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Imunochemické detekce tkáňového poškození

Disertační práce

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OBSAH

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SEZNAM ZKRATEK

Ab	protilátka
Ag	antigen
BSA	hovězí serumalbumin
COMP	trombospondin-5 (cartilage oligomeric matrix protein)
DTT	dithiothreitol
EIA	enzymoimunoesej
Ig	imunoglobulin
iNOS	inducibilní NO syntaza
KLH	hemocyanin z přílipky modravé (Patella caerulea)
MAb	monoklonální protilátka
MMP	tkáňové metaloproteinasy
mRNA	messenger ribonukleová kyselina
OA	osteoartróza
RA	revmatoidní artritida
RIA	radioimunoesej
SDS	laurylsíran sodný (dodecylsulfát sodný)
TIMP	tkáňový inhibitor metaloproteinas

ÚVOD

1. Tkáňové poškození

Primární podnět, vedoucí k tkáňovému poškození, je obvykle exogenního původu. Endogenního původu jsou zřejmě ta poškození tkáně, kdy dochází k soustavnému, dlouhodobému modifikování (např. vlivem stárnutí) některé její komponenty, která vede k ztrátě její funkčnosti (např. glykace proteinů čočky či racemisace jejich aminokyselin). Exogenní zásah, vedoucí k poškození tkáně, může být fyzikální podstaty (mechanický - poranění, tepelný - popálení, ionizující záření atd.), chemický (např. toxiny, alergeny, ale i přebytek či nedostatek kyslíku) nebo biologický (např. infekce).

Chemický či biologický podnět vyvolává primární odpověď a tou je obvykle uvolnění cytokinů do extracelulárního prostoru anebo produkce volných radikálů. Účinek cytokinů na ostatní buňky je velmi různorodý a závisí na jejich typu, ale cytokiny jako takové tkáně nedegradují. Oproti tomu volné radikály mohou sice mít vlastnosti hormonů (oxid dusnatý) a mít protektivní účinek, ale mnohem častěji tkáně poškozují, ať už jako takové nebo svými reaktivními produkty (po reakci s kovy, oxidanty nebo reduktanty). Volné radikály, díky své reaktivitě, mají krátkou dobu existence a proto jejich stanovení je obtížné a často se převádí na stanovení stabilního produktu vznikajícího reakcí volného radikálu. Příkladem může být měření produkce oxidu dusnatého na základě stanovení dusitanů, či měření současné produkce oxidu dusnatého a superoxidu, které spolu reagují za vzniku kyseliny peroxydusité. Ta je silným oxidačním a nitračním agens a produkt její reakce s tyrosinem - 3-nitrotyrosin - je stabilní a proto lze produkci kyseliny peroxydusité změřit na základě stanovení 3-nitrotyrosinu.

Další fází tkáňového poškození je exprese, uvolnění a zvýšená aktivita "lytických" enzymů v extracelulárním prostoru (např. tkáňové metaloproteinasy - MMP). Zvýšená aktivita může být

způsobena jak přeměnou již syntetizovaných enzymů v neaktivní formě (konverse proenzymu na enzym) či odstraněním jejich inhibitorů, tak i jejich synthesou de novo. Obecně lze říci, že zvýšená aktivita v první fázi je vyvolána aktivací již přítomných neaktivních enzymů. Zvýšená aktivita daného enzymu může být za pomocí vhodných substrátů změřena, ale pro bližší objasnění mechanismu vlivu daného enzymu na degradaci tkáně je nutné zjistit i analytické koncentrace enzymu a proenzymu ev. jeho důležitých inhibitorů (např. TIMP v případě MMP) a nebo alespoň kvantifikovat množství mRNA daného enzymu v studované tkáni .

V následující fázi jsou makromolekuly, modifikované reakcí s volnými radikály anebo degradované indukovanými enzymy, odstraňovány z poškozené tkáně. Velmi často tyto štěpy makromolekul dále stimulují "zánětlivou reakci" (např. štěpy kyseliny hyaluronové). Detekovat tuto fázi tkáňového poškození můžeme pomocí přítomnosti specifických markerů v tělních tekutinách, nejčastěji v krvi. Marker pro poškození dané tkáně je obvykle fragment bílkoviny specifické pro danou tkáň.

Konečně poslední fází tkáňového poškození je reparace. Příslušné buňky obvykle migrují do poškozené oblasti, proliferují a syntetizují novou extracelulární matrix. V případě větších poškození se obvykle úplně neobnoví původní tkáň, ale remodelací vzniká tkáň se změněnými funkčními vlastnostmi. Příkladem může být tkáňové poškození plic vlivem chronické hypoxie a následná remodelace plicních cév vedoucí k plicní hypertensi (Hampl and Herget 2000).

2. Imunochemické metody

Jednotlivé fáze tkáňového poškození lze detekovat různými biochemickými technikami, ale v posledním desetiletí nabývají na významu imunochemické metody, tj. metody využívající protilátky. Výhodou těchto metod je universalita, protože protilátky je možno připravit proti všem biologickým makromolekulám (výjimkou je hyaluronová kyselina) a i proti většině

nízkomolekulárních organických látek. Specifita a citlivost těchto metod závisí na příslušné protilátce. Obecně je specifita vysoká a metody patří k nejcitlivějším biochemickým metodám. Hlavní nevýhodou je nutnost získání příslušné protilátky. Je-li komerčně dostupná, je s výjimkou protilátek proti nejběžnějším antigenům poměrně drahá, není-li v nabídce firem, je nutné ji připravit, což bývá náročné jak časově, tak finančně.

Základním kamenem imunochemických metod je protilátka (Ab), biochemicky imunoglobulin. Imunoglobulin je glykoprotein skládající se z těžkých řetězců o molekulové váze 55-80kDa a lehkých řetězců (22-23kDa). Je známo pět tříd imunoglobulinů (viz Tabulka 1). V organismu jsou imunoglobuliny syntetizovány plasmatickými buňkami, které vznikají terminální diferenciací B lymfocytů. Každý B lymfocyt má specifický membránový receptor, kterým je imunoglobulin s extensí C-konce těžkého řetězce a touto extensí je ukotven v membráně lymfocytu. Po navázání antigenu na tento receptor se B lymfocyty dělí a dále uchovávají jako paměťové buňky nebo diferencují v plasmatické buňky syntetizující a sekretují imunoglobulin identický receptoru (bez extense těžkého řetězce). Každá plasmatická buňka pochází z jednoho klonu lymfocytu a produkuje pouze jediný typ imunoglobulinu - monoklonální protilátku. Protože v organismu je celá řada klonů lymfocytů s odlišným receptorem proti danému antigenu, je během imunizace stimulována celá řada klonů B lymfocytů, výsledné plasmatické buňky každého klonu produkují svůj specifický imunoglobulin a výsledkem je polyklonální protilátka, jejíž vlastnosti jsou průměrem vlastností a koncentrací jednotlivých imunoglobulinů různých tříd a specifit. Nelze proto opakovaně připravit naprosto shodnou polyklonální protilátku. Navíc isolované sérum z imunizovaných zvířat obsahuje všechny sérové bílkoviny a zvláště protilátky proti jiným antigenům interferují při většině metod . Pro použití v imunochemických metodách je obvykle nutné specifické protilátky složitými postupy isolovat.

Tabulka

Základní vlastnosti myších imunoglobulinů

	Třída imunoglobulinů				
	IgM	**IgG**	**IgA**	**IgD**	**IgE**
Podtřídy	1	4	1	1	1
M.v. [kDa]	900	160	170 - 500	180	190
Koncentrace v séru [mg/ml]	0,1 - 1,0	3 – 20	1 - 3	0,001-0,01	<0,001
Těžký řetězec	μ	γ	α	δ	ε
Lehký řetězec	κ či λ	κ či λ	κ či λ	κ či λ	κ či λ
Stechiometrie *	$(\kappa_2\mu_2)_5$	$\kappa_2\gamma_2$	$(\kappa_2\alpha_2)_{1-3}$	$\kappa_2\delta_2$	$\kappa_2\varepsilon_2$
	$(\lambda_2\mu_2)_5$	$\lambda_2\gamma_2$	$(\lambda_2\alpha_2)_{1-3}$	$\lambda_2\delta_2$	$\lambda_2\varepsilon_2$
Obsah cukrů [%]	9 - 12	2 – 3	7 - 11	12 - 15	12
Poločas v séru [dny]	1	2 (G_{2b})	1	<1	<1
		4 (ostatní)			

* Složení nativní molekuly

Nevýhody polyklonálních protilátek odstranil objev universální přípravy monoklonálních protilátek Köhlera a Milsteina (Kohler and Milstein 1975; Kohler and Milstein 1976). Jejich zavedení do bylo příčinou rychlého rozvoje imunochemických a imunohistologických metod v posledních dvou dekádách. Princip přípravy monoklonálních protilátek je jednoduchý a dobře ilustruje využití několika objevů základního výzkumu v následný komerčně využitelný objev. Jerneho teorie definovala, že každý lymfocyt je schopen syntetizovat jediný specifický imunoglobulin. Technika buněčných kultur umožňovala kultivaci buněk Metazoí v živném roztoku, odvození nesmrtelných linií buněk a také principy a techniky fúze buněk, tj. spojení dvou buněk v jedinou, která nese genetický materiál obou buněk. Konečně genetické techniky využívané v in vitro metodách poskytly různě mutované linie buněk a také zjistily způsob jejich selekce. Köhler a Milstein použili buněčnou linii myších myelomových buněk s poškozeným genem pro enzym hypoxanthinguaninfosforibosyl-transferasu (HGPRT). Pro takto mutované buňky je toxický analog kyseliny listové aminopterin. Buňky této nesmrtelné

linie fúzovaly s diferencujícími B lymfocyty (ze sleziny imunizované myši po provokující injekci antigenu) a takto získané sfúzované buňky - hybridomy - kultivovali v živném mediu obsahujícím aminopterin. Původní diferencující lymfocyty v kultuře (stejně jako v organismu) nerostly, původní myelomové buňky byly zabíjeny aminopterinem a po zhruba čtrnácti dnech rostly pouze hybridomy, které získaly růstový potenciál (a nesmrtelnost) z buňky myelomové linie a resistenci vůči aminopterinu a schopnost syntetizovat imunoglobulin z B lymfocytu. Po následném klonování a testování na produkci imunoglobulinu proti danému antigenu byly izolovány hybridomy, které produkovaly jedinou monoklonální protilátku (MAb) dané třídy a byly nesmrtelné.

Hlavní výhoda monoklonální protilátky oproti polyklonální je, že danou monoklonální látku můžeme přesně definovat (co se týče afinity, avidity, křížové reaktivity atd.) jednou provždy. Zdroj této monoklonální protilátky je neomezený a zaručuje nám reprodukovatelnost. Pokud je potřeba monoklonální protilátku purifikovat, stačí pouze jednoduchá universální afinitní chromatografie na imobilizovaném proteinu A nebo G (váží IgG v oblasti těžkého řetězce) oproti složitým purifikačním postupům na purifikaci polyklonálních protilátek. Výhodou je i fakt, že zvířata je možné imunizovat i velmi komplexní směsí antigenů (např. buňkami) a přesto izolovat monoklonální protilátku proti jedinému antigenu, což velmi napomohlo např. při výzkumu membránových antigenů. Nevýhodou monoklonálních protilátek je složitější způsob získávání monoklonální protilátky proti novým antigenům, příprava hybridomů je technicky i finančně náročnější než klasická imunizace zvířat.

Imunochemické metody mohou využívat jak monoklonální tak polyklonální protilátky všech tříd, ale v praxi se využívají hlavně imunoglobuliny třídy G. U IgM je nevýhodou jejich pentamerní charakter (molekula obsahuje 10 těžkých a 10 lehkých řetězců a tudíž 10 potenciálních vazných míst), navíc IgM jsou imunoglobuliny primární imunitní odpovědi, tudíž mají nižší afinitu a jsou produkovány v nižších množstvích než imunoglobuliny třídy G

(hlavní imunoglobuliny sekundární odpovědi). Imunoglobuliny IgA (hlavní imunoglobuliny sekretů) se proti specifickým antigenům izolují zřídka a imunoglobuliny D a E se vyskytují v organismu ve velmi nízkých koncentracích a hybridomy pro produkci těchto imunoglobulinů byly připraveny v omezeném množství specifickými postupy pro vlastní studium těchto imunoglobulinů.

Základním krokem všech imunochemických metod je tvorba komplexu antigen-protilátka (rovnováha je posunuta výrazně na stranu imunokomplexu) a jeho separace od volných komponent (tj. volného Ag a Ab). Níže uvedené imunochemické techniky, seřazeny dle jejich využitelnosti při studiu tkáňového poškození, jsou :

1) Imunoeseje
2) Imunoblotování
3) Imunoprecipitace
4) Imunoafinitní chromatografie

2.1. Imunoeseje

Imunoeseje jsou pravděpodobně nejužívanější a nejužitečnější imunochemickou metodou. Umožňují detekovat i kvantifikovat jak protilátky (Ab) tak antigeny (Ag) a studovat strukturu antigenů. Jsou poměrně rychlé a nepříliš složité a lze s nimi získat informace obtížně získatelné jinými metodami. Princip této kvantitativní metody je založen na přidání malého množství značené protilátky nebo antigenu k studované směsi antigen-protilátka a stanovení této značené komponenty ve výsledném imunokomplexu. Množství značené komponenty v imunokomplexu je závislé na koncentracích výchozích neznačených komponent. Nezbytnou podmínkou využití těchto metod pro analýzu antigenů je, aby antigen byl rozpustný v pufru zhruba fysiologického pH, osmolarity a bez detergentů rušících vazbu antigen-protilátka. Podle způsobů provedení si můžeme tyto metody rozdělit na metody homogenní a

heterogenní, dle způsobu značení a následné detekce na radioimunoeseje (RIA) a enzymoimunoeseje (EIA).

V homogenních systémech se vazba antigen-protilátka vytváří za podmínek, kdy obě složky (antigen a protilátka) jsou v roztoku a výsledný komplex se od volných komponent odstraní z roztoku pomocí precipitace či sorpce. Tento typ metod měl velký význam v RIA , ale dnes jsou dnes spíše historickou záležitostí. Heterogenní systém využívá toho, že jedna z komponent (antigen nebo protilátka) je adsorbována na povrchu pevné látky (obvykle povrchu jamky mikrotitrační destičky), takže po navázání druhé komponenty a vytvoření vazby antigen-protilátka lze volnou komponentu snadno odstranit („odmýt"). Tři základní uspořádání jsou :

a) s adsorbovaným antigenem - vhodný pro detekci a kvantifikaci jak antigenů tak specifických protilátek

b) s adsorbovanou protilátkou - vhodný pro detekci i kvantifikaci antigenů

c) sendvičový systém - vhodný pro detekci a stanovení bílkovin nesoucích dvě odlišné antigenní determinanty (proti nimž jsou protilátky)

Variací jednotlivých uspořádání je celá řada, detailnější informace jsou uvedeny v knize Harlowa a Laneho (Harlow and Lane 1988) či Crowthera (Crowther 2001). K detekci a kvantifikaci imunokomplexů se v radioimunoesejích využívají radioisotopy (tj. značení radioisotopem), ale modernější a vůči okolí šetrnější jsou metody, kde se molekuly (ať již antigenu, protilátky, nebo sekundární protilátky proti první protilátce) označí kovalentním navázáním vhodného enzymu (nejčastěji křenové peroxidasy) a detekce je provedena enzymatickou reakcí příslušného enzymu - ezymoimunoeseje (EIA někdy ELISA). Dlouho se zdálo, že EIA metody jsou sice šetrnější vůči životnímu prostředí i pracovníkům, ale méně citlivé. Zavedením fluorescenčních a luminiscenčních enzymatických reakcí se citlivost EIA

metod zvýšila natolik, že se citlivostí RIA metodám vyrovnají a ve všech ostatním směrech je předčí.

2.2. Imunoblotování

Imunoblotování, nazývané také Western blotting, je technika, která kombinuje separační účinnost gelové elektroforesy se specifitou detekce na basi vazby antigen-protilátka (Burnette 1981). V principu je to metoda semikvantitativní, ale umožňuje studovat i antigeny, které jsou v běžných pufrech nerozpustné a tudíž nestanovitelné pomocí imunoeseje. Základní postup spočívá v rozpuštění vzorku ve vhodném pufru (např. zvýšená teplota, detergenty a redukční činidla nejen že nevadí, ale často jsou i doporučeny), rozdělení antigenů vzorku vhodnou gelovou elektroforesou (nejčastěji SDS polyakrylamidovou gelovou elektroforesou), přenosu rozdělených polypeptidů z gelu na vhodnou membránu (nitrocelulosu, nověji PVDF), blokování nespecifických vazebných míst membrány, následné přidání specifické protilátky (primární protilátky) a její vazbě na ta místa membrány, která obsahují příslušným antigen. Buď je primární protilátka vhodně označena (enzym, radioisotop), ale častěji je označena druhá protilátka (tzv. sekundární Ab) proti imunoglobulinům zvířecího druhu primární protilátky (klasicky je primární protilátka myší MAb a sekundární protilátka je proti myším Ig) a detekce se provádí v případě značení radioisotopy fotografickou emulsí a při enzymovém značení barevnou nebo luminiscenční reakcí.

2.3. Imunoprecipitace

Imunoprecipitace je metoda, při níž se pomocí specifické protilátky isolují bílkoviny se stejnou antigenní determinantou a po precipitaci imunokomplexů se nerozpustné komplexy antigen-protilátka disociují pomocí SDS a proteiny se rozdělí pomocí SDS gelové

elektroforesy. Metoda je zvláště významná pro analysu různých bílkovin, které nesou společný antigen (např. fosfotyrosin, glykosaminoglykan atd.).

2.4. Imunoafinitní chromatografie

Imunoafinitní chromatografie je specifickým druhem afinitní chromatografie, která využívá tvorbu nekovalentní vazby mezi antigenem a protilátkou. Tato technika je velmi účinná při isolacích bílkovin, které jsou za patřičných podmínek v jednom kroku purifikovány o tři až čtyři řády. Je to metoda preparativní a při studiu tkáňového poškození je využitelná pro odstranění nežádoucích příměsí při studiu specifické bílkoviny (typickým případem může být isolace specifického enzymu a studium jeho enzymové kinetiky se sloučeninou, která v surové směsi je substrátem pro celou řadu kontaminujících enzymů). Specifickou modifikací této metody s EIA metodou je nekovalentní navázání specifické protilátky proti žádanému enzymu na povrch jamky mikrotitrační destičky a po následném inkubaci vzorku je příslušný enzym navázán na protilátku a po „odmytí" interferujících látek se provede enzymatická reakce s imunokomplexem.

Imunochemickými metodami se obecně zabývají daleko šířeji příslušné monografie (Goding 1986), (Harlow and Lane 1988). Konkrétní využití imunochemických metod pro detekce některých typů tkáňového poškození v různých fázích jsou uvedeny v následujících odstavcích.

3. Příklady sloučenin významných při detekci tkáňového poškození.

3.1. Kyselina peroxydusitá a 3-nitrotyrosin

Jak již bylo výše uvedeno, v počátečním stadiu tkáňového poškození hrají významnou roli volné radikály. Přírodně se vyskytující volné radikály typicky obsahují kyslík nebo dusík. Klasické příklady reaktivních kyslík-obsahujících radikálů jsou $\bullet O_2^-$ (superoxidový anion) a $\bullet OH$ (hydroxylový radikál). V případě radikálů dusíku je to $\bullet NO$ (oxid dusnatý). Reakce těchto radikálů s kovy, oxidanty nebo reduktanty vytváří v buňkách další velmi reaktivní molekuly (Lander 1997).

Příkladem interakce mezi kyslíkovým a dusíkovým radikálem je reakce superoxidu s oxidem dusnatým. Výsledný produkt je $ONOO^-$ (peroxydusitanový anion). Rychlost této reakce je velmi vysoká ($6,7x10^9$ $M^{-1}s^{-1}$, tj. je prakticky limitovaná rychlostí difuse reaktantů). Disociační konstanta kyseliny peroxydusité je pK=6,8 při $37^{O}C$, což znamená, že za fysiologického pH (7,4) je přibližně 20% kyseliny peroxydusité ve formě konjugované kyseliny. Zatímco anion je relativně stabilní, nedisociovaná kyselina peroxydusitá se rychle rozkládá (resp. isomerizuje) na dusičnan ($t_{1/2} < 1s$) (Beckman 1996).

Kyselina peroxydusitá je silné oxidační činidlo a jako taková oxiduje mnohé aminokyseliny v polypeptidickém řetězci. Typickým substrátem pro oxidaci je thiolová skupina. Oxidace probíhá dvěma mechanismy (jedno- a dvouelektronový), které navzájem kompetují. V kyselém prostředí převažuje jednoelektronová oxidace a výsledné působení je velmi podobné působení hydroxylových radikálů. V alkalickém prostředí převažuje dvouelektronová oxidace (výsledným produktem je dusičnan a odpovídající disulfid) a tento mechanismus převažuje i za fyziologických podmínek (Quijano, Alvarez et al. 1997; Scorza and Minetti 1998). In vivo je situace složitější, oxidace je inhibována např. bikarbonátovými anionty (resp. oxidem

uhličitým), bilirubinem či askorbátem (Gow, Duran et al. 1996; Minetti, Mallozzi et al. 1998) a zdá se, že více je inhibována dvouelektronová oxidace (Scorza and Minetti 1998).

Kyselina peroxydusitá oxiduje i funkční skupiny dalších aminokyselin (Kato, Kawakishi et al. 1997; Pollet, Martinez et al. 1998). Aromatická aminokyselina tyrosin je oproti tomu při neutrálním pH převážně nitrována a nově vzniklý 3-nitrotyrosin je in vivo relativně stabilní (Ischiropoulos and al-Mehdi 1995) i když rychlost nitrace tyrosinu je ovlivněna různými faktory (Gow, Duran et al. 1996; Radi, Denicola et al. 1999). Kyselina peroxydusitá oxiduje také lipidy a deaminuje, oxiduje a štěpí DNA (Tamir, deRojas-Walker et al. 1996; Cuzzocrea, Caputi et al. 1998). Všechny tyto modifikace vytvářejí nové funkční skupiny v makromolekulách.

Chemická modifikace metaloproteinas kyselinou peroxydusitou zvyšuje jejich aktivitu (Rajagopalan, Meng et al. 1996; Okamoto, Akaike et al. 1997; Owens, Milligan et al. 1997) a naopak inaktivuje α_1-antiproteinasu (Whiteman and Halliwell 1997) nebo TIMP-1 (Frears, Zhang et al. 1996). Seznam fyziologických účinků kyseliny peroxydusité se stále rozšiřuje, kyselina peroxydusitá vykazuje baktericidní vlastnosti (Zhu, Gunn et al. 1992) a obvykle cytotoxické účinky, např. inhibice syntézy proteoglykanů v kloubních chondrocytech (Oh, Fukuda et al. 1998).

Produkci kyseliny peroxydusité in vivo nelze díky jejímu krátkému poločasu změřit (Beckman 1996), ale produkt její reakce s tyrosinem (3-nitrotyrosin) je in vivo poměrně stabilní (Ischiropoulos and al-Mehdi 1995). Později byly nalezeny jiné dusíkaté reaktivní sloučeniny, které tyrosin přeměnily 3-nitrotyrosin. Proto se nyní 3-nitrotyrosin považuje za obecný marker reaktivních dusíkatých sloučenin. Tyrosin reaguje v kyselém prostředí s kyselinou dusičnou a dusitou (klasická elektrofilní substituce aromátů, probíhající snadno na fenolických sloučeninách) za vzniku 3-nitrotyrosinu, ale tato cesta má in vivo význam asi jen

v žaludku (ale je klíčová při stanovení vázaného 3-nitrotyrosinu po kyselé hydrolyse bílkovin). V poslední době se ukázalo, že 3-nitrotyrosin může in vitro vzniknout za přítomnosti dusitanů (tj. konečného produktu oxidace NO) i enzymaticky pomocí neutrofilové myeloperoxidasy (Sampson, Ye et al. 1998) nebo eosinofilové peroxidasy (Wu, Chen et al. 1999). Proti možnému účinku myeloperoxidasy in vivo na vznik 3-nitrotyrosinu hovoří fakt, že u myší deficientních na myeloperoxidázu (knockout) je obsah 3-nitrotyrosinu po ischemickém poškození mozku vyšší než u kontrolních myší s funkční myeloperoxidasou (Takizawa, Aratani et al. 2002). Je pravděpodobné, že naopak působením myeloperoxidasy vznikající kyselina chlorná destruuje 3-nitrotyrosin, jak bylo prokázáno v in vitro pokusech s nitrovaným serumalbuminem (Whiteman and Halliwell 1999). Také některé extrakty tkání (slezina, plíce) údajně snižují hladinu 3-nitrotyrosinu (resp. sníží jeho rozpoznávání protilátkami) (Kamisaki, Wada et al. 1998; Kuo, Kanadia et al. 1999).

Zvýšené hladiny 3-nitrotyrosinu byly nalezeny u celé řady chorob (přehled viz (Halliwell, Zhao et al. 1999)) a proto jsou důležité metody jeho stanovení (přehled viz (Herce-Pagliai, Kotecha et al. 1998)). Jedna skupina metod je založena na chromatografickém dělení aminokyselin a detekci 3-nitrotyrosinu buď na základě absorpce UV při 360nm (nejméně citlivé) nebo na základě fluorescence po redukci na aminotyrosin nebo elektrochemické detekci. Obdobně lze po derivatizaci stanovit 3-nitrotyrosin plynovou chromatografií. Metody jsou pracné, materiálně a technicky náročné, mají malou kapacitu a navíc je problematické stanovení vázaného 3-nitrotyrosinu, neboť při hydrolyse i stopy dusitanů či dusičnanů výrazně nitrují tyrosin. Slibné, ale technicky a finančně ještě náročnější, je stanovení 3-nitrotyrosinu pomocí hmotové spektrofotometrie (Pennathur, Jackson-Lewis et al. 1999). Pro účely analysy mnoha vzorků jsou tyto příliš pracné metody nevhodné, naopak imunochemické metody, konkrétně EIA, jsou přímo určené pro tyto účely. Citlivost i přesnost stanovení pomocí EIA je primárně závislá na použité protilátce. Při přípravě jak polyklonálních, tak monoklonálních

protilátek bylo pozorováno, že afinitní konstanta pro 3-nitrotyrosin vázaný peptidickou vazbou je o 2-3 řády vyšší než pro volný 3-nitrotyrosin (Crow and Ischiropoulos 1996), takže metody s dosavadními protilátkami stanovují 3-nitrotyrosin v bílkovinách. Bylo popsáno několik EIA metod, ale všechny trpí jedním nedostatkem, jsou málo citlivé a pro stanovení 3-nitrotyrosinu v séru je třeba používat nízká ředění sér (cca 10x), což vede k vysokým chybám metod způsobeným interferencemi ostatních látek (např. revmatoidní faktor, či přirozená hladina protilátek proti 3-nitrotyrosinu atd.) a velmi rozdílným výsledkům nalezeným jednotlivými autory (Herce-Pagliai, Kotecha et al. 1998; Khan, Brennand et al. 1998; Tanaka, Choe et al. 1998; ter Steege, Koster-Kamphuis et al. 1998) .

3.2. COMP (Trombospondin-5)

Glykoprotein COMP (cartilage oligomeric matrix protein) byl objeven v chrupavce. Je složen z pěti podjednotek (Morgelin, Heinegard et al. 1992),obsahujících vazebná místa pro kolagen I, II a IX (Rosenberg, Olsson et al. 1998) a pro vápník (Oldberg, Antonsson et al. 1992).V oblasti oligomerizující N-koncové domény může COMP vázat hydrofobní hormony (Guo, Bozic et al. 1998). COMP patří do rodiny trombospondinů a je proto někdy označován jako trombospondin-5 (Newton, Weremowicz et al. 1994). Jeho význam pro normální vývoj a funkci chrupavky potvrzuje fakt, že mutace genu pro COMP jsou příčinou dvou popsaných chondrodysplasií (Briggs, Hoffman et al. 1995). Dlouhou dobu se věřilo, že COMP je specifická bílkovina chrupavky,ale později byl objeven i ve šlachách (DiCesare, Hauser et al. 1994) a v synoviální tkáni (Hummel, Neidhart et al. 1998), a recentně byl detekován v hladké svalovině cév (Riessen, Fenchel et al. 2001). Nicméně při porovnání všech uvedených tkání je jeho relativní obsah v chrupavce nejvyšší a proto je stále považován za jeden ze slibných "markerů" poškození chrupavky, který je možné stanovit v tělních tekutinách (Garnero, Rousseau et al. 2000).

4. Patofyziologické stavy

Výše uvedené sloučeniny (3-nitrotyrosin, COMP) byly použity k studiu následujících stavů.

4.1. Poškození plic hypoxií

Valná většina plicních onemocnění souvisí se zánětlivými procesy. Při zánětlivých procesech jsou generována velká množství reaktivních kyslíkových a dusíkových sloučenin a není proto divu, že primární fáze poškození plic je zapříčiněna těmito látkami. Za hypoxie dochází k pochodům velmi podobným jako jsou během zánětlivého procesu. V počáteční fázi jsou produkována velká množství volných kyslíkových a dusíkových radikálů a následně jejich reaktivních sloučenin, které způsobí primární poškození tkáně. Příkladem je produkce velkého množství superoxidu a oxidu dusnatého, což vede k vzniku kyseliny peroxydusité, která se pravděpodobně významně podílí na poškození tkáně. Její produkci je možno sledovat pomocí jejího markeru - 3-nitrotyrosinu.V další fázi jsou aktivovány různé proteolytické enzymy a posléze dochází k odbourávání extracelulární matrix. Novotná a Herget (Novotna and Herget 1998) prokázali vznik štěpu kolagenu I za těchto podmínek. Nakonec dochází k remodelaci tkáně, která vede plicní hypertensi (Hampl and Herget 2000).

4.2. Radikálové poškození a stárnutí rostlin.

Stárnutí listů je konečná fáze jeho vývoje, v kterém buňky listu podléhají programované buněčné smrti (Nooden 1988). Celý proces je řízen specifickými senescenčními geny, které jsou regulovány hormony (Thomas, Ougham et al. 2003), ale také volnými radikály. Kyslíkové volné radikály způsobují ztrátu fysiologických funkcí během stárnutí. Oxid dusnatý hraje významnou roli během růstu, vývoje a stárnutí a je spojen s regulací rostlinnými hormony ethylenem (Leshem and Haramaty 1996), abscisovou kyselinou (Desikan, Griffiths et al. 2002) a cytokininy (Tun, Holk et al. 2001). Jelikož je v rostlinách přítomný jak oxid

dusnatý, tak superoxid, musí nutně vznikat i kyselina peroxydusitá. Produkci kyseliny peroxydusité během stárnutí je opět možné sledovat dle jejího stabilního markeru 3-nitrotyrosinu imunochemickými metodami.

4.3. Radikálové poškození a stárnutí kvasinek

I jednobuněčné organismy jako kvasinky stárnou obdobnými mechanismy jako vyšší organismy (zvláště za podmínek kultivace buněk Metazoí in vitro) – tj. je omezen počet mitotických dělení, kterými buňka může projít. Stárnutí kvasinek je dobře popsáno jak morfologicky, tak biochemicky a tento model stárnutí je výhodný jak z hlediska rychlosti růstu a krátké délky života, tak z hlediska poměrně snadné manipulace s jejich genetickým materiálem. Radikálová teorie stárnutí (Harman 1956) vyhovuje i tomuto modelu a bylo popsáno snížení délky života za podmínek zvýšeného množství volných radikálů (Laun, Pichova et al. 2001). Dosud se uvažovalo pouze o reaktivních kyslíkových radikálech, ale lze oprávněně předpokládat, že na radikálovém poškození kvasinek, které je spojeno se stárnutím, mají vliv i reaktivní dusíkaté sloučeniny, ale až do naší studie nebyl důkaz účasti reaktivních sloučenin dusíku v kvasinkách nalezen. Jelikož stabilním produktem působení těchto sloučenin na bílkoviny je 3-nitrotyrosin, nabízela se možnost zjistit přítomnost reaktivních sloučenin dusíku a jejich relativní množství pomocí imunochemické detekce 3-nitrotyrosinu v kvasinkových proteinech během stárnutí.

4.4. Degradace kloubní chrupavky

Kloubní chrupavka je jednoduchá avaskulární tkáň s jedinným typem buněk (chondrocyty) a převahou extracelulární matrix. Majoritními složkami extracelulární matrix hyalinní chrupavky jsou kolagen typu II a proteoglykan agrekan; kromě nich zde nalezneme několik

tzv. minoritních kolagenů, několik dalších proteoglykanů a řadu tzv. nekolagenních bílkovin a glykoproteinů - jedním z nich je i COMP, který tvoří asi 1% suché váhy kloubní chrupavky.

Destrukce kloubní chrupavky je hlavním společným rysem artróz a artritid. Osteoartróza (OA) je invalidizující onemocnění, běžné ve vyšším věku (ve věkové skupině nad 75 let je incidence OA zhruba 85%); degradace a postupná ztráta kloubní chrupavky je považována za klíčový rys jejího rozvoje. Revmatoidní artritida (RA) je chronické zánětlivé autoimunitní onemocnění, pro které je typická extensivní erosivní destrukce kloubní chrupavky.

Nejrozšířenější metodou používanou k posouzení destrukce a ztráty kloubní chrupavky je měření šířky kloubní štěrbiny na roentgenových snímcích; takto detekovatelná destrukce je ale nutně už značně pokročilá a jako taková je považována za ireversibilní. S cílem překonat tuto nevýhodu se řada laboratoří ve světě zabývá vývojem tzv biochemických markerů destrukce chrupavky, tj molekul přítomných ve chrupavce a uvolňovaných do tělních tekutin při její degradaci. Celosvětově jsou do tohoto výzkumu vkládány značné intelektuální i finanční prostředky, v očekávání že biomarkery pomohou při časné diagnóze OA (dříve než jsou změny viditelné na roentgenových snímcích), při identifikaci pacientů s vysokým rizikem progrese onemocnění, při monitorování terapie, při výběru pacientů do klinických pokusů (testování nových léčiv), a podobně. U revmatoidní arthritidy jakožto zánětu lze navíc detekovat průběh počáteční fáze zánětu (tvorba volných radikálů a kyseliny peroxydusité) pomocí sledování hladiny 3-nitrotyrosinu a indukci proteolytických enzymů pomocí sledování matrixinů v synoviální tekutině. Vhodnými sérovými markery poškození chrupavky jsou pak až výše uvedené degradační produkty hyalinní chrupavky.

