Lymphocyte Function-Associated Antigen-1 Expression on Plasma Cells Correlates With Tumor Growth in Multiple Myeloma

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Lymphocyte function-associated antigen-1 (LFA-1) (CD11a/CD18) expression on bone marrow–derived plasma cells from normal individuals, patients with monoclonal gammopathies of undetermined significance (MGUS), and patients with multiple myeloma (MM) was studied by immunofluorescence microscopy and flow cytometry using a new monoclonal antibody (MoAb) F8.8. This MoAb recognizes the α-chain (CD11a) of LFA-1 as determined by immunoprecipitation, and inhibits T-cell–induced cytotoxicity. Although the F8.8 MoAb stains unstimulated peripheral blood T cells with the same mean fluorescence intensity as other anti-CD11a MoAbs, it proved to be superior in detecting CD11a on plasma cells as compared with reference MoAbs. Using the anti-CD11a MoAb F8.8, a strong correlation was found between LFA-1 expression and disease activity in MM, as defined by clinical performance and serum M-protein level. Hardly any LFA-1+ plasma cells were detected in normal individuals, patients with MGUS, and MM patients in a nonactive phase of their disease, while plasma cells of some MM patients with active disease and all patients with fulminant disease expressed LFA-1. Plasma cell LFA-1 expression correlated well with the labeling index (LI) of the tumors in the individual patients. The relation between LFA-1 expression and the tumor growth suggests an involvement of this adhesion molecule in cellular interactions resulting in plasma cell proliferation.

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ADHESION MOLECULES play an important role in the immune system. By facilitating cell-cell interactions, they are involved in migration and homing of mononuclear cells and the induction and regulation of immune responses. The known cellular adhesion molecules can be divided into three families: the immunoglobulin supergene family, the selectins, and the integrin family.

The integrin family consists of several structurally related subfamilies of membrane-bound glycoproteins. They combine different α-subunits with a subfamily-specific β-subunit in a noncovalent association. One of these subfamilies, the β2-integrin subfamily, consists of three members: lymphocyte function-associated antigen-1 (LFA-1), Mac-1, and gp150/95. The expression of these molecules is limited to white blood cells.1

LFA-1, Mac-1, and gp150/95 consist of distinct α-chains (CD11a, CD11b, and CD11c, respectively) and a common β-chain (CD18). Mac-1 and gp150/95 are expressed by monocytes and granulocytes and are involved in adhesion to endothelial cells and localization at sites of inflammation.2 LFA-1 mediates contact between cells within the immune system through binding to cellular receptors called intercellular adhesion molecules (ICAMs), two of which have been described (ICAM-1 and ICAM-2). LFA-1/ICAM-1 interactions are a prerequisite for the induction of a broad spectrum of leukocyte functions, including cytotoxicity, proliferation, and differentiation,3 as well as lymphocyte binding to activated endothelial cells.4,5

Adhesion molecules may be involved in processes of growth, spread, and clinical behavior of lymphoid malignancies. In a mouse model system using lymphoma cells, LFA-1 expression could be related to an increased metastatic potential of such cells in vivo.6 In human lymphomas, LFA-1 expression is found on tumor cells in low- and medium-grade malignant lymphomas,7,8 while absence of LFA-1 seems to be a characteristic of high-grade malignant lymphomas.9,10 The presence of LFA-1 has been related to the aggressiveness and anatomical site of the tumor9,11 and its absence might contribute to an escape from immunosurveillance.9,11

Given the differential expression of LFA-1 in lymphomas, we studied whether malignant plasma cells in MM express LFA-1 and whether the expression of this adhesion molecule correlates with the proliferation of the plasma cells as the hallmark of the disease.

