Analysis of the activation state of α4β1 integrin in human B cell lines derived from myeloma, leukemia or lymphoma

Mercedes García-Gila[a], Carlos Cabañas[b], Angeles García-Pardo[a,*]

[a]*Departamento de Inmunología, Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid, Spain*
[b]*Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Complutense de Madrid, Madrid, Spain*

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Abstract Myeloma cells specifically localize in the bone marrow and rarely circulate in blood. To study whether this immobilization could be partially explained by the presence of constitutively activated integrins, particularly α4β1, we used the activation reporter HUTS-21 anti-β1 mAb. These analyses showed that β1 integrins on myeloma cells were moderately active and could be upregulated similarly to integrins on lymphoma or leukemia cells. Myeloma cells were also tested for their ability to attach to RGD-containing fibronectin fragments, a property of activated (but not resting) α4β1. Two cell lines adhered to these fragments and this was inhibited by anti-α5 but not by anti-α4 mAbs. These results show that myeloma cells bear low/moderately active α4β1 and support the notion that multiple interactions contribute to their localization in the bone marrow.

Key words: α4β1 integrin; Integrin activation; Myeloma cell; Fibronectin

1. Introduction

Integrins are a family of surface receptors which mediate cell adhesion to multiple ligands in the extracellular matrix (ECM), endothelium, or other cell surfaces [1]. Integrins are dynamic heterodimeric molecules (αβ) which exist in several states of activation. Transition from inactive to active forms can be modulated by signals from inside the cell as well as by external agents [1,2]. Among these agents are the divalent cation Mn^{2+} and some anti-β mAbs which induce a conformational change in the integrin resulting in a high affinity receptor [3–5]. Another group of anti-β mAbs such as HUTS [6], recognize only activated integrins and are therefore useful markers for activation.

Among B cell malignancies, myeloma cells represent an interesting system to study the presence of constitutively activated integrins. Multiple myeloma is a B cell neoplastic disease characterized by clonal expansion of the malignant plasma cells within the bone marrow (BM) [7]. In contrast to normal plasma cells, myeloma cells localize primarily in the BM and rarely circulate in the peripheral blood except at very advanced stages of the disease [7]. Previous reports have established that activation of α4β1, the major integrin in most normal and malignant B lymphoid cells [8,9], results in inhibition of cell migration on fibronectin (Fn) or vascular cell adhesion molecule-1 (VCAM-1), the two ligands for α4β1 [10–13]. Such inhibition is likely due to the increased adhesion strength induced by the high affinity interactions obtained with activated receptors. It is possible that one of the reasons that may contribute to the retention of myeloma cells in the BM is the presence of a highly activated form of α4β1.

α4β1 binds constitutively to the specific sequences CS-1 and H1, located in the carboxy-terminal region of Fn [4,12] and upon activation with the anti-β1 mAb TS2/16 or Mn^{2+}, also recognizes the RGD sequence in the central cell binding domain [14]. Therefore, binding to RGD serves as a criterium for evaluating the activation state of α4β1. In a previous study, the adhesion of several myeloma cell lines to Fn was inhibited by an anti-α4 mAb as well as by an RGD-containing peptide [15]. These authors concluded that interaction of myeloma cells with Fn involves two different receptors: α4β1 and an RGD-dependent receptor. Another interpretation however, is that α4β1 is present on myeloma cells in the activated form and may therefore recognize the RGD sequence in Fn.

In the present report we have tested this hypothesis by: (1) studying whether myeloma cell lines attach to Fn fragments containing the RGD sequence; (2) analyzing the expression of the activation-dependent HUTS-21 epitope on these cells. Our results show that α4β1 on myeloma cells is not constitutively activated and can be regulated similarly to integrins in other B cell malignancies such as lymphoma or leukemia.

2. Materials and methods

2.1. Fibronectin fragments

Proteolytic fragments of 80 kDa and 38 kDa were obtained from tryptic digests of Fn and purified as previously described [4,14]. The 80 kDa is derived from the central region of Fn (repeats $III4_{-1/2}11$) and contains the RGD cell adhesion site which interacts mainly with the α5β1 integrin. The 38 kDa fragment is derived from the carboxy-terminal region of Fn and contains the Hep II domain and the IIICS segment; both of these regions contain ligands for α4β1 integrin. The recombinant fragment FN-III7–10, expanding repeats III7–10 in Fn and therefore containing the RGD sequence, was a gift from Dr. Harold Erickson (Duke University Medical Center, Durham, NC, USA).

