

## ORIGINAL PAPER

**THE INFLUENCE OF FIBRONECTIN ON PROLIFERATION AND APOPTOSIS OF ACUTE LYMPHOBLASTIC LEUKAEMIA CELLS *IN VITRO***JOANNA WŁODEK<sup>1</sup>, ANNA PITUCH-NOWOROLSKA<sup>2</sup><sup>1</sup>Student of the doctoral programme in medical sciences, Department of Clinical Immunology, Medical College, Jagiellonian University, Krakow, Poland<sup>2</sup>Department of Clinical Immunology, Medical College, Jagiellonian University, Krakow, Poland

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The extracellular matrix (ECM) is a dynamic environment involved in the regulation of haematopoiesis. A crucial role of this structure is the promotion of proliferation, maturation, and differentiation of haematopoietic stem cells (HSC), and adhesion and migration of HSC in bone marrow.

In the present study the effect of ECM proteins (fibronectin, collagens, laminin, thrombospondin, and vitronectin) on proliferation and apoptosis of acute lymphoblastic leukaemia cells isolated from acute lymphoblastic leukaemia (ALL) patients (*in vitro*) was assessed. The leukaemia cells were obtained as interphase on Ficoll/Isopaque (Pancoll human, PAN-Biotech) density gradient and, after washing, counted in a chamber. Subsequently, cells were used for culture and apoptosis assay.

Presence of fibronectin, collagen type IV, and laminin was associated with inhibition of lymphoblastic leukaemia cell proliferation. Analysis of the culture of lymphoblastic leukaemia cells in the presence of ECM showed fibronectin as the most active protein.

**Key words:** acute lymphoblastic leukaemia, proliferation, apoptosis, fibronectin, collagen.

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**Introduction**

Acute lymphoblastic leukaemia cells are B lymphocyte precursors of leukaemia (B-ALL), in the majority cases in a stage of differentiation and maturation when CD10 (common ALL) antigen is expressed [1]. The regulation of B-cell ontogeny in bone marrow, like other cell lines, is based on morphological structures, e.g. niches with stromal cells (“osteoblastic”) and vascular niches localised at the sinusoidal walls. Vascular niches contain mesenchymal stromal cells, endothelial cells, and perivascular stromal cell [2]. Like cytokines, the extracellular matrix (ECM) proteins are another group of signalling molecules taking part in complex regulation of haematopoiesis [3]. The basic role of these proteins is the promotion

of proliferation, maturation, and differentiation of haematopoietic stem cells (HSC) with nutrient-rich microenvironment and growth factors. Several stromal cell types surrounding the vessels are regulating HSC with e.g. CXCL12 reacting with CXC-chemokine receptor type 4 (CXCR4) expressed on HSC. Signals from this axis are important for homing and maintaining the HSC pool in bone marrow [4]. Leukaemia initiating cells, similarly to normal haematopoietic stem cells, are dependent on signals coming from cells present in both type of niches and from their soluble products [5]. In B-ALL homing of leukaemia cells in bone marrow is supported by the CXCL12/CXCR4 axis because CXCR4 is present on leukaemia cells. Moreover, high expression of CXCR4 and phosphorylated form (active CXCR4) on B- leu-

kaemia cells is associated with worse patient outcome due to therapy resistance [6].

ECM components are important factors in functional network harmonising self-renewal of HSC, regulating cell adhesion, inflammatory response, angiogenesis, and homing of cells, e.g. tumour metastasis. Hyaluronic acid (HA), a major component of ECM, helps the recovery of peripheral white blood cells to stimulate HSC expressing HA receptors. Heparan sulphate proteoglycans play a role in establishing and regulating niche function by participating in patterning, compartmentalisation, growth, differentiation, and maintenance of tissue building HSC niches in bone marrow [3]. Fibronectin, collagens, laminin, thrombospondin, and vitronectin are a group of ECM proteins with lesser known function in haematopoiesis regulation. Fibronectin is involved in adhesion and migration of HSC in bone marrow. However, there are some data showing that fibronectin has an inhibitory effect on HSC proliferation [7]. Laminin bound to HSC supports proliferation and migration of these cells. In more detailed studies the supporting effect of laminin was shown in erythropoiesis [8]. It is noteworthy that, from ECM proteins, collagens and laminin enhance the proliferation and differentiation of myeloid cells not only in bone marrow but also in the extramedullary localisation [9]. From the collagen family the collagen type IV, especially denatured collagen IV, was shown to stimulate secretion of matrix metalloproteinase 9 (MMP-9) and regulate migration of cells in bone marrow, which may promote leukaemogenesis due to increased activation of AKT via LSC interaction [5]. The above data about the effects of ECM proteins on HSC, erythropoiesis, and myeloid cell ontogeny raise a question about the effects of ECM proteins on B cell line ontogeny.

