KONCENTRACIJA CITOKINA (IL-4, IL-12 I IFNγ) U PLAZMI I NJENA POVEZANOST SA APOPTOZOM LIMFOCITA PACIJENATA OBOLELIH OD HRONIČNE LIMFOCITNE LEUKEMIJE

ORIGINALNI RAD

ORIGINAL ARTICLE

CONCENTRATION OF CYTOKINES (IL-4, IL-12, IFNγ) IN THE BLOOD PLASMA AND ITS ASSOCIATION WITH LYMPHOCYTE APOPTOSIS IN PATIENTS SUFFERING FROM CHRONIC LYMPHOCYTIC LEUKEMIA

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SAŽETAK

Uvod: Hronična limfocitna leukemija (HLL) predstavlja malignu bolest hematopoetskog tkiva i najčešća je leukemija odraslih. Godinama je dominirao stav da je u patogenezi hronične limfocitne leukemije osnovni mehanizam nastanka i progresije bolesti poremećaj apoptoze i produženo preživljavanje malignih limfocita.

Cilj rada: Cilj našeg istraživanja je da se utvrdi povezanost procenta apoptotskih limfocita u perifernoj krvi pacijenta obolelih od hronične limfocitne leukemije sa koncentracijom antiapoptotskih citokina (IL-4, IL-12, IFNγ) u plazmi pacijenata.

Materijal i metode: U istraživanje je uključeno 29 pacijenata obolelih od hronične limfocitne leukemije (21 muškarac i 8 žena) koji u prethodnih 6 meseci nisu bili na hemioterapijskom režimu. Kod svih pacijenata su određivani klinički parametri (klinički stadijum bolesti, prisustvo limfadenopatije, splenomegalije), biohemijski parametri (LDH) i tip i procenat infiltracije koštane srži. Iz periferne krvi pacijenata određen je procenat apoptotskih limfocita i koncentracija citokina (IL-4, IL-12, IFNγ) u plazmi pacijenata.

Rezultati: U ispitivanoj grupi pacijenta su detektovane vrednosti plazmatske koncentracije IL-4 = 121,42 pg/ml (62,44 – 180,40), IL-12 = 7,62 pg/ml (4,36 – 10,87), IFNγ = 31,45 pg/ml (18,35 – 44,56). Među ćelijskom populacijom je detektovano ispod 1% apoptotskih ćelija, u rasponu od 0,03% do maksimalno 0,84%. Rezultati pokazuju da nema korelacije između koncentracije citokina i procenta apoptotskih limfocita u perifernoj krvi pacijenata, ali da koncentracija Il-12 pozitivno korelira sa stadijumom bolesti, kao i procentom infiltracije koštane srži malignim limfocitima. ($p < 0,001$ odnosno $p = 0,028$).

Zaključak: Plazmatske koncentracije IL-4, IL-12, IFNγ nisu u korelaciji sa procentom apoptotskih limfocita periferne krvi pacijenata obolelih od hronične limfocitne leukemije. Interleukin-12 ipak pokazuje pozitivnu korelaciju kod uznapredovale bolesti.

Ključne reči: hronična limfocitna leukemija, apoptoza, citokini

ABSTRACT

Introduction: Chronic lymphocytic leukemia (CLL) is a malignant disease of hematopoietic tissue and is the most common leukemia in adults. For years, the dominant view was that in the pathogenesis of chronic lymphocytic leukemia, the basic mechanism of the origin and progression of the disease is disruption in apoptosis and prolonged survival of malignant lymphocytes.

Aim: The aim of our study is to determine the association between the percentage of apoptotic lymphocytes in the peripheral blood of patients suffering from chronic lymphocytic leukemia and the concentration of antiapoptotic cytokines (IL-4, IL-12, IFNγ) in the patients' blood plasma.

Materials and methods: The study included 29 patients suffering from chronic lymphocytic leukemia (21 men and 8 women) who had not been on a chemotherapy regimen in the preceding 6 months. Clinical parameters (clinical stage of disease, presence of lymphadenopathy, splenomegaly), biochemical parameters (LDH), and the type and percentage of bone marrow infiltration were determined in all patients. The percentage of apoptotic lymphocytes and the concentration of cytokines (IL-4, IL-12, IFNγ) in the patients' plasma were determined from the patients' peripheral blood.

