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Pregledni članak
Review article

ULOGA EOZINOFILNIH LEUKOCITA U PATOGENEZI BRONHIJALNE ASTME

Danijela Vučević, Tatjana Radosavljević, Gordana Đorđević-Denić

Institut za patološku fiziologiju, Medicinski fakultet, Beograd

Kratak sadržaj: Patogeneza bronhijalne astme nije do kraja razjašnjena. U plućima, perifernoj krvi i sputumu astmatičara prisutan je povećan broj eozinofila. Eozinofilija je identifikovana kao faktor rizika za razvoj opstrukcije vazdušnih puteva. Izrazit eozinofilni zapaljenski infiltrat u bronhijalnoj sluznici i korelacija između broja eozinofilnih leukocita i težine bolesti podržava hipotezu prema kojoj su eozinofili glavne inflamacijske ćelije sposobne da izazovu patofiziološke promene karakteristične za astmu. Aktivirani eozinofili sekretuju široki spektar preformiranih i novosintetisanih medijatora koji dovode do oštećenja bronhijalnog epitela, spazma glatkih mišića bronhija, povećanja sekrecije sluzi i vazodilatacije. Dokazano je da se u toku astmatičnog napada povećava proizvodnja oksidanasa. Brojna istraživanja ukazuju da eozinofili u krvi i vazdušnim putevima osoba obolelih od bronhijalne astme stvaraju veću količinu oksidanasa u odnosu na zdrave osobe.

Ključne reči: eozinofilni leukociti, bronhijalna astma, inflamacija, oksidansi

Bronhijalna astma i hronične opstruktivne bolesti pluća

Bronhijalna astma je inflamacijska bolest koju karakteriše preosetljivost vazdušnih puteva sa povremenim periodima bronhospazma. Radi se o spazmu ili dužim kontrakcijama bronhijalne i bronhiolarne glatke muskulature. *Exstrinsic* astma (atopijska, alergijska astma) je mnogo češća od *intrinsic* astme (nealergijske, neatopijske, inflamacijske astme). Bronhijalna astma je veoma složeno oboljenje koje podrazumeva biohemijske, autonomne, imunske, infektivne, endokrine i psihičke faktore različitog stepena u različitih osoba (1).

Smatra se da je inflamacija koja nastaje kao posledica preosetljivosti ili hiperosetljivosti vazdušnih puteva osnovni patofiziološki razlog u svim tipovima astme. Oslobađanje medijatora inflamacije dovodi do spazma glatkih mišića bronhija, vaskularne kongestije, povećane propustljivosti krvnih sudova, edema, stvaranja guste, lepljive sluzi i prestanka funkcije (1).

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Po mnogima eozinofilni leukociti su ključne efektorne ćelije u patogenezi ove inflamacijske bolesti vazdušnih puteva (2–4, 9, 47, 48–59). Aktivirani eozinofili sekretuju široki spektar preformiranih i novosintetisanih medijatora koji razaraju integritet bronhijalne sluznice, zaustavljaju pokretanje cilija i dovode do oštećenja i jake sekrecije epitelnih ćelija. Oštećenje epitelnih ćelija je u korelaciji sa preosetljivošću vazdušnih puteva (1, 51, 57, 58). Brojni citokini koje produkuju eozinofili obezbeđuju lokalni mehanizam kojim se pojačava i modulira postojeći zapaljenski proces (5, 55, 56). Metaplazija peharastih ćelija, koja nastaje kao posledica inflamacije disajnih puteva, takođe umnogome doprinosi kliničkoj simptomatologiji, opstrukciji disajnih puteva i mortalitetu (6, 47, 51). Bitne patomorfološke karakteristike bronhijalne astme su i subepitelna fibroza, hipertrofija glatkih mišića i formiranje novih krvnih sudova, što u krajnjem ishodu dovodi do remodelovanja zida disajnih puteva (5, 7, 51, 56).

Osim bronhijalne astme, inflamacija je značajna za razvoj hroničnih opstruktivnih bolesti pluća (HOBP). Ipak, inflamacijski odgovor u HOBP se značajno razlikuje od odgovora u astmi, što je prikazano na *tabeli I*. Međutim, neki bolesnici imaju istovremeno i HOBP i astmu, pa inflamacija u njihovim plućima može pokazivati karakteristike obe bolesti (8).

Table I Karakteristike inflamacije u bronhijalnoj astmi i HOBP

Bolest pluća	Bronhijalna astma	HOBP
Inflamacijske ćelije	Eozinofili Mali porast broja makrofaga Porast broja CD4+ T limfocita Aktivacija mastocita	Neutrofili Veliki porast broja makrofaga Porast broja CD8+ T limfocita Leukotrien B_4 (LTB_4)
Medijatori inflamacije	Leukotrien D_4 (LTD4) Interleukin-4 (IL-4) Interleukin-5 (IL-5) Mnogi drugi medijatori	Interleukin-8 (IL-8) Faktor nekroze tumora α (TNFα) Mnogi drugi medijatori Skvamozna metaplazija epitela
Posledice	Fragilan epitel Zadebljanje bazalne membrane Metaplazija sluzi Uvećanje žlezda	Destrukcija parenhima Metaplazija sluzi Uvećanje žlezda
Odgovor na terapiju	Glikokortikoidi inhibiraju inflamaciju	Glikokortikoidi imaju mali efekat ili ne ispoljavaju svoje dejstvo

Table II Diferencijalna dijagnoza bronhijalne astme i HOBP

Bolest pluća	Znaci koji ukazuju na bolest
Bronhijalna astma	Početak u mladosti (često u detinjstvu) Simptomi se menjaju iz dana u dan Simptomi se javljaju noću ili rano ujutru Česta pridruženost alergije, rinitisa i/ili ekcema Pozitivna porodična anamneza za astmu Uglavnom reverzibilno ograničenje protoka vazduha
HOBP	Početak u srednjem životnom dobu Spora progresija simptoma Anamneza o dugotrajnom pušenju Dispnoja pri fizičkom naporu Uglavnom ireverzibilno ograničenje protoka vazduha

U nekih bolesnika s hroničnom bronhijalnom astmom jasno razlikovanje ove bolesti od HOBP nije moguće korišćenjem savremenih metoda za imidžing pluća i ispitivanje plućne funkcije. Znaci karakteristični za bronhijalnu astmu i HOBP, koji su važni za diferencijalnu dijagnozu ovih bolesti prikazani su na *tabeli II*. Međutim, ovi znaci se ne sreću kod svih bolesnika. Na primer, osoba koja nikad nije pušila može da se razboli od HOBP. Takođe, astma može da se razvije u odraslih, pa čak i u starijih osoba (8).

Eozinofilni leukociti i zapaljenje mukoze disajnih puteva

Postoje dokazi o akutnoj i hroničnoj inflamaciji koje su nepravilno raspoređene u disajnim putevima astmatičara (9). Opšte je prihvaćeno da su eozinofili dominantne efektorne ćelije u hroničnoj inflamaciji (2–4, 9, 47, 48–59). Svoju ulogu ostvaruju u toku procesa degranulacije oslobađanjem čitavog niza medijatora i faktora rasta (*Tabela III–V*).

Table III Proteinski sadržaj eozinofilnih granula

Protein	Vrsta granula
Glavni osnovni protein (MBP-major basic protein)	Sekundarne (specifične) granule
Eozinofilni katjonski protein	Sekundarne (specifične) granule
Eozinofilni neurotoksin	Sekundarne (specifične) granule
Eozinofilna peroksidaza	Sekundarne (specifične) granule
Lizozim	Sekundarne (specifične) granule
Kisela fosfataza	Sekundarne (specifične) granule
Arilsulfataza B	Sekundarne (specifične) granule
Katalaza	Sekundarne (specifične) granule
Enoil-CoA hidrataza	Sekundarne (specifične) granule
3-ketoacil-CoA tiolaza	Sekundarne (specifične) granule
β-glukuronidaza	Sekundarne (specifične) granule
Katepsin D	Sekundarne (specifične) granule
Elastaza	Sekundarne (specifične) granule
Granulocitno-monocitni faktor rasta (GM-CSF)	Sekundarne (specifične) granule
Interleukin-2 (IL-2)	Sekundarne (specifične) granule
Interleukin-4 (IL-4)	Sekundarne (specifične) granule
Interleukin-5 (IL-5)	Sekundarne (specifične) granule
Interleukin-6 (IL-6)	Sekundarne (specifične) granule
Faktor nekroze tumora α (TNFα)	Sekundarne (specifične) granule
RANTES	Sekundarne (specifične) granule
Tip II fosfolipaza A_2	Sekundarne (specifične) granule
Baktericidni protein koji povećava permeabilnost	Sekundarne (specifične) granule
Kisela fosfataza	Male granule
Arilsulfataza B	Male granule
Katalaza	Male granule
Citohrom b_{558}	Male granule
Elastaza	Male granule
Eozinofilni katjonski protein	Male granule
Lizofosfolipaza (Charcot--Leyden kristalni protein)	Primarne (nespecifične granule)
Ciklooksigenaza	Lipidna telašca
5-lipoksigenaza	Lipidna telašca
15-lipoksigenaza	Lipidna telašca
Leukotrien C_4 sintetaza	Lipidna telašca
Eozinofilna peroksidaza	Lipidna telašca
Esteraza	Lipidna telašca

Table IV Eozinofilni lipidni medijatori

Eozinofilni lipidni medijatori
Faktor aktivacije trombocita (PAF-platelet activation factor)
Leukotrien B_4 (LTB_4)
Leukotrien C_4 (LTC_4)
Tromboksan A_2 (TXA_2)
Prostaglandin E_2 (PGE_2)
Prostaglandin G_2 (PGG_2)
Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)
Prostaglandin I_2 (PGI_2)
5-hidroksieikosatetraenoična kiselina (5-HETE)
12-hidroksieikosatetraenoična kiselina (12-HETE)
15-hidroksieikosatetraenoična kiselina (15-HETE)
5,15-dihidroksieikosatetraenoična kiselina (5,15-diHETE)
8,15-dihidroksieikosatetraenoična kiselina (8,15-diHETE)
14,15-dihidroksieikosatetraenoična kiselina
(14,15-diHETE)
Lipoksin A_4 (LXA_4)
Lipoksin C_4 (LXC_4)
13-hidroksilinoleinska kiselina (13-HODE)

Table V Eozinofilni faktori rasta

Eozinofilni faktori rasta
Faktor nekroze tumora α (TNFα – tumor necrosis factor α)
Faktor nekroze tumora β (TNFβ – tumor necrosis factor β)
Faktor rasta poreklom iz trombocita (PDGF – platelet-derived growth factor)
Faktor rasta vaskularnog endotela (VEGF – vascular endothelial growth factor)
Heparin vezujući epidermski faktor rasta (HB-EGF → heparin-binding epidermal growth factor)
Nervni faktor rasta (NGF – nerve growth factor)
Endotelin (ET – endothelin)

Iz granula eozinofila oslobađaju se katjonski proteini (eozinofilni katjonski protein, eozinofilna peroksidaza, eozinofilni neurotoksin, i dr.), enzimi (fosfolipaza A_2, eozinofilna kolagenaza, katalaza, kisela fosfataza, histaminaza, itd.) i neuropeptidi (supstanca P, vazoaktivni intestinalni peptid – VIP, i dr.). Najzastupljeniji protein u granulama eozinofila (MBP – major basic protein), deluje toksično na epitel vazdušnih puteva. Pokazano je da MBP izaziva oštećenje pneu-

Tabela VI Eozinofilni receptori za hemokine i njihovi endogeni ligandi

Subfamilija hemokina	Nomenklatura	Endogeni ligandi
CXC hemokini	CXCR1	Interleukin-8 (IL-8)
CXC hemokini	CXCR1	Granulocitni protein hemotakse-2 (GCP-2 → granulocyte chemotactic protein-2)
CXC hemokini	CXCR2	Interleukin-8 (IL-8)
CXC hemokini	CXCR2	Granulocitni protein hemotakse-2 (GCP-2 → granulocyte chemotactic protein-2)
CC hemokini	CCR1	Makrofagni inflamacijski protein 1α (MIP1α)
CC hemokini	CCR1	RANTES
CC hemokini	CCR1	Monocitni protein hemotakse-2 (MCP-2 → monoocyte chemotactic protein-2)
CC hemokini	CCR1	Monocitni protein hemotakse-3 (MCP-3 →monoocyte chemotactic protein-3)
CC hemokini	CCR1	Monocitni protein hemotakse-5 (MCP-5 → monoocyte chemotactic protein-5)
CC hemokini	CCR1	Leukotaktin-1
CC hemokini	CCR3	Eotaksin I
CC hemokini	CCR3	Eotaksin II
CC hemokini	CCR3	Leukotaktin-1
CC hemokini	CCR3	Monocitni protein hemotakse-3 (MCP-3 → monoocyte chemotactic protein-3)
CC hemokini	CCR3	Monocitni protein hemotakse-4 (MCP-4 → monoocyte chemotactic protein-4)
CC hemokini	CCR3	RANTES

mocita (10, 11) i deskvamaciju epitela respiracijskog trakta (12, 13). MBP narušava transport jona u epitelu traheje, što može da izmeni zapreminu i sastav tečnosti koja oblaže vazdušne puteve (14).

Nezaobilazni element aktivacije i efektorne funkcije eozinofila su i novosintetisani lipidni medijatori ciklooksigenaznog puta (prostaglandini i tromboksan A_2) i lipoksigenaznog puta (leukotrieni, lipoksini i dr.). Osim toga, eozinofili stvaraju i citokine (eokine), i to pre svega interleukin-3 (IL-3), IL-5 i granulocitno-monocitni faktor rasta (GM-CSF), koji svojim autokrinim dejstvom održavaju i intenziviraju inflamaciju. U granulama eozinofila utvrđeno je i prisustvo IL-10, IL-11, IL-12, IL-16, interferona γ (INFγ) i faktora koji inhibira migraciju makrofaga (MIF – macrophage migration inhibitory factor). Zapaljenski proces moduliraju i eokini akutne inflamacije (IL-1α, IL-6, i faktor nekroze tumora α – TNFα), faktor aktivacije trombocita (PAF), kao i hemokini (IL-8, makrofagni inflamacijski protein 1α – MIF1α, i dr.) (2–4, 9, 47, 48–59).

Inflamacijski odgovor predstavlja kaskadu događaja koja se manifestuje sekvencionalnom akcijom integrina, selektina, superfamilije imunoglobulina, kadherina, adresina i drugih familija adhezivnih molekula i njihovih receptora. Zapaljenske ćelije se kotrljaju preko endotela posredstvom mehanizama koje obezbeđuju selektini (L, E i P selektini). Zahvaljujući integrinima ostvaruje se čvrsto vezivanje inflamacijskih ćelija za endotel. Naime, na neaktiviranim leukocitima integrini se nalaze u mirujućem stanju i eksprimiraju se na bazalnom nivou. Aktivacijom ćelija stimuliše se i ispoljavanje i aktivacioni status ovih glikoproteinskih membranskih molekula, kao i sekrecija L-selektina. Integrini su uključeni i u kontakte leukocita sa proteinima ekstracelularnog matriksa. Najznačajniji ligandi integrina među proteinima ekstracelularnog matriksa su kolagen, laminin, fibronektin i vitronektin (15, 67).

Eozinofili na svojoj površini ispoljavaju adhezivne molekule važne za povezivanje ovih leukocita u nastanku imunskog odgovora, za usmeravanje njihovog kretanja kroz krvne sudove i za interakciju sa ekstracelularnim matriksom. Subfamiliji β_1 integrina (raniji naziv VLA antigeni – very late activation) pripadaju VLA-4, VLA-5 i VLA-6. U β_2 subfamiliju integrina svrstani su Mac-1 (macrophage-1 antigen koji se još označava i kao CD11b/CD18), leukocitni funkcionalni antigen-1 (skraćeno označen kao LFA-1 ili CD11a/CD18) i p150/95 (CD11c/CD18). $\alpha_4\beta_7$ je takođe integrin ćelijske membrane eozinofila. Na površini eozinofila ispoljeni si u L-selektin i sijaloglikoprotein (Sialil Lewis X). PECAM-1, koji je dobio naziv od početnih slova engleskih reči platelet endothelium cellular adhesion molecule-1, i intercelularni adhezivni molekul-1 (ICAM-1) predstavljaju adhezivne molekule iz superfamilije imunoglobulina takođe eksprimirane na eozinofilima (2, 4, 67).

Eozinofili vezuju imunoglobuline (Ig) preko površinskih receptora. Tako se IgE i IgG vezuju za FcγRI, FcγRII i Mac-2 receptore, dok se IgA vezuje za FcαR (4).

Svoje specifične receptore na površini eozinofila imaju i C5a, CR1, CR3 i C1q komponenta komplementa (4).

Brojni površinski receptori eozinofila olakšavaju interakciju ovih ćelija sa citokinima. Na ovaj način svoje delovanje ostvaruju IL-2, CD25, IL-3, IL-5, IL-13, GM-CSF, interferon α (INFα), INFβ, INFγ, TNFα, i transformišući faktor rasta β (TGFβ) (4, 16).

Na površini eozinofila se mogu uočiti i receptori za hemokine. Vezivanjem za ove receptore hemokini učestvuju u migracijskim kretanjima eozinofila, aktivaciji integrina, indukciji respiracijskog praska, transkripciji citokina (neki od njih), angiogenezi, stvaranju kolagena i proliferaciji hematopoetskih prekursora. Najnovija istraživanja pokazuju da je RANTES (hemokin čiji naziv predstavlja kovanicu dobijenu od početnih slova engleskih reči r*egulated upon activation normal T cell expressed and secreted*) pravi hemotaktički faktor u bolesnika sa alergijskom astmom, dok je IL-5 neophodan kofaktor (4, 17).

Eozinofili i njihovi produkti su prisutni u krvi, disajnim putevima, pljuvački i u bronhoalveolarnom lavatu (BAL) osoba obolelih od bronhijalne astme. U astmatičara eozinofilija je u korelaciji sa stepenom opstrukcije i težinom bolesti (2, 4).

O procesima koji dovode do aktivacije eozinofila *in vivo* još uvek se relativno malo zna. Osnovna karakteristika eozinofilne funkcije je da je za nju neophodan »priming« eozinofila, što prevedeno sa engleskog jezika znači »prvi sloj, prvo premazivanje«. *Priming* bi mogao da se posmatra kao međusobni uticaj modulacijskih i aktivacijskih signala. Aktivacija eozinofilnih receptora preko adhezivnih molekula, komponenti komplementa i imunoglobulina bi mogla da bude uključena u ove događaje. Receptori eozinofila su potentni signalni molekuli *in vitro*, ali svoju optimalnu funkciju postižu tek nakon priminga sa citokinima i hemotaksinima. IL-5 i GM-CSF priming eozinofila neophodan je za odgovore ovih ćelija, uključujući sintezu i oslobađanje bioaktivnih medijatora, što doprinosi povećanoj bronhijalnoj reaktivnosti (18). Smatra se da cirkulišući eozinofili dobijaju povećanu sposobnost da sekretuju IL-5 kad stignu u pluća, gde su stimulisani i drugim citokinima, kao što su IL-2 i IL-4 (19).

Migracija eozinofila iz cirkulacije u plućno tkivo se odigrava nakon njihove adhezije za vaskularne endotelne ćelije, komponente ekstracelularnog matriksa i tkivne ćelije. Kretanje ćelija u zapaljensko područje se sastoji iz rolovanja (kotrljanja), čvrste adhezije i transendotelne migracije. Eozinofilna dijapedeza na mesto inflamacije je regulisana citokinima podstaknutom i pojačanom ekspresijom endotelnih ad-

hezivnih molekula. Na površini endotelnih ćelija utvrđeno je prisustvo vaskularnog adhezivnog molekula-1 (VCAM-1), ICAM-1, ICAM-2, PECAM-1, E-selektina, P-selektina, fibronektina i laminina (20). Svaki od ovih molekula ima svoj odgovarajući ligand na eozinofilima i bazofilima (LFA-1, Mac-1, VLA-4, L-selektin) (21). Specifična ekspresija VLA-4 na eozinofilima i limfocitima, i njeno odsustvo na neutrofilima doveli su do hipoteze da je VCAM-1 predominantni endotelni regulator hronične inflamacije bronhijalne mukoze (22). IL-4 dovodi do povećane ekspresije VCAM-1 na endotelnim ćelijama (23). Povećana endotelna ekspresija VCAM-1, E-selektina i ICAM-1 je povezana sa alergijskim zapaljenjem pluća (2). Lutman i saradnici (24) su eksperimentalno pokazali da IL-4 i IL-13 pojačavaju efekat TNF? na eozinofilnu aktivaciju, kao i sinergistički efekat ovih citokina sa IL-5 na eozinofilnu aktivaciju.

IL-5 pojačava adheziju humanih eozinofila za vaskularni endotel i dovodi do hiperreaktivnosti donjih disajnih puteva (25). Receptor IL-5 pripada familiji tipa 1 citokinskih receptora. α subjedinica (βc lanac koji koriste i IL-3 i GM-CSF za signalnu transdukciju) ovog visokoafinitetnog receptora se aktivira nakon vezivanja IL-5 za receptorski IL-5 α lanac (IL-5Rα), i time započinje serija intracelularnih događaja. Na ovaj način tirozinskom fosforilacijom Janus kinaze 2 (JAK2) i proteina STAT1α (signal transducer and activators of transcription 1α) aktivira se JAK-STAT put. Familiju STAT proteina čini sedam članova (STAT 1α, STAT 1β i STAT 2–6). Osim JAK2, IL-5 aktivira i druge tirozin kinaze (lyn, hck, yes, btk, tec, c-fes). JAK2 i c-fes su direktno udružene sa βc subjedinicom GM-CSF/IL-3/IL-5 receptora, sugerišući da je njihova aktivacija rani događaj citokinske signalizacije (26, 27). JAK kinaze su neophodne za aktivaciju STAT proteina koji se nalaze u latentnom obliku u citoplazmi. Nakon aktivacije, odnosno fosforilacije, STAT proteini formiraju homodimere i heterodimere, translociraju se u jedro, vezuju za određenu sekvencu dezoksiribonukleinske kiseline (DNK) i regulišu transkripciju. U humanim eozinofilima IL-5 indukuje dva DNK-vezujuća kompleksa koji sadrže tirozin fosforilisane proteine. Jedan od ova dva DNK-vezujuća kompleksa sadrži STAT1α verovatno kao dimer. Međutim, IL-5 pored indukcije STAT proteina indukuje i mnoge druge nuklearne proteine. Dugotrajna stimulacija ovim eokinom dovodi do indukcije transkripcionog faktora c-myc u humanim eozinofilima. IL-5 preko ras puta indukuje transkripcione faktore c-fos i c-jun, p21 ras, raf, MAPKK (p41 i p45) i MAPK (p44). S obzirom da do sada nije identifikovan apsolutno eozinofilni specifični regulacijski DNK element i transkripcioni faktor, moguće je da je specifična signalizacija rezultat specifične kombinacije transkripcionih faktora (25).

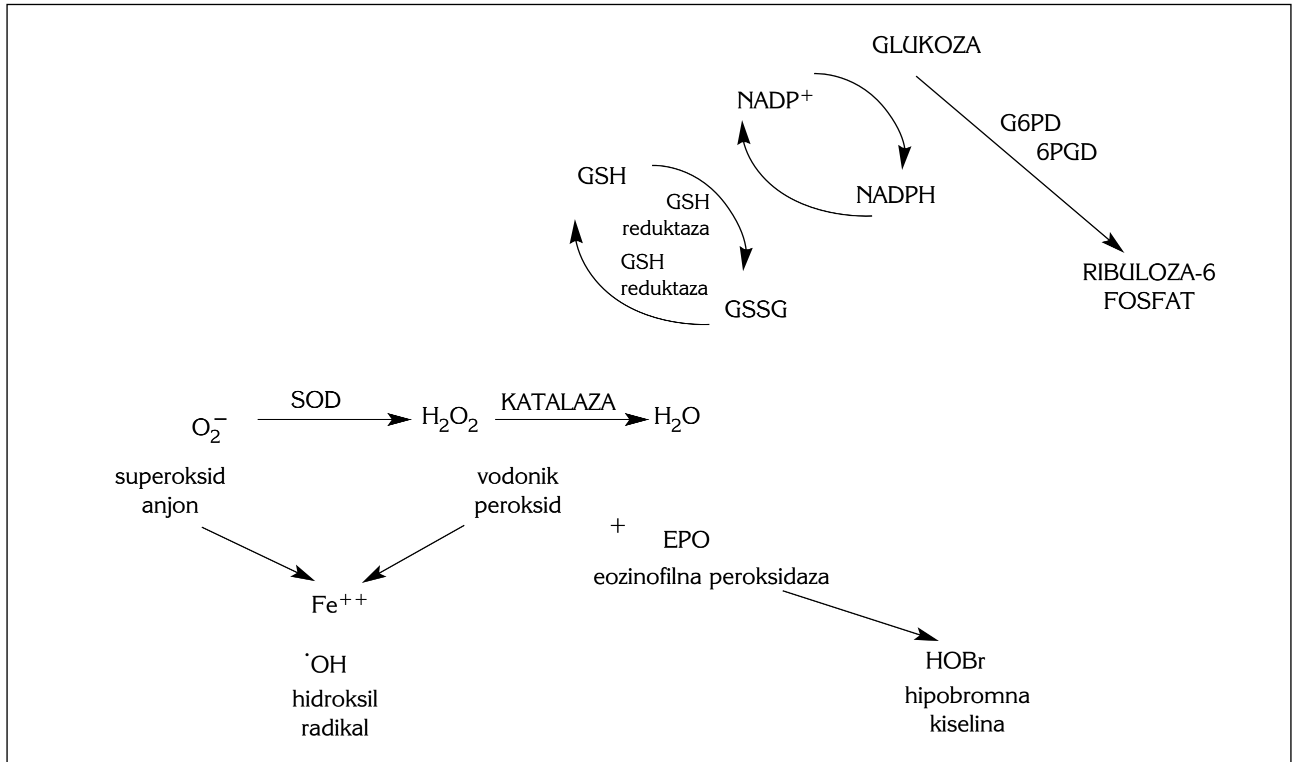
Eozinofilna apoptoza u hroničnoj inflamaciji bronhijalne mukoze

Broj eozinofila *in vivo* je regulisan ne samo produkcijom eozinofila u kostnoj srži, već i stepenom eozinofilne apoptoze, koja predstavlja najčešći oblik fiziološke ćelijske smrti. Bcl-2 (B-cell leukemia oncogene-2) familija gena kodira heterogenu grupu proteina koji predstavljaju ključne intracelularne modulatore (regulatore) ćelijskog umiranja po tipu apoptoze (28, 29). Otkrićem Bcl 2 gena, Bcl 2 familija proteina postaje posebno istraživana oblast zbog izuzetnog značaja za razumevanje molekulsko-bioloških mehanizama koji leže u osnovi procesa apoptoze. Poslednjih godina naročito se istražuju antiapoptozni članovi Bcl 2 familije proteina (29–34).

