*Revisão / Review*

# Interaction of leukemic cells with proteins of the extracellular matrix

## *Interações de células leucêmicas com proteínas da matriz extracelular*

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*The interaction of neoplastic cells with basement membrane molecules is the first step for the dissemination of tumor cells* in vivo. *Leukemic cells have a great ability to spread in the host, since cells are released from the bone marrow to the circulation. In this study we analysed whether CEM, U937, K562 and HL-60 cells were able to attach to different concentrations of laminin and/or fibronectin and/or type IV collagen. Attachment to type IV collagen was low, but it increased with the addition of laminin and occurred in all four leukemic cell lines. On the other hand, attachment to fibronectin was higher, but it decreased with the addition of laminin in the assays using U937 and HL-60 cells. The combination of type IV collagen and fibronectin was a good substratum for cellular attachment. However, the addition of laminin to this substratum impaired its attachment activity in U937, HL-60 and K562. These data suggest that laminin may control cellular attachment to the extracellular matrix during leukemic dissemination in hosts in different ways. Rev. bras. hematol. hemoter. 2004;**26**(3):206-211.*

***Key words:*** *Leukemia; extracellular matrix; attachment; fibronectin; laminin; type IV collagen.*

### Introduction

Hematopoiesis is a highly organized system of blood cells production. The control of hematopoietic cell growth and proliferation is controlled by complex molecular interactions of cells in the bone marrow microenvironment.[1] The microenvironment is formed by the ordered production of cytokines and extracellular matrix (ECM) components by bone marrow stromal cells.[2-4] There is increasing evidence that leukemic cells, like normal hematopoietic cells, may be dependent on a specific microenvironment for growth and dissemination in hosts. It has been demonstrated that the proliferation of neoplastic cells is dependent of the autocrine production of cytokines[5,6] as well as their interaction with ECM components.[7-9] It has been demonstrated that a specific microenvironment composed of ECM can support their migration and growth.[5,10]

Cell adhesion to the ECM regulates numerous biological processes in which migratory functions are of paramount importance. In this process cells interact with ECM components of basement membrane and of loose connective tissue. The adhesive functions involve several receptors present at the cell surface.[25]

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The spreading of tumor cells in hosts is dependent of several factors. The attachment of neoplastic cells to the basement membrane of the vascular system as well as migration and proliferation in a new organ's microenvironment may establish the successful formation of metastasis.[11] The interaction of neoplastic cells with basement membrane molecules is the first step of dissemination of tumor cells *in vivo*.[10] Leukemic cells have a great ability to spread in hosts since cells are released from the bone marrow into the circulation. The infiltration of circulating leukemic cells begins with the adhesion of the cells to ECM components to the vessel cell wall followed by cell migration into the surrounding tissue. This process involves specific interactions between integrins and the ligand that persistently sends the extracellular signals to the leukemic cells.[12] Dissemination of leukemic cells in many tissues is frequently observed during the evolution of the disease. Widespread leukemia dissemination comprises of the spleen, bone marrow, thymus, lymph nodes, brain and multiple visceral organs (liver, kidney, lung, small intestine and pancreas), as well as skeletal muscles.[13,24]

An investigation of the interaction of leukemic cells with proteins of the extracellular matrix is the objective of this study. We analysed the role of fibronectin, laminin and type IV collagen as substrates for the attachment of leukemic cell lines. We tested the following leukemic cells: U937, CEM, K562, HL-60 and FDC-P1, that is a normal cell line obtained by murine bone marrow, in attachment assays. Fibronectin, laminin and type IV collagen are commonly found in basement membranes and may drive leukemic cell interaction with tissues, causing leukemic cell dissemination in hosts.

## Material and Methods

### Cell Culture

The leukemic cells U937 (histiocytic lymphoma derived cells), HL-60 (acute promyelocytic leukemia), CEM (acute lymphoblastic leukemia), K562 (chronic myelocytic leukemia) and FDC-P1 (murine normal bone marrow) were maintained at a density of 3-10x$10^5$ cells/mL in tissue culture flasks (Costar, Cambridge, MA) containing Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS, Fazenda Pigue, Brazil).[16] The cultures were incubated at 37 ºC, under a 5% $CO_2$ atmosphere.

