INTERACTION OF LAMININ AND FIBRONECTIN WITH LEUKEMIC CELLS

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Stem cell proliferation and differentiation depends on critical interactions that occurs between stem cells and their microenvironment. Although poorly defined, this microenvironment is composed of both cellular and extracellular matrix components (ECM) (reviewed by Campbell & Wicha, 1988). Of the well characterized ECM glycoproteins, fibronectin and laminin have been clearly demonstrated in bone marrow ECM (Zuckerman & Wicha, 1983; Zipori et al., 1985). Laminin is related to some hematopoietic cell functions promoting attachment of polymorphonuclear neutrophils (Bryant et al., 1987; Terranova et al., 1986) and probably playing a role in the mechanism of natural cell mediated cytotoxicity (Laybourn, 1989). Several studies have indicated an important role of fibronectin in the normal development program of erytroid and myeloid cells (Giancotti et al., 1986; Patel & Lodish, 1986) in differentiation of thymocytes (Cardarelli & Pieschbacher, 1986) and in migration of hematopoeitic precursors (Savagner et al., 1986).

The ability of laminin and fibronectin to promote such diverse actions seems to be related to its interaction with cell surface-associated molecules. The best characterized laminin receptor is a 67 kDa receptor which binds to a laminin fragment P_1 derived from the intersection of the short arms of the cruciform laminin molecule, via the YIGRS peptide sequence located on the β_1 chain (Graf et al., 1987). Additional LN-binding proteins include 180 Kd and 110 — 120 Kd species (Kleinman et al., 1988; Smalheizer & Schwartz, 1987); a carbohydrate binding protein 35 (Mac-2) (Woo et al., 1990) and several integrins belonging to the VLA subfamily of integrins. Four of these VLA proteins, VLA-1 $(\alpha_1\beta_1)$, VLA-2 $(\alpha_2\beta_1)$, VLA-3 $(\alpha_3\beta_1)$ and VLA-6 $(\alpha_6\beta_1)$ have been shown to function as receptors for laminin (Wayner & Carter, 1987; Gehlsen et al., 1988; Ignatius & Reichardt, 1988; Sonnenberg et al., 1988; Languino et al., 1989; Turner et al., 1989; Elices & Hemler, 1989). Three fibronectin receptors have been

described belonging also to the VLA subfamily.: VLA-5 $(\alpha_5\beta_1)$, VLA-4 $(\alpha_4\beta_1)$ and VLA-3 $(\alpha_3\beta_1)$ (Takada et al., 1987; Wayner et al., 1988; Wayner et al., 1989). Other integrins with α –subunits distinct from α_1 might also function as FN receptors (Cheresh et al., 1989).

Little is known about the molecular mechanism by which developing blood cells are retained within the bone marrow. Cytoadhesiveness to ECM matrix proteins have been postulated to be involved in maintaining the cells anchored in the bone marrow until their release into the circulation (Patel et al., 1985). A clinical hallmark of leukemia is the inappropriate appearance in the circulation of immature cells. It will be of interest to determine whether alterations in the adhesive interactions between leukemia blasts and hematopoietic adhesion molecules may be involved in the pathogenesis of leukemia.

In the present study we have evaluated the levels of laminin and fibronectin receptors expressed in blast cells from leukemia patients representing immature hematopoietic cells and in lymphocytes and granulocytes from normal subjects as a model of *in vivo* differentiation.

MATERIALS AND METHODS

Patients — Laminin receptor was determined in the leukemic cells of 21 patients with acute myeloid leukemia (AML), (ten males and eleven females, age range, 2 — 73 years; three were children < 10 years, the adult patients were of age 18 — 73 years) and 22 patients with ALL, (12 males and 10 females; 18 were children < 10 years, the adult patients were of age 22 to 47 years). Our fibronectin receptor study comprised 18 patients with acute lymphocyte leukemia (ALL) (16 children < 16 years and 2 adults > 20 years) and 10 patients with AML (6 adults > 30 years and 4 children < 15 years). Acute leukemia patients were

classified according to the cyto-morphological criteria of French-British (FAB) Cooperative Group (Bennett et al., 1985). Patients were from the Hematology Division of Escola Paulista de Medicina and from the Pediatric Oncology Division of Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo.