Results: In the studied group of patients, plasma concentration values of IL-4 = 121.42 pg/ml (62.44 – 180.40), IL-12 = 7.62 pg/ml (4.36 – 10.87), IFNγ = 31.45 pg/ml (18.35 – 44.56) were detected. In the cell population, less than 1% of apoptotic cells were detected, ranging from 0.03% to a maximum of 0.84%. The results show no correlation between the concentration of cytokines and the percentage of apoptotic lymphocytes in the patients' peripheral blood, however, they show that Il-12 concentration positively correlates with the stage of the disease, as well as with the percentage of bone marrow infiltration by malignant lymphocytes ($p < 0.001$ or $p = 0.028$).

Conclusion: Plasma concentrations of IL-4, IL-12, IFNγ are not correlated with the percentage of apoptotic lymphocytes in the peripheral blood of patients with chronic lymphocytic leukemia. Interleukin-12 nevertheless shows a positive correlation in advanced disease.

Keywords: chronic lymphocytic leukemia, apoptosis, cytokines

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Primljeno • *Received*: November 27, 2023; **Revidirano • *Revised*:** December 4, 2023; **Prihvaćeno • *Accepted*:** December 5, 2023; ***Online first*:** December 25, 2023

DOI: 10.5937/smclk4-47931

UVOD

Hronična limfocitna leukemija (HLL) predstavlja malignu bolest hematopoetskog tkiva koja nastaje proliferacijom i akumulacijom klona, malih, naizgled zrelih, imunološki izmenjenih limfocita u perifernoj krvi, koštanoj srži, limfnim nodusima, slezini i drugim organima, i ovo je najčešća leukemija kod odraslih pacijenata [1].

Godinama je dominirao stav da je u patogenezi hronične limfocitne leukemije osnovni mehanizam nastanka i progresije bolesti poremećaj apoptoze i produženo preživljavanje malignih limfocita [1]. Danas se zna da je produženo preživljavanje limfocita bitan parametar u patogenezi bolesti, ali da napredovanje bolesti takođe zavisi i od povećane proliferacije malignog klona [2,3]. Produženo preživljavanje malignih limfocita delom je posledica genetskih promena unutar samih ćelija, a delom i spoljašnjih uticaja, kao što su ćelijsko-ćelijske interakcije u ciljnim tkivima (koštana srž, limfni čvorovi) i cirkulišući citokini koji kao jednu od uloga imaju i inhibiciju apoptoze [3 – 6].

Cilj našeg istraživanja je bio da se utvrdi povezanost procenta apoptotskih limfocita u perifernoj krvi pacijenata obolelih od hronične limfocitne leukemije sa ekspresijom *Bcl-2* u malignim limfocitima, kao i koncentracijom antiapoptotskih citokina (IL-4, IL-12, IFNγ) u plazmi pacijenata. Takođe, cilj je bio i da se ispitivani parametri uporede sa dostupnim kliničkim i laboratorijski parametrima bolesti.

MATERIJALI I METODE

Istraživanje je sprovedeno na Klinici za hematologiju Univerzitetskog kliničkog centra Kragujevac, kao prospektivna studija, u periodu od oktobra 2017. godine do februara 2018. godine. U istraživanje je uključeno 29 pacijenata (21 muškog i 8 ženskog pola) obolelih od hronične limfocitne leukemije, koji u prethodnih 6 meseci nisu primali hemioterapiju. Kod svih pacijenata su određivani: klinički parametri (klinički stadijum bolesti, prisustvo limfadenopatije, splenomegalije), biohemijski parametri (laktat dehidrogeneza – LDH), tip i procenat infiltracije koštane srži (nodularni, intersticijalni i difuzni), kao i parametri krvne slike. Kod svih pacijenata je u jednom aktu uzorkovana periferna krv u količini od 5 ml sa EDTA antikoagulansom.

U perifernoj krvi je određivan procenat apoptotskih limfocita, a iz plazme pacijenata je metodom *microbeads* tehnike određivana koncentracija IL-4, IL-12, i IFNγ, na protočnom citometru *Beckman Coulter FC500*.