CÍLE PRÁCE

K studiu tkáňového poškození byly vybrány dva markery, které jsou charakteristické pro první respektive třetí fázi tkáňového poškození. Jako marker počátečního poškození tkáně reaktivními sloučeninami dusíku byl vybrán 3-nitrotyrosin a jako marker třetí fáze tkáňového poškození chrupavky COMP (trombospondin-5).

V době vývoje metody pro stanovení 3-nitrotyrosinu byla sice na trhu monoklonální protilátka, ale citlivost imunoesejí s touto protilátkou byla příliš nízká, takže ji nebylo možno využít k studiu rozvoje tkáňového poškození. Bylo tedy nutné nejprve připravit monoklonální protilátku s vyšší afinitou a poté vyvinout citlivou a jednoduchou metodu, kterou by bylo možno stanovit 3-nitrotyrosin v dostatečně ředěných sérech (alespoň 40x) a v tkáňových extraktech při koncentracích bílkovin pod 1mg/ml.

Jako první z imunochemických markerů degradace kloubní chrupavky, detekovatelném v tělních tekutinách, byl testován keratansulfát. Časem bylo ale zjištěno, že keratansulfát není optimální marker, protože může být při různých fyziologických stavech různě modifikován (např. sulfatován), ale monoklonální protilátky jsou vždy proti jedinému epitopu (např. specificky sulfatovanému disacharidu). Jako další slibný marker byl vytipován COMP, protože bezprostředně po jeho objevu se předpokládalo, že je absolutně specifický pro chrupavku. Pouze jediná laboratoř ve světě disponovala v tomto počátečním stádiu polyklonálními protilátkami . Naším cílem tedy bylo připravit soubor monoklonálních protilátek proti různým epitopům COMP, z nich vybrat ty nejperspektivnější a vyvinout inhibiční a eventuelně sendvičovou ELISA, kterou by bylo možno měřit hladinu COMP v tělních tekutinách za různých fyziologických a patologických stavů.

VÝSLEDKY

Prvním studovaným markerem tkáňového poškození je 3-nitrotyrosin, který je stabilním produktem působení kyseliny peroxydusité na tyrosin. Po vypracování základní metodiky na stanovení 3-nitrotyrosinu inhibiční enzymoimunoesejí byl studován vliv čtyřdenní hypoxie (10% O_2) na produkci kyseliny peroxydusité, resp. jejího markeru – 3-nitrotyrosinu – v séru laboratorních potkanů (**Příloha 1**). Ukázalo se, že hladina 3-nitrotyrosinu je u potkanů v hypoxii významně zvýšena. Je zřejmé, že za hypoxie zvýšená produkce kyslíkových a dusíkových radikálů vede k vyšší produkci kyseliny peroxydusité, která se může významně podílet na nežádoucích modifikacích makromolekul v raných fázích tkáňového poškození.

Zvýšená hladina 3-nitrotyrosinu byla naměřena v sérech dětí s rozvinutou bronchopulmonární dysplasií a jeho hladina korelovala s obsahem vdechovaného kyslíku (Banks, Ischiropoulos et al. 1998), ale protože patogenese bronchopulmonární dysplasie není zatím plně známo, nebylo možné rozhodnout, zda zvýšený obsah vdechovaného kyslíku je příčinou zvýšené koncentrace 3-nitrotyrosinu v séru. Proto byl v další fázi studován vliv hyperoxie a experimentální pneumonie vyvolané carrageenanem na množství 3-nitrotyrosinu v plicní tkáni laboratorních potkanů (**Příloha 2**). U dvou experimentálních skupin byla vyvolána pneumonie intratracheální aplikací carrageenanu a jedna z nich vystavena po dobu 7 dnů hyperoxii (80%O_2) a druhá byla chována za normoxických podmínek (21%O_2). Z dvou kontrolních skupin byla jedna opět vystavena hyperoxii a druhá normoxii. Obsah 3-nitrotyrosinu v plicní tkáni byl signifikantně zvýšen za normoxických podmínek u zvířat s experimentální pneumonií (carrageenan 147±7 pmol/mg proteinu, kontrola 90±10 147±7 pmol/mg proteinu). V séru týchž zvířat byla obdobně zvýšená hladina dusičnanů+dusitanů, ale nikoliv 3-nitrotyrosinu. Hyperoxie nemá vliv na koncentraci 3-nitrotyrosinu v plicní tkáni kontrolních zdravých zvířat, ale u zvířat s experimentální pneumonií blokuje jak vzrůst 3-nitrotyrosinu v plicní tkáni, tak dusitanů a dusičnanů v séru a signifikantně sníží koncentraci 3-nitrotyrosinu

v séru. Důvodem, proč hyperoxie zdánlivě blokuje nitraci tyrosinu, je pravděpodobně zvýšené odbourávání již vzniklého 3-nitrotyrosinu.

Respirační vzplanutí, spojené s vysokou produkcí kyslíkových a dusíkových radikálů, je typickým jevem během fagocytosy makrofágů. Nadprodukce jak superoxidu, tak oxidu dusnatého, vede ke zvýšené produkci kyseliny peroxydusité a následně i k zvýšené nitraci tyrosinu. Erytrocyty jsou po uplynutí své délky života odstraňovány z krevního oběhu retikuloendotheliálním systémem, ale zdánlivě paradoxně se zdá, že během jejich fagocytování makrofágy k oxidačnímu vzplanutí nedochází. V naší studii jsme využili k studiu tohoto jevu myší makrofágové linie RAW 264.7 a membrány lidských erytrocytů jak intaktních, tak po 24 hodinové peroxidaci (**Příloha 3**). Výsledky ukázaly, že erythrofagocytosa jak kontrolních, tak peroxidovaných membrán nemění hladinu 3-nitrotyrosinu v makrofázích. Zdá se, že membrány erytrocytů účinně inhibují produkci reaktivních kyslíkových i dusíkových radikálů a inhibice synthesy a jev není zapříčiněn obsahem erytrocytů, jak se dříve předpokládalo (Commins, Goodger et al. 1990).

Další studie byla zaměřena na sledování vlivu hladiny cytokininů na produkci ethylenu a oxidu dusnatého u transgenních rostlin tabáku s geneticky zvýšenou či sníženou hladinou cytokininů během vývoje listu a jeho stárnutí (**Příloha 4**). Současně byla i sledována koncentrace 3-nitrotyrosinu v buněčných bílkovinách, jak rozpustných tak i nerozpustných, jako indikátor reaktivních dusíkových sloučenin. Byl potvrzen již dříve publikovaný fakt, že ve stárnoucích listech je produkce NO snížená. Ukázalo se, že koncentrace 3-nitrotyrosinu je lepším indikátorem reaktivních dusíkových sloučenin než měření množství NO. Vyšší hladiny 3-nitrotyrosinu byly naměřeny v nerozpustných proteinech, což naznačuje, že membránové bílkoviny jsou primárním cílem reaktivních dusíkových sloučenin. Rostliny se zvýšenou hladinou cytokininů vykazovaly nižší koncentraci 3-nitrotyrosinu jak v rozpustné, tak

nerozpustné frakci bílkovin. To ukazuje na protektivní účinek cytokininů proti reaktivním dusíkovým sloučeninám.

Obdobně jako v předchozí práci byl studován vliv reaktivních kyslíkových a dusíkových sloučenin během stárnutí kvasinek Sacharomyces cerevisiae (**Příloha 5**). Byla porovnávána linie divokého typu s mutantní linií, která měla konstitutivně aktivní Ras onkogen, který je odpovědný za zvýšenou produkci volných radikálů. Zvýšená produkce kyslíkových volných radikálů vedla k akumulaci lipofuscin-like pigmentů během stárnutí Ras mutantních kvasinek, zatímco divoký typ vůbec tyto pigmenty neobsahoval, což je ojedinělé mezi známými biologickými modely. Příčinou může být zvýšená koncentrace lipofilního antioxidantu α-tokoferolu. Oproti tomu Ras mutantní linie měla koncentraci α-tokoferolu sníženou dokonce i v mladých buňkách. Sledování 3-nitrotyrosinu jakožto markeru reaktivních dusíkových sloučenin ukázalo, že jeho koncentrace v rozpustných bílkovinách je signifikantně zvýšena v senescentních Ras mutantních kvasinkách jak oproti mladým mutantním buňkám, tak proti koncentraci 3-nitrotyrosinu v senescentních kvasinkách divokého typu.

Další soubor prací se zabýval detekcí poškození kloubní chrupavky a jako marker byl vybrán COMP. Počáteční studie (**Příloha 6**) se zabývala přípravou monoklonálních protilátek proti COMP a jejich charakterizací. Prokázali jsme omocí SDS-elektroforesy a imunoblotů se štěpy COMP připravenými trypsinovou digescí, že každá z tří vybraných monoklonálních protilátek reaguje s jinou částí molekuly. Pomocí imunoblotů jsme rovněž prokázali, že protilátky rozeznávají různé fragmenty COMP nacházející se v lidské synoviální tekutině. Vyvinuli jsme dvě nezávislé inhibiční EIA se dvěma z těchto protilátek a zjistili jsme, že koncentrace antigenního COMP je v lidské synoviální tekutině o dva řády vyšší než v séru a že koncentrace COMP v synoviální tekutině pacientů s osteoarthrosou jsou signifikantně vyšší než u pacientů s revmatoidní arthritidou.

Takto vyvinutá inhibiční EIA s jednou z připravených monoklonálních protilátek (17-C10) byla využita v klinické studii, která sledovala hladinu COMP v sérech pacientů s osteoarthrosou kolenního kloubu (**Příloha 7**). Koncentrace COMP v séru korelovala s věkem, synovitidou a kombinací synovitidy a míry osteoarthrotického poškození. Synovitida se ukázala jako nejvýznamnější faktor zvyšující hladinuCOMP.

V další studii byly mapovány epitopy COMP na které se váží tři vybrané monoklonální protilátky (**Příloha 8**). Tryptické fragmenty COMP byly rozděleny pomocí SDS-PAGE a pomocí imunoblotování byly určeny fragmenty specificky rozpoznávané vždy výhradně jedinou z protilátek. U těchto specifických fragmentů byla pomocí Edmanova odbourávání určena N-koncová sekvence aminokyselin. Porovnáním se známou primární strukturou COMP bylo prokázáno, že každá z testovaných protilátek se váže na jinou doménu v jeho podjednotce (N-terminální doména, oblast EGF-like a C-terminální oblast).Pomocí kompetetivní EIA bylo dále prokázáno, že mezi těmito protilátkami neexistuje stérická kompetetice. Bylo tudíž možno vyvinout sendvičovou imunoesej. Určili jsme základní parametry metody (inter- a intra-esej variabilita) a porovnali jí s dříve publikovanou inhibiční EIA. Sendvičovou metodou byly poté stanoveny základní údaje týkající se hladiny COMP u lidí (zdravých i revmaticky nemocných, v séru i synoviální tekutině, kolísání hodnot ze dne na den a cirkadiální variabilita). Výsledky prokázaly, že vyvinutá sendvičová EIA je citlivá a vysoce reprodukovatelná metoda vhodná pro stanovení COMP v lidském séru a synoviální tekutině.

DISKUZE

Tématem disertace je využití imunochemických metod pro studium tkáňového poškození. Základním kamenem každé imunochemické metody je specifická protilátka proti studované substanci. V souboru prací předložených ve výsledcích byly vždy použity naše vlastní monoklonální protilátky připravené popsanými postupy (Goding 1986) (Karsten, Stolley et al. 1993; Neil and Zimmermann 1993). V případě 3-nitrotyrosinu se jedná o nízkomolekulární sloučeninu (hapten) a bylo tedy třeba připravit MAb proti jedinému epitopu s těmi nejlepšími možnými vlastnostmi (z hlediska afinity a specifity), u COMP(trombospondinu-5) se naopak jedná o makromolekulu s mnoha epitopy a bylo tudíž možno připravit celý soubor MAb proti různým částem polypeptidického řetězce.

Stanovení 3-nitrotyrosinu

Stanovení 3-nitrotyrosinu je v principu možné třemi různými typy metod – optickými (spektrofotometrie a fluorimetrie), chromatografickými a imunochemickými.

Stanovení 3-nitrotyrosinu na základě rozdílu absorbance při 430nm v kyselém a alkalickém prostředí je velmi jednoduché, ale vhodné pouze pro stanovení čistého 3-nitrotyrosinu, protože citlivost je nízká (ε= 4400 $^{M-1}$ při pH>9,5, tj. okolo 1μM) a navíc interferují látky hemové podstaty. Citlivé fluorimetrické stanovení 3-nitrotyrosinu po jeho redukci na 3-aminotyrosin nelze též pro biologické vzorky použít, protože poměrně drastická redukce nitro-skupiny 3-nitrotyrosinu způsobuje denaturaci bílkovin a fluorescenční spektrum tryptofanu přítomného v proteinech má obdobné spektrální charakteristiky. Obě metody jsou proto vhodné pouze pro stanovení čistého 3-nitrotyrosinu nebo čisté bílkoviny s 3-nitrotyrosinem.

Druhá skupina metod je založena na chromatografické isolaci 3-nitrotyrosinu ze směsi ostatních látek. Pokud je stanovován 3-nitrotyrosin v bílkovinách, je nutné nejprve provést hydrolysu bílkoviny (buď kyselinou nebo enzymaticky). 3-nitrotyrosin je možné separovat ze směsi buď pomocí kapalinové chromatografie (HPLC) nebo po jeho derivatizaci plynovou chromatografií . Výhodou všech chromatografických metod je citlivost stanovení, která je navíc stejná pro volný i vázaný 3-nitrotyrosin (nezávisle na jeho lokalizaci v polypeptidickém řetězci) a proto je stanovení 3-nitrotyrosinu tímto způsobem bráno jako „zlatý standard". Hlavní nevýhodou je jednak složitá příprava vzorku (včetně možných artefaktů při hydrolyse bílkovin) a také jak časová , tak finanční náročnost vlastního stanovení (cena přístrojů i cena vlastního stanovení). Proto jsou tyto metody využívány hlavně pro výzkumné účely.

Z imunochemických metod jsou pro stanovení 3-nitrotyrosinu využívány imunoeseje a nejčastěji EIA a parametry stanovení (citlivost, selektivita) jsou dány hlavně použitou protilátkou proti 3-nitrotyrosinu. Do současné doby popsané protilátky proti 3-nitrotyrosinu mají výrazně vyšší citlivost (přibližně 100x) vůči 3-nitrotyrosinu vázanému v proteinech (Crow and Ischiropoulos 1996), což je pravděpodobně zapříčiněno způsobem přípravy protilátky (imunizace je prováděna nosičem (BSA nebo KLH) s nitrovaným tyrosinem ve vlastním polypeptidickém řetězci). Také naše protilátka NO-60-E3 se vykazovala citlivost o dva řády vyšší k vázanému 3-nitrotyrosinu oproti volnému. Proto i námi vyvinutá EIA je použitelná pouze pro stanovení 3-nitrotyrosinu vázaného v bílkovinách. Námi popsané stanovení se nevymyká výše uvedeným omezením, ale oproti dříve popsaným metodám využívá protilátku s vyšší afinitou k 3-nitrotyrosinu a proto je citlivější než dříve popsané metody (Herce-Pagliai, Kotecha et al. 1998; Khan, Brennand et al. 1998; Tanaka, Choe et al. 1998; ter Steege, Koster-Kamphuis et al. 1998). Fakt, že afinita vůči protilátek proti 3-nitrotyrosinu je různá pro jednotlivé nitrované tyrosinové zbytky, nevadí, protože míra nitrace tyrosinů v bílkovině je též různá a kyselinou peroxydusitou (a pravděpodobně i jinými

reaktivními sloučeninami dusíku) jsou více nitrovány tyrosinové zbytky na povrchu makromolekuly v hydrofilnějších částech polypeptidického řetězce.

Stanovení COMP

Množství bílkoviny lze někdy zjistit na základě jejich funkčních vlastností (např. enzymy či hormony), ale obecně využitelnými metodami stanovení bílkovin jsou imunoeseje. Máme-li k disposici jedinou protilátku proti dané antigenní determinantě, připadá v úvahu nějaká varianta kompetetivní imunoeseje, pokud disponujeme protilátkami proti dvěma různým epitopům vysokomolekulárního antigenu, je možné vyvinout i sendvičovou imunoesej, která je obecně citlivější a více specifická. Do přípravy našich monoklonálních protilátek (17-C10, 16-F12 a 12-C4) byl COMP stanovován EIA s polyklonálními protilátkami (Saxne and Heinegard 1992; Saxne and Heinegard 1995); využití monoklonálních protilátek umožnilo podle očekávání zvýšit citlivost, selektivitu a reprodukovatelnost stanovení.

3-nitrotyrosin v tělních tekutinách a tkáňových extraktech

Při stanoveních hladin 3-nitrotyrosinu v sérech experimentálních zvířat, v extraktech krysích plic, listů rostlin či kvasinek byl počátečním problémem standard. Pokud jsme používali jako standard in vitro nitrovaný BSA, jehož obsah 3-nitrotyrosinu jsme určili spektrofotometricky, závisely absolutní naměřené hodnoty na tom, do jaké míry byly tyrosinové zbytky v BSA nitrovány. Vysoce nitrovaný BSA (25-50% tyrosinových zbytků nitrováno) vykazoval podstatně nižší koncentraci 3-nitrotyrosinu (určenou spektrofotometricky) potřebnou pro 50% vazbu s protilátkou než BSA s jedním či dvěma 3-nitrotyrosinovými zbytky v molekule. Je známo, že lokalizace 3-nitrotyrosinu v makromolekule ovlivňuje výrazně schopnost vazby

protilátky , která záleží na tom, zda se modifikovaný tyrosin nachází na povrchu molekuly, či je „zanořen" uvnitř (a nedostupný pro protilátku) (Ischiropoulos 2003). Vlastní statistické hodnocení míry nitrace proteinů, tj. např. fakt, že za 4 denní hypoxie je signifikantně zvýšená hladina 3-nitrotyrosinu v séru krys atd. není touto skutečností ovlivněno, protože série pokusů v každé z předložených prací byla vždy prováděna se stejně nitrovaným standardem. Později byl jako standard používán synthetický peptid Asp-Ser-Gly-NTyr-Gly-Leu–Glu (**Příloha 4, Příloha 5**).

Vážným problémem pro validitu stanovení 3-nitrotyrosinu jako markeru množství produkovaných reaktivních sloučenin dusíku se zdály práce, které popisovaly denitraci 3-nitrotyrosinu. Gow a spol. (Gow, Duran et al. 1996) tvrdí, že objevili v lidské plazmě neznámý faktor, který je schopen „denitrovat" nitrotyrosin neproteolytickým mechanizmem. Inkubací čerstvé plazmy a nitrovaného hovězího serumalbuminu se v závislosti na čase, teplotě a koncentraci plazmy 3-nitrotyrosin ztrácel. Bylo popsáno, že kyselina peroxydusitá tento faktor inaktivuje - plazma, která nejprve reagovala s kyselinou peroxydusitou, schopnost „denitrace" neměla. Muradova skupina (Kamisaki, Wada et al. 1998) popsala ztrátu epitopu rozpoznávaného monoklonálními protilátkami proti 3-nitrotyrosinu na imunoblotech nitrovaného hovězího albuminu po jeho inkubaci se supernatanty z homogenátů krysích slezin a plic. Faktor, odpovědný za odbourávání 3-nitrotyrosinu byl citlivý k trypsinu, větší než 10kDa, labilní při vyšší teplotě a vykazoval určitou substrátovou specificitu, protože volný 3-nitrotyrosin ani nitrované proteiny vazbu protilátky nesnižovaly. Po intraperitoneálním podání lipopolysacharidu aktivita faktoru 2-3x vzrostla, což naznačuje, že aktivita je regulovatelná nebo inducibilní. Při použití homogenátů jater nebo ledvin se podobná aktivita neprojevila, což by svědčilo pro určitou orgánovou specificitu. V další práci autoři porovnávali reakce plic, sleziny a jater po intraperitoneálním podání lipopolysacharidu a zjistili v plicích těžké tkáňové poškození, výraznou aktivaci iNOS a myeloperoxidasy a

infiltraci zánětlivými buňkami (PMN, makrofágy) (Bian and Murad 2001). Hladina 3-nitrotyrosinu byla přitom nižší než v játrech. Naopak v játrech i ve slezině bylo poškození tkáně menší, ale nitrovaných proteinů více. I u kontrolních krys bez lipopolysacharidu bylo v játrech a ve slezině (s výjimkou bílé pulpy sleziny) více 3-nitrotyrosinu. Neenzymatická redukce 3-nitrotyrosinu na aminotyrosin byla popsána stejnou skupinou (Balabanli, Kamisaki et al. 1999) a je závislá na hemu a thiolových skupinách. Thiolové skupiny mohou být nahrazeny i jinými redukčními agens (kyselina askorbová) a hemová skupina může pocházet z různých hemoproteinů, nejen z hemoglobinu.

Domnívám se, že výše uvedené nálezy lze lépe a jednodušeji vysvětlit pomocí pracovní hypotézy postulující, že během přípravy a skladování vzorku dochází k změnám konformace nitrované bílkoviny a k interakcím různých proteinů mezi sebou navzájem. Tímto se dříve protilátce přístupné nitrotyrosinové zbytky se zanoří dovnitř molekuly proteinu či „zakryjí" jinou molekulou a jsou nedostupné. Také dojde k změnám náboje v okolí nitrotyrosinového zbytku a to může silně ovlivnit vazbu protilátky na 3-nitrotyrosin, stejně jako modifikace aminokyseliny v okolí 3-nitrotyrosinu. Následující fakta svědčí pro tuto hypotézu

a) Gow a spol. (Gow, Duran et al. 1996) popsali, že inkubací plasmy s nitrovaným BSA se epitop 3-nitrotyrosinu ztrácí a množství detekovaného 3-nitrotyrosinu polyklonální protilátkou pomocí dot-blotů více klesá se stoupající teplotou a množstvím přidané plasmy. Množství 3-nitrotyrosinu určené pomocí aminokyselinové analysy bylo také nižší u vzorku nitrovaného BSA po inkubaci s plasmou. Práce obsahuje mnoho metodických nejasností a nepřesností (např. (i) různé podmínky inkubace vzorků na dot bloty a na aminokyselinovou analysu, (ii) při vyšším obsahu plasmy ve vzorku se evidentně nebude část nitrovaného BSA na nitrocelulosu vázat a to je důvod snížených hodnot (iii) není jasné, jakým způsobem odstranili plasmu anebo isolovali albumin ze směsi před

hydrolysou na aminokyselinovou analysu atd.) a proto z uvedené práce nelze vyvodit žádný závěr

b) Muradovou skupinou popsané „denitrasa" je artefakt. Probíhající mizení nitrotyrosinového epitopu je způsobeno (i) neezymatickou redukcí merkaptoethanolem či DTT za katalysy hemem (slezinné extrakty jsou na ně obzvláště bohaté) při přípravě vzorků na SDS-polyakrylamidovou elektroforesu a blotování (Balabanli, Kamisaki et al. 1999), (ii) aktivací myeloperoxidasy během zánětu dochází k zvýšené produkci kyseliny chlorné; ta destruuje 3-nitrotyrosin (Whiteman and Halliwell 1999), takže k částečné destrukci 3-nitrotyrosinu dochází, ale není zapříčiněna specifickým „denitračním" enzymem.

c) vazbu protilátky ovlivňují aminokyseliny v okolí 3-nitrotyrosinu a také lokalizace 3-nitrotyrosinu, tj. zda je na povrchu molekuly, či je „zanořen" uvnitř a nedostupný pro protilátku (Ischiropoulos 2003).

d) rozptyl stanovení 3-nitrotyrosinu v sérech experimentálních zvířat je poměrně malý, rozptyl stanovení v lidském séru je výrazně vyšší. Séra a extrakty tkání experimentálních zvířat jsou připravena identickým způsobem a identicky skladována, protože jsme prokázali, že způsob odběru má vliv na naměřené hodnoty 3-nitrotyrosinu; stresovaná zvířata (odebíraná kardiální punkcí) měla koeficient variace(%CV) o 50% vyšší než standartně odebíraná zvířata. Také způsob přípravy séra může ovlivnit naměřené hodnoty (Herget a Vytášek, nepublikované výsledky). Koeficient variace stanovení 3-nitrotyrosinu byl v krysích serech okolo 15% (**Příloha 2**), ale identickou metodikou v stejném čase byl koeficient variace stanovení 3-nitrotyrosinu v lidském séru (poměrně standartně připraveném) z pupečníkové krve novorozenců 25% a dále se zvyšoval v sérech z krve odebírané v prvních dnech života dítěte (Fišárková 2005).

e) S výše uvedeným poznatkem koreluje i fakt, že identická lidská séra skladovaná po různě dlouhou dobu vykazují různou koncentraci 3-nitrotyrosinu při stanovení pomocí EIA (Vilím, osobní sdělení)

f) Byly připraveny čtyři různé peptidy, které obsahovaly uvnitř řetězce nitrotyrosinový zbytek s nimi provedena kompetetivní EIA a spočítány pomocí Rodbardovy čtyřparametrové rovnice koncentrace peptidu inhihující z 50% (Rodbard and McClean 1977). Zatímco potřebná koncentrace u peptidu s kyselými aminokyselinami (Asp-Ser-Gly-NTyr-Gly-Leu–Glu) byla 0,54μM, u peptidu s neutrální (Ala-Ala-Ala-NTyr-Ala-Ala-Ala) resp. aromatickou (Phe-Val-Gly-NTyr-Gly-Ala-Leu) AA byla 2,72μM resp. 3,48μM a u peptidu s bazickými aminokyselinami (Arg-Asn-Gly-NTyr-Gly-Ala-Lys) dokonce 7,53μM (Vytášek a Vilím nepublikované výsledky)

V současné podobě je naše EIA pro stanovení 3-nitrotyrosinu (**Příloha 3**) s využitím jako standardu synthetického peptidu s 3-nitrotyrosinem plně vhodná pro stanovení 3-nitrotyrosinu v různých biologických materiálech připravených standardním způsobem a validita metody byla ověřena stanoveními 3-nitrotyrosinu jak v sérech a tkáních experimentálních zvířat, tak v listech rostlin a extraktech z kvasinek. Zvýšení citlivosti této EIA metody je možné nahrazením barevné peroxidasové reakce reakcí chemiluminiscenční reakcí peroxidasy, což zvýšilo citlivost asi 10x (Vilím a Vytášek, nepublikované výsledky).

V budoucnosti by vlastní metoda mohla být vylepšena konstrukcí hybridomu produkujícího protilátku, která by měla stejnou nebo vyšší afinitní konstantu jako současná NO-60-E3, ale vázala by se stejně dobře na volný jako vázaný 3-nitrotyrosin. Také v přípravě vzorků pro EIA by bylo možné zlepšit přípravu vzorků tak, aby možné agregace proteinů či změny jejich konformace neměly vliv na rozpoznávání nitrotyrosinového epitopu protilátkou. Tak by bylo možné sledovat citlivěji poškození bílkovin reaktivních sloučenin dusíku v živých organismech.

COMP v séru a synoviální tekutině.

Diskuse vlastních výsledků, týkajících se vlastností monoklonálních protilátek, EIA (inhibiční i sendvičové) i měření hladiny COMP v lidském séru a synoviální tekutině za různých fyziologických a patologických stavů, jsou uvedeny v **přílohách 6,7,8**. Dle WEB Science byly do konce r. 2005 ostatními autory tyto práci citovány 12x, 9x a 6x ; vyčerpávající obecnou diskusi všech prací, popisujících využití našich mAb 16-F12 a 17-C10 při prognóze OA podala Jordanová (Jordan 2004). Další perspektiva využití těchto monoklonálních protilátek a EIA metod je ve zjištění Riessena a spol. (Riessen, Fenchel et al. 2001), že trombospondin-5 se vyskytuje ve hladké svalovině cév.

ZÁVĚR

Předložený soubor ukazuje, jak lze využít imunochemických metod a zvláště enzymoimunoesejí k studiu mechanismu tkáňového poškození. První série prací se zabývala stanovení stabilního produktu působení reaktivních dusíkových sloučenin – 3-nitrotyrosinu. Bylo jednak prokázáno, že hladina 3-nitrotyrosinu v seru potkanů se signifikantně zvyšuje během čtyřdenní hypoxie. Oproti tomu hyperoxie u zdravých zvířat hladinu 3-nitrotyrosinu v seru ani v plicní tkáni nemění. Při experimentální pneumonii vzrůstá signifikantně množství 3-nitrotyrosinu v plicní tkáni a hyperoxie tento vzrůst blokuje a dokonce signifikantně snižuje koncentraci 3-nitrotyrosinu v seru. Při studiu erythrofagocytosy makrofágovou linií bylo stanovení 3-nitrotyrosin jedním z důkazů, že inhibice respiračního vzplanutí během fagocytosy erytrocytů je zapříčiněno jejich membránami a nikoliv jejich obsahem.

Sledování 3-nitrotyrosinu během stárnutí modelových vyšších rostlin (Nicotiana) a kvasinek (Sacharomyces) ukázalo, že přítomnost 3-nitrotyrosinu po poškození reaktivními dusíkovými sloučeninami je universální marker u všech Eukaryontů a úzce souvisí s pochody spojenými se stárnutím. Účast reaktivních sloučenin dusíku na stárnutí kvasinek nebyla do této nikým popsána.

Soubor tří prací se věnoval vývoji monoklonálních protilátek proti COMP, jejich charakterisaci a využití v enzymoimunoesejích. Obě eseje – inhibiční i sendvičová EIA – jsou vhodné k stanovení hladiny COMP v séru i synoviální tekutině. Aplikovatelnost těchto stanovení byla dokumentována vyšetřením souboru pacientů s různým revmatickým postižením.

Souhrně lze říci, že imunochemické metody jsou citlivé, levné a relativně málo pracné metody vhodné k objasnění průběhu různých fází tkáňového poškození a při dalším studiu těchto mechanismů bude jejich význam stále stoupat.

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Příloha 1

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MINIREVIEW

A Possible Role of the Oxidant Tissue Injury in the Development of Hypoxic Pulmonary Hypertension

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Summary

Chronic sojourn in hypoxic environment results in the structural remodeling of peripheral pulmonary arteries and pulmonary hypertension. We hypothesize that the pathogenesis of changes in pulmonary vascular structure is related to the increase of radical production induced by lung tissue hypoxia. Hypoxia primes alveolar macrophages to produce more hydrogen peroxide. Furthermore, the increased release of oxygen radicals by other hypoxic lung cells cannot be excluded. Several recent reports demonstrate the oxidant damage of lungs exposed to chronic hypoxia. The production of nitric oxide is high in animals with hypoxic pulmonary hypertension and the serum concentration of nitrotyrosine (radical product of nitric oxide and superoxide interaction) is also increased in chronically hypoxic rats. Antioxidants were shown to be effective in the prevention of hypoxia induced pulmonary hypertension. We suppose that the mechanism by which the radicals stimulate of the vascular remodeling is due to their effect on the metabolism of vascular wall matrix proteins. Non-enzymatic protein alterations and/or activation of collagenolytic matrix metalloproteinases may also participate. The presence of low-molecular weight cleavage products of matrix proteins stimulates the mesenchymal proliferation in the wall of distal pulmonary arteries. Thickened and less compliant peripheral pulmonary vasculature is then more resistant to the blood flow and the hypoxic pulmonary hypertension is developed.

Key words

Pulmonary hypertension • Chronic hypoxia • Pulmonary vasculature • Structural remodeling • Oxygen free radicals • Nitrotyrosine

Introduction

Two basic mechanisms increase the pulmonary vascular resistance in hypoxic pulmonary hypertension: structural remodeling of peripheral pulmonary vasculature and vasoconstriction. In the developed hypoxic pulmonary hypertension the structural remodeling prevails. It consists of proliferation and hypertrophy of vascular smooth muscle cells in peripheral pulmonary arteries (muscularization) and excessive production of matrix

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proteins in the vascular wall. Vasoconstricticton may play a role in the early phases of exposure to hypoxia, although pharmacological vasodilatation was documented even in the "steady-state" of the disease (McMurtry *et al.* 1977, Dingemans and Wagenvoort 1978, Emery *et al.* 1981). We hypothesize that hypoxic injury to the pulmonary vascular wall is a mechanism, which triggers vascular remodeling (Herget and Ježek 1989, Hampl and Herget 2000). The production of reactive oxygen metabolites plays a crucial role in lung tissue injury (Kinnula *et al.* 1995). In the present paper, we discuss the evidence that the production of free radicals is increased in chronic hypoxia and that increased radical production may be pathogenetically involved in remodeling of the wall of peripheral pulmonary arteries.

Production of reactive oxygen species (ROS) in lung tissue hypoxia

Several studies have documented that exposure to low partial pressures of oxygen induces rapid changes in membrane lipid composition of a variety of mammalian cell lines (Coleman *et al.* 1976, Johnstone *et al.* 1985). These changes may be caused by a free radical-initiated lipid peroxidation, a view supported by an increase of malonaldehyde, one of the aldehydic products of lipid peroxidation, in the serum, heart, lung, liver and kidney of hypoxic rats (Nakanishi *et al.* 1995). Accordingly, indicators of lipid peroxidation have been observed in cultures of pulmonary artery endothelial cells exposed to hypoxia (Block *et al.* 1989).

The underlying mechanism of hypoxic oxidative damage may be multifactorial. The prerequisite of oxidative damage is the formation of oxidants and these may originate from several processes:

1. Superoxide leakage from mitochondrial respiration may play an important role. Under hypoxic conditions the electron transport chain may become more reduced, resulting in an increased rate of non-enzymatic autoxidation of electron transport components, producing superoxide (Cadenas *et al.* 1977).
2. Certain oxygen utilizing enzymes which use reduced flavins or semiquinones produce more superoxide at low oxygen tensions than at normal oxygen tension (Misra and Fridovich 1972).
3. Another possible source of increased radical production with hypoxia is xanthine oxidase, as both this enzyme and its substrate, hypoxanthine, are increased under hypoxic conditions (Fried *et al.* 1973).
4. The important source of reactive oxygen species is NADPH oxidase of phagocytes, which produces superoxide and hydrogen peroxide. White cells may be attracted to hypoxic tissue and activated by products of hypoxic tissue injury. NADPH oxidase was found in smooth muscle cells of pulmonary arteries and was activated by hypoxia to produce superoxide (Marshall *et al.* 1996).