Sixteen health subjects, nine females and three males (age range, 23 to 61 years) acted as controls. All patients were advised of procedures and gave informed consent.

Cells — Mononuclear cells and leukemic cells were obtained from heparinized peripheral blood by using Ficoll-Hypaque (Pharmacia and Winthrop) density centrifugation (Boyum, 1968). With this method, we have obtained samples with at least 95% lymphocytes and 80% leukemic cells. Neutrophils (95% purity) were obtained by using subsequently a hypotonic lysis. The viability of the cells always exceeded 95% as determined by trypan blue exclusion. Cases with less than 80% blasts in the final cell suspension were excluded.

Surface markers, including surface immunoglobulin (SIg) (Sigma Chemical Co., St. Louis, Mo), OKT monoclonal antibodies (OKT3, OKT8, OKT4, OKM1 and J5) were analyzed by direct and indirect immunofluorescence, respectively.

HL60 and K562 cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in RPMI-1640 (Gibco) suplemented with 10% heat-inativated fetal calf serum and penicilin and streptomycin (50 IU/ml and 50 mg/ml respectively) under a humified atmosphere at 37°C containing 5% CO_2 in air.

Cell binding studies — Mouse LN purified from Engelbreth-Holm-Swarm (EHS) murine tumor (Timpl et al., 1979) and fragments P_1 and E_8 were obtained as previously described (Timpl et al., 1979; Paulsson et al., 1987). Fibronectin and the 120 kDa fibronectin fragment were isolated by described methods (Pasqualini et al., 1989).

Purified laminin, laminin E_8 and P_1 fragments, the 120 kDa fibronectin fragment were labeled with $Na^{125}I$ (Amersham), Arlington Heights, IL) by the Chloramine T method (Fraker, 1978) to a specific activity of 5 $\mu Ci/\mu g$. The radioligand was eletrophoresed and autorradiographed to assess its purity and integrity.

Cell binding studies, as previously described (Yoon et al., 1987), were performed at 4°C in phosphate buffer saline (PBS, pH 7.4) supplemented with 1.8 nM $CaCl_2$ at a cell concentration 5 − 7 x 10^5 cells per tube. Cells were resuspended in PBS containing $CaCl_2$ 1.8 nM and incubated in triplicate with ^{125}I-laminin at a final concentration range of 0.5 − 3.5 nM, in the presence and absence of a 100 fold excess of cold laminin or with ^{125}I-FN-120 kDa at final concentration 5 − 45 nM in the presence or absence of a 50 fold excess of unlabeled fibronectin.

RESULTS

Figure 1 shows a representative equilibrium binding curve for the binding of ^{125}I 120 kDa fibronectin fragment to K562 cells as a function of free ligand concentration. Incubations were performed at 4°C for 45 min at which time the binding had reached equilibrium. Apparent dissociation constants (Kd) and number of cell binding sites for the fibronectin fragment were evaluated from Scatchard plots. The data is consistent with the presence of an uniform class of binding sites (35,000) which affinitty of 10 nM. A similar curve was obtained with ^{125}I-laminin binding to the HL60 cells (Fig. 2). It should be noted, however, that the HL60 cells displayed 8,300 binding sites/cell for laminin with a comparatively high affinity (Kd = 2.7 nM).

Table I shows the results of cell binding studies to the 120 kDa fragment of fibronectin obtained by Scatchard analysis. Lymphocytes expressed higher levels (18,301 ± 8,582 sites/cell, Kd 7.11 ± 5.18 nM) as compared to granulocytes which expressed few binding sites (2,705 ± 379 sites/cell) (p < 0.05). On the other hand, granulocytes displayed statistically higher laminin receptor levels (24,739 ± 13,516 sites/cell), as compared to lymphocytes (2,825 ± 2,007 sites/cell), p < 0.05. K562 and HL60 cells presented similar numbers of fibronectin and laminin binding sites. The affinities of binding to laminin or to 120 kDa fibronectin fragment were similar for all cells studied.