MATERIALS AND METHODS

Patients. Forty-four patients with multiple myeloma (MM) and six with monoclonal gammopathies of undetermined significance (MGUS) were included in this study. MM was diagnosed at the time of bone marrow sampling according to the Southwest Oncology Group (SWOG) criteria.12 MGUS patients showed a stable M-component for at least 2 years. According to the Durie and Salmon staging system, one patient was stage Iib, three stage IIa, 39 stage IIla, and one stage IIlb. The isotypes of the serum monoclonal immunoglobulins in the various patients were two IgM, 28 IgG, 10 IgA, two IgD, and eight Bence Jones protein. The median age (MM and MGUS patients) was 60.1 years, ranging between 43 and 81; 22 were men and 28 were women. The patient population was divided into three groups according to clinical performance and M-protein level. The first group included patients with nonactive disease having no or mild clinical symptoms and no increase in M-protein 3 months before or after the time of analysis. The second group contained the patients with active, symptomatic disease, including bone pain and an increase in M-protein in the 3 months before analysis. The third group consisted of patients with rapidly progressive disease, characterized by severe bone pain, hypercalcemia, and pancytopenia; in these patients, a decrease in M-protein production is observed (fulminant myeloma). In the third group, 12 patients progressed into a fulminant phase after a relatively benign disease course, and two patients already presented with fulminant disease. The mean labeling index (LI) in the three MM groups defined above were 0.6%, 1.4%, and 8.8% for the nonactive, active, and fulminant MM.
group, respectively. The median time of follow-up in these three MM groups was 26 months for nonactive MM (range, 12 to 36), 16 months for active MM (range, 0 to 36), and 24 months for fulminant MM (range, 0 to 40). The characteristics of the patients with MGUS and the three groups of MM are summarized in Table 1.

**Cells and cell lines.** Bone marrow cells of patients were isolated for diagnostic reasons by Ficoll-Isoopaque (Pharmacia, Uppsala, Sweden) density centrifugation of bone marrow aspirates. In addition, bone marrow cells from healthy individuals were analyzed.

Cells were washed twice in minimal essential medium (MEM) containing penicillin (100 IU/mL) and streptomycin (100 μg/mL) and used for immunofluorescence analysis (see below) or stored frozen for later analysis. Peripheral blood monocytes were purified from the Ficoll-Isoopaque density centrifugation isolated mononuclear cell fraction by adherence to plastic dishes. The human plasmacytoma cell line CRL 1484 was maintained in RPMI-1640 supplemented with 10% fetal calf serum (FCS; Gibco, Paisley, UK), glutamine (2 mmol/L), penicillin (100 IU/mL), and streptomycin (100 μg/mL) at 37°C in a 5% CO₂ humidified atmosphere. The effector cell line used for the cytotoxicity assay (see below) was AK4 (TCRγδ), an allogeneic cytotoxic T-cell clone (CTL). The target cell line was APD, an Epstein-Barr virus (EBV)-transformed B-cell line. The cell lines were free of mycoplasma contamination, which was determined as described by Spiereburg et al.

| Table 1. Clinical Characteristics of MGUS and MM Patients |
|-----------------------------|---------------------|-----------------|-----------------|-----------------|
|                             | MGUS (n = 6)        | Nonactive MM (n = 8) | Active MM (n = 22) | Fulminant MM (n = 14) |
| Sex (M/F)                   | 3/3                 | 2/6              | 8/14             | 9/5              |
| Stage                       | l                   | ll               | lll              | lll              |
| M-component                 | mgM                 | mgS              | mgA              | mgD              |
| IgM                         | 2                   | 3                | 1                | 1                |
| IgG                         | 3                   | 3                | 3                | 3                |
| IgA                         | 1                   | 3                | 4                | 2                |
| IgD                         | 1                   | 1                | 1                | 1                |
| Median age (yr) (range)     | 59 (43-71)          | 61 (43-81)       | 59.6 (36-71)     |
| Mean LI% (range)†           | 0.4 (0-0.8)         | 0.6 (0-1.4)      | 1.4 (0-8)        | 8.8 (2.8-29)     |
| Median time of follow-up (range)† | 26 (12-36)     | 16 (0-36)        | 24 (0-40)        |
| Disease status              | Newly diagnosed     | Plateau†         | Treatment§       | Relapse†         |
|                             | 5                   | 2                | 5                | 1                |
|                             | 2                   | Treatment§       | 12               |