### Aim of the study

The effect of ECM proteins (fibronectin, collagens, laminin, thrombospondin, and vitronectin) on proliferation and apoptosis of acute lymphoblastic leukaemia cells isolated from ALL patients was studied *in vitro*.

## Material and methods

### Patients

The bone marrow biopsy material of 18 consecutive patients with initial diagnosis of acute lymphoblastic leukaemia were included in study. Bone marrow was taken for flow cytometry analysis of leukaemic blast immunophenotype and classification of leukaemia, before introduction of therapy. The leukaemia cells remaining after complex diagnostic procedures were used for this study. The characteristics of patients and immunophenotype of leukaemia cells are shown in Table I.

### Isolation of acute lymphoblastic leukaemia cells

The bone marrow taken on EDTA under local anesthesia for diagnosis and classification of leukaemia was divided into two portions – one for flow cytometry standard diagnostic procedure (AIEOP-BFM<sup>1</sup>) and molecular biology, and the second for study of the influence of ECM proteins on proliferation and apoptosis of leukaemia cells. The leukaemia cells were obtained as interphase on Ficoll/Isopaque (Pancoll human, PAN-Biotech) density gradient. After washing, cells were counted in a chamber and used for culture and apoptosis assay. The viability of cells used for further tests was no less than 90%.

### Culture of cells

The leukaemia cells were cultured in 96-well microplates (Nunc, Denmark) in concentration  $2 \times 10^6$ /ml in RPMI1640 medium supplemented with antibiotic (gentamycin 10 mg/200 medium) and 10% foetal calf serum in a humid atmosphere of 5% CO<sub>2</sub>. The culture of leukaemia cells was in medium alone (control) or in the presence of the following ECM proteins (Sigma, Germany) after coating the wells:

- fibronectin polymer – 10 µg/ml of final concentration – 2 hours coating at room temperature,
- collagen type I and IV – 10 µg/ml final concentration – 24 hours coating at 4°C,
- laminin – 10 µg/ml final concentration – 1 hour coating at room temperature,

**Table I.** Characteristics of patients included in the study of ECM proteins and leukaemia cells

TYPE OF LEUKAEMIA	NO. OF PATIENTS	AGE (YEARS)	BOYS/GIRLS	CO-EXPRESSION OF OTHER DETERMINANTS
ALL-proB (progenitor B cell leukaemia)	3	3/12-6	1/2	
ALL-proB+My (progenitor B cell leukaemia + co-expression of myeloid determinants)	1	5	1	CD15
Common ALL	11	4-9	8/3	
Common ALL+ My (co-expression of myeloid determinants)	3	2-4	2/1	CD33, CD13, CD15, cytCD79a

- vitronectin – 10  $\mu\text{g/ml}$  final concentration – 2 hours coating at 37°C,
- thrombospondin was added to culture (0.1 ml per well) in a final concentration of 10  $\mu\text{g/ml}$  (without previous coating of wells) [10].

### Apoptosis induction and assay

The cells were seeded in triplicate 96-well microplates  $2 \times 10^5$  per well and cultured in medium or ECM protein precoated wells for 48 hours in standard conditions. After this, cells were harvested, washed in PBS, and incubated for 5 min with Annexin V (Annexin V/PI kit, Becton-Dickinson) at room temperature, in darkness, then washed and assayed in flow cytometry. Propidium iodide (PI – 5  $\mu\text{g/ml}$  final concentration, 10  $\mu\text{l/tube}$ ) was added before the acquisition of 10,000 events from each sample. The analysis was performed in dot plot for Fl 1 (annexin V – early apoptosis) and Fl 2 (PI – dead cells) or FL1 and FL2 (late apoptosis) simultaneously.

### Proliferation of leukaemia cells

Culture of cells for proliferation assay was performed for 72 hours and terminated with  $^3\text{H}$ -thymidine (Hartmann, Germany) added to each well for a final 8 hours, and then harvested on glass fibre with a cell harvester. Incorporation of  $^3\text{H}$ -Thymidine was measured in a liquid scintillation beta counter (Beckmann, USA). The index of stimulation or inhibition of proliferation was counted as the ratio of cpm (counts per minute) of cells cultured in the presence of ECM to cpm cells cultured in medium alone.