Mean 120 kDa fibronectin receptor in blast cells from ALL patients (Table II) were not different from the values found in normal lymphocytes. We were not able to establish a correlation between the 120 kDa fibronectin receptor levels and age, sex, FAB or immunological subgroup of these patients. Blast cells of AML patients presented a wide range of fibronectin receptor numbers, with higher lev-
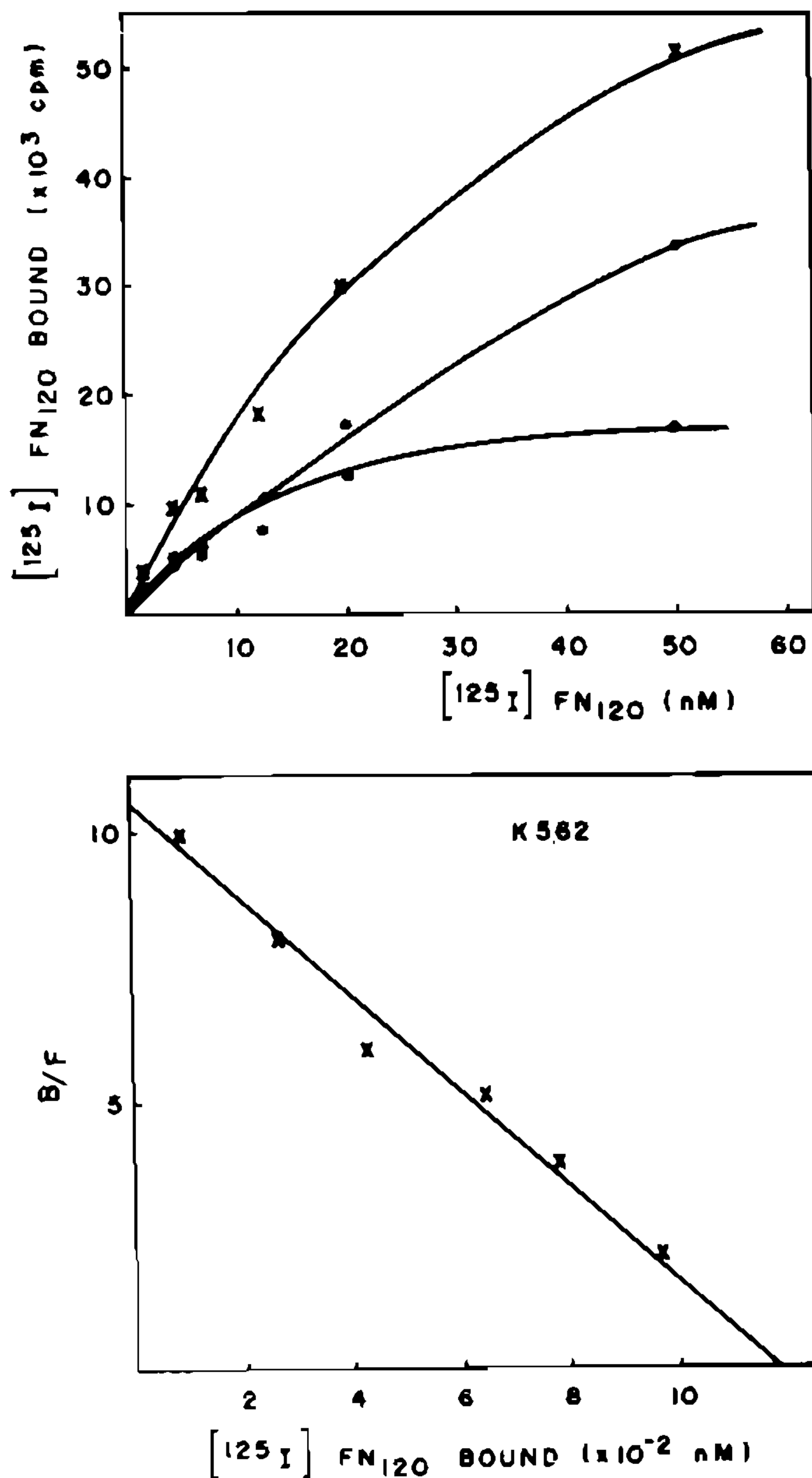
Fig. 1: equilibrium binding and derived Scatchard analysis for the binding of ^{125}I-labeled fibronectin fragment of 120 kDa to K562 cells (10^5 cells/tube) as a function of free ligand concentration. Specific binding (— • — • — • —) represents the difference between the binding in the absence (— x — x — x —) and presence of 100-fold (— o — o — o —) excess unlabeled ligand. The Scatchard plots of the specific binding data are shown with the ratio bound/free x 10^{-1}.