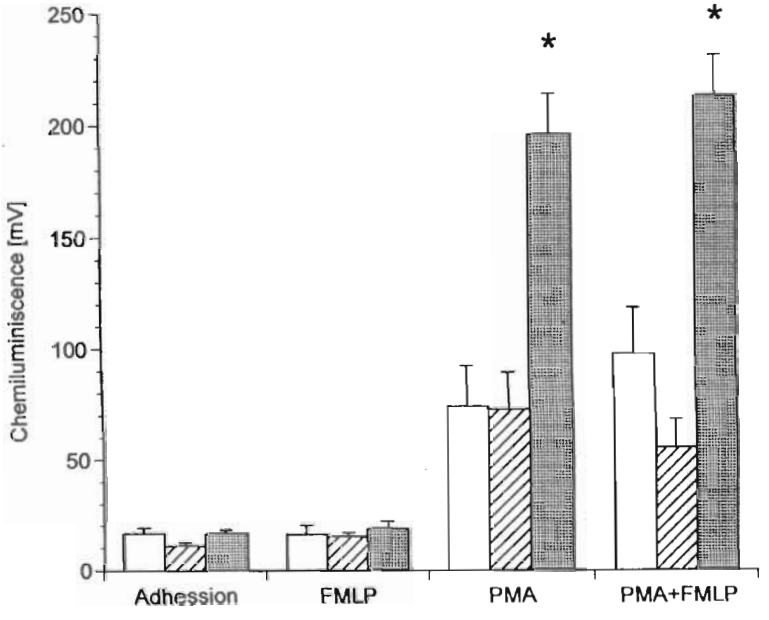

Fig. 1. *The increase of H_2O_2 production (chemiluminiscence) by alveolar macrophages isolated from control rats and from rats exposed to hypoxia. Adhesion – production of H_2O_2 after the cells adhered to measuring cuvette surface. FMLP – surface receptor mediated stimulation by chemotactic peptide N-formyl-methionyl-leucyl-phenylalanin. PMA – direct stimulation of protein kinase C by phorbol myristate acetate. *$P<0.05$. (Reproduced with permission from Wilhelm et al. 1996.)*

We have found an increased production of hydrogen peroxide by alveolar macrophages isolated from rats exposed to hypoxia (Wilhelm *et al.* 1996). Hydrogen peroxide production was measured by luminol-dependent chemiluminescence after stimulation of the macrophages by adherence, phorbol myristate acetate, or by chemotactic peptide. It was observed that a 3-day hypoxia primes alveolar macrophages for an enhanced production of H_2O_2 upon stimulation (Fig. 1). It is interesting that such an effect of hypoxia was not found in peritoneal macrophages (Wilhelm *et al.* 1997).

The production of reactive oxygen species by animals exposed to hypoxia was directly detected by measuring H_2O_2 in the expired breath (Wilhelm *et al.* 1999). Animals exposed to hypoxia for 3 days had an increased amount of H_2O_2 in their breath by 100 % in comparison to control animals. After 7 days of recovery in air, the exposed animals still produced significantly increased levels of H_2O_2 (Fig. 2). It was hypothesized that H_2O_2 is a product of enzymic reactions that are themselves sensitive to oxidative damage, because the production of H_2O_2 was inhibited under the conditions of severe oxidative stress induced by paraquat administration.

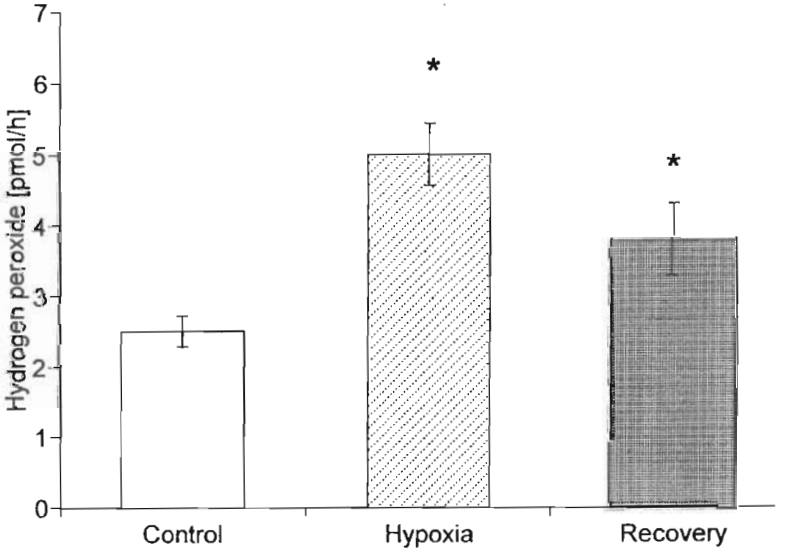

Fig. 2. *Production of hydrogen peroxide in the breath of rats exposed to and recovering from hypoxia. Ordinate shows the amount of hydrogen peroxide collected from the expired breath during 1 hour. Hypoxia indicates the group exposed to 10% oxygen for 3 days, recovery represents the measurement of the hypoxic group after 7 days of air breathing. * $P<0.001$. (Reproduced with permission from Wilhelm et al. 1999.)*

Oxidative damage to various tissues can be detected by measuring the concentration of fluorescent end products of lipid peroxidation. These are usually termed lipofuscin-like pigments (LFP). The exposure of rats to hypoxia for various time periods induced formation of LFP in both erythrocytes and the spleen (Wilhelm and Herget 1999a). The concentration of LFP depended on the duration of hypoxic exposure both in erythrocytes and the spleen. The higher concentration of LFP (compared with normoxia) was found in erythrocytes after 3 days of hypoxia. On the contrary, a decrease in LFP concentration was observed 3 h and 21 days after exposure. The decrease of LFP in erythrocytes was paralleled by an increase in the spleen.

In the next study, we focused on the effects of hypoxia on the lung tissue (Wilhelm and Herget 1999b). The LFP concentration was measured in relation to the activity of superoxide dismutase (SOD). This enzyme is known to be inducible by its substrate. This implies that the increase in its activity is to be expected under conditions of enhanced free radical production. There are principally two types of mammalian SOD that differ by the metal ion in their active center and by their subcellular localization. Cu,Zn-SOD is preferentially found in the cytoplasm, while Mn-SOD is an enzyme localized in the mitochondrial matrix. The experiment was organized in the following way. Adult male rats were exposed to normobaric hypoxia (10 % O_2) for 3 h, 1 day, 10 days and 21days. After 21 days of hypoxic treatment, samples were also taken after 3 and 14 days of recovery in the air. LFP were assayed in lipophilic extracts of the lungs. Four types of fluorophores were observed, labeled according to their excitation/emission maxima F263/376, F287/330, F330/370, and F345/458. These fluorophores had a different time-course of concentration during hypoxia and recovery in air. F287/330 increased after 3 h

of hypoxia ($P<0.0001$), then decreased deeply below control value after one day ($P<0.0001$), and with minor fluctuations remained at this level for up to 21 days of hypoxia. During recovery in air it returned to the control level. The other fluorophores were significantly reduced during hypoxia and they either reached the initial values during recovery (F345/458), or rose to significantly higher levels (F263/376, F330/370). The maximum increase was observed on the 14th day of recovery. The time-course of one of these fluorophores (F330/370) is illustrated in Figure 3 together with the changes of Cu,Zn-SOD and Mn-SOD activity. Its concentration slightly increased (to about 160 %) immediately after transition of the animals to hypoxia and then again after transition from hypoxia to normoxia. Its concentration was decreased throughout the 21 days of hypoxia, indicating attenuated production of free radicals during prolonged hypoxia. This view is supported by low levels of Cu,Zn-SOD. The activity of Mn-SOD started to rise immediately after transition to hypoxia and reached its maximum after one day of hypoxia. It then oscillated around the control value and started to rise significantly after transition of the animals to air, reaching the maximum activity after 14 days of recovery in air (Fig. 3). These data, taken together, indicate that free radicals are actively produced during the transition periods from air to hypoxia and *vice versa*. The site of their production appears to be localized in the mitochondria. We assume that F330/370 might represent a fluorophore produced during oxidative damage to lung mitochondria

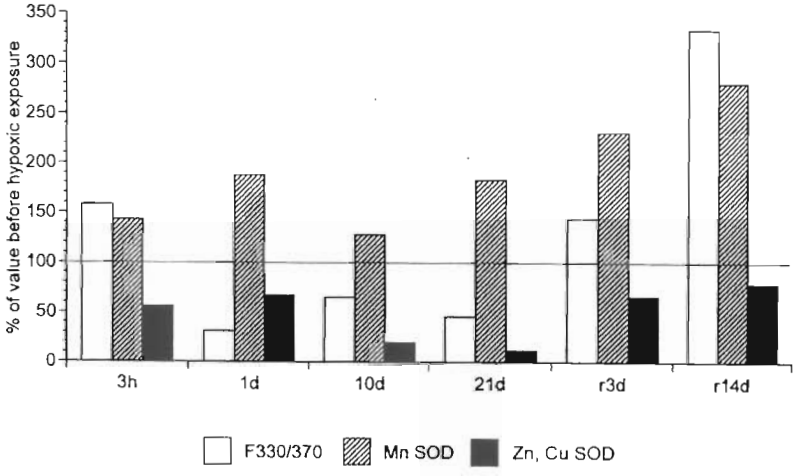

Fig. 3. *The time course of the fluorophore F330/370 in relation to the activity of Cu,Zn-SOD and Mn-SOD during hypoxia and return to air breathing. All the data are expressed in percentage of control values. The time of sampling is indicated below abscissa: 3h – 3 hours of hypoxia, 1d – 1 day of hypoxia, 10d – 10 days of hypoxia, 21d – 21 days of hypoxia, r3d – recovery in air for 3 days, r14d – recovery in air for 14 days.*

Collagen represents a possible target for free radicals in the extracellular matrix. It is difficult, however, to prove collagen oxidation *in vivo* by standard methods. We therefore initiated a study that aimed at the detection of oxidized collagen by means of its fluorescence. In a model oxidation of collagen initiated by UV irradiation *in vitro* we found new specific fluorophores which might be potentially used for the estimation of collagen damaged by free radical attack *in vivo* (Wilhelm *et al.* 1998).

The oxidative damage to the extracellular matrix might represent a factor responsible for the initiation of the remodeling of the vascular wall triggered by chronic hypoxia. We studied the effects of collagen type I oxidation on the proliferation of smooth muscle cells (VSMC) obtained from the rat aorta in culture (Bačáková *et al.* 1997). VSMC proliferated more rapidly on UV-irradiated collagen than on normal collagen type I. The oxidation of UV-irradiated collagen was detected by the assay of specific fluorophores described above. As we have previously observed lung macrophage activation by hypoxia (Wilhelm *et al.* 1996), we tested the effects of collagen type I exposed to activated macrophages *in vitro* on the proliferation of VSMC in culture (Bačáková *et al.* 1999). The responses were complex, as collagen exposed to activated macrophages was both slightly cytotoxic and also stimulated VSMC proliferation.

Effects of antioxidants on the development of hypoxic pulmonary hypertension

Several reports have documented inhibition of the development of experimental pulmonary hypertension by antioxidants in laboratory animals. Already 10 years

ago, the group of Dr. Reid reported that antioxidant dimethythiourea inhibits the structural reconstruction of peripheral blood vessels and right heart hypertrophy induced by 10-day exposure to 10% oxygen in rats (Langleben *et al.* 1989). Similar results have been reported recently (Lai *et al.* 1998). Furthermore, the latter authors measured the pulmonary arterial blood pressure and found that it was lower in hypoxic rats treated with dimethylthiourea than in not-treated hypoxic rats. Bottje *et al.* (1995) successfully treated the pulmonary hypertension syndrome in broilers by vitamin E implants applied after hatching. The incidence of the pulmonary hypertension syndrome in chickens was decreased and the authors reported a significant increase in the antioxidant capacity in the vitamin E-treated group.

Another antioxidant used for influencing experimental pulmonary hypertension was N-acetyl-L-cysteine (NAC), a precursor of reduced glutathione. Hoshikawa *et al.* (1995) reported attenuation of pulmonary hypertension in rats by NAC treatment during exposure to hypoxia. In addition, they estimated phosphatidylcholine hydroperoxide as a measure of the presence of lipid peroxidation. It was increased in rats exposed to chronic hypoxia and not treated with NAC. This increase was reduced by NAC treatment.

In our experiments, we exposed young male rats to chronic isobaric hypoxia and the animals were treated with 20 g NAC per 1 liter of drinking water. Compared with hypoxic rats offered tap water without NAC, their pulmonary arterial mean blood pressure and right ventricle heart weight related to the body weight were significantly reduced (Herget *et al.* 1999). However, they were still higher than in normoxic controls (both treated and not-treated with NAC) (Table 1). Hoshikawa *et al.* (1995) reported that NAC treatment in hypoxia inhibited even the muscularisation of peripheral pulmonary arteries. This was not the case in our study and the relative number of peripheral pulmonary arteries with smooth muscle cells in their media was not significantly different in NAC-treated hypoxic rats from that in non-treated hypoxic controls (Table 1).

Table 1. Effects of NAC treatment in rats with experimental hypoxic pulmonary hypertension

	Body weight (g)	P_{AP} (mm Hg)	P_S (mm Hg)	Cardiac output (ml)	RV/BW (mg/100 g)	(LV+S)/BW (mg/100 g)	DL vessels (%)
Hypoxic	$263 \pm 21^{**}$	$27 \pm 0.9^{*,**}$	111 ± 6	28 ± 5	$73 \pm 5^{*,**}$	208 ± 8	$34.5 \pm 3.4^{**}$
Hypoxic + NAC	$250 \pm 17^{**}$	$21 \pm 0.8^{*,**}$	91 ± 5	23 ± 3	$60 \pm 4^{*,**}$	196 ± 6	$27.8 \pm 1.1^{**}$
Normoxic	354 ± 20	15 ± 0.4	107 ± 4	29 ± 4	46 ± 6	187 ± 9	12 ± 1.6
Normoxic + NAC	305 ± 15	14 ± 0.4	103 ± 5	24 ± 2	55 ± 2	203 ± 5	13.4 ± 1.2

*P_{AP} – pulmonary arterial mean blood pressure, P_S – mean blood pressure in the aorta, RV/BW – relative weight of the right heart ventricle, LV+S/BW – relative weight of the left heart ventricle plus septum, DL – % of muscularized distal pulmonary arteries. * Hypoxic vs. Hypoxic + NAC, ** hypoxic group vs. relevant normoxic control group.*

Role of radical lung injury in the pathogenesis of hypoxic pulmonary hypertension

We hypothesized that the proliferative process in the walls of peripheral pulmonary arteries is triggered by an alteration of matrix proteins in their walls. Exposure to hypoxia increases collagenolytic activity in the peripheral pulmonary vasculature, which results in a presence of specific, low molecular weight cleavage products of collagen type I (Novotná and Herget 1998). It has been shown repeatedly that the presence of collagen cleavage turns on mesenchymal proliferation (Gardi *et al.* 1990, 1994, Bačáková *et al.* 1997). In our experiment with NAC treatment (Herget *et al.* 1999) collagenous proteins were extracted from isolated peripheral pulmonary arteries. The extracts were analyzed by SDS PAGE electrophoresis. A characteristic, ~76 kD cleavage product occurred in all extracts from non-treated rats exposed to hypoxia. However, this protein was not present in samples from hypoxic rats treated with NAC (Fig. 4).

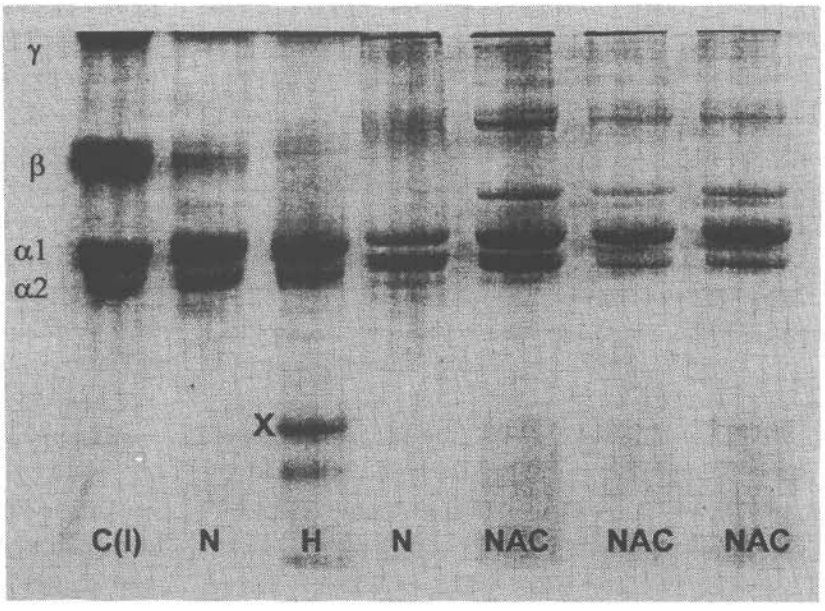

Fig. 4. *Gel electrophoresis profile of the collagenous fraction isolated from peripheral pulmonary arteries of rats exposed to hypoxia and of rats exposed to hypoxia and treated with N-acetyl-L-cysteine. C(I) – collagen type I standard from rat tail (Sigma), collagenous fraction extracted from peripheral pulmonary arteries of normal control rats (N), rats exposed for 3 weeks to hypoxia (H), rats exposed to chronic hypoxia and treated with N-acetyl-L-cysteine (NAC). γ – γ-fraction (chain polymers, collagens type I + III); β – β-fraction (chain dimers, collagens type I + III); α1 – mixture of individual α1 chains (collagens type I + III); α2 – α2 chains (collagen type I); X – small peptide present predominantly in the hypoxic peripheral pulmonary arteries (Novotná and Herget 1998).*

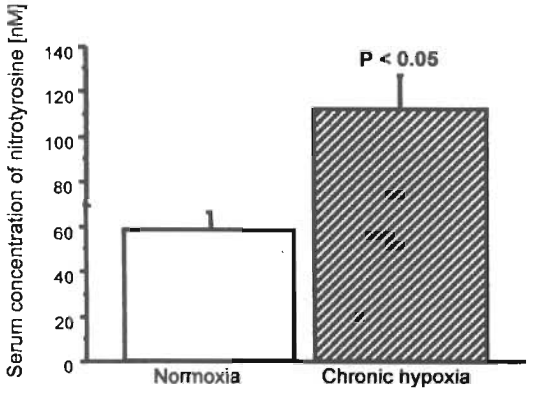

Fig. 5. *Concentration of nitrotyrosine in serum of normoxic and hypoxic rats. Nitrotyrosine was measured by inhibition ELISA. Polystyrene plates (Maxisorp, Nunc) were coated with nitrated serum albumin. Plates were incubated under gentle shaking for 90 min with monoclonal antibody (NO-60-E3, prepared in our laboratory) and diluted serum samples. After washing, the plates were then incubated with anti-mouse IgG conjugated with peroxidase (A-8924, Sigma) for another 90 min and developed with o-phenylenediamine.*

We hypothesize that the activation of collagenolysis in the peripheral pulmonary arteries is related to radical injury to the vascular wall (Hampl and Herget 2000). Increased NO production in the lungs after exposure to chronic hypoxia is well documented (Hampl and Herget 2000). Nitric oxide can react with superoxide yielding peroxynitrite and other radical products. Nitration of tyrosine in proteins to nitrotyrosine can be used as a marker of peroxynitrite production (Beckman 1996). We have recently found that the blood serum nitrotyrosine concentration is increased in rats exposed to chronic hypoxia (10 % O_2 for 4 days) (Fig. 5). This documents a production of peroxynitrite in the early phases of hypoxic exposure. Nitric oxide, superoxide and also peroxynitrite are potent activators of interstitial matrix metalloproteinase (Rajagopalan *et al.* 1996). The importance of increased collagenolytic activity for vasculature remodeling was further supported by our recent findings that pharmacological inhibition of collagenolysis by Batimastat suppressed the pulmonary vascular changes induced by chronic hypoxia (Novotná *et al.* 1999).

Hydrogen peroxide probably also plays a physiological role in the regulation of vascular tone. Vascular endothelial cells in culture release H_2O_2 into the extracellular space. This production has a constant rate under oxygen concentrations between 100 and 10 % and decreases when oxygen concentration is lowered below 10 %, reaching one third of the original activity at 0 % oxygen (Kinnula *et al.* 1993). H_2O_2 induces

vasoconstriction in preparations of isolated pulmonary arteries (Sheenan *et al.* 1993) as well as in isolated lungs (Burghuber *et al.* 1984, 1985, Tate *et al.* 1984). However, a vasodilator action of H_2O_2 was reported in other studies (Burke and Wolin 1987, Burke-Wolin and Wolin 1990, Monaco and Burke-Wolin 1995, Burke-Wolin *et al.* 1997)

We have found that high doses of hydrogen peroxide injected into the inflow cannula of isolated ventilated lungs produced a dose-dependent vasoconstriction in the range of 0.25-10 mM, with a maximum response between 2-5 mM. The effects of H_2O_2 were modulated by ionophores or specific inhibitors of ionic channels or pumps. A key role was deduced for sodium ions that regulate the subsequent inflow or outflow of calcium, i. e. an ion-mediated vasoconstriction (Wilhelm and Herget 1995). Therefore the participation of released ROS on changes of pulmonary vascular tone in hypoxic pulmonary hypertension cannot be excluded, although a direct experimental evidence of this action has not been well established yet.

In conclusion, there is growing evidence that the release of free radicals due to hypoxic lung injury participates in the onset of hypoxic pulmonary hypertension. The probable source includes NADPH oxidase of activated macrophages. The possibility that oxygen radicals are released from other cells due to the effects of hypoxia on their energy metabolism pathways has still not gained sufficient experimental support. The important pathogenetic mechanism probably concerns the role of radical substances, oxygen radicals and peroxynitrite, in the mesenchymal proliferation in peripheral pulmonary arteries.

Acknowledgements

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Reprint requests

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Příloha 2

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Hyperoxia Attenuated Nitrotyrosine Concentration in the Lung Tissue of Rats with Experimental Pneumonia

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Summary

Although nitrated proteins have been repeatedly used as markers of lung injury, little is known about their formation and metabolism under hyperoxia. We therefore measured 3-nitrotyrosine (3NTYR) concentrations in lung tissue and serum of rats with carrageenan-induced pneumonia exposed to hyperoxia. Twenty-nine Wistar male rats were assigned to one of 4 groups. Two experimental groups were treated by intratracheal application of carrageenan (0.5 ml of 0.7 % solution) and then one was exposed to hyperoxia for 7 days (F_IO_2 0.8), the other to air. Rats of two control groups breathed either hyperoxic gas mixture or air for 7 days. At the end of exposure the ventilation was determined in anesthetized, intubated animals in which 3NTYR concentrations were measured in the lung tissue and nitrites and nitrates (NO_x) were estimated in the serum. Carrageenan instillation increased 3NTYR concentrations in lung tissue (carrageenan-normoxic group 147±7 pmol/g protein, control 90±10 pmol/g protein) and NO_x concentration in the serum (carrageenan-normoxic group 126±13 ppb, control 78±9 ppb). Hyperoxia had no effect on lung tissue 3NTYR concentration in controls (control-hyperoxic 100±14 pmol/g protein) but blocked the increase of lung tissue 3NTYR in carrageenan-treated rats (carrageenan-hyperoxic 82±13 pmol/g protein), increased NO_x in serum (control-hyperoxic 127±19 ppb) and decreased serum concentration of 3NTYR in both hyperoxic groups (carrageenan-hyperoxic 51±5 pmol/g protein, control-hyperoxic 67±7 pmol/g protein, carrageenan-normoxic 82±9 pmol/g protein, control 91±7 pmol/g protein). The results suggest that hyperoxia affects nitration of tyrosine residues, probably by increasing 3NTYR degradation.

Key words

Nitrotyrosine • Hyperoxia • Experimental pneumonia • Carrageenan • Nitrites and nitrates

Introduction

3-nitrotyrosine (3NTYR) is formed in a biological process associated with NO and reactive oxygen species (ROS). It has therefore been repeatedly used as an indicator of increased oxidative stress, particularly in inflammation-related forms of lung injury like asthma (Hanazawa *et al.* 2000), cystic fibrosis (Balint *et al.* 2001), acute respiratory distress syndrome (Lamb *et al.* 1999) or airway inflammation in lung transplants (De Andrade *et al.* 2000). Interestingly enough, Banks *et al.* (1998) reported that plasma nitrotyrosine content was also increased in infants who have developed bronchopulmonary dysplasia and that its

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level correlated with the fraction of inspired oxygen that the infant was receiving. The latter finding suggests that 3NTYR could be a marker of possible adverse effects of high oxygen concentrations used during oxygenotherapy. However, because of the complicated and not fully understood pathogenesis of bronchopulmonary dysplasia, it is difficult to speculate whether increased 3NTYR levels were due to high inspired O_2 concentrations or to the severity of the disease necessitating higher O_2 concentrations during treatment. To elucidate the role of hyperoxia in 3NTYR formation, we exposed the rats with experimental pneumonia to hyperoxia lasting 7 days. We measured 3NTYR concentrations in lung tissue and serum after the exposure in these rats and in respective controls.

Methods

Studies were performed in adult male Wistar rats with initial body weight 228±3 g (mean ± SEM). All techniques used were compatible with the National Institute of Health Guidelines. The rats were divided into four groups and treated as follows:

Group	n	treatment
Car+O₂	6	carrageenan[1] + 7 days of breathing F_IO_2 0.78-0.84
Car+A	8	carrageenan[1] + 7 days of breathing air
O₂	7	7 days of breathing F_IO_2 0.78-0.84
C	8	7 days of breathing air

[1] 0.5 ml of 0.7 % solution intratracheally

Exposure to hyperoxia was performed in a normobaric chamber (Herget and Kuklík 1995) as described previously (Fišárková and Vízek 2003).

Measurements

The animals were anesthetized by thiopental (40 mg/kg, i.p.) and intubated (tracheal cannula ID 1.7 mm, OD 2.3 mm). Rats were then placed in the body plethysmograph (Maxová and Vízek 2001), the tracheal cannula was connected to an outer circuit ventilated with room air. Pressure changes in the plethysmograph were measured by pressure differential transducer (Elema-Schonander EMT 32). A specific computer program (Maxová and Vízek 2002) was used to calculate ventilatory parameters.

Blood samples (2 ml) were taken from the jugular vein, the animals were sacrificed by an overdose of anesthetic and the two lower lobes of the right lung were taken for determining 3-nitrotyrosine, nitrite and nitrate concentrations. The left lung was used to assess its wet and dry weight (Glogowska and Widdicombe 1973).

Nitrite and nitrate concentrations were analyzed using the chemiluminiscence determination of NO, based on its reaction with ozone (Hampl *et al.* 1996) using the Chemiluminiscence NO Analyzer ECO Physics CLD 77 AM. To convert nitrites and nitrates in serum to NO, vanadium, chloric acid and heating of the sample to 90 °C were used as described by Michelakis and Archer (1998)

ELISA estimation of 3-nitrotyrosine

Competitive ELISA for estimation of 3-nitrotyrosine in serum proteins was described by Herget *et al.* (2000). Briefly, polystyrene ELISA 96-well plates (Maxisorp, Nunc) were coated with BSA nitrated by TNM dissolved in PBS at a concentration 5 nM nitrotyrosine overnight. The plates were blocked by three 5 min incubation with PBS plus 0.05 %(v/v) Tween-20 (TPBS). Then 50 µl per well of 0.2 % gelatin in TBS (Tris buffered saline) pH 8.4 was pipeted and standard solution of nitrated BSA (prepared by peroxynitrite treatment) were serially diluted. Examined samples of rat serum were diluted 1:20 in the same buffer and 50 µl added to each well. Then 50 µl of 1:125 000 diluted ascites of monoclonal antibody NO-60-E3 (prepared in our laboratory) in the same buffer were added and mixture was incubated under gentle shaking at laboratory temperature for 60 min. After three washings with PBS the plates were incubated with 100 µl of antimouse Ig rabbit antibody conjugated with peroxidase (Sigma A-8924) diluted 1:1000 in 1 % BSA in PBS for 90 min. After five washings with TPBS (with duration of washings 15 min), the plates were developed with o-phenylenediamine and reaction was terminated by addition of sulphuric acid. Absorbance was read at 492 nm using a microplate reader.

All extraction and centrifugation steps were performed at 4 °C. About 100 mg of lung tissue (wet weight) was homogenized in 2 ml TBS with protease inhibitors (benzamidine, PMSF, EDTA) and centrifuged (48 000 g, 10 min). Supernatants were diluted 1:10 and concentration of 3-nitrotyrosine estimated by the same competitive ELISA. Concentration of proteins was measured by the bicinchoninic acid method (Smith *et al.* 1985).

Standard curves and concentrations of 3-nitrotyrosine in the samples were calculated according to Rodbard's four parameter equation (Rodbard and McClean 1977).

Data analysis and statistics

Each ventilatory variable was averaged over six consecutive respiratory cycles. Results are presented as means ± S.E.M. ANOVA and Fisher's PLSD test were used for statistical evaluation of the data. $P<0.05$ was considered significant.

Results

Ventilation

Ventilatory parameters of all groups are summarized in Table 1. Minute ventilation (V'_E) of both groups of rats exposed to hyperoxia was somewhat lower than that of the controls, the decrease being significant only for *Car+O₂* group. The rats of *Car+A* group reached the same V'_E as controls, but had a higher rate of breathing and lower tidal volume.

Table 1. Minute ventilation (V'_E), breathing frequency (f_R) and tidal volume (V_T) in control group, group exposed to 7 days of hyperoxia (O_2), group breathing air after carrageenan application (Car+A) and group exposed to 7 days of hyperoxia after carrageenan application (Car+O_2).

	control	O_2	Car + A	Car + O_2
n	8	7	8	6
Body weight (g)	293±5	256±5 *	268±8 *	240±11 *
V'_E *(ml/min)*	208.2±14.3	172.9±8.7	200.5±13.4	163.9±15.4 *
f_R *(c/min)*	145±8	119±7	175±12 $^{+}$	131±15
V_T *(ml)*	1.4±0.1	1.5±0.1	1.2±0.1 *†	1.3±0.1 †

Data are means S.E.M., *p<0.05 from the control group, $^{+}$p<0.05 from all other groups, †p<0.05 from the O_2 group

Serum nitrites and nitrates

The concentrations of NO_x^- in the serum are shown in Figure 1. The application of carrageenan or the exposure to hyperoxia increased NO_x^- concentration, which remained unaffected by the combination of both interventions.

3-nitrotyrosine in the serum

The application of carrageenan did not change the 3NTYR concentration in serum (Fig. 2), but its concentration decreased in both groups exposed to 7 days of hyperoxia. The differences between controls and *O_2 group* and between *Car+A* and *Car+O_2* groups were significant.

3-nitrotyrosine in the lung tissue

As expected, lung tissue concentration of 3NTYR was increased in carrageenan-treated rats breathing air (Fig. 3). The concentrations of 3NTYR in the control rats exposed to hyperoxia alone and rats exposed to carrageenan and hyperoxia did not differ. This means that exposure to hyperoxia blocked the carraggenan-induced increase, while it did not affect the 3NTYR concentration in normal lungs.

There was no correlation between concentration of 3NTYR in lung tissue and in serum.

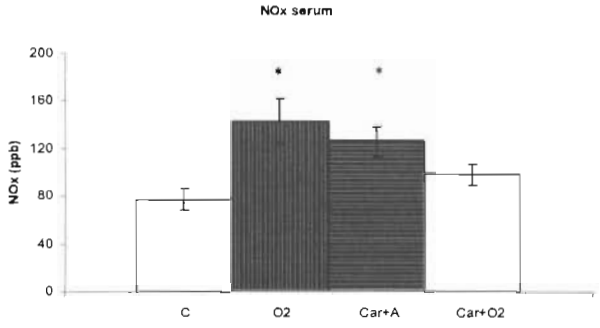

Fig. 1. Concentration of nitrites and nitrates (NOx) in serum of control rats (C), rats exposed to 7 days of hyperoxia (O_2), rats breathing air for 7 days after carrageenan application (Car+A) and rats exposed for 7 days to hyperoxia after carrageenan application (Car+O_2). *p<0.05 from the control (C) group

Weight of the left lung

The application of carrageenan increased both wet and dry weight of the left lung (*C group* 0.41±0.02; 0.09±0.01 g, *O_2 group* 0.42±0.01; 0.09±0.01 g, *Car+A group* 0.76±0.04; 0.16±0.01 g, *Car+O_2 group* 0.68±0.08;

0.15±0.02 g). There were no differences in dry to wet weight ratios of our groups.

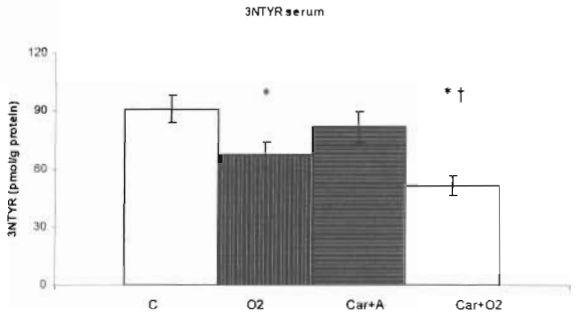

Fig. 2. Concentration of 3-nitrotyrosine (3NTYR) in serum of control rats (C), rats exposed to 7 days of hyperoxia (O_2), rats breathing air for 7 days after carrageenan application (Car+A) and rats exposed for 7 days to hyperoxia after carrageenan application (Car+O_2). *p<0.05 from the control (*C*) group, †p<0.05 between values in air and hyperoxia of the carrageenan treated rats.

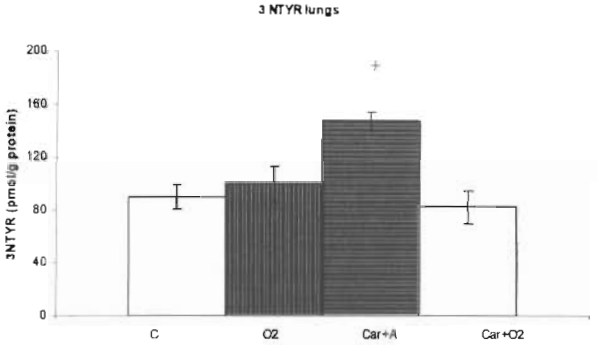

Fig. 3. Concentration of 3-nitrotyrosine (3NTYR) in the lung tissue of control rats (C), rats exposed to 7 days of hyperoxia (O_2), rats breathing air for 7 days after carrageenan application (Car+A) and rats exposed to hyperoxia for 7 days after carrageenan application (Car+O_2). †p<0.05 from all other groups

Discussion

This study was designed to test: 1) whether breathing of hyperoxic gas mixture affects 3NTYR concentrations in lung tissue and serum of rats with experimental pneumonia, and 2) whether concentration of 3NTYR in serum reflects changes in protein nitration in the lungs. High oxygen concentration surprisingly decreased 3NTYR levels in serum of control as well as carrageenan-treated rats and blocked the increase of 3NTYR concentration in lung tissue found in carrageenan-treated rats breathing air. The 3NTYR concentration in the serum and in the lung tissue did not correlate.