The division of patients with MM in groups with nonactive, active, and fulminant disease is based on criteria described in the Methods. The median time of follow-up before analysis in months. The plateau phase defined as M-component decrease greater than 50% after treatment lasting for at least 6 months without treatment. Treatment with chemotherapeutic agents. Relapse defined as an increase in M-component greater than 50% from the lowest value or clinical and bone marrow relapse in case the M-component did not reflect tumorload and disease activity. Relapse during treatment in four of the 12 cases.

Monoclonal antibodies (MoAbs). The CD11a MoAbs used in this study were F8.8 (see below), SPV-L7 (Monosan, Uden, The Netherlands), and CLB-LFA-1/2 (CLB, Amsterdam, The Netherlands). In addition, the following CD antigens were analyzed: CD11b (CLB-mon/gra-1; CLB), CD38 (OCT-10; Ortho, Raritan, NJ), CD20 (B1; Coulter, Mydrecht, The Netherlands), CD10 (CALLA; Becton Dickinson, Ereembegem, Belgium), CD3 (Leo-4, Becton Dickinson), and CD13 (MY7; Coulter). Antibromodeoxyuridine (α-BrdU) fluorescein isothiocyanate (FITC)-conjugated MoAb (Becton Dickinson) was used to determine the LI of the plasma cells (see below).

**Production of the F8.8 MoAb.** Six-week-old BALB/c mice were immunized twice, intraperitoneally, with 2 x 10⁶ cells of the surface IgM-negative EBV cell line Fl8B2, with a 1-week interval. Three days after the last immunization, splenic cells were fused as described previously. IgG-containing hybridoma supernatants were tested for their ability to inhibit homotypic adhesion of the EBV cell line N103 as described by Rothlein et al. The hybridoma selected this way (F8.8) was cloned twice by limiting dilution and injected intraperitoneally in pristane-primed mice for ascites production. IgG was isolated by protein A chromatography and the subclast was determined using goat anti-mouse IgG subclass-specific antibodies (SBA; Birmingham, AL) as described by Koolwijk et al.

**Immunofluorescence studies.** The analysis of the surface phenotype and the nature of the heavy and light chains present in the cytoplasm of the plasma cells using double immunofluorescence microscopy (DIFM) has been described elsewhere. In addition, bone marrow aspirates containing more than 50% CD38⁺ plasma cells were analyzed by flow cytometry (FACSCan, Becton Dickinson). For this purpose, cells were sequentially incubated with OKT10 (CD38), FITC-labeled goat anti-mouse IgG antibodies (Becton Dickinson), and blocked with normal mouse serum followed by incubation of biotinylated MoAb (CD11a, CD11d, CD20, or CD15) and streptavidin coupled to phycoerythrin (Becton Dickinson). LFA-1 expression on T cells in peripheral blood was analyzed by flow cytometry in a double-staining procedure as described above, using the anti-CD11a MoAb F8.8, CLB-LFA-1/2, or SPV-L7 and biotinylated CD3 D MoAb. The procedure for the biotinylation of MoAbs was performed exactly as described by Heitzmann and Richards. The phenotype of the plasma cells of the cell line CRL 1484 was analyzed by flow cytometry as described above.

**Determination of the labeling index.** The method to determine the LI of the bone marrow plasma cells has been described elsewhere. In short, cells were incubated with 10 μmol BrdU. After fixation in suspension with 70% ethanol and partial DNA denaturation with 4N HCI, the cells were cytocentrifuged and subsequently stained with FITC-conjugated anti-BrdU MoAb and tetramethylrhodamine isothiocyanate (TRITC)-conjugated, affinity-purified goat antibodies directed against human heavy or light chains (SBA).