2.2. Monoclonal antibodies

Anti-integrin subunit specific antibodies P1E6 (α2, purified Ab), P1B5 (α3, ascitic fluid), P1D6 (α5, ascitic fluid), and LM609 (αVβ3, purified Ab) were purchased from Calbiochem-Novabiochem Int., La Jolla, CA; mAb HP1/1 (α4, culture supernatant) and Alex1/4 (β1, culture supernatant) were obtained from Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain); mAb Act-1 (α4β7, purified Ab) was obtained from Dr. Douglas J. Ringler (Leukosite, Cambridge, MA, USA). mAb HUTS-21, specific for an activation epitope of β1 was obtained as described [6,13].

2.3. Cells and cell cultures

The human B cell lines IM-9, RPMI 8226, NCI-H929 (derived from the bone marrow, peripheral blood or malignant effusion respectively, of patients with multiple myeloma), Nalm-6 (pre-B from acute lymphoblastic leukemia), WIL2-NS (spherocytic anemia), Daudi and Na-

*Corresponding author. Fax: +34 (1) 562 7518.
E-mail: cibgp96@fresno.csic.es

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malwa (Burkitt's lymphoma) were purchased from the American type culture collection (ATCC). Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (ICN Pharmaceuticals, Costa Mesa, CA, USA), 2 mM L-glutamine, and 40 μg/ml gentamicin (Gibco BRL, Life Technologies S.A., Barcelona, Spain).

2.4. Flow cytometric analyses

5×10^5 cells were incubated for 30 min at 4°C with 100 μl of culture supernatants (1:2 dilution) or ascitis (1:100 dilution) containing the appropriate mAb. Cells were washed twice with cold PBS–1% BSA and resuspended in 100 μl of a 1:30 dilution of fluorescein-conjugated $F(ab')_2$ fragments of rabbit antibodies to mouse IgG (Dakopatts, Glostrop, Denmark). After 30 min at 4°C cells were washed twice, resuspended in 200 μl of PBS and analyzed by flow cytometry on an EPICS-CS (Coulter Científica, Móstoles, Spain). Analysis of the expression of the HUTS-21 activation epitope was done in the presence of Ca^{2+}+Mg^{2+}, or Mn^{2+} divalent cations exactly as described [6,13].

2.5. Cell attachment assays

These assays were performed as reported [4,12,14] using flat bottom high binding 96-well microtiter plates (Costar Co., Cambridge, MA). Assays were carried out for 2 h at 37°C, attached cells were stained with 0.1% toluidine blue and quantitated by reading the absorbance at 620 nm on a Multiskan Bichromatic plate reader (Labsystems, Helsinki, Finland), and visually on an inverted microscope (Nikon Diaphot, Japan). For inhibition experiments with anti-integrin mAbs, cells were incubated in a total volume of 400 μl, with appropriate supernatant (1:5 dilution) or ascitic fluid (1:100 dilution) for 30 min at room temperature; the cell suspension was then diluted to 5×10^5 cells/ml and 100 μl was added to each substrate-coated well.

3. Results and discussion

We first analyzed the expression of relevant integrin subunits on B cell lines derived from different pathologies, namely multiple myeloma, Burkitt lymphoma and leukemia. As shown in Table 1, all cell lines studied expressed moderate to high levels of α4 which was present in both forms, α4β1 or/and α4β7. The expression of α5, another Fn receptor, was either negative or very low, except for the myeloma cell line NCI-H929 which showed similar levels as α4, and for the leukemia-derived Nalm-6 cells (Table 1). This pattern is in agreement with the reported major role of α4 integrin as a Fn and VCAM-1 receptor in B cells [9,12].

To determine whether α4β1 integrin was present in the activated form and thus contributed to the selective retention of myeloma cells in the bone marrow, we performed two types of experiments: (1) we analyzed the expression of the HUTS-21 epitope on the β1 subunit; (2) we tested the ability of these cells to attach to Fn fragments containing the RGD site.