An increased production of nitrogen and oxygen related reactive species in carrageenan-induced inflammation have been described in previous studies (Oh-ishi *et al.* 1989, Salvemini *et al.* 1996, Cuzzocrea *et al.* 1997). The higher concentrations of nitrates and nitrites (NO_x^-) in serum as well as 3NTYR in the lung tissue after intratracheal application of carrageenan were therefore expected in our rats. In addition, the pattern of breathing of carrageenan-treated rats during air breathing was similar to that described by Wachtlová *et al.* (1975).

The fact that hyperoxia increased NO_x^- concentration but decreased 3NTYR concentration in the serum suggests that hyperoxia might have enhanced oxidation of NO to NO_2 and NO_3. If so, the NO produced by endothelial cells was oxidized to NO_x^- which restricted formation of peroxynitrite and 3NTYR.

In general, hyperoxia is believed to increase formation of ROS, however, this effect depends on its level and also on the duration of the exposure. In rats, Crapo *et al.* (1980) found marked injury after exposure to 100 % oxygen, but lesser changes at 85 % O_2. The pronounced signs of lung injury (and ROS production) were reported after 48-72 h of exposure to hyperoxia and were concomitant with the infiltration and activation of phagocytes (Narasaraju *et al.* 2003). We tested the changes after 7 days of hyperoxia when its effects probably abated.

Data about the effects of hyperoxia on NO production in the lungs are controversial. Schmetterer *et al.* (1997) found an increase in exhaled NO levels in human and Arkovitz *et al.* (1997) found an increase in NO_x^- concentration in the bronchoalveolar lavage fluid during hyperoxia, while Cucchiaro *et al.* (1999) showed that hyperoxia induced iNOS expression in the rat lung, but did not affect NO concentration in the exhaled air and 3NTYR concentration in lung tissue.

It is difficult to explain the effects of the combination of carrageenan administration and hyperoxia, in particular the fact that 3NTYR concentrations decreased. Formation of 3NTYR was originally proposed as a relatively specific marker of peroxynitrite formation (Beckman 1996). However, other reactions, e.g. a reaction of nitrite with hypochlorous acid (Eiserich *et al.* 1998) and reaction of hydrogen peroxide with NO_2^- catalyzed by myeloperoxidase (Van der Vliet *et al.* 1997, Narasaraju *et al.* 2003), could also be involved. The increased concentration of 3NTYR in the lung tissue of our carrageenan-treated rats probably resulted from the activation of all these pathways.

Because hyperoxia is known to enhance reactions mediated by free radicals in the rat lung (Freeman and Crapo 1981), the expected result of the combination of inflammation with hyperoxia would be an increase in 3NTYR formation. However, hyperoxia surprisingly attenuated 3NTYR concentration in the lung tissue and serum of carrageenan-treated rats. Such decrease in 3NTYR concentration could be a result either from reduced nitration of proteins or from faster breakdown of 3NTYR or 3NTYR containing proteins. Although we cannot exclude an effect of hyperoxia on 3NTYR formation, it is difficult to envisage a metabolic pathway activated by hyperoxia, which would turn NO to substance(s) other than NO_x^- or 3NTYR. Therefore, an increase in breakdown of 3NTYR seems to be more likely. In our experiments, the amount of 3NTYR was calculated per gram of protein. This indicates denitrification of tyrosine or an increase in degradation of 3NTYR containing proteins, rather than the increased breakdown of all proteins. There are some data about enzymatic denitrification of 3NTYR-containing proteins (Gow *et al.* 1996, Kamisaki *et al.* 1997), but it is not known whether this process can be enhanced by hyperoxia.

3NTYR concentration in the serum did not correlate with that in the lung tissue, which suggests that changes in 3NTYR production localized to the lungs were too small to modify the 3NTYR concentration in the serum.

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Reprint requests

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Příloha 3

Wilhelm, J.; Skoumalová, A.; Vytášek, R.; Fišárková, B.; Hitka, P.; Vajner, L. : Erythrocyte membranes inhibit respiratory burst and protein nitration during phagocytosis by macrophages. Physiol Res 54(2005) 533-539

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Erythrocyte Membranes Inhibit Respiratory Burst and Protein Nitration during Phagocytosis by Macrophages

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Summary

Phagocytosis is associated with respiratory burst producing reactive oxygen and nitrogen species. Several studies imply that erythrocytes can inhibit the respiratory burst during erythrophagocytosis. In this work we studied the mechanisms of this effect using control and *in vitro* peroxidized erythrocyte membranes. We demonstrated that autofluorescence of peroxidation products can be used for visualization of phagocytozed membranes by fluorescence microscopy. We also found that respiratory burst induced by a phorbol ester was inhibited by control membranes (5 mg/ml) to 63 % ($P<0.001$), and to 40 % by peroxidized membranes ($P<0.001$). We proved that this effect is not caused by the direct interaction of membranes with free radicals or by the interference with luminol chemiluminescence used for the detection of respiratory burst. There are indications of the inhibitory effects of iron ions and free radical products. Macrophages containing ingested erythrocyte membranes do not contain protein-bound nitrotyrosine. These observations imply a specific mechanism of erythrocyte phagocytosis.

Key words

Phagocytosis • Macrophages • Erythrocyte membranes • Respiratory burst • Nitrotyrosine

Introduction

The activation of macrophages associated with the generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) is of critical importance in the process of a nonspecific immunological response. It leads to the killing of invading bacteria and inflammatory tissue injury. ROS, comprising mainly of superoxide, hydrogen peroxide and the hydroxyl radical originate from a metabolic pathway referred to as a respiratory burst, characterized by non-mitochondrial oxygen consumption. The enzyme NADPH oxidase plays the key role in this process. It is activated during phagocytosis and generates superoxide as the primary product (Maly and Schurer-Maly 1995). Superoxide is the precursor of other ROS, such as hydrogen peroxide, which can be formed during superoxide dismutation, both spontaneous and enzymatic. We have documented production of hydrogen peroxide by activated macrophages in several studies (Wilhelm *et al.* 1996, 1997, 2003).

Macrophage activation is also accompanied by the induction of inducible nitric oxide synthase and

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sustained release of NO (MacMicking *et al.* 1997). An important compound among RNS is represented by peroxynitrite ($ONOO^-$), which produces a wide range of biological effects, including protein tyrosine nitration (Beckman and Koppenol 1996). Protein nitrotyrosine is thus a widely used indicator of RNS generation.

Erythrocytes have a limited life span of about 110 days and after this period are cleared from the circulation by the reticuloendothelial system. Free radical damage to erythrocytes leads to the loss of deformability of their membranes and increased their scavenging by spleen macrophages (Jain 1988). In this way, erythrocytes damaged by free radicals in various pathologies (Ramachandran and Iyer 1984, Laszlo *et al.* 1991, Peuchant *et al.* 1994, Wilhelm and Herget 1999, Skoumalová *et al.* 2003) could enhance the generation of ROS and RNS during their clearance by macrophages. On the other hand, it was found that phagocytosis of immunoglobulin G-coated erythrocytes was followed by a depression of macrophage functions including respiratory burst, whilst phagocytosis of erythrocyte ghosts did not depress macrophage function (Loegering *et al.* 1996). Inhibition of phagocytosis and oxidative burst were also observed after ingestion of *Plasmodium falciparum*-infected erythrocytes (Schwarzer *et al.* 1992). This inhibition is probably mediated by the stable end products of lipid peroxidation (Schwarzer *et al.* 2003).

Thus the mechanisms of inhibition of macrophage respiratory burst by erythrocytes are of great medical importance and therefore we addressed this question in this study. We investigated the effects of isolated human erythrocyte membranes, either control or peroxidized *in vitro*, on the respiratory burst of RAW macrophages and on the generation of nitrated proteins during phagocytosis. The results indicate inhibition of macrophage activities by erythrocyte membranes.

Methods

The remnants of blood samples left after blood donor testing were obtained from the Department of Hematology (Second Medical Faculty, Charles University, Prague). The blood, anticoagulated by K-EDTA, was pooled, 100 ml of blood was lyzed in ACK lyzing buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 0.0001 M Na_2 EDTA, pH 7.2) and left for one hour. The lyzed blood was centrifuged (40 000g, 40 min). The sediment was resuspended in 300 ml of PBS and centrifuged again under the same conditions. This procedure was repeated once more, then the sediment was resuspended in a small volume of PBS and dialyzed overnight against the large volume of PBS. The membranes were then sedimented by centrifugation, resuspended in a small volume of PBS and the protein concentration was assayed according to Lowry *et al.* (1951). One ml aliquots were stored at –70 °C until used.

Membranes were diluted to a concentration of 2 mg protein/ml in 10 mM Tris.HCl buffer containing 600 μM EDTA, pH 7.4. Lipid peroxidation was initiated by the addition of $FeSO_4$ (final concentration 300 μM) and ascorbate (final concentration 600 μM). The mixture was incubated at laboratory temperature in sealed conic flasks for 24 h. During this period, samples were taken at specific time intervals and oxidized and control membranes were frozen at –70 °C until used.

Mouse monocyte macrophages RAW 264.7 were obtained from ECACC, through Sigma-Aldrich (Prague). The cells were cultivated in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10 % fetal calf serum (BioClot, Germany). Cells were routinely grown at 37 °C in humid atmosphere equilibrated with 5 % CO_2. Experiments were performed with cultures near the confluency.

Macrophages after overnight cultivation with peroxidized or control erythrocyte membranes were studied using fluorescence microscope Nikon Eclipse 400 (excitation filter 330-380 nm, barrier filter 420 nm, dichroic mirror 400 nm).

The concentration of lipofuscin-like pigments (LFP) was assayed according to Goldstein and McDonagh (1976). The chloroform-isopropanol mixture (6 ml, 3:2, v/v) was added to 250 μl of control or peroxidized membranes. The mixture was extracted for 1 h on a motor-driven shaker. After extraction, 2 ml of water were added to achieve phase separation, the samples were centrifuged (100 g, 10 min) and the lower chloroform layer was used for the assay. The same procedure was adopted for the LFP assay of macrophages cultivated in the presence of erythrocyte membranes.

Fluorescence spectra of chloroform extracts were measured on Aminco-Bowman 2 spectrofluorometer. Tridimensional excitation spectral arrays were measured in the range of 250-400 nm for the emissions set between 300-500 nm with a step of 10 nm. For the quantitative assay of LFP the excitation and emission maxima found in the 3D spectra were used, instrument was calibrated with standard No. 5 from the instrument manufacturer. LFP concentration was expressed in

relative fluorescence units (RFU/mg protein).

Chemiluminescence measurements were carried out similarly as in our previous study (Wilhelm *et al.* 2003). We measured the luminol-dependent chemiluminescence originating from the reaction between hydrogen peroxide and luminol catalyzed by horse-radish peroxidase. For one assay $1x10^5$ cells was used. They were stimulated with protein kinase C activator phorbol-myristate-acetate (PMA), final concentration 1.6 μM. The measurements were carried out in plastic cuvette at 37 °C. The basic chemiluminescence (without PMA) was measured at the beginning of each experiment. Data are expressed as means ± SD of three separate experiments.

The measurement of the effects of erythrocyte membranes on the ROS production in xanthine/xanthine oxidase system was based on our previous study (Wilhelm and Vilim 1986). The reaction mixture contained in 1 ml of phosphate buffer (50 mM KH_2PO_4-KOH, 10 mM EDTA, pH 7.5) 0.25 mM luminol and 0.5 mM xanthine. The reaction was started by addition of 0.02 U of xanthine oxidase. The erythrocyte membranes (either control or peroxidized, 5 mg/ml) were added before xanthine oxidase.

The concentration of nitrotyrosine was measured in the fraction of soluble macrophage proteins. Cells were homogenized by freezing and thawing twice and extracted by TBS pH 8.4. The content of 3-nitrotyrosine was determined by previously described an enzyme immunoassay (EIA) method (Herget *et al.* 2000). Briefly, wells of polystyrene plates (Nunc) were coated with nitrated bovine serum albumin (BSA). Standard solutions of nitrated BSA (the content of 3-nitrotyrosine of standard was estimated from absorption at 430 nm) or extracts of RAW cells were mixed with monoclonal antibody NO-60-E3, that was developed in our laboratory, and incubated in coated wells of plate for 90 min under gentle shaking. After washing the wells were incubated with anti-mouse IgG antibody conjugated with peroxidase (P206, Dako) for next 90 min and developed with o-phenylenediamine. Standard curves and concentration of 3-nitrotyrosine in samples were calculated according to Rodbard's four parameter equation (Rodbard and McClean 1977).

Results

Firstly, the isolated erythrocyte membranes were subjected to lipid peroxidation *in vitro*. The free radical damage was detected by means of intrinsic fluorescence of the end products of lipid peroxidation. Figure 1 shows the 3D fluorescence spectra of control (part A), and peroxidized membranes (part B). The free radical damage results in the accumulation of fluorophore with maximum emission at 430 nm after excitation at 350 nm. The concentration of this fluorophore in peroxidized membranes increased almost fivefold, from 0.25±0.06 to 1.21±0.12 RFU/mg protein ($P<0.001$).

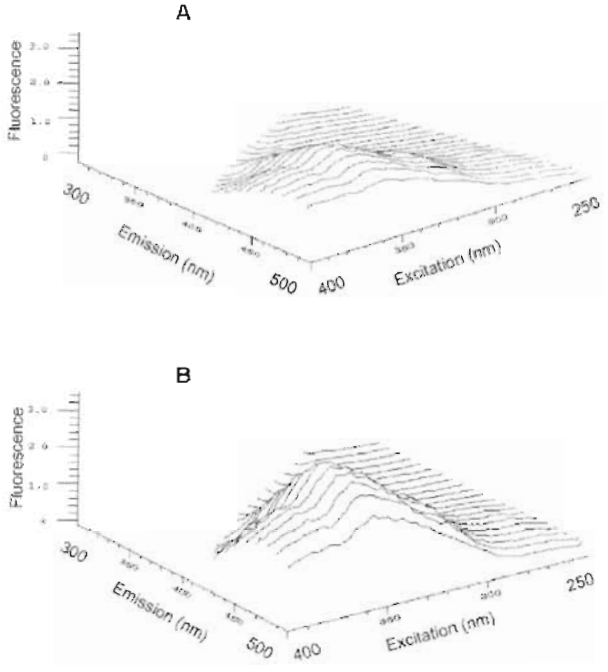

Fig. 1. Tridimensional spectral arrays of fluorescent products of lipid peroxidation. A - control erythrocyte membranes, B - membranes after 24 h of in vitro lipid peroxidation. Fluorescence intensity given in arbitrary units.

Both peroxidized and control membranes were incubated with RAW macrophages for 12 h. Figure 2 represents typical examples of fluorescence microscopy pictures of macrophages with ingested peroxidized membranes (upper part) and non-peroxidized control membranes (lower part). As this technique uses the autofluorescence of peroxidative products, the photo of the macrophage with ingested control membranes is much dimmer. Nevertheless, this picture documents that highly peroxidized erythrocyte membranes are eagerly phagocytozed by macrophages.

Secondly, we tested the effect of membrane phagocytosis on the respiratory burst. As the respiratory burst induced by membranes occurs in RAW cells in 1-5 h (Pfeifer *et al.* 2001) and it would be difficult to maintain the cell fully viable in the cuvette of

luminometer for this time period, we choose another approach. We triggered the respiratory burst by the addition of PMA either in the absence or in the presence of membranes. Figure 3 shows that the peak chemiluminescence was reached in 18 min in macrophages without membranes (curve 1). The addition of 1 mg/ml of membrane protein shortened the time to peak chemiluminescence to about 11 min. The peak intensity was reduced by 26 % with control membranes (curve 2) or by 37 % with peroxidized membranes (curve 3). The quantitative expression of three separate experiments gave 25.5±0.7 mV for the control macrophages, 19.1±0.5 mV for the non-peroxidized membranes ($P<0.05$), and 16.2±0.5 mV for the peroxidized membranes ($P<0.01$).

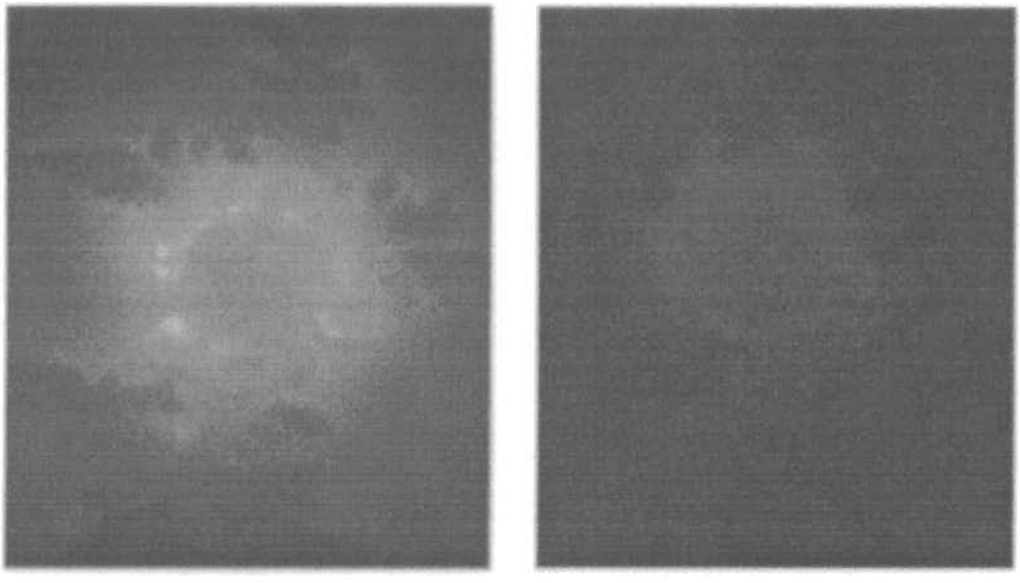

Fig. 2. Autofluorescence of phagocytes filled with erythrocyte membranes. Photos from fluorescence microscope showing a phagocytic cell with ingested peroxidized erythrocyte membranes (left part), or control membranes (right part).

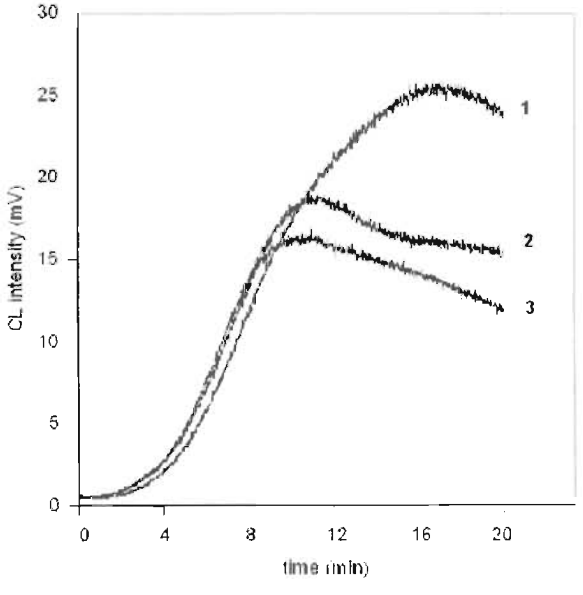

Fig. 3. Chemiluminescence tracing of respiratory burst induced by PMA. Curve 1 - without erythrocyte membranes, curve 2 - control erythrocyte membranes (1 mg/ml), curve 3 - peroxidized erythrocyte membranes (1 mg/ml).

Furthermore, we studied the effect of membrane concentration on chemiluminescence inhibition. When the membrane concentration was increased fivefold, the peak intensity was reduced by 10 % with control membranes (Fig. 4A), i.e. the inhibition of the total chemiluminescence was 37 % in relation to the control. In quantitative terms, the chemiluminescence intensity decreased from 19.1±0.5 to 17.6±0.7 mV and the decrease was not significant. The same increase in concentration of peroxidized membranes reduced the peak chemiluminescence by 40 % (Fig. 4B); in this case the inhibition of the total chemiluminescence was 60 % as compared to the control. Quantitatively expressed, the chemiluminescence intensity decreased from 16.2±0.5 to 10.1±0.7 mV ($P<0.01$).

One possible explanation for the observed inhibition of chemiluminescence by erythrocyte membranes might be the direct interaction of the membranes with the ROS generated by activated macrophages. We tested this possibility by measuring the effects of membranes on chemiluminescence produced in

xanthine/xanthine oxidase system, that generates similar mixture of ROS as activated macrophages. Figure 5 (curve 1) shows that the addition of xanthine oxidase results in a short burst of chemiluminescence. In the presence of erythrocyte membranes (curve 2), the initial peak is followed by sustained chemiluminescence. There was practically no difference between the control and the peroxidized membranes, both preparations increased the chemiluminescence intensity. Thus the inhibitory effect of membranes on chemiluminescence generated by activated macrophages cannot be explained by direct interaction of membranes with ROS.

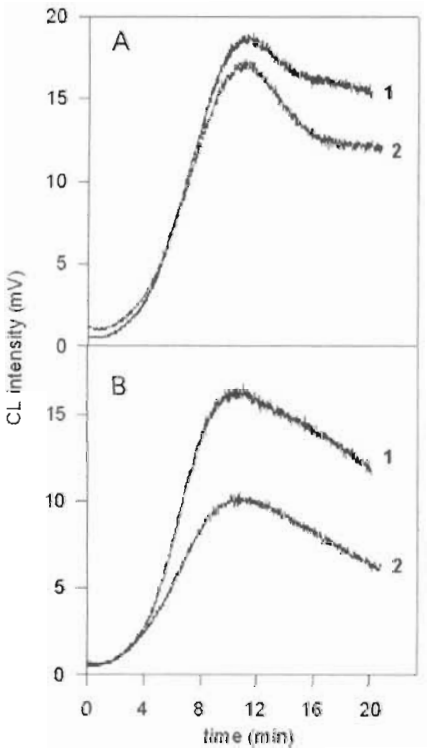

Fig. 4. The effect of membrane concentration on respiratory burst. Part A - control membranes, part B - peroxidized membranes. Curve 1 - protein concentration 1 mg/ml, curve 2 - protein concentration 5 mg/ml.

In order to evaluate the production of RNS during erythrocyte membrane phagocytosis we measured the concentration of protein nitrotyrosine. In RAW cells iNOS induction occurred with a lag phase of 4 h, and the fall in the rate of NO release was observed between 6 and 15 h (Pfeifer *et al.* 2001). Therefore, we measured protein nitrotyrosine after 12 h, when most membranes had already been ingested, as documented by Figure 2. The nitrotyrosine content in non-phagocytosing macrophages was 2.007±0.017 μmol/g of protein, in macrophages with phagocytozed control membranes 1.977±0.035 μmol/g of protein, and in macrophages with phagocytozed peroxidized membranes 2.095±0.059 μmol/g of protein. The differences were not statistically significant. Apparently, RNS production was not activated during erythrocyte membrane phagocytosis.

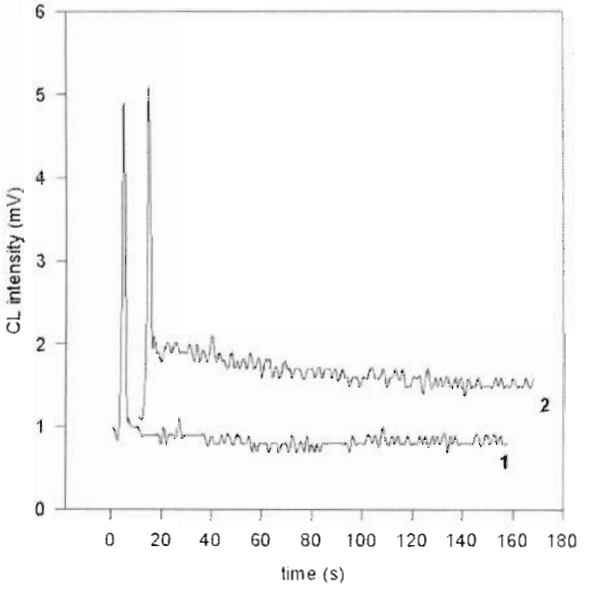

Fig. 5. Luminol chemiluminescence in xanthine oxidase system. Curve 1 - without erythrocyte membranes, curve 2 - in the presence of peroxidized erythrocyte membranes, protein concentration 5 mg/ml.

Discussion

Erythrophagocytosis in the reticuloendothelial system is the usual fate of erythrocytes at the end of its life span. From the teleological point of view it would be advantageous for the organism if erythrophagocytosis under physiological conditions would not comprise respiratory burst and tissue damage. However, this phenomenon might have its dark side. Several clinical studies have shown that increased erythrophagocytosis induces phagocyte dysfunction which may contribute to increased susceptibility to infection under situations such as burn injury (Pruitt and McManus 1984), salmonellosis (Hook, 1961), or malaria (Schwarzer *et al.* 1992).

It was deduced that the erythrocyte content is responsible for the macrophage dysfunction, as the ingestion of erythrocyte ghosts had no effect on macrophage function (Commins *et al.* 1990). However, our study shows that even the washed erythrocyte membranes inhibit respiratory burst induced by PMA. This could have been caused by oxidative inactivation of

protein kinase C, the effector of PMA signal. Currently, we do not know the specific site of the pathway leading from activated protein kinase C to superoxide production, which has been affected. However, our results correspond to the study which showed that phagocytosis of *Plasmodium falciparum* malarial pigment hemozoin, which induces free radical production inside the phagocyte, inactivates protein kinase C (Schwarzer *et al.* 1993). In our experiments even the non-peroxidized membranes contained some residual iron and it was suggested in another study that hemoglobin-derived iron interacts with ROS to cause oxidant damage to phagocytes (Loegering *et al.* 1996).

Inhibition of PMA-elicited respiratory burst was higher in the presence of peroxidized membranes. This preparation contained some extra iron from the *in vitro* lipid peroxidation experiment and also the products of membrane lipid peroxidation as revealed by fluorescence measurements. In the recent study (Schwarzer *et al.* 2003) it has been shown that some stable products of lipid peroxidation, specifically monohydroxy derivatives of fatty acids, are toxic to phagocytes. This could explain the increased inhibition by peroxidized membranes. The highest mebrane concentration used in our study (5 mg protein/ml) produced the same inhibition (60 %) as the optimum concentration of monohydroxy fatty acid in the afore mentioned study. We could not study higher membrane concentrations, because the opacity of the solution interfered with the chemiluminescence assay.

Inhibition of respiratory burst did not supress phagocytosis of the peroxidized membranes as revealed by fluorescence microscopy. At the time of completed phagocytosis we did not observe any change in protein nitration. This observation is different from the experiments which showed the increase of nitrotyrosine concentration in murine peritoneal macrophages activated *in vitro* with interferon-γ/lipopolysaccharide (Pfeifer *et al.* 2001). Thus it appears that phagocytosis of erythrocyte membranes does not activate iNOS and this effect is not dependent on membrane peroxidation.

In summary, we observed that erythrocyte membranes inhibit PMA-elicited respiratory burst by a mechanism that might involve oxidant dependent inhibition of protein kinase C, and/or the related pathway of superoxide production. The present study was not designed to localize the specific site. This question should be addressed in the future studies. Our experiments proved that this effect is not caused by interference of the membranes with the detection system which uses luminol chemiluminescence. We demonstrated that autofluorescence of peroxidation products can be used for visualization of phagocytozed membranes by fluorescence microscopy. Macrophages containing ingested erythrocyte membranes do not contain protein-bound nitrotyrosine. These observations imply a specific mechanism of erythrocyte phagocytosis.

Acknowledgements

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Příloha 4

Wilhelmová, N.; Fuksová, H.; Srbová, M.; Miková, D.; Mýtinová, Z.; Procházková, D.; Vytášek, R.; Wilhelm, J. : The effect of plant cytokinin hormones on the production of ethylene, nitric oxide, and protein nitrotyrosine in ageing tobacco leaves. BioFactors, přijato do tisku

The effect of plant cytokinin hormones on the production of ethylene, nitric oxide, and protein nitrotyrosine in ageing tobacco leaves.

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Abstract

In the present study we used transgenic plants with genetically increased or decreased levels of cytokinins. We investigated the effect of cytokinin level on the production of ethylene, a plant hormone with suggested role in senescence, and the production of nitric oxide, potentially important signalling and regulatory molecule. The production of these gases was followed during the course of leaf development and senescence. The production of ethylene and nitric oxide is under genetic control of genes other than those involved in regulation of senescence. The difference in basic ethylene and NO levels in different tobacco cultivars was higher than their changes in senescence. Our results do not indicate a direct link between ethylene production and cytokinin levels. In accordance with the previous studies we observed a decreased production of NO in senescent leaves. Low cytokinins level was associated with increased NO production during leaf development. Protein nitrotyrosine proved to be a better indicator of the reactive nitrogen species than measuring of the NO production. Higher nitrotyrosine concentrations were found in insoluble proteins than in the soluble ones, pointing to membrane proteins as the primary targets of the reactive nitrogen species. In plants with elevated cytokinin levels the content of nitrated proteins decreased both in soluble and insoluble fractions. This finding indicates an antioxidative function of cytokinins against reactive nitrogen species.

Keywords: cytokinin, ethylene, leaf ageing, nitric oxide, reactive nitrogen species, nitrotyrosine, tobacco

1. Introduction

Leaf senescence is the final stage of leaf development under which the leaf cells undergo a programmed cell death process, referred to as the senescence syndrome [17]. Certain hormones can trigger, accelerate, or even reverse the overall process of senescence by activating specific genes. Therefore intervening in the hormonal signalling can modulate senescence by many ways. It appears that signalling at the transcriptional level represents only a coarse regulation of the onset of senescence, whereas the fine tuning of the process takes place in post-transcriptional stages [21].

Senescence associated genes (SAG) can be also regulated by free radicals. Free radicals are produced throughout the whole life span of plants and their high reactivity might be responsible for the stochastic character of the loss of physiological functions with increasing age. Oxygen-derived free radicals originate from the partial reduction of oxygen molecules. The reactive nitrogen species are represented by NO and compounds formed during interactions of nitric oxide with oxygen-derived free radicals.

It was recognized that nitric oxide plays a role in plant growth, development and senescence [14, 12]. The effects of NO in plants may be associated with hormonal network, namely, ethylene [13], abscisic acid [5], and cytokinins signalling [23].

Nitric oxide can readily react with superoxide radical giving highly reactive and toxic peroxynitrite [18]. Peroxynitrite attacks protein tyrosine with the formation of protein-bound nitrotyrosine. It has been shown in an animal model that physiological protein nitration can be selective, dynamic, and reversible, indicating the possibility of its use for signalling purposes [2].

In the present study we have investigated the generation of ethylene, the supposed inductor of senescence, in relation to the production of nitric oxide and its late products,

protein-bound nitrotyrosines. We employed tobacco plants with genetically increased or reduced levels of cytokinins in order to elucidate the role of these plant hormones in reactive nitrogen species metabolism.

2. Material and methods

2.1. Plants and cultivation

Two kinds of transgenic tobacco (*Nicotiana tabacum* L.) plants with modulated cytokinin levels were used together with corresponding control cultivars. We used tobacco plants with prolonged life span of their leaves due to coupling of cytokinin synthesis to the promoter of senescence related genes. In the *Nicotiana tabacum* cv. Wisconsin 38 the gene for isopentenyl transferase was under control of SAG_{12} promoter [6], i.e. when leaves approached senescence, the synthesis of cytokinins was increased up to 100-fold [10] and the leaves were rejuvenated. These plants were labeled as SAG. We compared these plants with *Nicotiana tabacum* cv. Samsun NN in which cytokinin levels are decreased due to increased degradation. Cytokinin content was lowered due to inserted gene *AtCKX2* for cytokinin dehydrogenase from *Arabidopsis thaliana* positioned under the control of a constitutive 35S promoter. The cytokinin content in transgenic tobacco plants was approximately one third of that in controls [24]. The plants of this group were labelled as CKX.

After in vitro pre-cultivation the plants were grown in a glasshouse under temperature 25/18 °C day/night. Day irradiation (overall integrated mid-values were ca. 500 μmol (quantum) m^{-2} s^{-1}) was prolonged by the additional irradiation (PFD ca. 200 μmol (quantum) m^{-2} s^{-1}) to 16 hours.

We studied ageing within the age gradient of leaves of a single plant. After 13 weeks the leaves were numbered according to the order in which they have budded, from the bottom

to the top 1 - 8. The neighbouring leaves were grouped together creating four groups containing leaves 1+2 (the oldest), 3+4, 5+6, 7+8 (the youngest), respectively. In one experiment we combined the leaves from three plants. This cultivation and sampling scheme was repeated three times.

The detached leaves were used for the determination of NO and ethylene or frozen in liquid nitrogen and stored at -70 °C until they were analysed.

2.2. Measurement of NO emission

The whole leaves were detached and immediately placed into the Al-foil covered 250 ml bottles that were filled with nitrogen and closed with silicon rubber stoppers. The leaves were kept for 2 hours in the dark at 25 °C. Then the gaseous content of the bottles was pumped into the NO analyser CLD 77 AM (ECO PHYSICS, Switzerland). NO was determined by the method of ozone-stimulated chemiluminescence [8].

2.3. Measurement of ethylene emission

After NO analysis the bottles were filled with the air, sealed, and kept for next 2 hours in the dark at 25 °C. Then 60 ml of the gas was extracted by a gas-tight syringe [25] and applied on the Carbotrap 300 (Supelco, USA) desorption tubes. Tubes with the adsorbed sample were placed into the concentrator (Tekmar Dohrmann 6000, USA), directly coupled to the gas chromatograph Shimadzu GC 2010 (Japan). The samples were desorbed 4 min. at 250 °C and then were trapped at -165 °C. Thus concentrated samples were released by heating at 290 °C for 1 min and directly applied on the chromatographic column 23% SP-1700 (9.14 m x 2.1 mm, Supelco). As the carrier gas nitrogen was used at a flow rate of 20 ml/min. The column temperature was set at 60 °C, the FID detector was adjusted at 230 °C. Ethylene

concentration was calculated from calibration curve constructed from ethylene standard (Supelco).

2.4. Assay of nitrotyrosine

Nitrotyrosine was assayed in the fraction of soluble and insoluble proteins. The freeze-dried leaves were homogenized in a phosphate buffer (0.1 M, pH 7.4) and the soluble fraction was separated by centrifugation (10 000 g, 10 min.). The pellet was incubated in 0.5 M NaOH overnight, and then adjusted by HCl to pH 7.8. The proteins content was assayed according to Lowry [16]. Nitrated proteins were assayed by a competitive ELISA with the aid of monoclonal antibodies developed in our laboratory [9].