Odložena eozinofilna apoptoza se smatra jednim od najodgovornijih mehanizama koji doprinose eozinofiliji. U ljudi izgleda da je IL-5 specifičan faktor preživljavanja eozinofila, pa nije iznenađujuća činjenica da su eozinofilija i visoka ekspresija IL-5 udružene u hroničnoj inflamaciji bronhijalne mukoze. Eozinofilna apoptoza može biti odložena IL-om 5 kojeg sekretuju susedne ćelije, ali i autokrinom produkcijom ovog citokina. Izgleda da stimulacija eozinofila IL-om 5 dovodi do indukcije Bcl-xl (Bcl-2-regulated factor xl), koji je važan antiapoptotski gen. Postoje istraživanja gde je verifikovana povećana ekspresija Bcl-2 nakon tretiranja eozinofila IL-om 5 (35). Antiapoptozno dejstvo Bcl 2 proteina zasniva se na mogućnosti da veže Bax (Bcl 2-associated x) protein u formi heterodimera i tako onemogući stvaranje proapoptoznih Bax/Bax homodimera. Tokom indukcije procesa apoptoze, homodimeri Bax proteina formiraju kanale na membranama mitohondrija. Putem ovih kanala mitohondrije napuštaju molekuli citohroma c, koji su od posebne važnosti za aktivaciju izvršne faze procesa apoptoze (36). Na osnovu ovih saznanja, većina autora kao glavnu biološku ulogu Bcl 2 proteina u inhibiciji procesa apoptoze smatra njegovu sposobnost vezivanja za Bax protein i heterodimerizacionu neutralizaciju njegovog proapoptoznog dejstva (37–39). U *in vitro* uslovima je dokazano da je preživljavanje eozinofila produženo kada se inkubiraju u kulturama sa eozinofilnim citokinima kao što su IL-3, IL-5 i GM-CSF (35).

Eozinofilni leukociti i oksidacijsko oštećenje u bronhijalnoj astmi

Pluća predstavljaju primarno ciljno tkivo za oksidacijsko oštećenje zbog svoje lokalizacije, anatomije i funkcije. Kod naglog povećanja broja i količine oksidanasa, u uslovima tzv. oksidacijskog stresa, prirodni zaštitni mehanizmi popuštaju. Dokazano je da se u toku astmatičnog napada povećava proizvodnja oksidanasa. Brojna istraživanja ukazuju da eozinofili u krvi i vazdušnim putevima osoba obolelih od bronhijalne astme stvaraju veću količinu reaktivnih kiseoničkih



Slika 1 Reaktivne kiseoničke vrste i plućni antioksidacijski mehanizmi

vrsta (RKV) u odnosu na zdrave osobe (4, 8, 46, 50, 51, 57, 58, 61–66, 68, 69). Metaboliti kiseonika čiji izvor su i eozinofili, direktno oštećuju proteine plućnog matriksa i/ili slabe funkciju antiproteaza i/ili inaktiviraju enzime uključene u sintezu elastina i obnovu plućnog tkiva. RKV eozinofila podstiču sekreciju sluzi i bronhokonstrikciju. Tako npr., vodonik peroksid (H_2O_2) kontrahuje glatki mišić disajnih puteva *in vitro*, a izoprostan $F_{2\alpha}$-III, koji se stvara delovanjem slobodnih radikala u toku peroksidacije arahidonske kiseline, je snažan konstriktor disajnih puteva čoveka (8, 40, 41).

U izdahnutom vazduhu astmatičara zabeležene su povišene vrednosti H_2O_2 i azot monoksida (NO) (42, 43). NO je veoma reaktivan i nestabilan oksidans. U sintezi NO učestvuje enzim NO sintetaza (NOS). Inducibilna forma ovog enzima (iNOS), koja je odgovorna za citotoksične i biocidne efekte NO, prisutna je u eozinofilnim leukocitima. Aktivira se bakterijskim lipopolisaharidima i određenim citokinima (INFγ, TNFα i IL1β). Inducibilnoj NOS je za maksimalnu produkciju NO potrebno 8–12 sati, a količine NO koje se tom prilikom produkuju su znatne (15, 44).

Oksidansi eozinofila mogu da reaguju i sa lipidima i nukleinskim kiselinama, što može da dovede do disfunkcije ili smrti ćelije. Takođe, u uslovima oksidacijskog stresa aktivacijom transkripcionog faktora NF-kappaB koji upravlja ekspresijom različitih inflamacijskih gena važnih za bronhijalnu astmu, kao što su IL-8 i TNFα, dodatno se podstiče zapaljenski proces (8, 60).

Singlet kiseonik ($^1O_2$), superoksid anjon ($O_2^{\cdot -}$), H_2O_2 i hidroksil radikal ($OH^{\cdot}$) su RKV koje eozinofili stvaraju u toku respiracijskog praska. U prisustvu H_2O_2 katalitičkim dejstvom eozinofilne peroksidaze dolazi do oksidacije halogenih elemenata i stvaranja reaktivnih kiselina (hipobromne, bromovodonične i jodovodonične kiseline) (16). Istraživanje na miševima inficiranim Toksokarom kanis (*Toxocara canis*) je pokazalo da se eozinofilna peroksidaza taloži u plućnom parenhimu i miokardu, što može da dovede do oštećenja srca i pluća (45).

Zaključak

Patogeneza bronhijalne astme nije do kraja razjašnjena. Ono što se sa sigurnošću zna je da eozinofili bitno doprinose njenom nastanku. Kompleksan patofiziološki supstrat ove bolesti otkriva nova polja istraživanja interakcija eozinofilnih medijatora i citokina sa medijatorima i citokinima drugih inflamacijskih ćelija. Takođe, nameće se potreba za dodatnim proučavanjem kako inhibicije aktivnosti IL-4 i IL-5, tako i pojačavanja aktivnosti IL-10, IL-12 i IFNγ, tj. što uspešnijeg imitiranja njihovih dejstava u cilju pronalaženja efikasnijih lekova u terapiji bronhijalne astme.

THE ROLE OF EOSINOPHILIC LEUKOCYTES IN PATHOGENESIS OF BRONCHIAL ASTHMA

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Summary: Pathogenesis of bronchial asthma has not been completelly understood. Eosinophilic leukocytes accumulate in high numbers in the lungs, blood and sputum of asthmatic patients. Peripheral blood eosinophilia has been identified as a risk factor for the development of airway obstruction. Prominent eosinophilic inflammatory infiltrate in the bronchial mucosa and correlation between eosinophil numbers and disease severity supports the hypothesis that eosinophils are central inflammatory cells capable of inducing pathophysiological features of asthma. Activated eosinophils secrete a wide range of preformed and newly generated mediators that damage the bronchial epithelium, contract smooth muscle, increase mucous secretion and cause vasodilation. There is ample evidence that oxidants generation is increased during an asthma exacerbation. Many investigations indicate that airway and blood eosinophils produce more oxidants in asthmatic patients compared with control subjects.

Key words: eosinophilic leukocytes, bronchial asthma, inflammation, oxidants

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CELL DEATH IN IRRADIATED PROSTATE CANCER CELLS ASSESSED BY FLOW CYTOMETRY

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Summary: Despite the significant advances in cancer chemotherapy, radiotherapy still remains a method of choice for treatment of metastatic human prostate cancer. This study presents quantitative analysis of ^{60}Co gamma-radiation effects on cell growth and cell death of metastatic human prostate cancer PC-3 cell line, performed in time (24–72h) and dose (2–20 Gy) dependent manner. The irradiated PC-3 cells were mostly dying by necrosis at late time intervals (72h), while apoptotic cell death was negligible. The EC_{50} or 50% of cytotoxicity was not achieved within the radiation doses used (2–20 Gy), but significant cell growth inhibition with IC_{50} of 10.4 Gy was observed. It is concluded that the increase in the radiation dose may have an important cytostatic effect, but for the complete eradication of metastatic prostate cancer novel cytotoxic drugs and radiosensitizers should be introduced as adjuvant.

Key words: human prostate cancer, cell death, gamma-rays, flow-cytometry

Introduction

Despite the significant advances in the area of cancer chemotherapy, radiotherapy, applied either alone or as adjuvant, still remains a method of choice for treatment of many malignant diseases. One of them is prostate cancer, the most frequent cancer in men population, which unfortunately shows a continual casualty increase (1). The ultimate aim of radiotherapy is to efficiently eradicate tumor cells with minimal deleterious effects to the surrounding normal tissues and to the whole organism. In that view, induction of apoptosis is very desirable therapeutic endpoint (2, 3). However, much is yet to be learned about either systemic or individual biological effects of both conventional (gamma-, x-ray) or accelerated particle (proton, etc.) ionizing radiation in order to optimize clinical results of treatment of human prostate cancer.

Regardless of the routine use of simple test for prostate specific antigen in sera, that can detect disease at an early stage (4, 5), the number of men with metastatic prostate cancer is still high. The early stages of disease are usually managed by ionizing radiation and/or hormone therapy, but there is no successful therapy for metastatic prostate carcinoma. Advanced disease is mostly treated by radiation therapy, sometimes in combination with hormone or chemotherapy, but hormone withdrawal often leads to selection of hormone-independent clones (6). Dose-escalated (*i.e.* 70–80 Gy) radiotherapy is an important treatment option especially for men with intermediate-risk prostate cancer (7). On the other hand, radiotherapy is often inefficient due to radioresistance of prostate cancer cells.

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Recent studies suggest that some prostate cancer cells can undergo apoptosis (8). The response to ionizing radiation, depends on a number of factors such as the stage of differentiation, mutations in specific genes (such as p-53 and bcl-2) that will determine the ability of the target cells to enter apoptosis (9, 10). For clinical purposes (*i.e.* eradication of the tumor, but prevention of undesired inflammatory *sequelae*, radiation sickness and fibrosis), it is useful to investigate whether the cells of certain types are susceptible to apoptosis or necrosis, as well as to determine the time and dose dependence of the process. In addition to cell killing, radiation can also lead to cell cycle arrest and stopping of proliferation with significant decrease in cell growth (11).

The purpose of this study is to investigate radiation induced cell death in PC-3 prostate cancer cell line in time- and dose-dependent manner. The changes in cell growth following irradiation were also determined.

Materials and Methods

Cell lines. Human prostate cancer cell line PC-3 was purchased from American Type Culture Collection (CRL 1435, Rockville, MD). They are androgen independent and were established from bone metastasis, which is the most usual place for metastatic prostate cancer. PC-3 were maintained in RPMI 1640 medium supplemented by 10% heat inactivated fetal calf serum, 100 IU/mL penicillin/streptomycin and 2 mmol/L L-glutamine (Sigma Aldrich Chemie GmbH, Germany), at 37 °C under 5% CO_2 atmosphere. Cells were grown as monolayers in 75 cm^2 culture bottles supplied with 15 mL RPMI, and after a few passages cells were transferred in 25 cm^2 culture bottles (Nunk, Nalgene, Danmark).

Cell Irradiation. For investigation of radiation induced effects on PC-3, 3×10^5 cells were seeded in 25 cm^2 culture flasks, and after 72 hours were irradiated at room temperature with 2, 10 or 20 Gy gamma-rays from ^{60}Co gamma-source, at the dose rate of 20 Gy/h. The effects of irradiation on cell viability, morphology and genomic DNA structure were determined 24-, 48-, and 72 h after irradiation.

Trypan blue exclusion assay. For analysis of cell growth and spontaneous cell death in culture, cells were seeded at a density of 12×10^3 cells/cm^2 in 25 cm^2 culture flasks. Cell growth, viability and morphology were followed for 8 consecutive days, by trypan blue exclusion (TBE) assay. Medium from each bottle was collected, cells were harvested by trypsinization (1 mL 0.25% / 0.02% trypsine/EDTA, Sigma Aldrich, per bottle) and pooled with the medium. Cells were washed twice in phosphate buffered saline (PBS) and pelleted at 1800 rpm for 5 min at room temperature. Pellets were resuspended in fresh media and the number of viable (trypan blue negative), dead (trypan blue positive) and total cells (viable + dead) was counted in five squares at 320× magnification using Neubauer haemocytometer and Leitz-Wetzlar Orthoplan microscope. Cell viability was determined as % of cells that excluded trypan blue stain. The doubling time (td) was calculated according to the following formula:

$$td = \frac{\ln 2}{\mu}$$

$$\mu = (\ln x - \ln x_0)/t$$

where x represents cell number in time t, and x_0 cell number in time t_0.

The same assay was used for determination of viable, dead and total cell number in irradiated samples 24-, 48- and 72 h post-irradiation. The viability index (Vi) of each sample was calculated related to appropriate, unirradiated control, which was used as viability index 1 (100%).

Cell death analysis by flow cytometry. Double staining of cells by Annexin and propidium iodide (PI) enabled estimation of cell viability after irradiation and discrimination between two ways of cell death – apoptosis and necrosis. After trypsinization and centrifugation of cells, approximately 10^5 cells of each sample were mixed with 100 μL of Annexin V-FITC reagent (Travigen Inc., Gaithersburg, MD, USA) containing 5 μg/mL Annexin V-FITC and 5 μg/mL propidium iodide, and incubated at room temperature for 15 minutes in dark and then diluted with 400 μL of binding buffer. Multiparameter measurement of the cell sample in order to detect radiation-induced cell death was performed using a FACS-calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with 488 nm, 15 mW argon-ion laser. Staining of the cells with Annexin V-FITC (Annexin) permitted identification of cells in early apoptosis, while staining of the cells with Annexin and PI permitted quantification of cells in the late apoptosis and necrosis. Data were acquired immediately after staining by analyzing about 20,000 cells/sample. The data were further processed by Becton Dickinson LYSIS II software.

DNA fragmentation assay. This assay was used for confirmation of necrosis, detected by double staining and flow cytometryc analysis. It was performed as previously described with minor modifications (12). Cells were incubated in one volume of digestion buffer (100 mmol/L NaCl, 25 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.0, 0.5% SDS and 0.5 mg/mL RNA-se A) for 2 h at 50 °C, followed by the addition of proteinase K (0.6 mg/mL) and digestion was continued overnight. DNA was deproteinised using the phenol/chloroform/isoamil-alcohol reagent, for three times. The aqueous layer was transferred to a new tube and precipitated with one volume of isopropanol and 1/10 volume of ammonium acetate overnight at 4 °C. The DNA pellet was washed three times with ice-cold 95% ethanol and dried at room temperature. The final DNA pellet was resuspended in 20 μL TE buffer (10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA) and the concentration

of DNA was determined spectrophotometrically. Electrophoresis of 4–10 μg of each DNA sample, was carried out for 60 min at 60 V at room temperature on 1% agarose gel containing 1 μg/mL ethidium bromide. Gels were scanned by GelDoc apparatus.

Statistical analysis. For statistical analysis of time- and dose- dependent changes in viability index and flow-cytometric determination, two-way ANOVA was used. If a statistical significance was found, Tukey post-hoc test was used to determine which groups differ from each other. Statistical significance was accepted if $p<0.05$.

Results

Analysis of cell growth and spontaneous cell death in culture

PC-3 cells were plated in 25 cm^2 culture flasks, at the density of 12×10^3 cells/cm^2 (e.g. 3×10^5 cells per bottle), and cell growth, viability and morphology were monitored for 8 consecutive days (*Figure 1*). During the first day post plating the viable cell number increase from 12×10^3 cells/cm^2 to almost 13.5×10^3 cells/cm^2, suggesting that plating efficiency was very high. The log phase of cell growth occurred between 2nd and 6th day post plating. The confluence was reached on the 6th day at the cell density of about 116×10^3 cells/cm^2. Further incubation led to the slightly decrease in cell number. TBE assay showed that approximately 2–8% of all cells were TB positive at all time points, indicating that cell viability was high during the whole experiment. We also calculated PC-3 cell doubling time, which was 32.9 ± 2.8 h under conditions maintained in our laboratory.

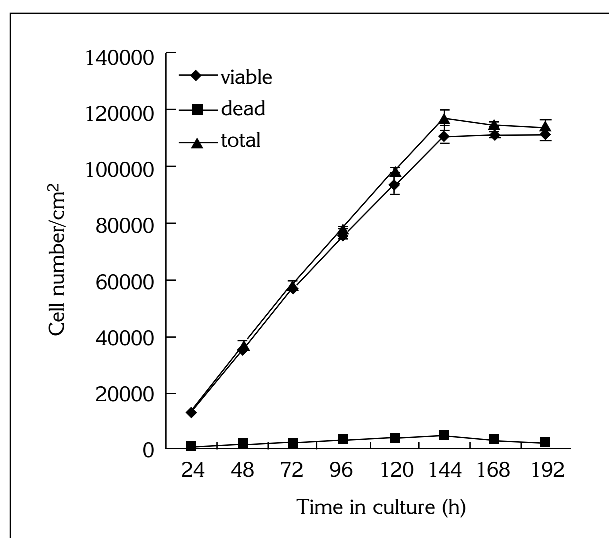

Figure 1. Growth and viability of PC-3 cells in culture determined by TBE assay. Data are the mean SEM from 2 distinct experiments.

Cell growth of irradiated PC-3 human prostate cancer cells

Effects of gamma-ionizing radiation on PC-3 growth were evaluated by TBE assay. The test is convenient for determination of Viability (V) and Viability index (Vi) after radiation treatment. TBE assay was performed 24-, 48- and 72 h after irradiation by 2-, 10- and 20 Gy. The obtained results (*Table I* and *Figure 2*) showed significant decrease in cell number and Viability Index (Vi), comparing with appropriate control, both by dose (F 190.1, $p<0.001$) and time (F 45.2, $p<0.001$) as determined by two-way ANOVA. This effect was most pronounced 72 h after treatment with 20 Gy, when the Vi decreased from 1, established in control, to 0.35. The radiation dose which caused decrease in cell V_i from 1 to 0.5, termed IC_{50} (mitotic cell death dose), was 10.4 ± 0.4 Gy. The dose dependent V_i (e.g. viable cell number) decrease was significant for all experimental points (*Table I*). The statistical differences between groups were analyzed by Tukey post hoc test, comparing irradiated samples with the control from the same time point, and significance was established at $^*p<0.05$. The number of TB positive cells, indicating cytotoxicity (actual cell death), was relatively low (up to 20 %, data not shown), which was in agreement with cytometric quantification of necrotic cells determined after double Annexin V-FITC/PI staining (*Figure 3*).

Table I Number of viable cells in the control and ^{60}Co gamma-irradiated human prostate cancer cells PC-3 measured by TBE assay 24–72 h post-irradiation. Data are the mean ± SEM from 3 distinct experiments. Statistical time- and dose-dependent differences were determined by two way ANOVA and Tukey post hoc test. ** $p<0.01$, *** $p<0.001$.

Dose → Time (h) ↓	control (0 Gy)	2 Gy	10 Gy	10 Gy
24	57.3 ± 1.6	** 43.7 ± 2.8	*** 40.9 ± 4.4	*** 40.9 ± 4.4
48	92.4 ± 4.9	*** 49.9 ± 9.7	*** 46.8 ± 5.9	*** 46.8 ± 5.9
72	84.6 ± 3.9	*** 69.6 ± 1.4	*** 44.7 ± 5.3	*** 44.7 ± 5.3

Radiation-induced cell death analysis by flow cytometry

All samples analyzed by TBE, were also stained by Annexin V-FITC and PI, and analyzed by FACS-Calibur flow cytometer. The results of quantification of cells in different states, such as viable cells, cells in early apoptosis, cells in late apoptosis or necrotic cells, and necrotic cells or cell aggregates, are presented in *Figure 3 A–D*. *Figure 3A* represents a flow cytometry scatter plot and the dots represent cells in different

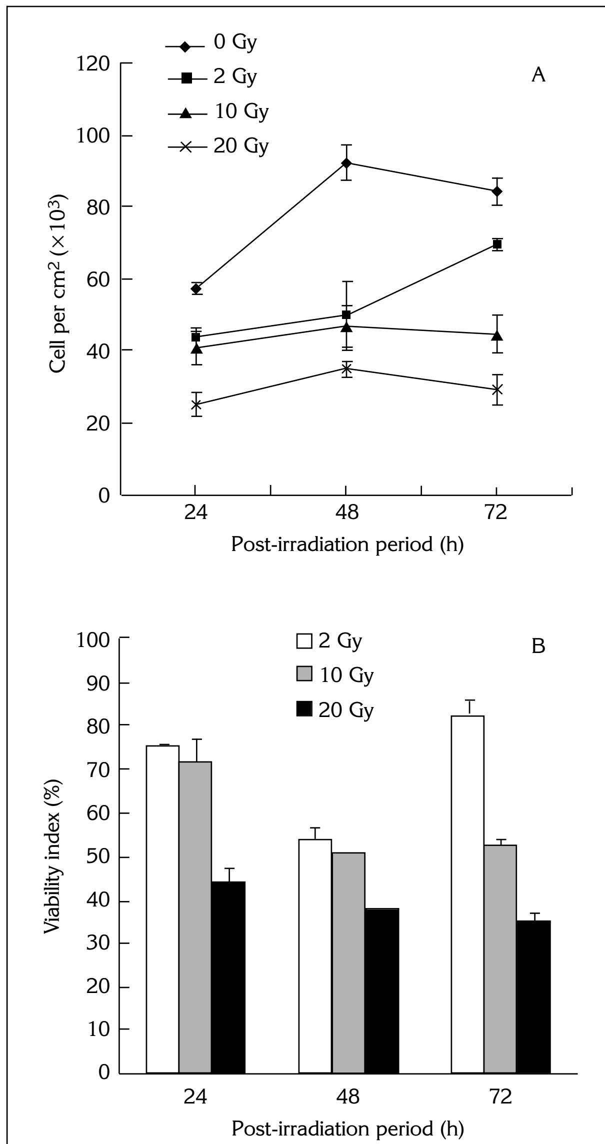

Figure 2. Time course of viable cells number in the control and ^{60}Co gamma-irradiated human prostate cancer cells PC-3 (A) and Viability index calculated according to the related control from the same time point (B) as measured in TBE assay. Error bars represent standard error of the mean (SEM). Data are the mean SEM from 3 distinct experiments.

states, depending on the place in scatter plot. Cells in early apoptosis were Annexin positive, while PI staining indicated late apoptosis or necrosis (*Figure 3A*). As it may be observed from *Figure 3B*, cell viability of the samples decreased with the increase in the radiation dose and with the period of incubation. Radiation-induced decrease of viability showed dose-dependent significance which was judged by two-way ANOVA analysis at $p<0.05$ (F 9.12, $p<0.001$), but not time-dependent significance (F 2.06, $p=0.142$). The Tukey post hoc test, used for comparison of irradiated samples with appropriate control, showed that only radiation dose of 20 Gy induced significant viability decrease, 48 and 72 h post irradiation. The evidence for apoptosis was minimal in either control or irradiated PC-3 cells. With the increase in the radiation dose, the percentage of cells in the state of early apoptosis increased from 0.2% up to 0.8 % (*Figure 3C*), but there were neither time- nor dose-dependent significance (F 1.35, $p=0.33$ and F 1.42, $p=0.33$ respectively).

The radiation dose and time dependent increase in number of PI positive cells in the late apoptosis or necrosis was also observed. The dose dependent increase in necrotic cells was significant (F 7.85, $p<0.001$), but time-dependence was not statistically significant (F 1.9, $p=0.17$). The Tukey post hoc analysis showed significant increase in number of dead cells after irradiation with 20 Gy, 48 and 72 h after treatment (*Figure 3D*).

Electrophoretic analysis of purified genomic DNA from irradiated cells

The genomic DNA from control and irradiated samples, including attached and floating cells, was analyzed on 1% agarose gel containing 1 μg/mL ethidium bromide. As it may be observed in *Figure 4* the initial fragmentation of PC-3 cell DNA to a high molecular size band (>10 Kb) was visible in all samples. In the case of irradiated samples, in addition to the fragmentation of PC-3 cell DNA to a high molecular size band (>10 Kb), smaller fragments (1< Kb) also appeared. The observed smear is most pronounced 72 h post irradiation with doses of 10 and 20 Gy. It correlated well with the highest percent of dead cells obtained by cytometry (*Figure 3D*). DNA ladder was not observed in examined samples, confirming flow cytometric data, that PC-3 do not die by apoptosis after gamma-irradiation.

Discussion

The hormone-independent metastatic prostate cancer is incurable at present. In the lack of efficient chemotherapeutic agents, ionizing radiation therapy still remains as a method of choice for the disease cure. It is known that apoptotic cell death plays an important role in the death of both normal prostate and androgen-dependent malignant prostate tissue following androgen withdrawal. Cancer cell death is leading to a decrease in either glandular or tumor volume, respectively. However, recent data indicate that apoptosis may not be the dominant form of cell death following radio- and chemotherapy in epithelial tissues (7, 13). Disruption of the pathways that lead to apoptosis is one of the major mechanism by which cancer cells become resistant to radiation or chemotherapy (14).

In this paper, we have analyzed ^{60}Co gamma-radiation-induced death of PC-3 human prostate cancer cells. PC-3 cells originates from epithelial cells.

