFA-1, VLA-4, and H-CAM occur simultaneously in patients with MM during the active phase. The relationship between the expression of LFA-1 and tumor growth suggests an involvement of this

adhesion molecule in cellular interactions, which is important to plasma cell proliferation.[8, 18, 32, 35]

Recently, Kawano *et al.*[30, 31] identified two subpopulations of myeloma cells: VLA-4$^+$VLA-5$^+$ and VLA-4$^+$ VLA-5$^-$. They reported that VLA-5$^-$ myeloma cells were immature or plasmablastic while VLA-5$^+$ cells were mature. Furthermore, they postulated that VLA-5$^-$ myeloma cells were proliferative and IL-6-responsive, whereas VLA-5$^+$ myeloma cells were higher producers of M-protein. In the present study, however, VLA-5 was expressed in most cases of both immature and mature myeloma cells. On the other hand, two myeloma cell lines, KPMM1 and KPMM2, did not express VLA-5. The growth of KPMM1 is weakly stimulated by exogenous IL-6.[24] KPMM2 has a plasmablastic morphology showing homotypic cell aggregation and its growth is dependent upon an external IL-6 autocrine loop.[25] An IL-6 autocrine-dependent myeloma cell line, U266,[36] expressed VLA-5 in the current study, though U266 was previously reported not to express VLA-5.[16, 31, 33] This discrepancy is possibly due to the use of different U266 sublines, as suggested by Klein *et al.*[37] Further investigations are needed to clarify the function and importance of VLA-5, especially in IL-6-dependent growth of myeloma cells.

Our report demonstrates that malignant myeloma cells express many adhesion molecules and that some of them are related to the progression and the specific type of this disease, suggesting that adhesion molecules play an important role in the myeloma proliferation. Further investigations are required to clarify the precise role of adhesion molecules in myeloma proliferation, which will lead to a better understanding of the biology of both normal and malignant plasma cells.

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