**Cytotoxicity assay.** Cytotoxicity was measured in a standard chromium release assay in a U-bottom, 96-well plate using complete medium. Briefly, varying numbers of effector cells (AK4) in 100 μL were mixed with 50 μL of diluted antibodies or for control values 50 μL medium in the wells of the plate. After 30 minutes, 51Cr-labeled target cells (APD, 2 x 10⁶/well) in 50 μL were added. Plates were centrifuged for 3 minutes at 100 x g and incubated for 3.5 hours at 37°C, 5% CO₂. Plates were centrifuged again and released 51Cr in 100 μL supernatant was measured in a gamma-counter (Minaxi, Auto-gamma counter, Packard, Warrenville, Downers Grove, IL). All tests were performed in triplicate. The percentage of specific 51Cr release was determined as: (experimen-
tal release − spontaneous release)/(total Triton-X100−induced release − spontaneous release) × 100.

Cell surface iodination and immunoprecipitation. Iodination and precipitation using the plasmacytoma cell line CRL 1484 was performed as described by Schwartz-Albiez et al. In short, cells were labeled with #131I (Amersham, Aylesbury, UK) in the presence of lactoperoxidase by pulses with hydrogen peroxide and subsequently solubilized in lysis buffer. After removing nuclei and cell debris by centrifugation, the cell lysate was cleared by preadsorptions with protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) and affinity-purified rabbit anti-mouse IgG and IgM antibodies (Dakopatts, Uithoorn, The Netherlands). To examine the specificity of the F8.8 MoAb for CD11a, sequential immunoprecipitation was performed. The lysate was divided in three aliquots. Aliquot I was precleared three times with a combination of 20 μg of rabbit anti-mouse IgG plus IgM, 100 μL protein A-Sepharose CL-4B, and 10 μg of the F8.8 MoAb, followed by immunoprecipitation with 10 μg of the anti-CD11a MoAb SPV-L7 or the CD38 MoAb OKT-10 as a positive control. Aliquot II was treated identically, except the lysate was precleared with the MoAb SPV-L7 and subsequently immunoprecipitated with the F8.8 MoAb or OKT-10. Aliquot III was precleared with an isotype- and subclass-matched control MoAb, followed by immunoprecipitation with the MoAb F8.8 or SPV-L7.

CD11a and CD11b were precipitated from 131I-labeled peripheral blood monocytes using protein A-Sepharose CL-4B, rabbit anti-mouse IgG plus IgM, and the MoAb F8.8 or CLB-mon/gran-1, respectively.

After extensive washing, samples were boiled in sample buffer and subjected to polyacrylamide slab gel electrophoresis under reducing conditions (sodium dodecyl sulfate [SDS]-PAGE, 7.5%) according to Laemmli. Dried gels were visualized by exposure to x-ray films using intensifying screens.

RESULTS

The F8.8 MoAb recognizes the α-chain (CD11a) of the LFA-1 molecule. The presence of adhesion molecule LFA-1 on plasma cells was investigated by immunoprecipitation. Since bone marrow samples usually contain nonplasmacytoid LFA-1+ cells as well, we performed the precipitations with the LFA-1+ human plasmacytoma cell line CRL 1484. This cell line phenotypically resembles malignant bone marrow−derived plasma cells in MM (see Table 3). Precipitation with the MoAbs F8.8 and CLB-LFA-1/2 shows two bands of approximately 180 and 95 Kd corresponding with the α- and β-chain of the LFA-1 molecule (Fig 1, lanes 5 and 6). The LFA-1 specificity of the F8.8 MoAb was confirmed in sequential immunoprecipitation with the CLB-LFA-1/2 MoAb. Immunoprecipitation with either one of the anti-CD11a MoAbs depleted the cell lysates of LFA-1 that could not be precipitated any more with the other antibody (Fig 1, lanes 2 and 4).