### ECM Proteins

Mouse type IV collagen (Coll IV), natural mouse laminin (LN) and human plasma fibronectin (FN) were purchased from Gibco BRL-Life Technologies (Grand Island, NY). LN and FN were prepared in phosphate-buffered saline (PBS). Coll IV was diluted in 0.5M acetic acid, as recommended by the supplier. Acid soluble Coll IV was prepared in PBS immediately before use. In some experiments, polyclonal antibodies against ECM proteins were used. Rabbit anti-human fibronectin (A0245, Dako, Carpinteria, CA) and rabbit anti-human laminin (a gift from Dr. Moura Neto, Departamento de Anatomia, UFRJ) were used in attachment assays. We treated substratum with anti-LN or anti-FN for two hours at 37°C in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA, Sigma). After washing 3 times with PBS, the attachment assay was performed as described in materials and methods.

### Cell Attachment Assays

The cell attachment assay was performed in 96-well flat-bottomed microtitre plates (#25801, Corning, NY). The plates were coated with 50 µL of FN (0,5 µg/well) and/or Cull IV (0,5 µg/well) associated or not with different concentrations of laminin (0,1; 0,5; 1 µg/well) (table 1) in phosphate-buffered saline (PBS) for 12 hr at 4 º C. Plates were washed with PBS and incubated at room temperature (22°-25°C) with 100 µL of 2% bovine serum albumin (BSA, Sigma) in PBS.

The plates were washed again with PBS and then 50µL of a $10^5$ cell suspension in attachment medium (DMEM) were added to each coated well. After 3 hours at 37° C, non-adherent cells were removed by gently washing three times with PBS. Attached cells were fixed with 4% paraformaldehyde (Sigma) for 10 min and stained with 0.5% crystal violet in 20% methanol.[12] After 10 min the plates were washed three times with PBS and the stain was eluted with 0.1M sodium citrate, pH 4.2 in 50% ethanol[14] and absorbance at 540 nm for each well was read using a

**Table 1**

| ECM PROTEINS ASSOCIATIONS |
|---|
| Coll IV (0.5 µ/well) |
| Coll IV (0.5 µ/well) + LN (0.1 µ/well) |
| Coll IV (0.5 µ/well) + LN (0.5 µ/well) |
| Coll IV (0.5 µ/well) + LN (1 µ/well) |
| FN (0.5 µ/well) |
| FN (0.5 µ/welll) + LN (0.1 µ/well) |
| FN (0.5 µ/well) + LN (0.5 µ/well) |
| FN (0.5 µ/well) + LN (1 µ/well) |
| Coll IV (0.5 µ/well) + FN (0.5 µ/well) |
| Coll IV (0.5 µ/well) + FN (0.5 µ/well)+ LN (0.1 µ/well) |
| Coll IV (0.5 µ/well) + FN (0.5 µ/well)+ LN (0.5 µ/well) |
| Coll IV (0.5 µ/well) + FN (0.5 µ/well)+ LN (1 µ/well) |
| Coll IV (0.5 µ/well) + FN (0.5 µ/well)+ LN (0.5 µ/well) + anti-LN |
| Coll IV (0.5 µ/welll) + FN (0.5 µ/well)+ LN (0.5 µ/well) + anti-FN |

Microplate Reader (Model ELX800, Bio-Tec Instruments). The adherence of cells was expressed in terms of absorbance. All values are quoted as the median of determinations made in quadruplicate and a difference of $p \leq 0.05$ was taken to indicate statistical significance.

**Statistical Analysis**

The Mann-Whitney U test was used to analyze differences between the different groups and a difference of $p \leq 0.05$ was taken to indicate statistical significance.

## Results

We verified the ability of different leukemic cells to adhere to ECM proteins. In our experimental system, the adhesion activities of leukemic cells to FN and/or Coll IV associated or not with laminin, were studied using the attachment assays method (Figure 1) In our other experiments (data not shown) the leukemic cells did not present adhesion to laminin alone. From then on, we do not use laminin alone in our experiments. We observed the differential ability of the cells analysed to adhere to different ECM substratum. As shown in Figure 1, all leukemic cell lines and FDC-P1 attached to a lower extent to a substratum composed by Coll IV only. The association of Coll IV with other ECM proteins increased the adhesion of all leukemic cell lines, except for FDC-P1. U937 cells showed greater capacity of binding to ECM substrata.