Mean LNR levels in blast cells from ALL patients (3.967 ± 3.406 sites/cell) and affinities (2.2 ± 1.2 nM) were not significantly different from the values found in normal lymphocytes. However, these LNR numbers were lower than levels presented by cells of AML patients (8,296 ± 6,007 sites/cell), p < 0.05 (Table III).

We were not able to establish any correlation between LNR, age and sex of ALL patients, peripheral leukocyte number, immunologic subtyping, or FAB subgroups L_1, L_2, L_3 (Bennett et al., 1985).

Blast cells of AML patients presented a wide range of LNR numbers but the average concentration and affinity were not statistically different from that found in normal granulocytes. Any correlation between LNR levels and positivity for
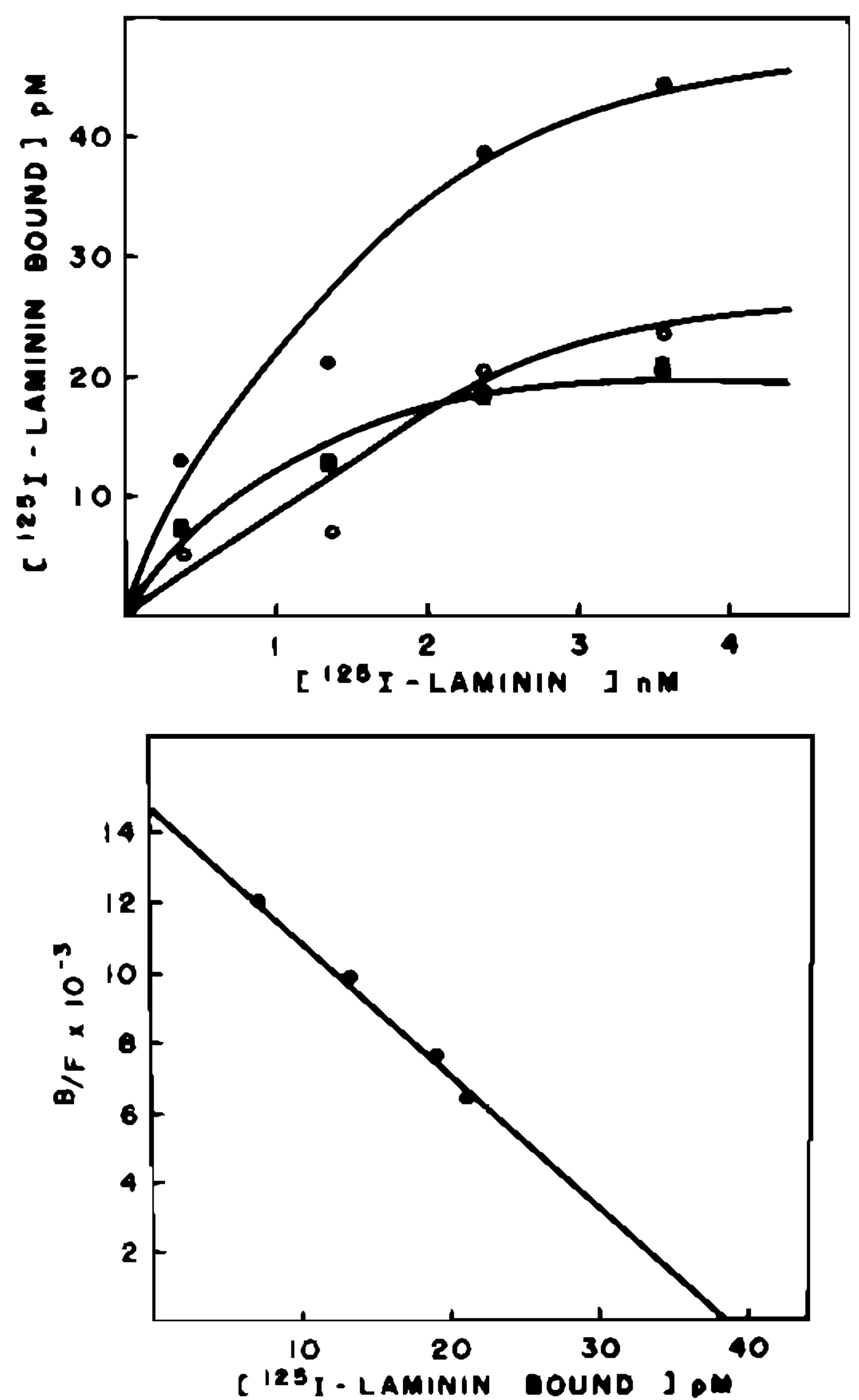
els than that found in normal granulocytes. However, mean levels showed a tendency to intermediate values as compared to granulocytes and lymphocytes or ALL patients. Cells obtained from patients classified as subtype M_1 showed statistically higher numbers as compared to M_2. Two patients in the M_6 sub-group showed also high levels. The values of FN-120 kDa receptor expressed by patients are in the same range displayed by the cell lines K562 and HL60.

