ANTIAPOPTOTIC PROTEINS MCL-1 AND BCL-2 AS WELL AS GROWTH FACTORS FGF AND VEGF INFLUENCE SURVIVAL OF PERIPHERAL BLOOD AND BONE MARROW CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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ANTIAPOPTOTSKI PROTEINI MCL-1 I BCL-2 KAO I VASKULARNI ENDOTELNI FAKTOR RASTA (VEGF) I FIBROBLASTNI FAKTOR RASTA (FGF) UTIČU NA PREŽIVLJAVANJE ĆELIJA HRONIČNE LIMFOCITNE LEUKEMIJE U PERIFERNOJ KRVI I KOSTNOJ SRŽI

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ABSTRACT

Apoptosis inhibition in chronic lymphocytic leukemia (CLL) is one of the most important mechanism in the disease onset, progression and therapy response and is dependent of interaction with different microenvironments.

Aim of our paper is to determine expression of antiapoptoic proteins mcl-1 and bcl-2 in CLL cells isolated from two different compartments (peripheral blood and bone marrow) and its relation to percent of apoptotic cells and concentration of growth factors (FGF and VEGF).

Our results showed that peripheral blood CLL lymphocytes have lower apoptotic rate then those isolated from bone marrow, though bone marrow CLL lymphocytes express higher levels of antipoptotic proteins bcl-2 and mcl-1. In bone marrow FGF concentration is 10-fold higher then in patients plasma but has an limited impact on mcl-1 expression. In contrary, VEGF concentration is higher in peripheral blood and corelate with percent of apoptotic cells and mcl-1 expression in this compartment.

CLL cells derived from two different microenvironmets acts differently when tested for apoptosis „ex vivo". In peripheral blood apoptosis is strongly connected with expression of antiapoptoic proteins (mcl-1 and bcl-2) and growth factors, but not in bone marrow.

Keywords: *CLL, apoptosis, mcl-1, VEGF, FGF*

SAŽETAK

Inhibicija apoptoze u hroničnoj limfocitnoj leukemiji (HLL) predstavlja jedan od najvažnijih mehanizama kako nastanka bolesti, tako i progresije ali i odgovora na primenjivanu terapiju i zavisi od interakcije malignog limfocita sa različitim mikrosredinama.

Cilj našeg rada je odrediti ekspresiju antiapoptotskih proteina mcl-1 i bcl-2 u HLL limfocitima izlovanim iz dve različite mikrosredine (periferne krvi i kostne srži) i njihovu povezanost sa procentom apopotičnih limfocita kao i koncentracijom faktora rasta (VEGF i FGF).

Naši rezultati su pokazali da HLL limfociti izolovani iz periferne krvi imaju manji procenat apoptoze nego oni izolovani iz kostne srži, iako im je ekspresija antiapopotskih proteina bcl-2 i mcl-1 niža. U mikrosredini kostne srži koncentracija FGF je 10 puta veća nego u plazmi pacijenata, ali je njen uticaj na ekspresiju mcl-1 minimalan. Sa druge strane koncentracija VEGF je veća u perifernoj krvi i korelira sa procentom apoptotskih limfocita kao i ekspresijom mcl-1 u HLL limfocitima izolovanim iz periferne krvi.

Limfociti hronične limfocitne leukemije izlovani iz dve različite mikrosredine pokazuju značajnu razliku u procentu „ex vivo" testirane apoptoze. U perifernoj krvi procenat apoptotskih ćelija snažno je povezan sa ekspresijom antiapopototskih proteina (mcl-1 i bcl-2) kao i koncentracijom faktora rasta, dok u kostnoj srži ove povezanosti nema.