Figure 2 demonstrates the adhesion of cells of leukemic lineages and FDC-P1 cells to the substratum formed by Coll IV (0.5µg/well) and increasing concentrations of LN (0.1; 0.5; 1µg/well). We observed that although the four leukemic cell lines do not have adhesion to LN alone (data not shown) when we tested the association of LN and Coll IV, we observed an increase in the number of attached cells. This increase is proportional to the concentration of LN used and all leukemic cells showed an increase in adhesion. When we verified the adhesion of leukemic cells and FDC-P1 cells to substrata formed by FN and increasing concentrations of LN (Figure 3), U937, HL-60 and FDC-P1 presented less cellular attachment, whereas the CEM and K562 cells presented an increase in adhesion. With the association of Coll IV-FN in increasing concentrations of LN (Figure 4), leukemic cells and FDC-P1 showed the same adhesion pattern as demonstrated in Figure 3, however, the adhesion intensity was smaller.

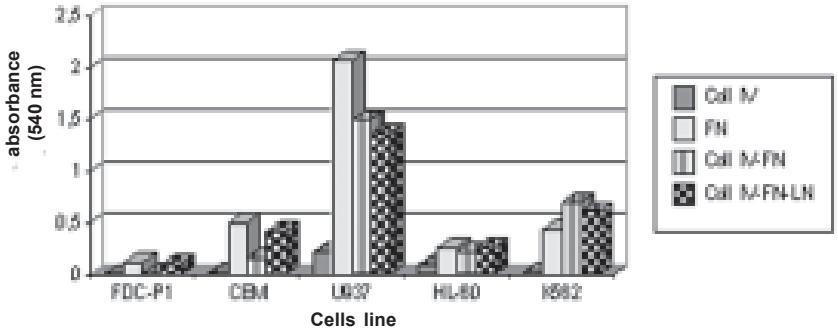


***Fig. 1** – Adhesion of leukemic cells to ECM proteins. Plates were prepared with LN, FN and Coll IV at concentrations of 0.5 µg/well. Differences were statistically significant using the Mann-Whitney test ($p<0.05$)*

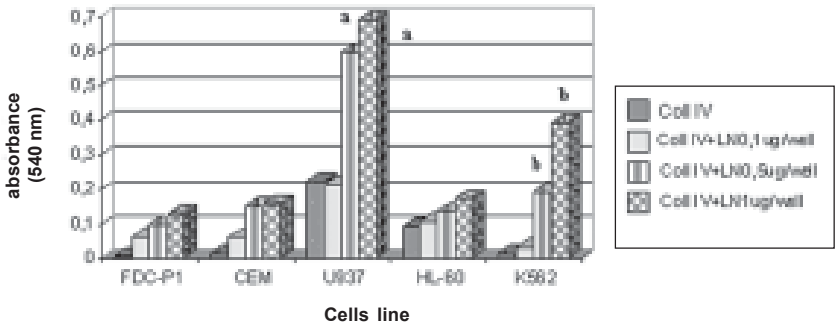


***Fig. 2** – Effects of increasing the concentration of laminin (LN) (0.1; 0.5 and 1µg/well) during preparation of substratum composed of 0.5 µg/well of type IV collagen (Coll IV) in adhesion of leukemic cells. Differences were statistically significant using the Mann-Whitney test ($p<0.05$)*
***a:** comparing the U937 adhesion to Coll IV-LN 0.5 µg/well and Coll IV-LN 1 µg/well with adhesion of this cell to Coll IV*
***b:** comparing the K562 adhesion to Coll IV-LN 0.5 µg/well and Coll IV-LN 1 µg/well with adhesion of this cell line to Coll IV*

To test the effects of LN on cellular attachment to FN and Coll IV, we treated different associations of LN, FN and Coll IV with an anti-LN antibody and we then measured the adhesion of leukemic cells to these prepared matrices (Figure 5). We observed that the treatment of matrices with anti-LN antibody abolished the impairment that the U937 cells had to attach to FN-LN or Coll IV-LN complexes. In HL-60 and CEM, the antibody had the same activity, however, at a lesser intensity. On the other hand, K562 adhesion was lower when compared to that observed with the substratum without antibody. These results suggest an anti adhesive activity of ECM components with LN. We observed that anti-FN antibodies increased the adhesion of U937, HL-60 and CEM cells.