2.5. Statistics Data represent the mean ± S.E. Statistical evaluations were made using ANOVA with Schefee post-hoc test.

3. Results

The tobacco plants were harvested when the oldest bottom leaves in control plants turned yellow. At this stage, the lowest leaves in SAG plants stayed green. The CKX plants differed markedly in their phenotype from their controls. This tobacco with lowered cytokinins had bushy appearance, smaller and thicker leaves.

The control plants to the SAG group produced ethylene from 75 to 110 pmol/g fresh weight/hour. This rate did not change significantly during ageing and the SAG plants did not differ significantly from the control group.

The situation in the CKX plant is illustrated in Fig. 1. Production of ethylene in the control cultivar of CKX tobacco was about four times higher than in controls of SAG tobacco.

The production of ethylene significantly increased in the leaves 5+6 in relation to the young controls (leaves 7+8) and then the level returned to that of the young leaves. In the transgenic CKX plants the production of ethylene of leaves 5+6 significantly decreased both in relation to the young leaves and in relation to the leaves 5+6 of the control cultivar. During further ageing the ethylene production approached the level of the young leaves.

The production of NO in the control cultivar of CKX tobacco was about three times higher than in controls of SAG tobacco. The course of NO production during leaf ageing of the CKX plants is shown in Fig. 2. In the control cultivar there was a statistically significant ($P< 0.05$) decrease of the NO production in the oldest leaves (7+8). In the transgenic CKX plants NO production increased in the leaves 3+4 more than four times, but this increase was not statistically significant due to high dispersion of the measured values. However, the subsequent decrease in the oldest leaves 1+2 was significant ($P< 0.05$) when compared to the value of the leaves 3+4.

The production of NO in the control plants to the SAG group did not change in the course of ageing and the SAG plants did not significantly differ from the control group.

Protein nitration represents another indicator of the activity of the reactive nitrogen species. As the intracelular compartmentalization may play a decisive role, we tested both soluble and insoluble protein fraction. Due to the minute amount of the material needed for the assay we analyzed individual leaves.

The course of protein nitration in the soluble fraction of CKX plants is shown Fig. 3A. In the control cultivar, statistically significant increase in protein nitration appeared in the old leaves No.2. In the oldest leaves No.1 it was slightly decreased, but still was significantly higher than in the young leaves. In the transgenic CKX plants the concentration of nitrated proteins was practically constant throughout the life span up to the leaf No. 2, in the oldest leaves No. 1 it was significantly increased.

The situation in the fraction of insoluble proteins is documented in Fig. 3B. Protein nitration was much more pronounced in this fraction, being about fifty times higher than in soluble proteins. In the control plants, protein nitration has steadily increased during ageing up to leaf No. 3, when it reached maximum. Then, in the oldest leaves No. 2, and No. 1 the level of nitrated proteins has returned to the values of young leaves.

The concentration of nitrated proteins in the SAG plants and their controls is shown in Fig. 4. It is apparent that there are major differences from the CKX plants. The maximum values in the soluble fraction are about four times higher than in the CKX plants, on the other hand, the SAG group values in the insoluble proteins were about three times lower than in the CKX plants.

The course of protein nitration of soluble proteins (Fig. 4A) indicates a steady growth in the control group; the maximum reached in the leaves No. 1 was statistically significant ($P< 0.05$). The SAG transgenic plants did not produce any significant changes throughout the whole life span.

In the fraction of insoluble proteins (Fig. 4B) there was a decreasing tendency in protein nitration with increasing age of control plants, reaching minimum in the leaves No. 2. The transgenic SAG plants had similar time course of protein nitration, however, in the leaves No. 6 - 4 the levels were significantly lower.

4. Discussion

Our data indicate that the genetic control of the production of ethylene and nitric oxide in tobacco goes beyond the senescence-associated genes, as the differences in the basic levels of these compounds in the cultivars Wisconsin 38 and Samsun NN were higher than the changes produced during ageing and senescence.

The production of ethylene increased only transiently in the cv. Samsun NN at the time when leaves were not showing any signs of senescence. In the cv. Wisconsin 38 no significant changes were observed throughout the whole life span of the leaves. These results can be used as an argument against the direct role of ethylene in induction of senescence suggested in previous studies [1, 20 11].

It appears that cytokinin level had little effect on the ethylene production during senescence and there is probably no direct link between them.

Most of the studies of NO effects on plants have been accomplished with application of artificial NO donors. Only few studies dealt with endogenous nitric oxide. In a recent study NO was found, in pea plants, to be generated by peroxisomes [19, 4]. In the senescent leaves the NO generation was reduced [4]. The concentrations of NO generated by leaves are very low and even with our highly sensitive chemiluminescence detection system we measured the values at the edge of instrument sensitivity and we obtained a great dispersion of the data. We have found an indication that low cytokinin level might increase NO production during leaf development; the terminal decrease of NO production was not dependent on the cytokinin level.

Protein nitrotyrosine appears to be a better indicator of the involvement of reactive nitrogen species. The ELISA technique is sufficiently sensitive and minimum amount of biological material is needed for the analysis. We found again the great effect of the kind of cultivar both on the overall nitrotyrosine concentration and its distribution between soluble and insoluble proteins. Generally, the higher nitrotyrosine concentrations were found in the insoluble proteins, suggesting that membrane proteins are exposed to higher fluxes of reactive nitrogen species. In the control cv. Samsun NN, there was a tendency for the terminal increase in protein nitration in the soluble fraction that did not depend on the cytokinin level. On the other hand, in the fraction of insoluble proteins, protein nitration increased during senescence

of plants with low cytokinins level. In the plants with elevated cytokinin levels there was a generally lower concentrations of nitrotyrosine both in soluble and insoluble protein fraction. This finding might represent the protective effect of cytokinins against the damage by reactive nitrogen species. Similar cytokinin action against reactive oxygen species has been found in previous studies [15, 3, 7, 22].

There was no correlation between NO generation and the concentration of protein nitrotyrosine. This may indicate that NO preferentially reacts inside the cell and the gas measured extracellularly represents only the remnants that escape from the intracellular reactions.

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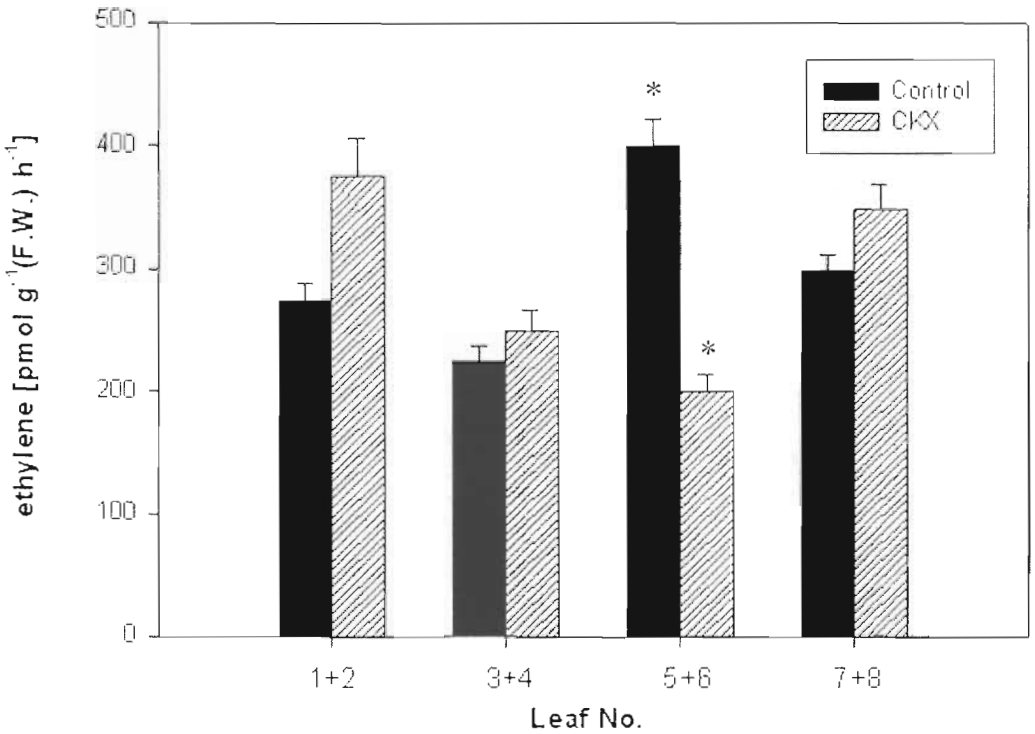
Captions to the figures

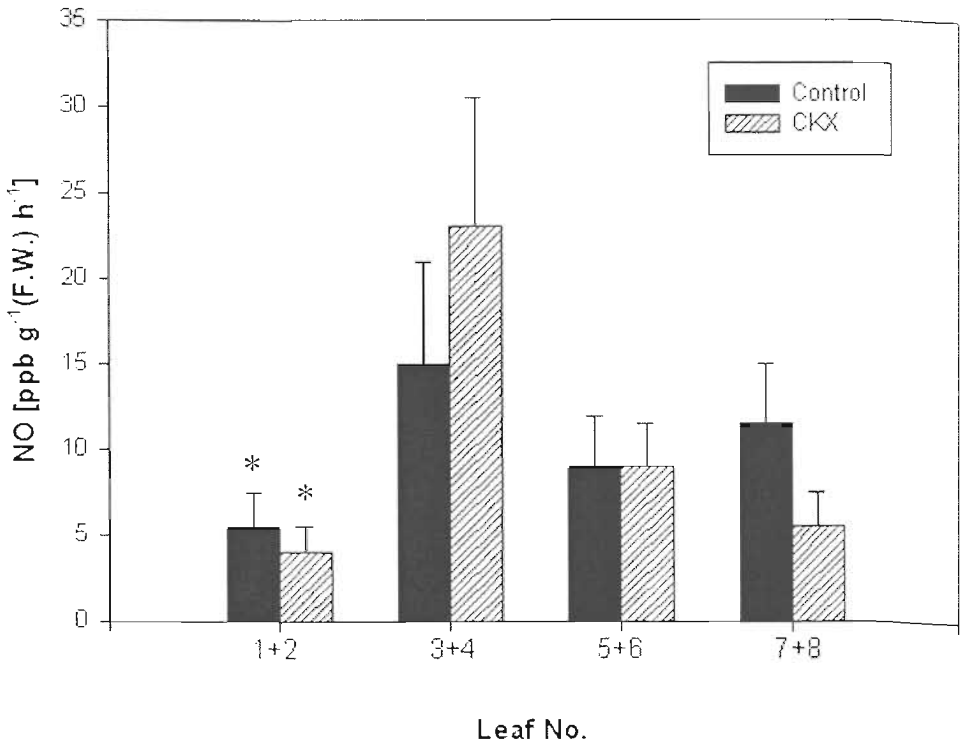
Fig. 1. Emission of ethylene from tobacco leaves of different age. Black columns = control tobacco cv. Samsun NN; shaded columns = transgenic tobacco CKX plants. Leaves were numbered from the bottom (the oldest) to the top (the youngest). Statistical significance: * $P<0.05$ related to the young leaves 7+8.

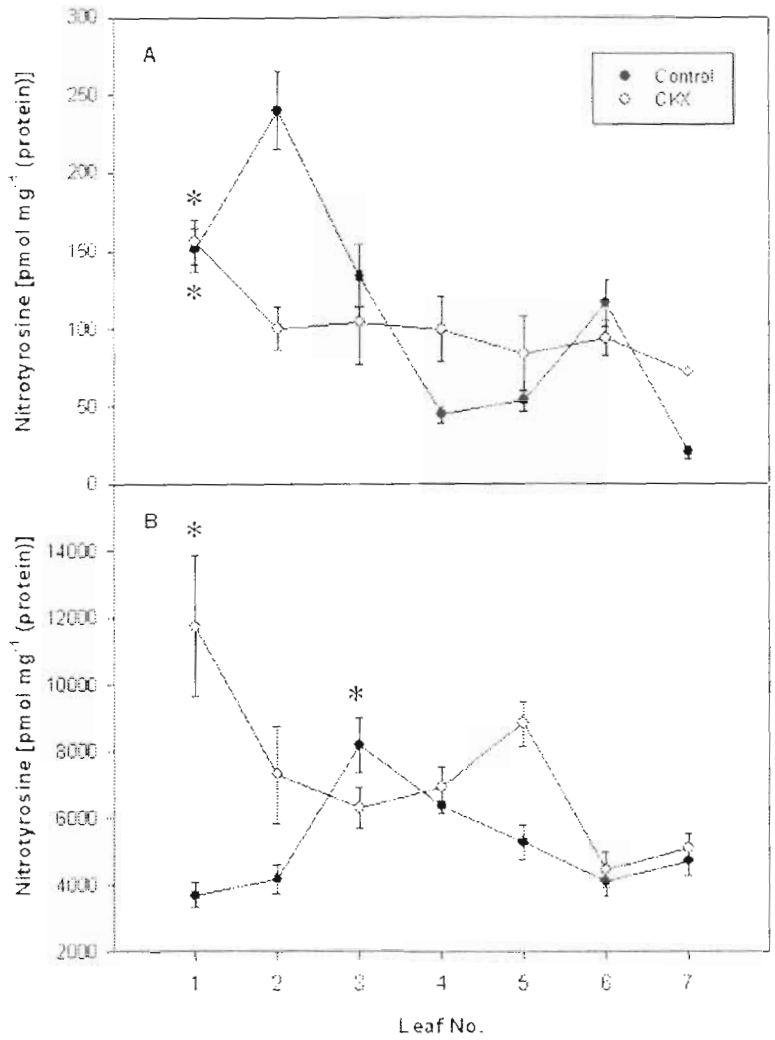
Fig. 2. Emission of NO from tobacco leaves of different age. Black columns = control tobacco cv. Samsun NN; shaded columns = transgenic tobacco CKX plants. Leaves were numbered as in Fig. 1. Statistical significance: * $P<0.05$ related to the young leaves 7+8.

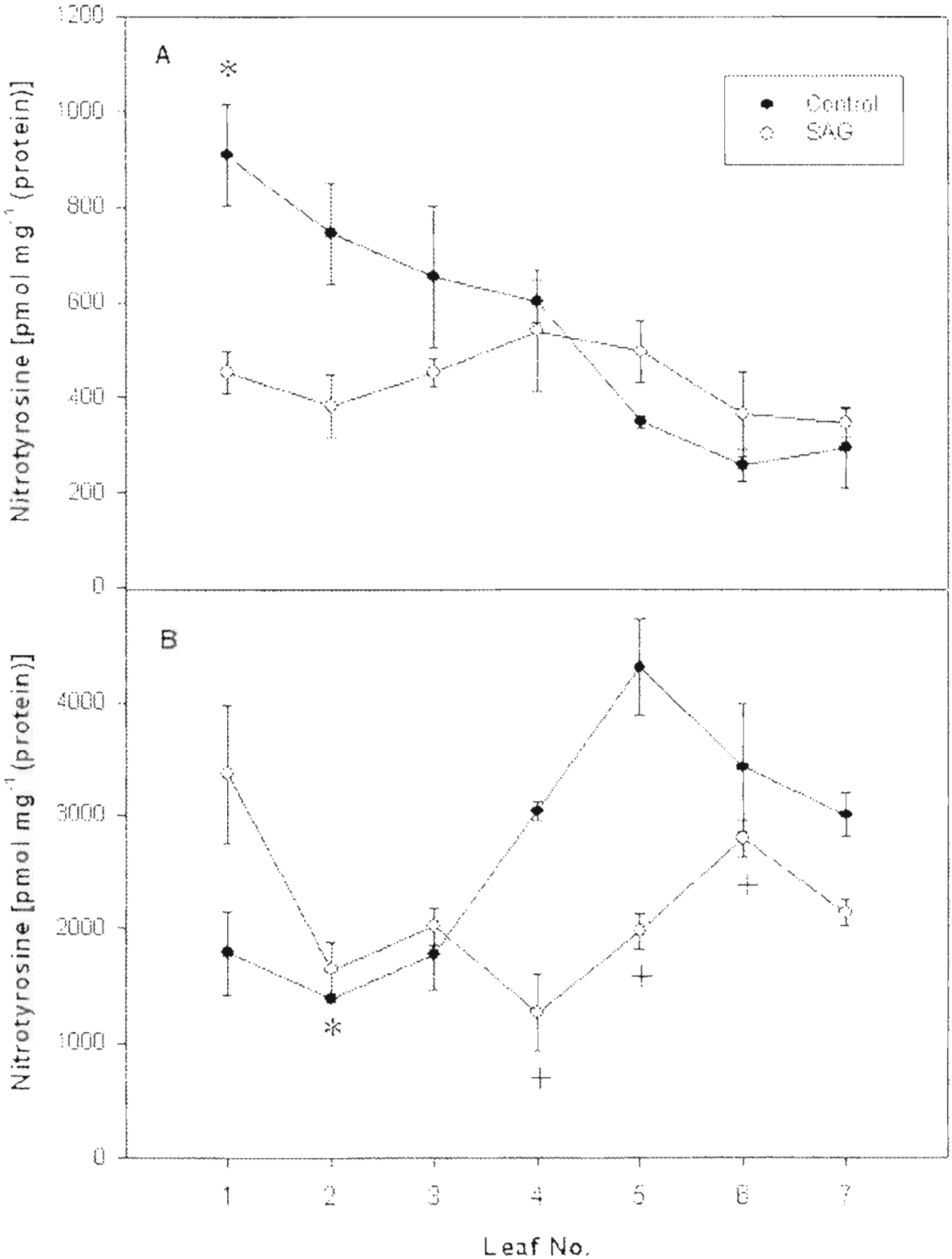
Fig. 3. Protein nitrotyrosine concentration during leaf ageing in CKX plants. Panel A – soluble proteins fraction, panel B – insoluble protein fraction. Full circles – control cultivar Samsun NN, open circles – transgenic plants. Statistical significance: * $P<0.05$ related to the young leaves.

Fig. 4. Protein nitrotyrosine concentration during leaf ageing in SAG plants. Panel A – soluble proteins fraction, panel B – insoluble protein fraction. Full circles – control cultivar Wisconsin 38, open circles – transgenic plants. Statistical significance: * $P<0.05$ related to the young leaves; + $P<0.05$ related to corresponding controls.


500
400
300
200
100
0
ethylene [pmol g-1(F.W.) h-1]
Control
CKX
*
*
1+2
3+4
5+6
7+8
Leaf No.


35
30
25
20
15
10
5
0
NO [ppb g-1(F.W.) h-1]
Control
CKX
*
*
1+2
3+4
5+6
7+8
Leaf No.


A
B
Control
CKX
Nitrotyrosine [pmol mg-1 (protein)]
Nitrotyrosine [pmol mg-1 (protein)]
300
250
200
150
100
50
0
14000
12000
10000
8000
6000
4000
2000
1
2
3
4
5
6
7
Leaf No.


A
B
Control
SAG
Nitrotyrosine [pmol mg-1 (protein)]
Nitrotyrosine [pmol mg-1 (protein)]
0
200
400
600
800
1000
1200
0
1000
2000
3000
4000
1
2
3
4
5
6
7
Leaf No.

Příloha 5

Wilhelm, J.; Fuksová, H.; Schwippelová, Z.; Vytášek, R.; Pichová, A. : The effects of reactive oxygen and nitrogen species during yeast replicative ageing. BioFactors , přijato do tisku

The effects of reactive oxygen and nitrogen species during yeast replicative ageing.

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Abstract. Free radicals are considered the most important cause of cellular ageing. We have investigated ageing process in the yeast *Saccharomyces cerevisiae*. We have compared the wild type strain with the mutant cells with constitutively active Ras oncogen, which generates increased amounts of free radicals. Increased generation of oxygen-derived free radicals resulted in the Ras mutant cells accumulation of lipofuscin-like pigments during ageing. Ageing wild type cells did not accumulate lipofuscin-like pigments. This is quite unique feature among known biological models. It may be caused by increased concentration of alpha tocopherol (the most prominent lipophilic antioxidant) in the wild type cells. In contrast, the Ras mutant cells contained decreased levels of alpha tocopherol even in the young cells. This observation indicates that the increased free radical generation can overwhelm the endogenous antioxidant system. We have documented the involvement of nitrogen-derived free radicals in the yeast metabolism. Protein nitrotyrosine, a marker of the reactive nitrogen species, has significantly increased in the senescent Ras mutant cells. The wild type cells contained basic level of nitrotyrosine corresponding to its concentration found in non-activated mammalian macrophages.

Keywords: Free radicals, Lipofuscin-like pigments, Nitrotyrosine, Yeast, Ageing

1. Introduction

Yeast represents a simple model for cellular ageing studies. It is similar to ageing of cells of higher organism as it exhibits replicative ageing (expressed as the limited number of mitotic divisions that an individual cell can complete). Yeast ageing is also well characterized in terms of morphological and biochemical changes [4, 9, 10]. This model is easy to handle because of the short life span and the ease of genetic analysis and manipulation. The old cells are bigger than young cells and can be separated by centrifugation.

The free radical theory of ageing, originally formulated by Harman [5], is also applicable to yeast. It has been shown in several studies that increased burden of free radicals on cells of the yeast *Saccharomyces cerevisiae* leads to a shortening of their replicative life span [11, 13].

Recently it has been shown that an accumulation of mutations in genomic DNA can be ruled out as a cause of yeast ageing [13]. This opens up the possibility that oxidative damage to cellular material other than DNA could play a decisive role in ageing mechanism.

We aimed our present study at the free radical-mediated oxidative damage to membranes, assessed on the basis of formation of the fluorescent end products of lipid peroxidation. These are the so called lipofuscin-like pigments (LFP) which characteristically accumulate in aged cells [16] and are used as a marker of oxidative damage [8-10]. Apart from oxygen-derived free radicals, we can also encounter the free radicals derived from nitrogen species, especially from NO in a cell. The principal damaging reactive nitrogen species is peroxynitrite. This, however, is difficult to measure due to its short half-life [1]. Therefore, the product of its reaction with protein tyrosine, 3-nitrotyrosine, is generally used as a marker of reactive nitrogen species [7]. The effects of free radicals are under physiological conditions balanced by elaborate antioxidant systems. As a measure of

antioxidant ability of the yeast we used the level of endogenous α-tocopherol, the principal non-polar antioxidant.

We compared the effects of ageing in the wild-type cells and in the mutant cells with constitutively active Ras allele, which were shown to generate increased amounts of free radicals throughout the life span [6].

2. Material and methods

2.1. Yeast strains and media

A wild-type yeast strain of *Saccharomyces cerevisiae* JC 482 and a mutant RAS2val19 derived from it with constitutively active protein kinase A [14] were used in the experiments. Liquid YPD medium (2% glucose, 1% yeast extract, 2% bactopeptone in H_2O) was used for growing of the yeast cells.

2.2. Cell separation

Cells were separated into fractions according to their diameter using the Beckman elutriation system and rotor JE-6B with a standard elutriation chamber as described in a previous publication [11]. For the present experiments we used young cells from fraction II (uniform small cells with a diameter of 5 - 7 μm, 70% of which were virgin cells) labelled as group Y, old presenescent cells from fraction IV (diameter of 8.5 - 10 μm) labelled as group P, and old cells undergoing senescence from fraction V (diameter10 - 15 μm) labelled as group S.

2.3. Fluorescence measurement

For the analysis of LFP we used the technique developed in our laboratory [17], based on the original method of Goldstein and McDonagh [3]. One ml of frozen cell suspension was added to 4 ml of chloroform-methanol mixture (2:1, v/v) and extracted for 1 hour on a motor-driven shaker. After extraction, 2 ml of distilled water was added, mixed, and the mixture was centrifuged (400 g, 10 min). After centrifugation, the lower chloroform phase was separated and used for measurements. Three-dimensional fluorescence spectra were measured on the spectrofluorometer Aminco Bowman series 2 (ChromSpec company, Prague). The excitation spectra were measured in the range of 300 - 420 nm for emission wavelengths adjusted between 370 - 550 nm. The quantitative estimation of LFP was based on the excitation and emission maxima found in 3D spectral arrays. The fluorometer was calibrated with the standard No. 2 of the instrument manufacturer and the LFP concentration was expressed in relative fluorescence units per mg of protein assayed according to Lowry [12]. The synchronous fluorescence emission spectra were measured in the range of 260 - 570 nm. The best resolution was obtained with constant difference of 35 nm between excitation and emission wavelength.

2.4. ELISA estimation of 3-nitrotyrosine

Cells were stored as a suspension at –70 °C. They were ruptured by repeating three cycles of thawing and re-freezing. The final solution was centrifuged at 8000 g for 10 min., and the supernatant was diluted four times with Tris/saline buffer pH 8.4 and taken for the analysis. We adapted a competitive ELISA for estimation of 3-nitrotyrosine in serum proteins as described in our previous study [2].

2.5. Assay of α-tocopherol

Alpha tocopherol was assayed by the HPLC method described in our previous study [15]. Briefly, one ml of cell suspension was extracted into n-hexane. The extract was evaporated under nitrogen and re-dissolved in methanol. The methanolic solution was analyzed on C-18 column with fluorescence detection (excitation 290 nm, emission 330 nm). The amount of α-tocopherol was calculated using external standard.

2.6. Statistics

The statistical evaluations were made using ANOVA with Scheffe post-hoc test, and the results are shown as means ± SEM.

3. Results

We have characterized the lipophilic fluorescent end products of lipid peroxidation by measuring the 3D spectral arrays. The typical example is illustrated in Fig. 1. There were no qualitative differences in the spectra of young or old cells, either from wild type or Ras mutant.

Therefore, we have chosen the major peaks at 340/400 nm, 317/420 nm, and 310/380 nm (excitation/emission) for quantitative evaluation. The results shown in Fig. 2 are expressed as a percentage of the value of the young cells that represents 100%. The upper panel documents the situation in the wild type cells, the lower panel in the Ras mutant. A striking characteristic of this analysis is that the concentration of fluorophores in the wild type cells decreases during ageing, whilst in the Ras mutant the levels of these fluorophores are

increasing. The decrease in the wild type cells was quite uniform for all the three fluorophores. On the other hand, changes of the individual fluorophores during ageing of the Ras mutant differed, the highest increase (208% of young cells) was found in the fluorophore 310/380 nm in the group P. In all fluorophores from the Ras mutant we observed a slight decrease in groups S relative to the maximum found in the group P.

The synchronous fluorescence spectra offer another tool of analysis of complex mixtures of compounds, such as we encounter in this case. The appearance of the spectra represents a "fingerprint" of a given mixture and it is sensitive to changes in the overall composition. Fig. 3 shows the spectra of the wild type cells. The upper panel represents the group Y, the lower panel the group P. These spectra were measured with a constant difference of 35 nm. The arrow indicates a fluorophore 455/490 nm, the intensity of which increased during ageing relative to fluorophores with emission around 350 nm.

When we compare these spectra to those of Fig. 4, which represent the situation in the Ras mutant, we see the increased amount of the fluorophore 455/490 nm already in the young cells. As even the young Ras cells produce increased amounts of free radicals, the fluorophore 455/490 nm might represent the specific product of free radical attack. Its concentration is not further increased during ageing.

Further we studied the products of the attack of reactive nitrogen species on soluble proteins as measured by protein nitrotyrosine. Our data represents the first detection of nitrated proteins in the yeast. The results are summarized in Fig. 5. It shows statistically significant ($P<0.001$) increase in protein nitrotyrosine in the group S of the Ras mutant cells. This value was significantly higher both to the young Ras cells and to wild type cells of the same age.

The overall oxidative status of the cells can be assessed on the basis of the levels of endogenous antioxidants. As we measured the oxidative damage in the lipidic fraction, we

assayed the levels of endogenous α-tocopherol, the principal lipophilic antioxidant. The results are summarized in Fig. 6. In the wild type cells the concentration of α-tocopherol was significantly higher in the group P than in the young cells and increased even further in the group S. In the Ras mutant cells the concentration of α-tocopherol in the young cells was about five times lower than in the young wild type cells and it did not change significantly during ageing. In all groups of the Ras cells the levels of α-tocopherol were significantly lower than in the corresponding groups of the wild type cells.

4. Discussion

The increased production of free radicals in the Ras mutant cells has its consequences at several organizational levels.

The lipofuscin-like pigments represent the stable end-products of free radical attack on the lipophilic fraction of the cell in several biological systems and their concentration increases with age (for a review see [20]). It appears that yeast can metabolize or degrade these compounds, as the wild type cells were able to decrease their concentration during ageing. This is a unique feature among so far described biological models.

On the other hand, the Ras mutant cells produced increased amounts of fluorescent end products during ageing. This phenomenon supports the view of cumulative damage effect of free radicals. A yeast-specific feature was represented by the terminal decrease in LFP of senescent cells relative to the aged "healthy" cells. Individual LFP fluorophores behaved independently in the course of ageing which suggests a specific metabolism of these compounds. The fact that the same fluorophore was observed in the synchronous spectra of aged wild type cells and in the young Ras cells supports the existence of a role of free radicals in cellular ageing.

This study documents the formation of protein nitrotyrosine in the yeast. The overall nitrotyrosine concentration in the yeast was found at the level of resting mammalian macrophages [19]. The significant increase of protein nitrotyrosine was detected in senescent Ras mutant cells indicating the interaction with the reactive oxygen species. Otherwise, the basic level of protein nitrotyrosine did not change during ageing and no significant changes were observed in the wild type cells.

The increased production of free radicals during ageing is revealed by the increase in tocopherol concentration in aged wild type cells. This might represent a compensatory mechanism protecting the ageing cells. On the other hand, tocopherol concentration was diminished even in the young Ras mutant cells. It appears that the antioxidative defences were overwhelmed in these cells as they were not able to elevate the decreased tocopherol level in the course of ageing.

5. Conclusion

In summary, the present study documents that increased production of free radicals can eliminate the endogenous antioxidant system which results in accumulation of LFP during ageing. The wild type yeast is evidently able to compensate for the increased free radical production during ageing and prevent accumulation of LFP. This study presents evidence of the generation of reactive nitrogen species in the yeast.

Acknowledgement

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Figure legends

Fig. 1. Tridimensional spectral array of the yeast chloroform extract. The ordinate shows fluorescence intensity in arbitrary units. This spectra represent the typical example of LFP fluorescence. It shows the situation in the young wild type cells. Spectra from other groups did not differ in their appearance from this one.

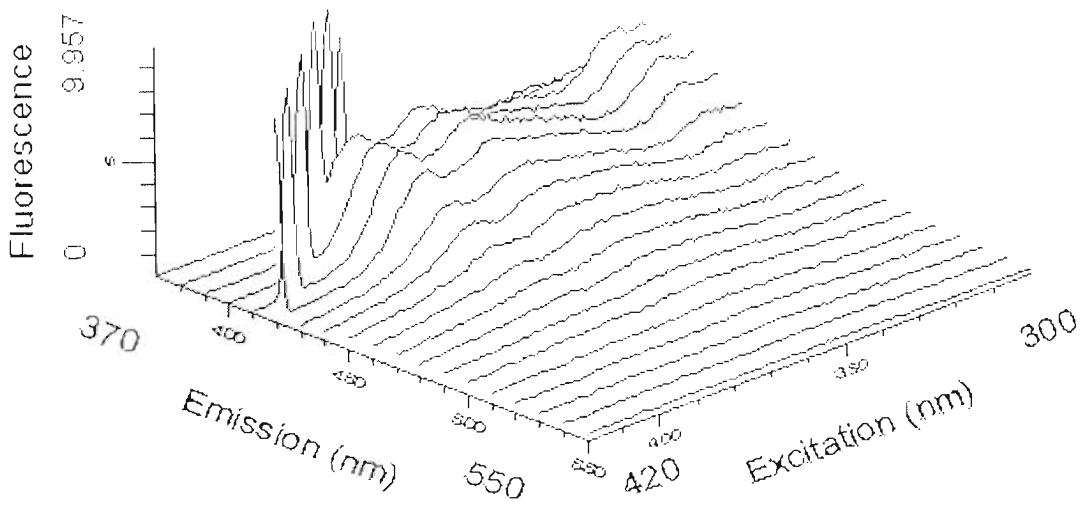
Fig. 2. Quantitative evaluation of individual fluorophores. The upper panel shows the changes in the wild type cells, the lower panel in the Ras mutant cells. The results are expressed in percentage related to the value of young cells (group Y). Statistical significance: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

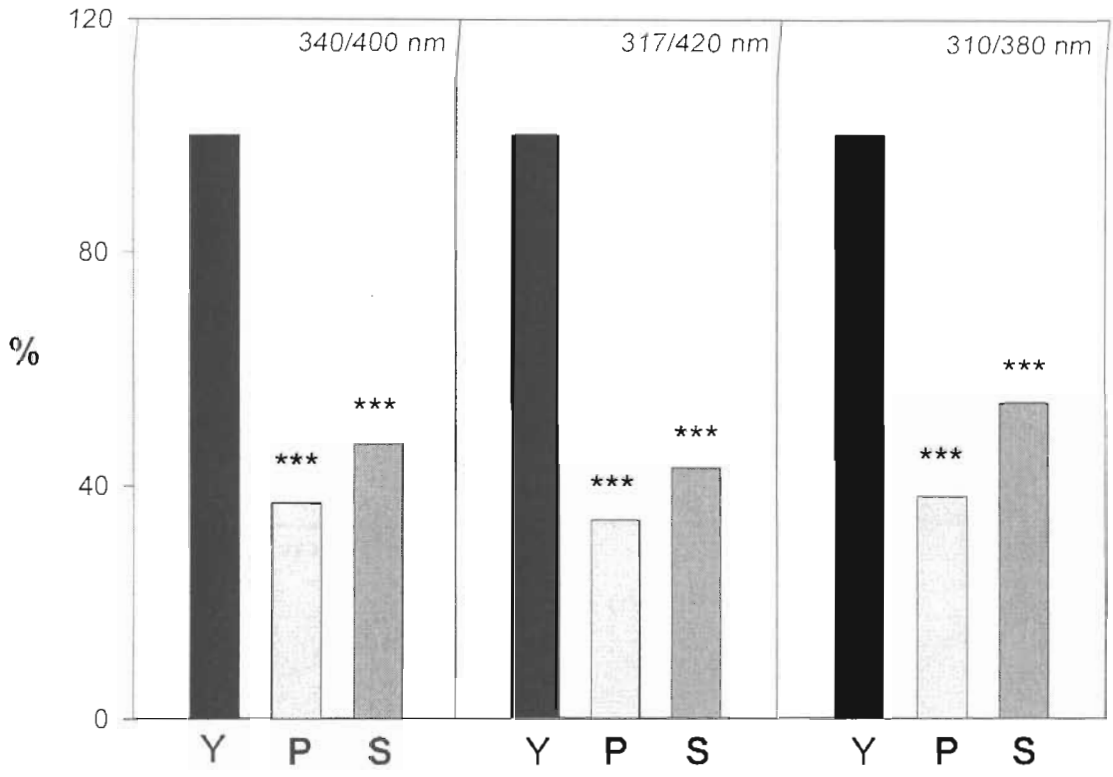
Fig. 3. Synchronous fluorescence emission scan of the chloroform extracts from the wild type cells. It was measured with difference of 35 nm between excitation and emission wavelength. The upper panel shows group Y, the lower panel group P. The ordinate shows fluorescence intensity in arbitrary units. The arrow indicates the position of the peak which increased during ageing.