In conclusion our data indicated that immature lymphoid cells expressed higher FN-120 kDa receptor levels than differentiated cells.

Fig. 2: saturation binding of ^{125}I-laminin to HL60 cells. Increasing concentrations of ^{125}I-laminin were incubated with 10^5 cells/tube. Specific binding (— □ — □ —) was determined by subtracting nonspecific (— o — o — o —) from total binding (— • — • — • —) and Scatchard analysis was also performed on these data.

monoclonal antibody OKM1, sex and age of the patients was verified. Cells obtained from patients classified, as subtype M_3 however, expressed higher LNR content (p $<$ 0.05) as compared to levels determined in M_3 and M_2 subgroups. The monocytic series showed apparently a similar tendency with lower binding sites in the M_4 subgroup as compared to one patients on M_5 subgroup.

In conclusion our data indicated that lymphoid cells express low LNR levels ($<$ 6,500 sites/cell). On the other hand, myeloid cells displayed a wide range of LNR, with higher levels being associated with the more differentiated FAB subgroups.

Apparent dissociation constants and number of cell binding sites for laminin fragments 1 and 8 were evaluated from Scatchard plots. A summary of the data for granulocytes, lymphocytes and HL60 cells (Table IV) indicated that HL60 cells and lymphocytes displayed a preferential binding for fragment P_1 and little binding of fragment E_8 could be detected. On the other hand, granulocytes bound equally well to fragments P_1 and E_8.

DISCUSSION

One of the major questions regarding hematopoesis is the understanding of the mechan-

isms underlyng the interaction of cells with their marrow environment. Various extracellular matrix glycoproteins receptors expressed on hematopoietic cells may exert a significant regulatory influence on cell state of proliferation and differentiation (Coulombel et al., 1988; Tsai et al., 1987). We have approached this question by studyng ligand binding affinity and specificity of laminin and fibronectin receptors in leukemic cells.

Our present study indicated that although normal hematopoietic cells and leukemic cells bind to radiolabeled laminin, the magnitude of LN receptor levels appears to be linked to lineage, the highest numbers of receptor being expressed by myeloid cells.

A wide range of laminin receptor levels was found in myeloid blasts. However, AML has been described as a very heterogenous disease, presenting cells arrested probably at various differentiation stages. It is possible that variability in laminin receptor numbers shown by AML cells could be a reflection of this subpopulation heterogeneity, resulting perphaps from subtle differences in the physiopathology of the disease.