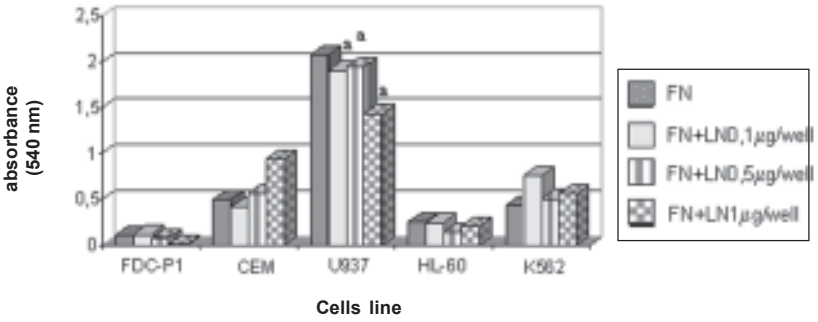


***Fig. 3 –*** *Effects of increased concentrations of laminin (LN) (0.1; 0.5 and 1 µg/well) during preparation of substratum composed of 0.5 µg/well fibronectin (FN) in adhesion of leukemic cells. Differences were statistically significant using the Mann-Whitney test (p<0.05)*
***a:*** *comparing the U937 adhesion to FN-LN 0.1 µg/well and FN-LN 0.5 µg/well and FN-LN 1 µg/well with adhesion of this cell to FN*

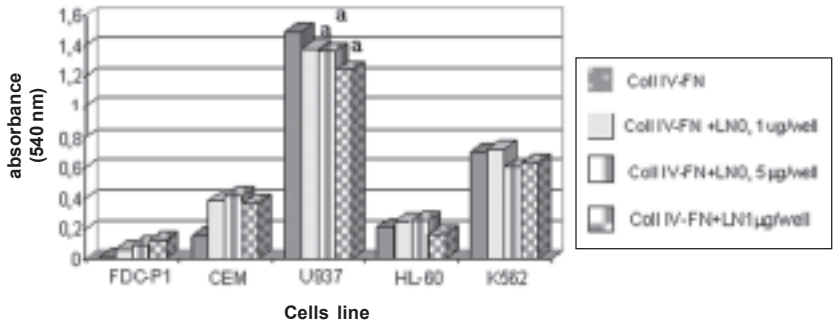


***Fig. 4 –*** *Effects of increased concentrations of laminin (LN) (0.1; 0.5 and 1 µg/well) during preparation of substratum composed of 0.5 µg/well of type IV collagen (Coll IV) and 0.5 µg/well of fibronectin (FN) in adhesion of leukemic cells. Differences were statistically significant using the Mann-Whitney test (p<0.05)*
***a:*** *comparing the U937 adhesion to Coll IV-FN-LN0, 1 µg/well, Coll IV-FN-LN 0.5 µg/well and Coll IV-FN-LN 1 µg/well with adhesion of this cell to Coll IV-FN*

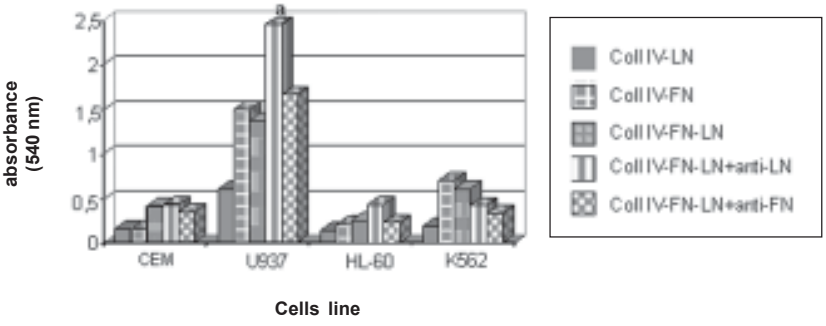


***Fig. 5 –*** *Effects of antibody anti-laminin (anti-LN) and antibody anti-fibronectin (anti-FN) on leukemic cells attachment to ECM substratum. Differences were statistically significant using the Mann-Whitney test (p<0,05).*
***a:*** *comparing the U937 adhesion to Coll IV-FN-LN treated with anti-LN with adhesion of this cell line to Coll IV-FN-LN.*

## Discussion

The ECM forms a specific microenvironment for individual cells in tissues of the body. The macromolecules that make up the ECM are basically constituted by collagen, elastin, glycoproteins (fibronectin, laminin) and proteoglycan packing elements.[17] It is now known that ECM components regulate cell migration, growth, differentiation and adhesion.[12,14,17,26] In long-term bone marrow cultures, the maintenance of hematopoiesis is dependent on the establishment of an intact layer of adherent cells that are derived from the marrow stroma.[18] The stroma is able to produce a microenvironment composed of growth factors and ECM that controls hematopoietic proliferation and/or differentiation.[18,19,20]