The expression of CD34 as another parameter associated with maturation of B lymphocyte precursors was assayed after 72 hours of culture only on cells expressing CD34 before culture. This assay was performed with flow cytometry method after staining with anti-CD34 FITC antibody and acquisition of 10,000 events (Becton-Dickinson).

## Results

### Apoptosis induction

The assay of early apoptosis based on annexin V staining did not show differences between medium and culture in the presence of ECM protein. In an analysis of 10 cultures of leukaemia cells isolated from patients, the effect of ECM proteins on late apoptosis was noted only with fibronectin and collagen type I presence. The mean value of the percentage of leukaemia cells stained with annexin V and PI (late apoptosis) showed an increase in the presence of fibronectin (from 28.47 to 49.04%) and collagen type I (up to 32.32%); however, the effect of collagen was weaker (Table II). The induction of early apoptosis (annexin V staining) was noted only in two cases in the presence of fibronectin. The analysis of cases with strong induction ( $> 50\%$ ) of apoptosis by fibronectin showed inhibition of proliferation of leukaemia in culture, which suggests an inhibitory role of fibronectin in the regulation of leukaemia cell proliferation.

### Proliferation

The mean values of stimulation index showed a weak effect of ECM proteins. However, collagen type I and vitronectin seemed to stimulate proliferation in contrast to fibronectin, collagen type IV, and laminin, leading to inhibition of proliferation in most samples isolated from patients (13, 11, and 14 out of 17, respectively; Table III). However, the modification of the proliferation index was in a narrow range due to low proliferation of lymphoblastic leukaemia cells (Fig. 1).

### Expression of CD34 after culture in the presence of ECM proteins

The analysis included 14 cases of ALL with expression of CD34 above 30% of leukaemia cells (in 3 cases expression of CD34 was below 20% and these cases

**Table II.** The induction of late apoptosis after 48 hours of culture of leukaemia cells from 10 patients in medium or the presence of ECM

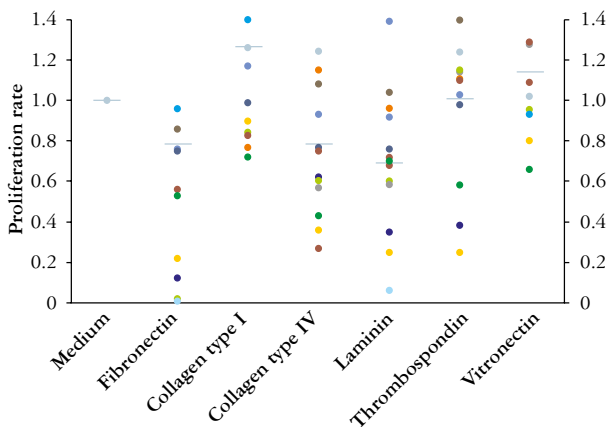
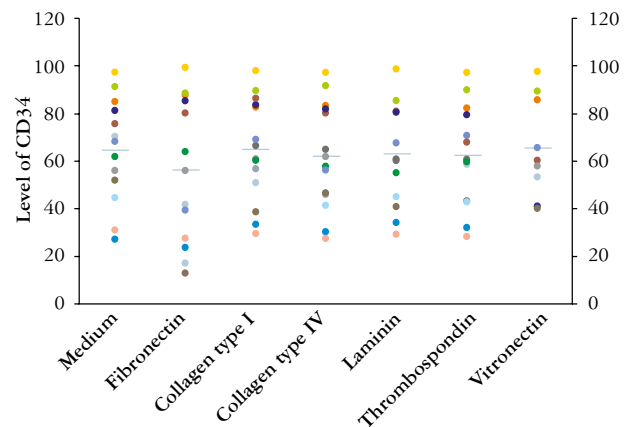
ECM PROTEIN	MEAN VALUE OF APOPTOTIC CELLS (%)	RANGE VALUE (%)	INDUCTION OF APOPTOSIS
Medium	28.47	8.0-30.8	0
Fibronectin	49.04	11.3-79.8	9/10
Collagen type I	32.32	12.5-64.7	7/10
Collagen type IV	30.11	13.9-58.1	8/10
Laminin	29.25	9.7-57.5	7/10
Thrombospondin	30.02	9.2-61.0	7/10
Vitronectin	30.19	6.7-56.3	8/10

*Results applicable for analysis were obtained only from 10 patients, so only these 10 cases were included.*