In spite of the small number of patients in each AML subgroups, higher receptor levels, seems to be associated with the more differentiated FAB

TABLE I

Number of specific receptor (R) sites and apparent dissociation constants (Kd) of various cells for laminin and fibronectin

Cells	N	Binding Parameters	Radioligand			
			Laminin		120 kDa Fibronectin Fragment	
Lymphocytes	16	Kd (nM)	1.6	$\pm$ 0.8	7.11	$\pm$ 5.8
		R (sites/cell)	4,097	$\pm$ 2,317	18,301	$\pm$ 8,582
Granulocytes	8	Kd (nM)	5.47	$\pm$ 0.6	12.57	$\pm$ 1.1
		R (sites/cell)	22,900	$\pm$ 17,837	2,705	$\pm$ 379
K562	8	Kd (nM)	5.47	$\pm$ 1.0	11	$\pm$ 2.7
		R (sites/cell)	9,000	$\pm$ 2,000	34,058	$\pm$ 17,703
HL60	20	Kd (nM)	3.18	$\pm$ 1.7	10.0	$\pm$ 0.6
		R (sites/cell)	8,204	$\pm$ 4,079	20,489	$\pm$ 12,745

Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *86*, Suppl. III, 1991

47

TABLE II

Number of fibronectin binding sites determined
in peripheral blast
cells of patients with acute lymphoid (ALL) and acute
myeloid leukemia (AML) subdivided according
to FAB criteria

	N	Sites/Cell
ALL	18	16,175 $\pm$ 12,964
L_1	13	12,697 $\pm$ 6,483
L_2	3	18,490 $\pm$ 9,633
L_3	2	30,521 $\pm$ 8,854
AML	10	12,281 $\pm$ 3,660
M_1	3	19,800 $\pm$ 4,000^a
M_3	3	6,680 $\pm$ 4,582^b
M_4/M_5	2	13,462 $\pm$ 2,633
M_6	2	19,595 $\pm$ 6,385

Values were determined by radioligand binding and data
were analyzed by Scatchard plots described in Materials
and Methods.
a versus b, p $<$ 0.05 — Mann Whitney test.

TABLE III

Number of laminin specific binding sites determined in
peripheral blast cells of patients with acute lymphoid
(ALL) and acute myeloid leukemia (AML) subdivideo
according to FAB criteria

	N	Sites/Cell
ALL	22	3,967 $\pm$ 3,406^a
L_1	19	4,070 $\pm$ 3,445
L_2	3	3,325 $\pm$ 3,772
AML	21	8,296 $\pm$ 6,007^b
M_1	8	4,787 $\pm$ 3,994^c
M_2	5	9,066 $\pm$ 7,272
M_3	2	17,119 $\pm$ 8,143^d
M_4	5	6,775 $\pm$ 3,710
M_5	1	38,693

Values were determined by radioligand binding and data
were analyzed by Scatchard plots described in Materials
and Methods.
a versus b, p $<$ 0.05; *c versus d*, p $<$ 0.05 - Mann
Whitney test.

TABLE IV

Apparent dissociation constants and number of receptor sites of various
cells for laminin fragments P_1 and E_8

Cells	Radioligand			
	LM fragment P_1 Sites/Cell	Kd (nM)	LM fragment E_8 Sites/Cell	Kd (nM)
Lymphocytes	3.010 $\pm$ 851	1.6 $\pm$ 0.8	903 $\pm$ 28	0.5 $\pm$ 0.1
Granulocytes	6.440 $\pm$ 2.000	5.4 $\pm$ 0.6	7.300 $\pm$ 135	2.0 $\pm$ 0.1
HL60	12.581 $\pm$ 7.776	4.0 $\pm$ 0.2	842 $\pm$ 170	0.5 $\pm$ 0.1

subgroups. Our results referring to normal granulo-
cytes agreed with previous finding about a high
content of LN receptors in granulocytes, macro-
phages and monocytes (Yoon et al., 1987; Huard
et al., 1986; Singer et al., 1989).

The ability of cells to interact with extracellu-
lar matrices is an important determinant of cell
function. It was found that polimorphonuclear
cells could respond chemotactically to laminin
(Bryant et al., 1987). The expression of laminin

receptors might be a part of the pleiotropy that characterizes the maturation of granulocyte in reponding to defend host. In this regard, it is to be noted that HL60 cells lack receptors for formyl peptide chemoattractants (Fontanta et al., 1980; Niedel et., 1980) which appear upon induction to a differentiated state.