Ćelije pune krvi su centrifugirane na 3.000 obrtaja tokom 10 minuta, nakon čega je beli tepih prebačen u epruvetu sa 2 ml *Lymphoprep*-a i centrifugiran 22 minuta na 3.000 obrtaja. Posle izolacije, ćelije su oprane tri puta u *PBS*-u i resuspendovane u ledeno hladnom ve-

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a malignant disease of hematopoietic tissue that results from the proliferation and accumulation of clones, small, seeminlgy mature, immunologically altered lymphocytes in the peripheral blood, bone marrow, lymph nodes, spleen, and other organs, and this is the most common leukemia in adult patients [1].

For years, the predominant view was that in the pathogenesis of chronic lymphocytic leukemia, the basic mechanism of the onset and progression of the disease lay in the disruption of apoptosis and in the prolonged survival of malignant lymphocytes [1]. Nowdays, it is known that the prolonged survival of lymphocytes is an important parameter in the pathogenesis of the disease, but that the progression of the disease also depends on the increased proliferation of the malignant clone [2,3]. Prolonged survival of malignant lymphocytes is partly due to genetic changes within the cells themselves, and partly to external influences, such as cell-cell interactions in target tissues (bone marrow, lymph nodes) and circulating cytokines, which also play a role in inhibiting apoptosis [3 – 6].

The aim of our study was to determine the association between the percentage of apoptotic lymphocytes in the peripheral blood of patients suffering from chronic lymphocytic leukemia and the expression of Bcl-2 in malignant lymphocytes, as well as the concentration of antiapoptotic cytokines (IL-4, IL-12, IFNγ) in the patients' plasma. Also, the aim was to compare the analyzed parameters with the available clinical and laboratory parameters of the disease.

MATERIALS AND METHODS

The study was conducted at the Clinic for Hematology of the University Clinical Center Kragujevac, as a prospective study, in the period between October 2017 and February 2018. The study included 29 patients (21 male and 8 female patients) suffering from chronic lymphocytic leukemia, who had not received chemotherapy in the preceding 6 months. Clinical parameters (clinical stage of the disease, presence of lymphadenopathy, splenomegaly), biochemical parameters (lactate dehydrogenase - LDH), type and percentage of bone marrow infiltration (nodular, interstitial and diffuse), as well as blood count parameters were determined in all patients. Five milliliters of peripheral blood, with ETDA anticoagulant, were sampled at once from all patients.

The percentage of apoptotic lymphocytes was determined in the peripheral blood, and the concentration of IL-4, IL-12, and IFNγ was determined from the patients' blood plasma using the microbeads technique, on a Beckman Coulter FC500 Flow Cytometer.

zujućem puferu do finalne koncentracije od 1.000.000 ćelija/ml. Nakon toga, u 100 µl radnog rastvora dodato je 10 µl FITC obeleženog Aneksina V (engl. *Annexin V*) i 20 µl 7-AAD, a potom inkubirano u mraku 15 minuta. Nakon inkubacije, sadržaj je resuspendovan u 400 µl vezujućeg rastvora i analiziran na protočnom citometru do 20.000 događaja, na populaciji limfocita koja je određena na *FS/SS* dijagramu. Smatra se da su *Annexin V* (-); 7-AAD (-) ćelije vijabilne, da su *Annexin V* (+); 7-AAD (-) ćelije u ranoj fazi apoptoze, da se *Annexin V* (+); 7-AAD (+) ćelije nalaze u kasnoj fazi apoptoze, dok su *Annexin V* (-); 7-AAD (+) ćelije nekrotične. Procenat rane i kasne apoptoze, kao i nekroze, određivan je korišćenjem *CXP Cytometer* softvera. Statistička obrada podataka je urađena je uz pomoć komercijalnog statističkog programa – *SPSS*, verzija 20.