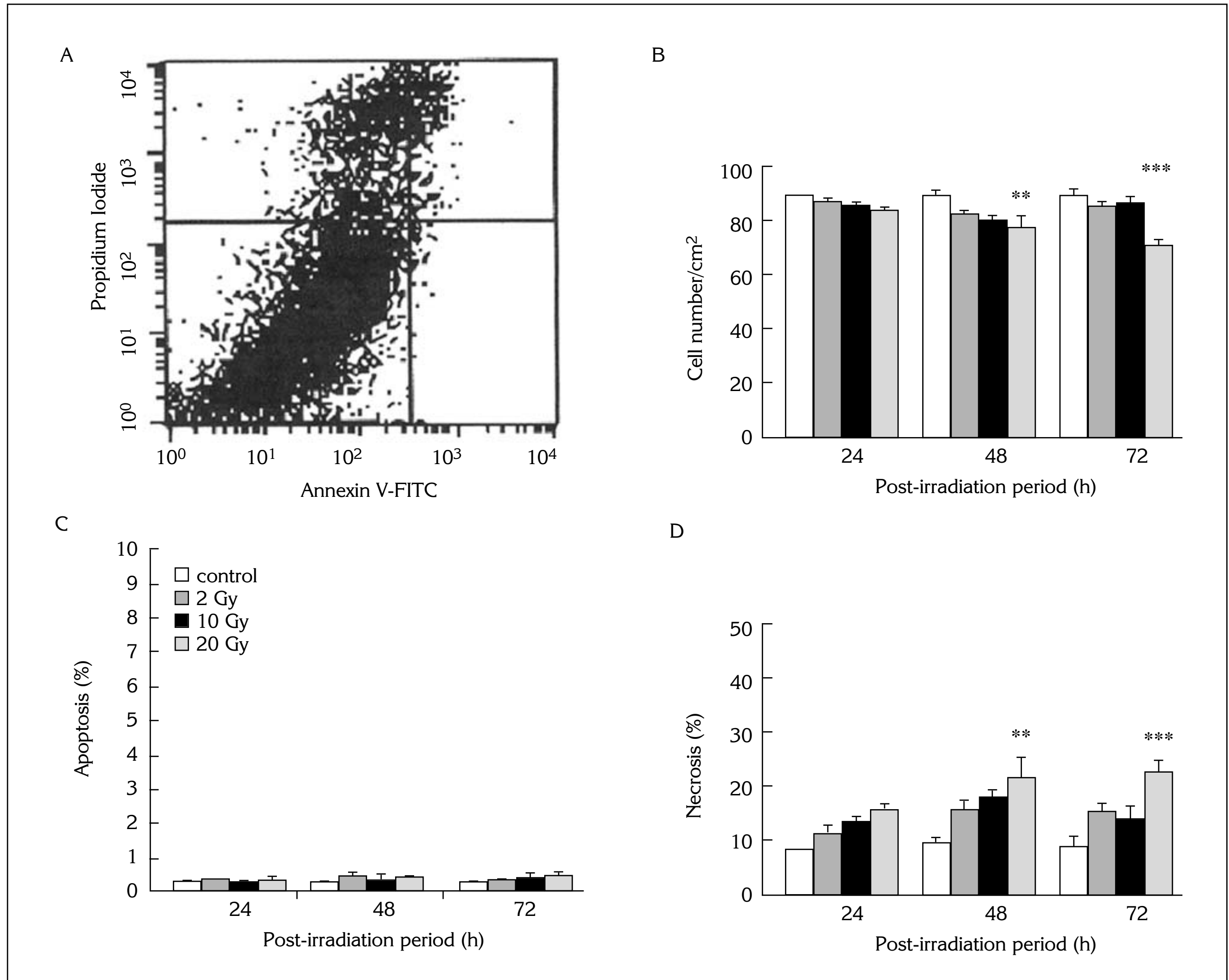

Figure 3. Time course of percent changes in PC-3 cell viability and death after 60Co gamma-irradiation as determined by flow cytometry. Viable cells (A), early apoptosis (B) or late apoptotic/necrotic cells (C). Data are the mean (n=3) and the error bars represent SEM. Statistical differences were determined by two-way ANOVA followed by Tukey post hoc analysis. ** $p<0.01$, *** $p<0.001$.

They are hormone-refractory cells derived from human bone metastasis of prostate adenocarcinoma, representing advanced prostate cancer (15). For these experiments, we chose doses of 2 and 10 Gy, to be representative of the 1.8–2 Gy daily clinical fractions given during curative radiotherapy and the 8–10 Gy single doses given in palliative radiotherapy, as well as the dose of 20 Gy which is in the range of cumulative curative dose for prostate carcinomas (70–80 Gy).

The growth curve of PC-3 cells showed sigmoid-like shape, with the population doubling time about 33 h. The viability of PC-3 cells was 92–98 % throughout the log phase of growth. The culture reach confluence 6th days post plating, at the density of 120 × 10^3 cells/cm^2, which is considerable less than other epithelial prostate cancer cell line, DU 145 (180 × 10^3 cells/cm^2), suggesting that PC-3 are bigger than DU 145 (to be published). After day 6th, number of cells slightly decreased, retaining surprisingly high viability (97–98%). Based on data from cell growth curve, all irradiation experiments were performed in the log phase of cell growth. Cells were irradiated with 2–20 Gy from ^{60}Co-source at the dose rate of 20 Gy/h. The irradiated PC-3 cell cultures were followed for three consecutive days *i.e.* through approximately two proliferation cycles. The data obtained by the TBE assay, showed significant decrease in cell Viability index, e.g. in the number of viable cells in irradiated samples relative to the control from the same time point. The process was dependent both on the radiation dose and on the incubation time. On the other hand, the number of TBE positive cells remained relatively low, indicating that radiation caused cell cycle arrest and blocking of cell proliferation, rather than actual cell

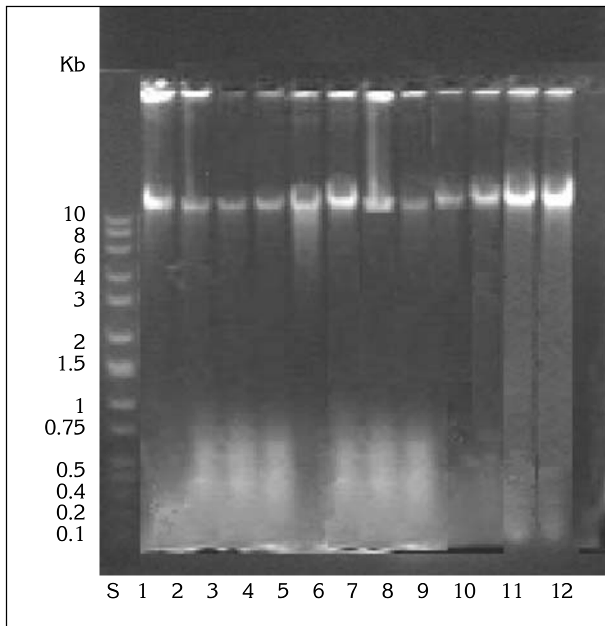

Figure 4. Agarose gel electrophoresis of genomic DNA extracted from ^{60}Co gamma-irradiated PC-3 cells. Lane S: DNA standard molecular size markers; lanes 1–12: genomic DNA pattern of samples irradiated with 0, 2-, 10 and 20 Gy respectively, isolated 24 hours (lines 1–4), 48 h (lines 5–8) or 72 h (lines 9–12) post-irradiation.

death. The obtained data enabled determination of doses which induced 50% of cell growth inhibition (named IC_{50}) and 50% of cytotoxicity, i.e. dose which induced 50% of trypan blue positive cells (EC_{50}). IC_{50} was achieved at the dose of 10.4 Gy, but EC_{50} was higher than applied doses, as 20 Gy induced only 20% of trypan blue positive cells. This indicated that the radiation treatment in the clinically relevant dose interval would predominantly inhibit PC-3 growth rather than induce cell killing. The similar results were obtained for other hormone-refractory epithelial prostate cancer cell line DU 145, derived from brain metastasis (to be published), but in the case of cervix epithelial cancer cells HeLa S3, the same doses of ionizing radiation induced cell death in up to 45% of irradiated cells (16). This comparison suggests that prostate cancer cells are much radioresistant than HeLa S3 cell line.

In order to determine the form of radiation-induced PC-3 cell death two different techniques were used: flow-cytometry analysis of cell morphological features after double staining with propidium iodide and Annexin-V-FITC, and DNA electrophoresis of purified DNA. Simultaneous staining of cells with Annexin V-FITC and propidium iodide enabled distinction of early apoptosis from late apoptosis and/or necrosis. After double staining and flow-cytometryc analysis of control and irradiated samples, the most of dead cells were in late apoptosis or necrosis (Annexin V+, PI+ cells). The early apoptosis (Annexin V+, PI- cells) occurred in insignificant number of cells, less than 1% of total cell number in each sample, and there were no significant changes in percent of apoptotic cells depending on time or dose. On the contrary, the cell necrosis was dose dependent, as determined by two-way ANOVA, and was most pronounced 72 hours post treatment. These results suggest that the prevailing form of ^{60}Co gamma radiation-induced PC-3 cell death was necrosis.

As it was not possible to distinguish the necrosis from the late apoptosis by double staining, it was necessary to perform the gel electrophoresis of purified genomic DNA from PC-3 cells. DNA fragmentation assay confirmed the presence of the necrosis process, showing the absence of DNA ladder characteristic for apoptosis in control (17), as well as in irradiated samples. One explanation for the absence of apoptosis following radiotherapy in PC-3 cell line is that these cells have mutant p53 gene. However, the impairment of other mechanisms necessary for initiation of the apoptotic process is not excluded. Namely, recent investigation showed that gamma-radiation activates acidic sphingomyelinase to produce ceramide, a catabolic product of membrane sphingolipids that is a cell death signal (14, 18, 19). It was suggested that the other epithelial prostate carcinoma LNCaP cells are highly resistant to induction of apoptosis by gamma-radiation due to a defect in ceramide generation (14, 20, 21). Likewise, resistance to apoptosis involves a defect in ceramide generation in the PC-3 prostate cancer cell line (20, 22).

In summary, the obtained results suggests that ^{60}Co gamma-ionizing radiation caused notable human prostate cancer PC-3 cell killing. The irradiated PC-3 cells were mostly dying by necrosis, while apoptotic cell death was negligible. Although within the radiation doses used in this study (2–20 Gy) the EC_{50} *i.e.* 50% of cytotoxicity was not achieved, we found significant cell growth inhibition with IC_{50} of 10.4 Gy. Thus, this *in vitro* study suggests, that the increase in radiation dose may have an important cytostatic effect, rather than eradicating the advanced prostatic carcinoma. It also suggests that, in addition to gamma irradiation, current antitumor strategies should introduce novel cytotoxic adjuvant or radiosensitizers, in order to achieve complete eradication of metastatic human prostate cancer.

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ĆELIJSKA SMRT U OZRAČENIM ĆELIJAMA KANCERA PROSTATE ANALIZIRANA PROTOČNOM CITOMETRIJOM

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Kratak sadržaj: Uprkos značajnom napretku u hemoterapiji kancera, radioterapija ostaje metod izbora u tretmanu metastaziranog kancera prostate. Ovaj rad predstavlja kvantitativnu analizu efekata ^{60}Co gama zračenja na ćelijski rasti i ćelijsku smrt PC-3 ćelijske linije humanog kancera prostate, pri čemu je praćena vremenska (2–72h) i dozna zavisnost (2–20 Gy). Ozračene PC-3 ćelije su uglavnom umirale nekrozom u kasnijem vremenskom intervalu (72h), dok je apoptoza bila zanemarljiva. Vrednost EC_{50} odnosno 50% citotoksičnosti nije dostignuta primenjenim dozama, ali je ustanovljena značajna inhibicija ćelijskog rasta, sa vrednošću IC_{50} od 10.4 Gy. Zaključeno je da povećanje doze može imati značajan citostatički efekat ali da je za kompletno odstranjivanje metastaziranog kancera prostate neophodno uvođenje novih citotoksičnih agenasa ili radiosenzitera kao adjuvanata.

Ključne reči: humani kancer prostate, ćelijska smrt, gama zračenje, protočna citometrija

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CFTR GENE ANALYSIS IN PATIENT WITH ATYPICAL CYSTIC FIBROSIS

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Summary: This paper reports a case of a patient presenting with atypical cystic fibrosis whose sweat test showes borderline values. In vast majority of cases the sweat test is essential diagnostic tool for establishing the diagnosis of cystic fibrosis, but only after the molecular genetic testing the diagnosis can be confirmed. The patient was found to be compound heterozygote for two CFTR mutations, F508del and D1152H. The presence of F508del mutation was analyzed by PSM method, while the screening for the second mutation was performed using DGGE. The strategy of mutation detection in cystic fibrosis patients, especially those with atypical presentations who carry less frequent mutations, should include both direct and indirect methods of molecular diagnostics.

Key words: atypical cystic fibrosis, CFTR gene, DGGE, molecular diagnostics

Introduction

Cystic fibrosis is one of the most common life-threatening autosomal recessive disorders that is usually estimated to affect 1 in 2000–3000 Caucasian newborns, with a carrier frequency of 1 in 26 individuals (1). In its classic and most common form, cystic fibrosis manifests with chronic obstructive lung disease, exocrine pancreatic insufficiency, elevated sweat chloride concentration and in males infertility due to obstructive azoospermia (2).

Cystic fibrosis is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, spanning 250kb at chromosomal region 7q31.3 and consisting of 27 exons. The gene was discovered in 1989, and it encodes a protein expressed at the apical membrane of exocrine epithelial cells (3). CFTR protein functions principally as a cAMP-induced chloride channel and appears capable of regulating other ion channels.

The most common mutation in the CFTR gene is F508del located in exon 10, and it is present on approximately two-thirds (66%) of all cystic fibrosis chromosomes. However, there is great mutational heterogeneity in the remaining one-third of all alleles. Nearly 1300 mutations within CFTR have been identified to date, and reported to Cystic Fibrosis Genetic Analysis Consortium (4). Although these mutations vary greatly in their frequency and distribution, the vast majority are present in either single individual or small number of individuals.

Mutations affect CFTR through a variety of molecular mechanisms which can produce little or no functional CFTR at the apical membrane. The phenotypic spectrum associated with mutations in the CFTR gene extends beyond the classically defined cystic fibrosis. Besides patients with atypical cystic fibrosis, there are large numbers of so-called monosymptomatic diseases, such as various forms of obstructive azoospermia, idiopathic pancreatitis or disseminated bronchiectasis associated with CFTR mutations uncharacteristic for cystic fibrosis (5).

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This paper reports a case of atypical cystic fibrosis that was diagnosed by the combination of direct and indirect mutation detection methods. It is shown that the patient is a compound heterozygote for two CFTR mutations.

Materials and Methods

Case history

Patient is a 38 year-old woman presented with a diagnosis of bronchiectasis. Her past medical history was noteworthy for the onset of respiratory symptoms such as: recurrent pneumonia and periods of cough and haemoptysis. Sweat test performed at the age of 6 showed borderline values. At the age of 33, computed tomography (CT) has shown the presence of bronchiectasis. At the age of 38, she presented with lung disease progression, which was observed by CT, and was referred for molecular testing for cystic fibrosis.

Methods

DNA was extracted from peripheral blood using GFX™ Genomic Blood DNA Purification Kit (Amersham Biosciences).

The presence of the most frequent CFTR mutation – F508del was detected by PCR-Mediated Site-Directed Mutagenesis (PSM) method (6). The 219bp long fragment was amplified with the following primers: 5'-GCACCATTAAAGAAAATATGAT-3' and 5'-CATTCACAGTAGCTTACCCA-3', and digested with *MboI*. Products were analyzed on 10% polyacrilamide gel.

The screening for the presence of variations in CFTR exons was performed by Denaturing Gradient Gel Electrophoresis (DGGE) method, as previously described (7). Exon 18 was amplified with the following primers: 5'-GTAGATGCTGTGATGAACTG-3' and 5'-GTGGCTATCTATGAGAAGGA-3' and sequenced with the primer 5'-TGCCCTAGGAGAAGTGTG-3' using Thermo Sequenase Cy™5 Dye Terminator Kit (Amersham Pharmacia Biotech).

Results and Discussion

The presence of CFTR F508del mutation was analyzed by PSM method. After digestion with *MboI* normal allele is digested giving fragments 202bp and 17bp long, while mutant allele remains undigested. This analysis has shown that the patient is heterozygous for F508del mutation (*Figure 1*).

The screening for the second mutation was performed using DGGE. After DGGE analysis was performed for several CFTR exons, altered band pattern was seen in exon 18. Mixing of the patient sample with control samples heterozygous for two mutations in exon 18, has shown that the mutation present in patient's sample is D1152H. Mixing with M1137V/N control gives extra heteroduplex bands, while no extra bands are seen after mixing with D1152H/N control (*Figure 2*).

The presence of D1152H mutation was confirmed by direct DNA sequencing (*Figure 3*).

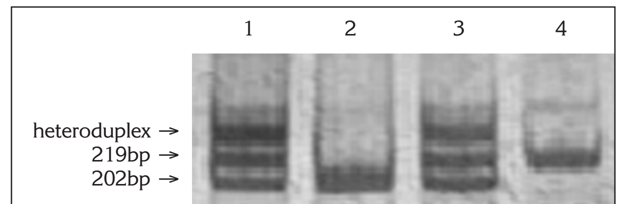

Figure 1. PSM analysis of patient's CFTR gene for the presence of F508del mutation:
1. Heterozygote for F508del
2. Homozygote for normal allele
3. Patient (heterozygote for F508del)
4. Homozygote for F508del

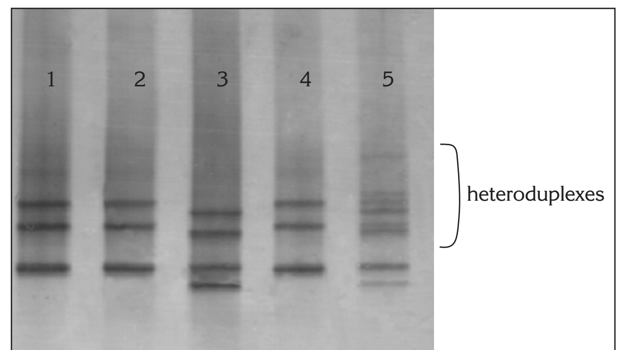

Figure 2. DGGE analysis of patient's CFTR gene for the presence of variations in exon 18:
1. patient's sample
2. control D1152H/N (heterozygote for D1152H)
3. control M1137V/N (heterozygote for M1137V)
4. mixed patient's sample and control D1152H/N
5. mixed patient's sample and control M1137V/N

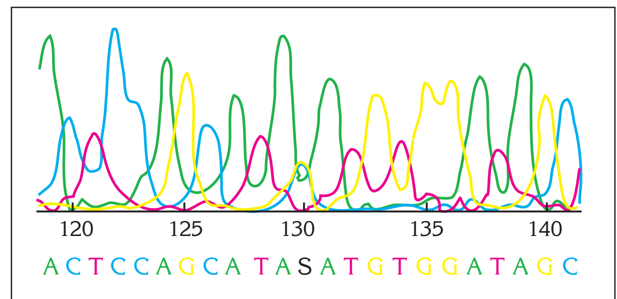

Figure 3. The part of the patient's CFTR gene exon 18 sequence containing D1152H mutation in heterozygous state (S = C/G)

The described patient, presenting with atypical cystic fibrosis, was found to be compound heterozygote for two CFTR mutations, F508del and D1152H. It has been previously reported that the F508del/D1152H genotype is associated with mild CF phenotype (8).

The CFTR mutation D1152H is caused by point mutation G to C at position 3586. Patients carrying D1152H mutation are usually diagnosed at advanced age, present mild pulmonary disease and pancreatic sufficiency. The mutation D1152H was found to be associated with normal sweat chloride values (9). Characterization at the protein and at the electrophysiological level has shown that this mutation does not alter the permeability sequence of the CFTR channels (10). However, it significantly reduces the whole cell cAMP activated chloride currents, indicating that this mutation interferes with the proper gating of the chloride channels.

In vast majority of cases, the sweat test remains the essential diagnostic tool for establishing the diagnosis of CF. Although the threshold of 60 mmol/L for the sweat chloride concentration has proven to be discriminating and useful in clinical practice, in described patient a borderline value was observed. Only after the molecular genetic testing, the diagnosis of cystic fibrosis was confirmed. In our opinion in borderline sweat chloride results, clinician should consider molecular genetics testing for cystic fibrosis. Further exhaustive genetic analysis is justified in patients with symptoms suggestive of CF and borderline sweat chloride concentration.

Although methods for direct detection of the most frequent CFTR mutations remain essential, methods for the screening of the whole gene are increasingly used for the purposes of cystic fibrosis molecular diagnostics. In our experience, the denaturing gradient gel electrophoresis is a method of choice, due to its reliability and sensitivity. It is followed by direct DNA sequencing, used for characterization of the detected aberrant pattern. The strategy of mutation detection in analysis of CF patients, especially those with atypical presentations who carry less frequent mutations, should include both direct and indirect methods of molecular diagnostics.

ANALIZA CFTR GENA KOD PACIJENTA SA ATIPIČNOM CISTIČNOM FIBROZOM

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Kratak sadržaj: U ovom radu je prikazan slučaj atipične cistične fibroze sa graničnom vrednošću znojnog testa. U većini slučajeva znojni test je glavni dijagnostički parametar za dijagnostikovanje cistične fibroze, ali se dijagnoza može potvrditi samo na osnovu rezultata molekularno-genetičkog testiranja. Utvrđeno je da je pacijent složeni heterozigot za dve CFTR mutacije, F508del i D1152H. Prisustvo mutacije F508del detektovano je PSM metodom, dok je za analizu prisustva druge mutacije korišćena DGGE metoda. Strategija detekcije mutacija kod pacijenata sa cističnom fibrozom, naročito onih sa atipičnim prezentacijama bolesti koji nose ređe mutacije, trebalo bi da uključuje i direktne i indirektne metode molekularne dijagnostike.

Ključne reči: atipična cistična fibroza, CFTR gen, DGGE, molekularna dijagnostika

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THE INFLUENCE OF ATOPY ON sICAM-1 SERUM LEVELS IN PATIENTS WITH ALLERGIC RHINITIS AND BRONCHIAL ASTHMA

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Summary: It has been shown that adhesive molecules are involved in inflammatory diseases of the lungs such as bronchial asthma. The purpose of the study was to measure and establish possible difference in serum levels of soluble ICAM-1 in 42 atopic patients (patients with allergic rhinitis and patients with bronchial asthma) in comparison with 28 patients without atopy (patients with asthma without rhinitis); whether there is a difference in sICAM-1 levels between groups of 26 patients with allergic rhinitis and asthma in comparison with group of 16 patients with allergic rhinitis only and also in comparison with 10 healthy controls. Results of the study have substantiated statistically significant difference in sICAM-1 levels between all groups of patients in comparison to healthy control, but no statistically significant difference in sICAM-1 levels between patients with and without atopy ($Z=-1.738$) or between patients with allergic rhinitis and bronchial asthma in comparison with group of patients with allergic rhinitis only ($Z=0.00$). ICAM-1 is an important marker of inflammation in patients with allergic rhinitis as well as in those with bronchial asthma. Atopic status does not influence differences in sICAM-1 levels. Although mean sICAM-1 levels were higher in patients with allergic rhinitis and bronchial asthma (312.71 ng/mL) in comparison with mean sICAM-1 levels in patients with allergic rhinitis only (279.69 ng/mL), no statistically significant difference was noted in sICAM-1 levels between these groups of subjects, i.e. asthma itself did not contribute to statistically significant increase of sICAM-1 levels.

Key words: intercellular adhesive molecule 1, allergic rhinitis, bronchial asthma, atopy

Introduction

Preliminary studies have suggested that intercellular adhesive molecule 1 (ICAM-1; CD54) may be involved in pathogenesis of asthma (1). Soluble form of the intercellular adhesive molecule 1 (sICAM-1) was detected in elevated levels in the sera of adult patients with some inflammatory, immune or malignant diseases (1). It originates from proteolytic cleavage of membrane ICAM-1 (m ICAM-1) (2). In asthmatic patients epithelial cells of airways may express increased amounts of ICAM-1 molecule, which is one of the responses to different stimuli such as local cytokines, infectious agents, air pollutants, allergens. Their response to these stimuli alters the immune and inflammatory environment that is critical in diseases such as bronchial asthma.

The aim of the study was to measure and establish the possible difference in sICAM-1 levels in sera of patients with atopy (patients with allergic rhinitis and patients with allergic rhinitis and bronchial asthma) in comparison with the group of patients without atopy (patients with asthma without rhinitis) as well as possible differences in sICAM-1 levels between groups of patients with allergic rhinitis and asthma in comparison with patients with allergic rhinitis only.

Material and Methods

Our study comprised patients with allergic rhinitis and bronchial asthma. The diagnosis of bronchial asthma was established following the guidelines for diagnosis and treatment of asthma of the international expert group of the National Institute of Health and

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National Institute of Heart, Lungs and Blood, Bethesda, May 1997, suppl. 2002 (3). All patients had positive results of metacholine and/or Ventolin test. Sensitization to standard inhaling allergens was tested by skin prick test in all patients.

All patients were subjected to physical medical examination. Pulmonary function before blood sampling was measured using Autospir Discom-14 Chest Corporation Tokyo, Japan. In addition to history, skin tests and determination of serum IgE, patients with allergic rhinitis were subjected to *in vivo* specific rhino-provocative tests to evidence the presence of allergic rhinitis.

The sICAM-1 levels were measured in blood samples obtained after the cubital vein puncture using vacutainer, without EDTA addition. The sample was left to coagulate at room temperature for 60–120 minutes. After that the samples were centrifuged at 1300 g for 10 minutes at room temperature. The separated serum was stored at –20 °C until the actual analysis. ELISA was used for the sICAM-1 serum level determination. The commercial Parameter human sICAM-1 immunoassay (ELISA, R&D Systems Inc. Minneapolis, USA) was used. The sICAM-1 serum levels were expressed in ng/mL. The lowest detectable value of serum sICAM-1 was 0.35 ng/mL (following the manufacturers instructions).

The subjects were classified into following groups: 16 with allergic rhinitis, 26 with allergic rhinitis and bronchial asthma (all of them combined composed the group with atopy); 28 patients bronchial asthma without allergic rhinitis (composing the group without atopy). The control group was composed of 10 healthy volunteers, mean age 37 years without history of allergy, asthma, allergic rhinitis, atopic dermatitis or any other significant disease.

Statistical analysis was conducted using EPI INFO ver. 10 program package. Statistical differences were calculated using non-parametric Mann-Whitney and Kruskal-Wallis tests. Correlations among different parameters were conducted using the Spearman's rank correlation coefficient (4, 5).

Results

The control group of healthy subjects had mean sICAM-1 values of 226.64 ng/mL, coinciding with control values stated by the manufacturer (R&D Systems). The mean sICAM-1 levels in patients with and without atopy were 300.10 ng/mL and 315.00 ng/mL, respectively. The correlation coefficient of sICAM-1 levels between groups of healthy subjects and patients with and without atopy was $H=12.072$, $p<0.01$, suggesting statistically significant difference of sICAM-1 levels between the groups of subjects.