CD18 reactivity of F8.8 was excluded by performing precipitation studies using 131I-labeled monocytes expressing CD11a/CD18 and CD11b/CD18. The F8.8 MoAb only precipitates bands corresponding to the CD11a and CD18 chains (Fig 2, lane 2) and no CD11b (Fig 2, lane 3). These results prove that F8.8 recognizes the α-chain (CD11a) of the LFA-1 molecule.

The F8.8 MoAb inhibits cytolytic activity. Interference in the LFA-1/ICAM-1 interaction pathway partially inhibits the activity of CTLs. Inhibition of the spontaneous cytolysis of the EBV-transformed B-cell line APD by the CTL clone AK4 by anti-CD11a MoAb F8.8 is as effective as the reference MoAb SPV-L7 (Table 2). This strengthens the evidence that the F8.8 MoAb recognizes the LFA-1 molecule.

LFA-1 expression on plasma cells. Bone marrow−derived plasma cells of seven normal individuals, six patients with MGUS, and 44 patients with MM were analyzed for the presence of CD11a using the MoAbs CLB-LFA-1/2, SPV-L7, and F8.8 as described in the Methods. Although binding of the three MoAbs to unstimulated peripheral
blood T lymphocytes results in comparable fluorescence patterns and intensities (Fig 3A), the F8.8 MoAb proved to be superior in staining CD11a on plasma cells as compared with the MoAbs SPV-L7 and CLB-LFA-1/2. This is exemplified in Fig 3B, where the flow cytometric analysis of the CD11a expression on CD38⁺ plasma cells of four representative MM patients is shown. Highest fluorescence intensities are obtained with the F8.8 MoAb. Furthermore, the histograms show that a considerable variation in LFA-1 expression on plasma cells is observed between individual patients. Plasma cells of patients K. and M. (Fig 3B-1 and B-3) are brightly stained, while plasma cells of patients B. and F. (Fig 3B-2 and B-4) only show a moderate LFA-1 expression. However, in all patients, staining with the F8.8 MoAb results in the highest LFA-1 fluorescence intensity.

**LFA-1 expression and clinical disease.** The patients with MM were divided into three groups based on clinical performance and M-protein level (see Methods). In the first group of MM patients (nonactive disease, n = 8), two patients could be identified as having low percentages of LFA-1⁺ plasma cells in their bone marrow. In the second group (active disease, n = 22), LFA-1⁺ plasma cells were detected in 10 patients. In this group, two patients had a high percentage of LFA-1⁺ plasma cells (23.4% and 30.3%). Interestingly, the tumors of these patients also had a high LI (5 and 8, respectively). In the bone marrow samples of all patients with fulminant disease (group 3, n = 14), moderate to high percentages of LFA-1⁺ plasma cells were present. Figure 4 shows the plasma cell LFA-1 expression

### Table 2. Anti-LFA-1 Antibody F8.8 Inhibits Spontaneous Cytolytic Activity of the Target APD by the CTL AK4

<table>
<thead>
<tr>
<th>Blocking MoAb</th>
<th>% Specific Release</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>65</td>
<td>50</td>
</tr>
<tr>
<td>F8.8</td>
<td>27</td>
<td>9:1</td>
</tr>
<tr>
<td>1 µg/mL</td>
<td>59</td>
<td>38</td>
</tr>
<tr>
<td>5 µg/mL</td>
<td>52</td>
<td>33</td>
</tr>
<tr>
<td>5 µg/mL</td>
<td>ND</td>
<td>36</td>
</tr>
</tbody>
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*Abbreviation: ND, not determined.*
in the three different groups of MM patients, in normal individuals, and in patients with MGUS.

**LFA-1 expression and LI in MM.** Both the LI and the plasma cell CD11a expression, as measured with the F8.8 MoAb, were determined in 36 bone marrow samples from patients with MGUS and MM in different stages of disease. A relation was found between both parameters; plasma cell LFA-1 expression was preferentially found in patients who had tumors with large growth fractions (Fig 5).