REZULTATI

Našu ispitivanu populaciju je činilo 70% pacijenata muškog pola i 30% pacijenata ženskog pola, prosečne starosti 66,9 godina (u rasponu od 53 – 87 godina). Što se tiče kliničkih parametara, 41% pacijenata je bilo u nižem kliničkom stadijumu bolesti (Rai 0 i 1), 28% je bilo u srednjem stadijumu (Rai 2), dok je 31% pacijenata bilo u uznapredovalom stadijumu bolesti (Rai 3 i 4). Pacijenti su većinom imali palpabilnu limfadenopatiju (oko 60%), dok je oko 43% pacijenata imalo palpabilnu slezinu. Što se infiltracije koštane srži tiče, ona je, po definiciji bolesti, bila prisutna kod svih pacijenata, ali je procenat infiltracije varirao od 25% – 95%. Prosečni broj leukocita kod pacijenata je bio 70,6 ± 47,1 x10^9/l, dok je koncentracija LDH bila 427,5 ± 216,5 U/l. Ostali ispitivani parametri su prikazani u Tabeli 1.

Iako je procenat ćelija u apoptozi bio u negativnoj korelaciji sa posmatranim parametrima (koncentracija IL-12, IFNγ i IL-4) nije bilo statističke značajnosti ($p > 0,05$). Pri analizi ćelija u pojedinačnim fazama apoptoze (rana apoptoza, kasna apoptoza i nekroza) takođe nije utvrđena statistička značajnost. Prilikom upoređivanja koncentracije citokina sa prisustvom limfadenopatije i uvećane slezine, takođe nije uočena korelacija. Ipak,

Whole blood cells were centrifuged at 3,000 rpm for 10 minutes, upon which the leukocyte precipitate was transferred to a test tube containing 2 ml of Lymphoprep and centrifuged for 22 minutes at 3,000 rpm. Upon isolation, cells were washed three times in PBS and resuspended in ice-cold binding buffer to a final concentration of 1,000,000 cells/ml. After that, 10 µl of FITC-labeled Annexin V and 20 µl of 7-AAD were added to100 µl of the working solution, and then incubated in the dark for 15 minutes. After incubation, the contents were resuspended in 400 µl of binding solution and analyzed on a flow cytometer at a maximum of up to 20,000 events, on a lymphocyte population determined on the FS/SS diagram. It is considered that Annexin V (-); 7-AAD (-) cells are viable, that Annexin V (+); 7-AAD (-) cells are in the early stage of apoptosis, that Annexin V (+); 7-AAD (+) cells are in the late stage of apoptosis, while Annexin V (-); 7-AAD (+) cells are necrotic cells. The percentage of early and late apoptosis, as well as necrosis, was determined using CXP Cytometer software. Statistical data processing was performed with commercial statistical software – SPSS, version 20.

RESULTS

Our study population consisted of 70% male patients and 30% female patients, of the average age of 66.9 years (range: 53 – 87 years). As to the clinical parameters, 41% of the patients had a lower clinical stage of the disease (Rai 0 and 1), 28% were in the intermediate stage (Rai 2), while 31% of the patients were in the advanced stage of disease (Rai 3 and 4). The majority of patients had palpable lymphadenopathy (about 60%), while the spleen was palpable in about 43% of patients. As far as bone marrow infiltration is concerned, it was, by virtue of the disease, present in all patients, but the percentage of infiltration varied from 25% – 95%. The average leukocyte count in patients was 70.6 ± 47.1 x 10^9/l, while the LDH concentration was 427.5 ± 216.5 U/l. Other tested parameters are presented in Table 1.

Although the percentage of cells in apoptosis was negatively correlated with the observed parameters

Tabela 1. Procenat apoptotskih limfocita u perifernoj krvi i koncentracija ispitivanih citokina

Table 1. The percentage of apoptotic lymphocytes in peripheral blood and the concentration of the analyzed cytokines

Parametar/ *Parameter*	**Srednja vrednost / *Mean value***
Procenat nekrotičnih limfocita / *Percentage of necrotic lymphocytes*	0.5 ± 0.5%
Procenat limfocita u ranoj apoptozi / *Percentage of lymphocytes in early apoptosis*	0.4 ± 0.3%
Procenat limfocita u kasnoj apoptozi / *Percentage of lymphocytes in early apoptosis*	1.0 ± 0.8%
Koncentracija IL-12 u plazmi / *Concentration of IL-12 in blood plasma*	70.6 ± 42.7 pg/ml
Koncentracija IFNγ u plazmi / *Concentration of IFNγ in blood plasma*	28.6 ± 16.4 pg/ml
Koncentracija IL-4 u plazmi / *Concentration of IL-4 in blood plasma*	121.4 ± 157.9 pg/ml

sva tri citokina pokazala su negativnu korelaciju sa procentom infiltracije koštane srži limfocitima (p = 0,028, p = 0,030, p = 0,042).