Fig. 4. Synchronous fluorescence emission scan of the chloroform extract from the Ras mutant cells. For the description see Fig. 3.

Fig. 5. Nitrotyrosine concentration in yeast soluble protein fraction. Black columns - wild type cells, grey columns - Ras mutant cells. Statistical significance: *** $P<0.001$ in relation to group Y, +++ $P<0.001$ in relation to Ras cells group S.

Fig. 6. Total concentration of α-tocopherol in the yeast. Black columns - wild type cells, grey columns - Ras mutant cells. Statistical significance: * $P<0.05$, ** $P<0.01$ in relation to the group Y of wild type cells; +++ $P<0.001$ in relation to the corresponding group of the wild type cells.


Fluorescence
9.957
0
370
Emission (nm)
550
420
Excitation (nm)
300


120
80
40
0
%
340/400 nm
317/420 nm
310/380 nm
***
***
***
***
***
***
Y
P
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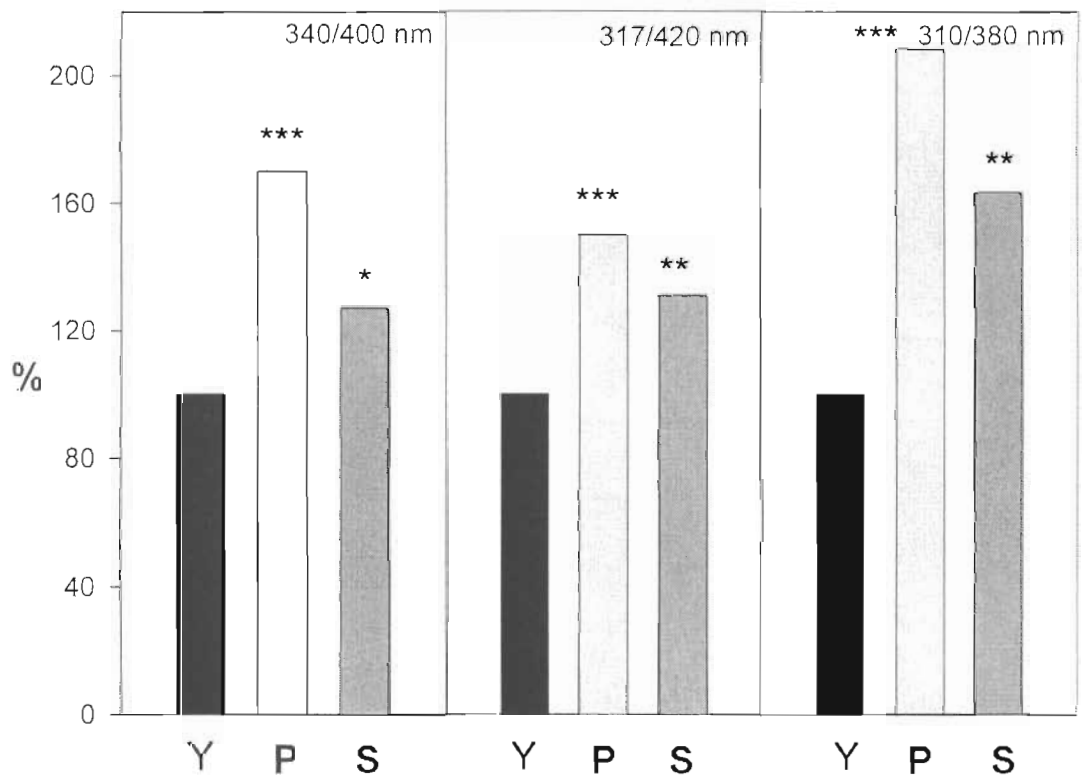
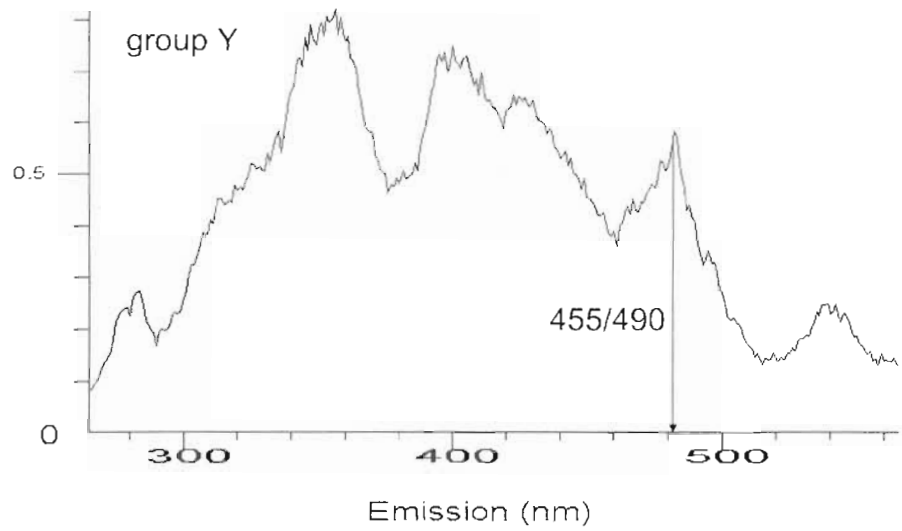
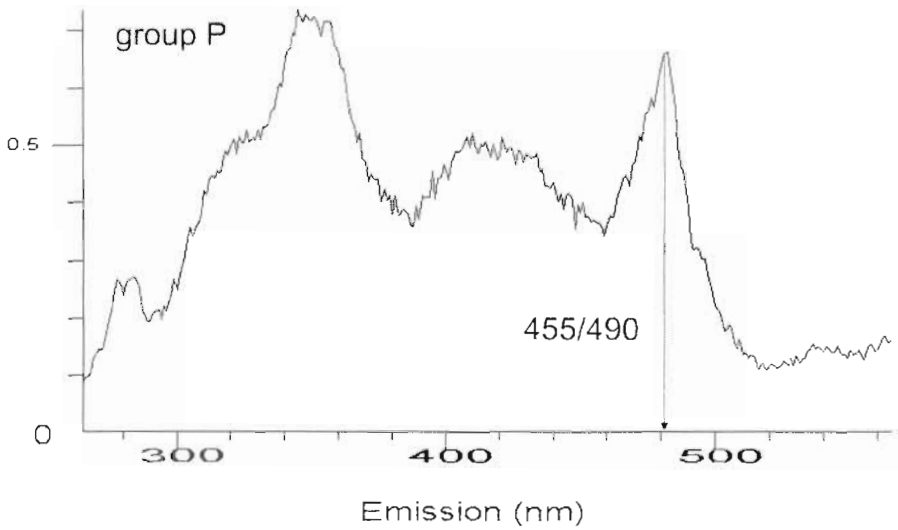
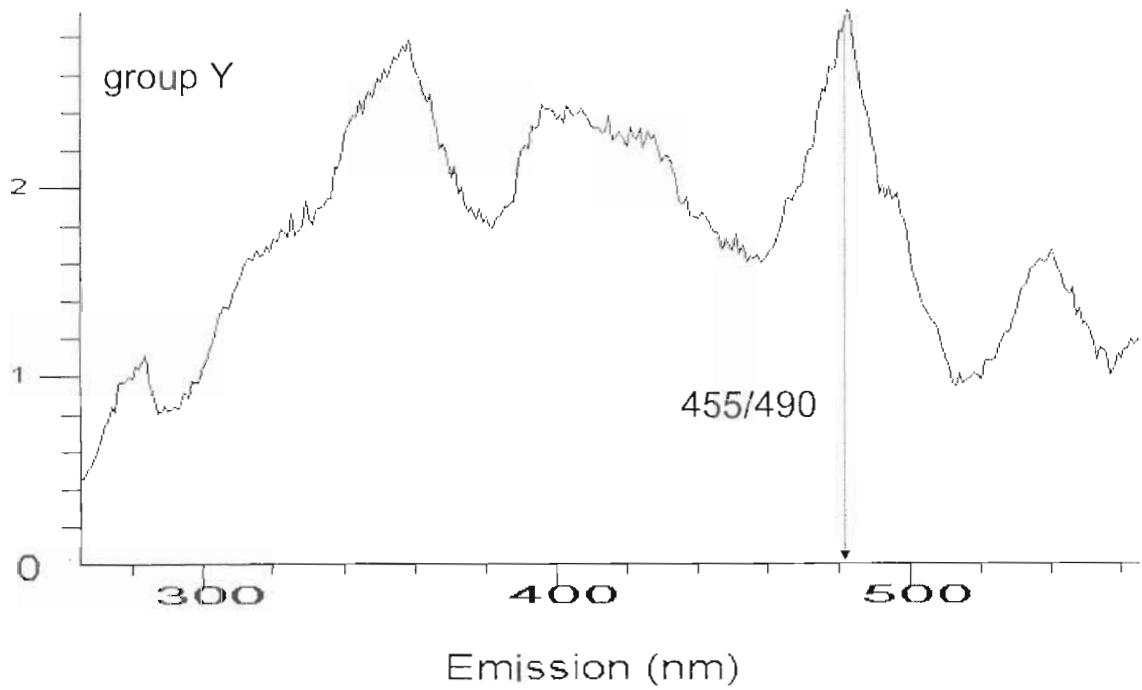
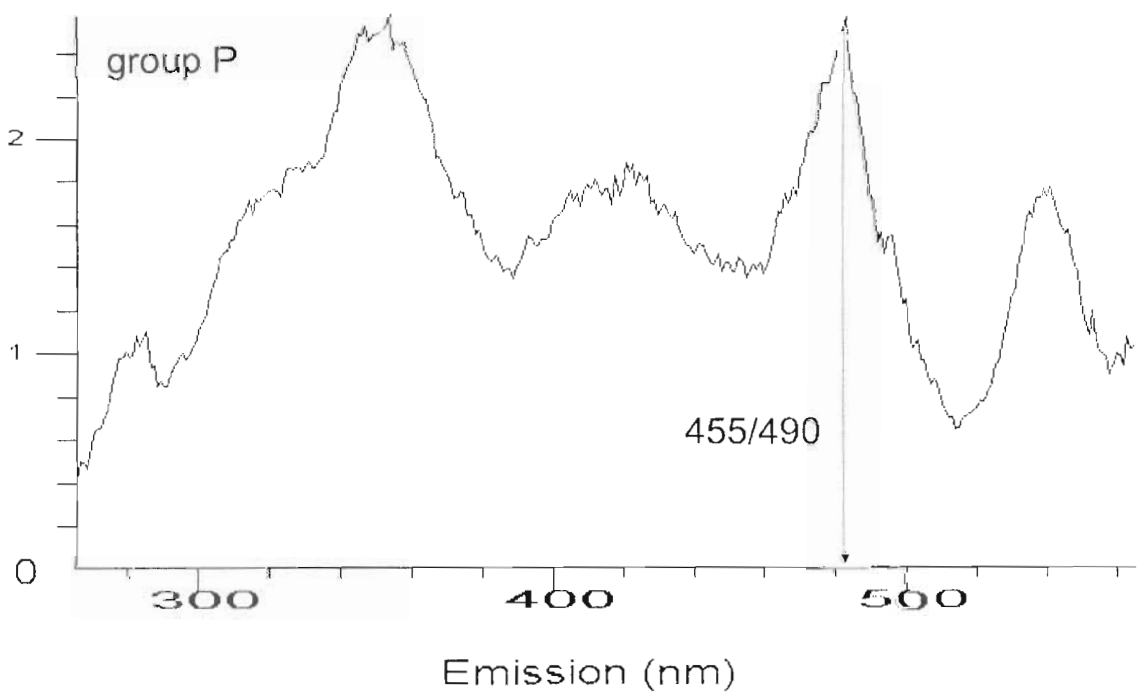
S
Y
P
S

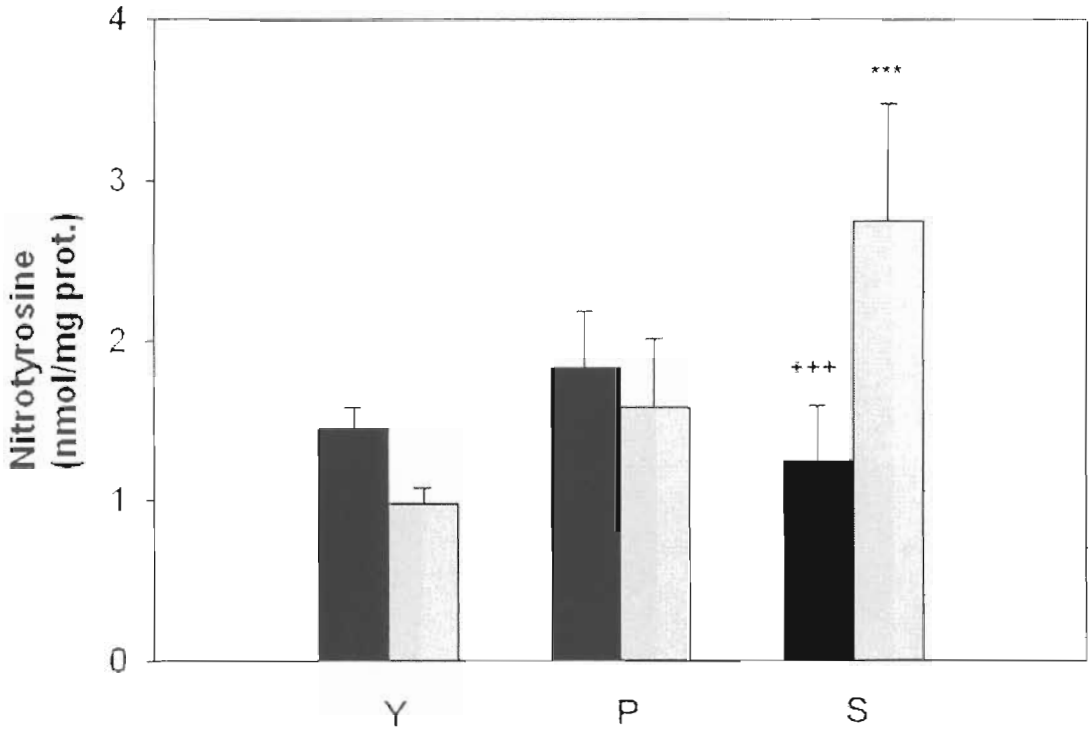

200
160
120
80
40
0
%
340/400 nm
317/420 nm
*** 310/380 nm
***
*
***
**
**
Y
P
S
Y
P
S
Y
P
S

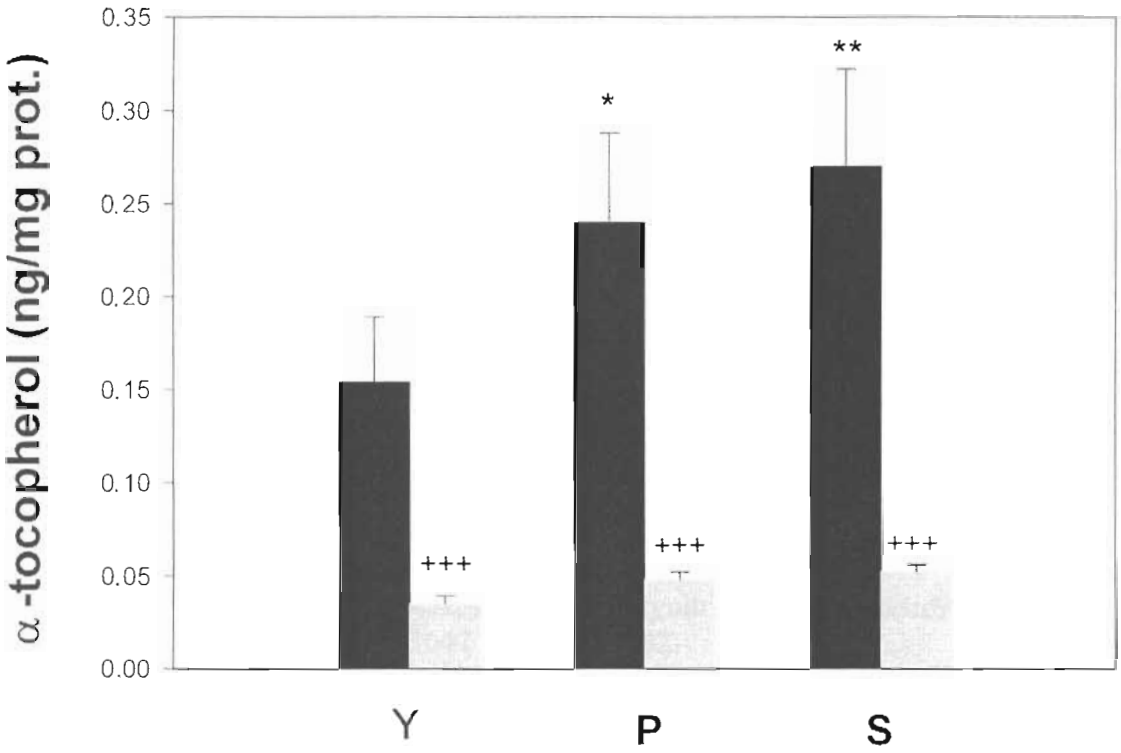

group Y
0.5
0
455/490
300
400
500
Emission (nm)


group P
0.5
0
455/490
300
400
500
Emission (nm)


group Y
2
1
0
455/490
300
400
500
Emission (nm)


group P
2
1
0
455/490
300
400
500
Emission (nm)


Nitrotyrosine
(nmol/mg prot.)
4
3
2
1
0
***
+++
Y
P
S


α -tocopherol (ng/mg prot.)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
**
+++
+++
+++
Y
P
S

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Příloha 6

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Characterization of Monoclonal Antibodies Recognizing Different Fragments of Cartilage Oligomeric Matrix Protein in Human Body Fluids

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Cartilage oligomeric matrix protein (COMP) is a high-molecular-weight glycoprotein found at a high concentration in articular cartilage. Recent studies have shown that the joint fluid and serum levels of antigenic COMP, measured by an enzyme-linked immunosorbent assay (ELISA) which uses a polyclonal antiserum raised against bovine COMP, provide important information about metabolic changes occurring in the cartilage matrix in joint disease. In this report, we describe the specificity of three monoclonal antibodies (mAbs) to human COMP and their usefulness in quantifying antigenic COMP fragments in body fluids. Two of the mAbs (16-F12 and 18-G3) recognized both oligomeric and monomeric forms of COMP, but the third (17-C10) reacted positively only with the former. Immunoblots of human COMP, predigested with trypsin for up to 6 h, showed that the three mAbs are directed against different epitopes identified on small tryptic fragments of 30 kDa (16-F12), 25 kDa (17-C10), and 40 kDa as well as 30 kDa (18-G3), respectively. The antibodies also recognized a different pattern of fragments in human pathological synovial fluids. This was particularly striking in the case of the medium size fragments (16-F12: 90 and 110 kDa; 17-C10: 70 and 90 kDa; 18-G3: up to five bands from 70 to 130 kDa). Competitive indirect inhibition ELISAs developed with mAbs 16-F12 and 17-C10 revealed further differences in the specificities of these antibodies. Thus, while mAb 16-F12 can be used only to quantify antigenic COMP in human synovial fluid and serum, mAb 17-C10 is useful in addition when analyzing canine and horse synovial fluid as well as canine serum. The results of analyses of synovial fluid samples from patients with osteoarthritis and rheumatoid arthritis provided preliminary evidence in support of the contention that measurement of the different COMP epitopes recognized by these mAbs in body fluids could prove useful in the clinical assessment of patients with joint disease. © 1997 Academic Press

Key Words: **cartilage oligomeric matrix protein (COMP); matrix degradation; monoclonal antibodies; synovial fluid; serum; ELISA.**

Cartilage oligomeric matrix protein (COMP)[2] is an anionic, noncollagenous protein of high molecular weight (>500 kDa) present in the extracellular matrix of articular, nasal, and tracheal cartilages (1–3), the Swarm rat chrondrosarcoma (4), and bovine tendon (5, 6). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), performed under reducing conditions, has shown that COMP is composed of five identical subunits exhibiting a molecular mass of approximately 120 kDa (1, 3). As the protein moiety of each subunit has a calculated molecular mass of only 82.4 kDa (7), it has been suggested that it is substituted with quite a few N-linked oligosaccharides (1, 3, 8). The subunits are assembled in a five-stranded α-helical bundle at their N-terminal end (9); the pentameric bouquet-like structure has been confirmed by electron mi-

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[2] Abbreviations used: COMP, cartilage oligomeric matrix protein; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; OA, osteoarthritis; RA, rheumatoid arthritis; SF, synovial fluid; HRP, horseradish peroxidase; ACL, anterior cruciate ligament; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ACLT, anterior cruciate ligament transection.

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roscopy (2, 4). COMP is a member of the thrombospondin family of extracellular calcium-binding proteins involved in cell proliferation, adhesion, and migration (7, 8, 10). Cloning and sequence analysis of COMP cDNA established that there is a high homology of COMP to the thrombospondins, both in the carboxyl-terminal domain and in the calcium-binding domain (7). The native form of COMP, like the thrombospondins, can be extracted from tissues in relatively high yield with buffers that contain ethylenediaminetetraacetic acid (EDTA) (2, 6).

The highest levels of COMP are found in articular cartilage where it constitutes approximately 1% of the wet weight (1). In immature rat (1) and fetal human (3) articular cartilage, COMP is preferentially localized in the territorial matrix directly adjacent to the chondrocyte. In normal adult human articular cartilage, however, COMP also has been detected in the interterritorial matrix, especially in the deeper layers (3). COMP is no longer thought to be a cartilage-specific glycoprotein since it also is present in significant amounts in tendon (5, 6). Immunostaining of adult and fetal bovine tendon revealed intense staining for antigenic COMP in the connective tissue surrounding the tendon bundles and in the areas around the collagen fibers (5); with age, the punctate staining around collagen fibers increased in intensity (5).

While the function(s) of COMP in cartilage is not clear, recent studies have suggested it plays important roles in chondrogenesis (11), the maintenance of the chondrocytic phenotype (1), and the interaction of the chondrocyte with its surrounding matrix (2). The structure of COMP predicts that it may bind to one or several interaction partners, a functional feature of all bouquet-like proteins (4), and that the binding may take place via calcium-dependent interactions (7). The importance of calcium binding to the function of COMP is supported by the recent discovery that mutations within the highly conserved calcium-binding domain of the COMP gene cause pseudoachondroplasia and multiple epiphyseal dysplasia (12, 13).

During the past decade, both basic and clinical studies have provided evidence in support of the contention that quantification of cartilage-derived molecules, such as antigenic COMP, in body fluids can prove useful in identifying metabolic changes occurring in the matrix of this shock-absorbing tissue (14). In joint fluid, these marker molecules provide information about specific catabolic or anabolic processes in the cartilaginous tissues within that joint (15). In blood or urine, they have helped detect systemic changes or abnormalities affecting most or all cartilaginous structures in the body (14). Measurements of these body fluid markers of metabolic processes already have helped identify increased catabolic as well as anabolic activities in articular cartilage from patients with joint disease (14). Recent reports support the contention that some body fluid markers of metabolic processes are potentially useful for monitoring disease activity (16), identifying metabolic alterations in articular cartilage prior to the development of clinical signs of disease (17), examining responses of the cartilaginous tissues to treatment (18), and evaluating long-term prognosis (19).

Antigenic COMP has received much attention for its role as a molecular marker of alterations in cartilage metabolism (20–22). Not unexpectedly, this molecular marker, measured by an enzyme-linked immunosorbent assay (ELISA) using a rabbit polyclonal antiserum directed against bovine articular cartilage COMP, is present at a higher concentration in synovial fluid (SF) than in serum (20). Importantly, the levels in these two body fluids correlate positively with one another (20–22). Recent reports that levels of antigenic COMP in SF and serum may have prognostic value in the clinical evaluation of patients with rheumatoid arthritis (RA), osteoarthritis (OA), and other joint diseases are exciting (16, 23–26). In one of these studies, a high serum level of antigenic COMP during the early stages of RA was shown to be predictive of rapid hip joint destruction (23). In another study, this time of patients with clinical knee OA, serum antigenic COMP levels significantly increased during the first year in those patients who showed decreased joint space or required knee surgery, compared to those who did not (26). These and other findings have led Saxne and Heinegard to suggest that at both early and later stages of OA, the measurement of antigenic COMP levels in serum might be useful for prognostic purposes (16).

Until now, levels of antigenic COMP in SF and serum have been quantified using a polyclonal anti-bovine COMP antiserum (16, 20–27). In this paper, we present the characterization of monoclonal antibodies (mAbs) to human COMP and demonstrate that they recognize different fragments of antigenic COMP in human joint fluid. We show that the antibodies can be used in quantitative ELISAs and provide evidence that the pattern of fragments present in joint fluids from OA and RA patients may be disease-specific.

EXPERIMENTAL PROCEDURES

Materials

Horseradish peroxidase (HRP)-conjugated porcine anti-mouse immunoglobulins used in the course of hybridoma screening were from USOL (Praha, Czech Republic). HRP-conjugated goat anti-mouse IgG used in the immunoblotting experiments and antibody typing kit were from Pierce Chemical Co. (Rockford, IL). HRP-conjugated goat anti-mouse immunoglobulins (polyvalent) used to develop the competitive indirect inhibition ELISAs, hyaluronidase from *Streptomyces hyalurolyticus* (EC 4.2.2.11), trypsin from bovine pancreas (TPCK-treated), and polyethylene glycol (Hybri-Max, MW 1300–1600) were purchased from Sigma Chemical Co. (St. Louis, MO). Microtiter plates (Nunc-Immunoplate 1F with certificate) and MicroWell Plates (low binding) were from Nunc (Kamstrup, Denmark).

Nitrocellulose membranes and chemicals for SDS–PAGE were from Bio-Rad Laboratories (Hercules, CA) and Q-Sepharose was from Pharmacia Biotech (Piscataway, NJ). All other chemicals were of analytical grade.

When appropriate for therapeutic or diagnostic reasons, SF samples were collected by aspiration from the knee joints of 35 patients attending outpatient clinics. Seventeen patients had primary OA of the knee: 7 women with a mean ± standard deviation (SD) age of 58 ± 8 years (range 46–74) and 10 men with a mean ± SD age of 66 ± 10 years (range 43–84). Seventeen patients had RA: 14 women with a mean ± SD age of 59 ± 13 years (range 27–78) and 3 men with a mean ± SD age of 66 ± 8 years (range 57–77). The SF from one male patient (age 21 years) presenting with anterior cruciate ligament (ACL) injury was also included. After collection, SF samples were centrifuged, aliquoted, and frozen at −70°C until utilized. All OA and RA patients fulfilled established diagnostic criteria of the American Rheumatism Association (28, 29). In this preliminary study with a very small number of patients, no attempt was made to characterize the patients with respect to disease severity, number of joints involved, etc. Normal human and animal sera and animal SFs were collected at the Department of Biochemistry, Rush-Presbyterian-St. Luke's Medical Center (Chicago, IL).

Preparation of Antigen

Full-depth slices of macroscopically normal human articular cartilage were removed from the femoral heads of two individuals aged 18 and 22 at the time of autopsy. Tissues were stored at −70°C until processed. The dissected cartilage slices were pooled, sectioned on a freezing cryostat at 20 μm, and extracted with Dulbecco's phosphate-buffered saline (PBS), pH 7.4 (without Ca^{2+} and Mg^{2+}), containing 10 mM EDTA and proteinase inhibitors (5 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine–HCl, 5 mM 6-aminohexanoic acid, and 5 mM *N*-ethylmaleimide). The extract was chromatographed on a column of Q-Sepharose eluted with a gradient of NaCl (0.1 to 1 M) in the presence of 7 M urea (30). The material that eluted as a sharp peak at 0.2–0.3 M NaCl was collected; its mobility in SDS–PAGE under reducing and nonreducing conditions confirmed its identity as COMP. The appropriate fractions were pooled, dialyzed against distilled water, and lyophilized.

Monoclonal Antibody Production

Six-week-old female BALB/c mice were immunized intraperitoneally with human articular cartilage COMP, isolated as described above, in complete Freund's adjuvant. Animals were boosted with an intrasplenic injection of the antigen in PBS, pH 7.2, 8 weeks later. Splenocytes of immunized mice were isolated after 4 days and fused with cells of myeloma line Sp2/0 (31). Hybridoma supernatants were screened by ELISA for the presence of mouse immunoglobulins and then for the presence of antibodies against the original immunizing antigen. Briefly, mice immunoglobulins in culture media were detected on microtiter plates coated with porcine anti-mouse immunoglobulins (2 μg/ml). Culture media from positive wells were further tested for the presence of specific antibodies by ELISA on microtiter plates coated with human COMP (1 μg/ml). Positive colonies of hybridomas were expanded and subcloned at least twice by the method of limiting dilution; culture media were further tested by immunoblotting against reduced and nonreduced antigen. Three hybridomas (16-F12, 17-C10, and 18-G3) were used to produce ascitic fluids by the intraperitoneal injection of 10^6 cells into male BALB/c mice previously primed with an injection of Freund's incomplete adjuvant. Ascitic fluids were harvested 10–14 days later.

Enzymatic Digestions of COMP

Trypsin. Nonreduced, oligomeric COMP was dissolved in 10 mM $CaCl_2$/50 mM Tris buffer, pH 8.0. The temperature of the solution was adjusted to 37°C, and TPCK-trypsin was added at the enzyme:substrate ratio of 1:50 (w/w). Aliquots of the digestion mixture were taken at the following time points: 0 min (prior to the addition of the enzyme), 5 min, 15 min, 45 min, 90 min, 3 h, and 6 h; the digestion was stopped by adding an equal volume of twice-concentrated nonreducing SDS–PAGE (32) sample buffer and boiling for 5 min.

Hyaluronidase. Samples of SF were first adjusted to 5 mM with EDTA; hyaluronidase from *S. hyalurolyticus* was dissolved in 0.1 M NaCl, 0.1 M sodium acetate, pH 6.0, at 200 U/ml. Twenty units (100 μl) of the enzyme was added to 1 ml of SF and the digestion was carried out at 37°C overnight.

Electrophoresis and Immunoblotting of COMP and COMP Fragments

The purity of the COMP preparation used as the immunizing antigen was ascertained by electrophoresis under reducing and nonreducing conditions in a 1.6–16% gradient gel (33). After SDS–PAGE the gel was stained with Coomassie brilliant blue R-250. The COMP preparation (reduced or nonreduced) used as immunizing antigen and samples of SF (nonreduced) were electrophoresed in 4–15% gradient gels (32) and transferred onto nitrocellulose membranes in 15 mM sodium borate at 25 V overnight. The membranes were blocked with 5% skim milk in PBS, pH 7.2, at 37°C for 1 h. Primary as well as secondary antibodies were diluted 1:1000 in 2% skim milk in PBS, pH 7.2. The peroxidase reaction was developed using 4-chloro-1-naphtol as a chromogen. Densitometric analysis of SF immunoblots was performed on a Macintosh Quadra 610 computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Quantification of Antigenic COMP by Competitive Indirect Inhibition ELISA

The competitive indirect inhibition ELISA for COMP was performed essentially as that previously described for the quantification of antigenic keratan sulfate (34, 35) with minor modifications.

Antibodies. Anti-COMP mAbs 16-F12 and 17-C10, as prepared above, were used in the COMP ELISAs. HRP-conjugated goat anti-mouse immunoglobulins (polyvalent) were used as the detector antibodies.

COMP standard antigen and plate-coating antigen. The same preparation of human articular cartilage COMP, isolated as previously described and used as the immunizing antigen, was also used for both COMP standard and plate-coating antigen in all ELISAs. The lyophilized material was dissolved in PBS containing 10 mM EDTA, pH 7.0, aliquoted, and frozen until needed.

Plate coating. Coating buffer [20 mM carbonate/bicarbonate, pH 9.2, 0.002% (w/v) sodium azide] containing human articular cartilage COMP at 5 μg/ml (50 μl) was introduced into each well of a Nunc-Immuno plate. The plate was covered with plate-sealing tape and kept at 4°C until used.

Inhibition step. Antibody titer procedures established that the appropriate initial dilution for both mAb 16-F12 and mAb 17-C10 ascites fluid was 1/40,000 in PBS–0.05% Tween 20, 1% bovine serum albumin (BSA), pH 7.0. Seventy microliters of the initial dilution of the mAb being used was added to 70 μl of the COMP standard or unknowns diluted in PBS–0.05% Tween 20, pH 7.0, in a Nunc Microwell plate. These monoclonal antibody–COMP inhibition mixtures were maintained at 4°C.

ELISA. The standard test was performed as follows: coated wells were washed (3× 5 min with PBS–0.05% Tween 20, pH 7.0) to remove excess antigen and blocked prior to use by incubating for 60 min with 100 μl of PBS, 5% BSA, pH 7.0. The blocked, coated wells

were washed and incubated for 60 min at 4°C with 100 μl of the inhibition mixture (monoclonal antibody plus antigen) prepared as described above. The plates were then washed and 100 μl of HRP-conjugated rabbit anti-mouse (polyvalent) antibody was placed into the wells. After incubating for 60 min at room temperature, unbound antibody was removed by washing and 100 μl of substrate (*o*-phenylenediamine plus H_2O_2) was placed into the wells for 30–60 min at room temperature. The production of chromophore was stopped by the addition of 25 μl of 2 M H_2SO_4. The plates were read in a plate reader at a wavelength of 490 nm. Concentrations of antigenic COMP were determined using the four-parameter logistic function equation $f(x) = (a - d)/[1 + (x/c)^b] + d$, where parameters a and d are the asymptotic maximum and minimum values, respectively, b is the slope parameter, and parameter c is the x value at the inflection point. The results are expressed as equivalents of a standard of human antigenic COMP (μg/ml), purified as previously described, and are reported as means ± SD of triplicate analyses.