The correlation coefficient of sICAM-1 levels between groups with atopy (patients with allergic rhinitis and patients with asthma and allergic rhinitis) and healthy subjects was $Z_1=-2.670$, $p<0.01$, suggesting highly statistically significant difference between the groups, i.e. sICAM-1 level was significantly higher in patients with atopy than in healthy controls.

The correlation coefficient of sICAM-1 levels between groups without atopy (patients with bronchial asthma without allergic rhinitis and negative skin prick test) and healthy subjects was $Z_2=-3.166$, $p<0.01$, suggesting highly statistically significant difference between the sICAM-1 levels in the groups, i.e sICAM-1 level was significantly higher in patients with asthma without atopy than in healthy controls. The correlation coefficient of sICAM-1 levels between groups with and without atopy was $Z_3=-1.738$, $p>0.05$, suggesting no statistically significant difference between the sICAM-1 levels in the groups, i.e. sICAM-1 serum levels were similar in patients with atopy (allergic rhinitis only and allergic rhinitis with bronchial asthma) and those without atopy (patients with bronchial asthma only), (*Table I*).

The mean value of sICAM-1 levels in patients with allergic rhinitis was 279.62 ng/mL, while in patients with allergic rhinitis and asthma the level of 312.71 ng/mL was recorded. The mean sICAM-1 level

Table I Mean values of sICAM concentrations in our studies groups

		Group		
		healthy	atopy	atopy free
ICAM-1 (ng/mL)	Valid N	N=10	N=42	N=28
	Mean	226.6	300.10	315.00
	Std Dev.	32.94	136.34	79.26
	Min.	197.0	167.05	190.04
	Max.	301.9	930.65	488.89
	$H=12.072$		$p<0.01$	
	$Z_1=-2.670$		$p<0.01$	
	$Z_2=-3.166$		$p<0.01$	
	$Z_3=-1.738$		$p<0.05$	

Table II Mean values of sICAM concentrations in our studies groups

		Group		
		healthy	rhinitis	asthma and rhinitis
ICAM-1 (ng/mL)	Valid N	N=10	N=16	N=26
	Mean	226.6	279.62	312.71
	Std Dev.	32.94	136.34	168.66
	Min.	197.0	183.14	167.05
	Max.	301.9	376.63	930.65
	$H=12.072$		$p<0.01$	
	$Z_1=-2.670$		$p<0.01$	
	$Z_2=-3.166$		$p<0.01$	
	$Z_3=-1.738$		$p<0.05$	

in healthy subjects was 226.64 ng/mL. The correlation coefficient of sICAM-1 levels between the groups was $H=7.172$, $p<0.05$, suggesting the statistically significant difference in sICAM-1 levels between the groups. The correlation coefficient of sICAM-1 levels between the groups of patients with allergic rhinitis and healthy controls was $Z_4=-2.821$, $p<0.01$, suggesting the statistically significant difference between the groups, i.e. patients with allergic rhinitis had statistically significantly higher sICAM-1 levels than the healthy controls. The correlation coefficient of sICAM-1 levels between the groups of patients with allergic rhinitis and asthma on one hand and healthy controls on the other was $Z_5=-2.172$, $p<0.05$, suggesting the statistically significant difference between the groups, i.e. patients with allergic rhinitis and asthma had higher sICAM-1 levels than the healthy controls. The correlation coefficient of sICAM-1 levels between the groups of patients with allergic rhinitis on one hand and bronchial asthma and allergic rhinitis on the other was $Z_6=0.00$, $p>0.05$, suggesting no statistically significant difference between the groups, i.e. sICAM-1 levels were similar in patients with allergic rhinitis only and those suffering from both allergic rhinitis and bronchial asthma (*Table II*).

Discussion

Nowadays, there is a general consensus that inflammation is crucial in pathophysiology of respiratory allergic diseases (6). The level of soluble adhesive molecules in the serum reflects the degree of systemic inflammation, but the dynamics of these molecules in the pathogenesis of allergic diseases and their evolution in the course of treatment remain to be evidenced. ICAM-1 may be induced on various cell types using miscellaneous inflammation stimuli (7). Allergic inflammation is associated with ICAM-1 expression on the surface of different cells such as the endothelium, bronchial epithelium and eosinophillic leukocytes ((8-11). Induction of expression of these molecules takes place under the influence of various proinflammatory cytokines such as IFN-gamma, IL-1, TNF-alpha and others.

The purpose of the study was to measure and compare levels of soluble ICAM-1 in the sera of atopic patients non-atopic patients and to compare the levels with the sICAM-1 levels in healthy controls. The mean sICAM-1 levels in healthy controls was 226.64 ng/mL. Shiota and associates reported somewhat higher mean sICAM-1 value in healthy volunteers, i.e. 260.90 ng/mL. It is possible that the difference in the number of healthy volunteers (10 vs. 39) contributed to the difference in the value obtained.

The mean value of sICAM-1 levels in our atopic patients (all patients with allergic rhinitis and patients with both allergic rhinitis and bronchial asthma) was 300.10 ng/mL as compared with 315.00 ng/mL measured non-atopic patients (bronchial asthma without rhinitis). Similar values in asthmatic patients were reported by Shiota and associates (12). Analysis of the levels of sICAM-1 between the groups of atopic patients and healthy controls as well as the difference between non-atopic group and healthy controls has revealed the presence of statistically significant difference. This actually means that atopic and non-atopic patients had statistically significant levels of sICAM-1 in the sera in comparison with healthy controls. This finding is in concert with referential data (1, 13, 14), substantiating the hypothesis on ICAM-1 as a marker of inflammation in respiratory diseases. However, analysis of correlation of sICAM-1 levels between groups of atopic vs. non-atopic patients failed to substantiate the statistically significant difference, i.e., both atopic and non-atopic patients had similar sICAM-1 levels in the respective sera. Laan and associates (15) obtained similar values in pediatric population, and Figen Do'Egu and associates (16) failed to identify any statistically significant differences in sICAM-1 levels between atopic and non-atopic children.

Results of our study have substantiated the presence of statistically significant difference in sICAM-1 values of patients with allergic rhinitis in comparison with healthy controls. In a study on a group of patients with allergic rhinoconjunctivitis Turgay et al. (17) reported no statistically significant difference between their sICAM-1 levels and healthy controls, but men with allergic rhinoconjunctivitis had higher values of sICAM-1 levels than in women, and in comparison with healthy controls. The sICAM-1 levels were higher in patients with allergic rhinoconjunctivitis that had higher symptom score (17). Kato and associates (18) reported that the level of sICAM-1 in the sera of patients with polynosis was up-regulated in the early season in comparison with healthy control. However, Chia-Ming Liu and associates (19) found overlapping values in sICAM-1 levels of patients with allergic perennial rhinitis and healthy controls. He explained the phenomenon by the target organ size (the nose) that is substantially lower in comparison with target surfaces of other organs such as the lungs and skin.

The reason for the differences in sICAM-1 levels in our allergic rhinitis patients in comparison with healthy control may lie in the »minimum persistent inflammation« present in patients hypersensitive to mite, that have it all the year round even in absence of symptoms (20). Also, the minimum exposure to allergens in natural conditions may lead to the occurrence of differences in sICAM-1 levels.

Our study has also substantiated the presence of statistically significant difference in sICAM-1 levels between groups of patients with atopy (allergic rhinitis and allergic rhinitis combined with asthma) and group of healthy volunteers, complying with referential data. However, in spite of the notable difference in mean sICAM-1 levels in patients with asthma versus patients

with allergic rhinitis only (312.71 ng/mL vs. 279.62 ng/mL) there was no statistically significant difference in sICAM-1 levels between these groups of patients. The reason may lie in heterogeneous composition of the groups where patients with bronchial asthma mainly used the therapy of inhaling glycocorticosteroids that may affect the down-regulation of ICAM-1 molecules (13, 16, 21–23).

The results of the study have substantiated that sICAM-1 is a marker of inflammation in both patients with bronchial asthma and those with allergic rhinitis, but that atopy itself does not influence the higher sICAM-1 levels in comparison with non-atopic patients. Also, in our study asthma did not contribute significantly to the total sICAM-1 levels. Naturally, future studies are necessitated for better elucidation of the function and regulatory mechanisms of serum ICAM-1.

UTICAJ ATOPIJE NA NIVO sICAM-1 U SERUMU KOD PACIJENATA SA ALERGIJSKIM RINITISOM I BRONHIJALNOM ASTMOM

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Kratak sadržaj: Poznato je da su adhezivni molekuli uključeni u inflamacione bolesti pluća kao što je bronhijalna astma. Cilj ovog rada je bio da se izmeri i utvrdi da li postoji razlika u koncentracijama solubilnog ICAM-1 u serumu 42 bolesnika sa atopijom (bolesnici sa alergijskim rinitisom i bolesnici sa bronhijalnom astmom) u odnosu na grupu od 28 bolesnika bez atopije (bolesnici sa astmom bez rinitisa) da li postoji razlika u koncentracijama sICAM-1 među grupama od 26 bolesnika sa alergijskim rinitisom i astmom u odnosu na 16 bolesnika samo sa alergijskim rinitisom kao i u odnosu na 10 zdravih kontrola. Rezultati rada su pokazali da postoji statistički značajna razlika u koncentracijama sICAM-1 svih pomenutih grupa ispitanika u odnosu na zdravu kontrolu, ali da ne postoji statistički značajna razlika koncentracija sICAM-1 među grupama bolesnika sa atopijom i bez atopije (Z = –1,738) kao ni među grupama ispitanika sa alergijskim rinitisom i bronhijalnom astmom u odnosu na grupu ispitanika samo sa alergijskim rinitisom (Z = 0,00). sICAM-1 predstavlja značajan marker inflamacije i kod bolesnika sa alergijskim rinitisom kao i kod onih sa bronhijalnom astmom. Atopijski status ne utiče na razlike u koncentracijama sICAM-1. Iako su srednje vrednosti koncentracija sICAM-1 bile više kod bolesnika sa alergijskim rinitisom i bronhijalnom astmom (312,71 ng/mL) u odnosu na srednje koncentracije sICAM-1 kod bolesnika samo sa alergijskim rinitisom (279,62 ng/mL), nije postojala statistički značajna razlika u koncentracijama sICAM-1 među ovim grupama ispitanika, tj. sama astma nije doprinosila statistički značajnijem povećanju koncentracija sICAM-1.

Ključne reči: intercelularni adhezivni molekul 1, alergijski rinitis, bronhijalna astma, atopija

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BIOCHEMICAL PHENOTYPE AND ORIGIN OF THE THREE MOST COMMON BETA-THALASSEMIA MUTATIONS IN SERBIA

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Summary: Molecular (DNA) characterization of thalassemia is the most reliable methodology for the diagnosis of this group of diseases. As thalassemias are very heterogeneous, hematological data and additional biochemical analysis are essential for their differential diagnosis. In this paper we present hematological and biochemical characteristics of the carriers of three most common beta-thalassemia mutations in Serbia (Hb Lepore, β°39 and β^+IVS-I-110), to be taken into consideration as the initial step of the diagnostic approach to the thalassemia patients. Also, this paper represents a detailed survey of the diversity of β-globin gene haplotypes in carriers of the most common β-thalassemia mutations and normal betaA/betaA individuals of Serbian descent. A novel haplotype associated with Hb Lepore-Boston-Washington gene has been identified in Serbian population. These data support the hypothesis of multicentric origin of this mutation. The mutation has arised *de novo* in the chromosomal background characteristic for Serbian population. Additionally, we have shown that two most common Mediterranean mutations, β°39 and β^+ IVS-I-110, have probably been introduced into Serbian population from Italy and Turkey, respectively, through historically documented migrations and settlements.

Key words: β-thalassemia, haplotype, Hb Lepore

Introduction

The β-globin gene cluster is located on chromosome 11. The alignment of genes in human β-globin locus represents the order of gene expression during ontogenic development: embryonal genes (ε) are located at the 5'end, then fetal genes (Aγ, Gγ) and finally, at the 3'end, adult genes (δ, β). Thalassemia syndromes are a group of hereditary disorders generally caused by mutations in globin genes. In Serbia, thalassemia syndromes are less frequent than in adjacent Mediterranean countries. The overall frequency of thalassemia syndromes in Serbia is 1.9% (1).

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Since 1998 thalassemia syndromes have been systematically characterized on molecular level in the population of Serbia (2). Nine different mutations have been detected: seven β-thalassemic mutations and two thalassemic hemoglobin (Hb) variants (Hb Lepore, Hb Sabine), among which 70% were Hb Lepore, β°39 and β^+ IVS-I-110 (2). Two studies showed that Hb Lepore is the most common cause of thalassemia in Serbia (30%) (1, 2).

Hb Lepore is an abnormal thalassemic hemoglobin variant, which consists of normal α-globin chains and fused δβ-globin chains. The δβ-globin chain is a product of an unequal recombination that joins the proximal end of the δ-globin gene with the distal end of the β-globin gene (3). Three types of Hb Lepore, that differ in the position at which the transition from δ to β DNA occurs, have been described: Hb Lepore-Hollandia, Hb Lepore-Baltimore and Hb Lepore-Boston-Washington (Hb Lepore BW) (3).

Substitution (C→T) alters codon 39 to a stop codon. Such chain termination (nonsense) mutation

aborts mRNA translation and leads to synthesis of a truncated polypeptide (4). Therefore, codon 39 results in β° thalassemic phenotype. Substitution (G→A) at position 110 in intron I (IVS-I-110) creates an alternative splice site. The incorrectly spliced mRNA leads to premature termination of translation but allows accumulation of some amount of normal globin mRNA (β^+ thalassemic phenotype) (5).

In this study we have analyzed hematological data and Hb content (Hb A, Hb A_2 and Hb F) in carriers of three most common beta-thalassemia mutations in Serbia. Also, we present a detailed study of the diversity of β-globin gene haplotypes in normal betaA/betaA and thalassemic individuals of Serbian descent, conducted with the aim of defining the origin of beta-thalassemia in Serbia.

The β-globin gene cluster is highly polymorphic. The pattern or combination of polymorphic restriction sites in β-globin locus for any chromosome is called a haplotype (6). There are nine common haplotypes (I-IX) known to be present in different Mediterranean populations with significant percentage (6). Additionally, four beta globin intragenic single base polymorphisms have led to the definition of three different beta globin gene frameworks (6). Haplotypes and frameworks of β-globin gene cluster are widely used nowadays to determine the chromosomal background on which a mutation arose, as well as its geographical pattern.

Material and Methods

Forty-five Serbian patients from 20 unrelated families affected with β-thalassemia, were studied: 21 Hb Lepore BW β-thalassemic variant carriers (from 8 unrelated families), 14 β°39 β-thalassemia mutation carriers (from 7 unrelated families) and 10 β^+IVS-I-110 mutation carriers (from 5 unrelated families). Healthy subjects related to the carriers were also analyzed.

Hematological parameters were obtained using an automated cell counter. Abnormal hemoglobin was detected by electrophoresis on cellulose acetate in Tris-borate-EDTA (pH 8.4) (7). Hb F was quantified by standard methods (8, 9) and Hb A_2 and Hb Lepore were estimated by elution from celogel electrophoretic strips.

DNA was extracted from peripheral blood collected with sodium citrate or EDTA as an anticoagulant (10). DNA samples were used as templates in polymerase chain reaction (PCR). For each sample eight PCR-RFLPs were performed, one for each polymorphism (*Hinc*II/ε, *Xmn*I/5'Gγ, *Hind*III/Gγ, *Hind*III/Aγ, *Hinc*II/ψβ, *Hinc*II/3'ψβ, *Ava*II/β, *Bam*HI/3'β). The conditions for PCR amplification were as described previously (11, 12), with changes in temperature of annealing for ε, 5'Gγ, Gγ, Aγ, ψβ, 3'ψβ, β, 3'β DNA fragments containing β-globin gene cluster polymorphic sites: 55, 55, 60, 55, 61, 61, 57 and 53 °C respectively. For haplotype analysis the polymorphic restriction enzyme sites were determined by digestion of PCR amplified fragments using the methodology of Sutton et al (12). Polymorphic sites were detected by agarose gel electrophoresis. The framework base substitutions were identified by dideoxy chain termination method using fluorescently labeled dideoxy nucleotides (Pharmacia Biotech, Uppsala, Sweden) on an automated DNA sequencer (ALFexpress DNA Sequencer, Pharmacia Biotech) using the primer 5'-AA TCA TTC GTC TGT TTC CCA-3'.

Results

All carriers of Hb Lepore had a clinical phenotype of thalassemia trait. Their MCV and MCH values were reduced (*Table I*). Hb A_2 level was normal or decreased, while Hb F was slightly increased. Hb analysis by electrophoresis on cellulose acetate revealed the presence of an abnormal Hb fraction, later characterized as Hb Lepore by PCR analysis. Heterozygous carriers of β° 39 mutation had evident anemia, but unusually mild microcytosis and hypochromia for this type of mutation (β°). As expected, Hb A_2 and Hb F were markedly elevated (5% and 3.2%, respectively). All carriers of β^+ IVS-I-110 mutation were clinically asimptomatic. They presented with relatively mild anemia and moderately reduced MCV and MCH values. Both Hb A_2 and Hb F levels were slightly elevated.

In order to elucidate the origin of the most common thalassemic mutations in the population of Serbia, haplotype analysis of Hb Lepore BW, β° 39 and β^+ IVS-I-110 mutation carriers was performed. We have studied eight linked polymorphic restriction sites along β-globin gene cluster (*Figure 1*). In order to establish the haplotype of thalassemic chromosomes with certainty, nonaffected relatives were included in the study.

Four different haplotypes associated with β-thalassemia mutations were detected: two of them were typical of Mediterranean countries (I and V), and two autochthonous haplotypes, specific for Serbian population, were discovered as well (haplotypes A and B) (*Table II*).

Table I Hematological data (average values and standard deviations), Hb A_2 and Hb F values for β-thalassemia heterozygotes

Mutation	Gender	No.	Hb (g/L)	MCV (fL)	MCH (pg)	Hb A_2 (%)	HbF (%)
Hb Lepore (δβ fusion)	M	11	114.5 ± 9.02	66.2 ± 3.51	20.35 ± 1.50	1.95 ± 0.82	2.15 ± 0.60
	F	10	108.5 ± 5.22	66.0 ± 1.53	20.55 ± 1.75	1.84 ± 0.62	2.16 ± 0.51
Codon 39 (C→T)	M	7	109.0 ± 1.50	67.4 ± 3.90	22.6 ± 0.98	5.0 ± 0.25	4.20 ± 4.08
	F	7	91.0 ± 0.73	67.0 ± 0.33	22.1 ± 0.33	4.6 ± 0.50	3.87 ± 0.43
IVS I-110 (G→A)	M	5	128.5 ± 0.50	66.1 ± 1.66	20.8 ± 0.50	4.3 ± 0.15	2.33 ± 0.77
	F	5	126.0 ± 0.90	64.1 ± 3.35	20.9 ± 1.05	4.2 ± 0.20	2.30 ± 0.60

Table II Haplotypes detected in the healthy and thalassemic population of Serbia

	5'subhaplotype						3'subhaplotype	
	ε	5'Gγ	Gγ	Aγ	ψβ	3'ψβ	β	3'β
Haplotype I [4]	+	–	–	–	–	–	+	+
Haplotype V [4]	+	–	–	–	–	–	+	–
Haplotype A	+	–	–	+	–	–	+	–
Haplotype B	+	–	–	+	–	–	+	+

Polymorphic restriction sites: *Hinc*II/ε, *Xmn*I/5'Gγ *Hind*III/Gγ, *Hind*III/Aγ, *Hinc*II/ψβ, *Hinc*II/3'ψβ, *Ava*II/β, *Bam*HI/3'β

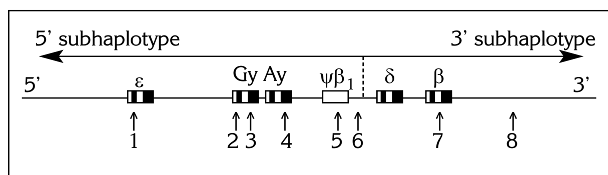

Figure 1. The location of eight polymorphic restriction sites in the β-globin gene cluster. Sites are numbered from 5' to 3': 1-*Hinc*II/ε, 2-*Xmn*I/5'Gγ, 3- *Hind*III/Gγ, 4-*Hind*III/Aγ, 5-*Hinc*II/ψβ, 6-*Hinc*II/3'ψβ, 7-*Ava*II/β, 8-*Bam*HI/3'β; 5' and 3' subhaplotypes are defined by restriction sites 1–6 and 7, 8, respectively.

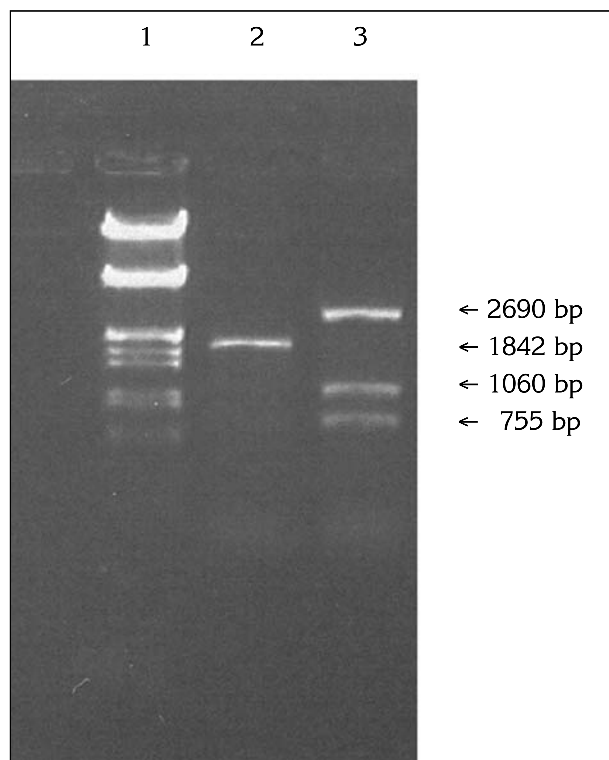

Figure 2. Detection of *Hind*III/Aγ polymorphic site by 1% agarose gel electrophoresis. 1. λ/*Hind*III/*Eco*RI DNA marker; 2. undigested PCR fragment; 3. homozygote for *Hind*III/Aγ polymorphism; 2690bp indicates pUC19 plasmid DNA digested with *Hind*III (control of the restriction enzyme activity)

All analyzed Lepore chromosomes were of the identical haplotype: haplotype A. Comparing this haplotype with Hb Lepore BW haplotypes reported to date showed that it differs from all of them. Thus, in this study a novel haplotype associated with Hb Lepore BW mutation was identified. The specificity of this haplotype is the presence of *Hind*III/Aγ restriction site (*Figure 2*).

Haplotype analysis revealed that β° 39 thalassemia mutation is mostly associated with haplotype I and framework 1. However, in one family, β° 39 chromosomes were found to be associated with framework 2. Two different haplotypes were associated with β^+IVS-I-110 thalassemic mutation: haplotype I and haplotype B. Also, we have detected the presence of haplotypes I and V, as well as haplotypes A and B specific for Serbian population, in normal betaA/betaA individuals.

Discussion

The results presented in this paper may help the clinicians as a guideline for differential diagnostics of thalassemia trait. The hematological values of particular importance are: MCV lower than 70fL and MCH lower than 24pg. If these values are consistently decreased (despite Fe supplementation therapy) in anemic patient, hemoglobin electrophoresis is recommended, followed by DNA analysis.

Haplotype analyses we present in this paper have been carried out on β-thalassemia carriers of common Mediterranean mutations (Hb Lepore BW, β° 39, β^+IVS-I-110). A number of similar studies have already provided insights into origin and distribution of these mutations. Beta-globin gene haplotypes associated with thalassemic mutations were sporadically studied in former Yugoslavia, too (13). However, chromosomal background of β-thalassemia mutations in Serbian population demonstrates specificity, not previously reported.

In this paper we present a novel haplotype associated with Hb Lepore BW gene. Hb Lepore BW is common in Mediterranean countries. Up to date, 6 haplotypes associated with Hb Lepore BW chromosomes were identified: haplotype V in 34 families of Spanish, Yugoslavian and Italian origin (14–16), haplotype I in 23 Italian families (15), haplotypes VI and VII in Italian families (15) and two unique haplotypes (in a Hungarian individual and in one Black family) (15). A multicentric origin of this mutation has been suggested (16). In order to elucidate the origin of Hb Lepore BW mutation in Serbia, the 5' and 3' subhaplotypes were taken into consideration as independent entities (see *Figure 1*). We have detected two different 5' subhaplotypes: the first one, typical for both I and V haplotypes, and the second, autochthonous 5' subhaplotype present in both haplotypes A and B. There were also two 3' subhaplotypes: the one typical

for haplotype I and the other typical for haplotype V (see *Table II*).

All Serbian Hb Lepore BW genes are associated with autochthonous 5' subhaplotype and 3' subhaplotype typical for haplotype V. We have previously shown that they are also associated with framework 2 (17). The Hb Lepore BW gene associated with haplotype V and framework 2 was virtually the only type found in Eastern Italy and former Yugoslavia (13, 16). Considering the fact that two different haplotypes containing autochthonous 5' subhaplotype (A and B) as well as haplotype V were detected in healthy Serbian population, two possible hypotheses could explain the origin of Hb Lepore BW mutation on a novel chromosomal background.

There is a possibility that unequal recombination or gene conversion occurred between two chromosomes with haplotypes A or B and Hb Lepore mutation associated with haplotype V, respectively, producing new chromosomal background for Hb Lepore mutation. The fact that the probable site of recombination could have been in the 9,1 kb segment, which has been defined by Chakravarti *et al.* (18) as a hot spot region for nonuniform recombination in β-globin gene cluster, supports this hypotesis.

However, the mutation might have arisen *de novo*, by a single independent mutational event in a healthy individual with haplotype A and spread in the population. The high frequency of Hb Lepore BW hemoglobin variant in Serbian population, the homogeneity of Hb Lepore BW haplotype (no haplotype V was detected), as well as its uniqueness, suggest its independent origin. The finding that Hb Lepore BW chromosomes are concentrated in particular geographical region of Serbia also supports this hypothesis.

It has been proposed that $\beta^{\circ}39$ mutation originates from Italy (19). Our data suggest Western Mediterranean (Italian) origin of this mutation in Serbia. Chromosomal background of $\beta^{\circ}39$ mutation in our population is similar to the one found in other Mediterranean countries. However, in one family $\beta^{\circ}39$ chromosomes are found to be associated with framework 2. Although mutation spread almost never involves a change of β-globin gene framework, the exception has been detected (19). To the best of our knowledge, this is the first report of the association between framework 2 and $\beta^{\circ}39$ mutation. This example of mutation spread from one beta-globin gene framework to another, within a population, may be due to inter-allelic gene conversion events or recurrent mutation (19).