Što se tiče upoređivanja koncentracije citokina sa stadijumom bolesti pacijenata, IFNγ i IL-4 nisu pokazali statistički značajnu povezanost, dok je koncentracija Il-12 pokazala značajnu negativnu korelaciju sa stadijumom bolesti pacijenata (p < 0,01). Kod viših stadijuma bolesti pacijenata, detektovane su niže vrednosti koncentracije IL-12. Visok stepen pozitivne korelacije sa stadijumom bolesti pokazala je koncentracija LDH u serumu (p < 0,01).

DISKUSIJA

Studije koje su proučavale *in vitro* preživljavanje limfocita u kulturama kod pacijenata obolelih od hronične limfocitne leukemije, nedvosmisleno su pokazale da kultivisani maligni limfociti u monokulturi imaju viši stepen apoptoze od kultivisanih nemalignih B limfocita [7,8], što je doprinelo zaključku da inhibicija apoptoze limfocita hronične limfocitne leukemije nije urođeni mehanizam same ćelije, već je posledica interakcije malignih ćelija sa protektivnom mikrosredinom. Naše istraživanje je posmatralo perifernu krv kao jednu od mikrosredina u kojoj cirkulišu limfociti periferne krvi, i u njoj koncentraciju citokina (IL-12, IFNγ i IL-6), te njihovu povezanost sa procentom apoptotskih ćelija i napredovanjem bolesti.

Studije koje su se bavile efektom IL-12 *in vitro* pokazale su da IL-12 inhibira apoptozu ćelija hronične limfocitne leukemije i tako doprinosi napredovanju bolesti [9], ali da ovaj efekat nije dovoljno potentan kada se limfociti hronične limfocitne leukemije kultivišu samo sa IL-12, već svoj pun potencijal pokazuje u kombinaciji sa drugim citokinima [10]. Kao antiinflamatorni citokin, IL-12 pokazuje i antitumorsko dejstvo, pa njegova niža koncentracija može biti povezana sa napredovanjem bolesti [11].

Takođe, ovoj teoriji doprinose i novi radovi, koji navode da je kod pacijenata obolelih od psorijaznog artritisa lečenih ustekinumabom (inhibitorom IL-12) bilo veće učestalosti pojave hronične limfocitne leukemije. Među ovim studijama je i Rad Gediza i saradnika koji je u saglasnosti sa našim rezultatima, koji pokazuju da se niže koncentracije IL-12 javljaju kod pacijenata sa uznapredovalom bolešću [12]. Sa druge strane Parfienčik i saradnici su pokazali da je koncentracija IL-12 kao i IL-6 viša kod obolelih pacijenata, ali njihovo istraživanje je obuhvatalo samo pacijente u nižem stadijumu bolesti [13].

Što se tiče IL-4 i IFNγ, naši rezultati povezivanja koncentracije ovih citokina i parametara apoptoze i napredovanja bolesti nisu pokazali direktnu povezanost. Ovi citokini su ipak literaturno više ispitivani, i iako se

(concentrations of IL-12, IFNγ and IL-4), there was no statistical significance (p > 0.05). When analyzing cells in individual stages of apoptosis (early apoptosis, late apoptosis and necrosis) statistical significance was also not determined. When the concentration of cytokines was compared with the presence of lymphadenopathy and an enlarged spleen, no correlation was also observed. However, all three cytokines showed a negative correlation with the percentage of bone marrow infiltration by lymphocytes (p = 0.028, p = 0.030, p = 0.042).

With regards to the comparison of the cytokine concentration with the disease stage in patients, IFNγ and IL-4 did not show a statistically significant association, while the Il-12 concentration showed a significant negative correlation with the disease stage of patients (p < 0.01). In patients with more advanced disease stages, lower values of the concentration of IL-12 were detected. The concentration of LDH in the serum showed a high degree of positive correlation with the stage of disease (p < 0.01).