**Phenotype and CD11a expression.** To investigate whether CD11a expression on plasma cells could be related to an aberrant phenotype, the expression of CD10, CD20, and CD13 on CD38+ plasma cells was determined in MM patients with nonactive, active, and fulminant disease. In Table 3, representative results are shown of these analyses, which indicate that no apparent correlation exists between plasma cell LFA-1 expression and the phenotype of the tumor cells.

**DISCUSSION**

In recent years, it has become clear that adhesion molecules play a pivotal role in mediating cellular contact. Studies on the expression of such molecules on tumor cells in lymphoid malignancies increase our knowledge about their ability to interact with themselves or other cell types, and in this way contribute to our understanding of tumor growth and spread. In MM, ICAM-1 is expressed on all plasma cells, while recently the expression of CD56 (N-CAM) on aberrant plasma cells has been described. In some cases of MM, LFA-1 expression has been reported, in contrast to normal plasma cells which do not express this adhesion molecule.

In this study, we have investigated the presence of the adhesion molecule LFA-1 (CD11a/CD18) on plasma cells of normal individuals and patients with plasma cell dyscrasias. CD11a expression was detected on plasma cells from
26 of 44 MM patients. The mean fluorescence intensity, as well as the percentage of LFA-1+ tumor cells, varied among patients. Little, if any, LFA-1 expression was detected on plasma cells from normal individuals and patients with MGUS. On the basis of clinical performance and the level of serum M-protein, the MM patients were divided into groups having nonactive, active, and fulminant disease (see Methods). The mean percentages of LFA-1+ plasma cells within these groups were 1.9%, 4.3%, and 35.6% for the nonactive, active, and fulminant MM patients, respectively. Plasma cell LFA-1 expression correlated well with the tumor LI. This suggests that a causal relationship exists between plasma cell proliferation and LFA-1 expression in MM. Studies are in progress to assess whether the proliferating plasma cells actually express the LFA-1 molecule.

LFA-1 expression was analyzed by double immunofluorescence microscopy and additionally by FACS analysis if more than 50% monoclonal plasma cells were detected in the bone marrow aspirates. Both analyses were routinely performed with a panel of anti-CD11a MoAbs. Both the results from the fluorescence microscopy and the flow cytometric analyses showed that the F8.8 MoAb proved to be superior in staining plasma cells as compared with the other two anti-CD11a MoAbs.

The LFA-1 expression on CD38+ bone marrow-derived plasma cells could not be related to the phenotype of the cells. No relation was found between LFA-1 expression and expression of the myelomonocytic antigen (CD13) and/or B-cell lineage antigens (CD20, CD10). These results argue against the existence of a relation between LFA-1 expression and stage of differentiation of the malignant cells in MM and indicate that LFA-1 can be expressed on these plasma cells.

Not much is known about the regulation of LFA-1 protein expression in lymphoid cells. Recently, ras oncoprotein activation has been implicated in the selective upregulation of the expression of the α-chain of the LFA-1 molecule in EBV-transformed B cells. Whether similar processes are responsible for LFA-1 expression on the malignant plasma cells in MM, which are found to harbor ras mutations, remains to be elucidated.

The results presented here indicate a novel correlation between LFA-1 expression on plasma cells of patients with MM and plasma cell proliferation. This relation suggests an involvement of this adhesion molecule in cellular interactions resulting in plasma cell proliferation. As plasma cells constitutively express ICAM-1- and this molecule can be induced on bone marrow adherent cells, one can envisage a mechanism in which the LFA-1/ICAM-1 adhesion pathway mediates adhesion among plasma cells, as well as with cells in the bone marrow microenvironment. Since lymphokines are important in plasma cell growth, LFA-1-mediated intimate cellular contact between plasma cells and lymphokine-producing cells might be a critical event in the regulation of plasma cell growth in MM. Therefore, studies on functional aspects and regulation of the expression of adhesion molecules involved in such cellular interactions will contribute to our understanding of the pathophysiology of the disease process.

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