Ključne reči: *HLL, apoptoza, mcl-1, VEGF, FGF*

ABBREVIATIONS

Bcl-2 - B cell lymphoma 2 protein
mcl-1- myeloid leukemia cell 1 protein
VEGF - vascular endothel growth factor
FGF - fibroblast growth factor
CLL - chronic lymphocytic leukemia

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INTRODUCTION

Apoptosis inhibition in chronic lymphocytic leukemia (CLL) cells stands as one of the most important mechanisms in the disease onset, but as well as in progression and therapy response. Multiple mechanisms of CLL cells apoptosis resistance have been discovered and described so far, but most important is definitely intrinsic pathway represented through bcl-2 family members (bcl-2, mcl-1, bcl-XL, Bax, Bad) (1). These antiapoptotic pathways are potent and keeps the cells long living. The bcl-2 protein is important for maintenance of B lymphocyte population in adults (long living memory cells) in normal hematopoesis while mcl-1 predominantely follows hematopoetic stem cell and B cell differentiation process (2 - 4). In CLL cells concentrations of these proteins are elevated due to enhanced syntesis, but also their reduced clevage. It is mostly due to hypomethylation of the promoter bcl-2 region as well as lack of microRNA 15, 16 and 29, which leads to accumulation of bcl-2 and mcl-1 in the CLL cells, resulting in the prolonged survival (1,5). Yet, when cultivated in vitro in monocultures, CLL cells undergo apoptosis in the higher percent than healthy B lymphocytes, which imposes a conclusion that prolonged survival is not characteristic od CLL cell itself, but is a product of interaction of CLL cell with protective microenvironment(6). The bone marrow and lymph node are two most important microenvirontems for proliferation, apoptosis inhibition and drug resistance of CLL cells. Predominantly stromal „nurse like" cells, but also endothelial cells in these microenvironments, through cell-cell interaction and soluble molecules modulate CLL cells in their apoptotic and proliferation signals. Pathogenesis of CLL is also impacted by angiogenesis in these protective microenvironments. Vascular endothelial growth factor (VEGF) is pro-angiogenic factor with multiple roles proved in CLL. Its stimultion of angiogenesis is associated with an advanced stage of the disease, resistance to apoptosis and cell motility. (7,8,9). Fibroblast growth factor (FGF) in tumors promotes angiogenesis, has important role in proliferation of stromal cells and thus modify microenviroment (10,11).

Aim of our paper is to determine expression of antiapoptoic proteins mcl-1 and bcl-2 in CLL cells isolated from two different compartments (peripheral blood and bone marrow) and its relation to percent of apoptotic cells and concentration of growth factors (FGF and VEGF) in these microenvironments.

PATIENTS AND METHODS

Study population and sample collection

In our study we evaluated 60 samples (30 peripheral blood and 30 matching bone marrow samples) from 30 patients diagnosed with chronic lymphocytic leukemia. Patients were at least 6 months without any chemotherapy and not suffering from other acute and chronic condition which could impact tested parameters. The study was approved by the Ethical Committee of the Clinical Center Kragujevac. All patients gave their written informed consent according to the Declaration of Helsinki. Democraphic data of our patients group refers to typical CLL patients, average age 67 years (53 – 87) with male predomination (22 males and 8 women). For study purpose we collected 5ml of peripheral blood and 4ml of bone marrow aspirate from each patient. Native samples were used for determination of expression of mcl-1 and bcl-2 in the CLL cells, while from the rest we isolated mononuclear cells and plasma and bone marrow supernatant. Plasma and bone marrow supernatant were quickly frozen to -70C and collected for determination of VEGF and FGF concentration. The mononuclear cells were isolated using comerrcial gradient LymphoPrep and used for the apoptosis detection.

Detection of antiapoptotic proteins

Peripheral blood and bone marrow aspirate samples of patients were analyzed using 5-color flow cytometry. Analyzing sample was prepared using 50 µl of whole blood, incubated 15min on room temperature with surface markers, and then dyed for intracellular markers (mcl-1 and bcl-2) using commercial intarcellular kit *IntraPrep* (*Beckman Coulter 2389*). Antibody sources were as follows: CD19 fitc Beckman Coulter (Cat.No A07768), CD5 PE-Cy7 Beckman Coulter (Cat.No A21690), *bcl-2* pe (*Invitrogen MHBCL04*), *mcl-1 8C6D4B1* (*Abcam ab31948*) and *Goat anti mouse IgG pe* (*Abcam ab97041*). Cells were analysed in the CD19/CD5 gate and expression was determined as postive/negative and low/high according to the isotypic control and MFI values. Samples were analysed on BC FC 500.