The HUTS-21 mAb recognizes an epitope in the β1 subunit which is expressed upon integrin activation. Therefore, constitutive expression of this epitope would be an indication of the presence of an activated β1 integrin. As shown in Table 2, with the exception of IM-9 cells, at 37°C all cell lines showed

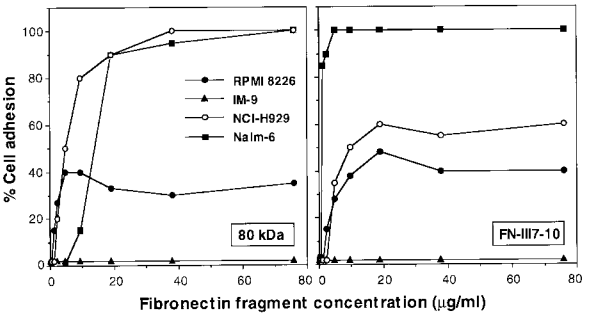

Fig. 1. Adhesion of human B cell lines to the 80 kDa and FN-III7–10 Fn fragments. Cells (6×10^4) were added to wells coated with the indicated concentrations of 80 kDa or FN-III7–10 fragments. After 2 h at 37°C, attached cells were quantitated as described in Section 2. Values are the means of at least three different experiments.

low/moderate basal expression of HUTS-21 epitope under resting conditions (Mg^{2+} and Ca^{2+}) compared to the total amount of β1 detected by Alex1/4 mAb, with higher levels for WIL2-NS (36.2%) and RPMI 8226 (24.5%) cells. In the presence of Mn^{2+}, expression of HUTS-21 increased in all cell lines in different proportions with maximal ratios for NCI-H929 (9.1 to 40.7%) and Nalm-6 (15.7 to 46.2%) cells. Interestingly, these two cell lines were the only ones that expressed significant amounts of α5 integrin (Table 1). In conclusion, β1 integrins on the myeloma cell lines studied displayed a similar pattern of activation as that shown by cell lines derived from lymphoma, leukemia or anemia.

The preceding results referred to all β1 integrins present on these cells. To specifically determine the activation form of α4β1, we took advantage of the ability of activated (but not resting) α4β1 to recognize the RGD sequence in Fn [14]. Two previously described Fn fragments containing this sequence were used as substrata for cell adhesion: an 80 kDa tryptic fragment which comprises repeats III4–1/2 11 [14], and a recombinant fragment comprising repeats III7–10 [16]. Another tryptic fragment of 38 kDa (IIICS and repeats III12–14) [4,12,14] which contains the α4β1 high affinity ligand CS-1 was used as positive control. As shown in Fig. 1, the three myeloma cell lines tested gave different patterns of adhesion to these two fragments. NCI-H929 cells bound very efficiently to the 80 kDa fragment and moderately (60% adhesion) to FN-III7–10; RPMI 8226 showed moderate adhesion to both fragments with maximum levels of 40–50%; and IM-9 cells did not bind to any fragment. This different behavior may be related to the different body compartment from which these cell lines were established (malignant effusion, peripheral blood, or BM respectively), which may in turn reflect distinct cell maturation stages. The pre-B Nalm-6 cell line also at-

Table 1
Expression of integrins on the B lymphoid cells studied

Cells	Mean fluorescence intensity							
	C	α2	α3	α4	α5	α4β7	αVβ3	β1
IM-9	0.17	0.19	1.53	2.42	0.37	2.02	3.27	4.51
RPMI 8226	0.30	0.32	1.35	0.72	0.63	4.19	2.12	1.51
NCI-H929	0.37	0.37	0.67	39.8	32.5	2.69	2.34	88.2
Daudi	0.27	0.36	0.30	1.43	0.31	12.0	0.38	2.58
Namalwa	0.20	0.20	1.45	3.20	0.57	0.35	0.25	3.73
Nalm-6	0.60	0.58	0.71	12.7	2.98	1.13	0.82	24.0
WIL2-NS	0.20	0.21	0.22	14.4	0.26	9.72	2.02	9.56