β^{+}IVS-I-110 mutation is previously reported to be of Eastern Mediterranean (Turkish) origin and associated with haplotype I (20). In this study, β^{+} IVS-I-110 mutation is found to be associated with two different haplotypes. Actually, these two haplotypes differ only in their 5' subhaplotypes: one is typical for haplotype I, and the other is 5' subhaplotype from haplotypes A or B. The likelihood that this haplotype dimorphism associated with the same β-thalassemia mutation is a consequence of random mutation is very low. The diversity of haplotypes associated with β^{+} IVS-I-110 mutation, would then be generated by recombination events (crossing-over or gene conversion) between the original β-thalassemic chromosome (haplotype I) and the 5' subhaplotype characteristic for normal population of Serbia.

Although thalassemia syndromes are sporadic disorders in Serbia, they reflect numerous historically documented migrations over Balkan Peninsula and yet show autochthonous features.

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BIOHEMIJSKI FENOTIP I POREKLO TRI NAJČEŠĆE BETA-TALASEMIJSKE MUTACIJE U SRBIJI

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Kratak sadržaj: Molekularna (DNK) karakterizacija talasemija je najpouzdaniji metod za dijagnostiku ove grupe oboljenja. Kako su talasemije vrlo heterogene, hematološki podaci i dodatne biohemijske analize su od esencijalne važnosti za njihovu diferencijalnu dijagnostiku. U ovom radu su prikazani hematološki parametri i biohemijske karakteristike nosilaca tri najčešće β-talasemijske mutacije u Srbiji (Hb Lepore, β°39 i β^{+}IVS-I-110) koje je neophodno uzeti u obzir u početnom koraku postavljanja dijagnoze kod talasemičnih pacijenata. Pored toga, ovaj rad predstavlja detaljnu analizu diverziteta haplotipova β-globinskog lokusa kod nosilaca najčešćih β-talasemijskih mutacija i zdravih betaA/betaA osoba u Srbiji. Identifikovan je novi haplotip asociran sa Hb Lepore-Boston-Vašington genom u srpskoj populaciji. Ovi podaci idu u prilog hipotezi o multicentričnom poreklu ove mutacije. Mutacija je nastala *de novo* u hromozomskom kontekstu koji je karakterističan za populaciju u Srbiji.Takođe je pokazano da su dve najčešće mediteranske mutacije, β°39 i β^{+}IVS-I-110, verovatno »uvezene« u populaciju Srbije iz Italije, odnosno Turske, istorijskim migracijama i seobama naroda.

Ključne reči: β-talasemija, haplotip, Hb Lepore

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EFFECT OF THYROXINE ON GLUTATHIONE-DEPENDENT ANTIOXIDANT ENZYME ACTIVITIES AND GLUTATHIONE CONTENT IN THE INTERSCAPULAR BROWN ADIPOSE TISSUE OF DIFFERENT MATURATED RATS

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Summary: Effect of thyroxine on glutathione-dependent antioxidant enzyme activities and glutathione (GSH) content in the interscapular brown adipose tissue (IBAT) of different aged rats were studied. Male Mill Hill hybrid hooded rats aged 15, 45 and 75 days were treated with L-thyroxine, T_4 (40 μg/100 g body mass), s.c., one dose per day, 14 days (finally aged 30, 60 and 90 days, respectively). Effect of T_4 on GSH-dependent antioxidant enzyme activities in the IBAT differs with respect to age. T_4 treatment gradually decrease activities of all GSH-dependent antioxidant enzymes in 60 and 90 days old rats in comparison to young ones. GSH content in animals of 30 and 60 days old rats are lower in comparison with 90 days old rats, but the effects are oppposite. L-thyroxine treatment significantly increase GSH content in 30 days old rats ($p<0.001$) in respect with coresponding controls, while decrease in 60 and 90 days old animals were detected ($p<0.01$). Different response of non-mature rats to thyroxine comparing to older rats could be attributed to the difference in thyroxine metabolism and developmental phase of regulatory physiological systems maturation including antioxidative.

Key words: thyroxine**,** glutathione-dependent antioxidant enzymes, glutathione, interscapular brown adipose tissue, rats

Introduction

Thermogenesis is the major function of interscapular brown adipose tissue (IBAT) which is found in small mammals. Thyroid hormone (TH) is essential for normal development in vertebrate species. Normal thyroid gland activity is concerned mainly with energy metabolism in nearly all tissues of the body. TH is a major regulator of energy homeostasis with hyperthyroidism increasing basal metabolic rate and body temperature. The development of a hyperthyroid state in vertebrates elevates basal metabolic rate due to increments in the rate of O_2 consumption in target tissues (1) an effect accomplished by both (a) short-term mechanism activating mitochondrial cytochrome *c* oxidase and (b) long-term pathway involving changes in nuclear and mitochondrial gene expression through 3,3,5-triiodothyronine signaling. In the latter mechanism, respiratory genes may be upregulated through liganded TH receptor which binds to a TH-responsive element in the promoter regions (2). In IBAT, as well as in other aerobic tissues acceleration of aerobic metabolism by thyroxine enhances the generation of reactive oxygen species (ROS) in mitochondrial and microsomal sites (3). These conditions determine a higher consumption of cellular antioxidants (4, 5) and inactivation of antioxidant enzymes (6) thus inducing oxidative stress (7) with the concomitant increase in lipid peroxides and protein oxidation (1).

The data concerning changes in the amount of low molecular antioxidants such as glutathione (GSH), (8, 9), as well as the activity of antioxidative enzymes in different hyperthyroid rat tissues were investigated by Petrović et al. (10), Asayama et al. (8), Mijalković (11), Saičić et al. (12, 13).

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GSH represents a major non-enzymatic antioxidant and the most abundant non-protein thiol source in the cell (14, 15). GSH serves as a substrate for glutathione peroxidase (GSH-Px) and glutathione-S-transferase (GST) and under physiological conditions, glutathione reductase (GR) will rapidly reduce any oxidized glutathione (GSSG) to reduced form (GSH). Glutathione has several major functions: it detoxifies ROS under normal and impaired homeostasis, detoxifies drugs and maintains an essential thiol status of proteins and other molecules and provides the main molecular form in which cysteine can be stored within the organism and used for transfer between tissues (16, 17).

TH are known to act directly on multiple sites in vertebrate cells, but it seems that mitochondria are the most striking target. IBAT is very rich with mitochondria and potentially important source of free radicals.

In the present work we examine the effect of L-thyroxine, T_4 on total glutathione content (GSH, reduced + GSSG,oxidized) and glutathione-dependent antioxidant enzyme activities: glutathione peroxidase (GSH-Px, EC 1.11.1.9), glutathione-S-transferase (GST, EC 2.5.1.18) and glutathione reductase (GR, EC 1.6.4.2.) in IBAT of different aged rats, 30, 60 and 90 days.

Materials and Methods

The experiments were carried out with the male *Mill Hill hybrid hooded* rats. Animals were housed from birth to 30th day of age near by their mothers. After 30 days they were transfered to individual cages (four animals per cage). All animals were held under controlled conditions of illumination (lights on: 5 a.m.-5 p.m.) and temperature (23 °C) and were allowed free access to water and food. Animals at the 15th, 45th and 75th day of age were treated with L-thyroxine, T_4 (40 *mg* dissolved in 9 mmol/L NaOH/100 g body mass), s.c., one dose per day, during the next 14 days before sacrificing (finally aged 30, 60 and 90 days, n=31) as performed earlier by Wooten and Cascarino (18). The study was performed using double control group protocol. One control group was consisted of non-treated (intact) animals (n=29). The second control group received 9 mmol/L NaOH/100 g body mass, the same way as T_4 treated animals (n=26).

All animals were sacrificed by decapitation always between 8 and 10 a.m. to avoid any possible rhytmic variations in the antioxidant enzyme level. Immidiately after the decapitation IBAT were extracted and washed out with saline solutions (154 mmol/L NaCl). Homogenization was performed with a Janke and Kunkel (Staufen,Germany) Ika-Werk Ultra-Turrax homogenizer at 0–4 °C in 0.25 mol/L sucrose, 1 mmol/L EDTA and 0.05 mol/L TRIS-HCl solution, pH 7.4 (19, 20). The homogenates were sonicated for 30s at 10 kHz on ice to release enzymes (21) and used to determine the content of total glutathione (GSH + GSSG). The remaining sonicates were centrifuged (90 min, 85000 × g, 4 °C) and the supernatant was used for GSH-dependent antioxidant enzyme activity assays and total protein determination. All chemicals were Sigma (St. Louis, MO, U.S.A.) products.

GSH-Px activity was assayed using t-butyl hydroperoxide as substrate (22, 23) and the activity was expressed in nanomoles of NADPH oxidized/min/mg protein. For the determination of GST activity, 1-chloro-2,4-dinitro benzene (CDNB) was used as a substrate (24) and the activity was expressed in nmol GSH used/min/mg protein. GR activity was measured as suggested by Glatzle et al. (25) and expressed in nmol oxidized NADPH/min/mg protein. For the GSH assay sonicated samples were deproteinized by 10% sulfosalycilic acid (2:1, v/v) and centrifuged 10 min on 3020 × g. Content of total GSH (GSH,reduced + GSSG,oxidized) was determined by enzymatic method suggested by Tietze (26) as modified by Griffith (27) and expressed as nmol GSH/g wet mass. All GSH-dependent antioxidant enzyme assays were performed at 25 °C and expressed as specific activity (units per mg protein) and as total activity (units per g wet mass). Protein content was measured by the method of Lowry et al. (28) using bovine serum albumin as a reference.

Statistical analysis was performed using protocols suggested by Hinkle et al. (29). In experimental design here applied treatment was performed on different maturated rats, thereby the effects were statisticaly analyzed considering two factors: treatment and age using two-way analysis of variance (two-way ANOVA).

Results

The GSH-dependent antioxidant enzyme activities after T_4 treatment were presented in *Table I* (activity expressed both per mg protein – as specific and per g wet mass – as total). Statistical data are presented in *Tables II* and *III*.

GSH-Px specific activity was significantly inreased ($p<0.01$) in T_4 treated 90 days aged rats (33.3 ± 3.2) in comparison with corresponding controls (22.4 ± 2.0). At the same time, the activity of GSH-Px in 30 and 60 days aged rats did not show any differences between compared groups (*Table I*). On the other hand, when activity of GSH-Px expressed both as specific and as total (*Table I*) decrease during development in rats (significant age effect- (A); $p<0.001$; *Tables II* and *III*). This effect is evident in 30 days aged rats in respect to old ones.

We observed the similar effect in GST activity which is not statistically significant, but shows the

Table I The activities of glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST) and glutathione reductase (GR) in the IBAT of 30, 60 and 90 days old rats treated with L-thyroxine (T_4), internal controls (Ki) and controls (K). Enzyme activities are expressed in units per mg protein as specific and in units per g wet mass as total. The results are presented as Mean ± SD.

	30 days			60 days			90 days		
	Controls (K)	Internal Controls (Ki)	Treatment (T_4)	Controls (K)	Internal Controls (Ki)	Treatment (T_4)	Controls (K)	Internal Controls (Ki)	Treatment (T_4)
Specific activity									
GSH-Px	34.4 ± 16.4	44.0 ± 14.3	44.2 ± 14.5	12.8 ± 4.2	22.4 ± 7.7	14.0 ± 2.9	22.4 ± 4.4	23.4 ± 6.9	33.3 ± 6.5
GST	53.9 ± 20.9	54.3 ± 15.3	53.5 ± 13.1	31.3 ± 7.7	41.1 ± 11.2	37.3 ± 7.2	36.6 ± 3.4	36.5 ± 7.7	43.7 ± 11.3
GR	113.5 ± 38.3	98.9 ± 29.0	91.7 ± 34.8	50.5 ± 5.9	51.8 ± 16.3	51.1 ± 9.4	61.1 ± 8.5	69.4 ± 5.9	60.4 ± 8.7
Total activity									
GSH-Px	548 ± 305	880 ± 625	873 ± 313	576 ± 223	940 ± 181	424 ± 91	807 ± 178	798 ± 201	1244 ± 264
GST	966 ± 532	951 ± 603	1072 ± 338	1380 ± 255	1586 ± 345	1133 ± 257	1314 ± 32	1304 ± 239	1681 ± 607
GR	1749 ± 714	1740 ± 748	1632 ± 311	2156 ± 252	2078 ± 720	1523 ± 135	2281 ± 152	2392 ± 236	2240 ± 260

Table II Two-way analysis of variance (ANOVA) for GSH-dependent antioxidant enzyme activities in rats of different age treated with T_4 or corresponding controls. Results are presented as specific activity and expressed in units per mg protein. Df – degree of freedom, MS – mean square. *** p < 0.001

		(A)	(T)	A × T	Error
GSH-Px	Df	2	2	4	65
	MS	3935	353	138	139
	F	28.4***	2.54	1.00	
GST	Df	2	2	4	62
	MS	2304	106	78.9	175
	F	13.2***	0.61	0.45	
GR	Df	2	2	4	71
	MS	20656	329	512	692
	F	29.9***	0.48	0.74	

Table III Two-way analysis of variance (ANOVA) for GSH-dependent antioxidant enzyme activities and GSH content in rats of different age treated with T_4 or corresponding controls. Results are presented as total activity and expressed in units per g wet tissue. Df – degree of freedom, MS – mean square. *** p < 0.001 * p < 0.05

		Age (A)	Treatment (T)	A × T	Error
GSH-Px	Df	2	2	4	65
	MS	403970	335015	375831	119516
	F	3.38*	2.8	3.14*	
GST	Df	2	2	4	62
	MS	1392245	33731	295041	179740
	F	7.75***	0.19	1.64	
GR	Df	2	2	4	74
	MS	2072760	542947	233355	260631
	F	7.95***	2.08	0.9	
GSH	Df	2	2	4	36
	MS	44867	10605	44686	1194
	F	37.6***	8.88***	37.4**	

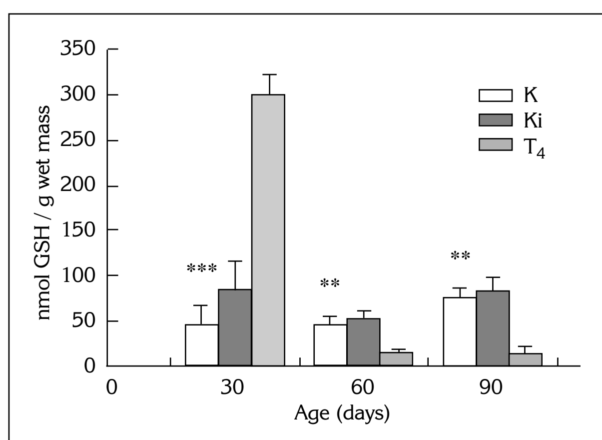

Figure 1. Glutathione content (GSH) in the IBAT of 30, 60 and 90 days old rats treated with L-thyroxine (T_4), internal controls (Ki) and controls (K) expressed in nmol GSH per g wet mass. Columns represent mean values and vertical bars are S.E.M. ** p < 0.01 *** p < 0.001

same trend, e.g. decreasing during development (significant age effect – (A); p<0.001; *Tables II* and *III*).

At the same time, specific GR activity appeared to be lower (p<0.05) in T_4 treated 30 days aged rats (91.7 ± 9.3) in comparison with the corresponding controls (113.5 ± 10.6). GR activity also decrease (p<0.001) during development in rats (significant age effect - (A), *Tables II* and *III*).

The effect of treatment is presented in *Tables II* and *III* as (T) effect. Treatment is strongly age dependent (interaction A × T, in *Tables*) only in GSH content (*Tables II* and *III*; *Figure 1*).

Our results showed, that T_4 treatment had opposite effects on GSH content. While in 30 days old animals we found a significant increase (p<0.001) in

GSH content between T_4 treated (300.7 ± 20) and corresponding controls (45.7 ± 5.9) in 60 and 90 days old rats we found decrease in GSH content ($p<0.01$). Detailed analysis of grand means reveiled, that T_4 direct effects should be viewed as clear change of T_4 treated animals in comparison with control and T_4 disolving buffer treated animals. Furthermore, animals at 30 days of age responded different to treatment in respect to old animals. These results suggest endogenous developmental pathern of antioxidant enzyme expression which could be modified by external factors.

Discussion

From experimental studies, as well as an epidemiological data, it can be inferred that hyperthyroidism is associated with a general increase in tissue oxidative stress. On the other side, great controversy exists as to whether hyperthyroidism is associated with an increase or decrease in the activity of antioxidant enzymes (8, 30). It was shown, that antioxidative defence system is endogenous dynamic system incorporated in homeostatic regulation lead by internal regulatory signals (31).

ROS have been related with many physiological and pathophysiological processes. Under physiological conditions, it is estimated that approximately 80% of stationary oxygen uptake depends on mitochondrial respiratory chain activity (32). However, it's been shown that mitochondrial respiratory chain generates superoxide anion radical ($O_2^{\cdot -}$) at two different places, such as in the proximity of NADH dehydrogenase and of ubiqinon-cytochrome b. Superoxide anion radical is free radical species which is hydrogen peroxide precursor during its generation in mitochondria (33). In that regard, treatment with TH increase activity of several enzymes coupled with mitochondrial respiratory chain, content of cytochrome c, as well as the size and number of mitochondria. Therefore we may conclude, that TH influence on respiration in mitochondria by changing the concentration of some components in electron-transport chain, as well as redox state of its components (34–36).

BAT is anatomically distinct from white adipose tissue and is located in a number of regions of the body. It is particularly abundant in the interscapular, axillary and perirenal regions. The brown adipocyte contains several lipid droplets and the fat-free cytoplasm is occupied almost exclusively with mitochondria packed with cristae (37). Proliferation and hypertrophy of BAT occurs in response to increased thermic need (i.e. cold exposure). This growth is accompanied by increases in total protein, amount of mitochondria (37–39) and alteration in mitochondrial ultrastructure (40). The factors controlling brown fat proliferation are not clearly defined. Norepinephrine (or an intact sympathetic nervous system) is required, but is not a sufficient agent alone. Thyroid hormone is required as a permissive synergistic agent (41–43) for the BAT adaptive thermic response, but only at low concentrations (44, 45).

Therefore, we choose to examine the effects of induced hyperthyroidism on GSH-dependent antioxidant enzymes, as well as GSH content in the IBAT of different maturated rats, since this tissue, as we mentioned before, are rich with mitochondria and therefore might be significant source of ROS generation under T_4 stimulation.

Changes in GSH-Px and GST activity in 90 day old rats suggest that in maturated rats enzymatic antioxidant activities in the IBAT depend on age ($p<0.001$, *Tables II* and *III*) more than treatment itself. The slight rise in activity of GSH-Px and GST in 90 day old rats is followed with statistically significant decrease in total amount of GSH in T_4 treated animals compared with corresponding controls. This distinct fall in GSH content is age and treatment dependent ($p<0.001$, *Table III*).

There were no changes in GSH-Px and GST activity in 30 day old rats. On the other side, GSH content in 30 day old rats in T_4 treated animals were significantly higher than corresponding controls ($p<0.001$, *Table III*). The diminished levels of tissue GSH have generally been correlated with the covalent binding of xenobiotics to tissue macromolecules (46). The occurrence of lower concentrations of GSH in older animals can be explained by the following possibilities. Firstly, the actual loss of GSH may be a result of increased rate of oxidation due to higher consumption of oxygen an concomitant higher generation of hydrogen peroxide and hydroperoxides. Secondly, the diminished GSH concentration may be due to either increase degradation or decreased synthesis of GSH. In fact, activity of GSH-dependent antioxidant enzymes have been found to be higher in the T_4 treated animals in comparison to controls, which implies higher consumption of reduced GSH. In the same time, GR activity was not changed. Thirdly, the lower concentration of GSH may be due to increased utilization of GSH in the removal of lipid and other peroxides.

Also, we must take in consideration that during maturation there may be an accumulation of toxic substances which would elevate the activity of enzymes such as GSH-Px and GST, resulting in the intracellular depletion of reduced GSH. Higher concentrations of TH itself in T_4 treated animals, may induce their own removal by GSH-dependent antioxidant enzymes, particularly with GST.

On the other side, quite opposite effects were observed in 30 day old T_4 maturated animals. Statistically significant rise in GSH content ($p<0.001$, *Table III*) may be explained different in comparison to old rats. Elevated GR activity in T_4 treated 30 day

old rats is probably adaptive response to overall biochemical and physiological processes within the cells, rather than direct effect of antioxidaive regulatory elements. This suggests, increased turnover between GSSG and GSH and maintaining of stable redox environment. It has been postulated that redox environment obtained by redox couples is one of developmental determinant (47). One of cellular redox couples is GSH/GSSG and its optimal ratio is considered as developmental causal.

In this investigation, we have demonstrated that during maturation of rats T_4 treated animals exhibit a diminshed reducing potential. This observations suggests, that during the period of lower GSH concentrations in hyperthyroid rats IBAT becomes susceptible to oxidative damage due to higher generation of oxidative molecules such as H_2O_2, hydroperoxides etc. Thus, older animals could be at risk and more vulnerable to deleterious effects of hyperthyroid state.

It can be concluded from presented results, which under normal conditions there are a delicate balance between the rate of formation and the breakdown of ROS in the IBAT which is partially under the control of TH. Alteration in the thyroid state of the body influences the antioxidative defence in the IBAT and can lead to a pathophysiological state. Different response of non-mature rats to thyroxine comparing to older rats, could be attributed to the difference in thyroxine metabolism and developmental phase of regulatory systems maturation including antioxidative. Direct effects of T_4 on mature rats might be summarized as part of its overall catabolic role.

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EFEKAT TIROKSINA NA AKTIVNOST ANTIOKSIDACIONIH GLUTATION-ZAVISNIH ENZIMA I KOLIČINU GLUTATIONA U INTERSKAPULARNOM MRKOM MASNOM TKIVU PACOVA RAZLIČITE STAROSTI

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Kratak sadržaj: Ispitivan je efekat tiroksina na aktivnost antioksidacionih glutation-zavisnih enzima i količinu glutationa (GSH) u interskapularnom mrkom masnom tkivu (IBAT) pacova različite starosti. Mužjaci Mill Hill hybrid hooded pacova starih 15, 45 i 75 dana tretirani su sa L-tiroksinom, T_4 (40 mg/100 g telesne mase), s.c., jedna doza dnevno, tokom 14 dana (do finalne starosti 30, 60 i 90 dana). Efekat T_4 na aktivnost GSH-zavisnih antioksidacionih enzima u IBAT-u se razlikuje u odnosu na starost.Tretman sa T_4 smanjuje aktivnost svih GSH-zavisnih antioksidacionih enzima kod pacova 60 i 90 dana starosti u poređenju sa mladim jedinkama. Količina GSH kod životinja starih 30 i 60 dana je niža u poređenju sa pacovima starih 90 dana. Tretman tiroksinom značajno povećava količinu GSH kod pacova starih 30 dana ($p<0,001$) u odnosu na odgovarajuće kontrole, dok kod pacova starosti 60 i 90 dana izaziva smanjenje ($p<0,01$). Različit odgovor ne-maturiranih pacova na tiroksin u odnosu na maturirane životinje može se pripisati razlikama u metabolizmu tiroksina i razvojnoj fazi regulatornih fizioloških sistema uključujući i antioksidacioni.

Ključne reči: tiroksin, glutation-zavisni antiksidacioni enzimi, glutation, interskapularno mrko masno tkivo, pacovi

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INFLAMMATORY RESPONSE IN RHEUMATOID ARTHRITIS

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Summary: The aim of the research is to determine the clinical significance of cytokines TNF-alpha, IL-12, IL-15 and IL-18 in evaluation of the activity of rheumatoid arthritis (RA). By comparing the concentrations in 30 patients with high, 14 patiens with moderate and 20 patiens with mild activity of RA it is established that the patients with high degree of disease activity, have significantly high ($p<0.01$; $p<0.05$) concentrations of examined cytokines and rheumatoid factor in blood and synovial fluid as well as C-reactive protein in serum in relation to patients with moderate and mild active disease. We have concluded that the cytokines concentrations can be good indicators of the degree of the general activity of RA. This research can contribute to interpretation of insufficiently well known views of pathogenesis role of cytokines in active disease.

Key words: cytokines, arthritis, rheumatoid factor, C-reactive protein

Introduction

Rheumatoid arthritis (RA) is chronic inflammatory disease of unknown etiology and multifactor pathogenesis, of changing clinical course and unpredictable prognosis in which base is the progressive destructive synovitis and the impairment of the joint structure (1). Symmetric pains and swelling of small peripheral joints, long-term morning stiffness, general symptoms, the occurrence of rheumatoid nodules, rheumatoid factor (RF) in blood and characteristic radiological changes in joints are the primary clinical features of the disease. The appearance of extraarticulary manifestations, autoimmunoglobulin antibodies and immune complexes in the circulation with activation of the complement system imply to systemic nature of the disease, which justifies the term rheumatoid disease (2). The most epidemiological, pathophysiological, immunological and genetic aspects of the disease are well known, but the precise starter and factors, which maintain the destructive inflammatory process, still haven't been determined. Because of that it is not unusual to claim that RA is the disease with many causes or many diseases with one cause (2, 3).

Immunopathological process in synovial tissue develops in five phases (4): in the first phase the presentation and recognition of the unknown antigen is being done by T lymphocyte; in the second phase occurs the proliferation of T and B lymphocytes, migration of inflammatory cells in to the joint and angiogenesis. The cells components of the joint react to the injury by changing the functional profile. Changes occur in endothelium of small blood vessels and hyperplastic reaction of synovia with proliferation of synovial cells – the third immunological phase of the disease. In the fourth phase of the disease the panus is formed. It is metabolically active and autonomous joint tissue. Chondrocytes and osteoclasts are activated. Cytokines interleukine (IL)-1, the tumor necrosis factor-alpha (TNF-alpha), interferon-gamma (INF-gamma) and many others, which are produced by macrophages and activated T cells, stimulate synovial cells to create hydrolytic enzymes, proteinases. The initial damage of the joint occurs. In the fifth immunological phase many mediators such as prostaglandins, activated components of complement, proteases, free oxygen and nitrogen radicals bring to the definitive destruction of joint cartilage, bones and surrounding structures (5).