Growth factors mesaurments

Concentration of VEGF and FGF in plasma and bone marrow supernatant were determined using a commercial flowcytometric kit Human VEGF-A Flowcitomix Simplex Kit (e-bioscience, BMS80277FF), Human FGF-2 Flowcitomix Simplex Kit (e-bioscience, BMS82074FF) on a FC500 Beckman Coulter Flow Cytometer according to the manufacturer's instructions. Collected data were analyzed using FlowCytomix™ Pro 3.0 Software.

Detection of apoptotic lymphocytes

Apoptotic cells were detected on flow cytometer on isolated mononulclear cells, dyed using commercial kit Annexin V-FITC/7-AAD kit (BC IM3614) according to the manufacturer's instructions. Finally, cells were analyzed on an FC500 Beckman Coulter flow cytometer to the number of 20000 events, gating lymphocytes and CD19+ cells. In the analysis Annexin V negative and 7-AAD negative cells are viable, Annexin V positive and 7-AAD negative cells are in the early stages of apoptosis, Annexin V positive and 7-AAD

Table 1. Median values of tested parametrs in peripheral blood and bone marrow

	Peripheral blood	Bone marrow	p value
% of CD19+ cells in early apoptosis	0,03 (0,0001-0,22)	0,155 (0,025-0,38)	**0,068**
% of CD19+ cells in late apoptosis	0,001(0,0001-0,18)	0,13 (0,03-0,58)	**0,005**
% of cells with high bcl-2 expression	12,29 (0,71-52,74)	1,36 (0,15-66,36)	0,323
% of cells with mcl-1 expression	52,06 (20,9-65,8)	37,22 (15,7 - 58,39)	0,13
VEGF (pg/ml)	24,44 (21,16-58,05)	21,69 (17,14 - 25,10)	0,791
FGF (pg/ml)	103,75 (86,67-119,51)	1106,24 (299,64 - 2360,87)	**<0,005**

positive cells are in late stages of apoptosis, while Annexin V negative and 7-AAD positive cells are necrotic. The percentages of early and late apoptotic cells, as well as necrotic cells were determined using CXP Cytometer software.

RESULTS

We determined percent of apoptotic CD19+ cells in peripheral blood (PB) and bone marrow (BM), as well as expression of bcl-2 and mcl-1 in CD19+/CD5+ CLL cells and concentration of VEGF and FGF in both patients plasma and bone marrow supernatnt. These results comparing values from both compartments were presented in the table 1.

Peripheral blood lymphocytes show lower percentage of cells in both early and late apoptosis when tested „ex vivo" compared to the lymphocytes isolated from bone marrow, though the cells does not have significant difference in expression of antiapoptotic proteins (bcl-2 and mcl-1). (Figures 1 and 2). Concentration of VEGF in PB and BM does not differ significantly, while concentration of FGF in BM is 10-fold higher then in PB.