Table 2
Expression of HUTS-21 epitope on B lymphoid cells of different origen

Cells	4°C						37°C					
	Ca^{2+}+Mg^{2+}			Mn^{2+}			Ca^{2+}+Mg^{2+}			Mn^{2+}		
	C	Alex1/4	HUTS-21	C	Alex1/4	HUTS-21	C	Alex1/4	HUTS-21	C	Alex1/4	HUTS-21
IM-9	0.21	5.73	0.55	0.79	5.42	1.20	0.18	3.42	0.18	0.18	2.74	0.28
RPMI 8226	0.24	1.72	0.28	0.24	2.00	0.35	0.28	2.28	0.56	0.32	2.35	0.76
NCI-H929	0.23	48.3	4.07	0.14	47.0	16.1	0.16	47.4	4.33	0.16	47.2	19.2
Daudi	0.14	2.15	0.44	0.17	1.95	0.69	0.16	1.85	0.42	0.16	1.82	0.82
Namalwa	0.44	3.65	0.26	0.26	4.29	0.43	0.28	4.26	0.62	0.31	3.94	1.39
Nalm-6	0.97	30.1	2.12	0.58	27.0	9.74	0.91	30.1	4.74	1.20	26.4	12.2
WIL2-NS	0.28	0.85	0.44	0.26	0.94	0.53	0.29	1.05	0.38	0.26	1.30	0.50

tached very efficiently to the 80 kDa and FN-III7–10 fragments (Fig. 1), while all other cell lines tested gave negative results (not shown). As expected, all cell lines tested attached very efficiently to the 38 kDa fragment indicating that α4β1 integrin on these cells was able to recognize its constitutive ligands (results not shown).

Because the cell lines showing attachment to RGD-containing fragments express α5 integrin on their surface (Table 1) we analyzed whether adhesion to the 80 kDa fragment was mediated by α5β1 or α4β1 integrins or both. For these experiments, cells were pre-incubated with the specific mAbs P1D6 or HP2/1 prior to the adhesion assay. As shown in Fig. 2, the anti-α5 mAb P1D6 completely inhibited adhesion of the three cell lines tested, while HP2/1 (anti-α4) had no significant effect. These results indicated that adhesion of NCI-H929, RPMI 8226, and Nalm-6 cells to the RGD region of Fn was mostly mediated by the α5β1 integrin and not by activated α4β1.

The results obtained in the present study therefore indicate that α4β1 on myeloma cells (and some pre-B cells) is apparently present in an intermediate activation form, unable to recognize the RGD sequence under resting conditions but susceptible of further activation with Mn^{2+}. Constitutively activated α4β1 was shown to be present on T cells infiltrating inflamed synovium in rheumatoid arthritis [17]; likewise, resident B cells acquired an active α4β1 integrin upon stimulation in contrast to peripheral blood B lymphocytes which bore an inactive integrin [18]. The myeloma cell system was chosen in the present study because of the characteristic retention of malignant cells in the BM and their consequent lack of circulation. However, we could not detect significant differences with respect to the activation form of α4β1 on myeloma or other types of B cell malignancies. This suggests the existence of other factors or/and signals in the BM microenvironment that contribute to the specific immobilization of malignant plasma cells. For example, myeloma cells express other non-integrin adhesion molecules, such as proteoglycans or selectins, which may mediate adhesive interactions to ligands in the BM stroma [8,15]. Additional signals such as interleukin-6, which is an autocrine growth factor for myeloma cells and inhibits their chemotaxis [19] may also be necessary to maintain strong adhesive interactions within the BM. In conclusion, the results reported here show that the state of activation of α4β1 on myeloma-derived cell lines is low to moderate and support the notion that a complex set of interactions between malignant plasma cells and BM takes place in multiple myeloma.

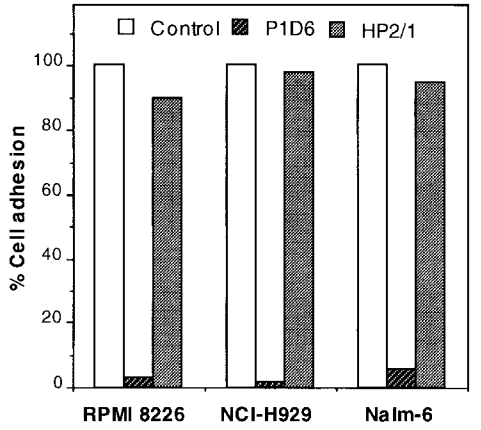

Fig. 2. Effect of anti-α4 (HP2/1) and anti-α5 (P1D6) mAbs on cell adhesion to the 80 kDa Fn fragment. Cells (6×10^4) were incubated for 30 min with HP2/1 or P1D6 mAbs and added to wells coated with 80 kDa fragment (76 μg/ml). After 2 h at 37°C, attached cells were quantitated. Values are the means of at least two different experiments.

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