**Table III.** The influence of ECM proteins on proliferation of ALL cells in 72-hour culture

ECM PROTEIN	MEAN VALUE OF STIMULATION INDEX (S.I.)	RANGE OF S.I.	INHIBITION/STIMULATION OF PROLIFERATION
Medium	1.0	0	0
Fibronectin	0.743	0.1-2.17	13/4
Collagen type I	1.188	0.21-2.56	7/10
Collagen type IV	1.08	0.123-2.62	11/6
Laminin	0.866	0.26-1.7	14/3
Thrombospondin	0.899	0.25-1.64	8/9
Vitronectin	1.029	0.52-1.536	8/9

*The cells from one patient did not proliferate and the number after culture was too small for analysis. Consequently, only 17 cases were evaluated.*

**Fig. 1.** Proliferation rate of leukaemia cells in the presence of ECM**Fig. 2.** Level of CD34 on leukaemia cells in the presence of ECM

were excluded). The lack of influence of ECM proteins on CD34 expression was noted in eight cases, in the remaining six cases only fibronectin modified CD34 expression, showing a decrease in the percentage of leukaemia cells and the mean value of fluorescence. Interestingly, CD34 expression was slightly decreased after co-culture with thrombospondin in two cases (Fig. 2).

## Conclusions

From the studied ECM proteins, only fibronectin, collagen type IV, and laminin showed a tendency towards inhibition of lymphoblastic leukaemia cell proliferation. The influence of the remaining ECM proteins on proliferation and apoptosis of leukaemia cells did not show a unique regulatory tendency.

## Discussion

The results of this study suggest the regulatory effect of fibronectin, collagens, and laminin from ECM proteins on acute lymphoblastic leukaemia cells being due to leukaemic progenitors of B lymphocytes. The effect of collagens, especially collagen type IV, on

myeloid leukaemia cells was shown [4, 5]; the influence of collagen type IV on lymphoid line ontogeny is not known. The general idea about the regulatory role of ECM was discussed in association with other problems of the bone marrow environment and the importance of this environment for haematopoietic stem cell transplantation and reconstitution of cell lines [3]. The latest studies were directed towards the structure and function of bone marrow niches; osteoblastic and vascular are more concentrated on local production and release of soluble cytokines, growth factors, and other regulatory molecules than on circulation of active molecules reacting in the whole bone marrow space [6].

The results of culture of lymphoblastic leukaemia cells in the presence of ECM proteins showed fibronectin as the most active protein, followed by collagen type I and IV. Fibronectin is implicated in many different cell processes, and not only within bone marrow. There are some data indicating the association between molecular conformation and the role of fibronectin in regulation e.g. wound healing, cell adhesion, and migration. Fibronectin is important for the process of collagen matrix deposition. In our culture, fibronectin and collagen were used as single factors, but if both

molecules are interacting, the effect of fibronectin and collagen in bone marrow may be stronger based on synergy. Moreover, our results demonstrating inhibitory activity of fibronectin on proliferation of leukaemia cells might support the fibronectin role in growth factor signalling function by binding the present growth factors to adherent cells [7]. Taken together, it may be that the effect of stimulation of maturation of cells is based on presentation of growth factors and inhibition of precursor proliferation.

Collagens inhibitory activity on immune cells is mediated by specific surface receptors – LAIR (leukocyte-associated Ig-like receptor) expressed on the surface of immune cells. The role of this receptor is binding collagen and conveying regulatory signals following this binding. The expression of LAIR on stages of myeloid cell ontogeny is inversely associated with more mature stages of myeloid cell differentiation up to the absence of LAIR on mature neutrophils present in bone marrow and in peripheral blood [4]. Collagen type IV, especially in remodelled form, is a potent activator of DDR1 (discoidin domain receptor-1) mediating and modifying both migration and adhesion of acute myeloid leukaemia cells [5]. The inhibitory effect of collagens present in culture on lymphoblastic leukaemia cells may suggest its presence on LAIR on B-cell precursors. The inhibitory effect on proliferation was noted when laminin was present in a medium of lymphoblastic leukaemia cell culture. This effect is similar to the results of a haematopoietic stem cell study for a short-term (24 hours) culture in the presence of prepared matrix, when stimulation of proliferation by laminin was lowest and almost absent compared to collagens and fibronectin [2].

Summarising, our results support data indicating the important role of ECM proteins in regulation of haematopoiesis, including myeloid line and B-cell line ontogeny.

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*The authors declare no conflict of interest.*

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