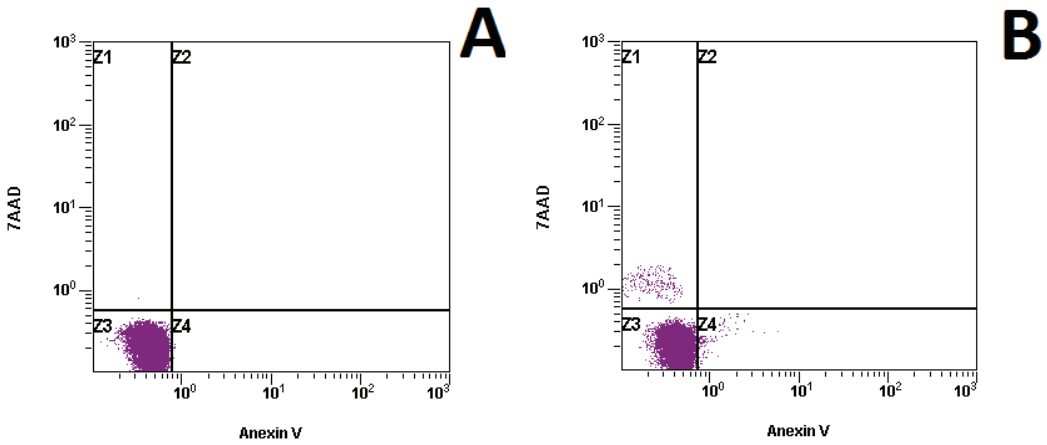

Figure 1. Dot plots showing apoptotic rate of lymphocys derived from peripheral blood (A) and bone marrow (B)

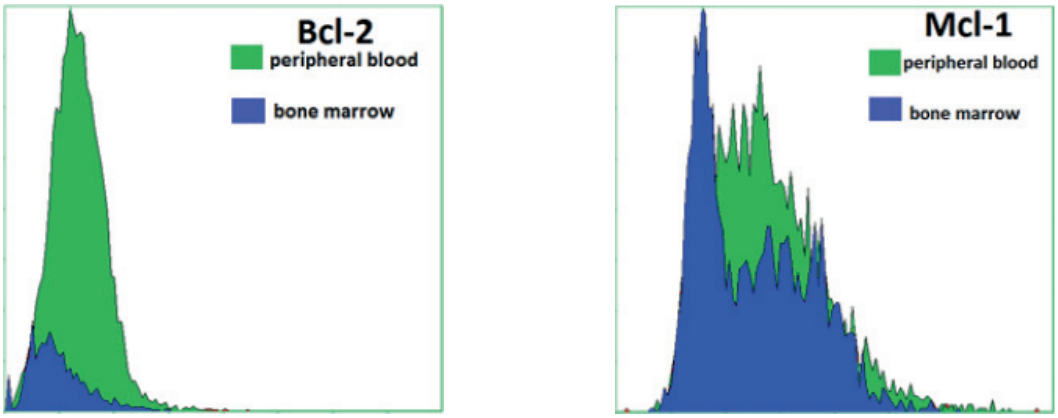

Figure 2. Overlay histograms showing expression of bcl-2 (left) and mcl-1 (right) in the CLL lymphocytes derived from peripheral blood and bone marrow

Expression of mcl-1 in bone marrow derived CLL cells correlate positivly with percent of both early and late apoptotic cells in bone marrow r^2= 0,453 p= 0,016, while it does not correlate with percent of apoptotic cells in peripheral blood r^2=0,191 p=0,313.

Early and late apoptosis in bone marrow are in strong positive correlation with concentration of VEGF r^2= 0,704 p< 0,0001 and r^2=0,554 p=0,002 respectivly. Also there is a strong positive correlation between FGF concentration in bone marrow with CLL cells early apoptosis in bone marrow r^2=0,611 p=0,001, as well as with CLL cells late apoptosis in bone marrow r^2= 0,620 p<0,0001