The tumor necrosis factor-alpha and IL-1 have almost identical biological activity, mutually stimulate

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production and act synergistic in the induction of inflammatory reaction (6, 7). Proinflammatory effects IL-18, IL-15 and IL-12 have been examined and demonstrated in experimental conditions *in vitro* and *in vivo* with mice on model of arthritis induced by collagen (8–13). According to us available literature great number of research with patients with RA has not been conducted. It is unknown if the production of cytokines in blood, and especially in synovial fluid (SF) is connected with the severity of RA and if their levels can reflect the disease activity, to imply to progression and foresee the course of the disease. These have just been the basic motives to conduct the research with the aim to determine clinical significance of cytokines for the evaluation of activity of rheumatic arthritis. In this region such research has not been done.

Methods

In four year period, total of 89 patients have been analyzed, 64 patients with RA, newly formed or in the phase of deterioration of the disease. The diagnosis of all patients has been set according to revised criteria for classification and diagnosis of RA (ARA/ACR, 1987). We have analyzed clinical manifestations of patients (P group) and have grouped the in relation of the disease activity in three groups: patients with highly active RA (HiA), 30 patients, patients with moderately active RA (MoA), 14 patients and patients with mildly active RA (MiA), 20 patients. The control group was consisted of 25 subjects with the arthritis of the knee during the deterioration of osteoarthrosis (OA), for whom we have presumed not to have deranged immunological parameters in blood and SF. The total evaluation of activity of rheumatic arthritis is determined on the basis of total number of swollen joints (SJ), total number of tenderness joints (TJ), values on visible analogical scale for pain (VAS) and rapidity of erytrocite sedimentation rate (ESR) by means of is calculated index Disease Activity Score 28 (DAS 28), numerical indicator of the degree of disease activity (14). High disease activity have represented the given values DAS >5.1, moderate >3.2, and mild disease activity have showed given values of DAS>2.6. In both examined groups the analyzes of the samples of serum (S) and SF has been done and the concentrations of C-reactive protein (CRP) (mg/L) have been determined by immunonephelometry method, IgM-RF (IU/mL) Latex nephelometriy (DADE Behring, Germany) and concentrations of cytokines (pg/mL) TNF-alpha, IL-12, IL-15 and IL-18 immunoenzymatic (ELISA) methods by using kits for humane interleukines (R&D, USA). The value of ESR (mm/h) was determined by standard laboratory procedure according to Westergreen.

For evaluation of statistical significance of difference between characteristics of observation in control group and group of patients with RA Mann-Whitney U test was administered. For comparing between groups of patients divided according to the degree of activity both Kruskal-Waliss test and Mann-Whitney U tests were used. The level of significance in all administered methods was on in the limit of 0.05.

Results

Comparing the number of subjects in control group and group of patients with RA in relation to sex, showed that there is highly significant difference, caused by larger number of female patients in the group of patients with RA ($p<0.01$) (*Table I*).

In groups with highly and moderately active RA examined patients have had significant increase of serum values RF in relation to the group of patients with mild case of RA ($p<0.01$) (*Table II*).

Activity of RA in the group of patients was determined and numerically presented on the basis of values of DAS 28 index. Clinical manifestations have been compared between the two groups of examinees, and between groups of HiA, MoA and MiA, by comparing clinical parameters, individual factors of DAS: average values of number of tenderness joints, swollen joints and average values ESR and VAS.

Values of all examined clinical parameters (SJ, TJ, VAS, DAS), have high significantly differentiated between the groups of patients with different activity of RA, with the highest values in HiA group, smaller in MoA and the least in the group of patients with mild RA activity (*Table III*). The examined subjects in control group have had significantly lower ($p<0.01$) average values clinical parameters in relation to patients with RA (results are not show).

Table I General characteristics of the study population

Characteristics	Group	
	Control	Patients
Number patients (% of total number examiness)	25 (28)	64 (72)
Age (yr.)	59.2	57.8
Female	15	45
Male	10	19
Mean duration of disease (m.)	45.2	74.4
Mean duration of presently hardships (m.)	5.4	6.0

Table II The division of the group of patients in relation to disease activity and seropositivity

Group	P		HiA		MoA		MiA	
	N	%	N	%	N	%	N	%
Number of »seropositive« pat.	57	89	30	100	14	100	13	65
Number of »seronegative« pat.	7	11	0	0	0	0	7	35
Total number patients	64	100	30	100	14	100	20	100

Table III Clinical parameters values in groups of patients with RA in relation to disease activity

Group	HiA	MoA	MiA	
Parameters	$\bar{x} \pm SD$	$\bar{x} \pm SD$	$\bar{x} \pm SD$	p
TJ	9.8 ± 6.1	4.2 ± 0.9	2.4 ± 0.5	0.00**
SJ	8.4 ± 5.7	2.8 ± 0.7	1.8 ± 0.6	0.00**
ESR	78.0 ± 24.9	47.4 ± 10.7	27.2 ± 11.3	0.00**
VAS	70.3 ± 13.8	31.4 ± 4.6	12.0 ± 5.7	0.00**
DAS	10.1 ± 5.2	4.7 ± 0.5	2.9 ± 0.1	0.00**
* p < 0.05; ** p < 0.01				

Table IV Comparing of mean values CRP and RF in serum and synovial fluid and ESR between control group and group of patients with RA

Group		CRP (mg/L)	CRP (mg/L)	RF (IU/mL)	RF (IU/mL)	SE (mm/h)
		S	SF	S	SF	
Patients	Mean	43.95	25.41	489.96	316.14	55.5
	SD	38.72	21.26	546.71	327.82	29.3
Control	Mean	6.67	4.77	14.08	12.36	14.8
	SD	7.15	4.46	10.02	6.98	9.6
z		5.79	5.74	6.22	5.95	6.44
p		0.00**	0.00**	0.00**	0.00**	0.00**
* p < 0.05; ** p < 0.01						

Table V Cytokines concentrations (TNF-alpha, IL-18, IL-15, IL-12) in the serum samples and synovial fluid of the control group and group of patients with RA

Group		TNFα (pg/mL)		IL-18 (pg/mL)		IL-15 (pg/mL)		IL-12 (pg/mL)	
		S	SF	S	SF	S	SF	S	SF
Patients	Mean	5.59	13.96	156.69	281.11	1.77	10.39	11.80	10.52
	SD	3.53	19.36	90.39	317.23	3.03	9.78	8.09	7.58
Control	Mean	2.32	3.45	89.68	112.18	0.59	3.34	4.92	6.08
	SD	0.80	1.22	17.23	84.83	0.20	1.17	2.14	2.11
z		4.66	5.18	4.40	3.24	3.88	5.13	4.77	3.73
p		0.00**	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**	0.00**
* p < 0.05; ** p < 0.01									

Comparing mean values of CRP, RF and ESR between control group and group of patients with RA, indicated significant differences in the concentrations CRP, RF and values ESR (p<0.01). RF and CRP values were higher in the samples of synovial fluid and samples of RA patients' serums as well as ESR values (*Table IV*).

Comparing mean values of TNF-alpha, IL-18, IL-15, IL-12 has showed that the values of these cytokines are significantly higher (p< 0.01) in serum samples and synovial fluid of the patients with RA in relation to samples of the control group (*Table V*).

Average serum values of CRP were the highest in HiA group, significantly lower (p<0.01) in MoA group and the lowest (p<0.01) in MiA group. Average values of CRP in synovial fluid were the highest in MoA group, then in MiA group while the lowest were in the group with the highest RA activity, but without any significant differences (*Figure 1*). Mean values of RF in serum samples and in the synovial fluid samples were highest in HiA group, significantly lower in MoA group (p <0.01) and again significantly the lowest in MiA group (p<0.01) (*Figure 2*).

Comparing the mean values of cytokines of the patients with RA according to disease activity indicates that with all observed parameters exist highly statistically significant differences (p<0.01), which appear because mean concentrations of cytokines were always higher in HiA group in relation to MoA and MiA groups. Only for the IL-12 concentrations in syno-

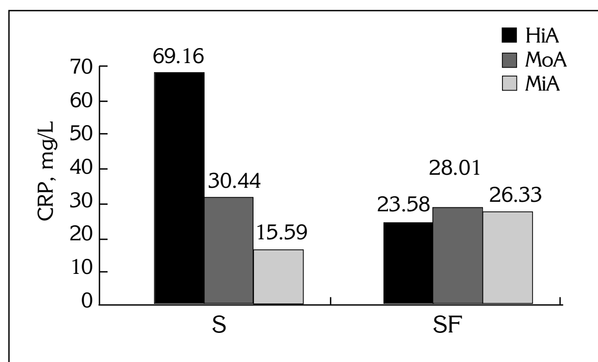

Figure 1. Concentrations of CRP in serum and synovial fluid samples of the patients according to degree of RA activity

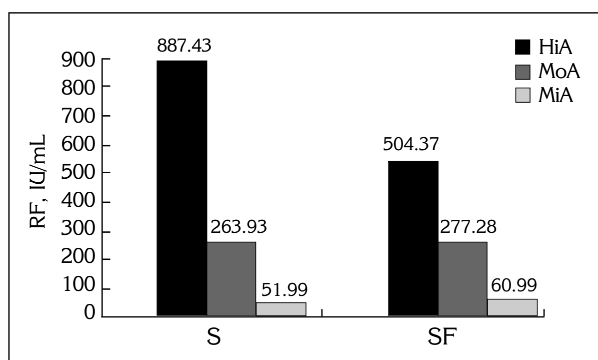

Figure 2. Concentrations of RF in serum and synovial fluid samples of the patients according to degree of RA activity

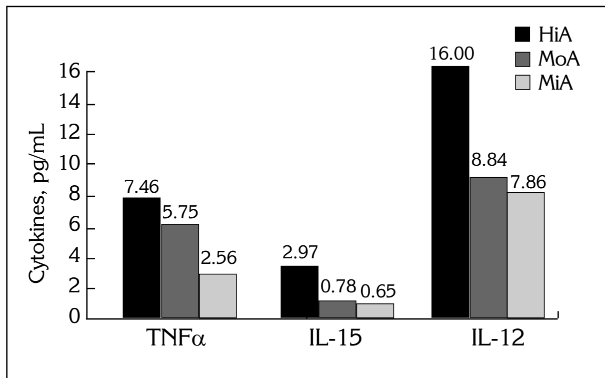

Figure 3. Concentrations of TNF alpha, IL-15 and IL-12 in serum samples of patients according to the degree of RA activity

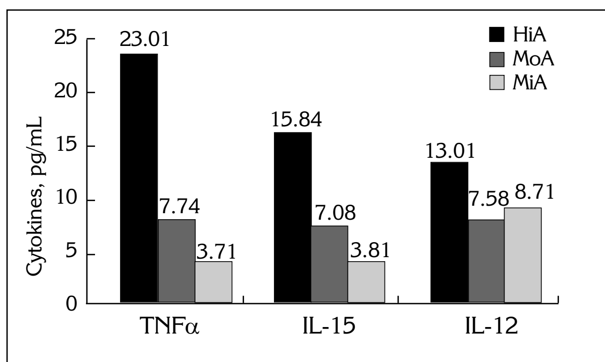

Figure 4. Concetrations of TNF-alpha, IL-15 and IL-12 in synovial fluid samples of patients according to the degree of RA activity

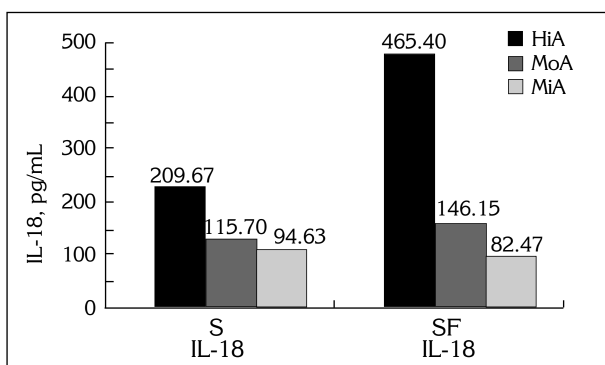

Figure 5. Concentrations of IL-18 in serum and synovial fluid samples of patients according to the degree of RA activity

vial fluid the difference was statistically significant ($p<0.05$) (*Figure 3–5*).

Intergroup comparisons between three groups according disease activity within the group of patients with RA, has showed the existence of statistically significant difference in all cases and concentration of cytokines in serum and synovial fluid have always been the highest in HiA and the smallest in MiA. Only situations when the intergroup differences haven't been significant ($p>0.05$) are with TNF-alpha in serum between HiA and MoA, IL-12 in serum and in synovial fluid where the difference haven't been significant between HiA and MoA.

Discussion

In pathogenesis of RA TH1 immune response prevails. TH1 cells create special profile of cytokines, which together with products of synovicites disturbs natural balance in cytokines net inside the synovial tissue. It leads at last to immunoinflmmatory reaction and joint damage. Many up today researches showed that IL-12 has major role in the differentiation of TH1 cells response, by stimulating differentiation of T cell's precursor (TH0) in TH1 phenotype. In the arisen TH1 reaction IL-12 increases the proliferation of T cells and production of IFN-gamma and other T cells cytokines in synergism with Il-18. Two cytokines create synergistic effect by reciprocal increase expression of T cells receptor and by different mechanism of IFN-gamma genetic expression (on transcription level). They are produced by synovial macrophages. Lately in synovial tissue of patients with RA has been discovered the IL-15, mediator with pleitropic effects on many immune system cells. In RA IL-15 activates T cells and stimulates their intercells contact with macrophages. That's how it indirectly induces large synthesis of TNF-alpha and other cytokines of the macrophages. With the help of IL-15 activated synoivial T cells secrete TNF-alpha directly. Interleukine-15 stimulates the proliferation of T cells, induces the expression of adhesive molecules and has significant effect of chemotaxis. It stimulates the proliferation of B cells with the consequential production of immunoglobulin including RF. It intensifies the cytotoxicity of natural killer cells, activates neutrophiles and stimulates the differentiation of osteoclasts and angiogenesis. Interleukine-15 is produced mainly by T cells and macrophages.

The discovery of new proinflammatory cytokine IL-18 is added to the list of mediators which stimulate activity and development of TH1 cell response with ability to induce the production of IFN-gamma in T cells and NK cells. Synergistic effect with IL-12 and IL-15 in the induction of IFN-gamma defines it as the member of the cytokine family, which induce TH1 (IFN-gamma, IL-2, IL-12 and IL-15). Interleukine-18 is the member of IL-1 family, because it shows the structural and functional similarity with this cytokine. It also owns pleiotropic effects and the role in innate and acquired immunity. Interleukine-18 is synthesized by macrophages, dendritical cells, chondrocytes, osteoclasts, keratinocytes, Kupffers cells, adrenal gland cortex cells and pituitary gland cells. The presence of IL-18 is proved in many diseases in human population, but his role in stimulating or repression the disease is

different. In RA it contributes to the development of TH1 response and induces the IFN-gamma and TNF-alpha synthesis in T and NK cells with consequential stimulation of production and secretion of proinflammatory cytokines of monocytes. The most important role of IL-18, pleiotropic proinflammatory cytokine in synovitis is most probably to facilitate and enable the creation of IFN-gamma dominant T cell response, which is induced by IL-12. There will be necessary further research to establish how much is the real contribution of IL-18 in process of immunopatogenesis of RA. The dilemma about the positive and negative effects of IL-18 in inflammation and synovitis and if there is a need to block or not to his effects because of the potentially positive effects still remains. These and other unknown facts invite us for further researches concerning IL-18.

In our research first was done the analyze of clinical manifestations of diseases inside the group of the patients with active RA, by comparing several clinical parameters which form DAS (average values of the number of tenderness joints and number of swollen joints and average values of ESR and VAS). Patients in the group with highly active disease had significantly higher number of tenderness and swollen joints in relation to group with moderate and mild activity of RA. The mean values of ESR and VAS of patients with the most severe case of the disease were significantly higher in relation to mean values in groups with lower degree of RA activity. Average values of DAS index of RA patients were 6.7. The highest DAS value was in HiA group (10.1) while the patients in MoA group (4.7) and MiA (2.9) had significantly lower DAS values. Patients with RA were divided according to the degree disease activity measured by values of DAS index, were significantly different in relation to the severity of clinical manifestations of the disease.

Between the patients with RA and OA as a control group and between the groups of patients divided according to degree of disease activity inside the group of patients with RA we have compared concentrations of TNF-alpha, IL-18, IL-15 and IL-12 in the samples of S and SF. The average values of all 4 cytokines were high significantly different between the control group and group of patients with RA. Patients with erosive arthritis have higher average concentrations of examined cytokines in blood as well in fluid of joint in relation to examinees with OA. This indicates that pathophysiological mechanism and the degree of inflammatory reaction is different in RA and OA. We have confirmed that TNF-alpha, IL-18, IL-15 and IL-12 have significant role in pathogenesis of rheumatoid synovitis.

Comparing the results of patients with different degree of RA activity significant difference was achieved. Average concentrations of TNF-alpha, IL-18, IL-15 and IL-12 in S and SF were highly and statistically different between the groups of patients with high, moderate and mild active RA. Patients with high active RA had higher average concentrations of all examined cytokines in serum and synovial fluid in relation to patients with moderate and mildly active RA. When we want to look at the intergroup comparisons between groups of HiA and MiA patients, high and statistically significant differences were established in cytokines concentrations, except for the average values of IL-12 in S ($p=0.62$) and SF ($p=0.43$). Concentrations of TNF-alpha of HiA and MoA patients' serum have also been approximately equal ($p=0.11$), while for other examined parameters highly significant differences came up. Comparing mean values of cytokines concentrations between HiA and MiA groups, highly and statistically significant differences came up. Higher cytokines concentrations in more severe disease cases and their lower values in the disease of the lesser activity indicate that levels of TNF-alpha, IL-18, IL-15 and IL-12 in S and in SF influence clinical manifestations and severity of RA and can be good indicators of general activity of RA as well as parameters to evaluation of the disease severity. We can expect high values of cytokines in S as well in SF in the patients with active RA. We have concluded that the concentrations of cytokines can predict with great accuracy the severity of RA. Concentrations of TNF-alpha, IL-18 and IL-15 in SF reflect the degree of general activity of the disease and severity better than their serum values.

In active disease in laboratory analyzes acute phase parameters of inflammation CRP, fibrinogen and speeded ESR were usually increased. For now there isn't specific biohumoral indicator of disease activity, except of RF. On the basis of comparison of concentrations of CRP and RF in serum and synovial fluid, we have tried to estimate if there is general and local activity of the disease. Patients in HiA group had higher average concentrations of CRP and RF in S and RF in SF in relation to MoA and MiA patients. It implies that RF concentrations in blood and ST and CRP concentrations in S were good indicators of the general activity degree and severity of the disease. Local concentrations of CRP in the knee joint haven't showed any difference among patients groups and it isn't the system inflammatory reaction indicator.

Our research has indicated to the importance of determining several cytokines concentrations in blood and synovial fluid in patients with active RA and can contribute to explanation of insufficiently known views of role in pathogenesis and significance of TNF-alpha, IL-12, IL-15 and especially IL-18 cytokines in the active disease. This research confirmed that examined cytokines could be indicators of the degree of RA activity of the same value and specific quality as up to now used standard biochemical parameters. Concentrations of cytokines can be useful for the early assessment of disease activity. The research can serve as the basis for further studies and evaluation of the administered therapy's effect.

INFLAMATORNI ODGOVOR U REUMATOIDNOM ARTRITISU

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Kratak sadržaj: Cilj istraživanja je da se utvrdi klinički značaj citokina faktora nekroze tumora-alfa (TNF-alfa), interleukina (IL)-12, IL-15 i IL-18 u proceni aktivnosti reumatoidnog artritisa (RA). Poređenjem koncentracija kod 30 bolesnika sa visoko, 14 sa umereno i 20 bolesnika sa blago aktivnim RA utvđeno je da bolesnici sa visokim stepenom aktivnosti oboljenja imaju značajno veće ($p<0,01$; $p<0,05$) koncentracije ispitivanih citokina i reumatoidnog faktora u krvi i sinovijskoj tečnosti kao i C-reaktivnog proteina u serumu u odnosu na bolesnike sa umereno i blago aktivnim oboljenjem. Zaključili smo da koncentracije citokina utiču na težinu RA i mogu biti dobri pokazatelji stepena opšte aktivnosti RA. Rad može pružiti doprinos razjašnjenju nedovoljno poznatih stavova o patogenetskoj ulozi i značaju određivanja IL-18, IL-15, IL-12 i TNF-alfa u aktivnoj bolesti.

Ključne reči: citokini, artritis, reumatoidni faktor, C-reaktivni protein

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THE BASIC HAEMATOLOGICAL MEASUREMENTS IN PERIPHERAL BLOOD FROM WORKERS EXPOSED TO MERCURY VAPOURS

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Summary: In the present study was assessed the influence of occupational exposure to mercury vapours on the basic haematological parameters (erythrocyte, leukocyte and platelet count, haemoglobin concentration, haematocrit, MCV, MCH and MCHC). Studies were carried out on 138 workers involved in the production of chlorine using the mercuric electrolysis method (divided into three groups: permanently, periodically and earlier exposed to mercury vapours), as well as on 38 healthy workers. The shift time - weighted averages for mercury was determined in the workplace air before research; mean value was significantly over maximum tolerated dose. The mercury content in the blood and urine of exposed workers was determined by atomic absorption spectrophotometry. In all three groups 95th percentile values of mercury in blood and urine are significantly over MTD. Peripheral blood cell parameters were determined using an automatic cell counter. In the group exposed to mercury vapours, was found a statistically significant increase of erythrocyte count with a concomitant decrease in MCV. The mean values of haemoglobin concentration, MCHC and platelet count were higher in the group of workers exposed to mercury vapours, but the difference was not statistically significant. There were no significant differences in haematocrit, MCH and leukocytes between the studied groups. Our results indicate that long-term and permanent exposure to mercury vapours induces changes in the important haematological parameters of the peripheral blood.

Key words: mercury, haematological parameters

Introduction

A large group of workers exposed to mercury vapours may be found in the chlor alkali industry, where chlorine and caustic soda are produced using the electrolysis of brine in mercury cells. A typical mercury cell, 10–20 m^2, may contain up to 3 tons of mercury, and there are often about 100 mercury cells at a plant. Although the process is closed, leakage of mercury occurs as a result of technical faults, and also during repairs and maintenance. The workers of these plants are exposed to mercury mainly in the form of vapour but to some degree also as the dust of mercury salts (1).

It is relatively easy to diagnose mercurialism with an acute or subacute course by such symptoms as metallic taste in the mouth, excessive salivation, gingivitis, stomatitis, diarrhoea and nephritis. CNS symptoms include nervousness, timidity and uncontrolled fierceness. It is much more difficult to detect the initial symptoms of intoxication showing gradual, yet deepening changes in various metabolic processes. At the beginning, they consist of the elimination of the microelements necessary for life (based on competitive interactions) such as Fe, Ca and Zn (2). A recent review of world literature collected by Moszczynsky (3) irrefutable shows the strong, negative influence of mercury on the immune system.

The aim of this investigation was to determinate (on easily accessible material such as the blood of workers exposed to mercury vapours) changes concerning the basic haematological measurements.

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Subjects and Methods

Subjects

The study has been performed on 138 workers involved in the production of chlorine using the mercuric electrolysis method, which were divided into three groups:

Group I, GI – workers (N = 33, average age 36.4 ± 7.21 yrs) permanently exposed to mercury vapours for 8.4 ± 4.7 yrs;

Group II, GII – workers (N = 87, average age 33.9 ± 7.60) periodically exposed to mercury vapours for 7.7 ± 5.3 yrs;

Group III, GIII – workers (N = 18, average age 42.3 ± 5.8 yrs) earlier (minimum 5 years ago) exposed to mercury vapours for 10.3 ± 4.9 yrs.

Control group, CG – (N = 38, average age 31.8 ± 9.4 yrs) consisted of healthy workers, not exposed to mercury vapours.

Methods

The metallic mercury concentrations in workplace air were determined using Analyser of mercury vapour »Jerome instrument corporation S« . The mercury content in the blood and urine of workers was determined by atomic absorption spectrophotometer »Perkin-Elmer«. Blood samples were obtained from fasting subjects together with blood samples for determination complete blood counts. The eight haematological parameters were determined using haematological analyser »Coulter counter«.

The results were subjected to statistical analysis by analysis of variance and Student-t test.

Results

Mercury in blood and urine

The air for the determination of the mercury concentrations was taken in different areas of the workplace on morning and during work time. The shift time – weighted averages determined for mercury in the workplace air before research were 0.045 ± 0.072 mg/m^3 (0.006 to 0.293 mg/m^3); mean value was significantly over maximum tolerated dose, MTD (0.01 mg/m^3).

Maximum tolerated dose for mercury in blood is 0.175 μmol/L and in urine 0.1 μmol/L. Workers in group permanently exposed to mercury vapours have mean value of mercury in blood close to MTD, but mean in urine are significantly over MTD (*Table I*). Mean values in blood in two other groups are bellow MTD, mean value in urine in group periodically exposed is slightly higher than MTD and in group earlier exposed is bellow MTD. In all three groups 95th percentile values of mercury in blood and urine are significantly over MTD.

Mercury concentration in blood and urine of workers significantly correlated with mercury concentration in workplace air: blood: $r = 0.415$, $y = 0.0094 + 0.382x$; urine: $r = 0.474$, $y = 0.069 + 0.354x$.

Haematological parameters

There were no significant differences in haematocrit, MCH and leukocytes between the studied groups (*Table II*).

Erythrocytes count was significantly higher in the group permanently exposed to mercury vapours, 4.74 × 10^{12}/L vs. 4.47 × 10^{12}/L in the control group ($t = 2.09$, $p = 0.04$). The other two groups of workers were not statistically different from the control group (*Figure 1*).

There was substantial drop in the mean corpuscular volume (MCV) value from 102.0 fL in the control group to 97.4 fL in the group permanently exposed to mercury vapours ($t = 2.044$, $p = 0.044$). The values in the group of workers periodically exposed to mercury vapours were slightly higher than the values in the other groups with extremely high 95th percentile value (*Figure 2*).

The mean values of haemoglobin concentration, MCHC and platelet count were higher in the group of workers permanently exposed to mercury vapours, but the difference was not statistically significant.