Currently, cell adhesion to ECM is recognized to be not merely a mechanical attachment, but a specific interaction between integrins and the ligand.[12,25] It has become clear that signals that result from the interaction between tissue adhesive proteins and their specific integrin receptors play a major role in the tissue-specific localization of migratory cells of all types.[14]

In this study, we evaluated the adhesion of human leukemic cell lines to ECM molecules. We compared the ability that leukemia cells have to bind to FN and/or Coll IV associated or not with LN. We studied the following types of leukemic cells: acute promyelocytic leukemia cells (HL-60), histiocytic lymphoma derived cells (U937), chronic myelocytic leukemia cells (K562), CEM (acute lymphoblastic leukemia) cells and a normal cell line of murine bone marrow (FDC-P1). All of these cells showed different patterns of adhesion to ECM components. The best attachment activity of U937, HL-60, K562, CEM and FDC-P1 was achieved with FN and a moderate activity was achieved with Coll IV. The adhesion of all leukemic cell lines and FDC-P1 to Coll IV increased with the

addition of increasing concentrations of LN. In FN-LN substrata, U937, HL-60 and FDC-P1 cells had their attachment decreased and anti-LN antibody abolished the obstruction of the adhesion of the U937, HL-60 and CEM cells to FN-LN or Cull IV-LN complexes.

These results demonstrate that the leukemic cell lines studied exhibited different attachment activities in the studies with ECM molecules. These molecules are commonly found in basement membranes.[17] U937, K562, CEM, HL-60 and FDC-P1 cell lines presented different binding properties to FN and/or LN associated or not with Coll IV. Strong adhesion was observed to FN whereas only moderate adhesion was observed to Coll IV. These results suggest that the expression of specific receptors at the cell surface may specifically drive the binding of the leukemic cell to individual extracellular matrix components. When FN was combined with Coll IV an enhancement in adhesion was observed when compared with the adhesion results of cells to Coll IV. This may be due to synergic effects, suggesting that different sets of ECM receptors may be used for interaction with FN and Coll IV. Interestingly, the addition of LN to the complex FN-Coll IV significantly impairs adhesion in U937, Hl-60 and K562, suggesting that preparation of matrices containing LN may impair cellular attachment. This may represent a transient attachment of the cell to this substratum, since the washing of cells may dislodge weakly bound cells. This observation may be important if we consider that LN may be used preferentially for cellular migration instead of cell binding.[21] During the dissemination of leukemia cells into the tissues, weak interactions with ECM may not favor the proper infiltration of the leukemic cells into the tissue since blood vessel pressure may dislodge loosely attached cells.[22] Since binding of leukemia cells is the first step in the dissemination of tumor cells in hosts a suitable microenvironment in tissues may determine the organ and tissue preference for metastasis by neoplastic cells.[5,23]

Taken together, these results suggest that a differential binding of leukemia cells takes place in each individual extracellular matrix component. The combination of these molecules in ECM may drive a differential ability of interaction with this extra cellular environment. Depending on the matrix composition of the organ, this may facilitate the interaction of leukemic cells with the vessel wall and the infiltration into tissues.

***Resumo***

*A interação de células neoplásicas com moléculas da membrana basal é a primeira etapa para a disseminação,* in vivo*, de células tumorais in vivo. As células leucêmicas possuem grande capacidade de espraiamento e disseminação no organismo uma vez que as mesmas são liberadas da medula óssea para a circulação. Neste trabalho avaliamos a capacidade das linhagens celulares CEM, U937, K562 and HL-60 em aderirem a uma matriz extracelular constituída por diferentes concentrações de laminina e,ou fibronectina e sobre colágeno IV. A adesão de todas a linhagens leucêmicas a colágeno IV foi baixa, mas aumentou com a associação à laminina. Por outro lado, as células U937 e HL-60 apresentaram alta ligação à fibronectina porém, foi reduzida com a adição de laminina. A associação de colágeno IV e fibronectina possibilitou um bom substrato para a adesão celular. Entretanto, a adição de laminina a este substrato reduziu a adesão das células U937, HL-60 e K562. Estes resultados sugerem que a adesão à matriz extracelular e a disseminação de células leucêmicas pode ser modulada pela laminina. Rev. bras. hematol. hemoter. 2004;**26**(3):206-211.*

***Palavras-chave:*** *Leucemia; matriz extracelular; adesão; fibronectina; laminina; colágeno tipo IV.*

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