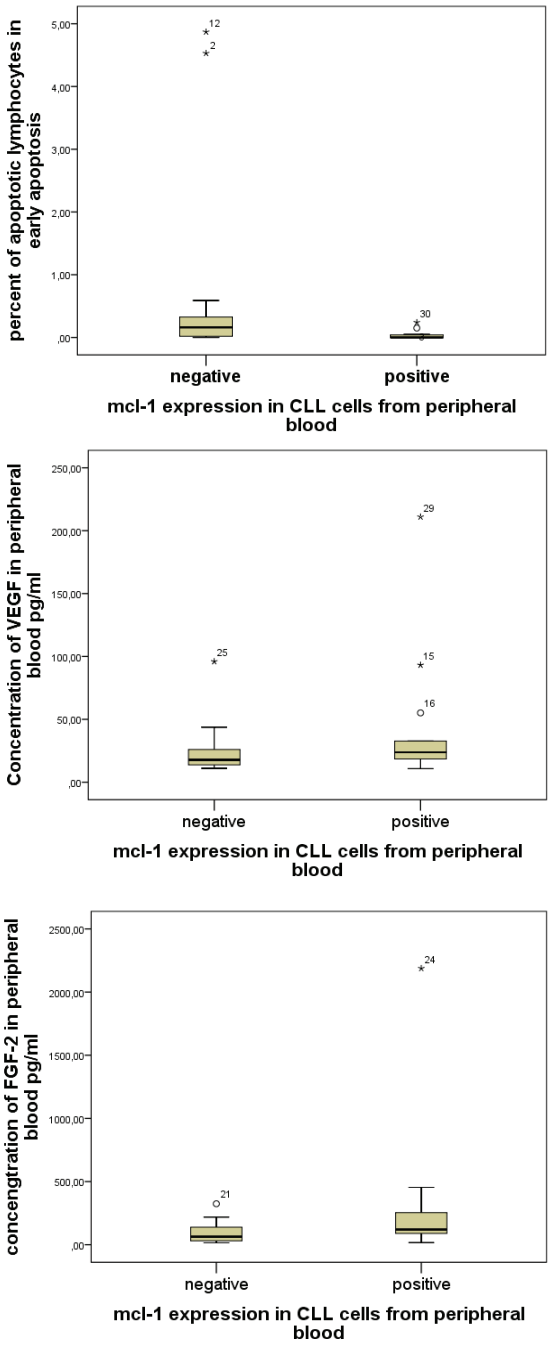

Figure 3. Apoptosis of CLL cells, VEGF and FGF concentration in peripheral blood depending on positivity of CLL cells to mcl-1

Results showed that when CLL cells in peripheral blood exprime mcl-1 molecule we have higher concentration of VEGF in peripheral blood (24,8±21,14 versus 42,98±52,73 pg/ml, p=0,077), higher concentration of FGF in peripheral blood (98,66±88,1 versus 309,4±552,84 pg/ml, p= 0,031), and lower early apoptotic rate (0,73±1,56% versus 0,038±0,07%, p=0,006) (Figure 3). There is no statisticaly significant difference in late apoptotic rate or bcl-2 expression in these cells.

There is no statisticaly significant difference in bcl-2 molecule expression, rate of early and late apoptosis, or concentration of VEGF and FGF in bone marrow supernatant between the mcl-1 positive and negative CLL cells.

Though there has been detected significant medium to strong correlation between mcl-1 expression in peripheral blood and bone marrow samples, r^2= 0,524 p=0,004.

DISCUSSION

Microenviroment studies in CLL has been in progress in the last decades thanks to which our view to CLL patients and its treatment has been changed. Protective role of microenvironments are the reasons for this disease to have so many faces.

CLL cells undoubtly have lower apoptotic rate comparred to lymphocytes isolated from peripheral blood of healthy subjects (12). But unlike most other studies that promote better survival of CLL cells in the protective microenvironments (bone marrow and lymph nodes), our results show that bone marrow derived CLL lymphocytes have higher apoptotic rate then those isolated from peripheral blood. Those results indirectly points that protective microenvironment acts in site, but does not make a permanent change in CLL cell which make it more prone to apoptosis when taken away from microenviromnent signals. So Siekluska at al, and Witkowska et al showed that there is a greater apoptotic rate when CLL cells are tested „ex vivo", just as our work showed, and that it can be correlated with the disease progression (13,14).