Our experiments showed further that a dual binding pattern to P_1 and E_8 fragments of laminin was displayed by granulocytes whereas the P_1 binding profile seems to be a characteristic of immature cells in the myeloid lineage. P_1 is also normally expressed by lymphoid cells irrespective of their maturation grade. The 67 kDa laminin receptor seems to be the major laminin binding protein in polymorphonuclear cells and macrophages (Bryant et al., 1987; Yoon et al., 1987; Singer et al., 1989; Huard et al., 1986; Mercurio & Shaw, 1988).

However, recently at least two more cell surface receptors have been described that can mediate adhesion to P_1. One of these is the vibronectin receptor that belongs to the β_3 family of integrins (Sonnenberg et al., 1990), and was expressed by polymorphonuclear leukocytes (Singer et al., 1989). The other putative P_1 receptor is the $\alpha_1\beta_1$ integrin that is a dual receptor for collagen IV and laminin (Hall et al., 1990).

It has been suggested previously that the P_1 cell-binding site is a cryptic cell-binding site (Nurcombe et al., 1989). Whether the P_1 cell binding site is exposed and used *in vivo* is not known yet. It is possible that the lymphoid lineage presents a nonfunctional type of receptor.

On the other hand granulocytes displayed also binding to the E_8 fragment of laminin, which contains most of the long arm of laminin (Edgar et al., 1984). Recently it has been demonstrated that the major $\alpha_6\beta_1$ (VLA6) attachment domain is present in fragment E_8. Alterations in LN receptor type might reflect the development of specific cell functions during maturation events. There is evidence in favor of a role for $\alpha_6\beta_1$ in attachment of macrophages to laminin (Shaw et al., 1990).

We have found that lymphocytes express high numbers of fibronectin receptors. It is possible that the presence of receptors may endow these cells with the ability to migrate and to localize to appropriate lymphoid tissues. Although two differ-

ent fibronectin receptors ($\alpha_4\beta_1$ and $\alpha_5\beta_1$) have been described in peripheral lymphocytes $\alpha_4\beta_1$ is more abundantly expressed (Wayner et al., 1989). However, VLA4 recognizes the COOH terminal 10 amino-acids of the alternatively spliced V segment of the FN molecule (Wayner er al., 1989) which are not included in the 120 KDA fibronectin fragment utilized in the present study. Therefore our results refers *probably* to VLA5.

Fibronectin receptors were found in blast cells of all ALL patients investigated. In spite of the wide range of values displayed there was a trend for higher values than those presented by lymphocytes. This variation may reflect different stages of maturation. A preferencial expression of fibronectin receptors on immature thymocytes has been described (Carderelli et al., 1988).

High values found in two cases of erythroleukemia and in K562 cells are according to previous works demonstrating that erythroid progenitors and leukemic cells attach highly to fibronectin and express fibronectin receptors (Tsai et al., 1987; Patel & Lodish, 1987; Vuillet-Gaugher et al., 1990). Monocytic leukemias (M_4 + M_5) presented also high levels of fibronectin receptor. Several studies demonstrated that fibronectin receptors are strongly expressed on mature monocytes (Hajman et al., 1983; Parker et al., 1988; Brown et al., 1989). Therefore the fibronectin receptor seems to be retained throughout the differentiation stages.

The granulocytic leukemias and the promyelocytic HL60 cells do not bind to fibronectin as strongly as erythroid leukemias. Moreover, there is a tendency for loss of binding as they differentiate and mature into functional granulocytes. The regulation of expression of fibronectin receptors by erythroid and myeloid progenitors cells as differentiation proceeds have been demonstrated (Vuillet-Gaugher et al., 1990).

It was found that fibronectin receptors differ in immature and normal monocytes and are altered by glycosylation in the course of cellular maturation (van der Water et al., 1988). Therefore it is possible that not only the number, but different types or differentially processed integrins may serve an important role in the disruption of normal interactions of progenitors cells with bone marrow stromal elements in leukomogenesis. Experiments along this line are in progress in our laboratory.

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