Statistical Analysis

Differences between SF levels of antigenic COMP in patient populations (OA vs RA) were assessed using the nonparametric Mann–Whitney *U* test. Differences between the levels in the two ELISAs (16-F12 vs 17-C10) were compared by the Wilcoxon matched-pairs signed-ranks test. Correlations between SF levels of antigenic COMP determined by the two ELISAs (mAb 16-F12 vs 17-C10) were calculated using the Pearson product moment correlation in each patient population (OA and RA).

RESULTS

Characterization of Monoclonal Antibodies to Human COMP

Three hybridoma clones (16-F12, 17-C10, and 18-G3) were identified based on the ability of the antibody they synthesized to recognize oligomeric human COMP. All three mAbs were IgG1 (κ chain) and recognized the nonreduced oligomeric antigen on immunoblots (Fig. 1). The reduced COMP monomer, with a molecular mass of approximately 100 kDa, was recognized by mAbs 16-F12 and 18-G3 but not by mAb 17-C10 (Fig. 1). Interestingly, the immunoblots performed on the reduced standard also revealed a fainter band (with a molecular mass of approximately 80 kDa): this molecule that was also recognized only by mAbs 16-F12 and 18-G3 probably represents the unglycosylated form of the COMP monomer. To help further probe possible differences in specificity of the three mAbs, immunoblots also were performed on fragments produced by trypsin digestion (5 min to 6 h) of oligomeric COMP in the presence of 10 mM calcium (2). The three mAbs detected different patterns of trypsin-generated fragments (Fig. 2), thereby demonstrating that they are directed against different epitopes. One or, in the case of mAb 18-G3, two small fragments predominated at the later time points. Importantly, the size of the fragment varied from mAb to mAb (16-F12: ~30 kDa; 17-C10: ~25 kDa; 18-G3: ~40 kDa with a smaller fragment of ~30 kDa). The 30-kDa fragment recognized by mAb 16-F12 reached near maximum concentration within 5 min and was resistant to further cleavage: the intensity of staining of this band did not diminish even after 6 h incubation with trypsin (Fig. 2A). This fragment was also recognized by mAb 18-G3 (Fig. 2C). The major fragment recognized by mAb 18-G3 was, however, a 40-kDa band that first appeared in the digest after 45 min (Fig. 2C, lane 4). Neither the 30-kDa nor the 40-kDa fragment was detected by mAb 17-C10 (Fig. 2B). The major fragment recognized by mAb 17-C10 first appeared in the digest after 45 min (Fig. 2B, lane 4); its molecular mass was 25 kDa. Interestingly, only mAb 18-G3 recognized tryptic fragments after reduction (data not shown).

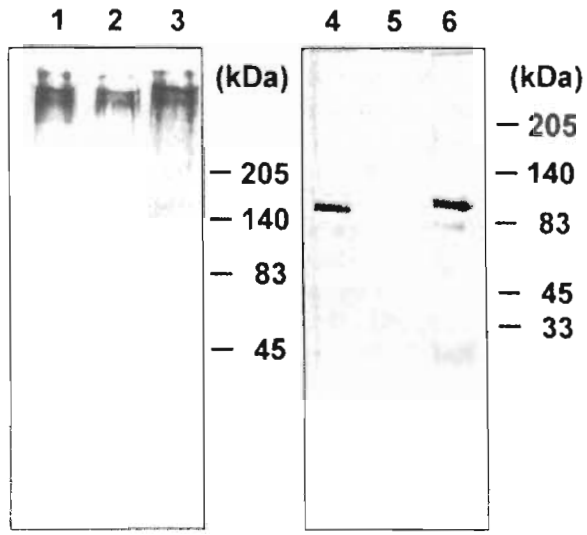

FIG. 1. Western blot analysis of human antigenic COMP. The human antigenic COMP preparation used as the immunizing antigen was subjected to SDS–PAGE (2 μg protein/lane) in 4–15% gradient gels under nonreducing (lanes 1–3) and reducing (lanes 4–6) conditions, transferred onto nitrocellulose, and immunodetected using anti-human COMP mAbs 16-F12 (lanes 1 and 4), 17-C10 (lanes 2 and 5), and 18-G3 (lanes 3 and 6), respectively, as described under Experimental Procedures. The positions of migration of the molecular weight standards are indicated.

Analysis of Antigenic COMP Fragments in Human SFs by Immunoblotting

Samples of human SF were digested overnight with hyaluronidase from *S. hyalurolyticus* and diluted with SDS–PAGE sample buffer; aliquots corresponding to 5 μl of undiluted SF were loaded on gels. Separated samples were blotted onto nitrocellulose and immunodetected with the mAbs described above. Each mAb recognized numerous fragments that were defined as belonging to one of three major groups [i.e., very large (>250 kDa), large (130–250 kDa), and medium size (70–130 kDa)] (Fig. 3A and Fig. 4). The very large size group consisted of many bands that appeared as a ladderlike pattern of bands, with two bands often

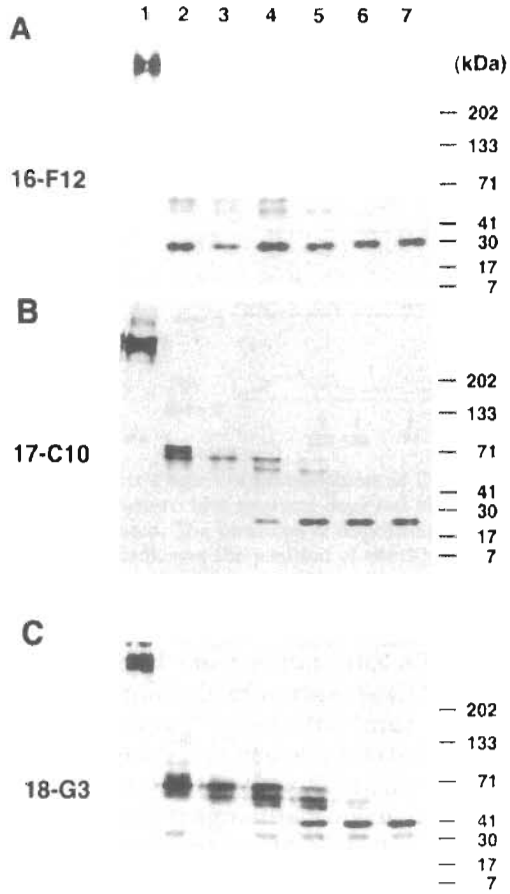

FIG. 2. Western blot analysis of antigenic fragments of human COMP produced by trypsin digestion. Nonreduced oligomeric human COMP was digested with TPCK-trypsin (1:50, w/w) for up to 6 h and aliquots (1 μg COMP/lane) corresponding to individual time points were subjected to SDS–PAGE in 4–15% gradient gels under nonreducing conditions, transferred onto nitrocellulose, and immunodetected using anti-human COMP mAbs 16-F12 (A), 17-C10 (B), and 18-G3 (C), as described under Experimental Procedures. Intervals of digestion were lane 1, 0 min (aliquot taken prior to the addition of trypsin); lane 2, 5 min; lane 3, 15 min; lane 4, 45 min; lane 5, 90 min; lane 6, 3 h; and lane 7, 6 h. The positions of migration of the molecular weight standards are indicated.

predominating (see Fig. 3A and Fig. 4). The group of large fragments consisted in most samples of a doublet of approximately 200 kDa that was detected by mAbs 17-C10 and 18-G3; mAb 16-F12 detected a slightly smaller doublet of approximately 175 kDa (Fig. 3A). The group of large fragments also included a sharp 140-kDa band that was present only in the sample of the patient with an ACL injury; this band was stained preferentially with mAbs 17-C10 and 18-G3 (Fig. 3A, lane 10). Otherwise, the patterns of bands recognized by the three different mAbs in each individual SF sample in the areas of large and very large fragments were similar, although not identical.

On the other hand, striking differences were detected in the pattern of medium size antigenic COMP fragments recognized by the different mAbs (Fig. 3A and Fig. 4). In the majority of the samples, mAb 16-F12 detected two bands with molecular masses of approximately 90 and 110 kDa and mAb 17-C10 detected two bands with molecular masses of approximately 70 and 90 kDa. In contrast, mAb 18-G3 detected up to five bands with varying intensities and sizes ranging from 70 to 130 kDa. Some, but not all, appeared to be of identical size to fragments recognized by mAbs 16-F12 and 17-C10 (Fig. 3A and Fig. 4). The precise molecular masses of the major medium size fragments recognized by each mAb were confirmed by electrophoresing one SF sample in three adjacent lanes flanked by lanes containing the molecular mass standards and, after transfer onto nitrocellulose, cutting out the lanes and probing each with one of the mAbs (Fig. 3B).

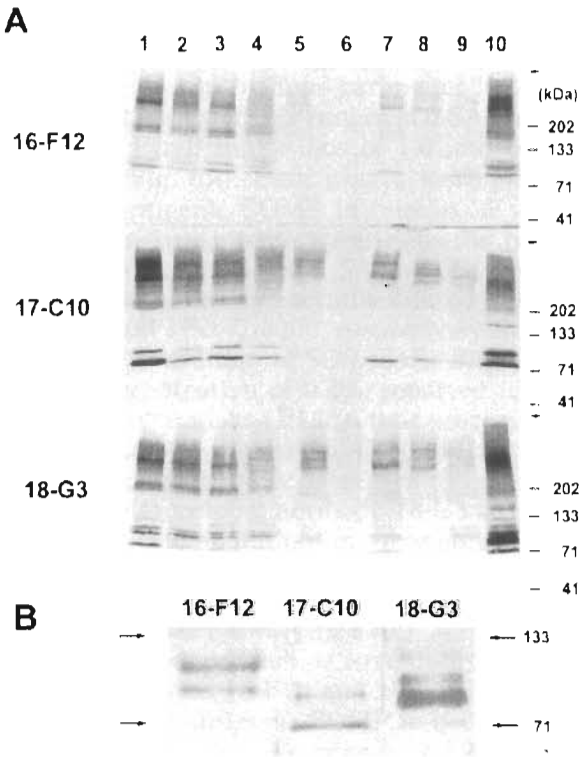

FIG. 3. Western blot analysis of antigenic COMP fragments in human SF samples. (A) Synovial fluid samples from patients with OA (lanes 1–4), RA (lanes 5–9), and ACL injury (lane 10) were digested with hyaluronidase from *Streptomyces hyalurolyticus*, separated by SDS–PAGE in 4–15% gradient gels under nonreducing conditions, transferred onto nitrocellulose, and immunodetected using anti-human COMP mAbs 16-F12, 17-C10, and 18-G3, as indicated. The same sample volume, corresponding to 5 μl of SF before hyaluronidase treatment, was loaded in each lane. (B) To precisely align medium size bands, the sample in lane 10 was electrophoresed in triplicate within the same gel in adjacent lanes flanked by lanes containing molecular mass standards and, after transfer onto nitrocellulose, the lanes were cut and immunostained using the different mAbs as indicated.

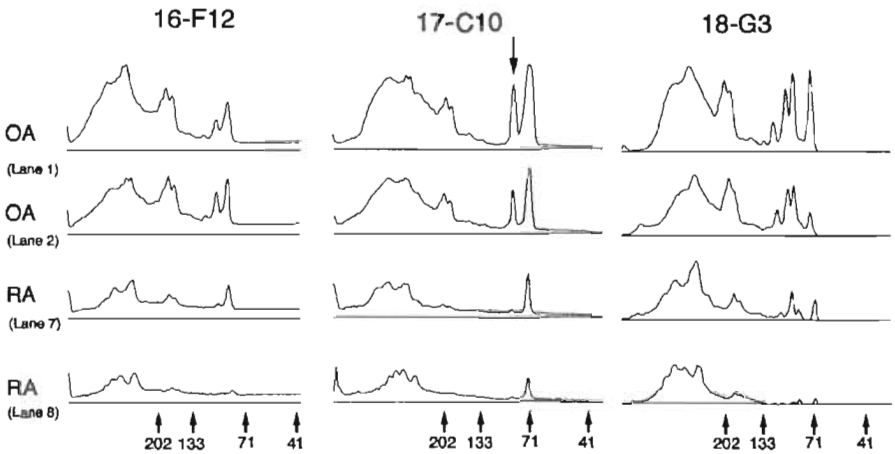

FIG. 4. Densitometric scans of immunoblots of OA and RA SFs. Using the NIH Image program, densitometric scans of selected OA and RA SFs from the Western blot analysis depicted in Fig. 3 (OA, lanes 1 and 2; RA, lanes 7 and 8) were obtained for mAbs 16-F12, 17-C10, and 18-G3, as indicated. The positions of migration of the molecular weight standards are marked by small arrows. The large arrow in the mAb 17-C10 panel indicates the position of the 90-kDa fragment.

Synovial fluids from RA patients all appeared to contain lower amounts of antigenic COMP than SFs from OA patients or SF from the knee of the patient with an ACL injury. However, no attempt was made to use the immunoblot approach to quantify COMP or any of its antigenic fragments. Interestingly, medium size fragments present in OA SFs, but especially the 90-kDa fragment recognized by all three mAbs, seemed to be less abundant in RA SFs (Fig. 3A and Fig. 4). This is best illustrated in Fig. 4, depicting the results of densitometric scans of the immunoblots of typical OA and RA samples: when using mAb 17-C10 the 90-kDa fragment, migrating in the position of the arrow, is noticeably less abundant in the RA SFs than in the OA SFs.

Surprisingly, although mAbs 16-F12 and 18-G3 recognized intact antigenic COMP after reduction (Fig. 1) and, in the case of mAb 18-G3, also reduced tryptic fragments (data not shown), none of the three mAbs reacted with reduced samples of SF (data not shown).

Competitive Indirect Inhibition ELISAs for the Quantification of COMP

SDS–PAGE, performed under both reducing and nonreducing conditions in a 1.6–16% gradient gel, confirmed that the preparation of human articular cartilage antigenic COMP isolated as described above and used both as the standard and for plate coating was relatively pure (~95%). In both cases, the major band migrated with the expected mobility of COMP (~100 kDa under reducing conditions and >200 kDa under nonreducing conditions) (Fig. 5A, inset). The inhibition curves for both mAbs 16-F12 and 17-C10 (used at a 1:40,000 dilution) exhibited steep slopes ($b = -2.2$ and -1.5, respectively, in the four-parameter logistic function equation) with working ranges of 0.5–2.0 and 0.1–0.5 μg/ml and half inhibitions of 1.0 and 0.3 μg/ml, respectively (Fig. 5A). Both assays were effective in quantifying antigenic COMP in human SF and sera; the inhibition curves were parallel to the curves obtained for the standard (Figs. 5A and 5B). Human serum and SF samples were serially diluted in the range of 1/2–1/64 and 1/50–1/5000, respectively. It is worth noting that the addition of SDS to the diluting buffer at a final concentration of 0.4%, reported to improve accuracy in the published ELISA that uses a polyclonal anti-bovine COMP antiserum (20), did not improve quantification and actually decreased the sensitivity of our ELISAs. Monoclonal antibody 18-G3 at a dilution of 1:10,000 yielded an inhibition curve almost identical to that of 17-C10 (data not shown), but the assay was not further developed because substrate color development proceeded very slowly (>3 h).

The newly developed competitive indirect inhibition ELISAs using mAbs 16-F12 and 17-C10 were used to measure levels of antigenic COMP in body fluids from several species. The ELISA using mAb 17-C10, but not that using mAb 16-F12, detected antigenic COMP in measurable levels in a canine serum (4.1 μg/ml) (Fig. 5B). While the ELISA using mAb 17-C10 detected antigenic COMP at a high concentration (261 μg/ml) in a canine SF (Fig. 5B), the ELISA using mAb 16-F12 detected it only at a very low level (0.9 μg/ml). Neither ELISA was useful in detecting antigenic COMP at a level above the limit of detection (mAb 16-F12: ~0.5 μg/ml; mAb 17-C10: ~0.1 μg/ml) in adult bovine and rabbit SFs or in adult bovine, goat, rabbit, guinea pig, and fetal bovine sera (data not shown). On the other hand, mAb 17-C10, but not mAb 16-F12, detected anti-

genic COMP at a very low level (0.2 μg/ml) in a horse SF (data not shown). These differences in the abilities of these two mAbs to recognize antigenic COMP in some species provide further evidence that they recognize epitopes present on different fragments in body fluids.

Determination of Levels of Antigenic COMP in Human SFs by ELISA

In both ELISAs, levels of antigenic COMP were significantly higher in the hyaluronidase-digested SFs of patients with OA than in patients with RA [mAb 16-F12 (OA SFs: 199 ± 84 μg/ml vs RA SFs: 76 ± 34 μg/ml, $P < 0.0001$) and mAb 17-C10 (OA SFs: 121 ± 64 μg/ml vs RA SFs: 36 ± 26 μg/ml, $P < 0.0001$)] (Table I). Importantly, within each patient population, the values obtained for levels of antigenic COMP using the two ELISAs were significantly different (OA: $P = 0.0015$; RA: $P = 0.0003$) (Table I). Within each population, there was a positive linear correlation between the levels of antigenic COMP measured by the two ELISAs (OA: $r = 0.743$, $P = 0.0113$; RA: $r = 0.604$, $P = 0.0368$). However, expression of the values obtained for levels of antigenic COMP in the two ELISAs as a ratio (i.e., mAb 16-F12/mAb 17-C10) revealed considerable individual variation within each population but especially in patients with RA (OA: mean ratio = 1.73, range = 0.94–2.60; RA: mean ratio = 2.80, range = 1.17–9.75).

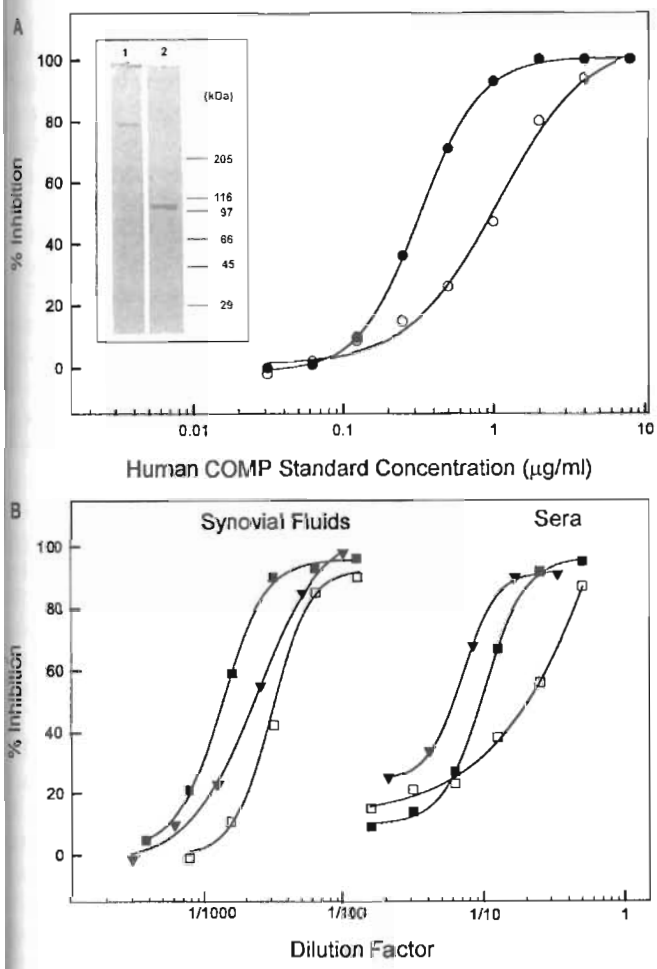

FIG. 5. ELISA inhibition curves obtained with mAbs 16-F12 and 17-C10. (A) Inhibition curves produced by the purified human articular cartilage COMP preparation used as the immunizing antigen in the ELISAs developed using mAb 16-F12 (○) and mAb 17-C10 (●). (Inset) The COMP preparation used as the immunizing antigen was electrophoresed under nonreducing (lane 1) and reducing (lane 2) conditions in a 1.6–16% gradient gel and then stained with Coomassie brilliant blue R-250. The positions of migration of the molecular weight standards are indicated. (B) Inhibition curves produced by SFs and sera analyzed at various dilutions with mAb 16-F12: human SF and serum (□) and mAb 17-C10: human SF and serum (■); dog SF and serum (▼).

TABLE I

Concentrations of Antigenic COMP in the SF of Patients with OA and RA

		Antigenic COMP (μg/ml)	
Diagnosis	*n*	mAb 16-F12 (range)	mAb 17-C10 (range)
OA	17	199 ± 84 (46–400)	121 ± 64*** (35–325)
RA	17	76 ± 34* (23–138)	36 ± 26**† (10–118)

* $P < 0.0001$ when compared to OA (mAb 16-F12).
** $P < 0.0001$ when compared to OA (mAb 17-C10).
*** $P = 0.0015$ when compared to OA (mAb 16-F12).
† $P = 0.0003$ when compared to RA (mAb 16-F12).

DISCUSSION

This study presents three anti-human COMP mAbs (16-F12, 17-C10, and 18-G3), each recognizing a different epitope in the COMP molecule. Immunoblots performed with these mAbs provided evidence that COMP-related epitopes in the SF of patients with OA and RA are present principally on COMP fragments. The presence of these antigenic fragments in large amounts in the pathological fluids studied is a significant finding as it strongly supports the contention that the rise in the SF level of antigenic COMP in these diseases (20, 22) provides first and foremost a measure of the upregulation of the catabolism of this molecule in connective tissues within the joint. As COMP is found at a higher concentration in articular cartilage than in other joint tissues (1), it is likely that a large proportion of the fragments reflect catabolic processes occurring in that tissue (1). However, since COMP is also present in tendon (5, 6) and, according to a recent

eport,[3] mRNA for COMP is detectable in human syno-ial cells, it is possible that other connective tissues ithin the joint contribute significantly to the level of OMP in SF and to the fragmentation of this molecule.

The fragmentation of oligomeric COMP is most likely nitiated in articular cartilage and the other tissues in hich this molecule is found. However, additional work s needed before one can state with some certainty that e process of fragmentation is essentially complete by e time the fragments are released into the SF. Metal-proteinases with a variety of substrate specificities re found in elevated amounts in OA and RA SFs (36–8). Further, recent reports have suggested that the atio of these enzymes to their natural inhibitor, TIMP-also is higher in OA and RA SFs than in normal SF 7–39). Consequently, one cannot rule out the possi-ility that some of the fragments undergo additional roteolytic cleavage while in transit in this body fluid mpartment. Studies of human articular cartilage ex-lants stimulated *in vitro* with catabolic cytokines such s IL-1 and TNF-α are currently in progress to help hed more light on the kinetics of the fragmentation rocess.

The discovery that each mAb recognized at least one ntigenic COMP fragment not recognized by the other wo mAbs is exciting as it suggests that it may be possi-le to raise mAbs capable of distinguishing between ntigenic COMP fragments produced in different dis-ases or at different stages of a disease. This contention supported by our observation that the three mAbs haracterized each recognized a different pattern of an-genic COMP fragments in OA and RA SFs. As each ecognized a different COMP-related epitope, it is ikely that at least some of the anti-human COMP nAbs raised in the future will be found to be specific or yet different antigenic sequences on the COMP mol-cule.

Two of the mAbs (16-F12 and 17-C10) exhibited high ffinity for antigenic COMP when used in a competitive ndirect inhibition ELISA. It is worth noting that both f these ELISAs yielded steep inhibition curves (i.e., a mall change in the concentration of antigenic COMP ielded a significant decrease in color development) hat made it possible to distinguish between two con-entrations differing by as little as 10%. In the ELISAs e have developed, compared to the well-characterized LISA using a polyclonal anti-COMP antiserum, it is ess important to ensure that the preparation of anti-genic COMP used for plate coating is absolutely free of ther matrix molecules because the specificity of the ssay resides in the mAb. The ELISA developed with nAb16-F12 detected higher levels of antigenic COMP n both OA and RA SFs than that which used mAb 17-C10. The significance of this finding is not clear at this time. However, it illustrates how important it is to report the results of the analyses of this molecular marker in terms of concentrations of antigenic COMP.

An important advantage of the ELISA which uses mAb 17-C10 is that, unlike the ELISA that uses mAb 16-F12, it detects with great sensitivity antigenic COMP or COMP fragments in canine SF and serum. This ELISA should thus prove useful to quantify antigenic COMP fragments in the body fluids of dogs which have undergone anterior cruciate ligament transection (ACLT). Such studies could be used, for example, to test a recent postulate, based on the observation that ACLT is accompanied by a measurable and reproducible rise in the serum level of antigenic keratan sulfate, that the injury gives rise to a state of chondrocyte hypermetabolism which, while most pronounced locally, develops systemically as well (17).

Measurement of antigenic COMP in serum has demonstrated that OA as well as RA patients with a high level of this molecular marker had a much greater chance of developing rapid joint destruction (16, 23, 24, 26). This most important finding has attracted much attention as it suggests that measurement of antigenic COMP in serum could enable clinicians to select patients most likely to develop rapid destruction of articular cartilage for the most aggressive therapeutic treatment. Based on our findings, we postulate that a sandwich ELISA that uses mAbs such as the ones characterized in this study, or others yet to be raised, could prove even more useful in identifying subsets of patients requiring aggressive intervention during the early stages of the disease. This is not an unrealistic aspiration considering our observation that many of the antigenic COMP fragments in SF differed in their reactivity with the different antibodies and that, in both OA and RA, the relative contribution of some of the fragments to the antigenicity detected varied within the population.

Studies are in progress to characterize the epitopes recognized by the mAbs and to determine the sequence of disease-specific fragments; these studies will provide information that will be critical in devising sandwich ELISAs with either diagnostic or prognostic values in the assessment of patients with joint diseases.

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Příloha 7

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Serum cartilage oligomeric matrix protein reflects the presence of clinically diagnosed synovitis in patients with knee osteoarthritis

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Summary

Objective: Cartilage oligomeric matrix protein (COMP) is a component of articular cartilage whose serum levels show a strong correlation with radiographic osteoarthritis (OA) status. It has recently been found, however, that COMP is also produced in synovium. To assess the hypothesis that synovitis affects serum COMP levels in patients with knee OA, we collected sera for COMP simultaneous with a clinical examination for synovitis.

Design: Sera were collected from OA patients who fulfilled the American College of Rheumatology criteria for knee OA. Radiographs were classified according to the grading system of Kellgren and Lawrence. Synovitis was diagnosed clinically by joint tenderness plus swelling and/or increased warmth over the joint. COMP levels in sera were measured by inhibition ELISA with monoclonal antibody (mAb) 17-C10.

Results: Serum COMP levels were significantly correlated with age, synovitis and an interaction of synovitis and OA severity. Synovitis showed the strongest effect on COMP levels (R=0.1587, P<0.01), in contrast to C-reactive protein, duration of OA and OA severity score which showed no significant effect on COMP levels. Individual signs of synovitis, namely, joint tenderness and warmth had a significant effect on serum COMP levels while swelling alone did not.

Conclusion: Synovitis exerts a significant effect on serum COMP levels measured with mAb 17-C10 in OA patients. These findings underscore the importance of the clinical joint examination to assess for synovitis, when attempting to apply objective measures, such as COMP, to the clinical setting. © 2001 OsteoArthritis Research Society International

Key words: Osteoarthritis, COMP, Serum, Synovitis.

Introduction

Cartilage oligomeric matrix protein (COMP) is a member of the thrombospondin family of extracellular calcium-binding proteins that was initially isolated from cartilage[1]. COMP is a non-collagenous glycoprotein of high molecular weight (>500 kDa), composed of five identical subunits[2]. Primary sequences of rat, mouse, and human COMP have been determined so far[2,3]. The carboxy-terminal globular domain of native COMP binds to collagens I and II[4]. A five-stranded coiled-coil domain, formed when subunits assemble their amino-terminal ends into an oligomer[5], may have a storage and delivery function for hydrophobic cell signaling molecules such as vitamin D[6]. The significance of COMP for normal development and function of cartilage has been underscored by the discovery that mutations of the COMP gene result in pseudoachondroplasia and some forms of multiple epiphyseal dysplasia[7].

Osteoarthritis (OA) is a common disease causing pain and disability in a significant proportion of the adult population[8]. It is characterized by the progressive destruction of articular cartilage and concomitant changes in subchondral bone[9]. A diagnosis of knee OA, traditionally based upon weight-bearing radiographs and pain, is usually made by the time tissue destruction is already advanced. Therefore, much attention has been focused on developing assays for cartilage derived macromolecules or their fragments whose release into the circulation from the joint may reflect disturbances in joint tissue turnover. Although many unresolved questions remain[10], such 'markers' may provide insight into the early stages of the disease[11] as well as facilitating diagnosis, prognosis and disease severity evaluation[12]. COMP has been suggested as a promising candidate molecular marker of OA[13], especially because it has been shown to be relatively specific to joint tissues.

Most previously published studies reporting serum COMP levels in OA have concentrated on its use as a potential prognostic marker; in all these studies COMP has

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been measured by enzyme linked immunosorbent assay (ELISA) with polyclonal antibodies. Two of these studies have demonstrated an association of increasing serum COMP levels with progression of OA monitored radiographically. In the study by Sharif *et al.*[14], serum COMP increased significantly in patients with clinical knee OA who showed decreased joint space or required knee surgery during a five-year follow-up period. Petersson *et al.*[15] determined serum concentrations of COMP over a 3-year period in individuals with chronic knee pain (with or without radiographic evidence of knee joint OA at study entry) and found that COMP levels increased significantly only in the individuals with radiographic OA at follow-up, while remaining unchanged in the individuals with normal radiographs at follow-up. Conrozier *et al.*[16] showed that baseline serum COMP correlated with mean joint space narrowing over 1 year in symptomatic hip OA patients. No correlation has been found in another study comparing baseline serum COMP levels to disease progression defined arthroscopically over a 1-year period[17]. In addition, serum concentrations of COMP, measured in a group of individuals with chronic knee pain, has correlated positively with the extent of bone scan abnormalities[18].

Taken together, the results of studies performed so far are promising with regard to COMP as an OA biomarker; their interpretation however, has been complicated by recent discoveries that, besides cartilage, COMP is produced by practically all tissues within a joint, namely tendon[19], ligament and meniscus[20], and synovium[21–25]. This finding has led some authors to speculate that synovitis may affect serum COMP level by influencing synovial tissue synthesis and release of COMP into the circulation[24,25].

We have developed a panel of monoclonal antibodies (mAbs) to human COMP and we used one of them, 17-C10, to develop a competitive indirect inhibition ELISA for COMP[26]. The mAb 17-C10 recognizes a conformational epitope in a domain of EGF-like repeats[27] that is located near the center of a COMP subunit, and as such it should be unaffected by partial, N- or C-terminal, cleavage of the antigen. Like polyclonal antibodies used by other authors previously, the 17-C10 mAb does not appear to distinguish between COMP produced by chondrocytes and COMP produced by synovial fibroblasts[28]. Our previous studies on sera have demonstrated that mAb 17-C10 is capable of distinguishing groups of OA-affected and unaffected (no radiographic hip or knee OA) subjects, and also subgroups of OA patients sorted by severity of OA and number of knee and hip joints involved[29]. The goal of this study was to assess the influence of synovitis, a common and important clinical sign often manifested with OA, on serum COMP levels.

Patients and methods

PATIENTS

This study was approved by the local research ethics committee. Patients seen consecutively over a 6-month period in the outpatient clinic of the Institute of Rheumatology, Prague, Czech Republic, with knee pain and who fulfilled the American College of Rheumatology criteria for knee OA[30] were recruited into the study. All eligible patients (N=196) chose to participate. Blood was collected for serological studies, and standing anteroposterior radiographs of both knees were performed. Radiographs of tibiofemoral joints were classified according to the grading system of Kellgren and Lawrence (K–L)[31]. A single radiologist (JG) scoring the radiographs was blinded to the results of the clinical exam. Patients were examined by a single rheumatologist blinded to the radiographic results, who clinically assessed three signs of knee joint inflammation, tenderness (T), non-bony swelling (S), and warmth (W), and graded each sign on a scale of 0–3 according to the method of Thompson[32] resulting in total scores ranging from 0 to 9. Tenderness was assessed by palpation along the joint-line (capsular/joint-line tenderness, which suggested capsular/intracapsular origin of pain). Swelling was assessed by clinical tests used for detection of synovial fluid presence (palpation, bulge sign). Warmth was assessed by the investigator's palm after patients accommodated to the local room temperature. Synovitis of the knee was defined as the presence of joint tenderness of at least grade 1 plus swelling and/or warmth of at least grade 1 for the same knee. The reproducibility of the determination of all three clinical signs of inflammation and of the presence of clinical synovitis overall was estimated by repeating the examination of 15 patients (30 knees) by the study rheumatologist on two consecutive days. For particular signs of knee joint inflammation, i.e., T, S, and W, the reproducibility was 83%, 90%, and 86.7%, respectively; the intra-observer reproducibility for presence of clinically diagnosed synovitis was 80%. Patients were interviewed to obtain a global patient assessment of disease severity referred to here as the ISK score or 'Index of Severity for Knee Osteoarthrosis'[33] where higher scores are indicative of more severe disease. The clinical examination and blood collection were performed concurrently on fasting patients instructed to refrain from the use of their arthritis medications on the morning of the evaluation.

IMMUNOASSAYS

Sera were obtained from blood samples collected in sterile tubes without additives, and stored at −80°C. C-reactive protein (CRP) levels were measured simultaneously by two methods: (a) by immunoturbidimetric assay kit commercially available from Boehringer Mannheim Systems and (b) by sensitive sandwich ELISA supplied as a kit (UBI MAGIWELL® CRP QUANTITATIVE AD-401) by UBI, Mountain View, CA, U.S.A. CRP levels determined by both methods were correlated (P<0.0001) when compared using the Spearman correlation coefficient (r) (not shown).