Main mechanism of CLL cell survival is apoptosis inhibition. All known apoptotic pathways were investigated in CLL, with conclusion that most impact deffinitely belongs to bcl-2 family proteins (15). This mechanism is widely used in the field of introducing novel therapies (15). Mcl-1 protein together with Act and in lot less percent other members of bcl-2 family (bcl-2 and bcl-XL) are the main regulators of apoptosis resistance in CLL cells. (16) Mcl-1 can be upregulated by several mechanisms, most of which include B cell receptor activation, which suggest that extrinsic as well as intrinsic factors cooperate in disease onset, progression and therapy resistance (16). All these mechanisms depends on stromal-mediated increase in RNA synthesis which could be a result of activation of transcription factors such as c-myc and NFκB (17). Since mcl-1 is an early responder and a fast turnover molecule it is amplified more then other proteins. Being a short living

molecule, there is a constant equlibrium between stabilisation and degradation of mcl-1, but in CLL cell it outweigh in favor of mcl-1 accumulation (17). Our results show that peripheral blood CLL lymphocytes have higher expression of mcl-1 then those derived from bone marrow, which suggest that mcl-1 expression is more important in antiapoptoic effect in peripheral blood.

Answer to the question of origin of VEGF in CLL is not simple. While Kay et al, and Chen et al came to the conclusion that CLL cells themselves secrete VEGF, Gehrke et al proved that only stromal cell derived VEGF have protective effect on CLL cells (18 - 20). Our results showing that plasma concentration of VEGF is higher that in bone marrow supernatant, give advantage to the fact that CLL cells themselves produce VEGF. Our results also showed that *ex vivo* apoptosis is accelerated in bone marrow comparing to peripheral blood CLL cells. CLL cells have receptors for VEGF on the cell membrane, expriming both VEGFR1 and VEGF R2 (13). When binded to these receptors VEGF increase apoptotic resistance of cells interact with STAT1 and STAT3 which ends up in upregulation of XIAP and mcl-1 expression (8,21,22). Also VEGFR expression in CLL cells are stimulated by endothelin -1 receptor signaling through hypoxia inducible factor 1, suggesting the impact of endotel cells in CLL survival (23, 24). Our results show greater concentration of VEGF in peripheral blood then in bone marrow, as well as a strong connection between mcl-1 expression and concentration of VEGF in peripheral blood, which also suggest that VEGF is secreted in peripheral blood and not overflow from bone marrow. Also VEGF mRNA levels in CLL cells are in strong positive correlation with mcl-1 expression (25).

Basic FGF (FGF-2) has an important role in early hematopoesis in proliferation of hemangioblasts, common progenitor cells for hematopoetic and endotel cells (26). For those reasons FGF is mostly investigated for its effect on endothelial cells. Concerning survival of endothelial cells in culture, adding FGF promote longer survival in the early phase because of the better cell adherence, and latter due to activation of MAP kinase, FGF promotes upregulation of antiapoptotic proteins (27). Out of bcl-2 protein family, FGF is proven to upregulate only bcl-2 protein (27). In our work we have proved that there is a relatively high concetration of FGF in patients plasma, but not significantly different from those in healthy subjects, but even 10-folder higer in bone marrow supernatant. (12) Several investigator proved that FGF is mostly secreted by stromal and endotel cells, and that its major role is in proliferation of bone marrow stromal cells (28). Also its concentration in CLL patients is higher than in the healthy control subjects (26,29,30). Similary to its effect in endothelial cells it is proven that FGF have a protective role in CLL cell survival, and that it has an impact on bcl-2 upregulation, but no effect on levels of mcl-1. (30). Beside longer survival, higher levels of FGF and presence of upregulated FGF-2 receptors on CLL cells are connected with resistance to standard fludarabine therapy (28). Our results show that levels of FGF in bone marrow strongly correlates with percent of apoptotic CLL cells, as well as with level of mcl-1, and not level of bcl-2 which is not in concordance with other results. Our results indirectly point to conection between FGF level in bone marrow and mcl-1 expression, which could be possible mechanism of FGF effect on CLL cell. Though the FGF effect should be further investigated towards its connection to mcl-1 molecule.

CONCLUSION

CLL cells derived from two different microenvironmets show different rate of apoptosis „ex vivo". In peripheral blood CLL cells apoptosis is strongly connected with expression of antiapoptoic proteins (mcl-1 and bcl-2) and growth factors, but not in bone marrow. However, these mechanisms are just a part of complex regulatory system.

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There is no conflict of interest concerned in this paper.

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