Table I Mercury concentrations in blood and urine of control group and workers exposed to mercury vapours

Group	Blood (μmol/L)		Urine (μmol/L)	
	mean ± SD	5th–95th P	mean ± SD	5th–95th P
CG	0.009 ± 0.0080	0–0.022	0.015 ± 0.033	0–0.078
GI	0.172 ± 0.123	0.055–0.457	0.379 ± 0.388	0.069–1.017
GII	0.102 ± 0.087	0–0.237	0.103 ± 0.098	0.006–0.280
GIII	0.087 ± 0.093	0–0.202	0.045 ± 0.053	0.006–0.132

Table II The values of haematological parameters in the studied groups

Group	Haemoglobin (g/L)		Erythrocytes count ($\times 10^{12}$)	
	mean ± SD	5th – 95th P	mean ± SD	5th – 95th P
CG	141.6 ± 15.86	111.9 – 160.1	4.47 ± 0.61	3.58 – 5.40
GI	148.0 ± 12.05	124.0 – 160.0	4.74 ± 0.46	3.97 – 5.50
GII	144.7 ± 11.98	125.7 – 160.0	4.54 ± 0.49	3.84 – 5.37
GIII	145.0 ± 15.79	125.6 – 160.0	4.68 ± 0.60	3.63 – 5.41
Group	Haematocrit (L/L)		MCV (fL)	
	mean ± SD	5th – 95th P	mean ± SD	5th – 95th P
CG	0.45 ± 0.045	0.39 – 0.50	102.0 ± 9.91	88.9 – 116.7
GI	0.46 ± 0.029	0.40 – 0.49	97.4 ± 9.27	85.7 – 112.3
GII	0.46 ± 0.029	0.41 – 0.50	103.0 ± 11.99	89.3 – 122.2
GIII	0.46 ± 0.042	0.39 – 0.50	100.2 ± 9.76	87.2 – 114.4
Group	MCH (pg)		MCHC (g/L)	
	mean ± SD	5th–95th P	mean ± SD	5th – 95th P
CG	31.8 ± 1.49	28.4 – 35.4	313.2 ± 22.07	275.5 – 350.0
GI	31.3 ± 2.45	28.0 – 36.0	323.0 ± 22.38	293.1 – 351.2
GII	32.1 ± 3.74	27.6 – 37.8	311.9 ± 23.14	278.9 – 354.5
GIII	31.2 ± 2.90	27.7 – 35.4	311.6 ± 18.33	281.4 – 340.4
Group	Leukocytes ($\times 10^9$)		Platelet ($\times 10^9$)	
	mean ± SD	5th – 95th P	mean ± SD	5th – 95th P
CG	7.50 ± 1.96	4.8 – 11.1	219.4 ± 33.0	169.7 – 281.0
GI	7.58 ± 1.57	4.8 – 9.8	240.3 ± 43.5	171.8 – 300.0
GII	7.52 ± 1.98	5.0 – 10.3	236.6 ± 42.4	166.3 – 296.8
GIII	7.60 ± 2.00	5.0 – 10.9	234.3 ± 39.9	178.5 – 290.0

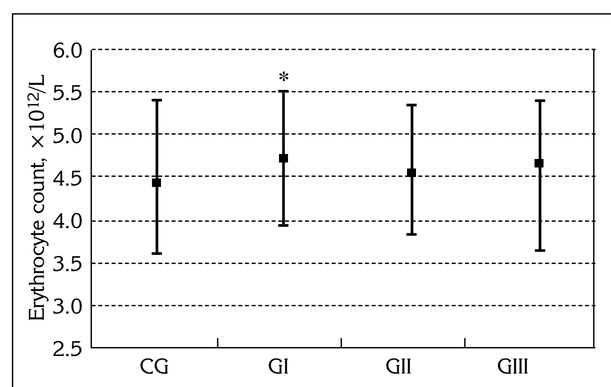

Figure 1. Mean value and 5th and 95th percentile values of erythrocyte count in control group and in groups of workers exposed to mercury vapours; * $p < 0.05$

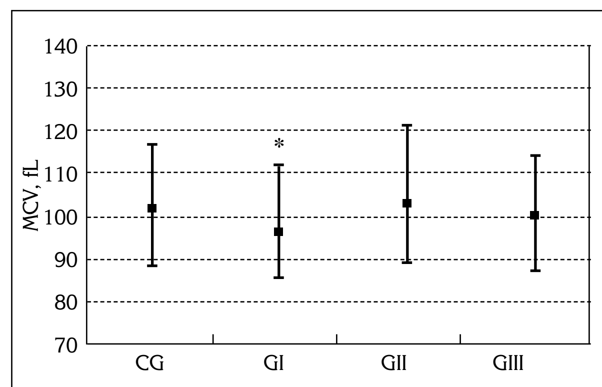

Figure 2. Mean value and 5th and 95th percentile values of MCV in control group and in groups of workers exposed to mercury vapours; * $p < 0.05$

Discussion

Elemental mercury has been detected in the urine of workers exposed to mercury vapour. The occurrence of trace amounts of elemental mercury is considered to be due to reduction of mercuric ion by certain types microorganisms present in the urine and direct urinary excretion following glomerular filtration of elemental mercury persisting in the blood. But determinations of the inorganic species of mercury in urine samples can provide information on both recent exposure and the body borden resulting from chronic exposure to mercury vapour (4). Our results support this assertion. Almost all workers have had increase values of mercury in urine.

Data collected for all studied groups, i.e. groups exposed to mercury vapours and control subjects are generally contained within normal range. However, when we compared group of workers permanently exposed to mercury vapours with control subjects, the mean value of erythrocyte count was significantly higher and mean value of MCV was significantly lower in the group permanently exposed to mercury. The

quantitative health status results obtained after treatment with toxic compounds or heavy metals where studied parameters fall below the physiological values are relatively easy to explain. It is not easy to comment results obtained in the present study. One possible explanation is that concerning differences in the level of physical exercise, which is higher in the group permanently exposed to mercury than the control group consisting of not physically hardworking people. It is known that physical exercise (6–8 h) increases loss of water from the body, which entails a »thickening« of the peripheral blood (5, 6). The blood »thickening« is accompanied by increased water reapsorption in the renal tubules and increased oxygen consumption by the renal tissue, resulting in local hypoxia which increases the erythropoietin synthesis. That effect is revealed by the increased number of erythrocytes (7).

The decrease of MCV in the group permanently exposed to mercury vapour may result from the diminished iron incorporation into erythrocytic cell lineage, as indicated by increased values of serum ferritin, transferrin and TIBC (8, 9). However, in further studies on cases of mercury intoxication, the level of Cu as well as cerulloplasmin activity in the serum should be taken into account. Copper deficiency is frequently the cause of decrease in MCV and MCHC (10).

Our results indicate that long-term and permanent exposure to mercury vapours induces changes in the important haematological parameters of the peripheral blood, but explanation that phenomenon requires further, broader research.

VREDNOSTI OSNOVNIH HEMATOLOŠKIH PARAMETARA U PERIFERNOJ KRVI RADNIKA IZLOŽENIH UTICAJU PARA ŽIVE

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Kratak sadržaj: U radu je ispitivan uticaj izloženosti parama žive iz radne sredine na osnovne hematološke parametre (broj eritrocita, leukocita i trombocita, koncentraciju hemoglobina, hematokrit, MCV, MCH i MCHC). U studiju je uključeno 138 radnika koji rade u proizvodnji hlora procesom elektrolize (podeljeni su u tri grupe: stalno izloženi, povremeno izloženi i ranije izloženi uticajima para žive), kao 38 zdravih radnika. Pre studije su određene koncentracije žive u vazduhu radne sredine; srednje vrednosti su bile znatno iznad maksimalne doze tolerancije (MTD). Koncentracije žive u krvi i urinu radnika koji su izloženi živi određena je atomskom apsorpcionom spektrometrijom. U sve tri grupe je vrednost 95-tog percentila bila znatno iznad MTD. Hematološki parametri su određeni uz pomoć automatskog brojača. U grupi koja je stalno izložena parama žive, dobijeno je značajno povećanje srednje vrednosti broja eritrocita, kao i značajno smanjenje vrednosti MCV. Srednje vrednosti koncentracije hemoglobina, MCHC i broja trombocita u grupi radnika koji su stalno izloženi uticaju žive bile su viće nego u kontrolnoj grupi, ali ta razlika nije statistički značajna. Između posmatranih grupa nije bilo značajne razlike u vrednostima hematokrita, MCH i broja leukocita. Dobijeni rezultati pokazuju da dugotrajna i stalna izloženost parama žive može da dovede do promena u vrednostima važnih hematoloških parametara.

Ključne reči: živa, hematološki parametri

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NOVI PROTOKOL ZA LABORATORIJSKO ISPITIVANJE PACIJENATA SA KALKULOZOM URINARNOG TRAKTA

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Kratak sadržaj: Kalkuloza urinarnog trakta je bolest koja je još uvek značajan socio-medicinski problem zbog visoke stope recidiva (50–100% u nelečenih pacijenata), kao i mogućnosti pojave hronične bubrežne slabosti (u 20% pacijenata). Iz tih razloga je pravilna klinička evaluacija pacijenata od neprocenjivog značaja. U cilju efikasnijeg i racionalnijeg pristupa u utvrđivanju mogućih uzroka nastanka kamena u urinarnom traktu i izbora najbolje terapije i mera prevencije važno je sprovođenje laboratorijskih ispitivanja prema inoviranom Protokolu koji je usaglašen sa preporukama Evropskog udruženja urologa (EAU) iz 2003. god. U zavisnosti od hemijskog sastava kamena, kliničke kategorije bolesti kao i specifičnih faktora rizika, program ispitivanja može biti: *minimalan* (analiza prvog jutarnjeg uzorka urina i urinokulture; određivanje kalcijuma, albumina, kreatinina i mokraćne kiseline u serumu i analiza sastava kamena) i *proširen* (kvantifikacija svih relevantnih parametara za nastanak kamena i procena bubrežne funkcije u serumu i 24-oro časovnom urinu (ispitivanjem dva uzastopna uzorka urina). Diferencijalna dijagnoza utvrđenog metaboličkog poremećaja, zasniva se na određivanju parathormona u serumu, cAMP u urinu i testova opterećenja kalcijumom, amonijum hloridom i [$^{13}C_2$] oksalatom.

Ključne reči: kalkuloza urinarnog trakta, metabolički poremećaj, protokol laboratorijskog ispitivanja, testovi opterećenja

Uvod

Kalkuloza urinarnog trakta je veoma često oboljenje sa prosečnom prevalencijom od 4–10% (1) i godišnjom incidencijom od 0,5% u zemljama Evrope (2). Od ove bolesti nije pošteđeno nijedno geografsko područje, etnička grupa niti jedno životno doba. Klinički je najmanifestnija između treće i šeste decenije života. U osnovi stvaranja kamena je proces kristalizacije u prezasićenom urinu. On nastaje kao rezultat poremećene ravnoteže između promotera (kalcijum, okslat, mokraćna kiselina i cistin) čiji je stepen ekskrecije u urinu povećan i inhibitora (Mg, citrat, pirofosfat i glikozaminoglikani) čiji je nivo ili inhibitorni potencijal u urinu snižen. Udružena promena pH vrednosti i zapremine urina dovode do povoljnih uslova za stvaranje novih kristala, agregata kristala i velikih kristalnih čestica koje u kasnijem toku prerastaju u vidljiv konkrement (3). Rezultati *in vitro* istraživanja (4, 5) su potvrđeni i u kliničkim studijama, koje su pokazale značajno snižene vrednosti inhibitora kristalizacije u urinu osoba sa kalkulozom u poređenju sa zdravima (6–9). Prisustvo infekcije izazvane mikroorganizmima koji razlažu ureu, poremećaji u morfologiji urinarnog trakta (stenoze, dilatacije, oštećenja urotela) i protoka urina (staza, refluks) takodje deluju kao faktori rizika za stvaranje kamena ili pojavu njegovog recidiva.

U proteklih nekoliko decenija načinjen je značajan napredak u lečenju ove bolesti primenom manje invazivnih metoda – ekstra i intrakorporalne dezintegracije kamena. Međutim, rizik od pojave recidiva ostaje i dalje visok, 50–100% u nelečenih i 10–15% u lečenih bolesnika (10). Novostvoreni kamen u urinarnom traktu značajno otežava kliničku sliku i evoluciju bolesti jer povećava mogućnost razvoja arterijske hipertenzije, a u 20% bolesnika i razvoj hronične bubrežne slabosti (11). Visok procenat recidiva, pored toga što je terapijski problem, ukazuje i na nepotpuno utvrđenu etiologiju bolesti kao i na neadekvatnu primenu mera prevencije. Zbog navedenog, pravilna klinička evaluacija pacijenata je od neprocenjivog značaja.

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Tabela I Kliničke kategorije kalkuloze urinarnog trakta

KAMEN KOJI NE SADRŽI KALCIJUM	
Infekcijom izazvan kamen (Infection stone)	INF*
Kamen mokraćne kiseline i njenih soli (Uric acid/sodium, ammonim urate stone)	UR
Cistinski kamen (Cystine stone)	CY
KALCIJUMSKI KAMEN	
Prva pojava kamena bez rezidualnih fragmenata (First time stone former without residual stone or stone fragments)	S^{o}
Prva pojava kamena sa rezidualnim fragmentima (First time stone former with residual stone or fragments)	S^{res}
Recidivna kalkuloza umerene težine bez rezidualnih fragmenata (Recurrent stone former with mild disease without residual stone)	$R^{m\text{-}o}$
Recidivna kalkuloza umerene težine sa prisutnim fragmentima (Recurrent stone former with mild disease witht residual stone)	$R^{m\text{-}res}$
Recidivna kalkuloza teškog stepena sa ili bez rezidualnih fragmenata (Recurrent stone former with severe disease with or without residual stone)	R^{s}
Kalkuloza+specifični faktori rizika (Stone forming patient with specific risk factor)	Risk

* Međunarodne skraćenice prema preporukama EAU-a

Tabela II Specifični faktori rizika za pojavu kamena urinarnog trakta

Početak bolesti pre 25. godine života
Prisustvo fosfata u hemijskom sastavu kamen
Jedan funkcionalan bubreg
Udružene bolesti: hiperparatireoidizam, hipertireoza, renalna tubularna acidoza, Crohn-ova bolest, jejuno-ilealni by-pass, stanja malapsorpcije, sarkoidoza
Unošenje visokih doza: kalcijuma, vitamina D, C, sulfonamida, triamterena i Indinavir-a.
Morfološke promene urinarnih puteva praćene zastojem mokraće ili veziko-ureteralnim refluksom (urođene ili stečene).

U cilju što efikasnijeg i racionalnijeg pristupa u utvrđivanju mogućih uzroka nastanka urinarnog kamena i/ili pojave njegovog recidiva, važno je, pre svega, utvrditi kojoj kliničkoj kategoriji pripada pacijent. Na osnovu poznatog hemijskog sastava kamena kao i određenih parametara koji ukazuju na težinu bolesti Odbor za zdravstvenu zaštitu Evropskog udruženja urologa (EAU) je 2003. god. preporučio kliničku kategorizaciju pacijenata sa kalkulozom, koja omogućava ciljanu primenu dijagnostičkih protokola i adekvatniju terapiju (12, 13), (v. *Tabelu I*).

Postoji više parametara koji se javljaju kao faktori rizika za pojavu recidiva. Na osnovu stepena njihovog prisustva može se predvideti težina bolesti i pojava recidivne kalkuloze. Koristeći analizu specifičnih faktora rizika pacijent se može pravilnije svrstati u određenu kategoriju bolesti (*Tabela II*).

Cilj ovog rada je da prikaže novi Protokol za laboratorijsko ispitivanje pacijenata sa kamenom urinarnog trakta u nas koji je usaglašen sa preporukama Evropskog udruženja urologa iz 2003. godine, kao i da se ukaže na korist od njegovog uvodjenja kao standardnog postupka u kliničku praksu.

Protokol laboratorijskih ispitivanja

Ispitivanje pacijenata sa kalkulozom urinarnog trakta sprovodi se u ambulantnim i/ili bolničkim uslovima (kada ne postoji mogućnost adekvatnog transporta 24h uzoraka urina do laboratorije, kao i u slučajevima kada se radi o proširenom programu ispitivanja).

Optimalno vreme za započinjanje ispitivanja je najmanje mesec dana od prestanka bubrežne kolike ili posle uklanjanja kamena. Protokol se ne nastavlja i u slučajevima kada pregled jutarnjeg uzorka urina pokaže hematuriju ili piuriju.

U zavisnosti od hemijskog sastava kamena, kliničke kategorije bolesti kao i prisustva specifičnih faktora rizika, program ispitivanja može biti *minimalan* i *proširen*.

Minimalan program ispitivanja obuhvata:

1. Pregled prvog jutarnjeg uzorka urina
   - pH, eritrociti, leukociti, nitriti (test trakama)
   - Određivanje specifične težine (test traka/urometar)
   - Pregled sedimenta urina (kristalurija)
2. Urinokultura

Tabela III Granične vrednosti parametara u urinu i serumu

Parametri	24^h urin	Serum
pH (dnevni profil)	< 5,8 ili >6,8	
Specifična težina	>1,010 g/cm^3	
Zapremina	< 2,0 L	
Kalcijum	>5,0 mmol/dU	2,0–2,5 mmol/L
Fosfat	>35 mmol/dU	0,81–1,29 mmol/L
Mokraćna kiselina	>4,0 mmol/dU	119–380 µmol/L
Magnezijum	<3,0 mmol/dU	
Citrat	<2,5 mmol/dU	
Oksalat	>0,5 mmol/dU	
Cistin	>0,8 mmol/dU	
Kreatinin	7,0–13,0 mmol/L (ž) 13,0–18,0 mmol/L (m)	25–100 µmol/L

3. Pregled seruma: kalcijum, albumin, kreatinin, mokraćna kiselina
4. Analiza kamena

Ovaj program ispitivanja se sprovodi kod svih kategorija bolesnika sa kalkulozom urinarnog trakta, a na osnovu analize parametara indikuju se dalja ispitivanja. Vrednosti pH urina veće od 5,8 ukazuju na mogućnost postojanja renalne tubularne acidoze a vrednosti pH preko 7,0 na urinarnu infekciju. Pozitivan nalaz na test trakama za leukocite i nitrite ukazuje takođe na prisustvo infekcije. Ukoliko pregled sedimenta urina pokaže povećan broj eritrocita, moguća je mehanička lezija mukoze urinarnih puteva usled prisustva kamena, infekcije ili glomerulonefritisa. Povećan broj LE (više od 5) u sedimentu urina takođe ukazuje na infekciju. Vrednosti kalcijuma i mokraćne kiseline u serumu iznad gornjih granica referentnih vrednosti postavljaju sumnju na hiperparatireoidizam i hiperurikemiju i zahtevaju dalja metabolička ispitivanja (*Tabela III*).

Analiza kamena se radi posle njegove spontane eliminacije ili posle primenjene terapije – dezintegracije ili operacije. Prema preporukama Odbora za zdravstvenu zaštitu EAU, referentne metode za ispitivanje sastava kamena su infracrvena spektroskopija ili difrakcija x-zracima.

Proširen program ispitivanja

Ovaj se program sprovodi kod pacijenata koji pripadaju kliničkim kategorijama komplikovane kalkuloze (S-res,R m-res,R-s i Risk, *Tabela I*).

Pored svih analiza navedenih u *minimalnom programu ispitivanja*, neophodan je i pregled dva uzorka 24^h urina koji se sakupljaju pri uobičajenom režimu ishrane i unosu tečnosti i uzorci se čuvaju na hladnom mestu (+4 °C).

UZORAK I – sakuplja se bez konzervansa ili uz prethodni dodatak 10 mL 5% rastvora timola u izopropanolu (za svaku bocu od 2,0 L)

Određuje se: 1. zapremina urina

2. urea, kreatinin, albumin, mokraćna kiselina, kalijum, fosfat, magnezijum i citrat

UZORAK II: sakuplja se uz prethodni dodatak 15–30 mL 6 mol/L rastvora HCL (za svaku bocu od 2,0 L)

Određuje se: 1. zapremina urina

2. kalcijum i oksalat

Zakišeljavanje urina je neophodno da bi se sprečilo taloženje soli kalcijuma (u formi oksalata i fosfata) kao i oksidacija askorbinske kiseline u oksalat. Urea, kreatinin, mokraćna kiselina, K, Ca, i fosfat u serumu određuju se na dan donošenja prvog 24^h uzorka urina, čime je omogućeno odredjivanje klirensa kreatinina. Pored navedenih parametara, koji su obavezni, određuju se Na i Cl u serumu i u urinu prvog uzorka koji uz vrednosti uree, fosfata i K ukazuju na navike u ishrani.

Određivanje navedenih parametara u urinu je moguće i iz uzoraka koji se sakupljaju u kraćim vremenskim intervalima (4 ili 6 sati). U tom slučaju se vrednosti prikazuju na gram kreatinina a ne na ukupnu diurezu.

Na osnovu seta parametara dobijenih analizom 24 h uzoraka urina moguće je izračunati indekse zasićenosti urina kalcijum oksalatom i kalcijum fosfatom (14) kao i prediktivni indeks rizika – PRI (15). Vrednosti ovih indeksa daju uvid u stepen poremećene ravnoteže između promotera i inhibitora kristalizacije. Takođe predviđaju mogućnost pojave recidiva, što je od značaja za kliničko praćenje pacijenata sa kalkulozom.

Kada vrednosti ispitivanih parametara odstupaju od referentnih, ispitivanje se ponavlja. Poznato je da na ekskreciju supstanci od značaja za stvaranje urinarnog konkrementa može da utiče i način ishrane bilo da prikrije, smanji ili poveća postojeći metabolički poremećaj. Iz tih razloga, protokol proširenog ispitivanja se ponavlja posle sprovođenja standardne dijete (2) u trajanju od tri dana, kada se u kontrolnim, bolničkim uslovima postiže stabilizacija metabolizma. Poređenjem vrednosti analiziranih parametara iz uzoraka urina I i II pre i posle dijete, moguće je otkriti one poremećaje u ekskreciji, na koje utiče način ishrane.

Ukoliko se ispitivani parametri u urinu vrate na normalne vrednosti posle standardne dijete uzrok po-

remećaja su nepravilnosti u ishrani. Održavanje vrednosti urinarnih parametara izvan referentnih granica ukazuje na metabolički poremećaj i zahteva sprovođenje testova opterećenja kalcijumom, amonijum hloridom ili test apsorpcije [$^{13}C_2$] oksalata.

Test opterećenja kalcijumom

Test opterećenja kalcijumom modifikovan prema Pak-u (17) izvodi se na sledeći način:

I dan: dijeta bez mlečnih proizvoda

18^h – večera

20^h unos 300 mL vode osiromašene kalcijumom

23^h unos 300 mL vode osiromašene kalcijumom

II dan: 07^h – uriniranje, zatim unos 600 mL vode osiromašene kalcijumom

$07–09^h$ prvi period sakupljanja urina (vrednosti posle gladovanja)

09^h – doručak (1 sendvič, puter, džem, 2 šolje voćnog čaja + 1 tbl od 1000 mg kalcijuma

11^h – unos 300 mL vode osiromašene kalcijumom

$09–13^h$ drugi period sakupljanja urina (vrednosti posle opterećenja)

U uzorcima urina I i II odredjuje se koncentracija kalcijuma (mmol/L) i kreatinina (mmol/L) i izračunava se indeks Ca/Cr.

Testom opterećenja kalcijumom omogućava se razlikovanje tri tipa hiperkalciurije i to:

1. *Apsorptivna hiperkalciurija* koja nastaje kao posledica povećanog stepena apsorpcije kalcijuma iz intestinalnog trakta. Ona može biti nezavisna od unete količine kalcijuma (tip I) i zavisna, kada se pojavljuje samo pri povećanom unosu kalcijuma (tip II).

2. *Renalna hiperkalciurija* koja je rezultat gubitka kalcijuma na nivou distalnih tubula usled poremećaja u njegovoj reapsorpciji. Tada se, zbog povećanog gubitka kalcijuma urinom javlja sekundarni hiperparatireoidizam.

3. *Resorptivna hiperkalciurija* koja je posledica primarnog hiperparatireoidizma koji se karakteriše značajnom resorpcijom kalcijuma iz koštanog tkiva i njegovom povećanom apsorpcijom iz intestinalnog trakta. Određivanje koncentracije PTH u krvi i cAMP u urinu olakšava diferencijalnu dijagnozu hiperkalciurije.

U 20% pacijenata i pored sprovedenog Protokola laboratorijskih ispitivanja uzrok hiperkalciurije ostaje neotkriven (*idiopatska hiperkalciurija*).

Tabela IV Indeks Ca/Cr

	Pre opterećenja	Posle opterećenja
Normalno	do 0,337	do 0,563
Apsorptivna hiperkalciurija	do 0,337	≥ 0,564
Renalna/resorptivna hiperkalciurija	≥ 0,338	≥ 0,564
Renalna hiperkalciurija: cAMP povećan		
Resorptivna hiperkalciurija: povećan PTH		

Test opterećenja amonijum hloridom

Indikacija za sprovođenje ovog testa je sumnja na postojanje renalne tubularne acidoze, tj. stanja neadekvatne sekrecije vodonikovih jona na nivou distalnih tubula. Sumnja na postojanje ovog oblika metaboličke acidoze postoji u slučajevima kada se pH vrednost urina ne snižavaju ispod 5,8 u toku nekoliko uzastopnih određivanja dnevnog profila pH (18).

Test se izvodi ambulantno, a pH vrednost urina se odredjuje isključivo pH-metrom, neposredno po sakupljanju svakog uzorka urina.

Test se izvodi na sledeći način:

08^h: doručak + 0,1g NH_4Cl/kg tt + 150 mL voćnog čaja
09^h: sakupljanje I uzorka urina i unos 150 mL voćnog čaja
10^h: sakupljanje II uzorka urina i unos 150 mL voćnog čaja
11^h: sakupljanje III uzorka urina i unos 150 mL voćnog čaja
12^h: sakupljanje IV uzorka urina i unos 150 mL voćnog čaja
13^h: sakupljanje V uzorka urina

Ukoliko se pH vrednosti urina snize do 5,4 isključuje se postojanje renalne tubularne acidoze, a ukoliko nisu ispod 5,4 da bi se dokazao tip renalne tubularne acidoze treba uraditi gasne analize krvi. Niske vrednosti bikarbonata (ispod 22 mmol/L) i pH krvi (ispod 7,35) ukazuju na kompletnu, dok normalne vrednosti ukazuju na inkompletnu renalnu tubularnu acidozu (v. *Tabelu V*).