DISCUSSION

Studies analyzing in vitro survival of lymphocytes in cultures taken from patients with chronic lymphocytic leukemia, unequivocally showed that cultivated malignant lymphocytes in monoculture had a higher degree of apoptosis than cultivated non-malignant B lymphocytes [7,8], which led to the conclusion that inhibition of the apoptosis of lymphocytes in chronic lymphocytic leukemia is not an innate mechanism of the cell itself, but a consequence of the interaction of malignant cells with the protective microenvironment. Our study considered peripheral blood as one of the microenvironments wherein peripheral blood lymphocytes circulate, the concentration of cytokines (IL-12, IFNγ and IL-6) in peripheral blood, as well as the association of cytokines with the percentage of apoptotic cells and the progression of the disease.

Studies dealing with the in vitro effect of IL-12 showed that IL-12 inhibits the apoptosis of chronic lymphocytic leukemia cells and thus contributes to the progression of the disease [9], but that this effect is not potent enough when chronic lymphocytic leukemia lymphocytes are cultured only with IL-12, rather it shows its full potential in combination with other cytokines [10]. As an anti-inflammatory cytokine, IL-12 also exhibits an antitumor effect, therefore, its lower concentration may be associated with disease progression [11].

Recent studies also contribute to this theory, reporting that patients suffering from psoriatic arthritis who were treated with ustekinumab (IL-12 inhibitor) had a higher incidence of chronic lymphocytic leukemia. Amongst these studies is the one by Gediz et al., which is in agreement with our results showing that lower con-

literaturno same koncentracije u plazmi nisu pokazale kao statistički značajne, gustina receptora za IFNγ i IL-4, kako na samim leukemijskim limfocitima tako i na drugim imunim ćelijama, ukazuju da je imuni odgovor pomeren od Th1 prema Th2 odgovoru, (povećanje IL-4 a smanjenje IFNγ receptora), što otvara dovoljno mesta za postepeni razvoj hronične limfocitne leukemije [14,15].

ZAKLJUČAK

Plazmatske koncentracije IL-4, IL-12 i IFNγ nisu u korelaciji sa procentom apoptotskih limfocita periferne krvi pacijenata obolelih od hronične limfocitne leukemije, ali su u značajnoj negativnoj korelaciji sa infiltracijom koštane srži malignim limfocitima. Kao poseban parametar, IL-12 je u negativnoj korelaciji sa stadijumom bolesti, što ga izdvaja kao antiapoptotski citokin o kom treba razmišljati u patogenezi i toku hronične limfocitne leukemije i uvrstiti ga u dalja istraživanja u ovoj oblasti.

Sukob interesa: Nije prijavljen.

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centrations of IL-12 occur in patients with advanced disease [12]. On the other hand, Parfieńczyk et al. showed that the concentrations of IL-12 and of IL-6 were higher in patients suffering from CLL, but their study included only patients with lower stage of the disease [13].

Regarding IL-4 and IFNγ, our results linking the concentrations of these cytokines to parameters of apoptosis and disease progression did not show a direct association. These cytokines have, in fact, been studied to a greater extent in literature, and although the plasma concentrations themselves have not been shown to be statistically significant, the density of receptors for IFNγ and IL-4, both on the leukemic lymphocytes themselves as well as on other immune cells, indicate the shifting of the immune response from Th1 response to Th2 response, (increase in IL-4 and decrease in IFNγ receptors), which opens up enough room for gradual development of chronic lymphocytic leukemia [14,15].

CONCLUSION

Plasma concentrations of IL-4, IL-12 and IFNγ are not correlated with the percentage of apoptotic lymphocytes in the peripheral blood of patients with chronic lymphocytic leukemia, but they are significantly negatively correlated with bone marrow infiltration by malignant lymphocytes. As a separate parameter, IL-12 is negatively correlated with the stage of the disease, which distinguishes it as an antiapoptotic cytokine that should be considered in the pathogenesis and course of chronic lymphocytic leukemia and included in further research in this area.

Conflict of interest: None declared.

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