Test apsorpcije [$^{13}C_2$] oksalata

Hiperoksalurija je važan faktor rizika u nastanku kalcijum oksalatne kalkuloze. Nastaje kao posledica naslednih poremećaja u metabolizmu oksalne kiseline (endogena ili primarna hiperoksalurija) ili usled povećanog unosa oksalne kiseline hranom, kao i zbog povećanog stepena apsorpcije pri različitim pa-

Tabela V Diferencijalna dijagnoza renalne tubularne acidoze

Biohemijski parametri	Kompletna	Inkompletna
pH krvi	snižen	normalan
Bikarbonati plazme	sniženi	normalni
Kalijum	snižen	normalan
Hloridi	povišeni	normalni
Ca i fosfor u urinu	povišeni	normalni
Citrat u urinu	snižen	snižen

tološkim stanjima u intestinalnom traktu (egzogena ili sekundarna hiperoksalurija).

Test apsorpcije [$^{13}C_2$] oksalata se upravo i izvodi kod pacijenata sa dokazanom hiperoksalurijom u cilju razlikovanja osnovnog uzroka nastanka poremećaja i pravilnog izbora načina lečenja. Test se sprovodi u hospitalnim uslovima, pri unosu 2400 mL tečnosti na dan i posle 2-dnevne standardne dijete (19), na sledeći način:

I dan 08–20^h: sakupljanje urina (+30 mL 2,5 mol/L HCl)

20–08^h: sakupljanje urina (+30 mL 2,5 mol/L HCl)

II dan 08^h: unos 33,8 mg [$^{13}C_2$] oksalne kiseline u formi natrijumove soli u kapsuli od 50 mg

09^h: doručak

08–14^h: sakupljanje urina (+15 mL 2,5 mol/L HCl)

14^h: ručak

14–20^h: sakupljanje urina (+15 mL 2,5 mol/L HCl)

20–08^h: sakupljanje urina (+30 mL 2,5 mol/L HCl)

Nivo ekskretovanog oksalata obeleženog radioizotopom ugljenika u 24^h uzorcima urina sakupljanih I i II dana određuje se gasnom hromatografijom. Razlika u nivoima veća od 10% smatra se značajnom.

Značaj protokola

Primena novog Protokola obezbeđuje racionalnost i efikasnost u kliničkoj evaluaciji pacijenata sa kalkulozom urinarnog trakta zbog sasvim preciznih indikacija za izvođenje testova laboratorijskih ispitivanja za svaku kliničku kategoriju bolesti. Kontrolisanost uslova pri skupljanju uzoraka materijala za analizu, sprovođenje standardnih dijeta i adekvatno čuvanje uzoraka urina povećavaju pouzdanost rezultata. Poštovanje algoritma ispitivanja omogućava da se na najbrži način utvrdi postojeći metabolički poremećaj, odnosno otkrije egzogeni faktor (greške u ishrani ili patološka intestinalna apsorpcja materija odgovornih za nastanak kamena). Utvrđivanje uzroka bolesti obezbeđuje da se u svakom konkretnom slučaju odredi najadekvatnija terapija, odnosno da se primene mere profilakse u cilju sprečavanja pojave recidiva bolesti.

Izračunavanje određenih indeksa rizika, na osnovu parametara dobijenih primenom ovog Protokola, omogućava precizniju procenu težine bolesti, predviđanje njenog toka kao i praćenje efekata primenjene medikamentozne terapije.

UPDATE ANALYTIC PROGRAM FOR LABORATORY INVESTIGATION IN URINARY TRACT CALCULOSIS

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Summary: Urinary tract calculosis due to it's high recurrence rate (50–100% in untreated patients) and possibility to cause chronic renal failure in 20% stil represents a great social and medical problem. From that aspect, an adequate clinical evaluation in stone forming patients is of paramount importance. An effective and rational attitude in detecting possible causes of stone formation demands the adequate analytic program in biochemical investigations. Presented program is adjusted to the guidelines on urolithiasis, recommended by Board EAU (European Association of Urologists) Healthcare Office, for year 2003. Regarding to known chemical stone composition, category of stone former and presence of an specific risk factor, analytic program could be *minimal* (fasting morning spot urine sample analysis, urineculture and blood analysis (calcium, albumine, creatinine and urate and stone analysis) and *extended* (quantification of all parameters in serum and in two consequtive 24^h urine collections, relevant for stone formation and evaluation of renal function). In order to identify metabolic disorders, an quantification of parathormone in serum, cAMP in urine and absorption/loading tests with calcium, NH_4Cl and [$^{12}C_2$] oxalate are recommended.

Key words: urinary tract calculosis, metabolic disorder, analytic program, absorption/loading tests

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SOMATOSTATIN U OBOLJENJIMA GASTROINTESTINALNOG TRAKTA

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Kratak sadržaj: Somatostatin u serumu određen je RIA metodom, kod 50 pacijenata sa cirozom jetre, 15 sa pernicioznom anemijom i atrofičnim gastritisom, 31 sa inflamatornim oboljenjem creva, 32 sa kolorektalnim tumorima i 40 kontrolnih osoba. Kod pacijenata sa cirozom jetre nađene su značajno više vrednosti somatostatina u odnosu na kontrolnu grupu ($p<0,01$). Pacijenti sa pernicioznom anemijom i ulceroznim kolitisom u akutnoj fazi bolesti imali su značajno niže vrednosti somatostatina ($p<0,005$). Kod pacijenta sa kolorektalnim tumorima somatostatin je bio značajno niži nego u kontrolnoj grupi ($p<0,01$). Dobijeni rezultati pokazuju da je jetra uključena u metabolizam somatostatina. Atrofični gastritis, ulcerozni kolitis i M. Crohn, kao i kolorektalni tumori bili su udruženi sa značajnim promenama u nivou somatostatina što sugeriše moguću patofiziološku i terapeutsku ulogu somatostatina u ovim oboljenjima.

Ključne reči: somatostatin, ciroza jetre, atrofični gastritis, ulcerozni kolitis, M. Crohn, kolorektalni tumori

Uvod

Somatostatin je tetradekapeptid, prvo izolovan iz hipotalamusa ovce 1973. godine, a kasnije je otkriven u gastrointestinalnom traktu, pankreasu, centralnom i perifernom nervnom sistemu i tireoidnoj žlezdi (1). Poznato je da izaziva inhibiciju većeg broja drugih hormona, kao i inhibiciju sekrecije i motiliteta u gastrointestinalnom traktu. Eksperimentalne studije ukazuju da se somatostatin metaboliše u jetri i bubrezima i dokazana je degradacija somatostatina u izolovanim hepatocitima (2, 3). Ispitivanja u ljudi za sada daju kontradiktorne rezultate.

U većini patoloških stanja kao što su oboljenja želuca, tankog i debelog creva dolazi do odstupanja pojedinih gastrointestinalnih peptida, pa i somatostatin (4). Objavljene studije ukazuju na hipotetički značaj somatostatina u patofiziologiji gastritisa, inflamatornih oboljenja creva i kolorektalnog kancera s jedne strane, i mogućnosti primene u terapijske svrhe (5–7). Cilj ovog rada je bio da se razjasni moguća uloga jetre u metabolizmu somatostatina merenjem njegovih vrednosti u serumu bolesnika sa različitim stepenom oštećenja jetre, utvrdi veza između razvoja intestinalne metaplazije i vrednosti somatostatina, ispita vrednost somatostatina, u bolesnika sa ulceroznim kolitisom i Kronovom bolešću u odnosu na fazu i lokalizaciju bolesti i eventualna odstupanja vrednosti somatostatina u bolesnika sa kolorektalnom neoplazijom.

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Materijal i metode

Ispitano je ukupno 50 (35 muškog pola i 15 ženskog pola) bolesnika sa histološki potvrđenom alkoholnom cirozom jetre, prosečne starosti 49 godina. Dobijeni rezultati posmatrani su sa dva aspekta: u odnosu na prisustvo ciroze i u odnosu na stepen hepatalne insuficijencije. Izvršena je podela prema kliničkim kriterijumima na kompenzovani i dekompenzovani oblik ciroze.

Sa oboljenjem perniciozne anemije i atrofičnog gastritisa sa ahlorhidrijom bilo je ukupno 15 bolesnika, prosečne starosti 59 godina. Dijagnoza perniciozne anemije postavljena je na osnovu megaloblastne kostne srži i pozitivnog Schilling testa, a atrofičnog gastritisa gastroskopski uz histopatološki nalaz.

Endoskopskim ispitivanjem uz histopatološku analizu postavljena je dijagnoza ulceroznog kolitisa u ukupno 31 bolesnika, od čega 17 u akutnoj fazi bolesti, a 14 u remisiji. Takođe je praćeno i 32 bolesnika sa M. Crohn različite lokalizacije (21 na tankom, 5 na

debelom crevu i 6 istovremeno na tankom i debelom crevu).

Moguća veza između somatostatina i kolorektalne neoplazije ispitivana je na 42 bolesnika, prosečne starosti 60 godina. Dijagnoza je postavljena kolonoskopski uz histopatološku verifikaciju i to u 16 adenomatozni polip, u 8 vilozni adenom i u 18 adenokarcinom.

Svih 138 bolesnika kao i 40 kontrolnih osoba bilo je hospitalizovano na Klinici za internu medicinu i detaljno ispitano (kompletne biohemijske analize i krvna slika, rendgenska ispitivanja, ultrazvučna dijagnostika i endoskopski pregledi uz histopatološke analize). Izuzimajući grupu sa alkoholnom cirozom jetre, svim ostalim bolesnicima isključena su druga oboljenja, pre svega bubrežna i hepatalna insuficijencija.

Svi ispitanici bili su bez medikamentne terapije i ujutru našte uzimana je krv za određivanje bazalnih vrednosti somatostatina. Analiza je vršena RAI metodom uz korišćenje laboratorijskog, eksperimentalnog test reagensa (dobijenog iz Laboratorije Hammersmith Hospital u Londonu). Referentne vrednosti za somatostatin bile su 17–150 pmol/L.

U statističkoj obradi korišćeni su Studentov t test i druge uobičajene statističke metode.

Rezultati

Alkoholna ciroza jetre

Bazalne vrednosti somatostatina u serumu bile su značajno više kod svih bolesnika sa alkoholnom cirozom jetre u odnosu na kontrolnu grupu (p<0,01) *(Tabela I)*. Vrednosti somatostatina u grupi bolesnika sa dekompenzovanim oblikom ciroze bile su značajno više u odnosu na kompenzovani oblik (p<0,05) *(Tabela I)*.

Tabela I Nivoi somatostatina u plazmi bolesnika sa alkoholnom cirozom jetre

Ispitivane grupe	N	Somatostatin, pmol/L
Kompenzovana ciroza	20	44,0 ± 9,8^{x}
Dekompenzovana ciroza	30	68,6 ± 20,1^{xx}
Sve ciroze jetre	50	50,7 ± 30,3**
Kontrolna grupa	40	28,0 ± 10,1

** p < 0,01 nivo statističke značajnosti razlika u odnosu na kontrolnu grupu
^{x} p < 0,05 nivo statističke značajnosti razlika bolesnika sa dekompenzovanom cirozom jetre u odnosu na grupu sa kompenzovanom
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Perniciozna anemija i atrofični gastritis

Svi bolesnici sa pernicioznom anemijom imali su hronični atrofični gastritis, a u 7 bolesnika histološki je potvrđena i intestinalna metaplazija. Nivoi somatostatina u serumu bili su značajno niži u bolesnika sa pernicioznom anemijom i hroničnim atrofičnim gastritisom u odnosu na kontrolnu grupu (p<0,01). Sve vrednosti u ispitivanih bolesnika bile su ispod normalnih vrednosti za somatostatin. Kod bolesnika sa intestinalnom metaplazijom nađene su značajno niže vrednosti u odnosu na bolesnike sa samo hroničnim atrofičnim gastritisom (p<0,01) *(Tabela II)*.

Tabela II Somatostatin u serumu bolesnika sa pernicioznom anemijom

Grupe bolesnika	N	Somatostatin, pmol/L
Perniciozna anemija	15	9,4 ± 10,6*
Samo atrofični gastritis	8	13,6 ± 12,1
Atrofični gastritis i interstinalna metaplazija	7	3,2 ± 2,3**
Kontrolna grupa	40	49,5 ± 20,1

* značajna razlika (p<0,01) za bolesnike sa pernicioznom anemijom u odnosu na kontrolnu grupu
** značajna razlika (p < 0,01) podgrupa perniciozne anemije sa i bez inestinalne metaplazije

Ulcerozni kolitis i Kronova bolest

Ispitivanjem su utvrđene značajno više vrednosti somatostatina u obe grupe bolesnika (105,5 ± 21,4 pmol/L) u odnosu na kontrolnu grupu (50,3 ± 10,2 pmol/L) (p<0,01). U odnosu na fazu bolesti vrednosti bazalnog somatostatina bile u značajno niže u akutnoj fazi u odnosu na remisiju (p<0,005). U odnosu na lokalizaciju Kronove bolesti vrednosti somatostatina bile su značajno više u grupi sa promenama na tankom crevu (p<0,001) *(Tabela III)*. Sve ispitivane vrednosti somatostatina bile su unutar referentnih vrednosti za somatostatin.

Tabela III Somatostatin u serumu bolesnika sa ulceroznim kolitisom i M. Crohn

Ispitivane grupe	N	Somatostatin, pmol/L
Ulcerozni kolitis	31	
– u akutnoj fazi	17	8,1 ± 4,4*
– u remisiji	14	39,9 ± 14,7
Kronova bolest	32	
– tanko crevo	21	44,1 ± 15,2**
– debelo crevo	5	28,7 ± 13,2
– oba	6	44,7 ± 2,9
Kontrolna grupa	40	19,5 ± 20,1

* p < 0,005
** p < 0,001

Kolorektalni tumori

U grupi bolesnika sa kolorektalnim adenokarcinomom i viloznim adenomom vrednosti somatostatina bile su značajno niže u odnosu na kontrolnu grupu (p<0,01), ali unutar normalnih vrednosti.

Diskusija

Do sada saopšteni kontradiktorni rezultati u literaturi (3, 8) ovo ispitivanje čine aktuelnim. Rezultati povišenih bazalnih vrednosti somatostatina u skladu su sa pretpostavkom da se somatostatin metaboliše u jetri, što ne isključuje njegovu ulogu kao neurotransmitera i lokalnog endokrinog regulatora pa i mogućnosti da se metaboliše na mestima gde se i oslobađa. Povećanje somatostatina u cirozi jetre, a posebno dekompenzovane, moguće je objasniti uticajem intrahepatične holestaze, portalne hipertenzije i postojanjem intra i ekstrahepatičnih šantova (10). Navedene rezultate treba posmatrati i u svetlu upotrebe somatostatina u farmakološkom tretmanu portalne hipertenzije (11).

Poznato je prisustvo hipergastrinemije kod bolesnika sa pernicioznom anemijom i hroničnim atrofičnim gastritisom, što se objašnjava sekundarno povećanim oslobađanjem gastrina zbog ahlorhidrije, uzrokovane upravo atrofičnim gastritisom, a ne isključuje se ni uloga imunoloških mehanizama (postojanje autoantitela na gastrinske receptore) (14). Niske vrednosti somatostatina mogu se objasniti pre povećanim odnosom G/D ćelija što je i dokazano, a što sugeriše da su niske vrednosti pre posledice ahlorhidrije, nego tipičnog efekta hipergastrinemije, jer je poznato da se somatostatin oslobađa pri niskim antralnim pH (15–17).

Nalaz značajno nižih vrednosti somatostatina u akutnoj fazi bolesti je u saglasnosti sa ispitivanom literaturom (18). Takođe veće vrednosti somatostatina u bolesnika sa intestinalnom lokalizacijom odražavaju mesto oslobađanja somatostatina uzrokovano najverovatnije nekrotičnim promenama. S druge strane od značaja je razmatranje upotrebe somatostatina u bolesnika sa teškim oblicima kolitisa (18, 19).

Poslednjih godina otkriveno je prisustvo receptora za somatostatin i druge peptide kod eksperimentalnog kancera kolona (20). Takođe potvrđena je imunoreaktivnost ćelija serotonina, enteroglukagona, peptida YY i dr. (21, 22). Niže vrednosti somatostatina uz povišene vrednosti gastrina, IGF-1 i drugih peptida uklapaju se u patofiziološki koncept rasta kolorektalnih tumora, gde endokrini sistem očigledno ima određenu ulogu. S obzirom na navedene promene moglo bi se razmišljati o upotrebi neuroendokrinih peptida, pa i somatostatina u dijagnostici i terapiji kolorektalnog kancera (23).

Iz svega izloženog može se zaključiti da pored poznatog opšteg inhibitornog dejstva somatostatina na sekreciju neuroendokrinih tumora, kao i na sekreciju i motilitet gastrointestinalnog trakta, somatostatin očigledno ima ulogu i u patofiziologiji gastritisa, inflamatornih oboljenja creva, kolorektalnog kancera. Ovo je od posebnog značaja kada se razmatraju mogućnosti upotrebe somatostatina u terapijske svrhe.

SOMATOSTATIN LEVELS IN GASTROINTESTINAL DISEASE

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Summary: Serum levels of somatostatin were determined by RIA method in 50 patients with liver cirrhosis, 15 with pernicious anemia and atrophic gastritis, 31 with inflamatory bowel disease, 32 with colorectal tumors and in 40 control persons. In patients with liver cirrhosis somatostatin levels were significantly higher than in control group (p<0.01). Patients with pernicious anemia and ulcerative colitis in acute phase of the disease had significantly lower levels of somatostatin (p<0.01) (p<0.005). In patients with colorectal tumors somatostatin were significantly lower then in control group (p<0.01). Our results show that the liver is involved in somatostatin metabolism. Atrophic gastritis, ulcerative colitis and M. Crohn, so as colorectal neoplasia were associated with significantly changes in somatostatin levels which suggest the potential pathophysiologic and terapeutic role of somatostatin in those disease.

Key words: somatostatin, liver cirrhosis, atrophic gastritis, ulcerative colitis, M. Crohn, colorectal tumors

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SOMATOSTATIN U OBOLJENJIMA GASTROINTESTINALNOG TRAKTA

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Kratak sadržaj: Somatostatin u serumu određen je RIA metodom, kod 50 pacijenata sa cirozom jetre, 15 sa pernicioznom anemijom i atrofičnim gastritisom, 31 sa inflamatornim oboljenjem creva, 32 sa kolorektalnim tumorima i 40 kontrolnih osoba. Kod pacijenata sa cirozom jetre nađene su značajno više vrednosti somatostatina u odnosu na kontrolnu grupu ($p<0,01$). Pacijenti sa pernicioznom anemijom i ulceroznim kolitisom u akutnoj fazi bolesti imali su značajno niže vrednosti somatostatina ($p<0,005$). Kod pacijenta sa kolorektalnim tumorima somatostatin je bio značajno niži nego u kontrolnoj grupi ($p<0,01$). Dobijeni rezultati pokazuju da je jetra uključena u metabolizam somatostatina. Atrofični gastritis, ulcerozni kolitis i M. Crohn, kao i kolorektalni tumori bili su udruženi sa značajnim promenama u nivou somatostatina što sugeriše moguću patofiziološku i terapeutsku ulogu somatostatina u ovim oboljenjima.

Ključne reči: somatostatin, ciroza jetre, atrofični gastritis, ulcerozni kolitis, M. Crohn, kolorektalni tumori

Uvod

Somatostatin je tetradekapeptid, prvo izolovan iz hipotalamusa ovce 1973. godine, a kasnije je otkriven u gastrointestinalnom traktu, pankreasu, centralnom i perifernom nervnom sistemu i tireoidnoj žlezdi (1). Poznato je da izaziva inhibiciju većeg broja drugih hormona, kao i inhibiciju sekrecije i motiliteta u gastrointestinalnom traktu. Eksperimentalne studije ukazuju da se somatostatin metaboliše u jetri i bubrezima i dokazana je degradacija somatostatina u izolovanim hepatocitima (2, 3). Ispitivanja u ljudi za sada daju kontradiktorne rezultate.

U većini patoloških stanja kao što su oboljenja želuca, tankog i debelog creva dolazi do odstupanja pojedinih gastrointestinalnih peptida, pa i somatostatin (4). Objavljene studije ukazuju na hipotetički značaj somatostatina u patofiziologiji gastritisa, inflamatornih oboljenja creva i kolorektalnog kancera s jedne strane, i mogućnosti primene u terapijske svrhe (5–7). Cilj ovog rada je bio da se razjasni moguća uloga jetre u metabolizmu somatostatina merenjem njegovih vrednosti u serumu bolesnika sa različitim stepenom oštećenja jetre, utvrdi veza između razvoja intestinalne metaplazije i vrednosti somatostatina, ispita vrednost somatostatina, u bolesnika sa ulceroznim kolitisom i Kronovom bolešću u odnosu na fazu i lokalizaciju bolesti i eventualna odstupanja vrednosti somatostatina u bolesnika sa kolorektalnom neoplazijom.

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Ispitivanjem su utvrđene značajno više vrednosti somatostatina u obe grupe bolesnika (105,5 ± 21,4 pmol/L) u odnosu na kontrolnu grupu (50,3 ± 10,2 pmol/L) ($p<0,01$). U odnosu na fazu bolesti vrednosti bazalnog somatostatina bile u značajno niže u akutnoj fazi u odnosu na remisiju ($p<0,005$). U odnosu na lokalizaciju Kronove bolesti vrednosti somatostatina bile su značajno više u grupi sa promenama na tankom crevu ($p<0,001$) *(Tabela III)*. Sve ispitivane vrednosti somatostatina bile su unutar referentnih vrednosti za somatostatin.

Tabela III Somatostatin u serumu bolesnika sa ulceroznim kolitisom i M. Crohn

Ispitivane grupe	N	Somatostatin, pmol/L
Ulcerozni kolitis	31	
– u akutnoj fazi	17	8,1 ± 4,4*
– u remisiji	14	39,9 ± 14,7
Kronova bolest	32	
– tanko crevo	21	44,1 ± 15,2**
– debelo crevo	5	28,7 ± 13,2
– oba	6	44,7 ± 2,9
Kontrolna grupa	40	19,5 ± 20,1

* $p < 0,005$
** $p < 0,001$

Kolorektalni tumori

U grupi bolesnika sa kolorektalnim adenokarcinomom i viloznim adenomom vrednosti somatostatina bile su značajno niže u odnosu na kontrolnu grupu (p<0,01), ali unutar normalnih vrednosti.

Diskusija

Do sada saopšteni kontradiktorni rezultati u literaturi (3, 8) ovo ispitivanje čine aktuelnim. Rezultati povišenih bazalnih vrednosti somatostatina u skladu su sa pretpostavkom da se somatostatin metaboliše u jetri, što ne isključuje njegovu ulogu kao neurotransmitera i lokalnog endokrinog regulatora pa i mogućnosti da se metaboliše na mestima gde se i oslobađa. Povećanje somatostatina u cirozi jetre, a posebno dekompenzovane, moguće je objasniti uticajem intrahepatične holestaze, portalne hipertenzije i postojanjem intra i ekstrahepatičnih šantova (10). Navedene rezultate treba posmatrati i u svetlu upotrebe somatostatina u farmakološkom tretmanu portalne hipertenzije (11).

Poznato je prisustvo hipergastrinemije kod bolesnika sa pernicioznom anemijom i hroničnim atrofičnim gastritisom, što se objašnjava sekundarno povećanim oslobađanjem gastrina zbog ahlorhidrije, uzrokovane upravo atrofičnim gastritisom, a ne isključuje se ni uloga imunoloških mehanizama (postojanje autoantitela na gastrinske receptore) (14). Niske vrednosti somatostatina mogu se objasniti pre povećanim odnosom G/D ćelija što je i dokazano, a što sugeriše da su niske vrednosti pre posledice ahlorhidrije, nego tipičnog efekta hipergastrinemije, jer je poznato da se somatostatin oslobađa pri niskim antralnim pH (15–17).

Nalaz značajno nižih vrednosti somatostatina u akutnoj fazi bolesti je u saglasnosti sa ispitivanom literaturom (18). Takođe veće vrednosti somatostatina u bolesnika sa intestinalnom lokalizacijom odražavaju mesto oslobađanja somatostatina uzrokovano najverovatnije nekrotičnim promenama. S druge strane od značaja je razmatranje upotrebe somatostatina u bolesnika sa teškim oblicima kolitisa (18, 19).

Poslednjih godina otkriveno je prisustvo receptora za somatostatin i druge peptide kod eksperimentalnog kancera kolona (20). Takođe potvrđena je imunoreaktivnost ćelija serotonina, enteroglukagona, peptida YY i dr. (21, 22). Niže vrednosti somatostatina uz povišene vrednosti gastrina, IGF-1 i drugih peptida uklapaju se u patofiziološki koncept rasta kolorektalnih tumora, gde endokrini sistem očigledno ima određenu ulogu. S obzirom na navedene promene moglo bi se razmišljati o upotrebi neuroendokrinih peptida, pa i somatostatina u dijagnostici i terapiji kolorektalnog kancera (23).

Iz svega izloženog može se zaključiti da pored poznatog opšteg inhibitornog dejstva somatostatina na sekreciju neuroendokrinih tumora, kao i na sekreciju i motilitet gastrointestinalnog trakta, somatostatin očigledno ima ulogu i u patofiziologiji gastritisa, inflamatornih oboljenja creva, kolorektalnog kancera. Ovo je od posebnog značaja kada se razmatraju mogućnosti upotrebe somatostatina u terapijske svrhe.

SOMATOSTATIN LEVELS IN GASTROINTESTINAL DISEASE

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Summary: Serum levels of somatostatin were determined by RIA method in 50 patients with liver cirrhosis, 15 with pernicious anemia and atrophic gastritis, 31 with inflamatory bowel disease, 32 with colorectal tumors and in 40 control persons. In patients with liver cirrhosis somatostatin levels were significantly higher than in control group (p<0.01). Patients with pernicious anemia and ulcerative colitis in acute phase of the disease had significantly lower levels of somatostatin (p<0.01) (p<0.005). In patients with colorectal tumors somatostatin were significantly lower then in control group (p<0.01). Our results show that the liver is involved in somatostatin metabolism. Atrophic gastritis, ulcerative colitis and M. Crohn, so as colorectal neoplasia were associated with significantly changes in somatostatin levels which suggest the potential pathophysiologic and terapeutic role of somatostatin in those disease.

Key words: somatostatin, liver cirrhosis, atrophic gastritis, ulcerative colitis, M. Crohn, colorectal tumors

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Eisen HN. Immunology: an introduction to molecular and cellular principles of the immune response. 5th ed. New York: Harper and Row, 1974: 406.

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