COMP was analysed by inhibition ELISA essentially as previously described[26], with several minor modifications. Briefly, plate coating was performed with coating buffer (20 mM carbonate/bicarbonate, pH 9.2, 0.002% sodium azide) containing human articular cartilage COMP at 5 μg/ml. Nunc-Immuno (MaxiSorp) plates (Nunc, Denmark) were coated with 50 μl/well, covered with plate sealing tape and kept at 4°C until used. COMP standard, purified as previously described[26] from human articular cartilage, was initially diluted to 1 μg/ml. An initial serum dilution of 1:2 was used. Standards as well as samples were further diluted in PBS-0.05% Tween 20, pH 7.3 (TPBS) in a Nunc Microwell (low-binding) plate (Nunc, Denmark). Anti-(human)COMP Mab 17-C10[26] was diluted 1:40,000 in TPBS/1% bovine serum albumin (TPBS/BSA). Primary antibody (70 μl) was added to 70 μl of the COMP standard or patient sera. The inhibition mixtures were incubated at 4°C overnight. A 100 μl aliquot of these inhibition mixtures

was transferred into washed coated wells of the Nunc-Immuno plate and incubated for 60 min at 4°C. The plates were then washed and received 100 µl/well of a solution of horse radish peroxidase-conjugated rabbit anti-mouse immunoglobulin antibody, absorbed with human immunoglobulins (Dako A/S, Denmark) diluted 1:1000 with TPBS/BSA. After incubating for 60 min at room temperature, unbound antibody was removed by washing and 100 µl of substrate (o-phenylenediamine plus H_2O_2) was placed into the wells for 30–60 min at room temperature. The production of chromophore was stopped by the addition of 50 µl of 2 M H_2SO_4. The plates were read at a wavelength of 490 nm. Results are expressed as equivalents of the standard human antigenic COMP (µg/ml). Each plate contained four identical samples of 'control' sera which were kept aliquoted and frozen at −70°C. Each day a fresh aliquot of these control samples was thawed and used on every plate to calculate intra- and interassay variance of the assay[34]. We found intra- and interassay variance of the assay to be less than 4% and less than 5%, respectively. Analyses were performed without knowledge of the clinical data.

STATISTICAL METHODS

Geometric means and their 95% confidence intervals (95% CI) were calculated. Differences between ln-transformed serum COMP levels [ln(COMP)] in groups of patients sorted according to K–L grade and synovitis were assessed by two-way ANOVA. Natural logarithm transformation of serum COMP levels was done in order to satisfy the assumptions of a Gaussian distribution of the data underlying the use of ANOVA methods. To describe dependency of ln(COMP) on age and gender and to test for an effect of other factors on ln(COMP) we used multiple linear regression. From each of the models, regression coefficients and their 95% confidence intervals were calculated. Linear regression model was also used for adjusting the geometric mean to the mean age. Scores of three clinical signs of knee joint inflammation (T+S+W) were compared to CRP levels using the Spearman correlation coefficient (r). A P-value of <0.05 was considered significant.

Results

Clinical and radiographic data were obtained at the time of sera collection on 196 patients. The patients were predominantly female with bilateral knee OA (Table I). The most severely diseased knee was used for purposes of grading, when bilateral knee OA of different grades was present. This occurred in 51 of the 156 bilateral knee OA cases and in 39 of the bilateral knee OA cases in the female subgroup. Synovitis at the time of sera collection was present in 68% of patients and was slightly more prevalent in the more severely affected OA knees (synovitis in 74% of K–L grade >2) compared with the less severely affected OA knees (synovitis in 65% of K–L grade ≤2). Although a total of 51 cases of bilateral knee OA demonstrated more severe OA in one knee compared with the other, the majority of clinically detected synovitis occurred in the more severely affected knee (44 of 51 cases or 86%).

Table II summarizes unadjusted serum COMP levels by OA severity and presence of synovitis. COMP levels were higher in the group with K–L grade >2 knee OA (1.197 µg/ml) compared with the group with less severe K–L grade ≤2 OA (1.029 µg/ml) (P=0.002 by two-way ANOVA using ln transformed COMP). COMP levels were also higher in the group with synovitis (1.127 µg/ml) compared with the group lacking synovitis (1.007 µg/ml) (P=0.038).

The effect of age, gender, and interaction of age and gender on ln(COMP) was tested by multiple regression. The multiple correlation coefficient was 0.482 (P<0.001). The effect of age on ln(COMP), when tested by means of linear regression, was different for males (correlation coefficient 0.121, NS) and females (correlation coefficient 0.524, P<0.001) (Fig. 1). Non-parallelism of both gender groups was highly significant (P<0.001). Thus, age and gender have to be considered confounding variables with regard to serum COMP levels.

Since the proportion of females recruited into the study was much higher than that of males, and since the characteristics of the subgroup of female patients were similar to the characteristics of the group as a whole (Table I), we

Table I
Characteristics of all patients with knee OA and the subgroup of female patients

Characteristic	All patients (N=196)	Female patients (N=153)
Age, mean (range) years	62 (44–76)	60 (44–76)
OA duration, mean (range) years	10 (1–50)	10 (1–40)
ISK*, mean (range)	9 (3.5–13)	9 (4–13)
Knee OA, number (%)		
Unilateral	40 (20%)	27 (18%)
Bilateral	156 (80%)	126 (82%)
Kellgren–Lawrence grade, number (%)		
I	38 (19%)	34 (22%)
II	87 (44%)	71 (46%)
III	63 (32%)	42 (27%)
IV	8 (4%)	6 (4%)
Knee synovitis, number (%)		
Total	133 (68%)	106 (69%)
Unilateral	102 (52%)	81 (53%)
Bilateral	31 (16%)	25 (16%)

*ISK = The Index of Severity for Knee OA according to Lequesne *et al.*[30].

Table II
*Serum COMP levels for all OA patients by OA severity and synovitis**

Group	N	COMP level, mean (95% CI)
Total	196	1.087 (1.038, 1.137)
K–L ≤2	125	1.029 (0.973, 1.088)
K–L >2	71	1.197 (1.112, 1.289)†
Syn (−)	63	1.007 (0.934, 1.085)
Syn (+)	133	1.127 (1.065, 1.192)‡
K–L ≤2, Syn (−)	44	0.994 (0.908, 1.089)
K–L ≤2, Syn (+)	81	1.048 (0.975, 1.125)
K–L >2, Syn (−)	19	1.036 (0.896, 1.199)
K–L >2, Syn (+)	52	1.262 (1.160, 1.372)

*Values for COMP levels are expressed in µg/ml.
Syn (−)=synovitis absent clinically; Syn (+)=synovitis present clinically.
†P<0.01 compared with K–L ≤2.
‡P<0.05 compared with Syn (−).

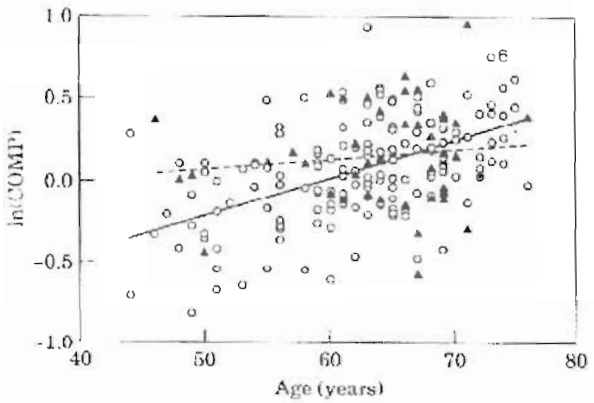

Fig. 1. An effect of age on COMP levels [expressed as ln(COMP)] measured by ELISA with mAb 17-C10 in sera of OA patients. (○)—observed values, females; (▲)—observed values; males. Best-fit regression lines were calculated independently for female (——) and male (– – –) patients using linear regression model.

chose to analyse the subgroup of female patients in more detail. Table III summarizes serum COMP levels in the subgroup of 153 female OA patients by radiographic grade knee OA and by knee synovitis. Unadjusted COMP levels were highest in the cases with more severe OA (K–L grade >2) and synovitis (P=0.001 and P=0.03, respectively). However, after adjusting for age, only the increase by synovitis remained significant (P=0.017).

The effects of synovitis, K–L grade, age, duration of the disease, ISK value, and CRP level on ln(COMP) for the subgroup of female patients was tested by multiple regression. The results are summarized in Table IV. Besides age, serum COMP was influenced by synovitis and by an interaction of synovitis and K–L grade. K–L grade alone however was not significantly correlated to ln(COMP) levels whereas synovitis alone was significant, showing the strongest effect of all the variables examined with a regression coefficient of 0.1587 (P<0.01). There was no significant effect of CRP level, ISK or disease duration on ln(COMP). The effect of three clinical signs of synovitis (T, S, and W) on ln(COMP), was tested for the subgroup of female patients using two independent models of linear regression, including age as a confounding variable. For each patient, the indices of synovitis were summed across both knees to give a total grade for T, S, and W. The simple regression model was used for sum of grades of all three signs (T+S+W); the multiple regression model was used to test for the effect of three signs separately (Table V). The linear regression model was significant for: (i) sum of total grades of all three signs (T+S+W); (ii) total grade of tenderness (T); (iii) total grade of warmth (W). The effect of the total grade of swelling (S) was not significant. The sum of grades of all three clinical signs of synovitis (T+S+W) did not correlate significantly with CRP level (P=0.079), when compared using the Spearman correlation coefficient.

Discussion

We have detected significant differences in unadjusted serum COMP levels, measured by ELISA with mAb 17-C10, between groups of patients with knee OA sorted according to radiographic grade (K–L grade ≤2 vs K–L grade >2) or according to the presence of clinically diagnosed synovitis of the knee. However, we have identified age as a confounding variable. After adjusting for age in the subgroup of female patients, only the effect of synovitis on COMP level remained statistically significant. Thus, our study demonstrates a correlation of clinically evident synovitis with serum COMP. Although the differences reported are statistically significant, they are small in terms of actual numerical difference. There is overlap between individual groups differentiated by synovitis thus serum COMP cannot be used as a discriminating test in individuals.

We used two assays to measure blood CRP level. We were concerned that the turbidimetric assay might not be sensitive enough to detect small elevations in CRP level associated with low-grade inflammation accompanying OA and detected by high-sensitivity immunoassays as was described recently[35,36]. Thus we also measured CRP levels by a very sensitive sandwich ELISA assay. Although blood CRP levels determined by the two different methods correlated highly, neither of them correlated to blood COMP levels or to clinical indicators of knee inflammation. We suggest that serum COMP probably reflects local, OA-associated inflammation localized to a joint, while CRP reflects a systemic inflammatory response, like general infection.

Using a larger sample size and the same mAb to COMP, we have previously observed higher serum COMP levels in individuals with knee OA of K–L grade ≤2 compared to a control population without knee or hip OA[29]. However, in the present study, the difference in serum COMP levels between two groups with knee OA (mild and severe) was not large enough to be statistically significant although the same trend was apparent, with higher mean serum COMP in the more severely affected group. Both studies have shown that serum COMP level increases with age, but this effect is more pronounced in females than in males. The difference between female and male subjects may relate to post-menopausally increased bone metabolism characterized by up-regulated osteoclastic and osteoblastic activity, since, according to a recent finding[37], COMP is also expressed by osteoblasts.

Although the study populations in these two studies (this and Clark *et al.*[29]) were very different, one a rural population in North Carolina, the other an urban population in middle Europe (both Caucasian), the 17-C10 ELISA measured very similar levels of serum COMP. This agreement was achieved by using the same mAb to COMP in addition to the same standard of human cartilage COMP in both studies. Although an internationally recognized COMP standard is not available, the advent of mAbs for COMP may aid in the standardization of COMP assays whose feasibility is demonstrated here. Compared to studies that measured COMP by ELISA with polyclonal antibodies[13–18], we report lower (by approximately 10-fold) levels of serum COMP. There are several possible explanations of this difference; the 17-C10 mAb may detect a narrower range of serum COMP fragments than is detected by polyclonal antibodies, and the purity of our standard may be greater, yielding overall lower COMP values.

Synovium is known to produce COMP[21–25] and in theory may contribute measurable levels of COMP to the serum. We have detected measurable amounts of COMP in hypertrophic synovium collected at the time of knee surgery from three OA patients[28]. The concentration of COMP in these synovial specimens, determined by ELISA with mAb 17-C10, ranged from 4 to 64 μg/g wet weight of tissue. Neidhart *et al.*[38] detected COMP at concentrations of

Table III
*Serum COMP levels for subgroup of female OA patients by OA severity and synovitis**

Group	*N*	COMP level, mean (95% CI)	
		Unadjusted	Adjusted for age
Total	153	1.066 (1.012, 1.123)	1.066 (1.020, 1.115)
K–L ≤2	105	1.004 (0.946, 1.065)	1.049 (0.996, 1.105)
K–L >2	48	1.217 (1.106, 1.340)‡	1.104 (1.014, 1.203)[NS]
Syn (–)	47	0.979 (0.897, 1.069)	0.985 (0.912, 1.064)
Syn (+)	106	1.107 (1.039, 1.180)†	1.105 (1.047, 1.165)†
K–L ≤2, Syn (–)	35	0.963 (0.873, 1.063)	1.019 (0.935, 1.111)
K–L ≤2, Syn (+)	70	1.025 (0.951, 1.104)	1.065 (0.997, 1.137)
K–L >2, Syn (–)	12	1.028 (0.830, 1.272)	0.891 (0.745, 1.066)
K–L >2, Syn (+)	36	1.288 (1.158, 1.433)	1.186 (1.083, 1.298)

*Values for COMP levels are expressed in μg/ml.
Syn (–)=synovitis absent clinically; Syn (+)=synovitis present clinically.
[NS]Not significant compared with K–L ≤2.
†$P<0.05$ compared with Syn (–).
‡$P<0.01$ compared with K–L ≤2.

Table IV
An effect of various factors on ln(COMP): multiple regression model for female OA patients

Factor	*b*	95% CI
Age	0.022	(0.015, 0.280)***
Synovitis	0.159	(0.051, 0.267)**
K–L grade	–0.007	(–0.123, 0.110)[NS]
Interaction of K–L grade and Synovitis	0.116	(0.011, 0.222)*
CRP	0.000	(–0.006, 0.006)[NS]
ISK	0.006	(–0.016, 0.026)[NS]
Duration of OA	0.000	(–0.007, 0.006)[NS]

Standard error of regression model=0.2688; multiple correlation coefficient r=0.5619.
Synovitis: patients were sorted into two groups, i.e., synovitis present clinically vs synovitis absent clinically (for definition of synovitis, see Methods)
K–L grade: patients were sorted into two groups, i.e., K–L grade ≤2 vs K–L grade >2.
ISK=The Index of Severity for Knee OA according to Lequesne et al.[30].
CRP=serum level of C-reactive protein.
b=Regression coefficient.
[NS]Not significant; *$P<0.05$; **$P<0.01$; ***$P<0.001$.

3.40 mg/g and 2.17 mg/g wet weight of human tibial and femoral cartilage, respectively. It can be estimated from these data that the concentration of COMP (per wet weight of tissue) in synovium is about 100 times lower then the concentration in articular cartilage. Supporting this contention, Saxne and Mansson report a similar estimation cited as an unpublished observation[15]. There is no available information at present on the turnover rate of COMP in synovial tissue and in cartilage. These turnover rates may vary by tissue type and by pathological condition. Dodge *et al.*[24] have reported that cultured human synovial cells produce more COMP (per μg DNA) than cultured articular chondrocytes maintained under conditions which promote retention of a cartilage-specific phenotype. DiCesare *et al.*[25] have found that COMP mRNA levels are three-fold higher in rheumatoid synovium compared to osteoarthritic synovium; the authors concluded from this comparison that inflammatory synovium should be considered a potential source of serum COMP. It has also been shown that a pro-inflammatory cytokine, TGF-β, can induce COMP mRNA and protein in synovial fibroblasts[23].

The clinical estimation of inflammation (synovitis) still remains an unresolved problem. Some objective methods (thermocameras, isotopes) have failed, while other modern objective methods (MRI, ultrasound) are not validated for clinical studies yet. Both synovial biopsy and arthroscopy are invasive and their routine use in studies like this are difficult to justify for ethical reasons. Therefore, the recommended tools for clinical detection of synovitis are various joint indices (tender joint count, swollen joint count). We decided to use standard measures of tenderness, swelling, and warmth, performed by a single rheumatologist, to make a clinical determination of synovitis. The reproducibility of clinically determined synovitis in this study was 80%, thus validating the clinical relevance of our serum COMP findings. The association of higher synovitis scores with higher COMP levels might strengthen the evidence of an association of COMP levels with synovitis. However, the associations of the three clinical signs of synovitis with serum COMP levels were not the same. The association with tenderness was the most pronounced while the association with swelling was not significant (Table V). We concluded that we could not divide patients further into subgroups based upon various levels of the total score for further statistical analysis in a sample of this size.

Episodic inflammation of affected joints is frequent in OA patients and synovitis is common in patients with advanced OA. Synovitis, defined as mononuclear cell infiltration on synovial tissue biopsy, was found in 50% of patients with early knee OA of K–L grade <3 by Myers *et al.*[39], while only 38% of all their patients (i.e., 76% of patients with histologically diagnosed synovitis) would have been simultaneously diagnosed clinically by our criteria (pain and effusion and/or warmth), indicating that we have probably missed some cases of inflammation, presenting with less striking clinical symptoms.

Our results suggest that inflamed synovium may either (1) produce COMP in amounts significant enough to be detected in serum by immunoassay with mAb 17-C10 or (2) accelerate cartilage loss of antigenic fragments of COMP, or (3) affect joint clearance of cartilage-derived COMP. All these mechanisms may operate simultaneously and all of them may be partially responsible for the serum COMP level increase. Our results do not necessarily suggest extra-cartilage origin of blood COMP, as it has been shown

Table V
An effect of three clinical signs of knee joint inflammation, tenderness (T), non-bony swelling (S), and warmth (W), on ln(COMP); regression models for female OA patients†

Clinical sign	*b*	95% CI	*b*	95% CI
T+S+W	0.032	(0.012, 0.051)**		
T			0.053	(0.017, 0.089)**
S			−0.011	(−0.540, 0.032)^{NS}
W			0.077	(0.017, 0.137)*

†The table describes two regression models, i.e., the simple regression model (T+S+W) used for the sum of total grades of all three signs of synovitis, and the multiple regression model (T, S, W) used to test for the effect of three signs of synovitis separately. Standard errors of regression models T+S+W and T, S, W equal to 0.269 and 0.267, respectively; correlation coefficient r(T+S+W) equals to 0.567; multiple correlation coefficient r(T, S, W) equals to 0.586.
b=Regression coefficient.
^{NS}Not significant; *$P<0.05$; **$P<0.01$.

that even mild synovitis may significantly increase the clearance of a protein from the synovial fluid[10,11]. On the other hand, COMP produced in synovium may enter the bloodstream directly, and thus may influence serum level more than COMP produced in cartilage, that has to enter the bloodstream via the synovial fluid and may therefore be subject to greater lymphatic uptake. Thus, although the actual mechanism causing the observed effect is unclear, synovitis should be considered a factor that affects serum COMP levels.

In summary, in this study we have identified another factor, besides age and OA severity, that influences the level of serum COMP in patients with knee OA, namely synovitis of the knee. This finding invites consideration of the role that synovitis may play in OA progression in light of studies showing that higher levels of serum COMP indicate patients with faster progressing OA. These results underscore the importance of assessing for synovitis by clinical joint examination in future studies of OA and COMP.

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Příloha 8

Vilím, V.; Vobůrka, Z.; Vytášek, R.; Senolt, L.; Tchetverikov, I.; Kraus, V. B.; Pavelka, K.: Monoclonal antibodies to human cartilage oligomeric matrix protein: epitope mapping and characterization of sandwich ELISA. Clin Chim Acta 328(2003) 59-69

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Monoclonal antibodies to human cartilage oligomeric matrix protein: epitope mapping and characterization of sandwich ELISA

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Abstract

Background: Cartilage oligomeric matrix protein/thrombospondin 5 (COMP/TSP 5) is one of the most promising serologic markers with regard to an ability to prognose development of osteoarthritis (OA). Our aim was to map the epitopes of three monoclonal antibodies (mAb) to COMP and to develop and characterize a sandwich enzyme-linked immunosorbent assay (ELISA) for measuring COMP levels in human body fluids. *Methods*: COMP was digested with trypsin and the NH_2-terminal sequence of the fragments recognized by each of the mAbs was determined. Steric competition among the mAbs was tested with an antibody capture assay. A sandwich ELISA was developed using unlabeled mAb 16-F12 as a capture antibody, and mAb 17-C10 labeled with biotin as the second antibody. *Results*: Epitopes of the three mAbs were mapped to three different domains within the COMP subunit (16-F12, NH_2-terminal domain; 17-C10, EGF-like domain; 12-C4, COOH-terminal domain). These epitopes did not overlap. mAbs 17-C10 and 12-C4 yielded similar serum COMP results when used as the secondary antibodies. Serum COMP levels measured with the new sandwich ELISA using mAbs 16-F12 and 17-C10 correlated strongly with results based on an inhibition ELISA with mAb 17-C10 alone ($r^2 = 0.836$; $P < 0.0001$). We characterized the new sandwich ELISA with regards to inter- and intra-assay variability, the range of COMP levels that can be expected in human synovial fluids (SF) and sera (controls and OA and rheumatoid arthritis (RA) patients), and the day-to-day and diurnal variability of COMP levels in sera. *Conclusions*: We have developed and characterized a sandwich

Abbreviations: ANOVA, analysis of variance; COMP, cartilage oligomeric matrix protein; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; OA, osteoarthritis; RA, rheumatoid arthritis; SF, synovial fluid.

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ELISA for COMP that is sensitive and yields highly reproducible COMP results upon analysis of human sera and synovial fluids.

Keywords: COMP; Thrombospondin 5; Monoclonal antibodies; Epitope mapping; Sandwich ELISA

1. Introduction

Cartilage oligomeric matrix protein (COMP), also designated thrombospondin 5 (TSP 5) [1], is a member of the thrombospondin family of extracellular proteins. COMP is a calcium-binding protein [2] of high molecular weight (>500 kDa) composed of five identical subunits [3]. The carboxy-terminal globular domain of native COMP binds to collagens I, II, and IX [4–6]. A five-stranded coiled-coil domain, formed when the subunits assemble their amino-terminal ends into an oligomer [7], may have a storage and delivery function for hydrophobic cell-signaling molecules such as vitamin D [8]. The primary sequences of rat, mouse, and human COMP have been determined so far [3,9,10]. Mutations in the domain of type 3 calcium-binding repeats or in the carboxy-terminal globular domain cause two types of skeletal dysplasias, pseudoachondroplasia and some forms of multiple epiphyseal dysplasia [11].

COMP is one of the most abundantly expressed genes in both normal and osteoarthritic human cartilage [12]. COMP is also a putative marker of cartilage catabolism [13] and results of several prospective studies reporting serum COMP levels in patients with osteoarthritis (OA) have been promising with regard to the ability to characterize disease severity and to prognose disease development [14–16]. Our previous studies on sera have demonstrated that an inhibition enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody (mAb) 17-C10 to COMP is capable of distinguishing groups of OA-affected and unaffected (no radiographic hip or knee OA) subjects, and also subgroups of OA patients sorted by the severity of OA and number of knee and hip joints involved [17]. The goal of this study was to map the epitopes of three mAbs raised against human COMP, to use them to develop a sandwich ELISA, and to test the feasibility of using this new assay system for measuring COMP levels in human body fluids. In a study simultaneous with this, we measured serum COMP levels in OA patients followed for 3 years and we have observed promising results with regard to the ability of the new sandwich ELISA to identify patients at high risk for progression [18].

2. Materials and methods

2.1. Production, purification, and labeling of mAbs

Three hybridomas producing anti-(human)COMP monoclonal antibodies, 16-F12, 17-C10, and 12-C4 [19], were grown in the oil-irritated intraperitoneal cavities of Balb/c mice. mAbs were isolated from ascitic fluids on a column of immobilized Protein G, concentrated on Centricon YM-100 filters (Amicon), and labeled by LC-biotin using EZ-Link™ NHS-LC-Biotin (Pierce) to give a molar ratio IgG/biotin of approximately 1:50.

2.2. Epitope mapping

A preparation of purified COMP was digested by TPCK-trypsin (Sigma), at an enzyme/substrate ratio 1:100, at calcium concentrations ranging from 0 to 100 mmol/l Ca^{2+}, at three different temperatures (0, 20, and 37 °C), and for varying time intervals ranging from 5 min to overnight. Based on pilot experiments, we chose three final sets of digesting conditions—Digest I: 0 mmol/l Ca^{2+}, 0 °C, 30 min; Digest II: 12.5 mmol/l Ca^{2+}, 37 °C, 6 h; Digest III: 12.5 mmol/l Ca^{2+}, 0 °C, 30 min. COMP fragments were separated by SDS/PAGE in 10% gels and transferred onto PVDF-membrane (Immobilon-P, Millipore) in 10 mmol/l CAPS, 10% methanol, pH 11.0, at 50 mA, 4 °C, overnight. Blots were either stained with Coomassie Blue or blocked with 5% low-fat milk and immunostained with the mAbs. Selected bands were cut out of Coomassie Blue-stained blots and sequenced by Edman degradation that was performed

in the protein sequencer (Procise Protein Sequencing System, PE Applied Biosystems) according to the standard program (PL PVDF Protein). NH_2-terminal pyroglutamic acid was removed by treating the blot, blocked with polyvinylpyrrolidone, with pyroglutamate aminopeptidase (Calbiochem) [20].

2.3. Antibody competition experiments

The immunoassay used to test for potential steric competition of mAbs 16-F12, 17-C10, and 12-C4 was set up in the format of an antibody capture assay. Briefly, Nunc-Immuno (MaxiSorp) plates were coated with COMP at 2 μg/ml and blocked with 3% BSA for 2 h before use. Sequential dilutions of non-labeled mAbs (10,000 ×, 20,000 ×, 40,000 ×, 80,000 ×, 160,000 ×, and 320,000 ×), from a stock of approximately 4 mg/ml, were prepared on a low-binding Nunc MicroWell plate and then transferred to the coated and blocked MaxiSorp plate, 50 μl/well. Plates were incubated for 2 h on an orbital shaker at room temperature. The same non-labeled mAbs, at the same volume and sequential dilutions as above, were added in combination with a biotinylated mAb (diluted 5000 ×) without removing the previous contents of the wells. The final reaction mixture contained biotinylated mAb diluted 10,000 × and one of the six sequential dilutions of non-labeled mAbs (from 10,000 × to 320,000 ×) in a final volume of 100 μl. The mixture was incubated for 2 h on the orbital shaker at room temperature. Plates were washed and incubated for 30 min with streptavidin-biotinylated horseradish peroxidase complex (Amersham Pharmacia Biotech), and a substrate for peroxidase (*o*-phenylenediamine plus H_2O_2) was added. The color that developed after 30 min was read at a wavelength of 492 nm.

2.4. COMP ELISA

2.4.1. Competitive inhibition ELISA with mAb 17-C10

This was done essentially as described [19,21] with COMP standard concentrations ranging from 400 to 12.5 ng/ml and serum samples measured simultaneously at three dilutions (10 ×, 20 ×, and 40 ×).

2.4.2. Sandwich ELISA

This was done exactly as described [18] with COMP standard concentrations ranging from 400 to 12.5 ng/ml, serum samples diluted 20 ×, and synovial fluid (SF) samples diluted 200 ×.

2.5. Standard COMP preparation

The preparation of standard COMP used in ELISA was purified from macroscopically normal human hip cartilage according to a protocol that has been described elsewhere [19]. By SDS/PAGE gel under non-reducing and reducing conditions, the preparation represented high molecular weight oligomeric COMP and did not contain low molecular-weight fragments or non-oligomerized subunits (not shown).

2.6. Human body fluid samples

To characterize the sandwich ELISA, we measured COMP levels in four sets of samples. Set I: 93 samples total—sera samples were obtained from 46 OA and 47 rheumatoid arthritis (RA) patients based upon the diagnosis of record on an inpatient admission; these samples were used to characterize the new assay. Set II: 20 pairs of matched synovial fluid and sera samples were obtained from 10 OA and 10 RA patients at the time of an outpatient visit for knee joint pain and swelling necessitating therapeutic arthrocentesis; control serum samples were obtained from 15 volunteers lacking obvious joint pathology; and control SF samples were aspirated post-mortem from nine individuals lacking obvious joint pathology; these samples were used to assess COMP levels in OA, RA, and control sera and synovial fluids. Set III: serial serum samples from five volunteers lacking obvious joint pathology were collected on days 1, 2, 4, 8, 16, and 32; these samples were used to assess day-to-day variability of serum COMP levels. Set IV: a pair of serum samples was collected from 20 inpatient volunteers with various diseases who agreed to donate two blood samples during a single day. The first sample was obtained fasting in the morning after the patients arose from bed but before breakfast, while the second sample was obtained approximately 2 h after lunch; these samples were used to assess diurnal variability of serum COMP levels. Collection and analysis of all four sets of samples were approved by local research ethic committees.

2.7. Statistical methods

Correlation between two continuous variables (COMP concentrations measured with the two assays) was assessed by linear regression analysis. Differences in mean COMP levels among groups of subjects were compared using analysis of variance (one-way ANOVA), and differences between particular groups were compared using the Bonferroni multiple comparisons post hoc test. P-value (two-tailed) of <0.05 was considered significant. The intra- and inter-assay variability of the ELISA was calculated as described [22].

3. Results

3.1. Epitope mapping by NH_2-terminal sequencing of tryptic fragments

Native human COMP was digested with TPCK-trypsin. We tested various digestion conditions (varying concentrations of Ca^{2+}, time, and temperature) in several pilot experiments to find conditions that would generate COMP fragments with unique immunoreactivity to the individual mAbs. We found that in the presence of calcium, the digestion of COMP with trypsin proceeded for several hours. In contrast, in the absence of calcium, antigenic fragments recognized uniquely by mAbs 16-F12 and 17-C10 were generated within 15 min, and antigenicity of COMP to mAb 12-C4 was completely destroyed within the first 5 min of the digestion (Vilim, unpublished results). Selected antigenic fragments generated during particular digests were cut out of Coomassie Blue-stained Western blots and identified by NH_2-terminal amino acid sequencing (Fig. 1).

Regardless of temperature, time, and the presence or absence of calcium, digests run under non-reducing conditions always contained a fragment with M_r of approximately 30 kDa which disappeared in gels run under reducing conditions (Fig. 1, gels 1 and 2, band 1). This fragment was recognized exclusively by mAb 16-F12 and was resistant to sequencing. The mature secreted subunit of COMP starts with Gln^{21}, after a signal peptide of 20 amino acids is cleaved off [10]; the NH_2-terminal glutamine can be blocked by forming pyroglutamic acid. Indeed, when Western blots of the 30-kDa fragment were deblocked by treatment with pyroglutamate peptidase, NH_2-terminal amino acid sequence was obtainable yielding the sequence GQSPLGSD matching the NH_2-end of the COMP

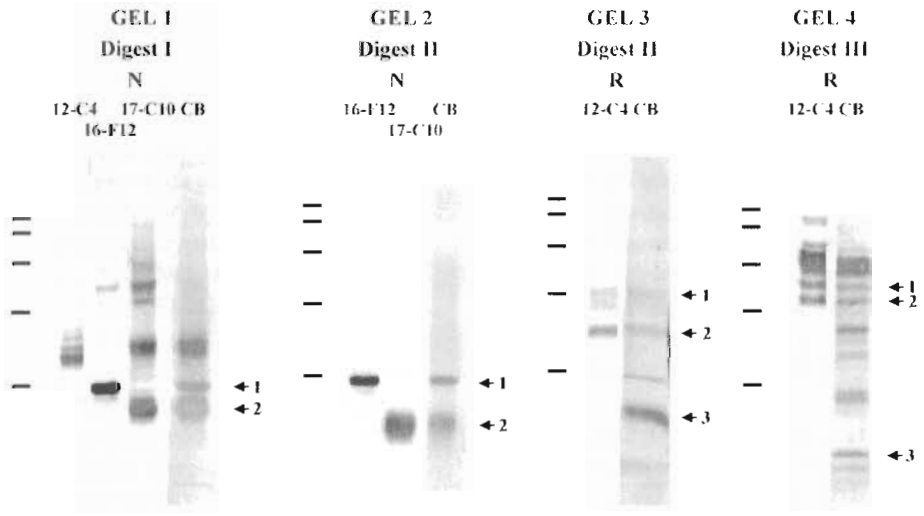

Fig. 1. Epitope mapping. Western blots of tryptic digests of COMP were stained with Coomassie Blue (CB) or immunostained with a particular mAb to COMP as indicated. Conditions of the digests were as follows: Digest I, 0 mmol/l Ca^{2+}, 0 °C, 30 min; Digest II, 12.5 mmol/l Ca^{2+}, 37 °C, 6 h; Digest III, 12.5 mmol/l Ca^{2+}, 0 °C, 30 min. SDS/PAGE in 10% gels was run under non-reducing (N) or reducing (R) conditions as indicated. Positions of molecular mass markers (116,000, 97,400, 66,000, 45,000, and 29,000) are indicated with bars on the left site of each panel. Fragments that were identified by sequencing their NH_2-end are indicated with arrows on the right site of each panel.

subunit starting with Gly^{22}. The subsequent trypsin-generated fragment, starting with Thr^{80}, was not recognized by mAb 16-F12. This localized the epitope for the mAb 16-F12 to the pentameric helical NH_2-terminal domain of COMP and also explained the disappearance of the 30-kDa band after reduction by disintegration of the pentamer in five much smaller fragments of subunits. Immunoprecipitation of COMP by mAb 16-F12 is almost completely inhibited by treatment of COMP with reagents reactive towards amino and sulfhydryl groups (DSP and NEM), suggesting that Lys^{63} and Cys^{69} and/or Cys^{72} may form part of the epitope (M. Briggs and E. Canty, personal communication).

The smallest fragment recognized exclusively by mAb 17-C10 on non-reduced Western blots had a M_r of approximately 22–26 kDa (Fig. 1, gels 1 and 2, band 2). Its NH_2-terminal amino acid sequence (TGLPSVRPLL) started with Thr^{80}. This particular fragment was rather broad after SDS/PAGE, possibly due to glycosylation, since two of three putative *N*-glycosylation sites exist within this region of the COMP subunit [23]. A COMP fragment of similar M_r and the same NH_2-terminal amino acid sequence was present on reduced Western blots (Fig. 1, gel 3, band 3); however, recognition by mAb 17-C10 was lost upon reduction of the antigen, since mAb 17-C10 binds to a conformational epitope [19]. The subsequent trypsin-generated fragment starting with Ala^{225} was not recognized by mAb 17-C10. This localized the epitope for the mAb 17-C10 to the EGF-like domain of the COMP subunit between Thr^{80} and Ala^{225}.

Two fragments of approximately 36 and 45 kDa were recognized exclusively by mAb 12-C4 on reduced blots of extensive trypsin digests (Fig. 1, gel 3, bands 1 and 2). Two NH_2-terminal sequences RAQRFXPDGSP and AQRFXPDGSP were present in both bands. The cleavage site contains two arginines in tandem at positions 223 and 224 and trypsin can obviously cleave behind each one of them. Even the smaller of these two fragments spanned the whole domain of calcium-binding type 3 repeats (Gly^{267}–Ala^{524}) and extended into the first third of the COOH-terminal globular domain, approximately up to amino acid 620. Two more fragments of higher M_r (Fig. 1, gel 4, bands 1 and 2) were recognized by mAb 12-C4; both of them started with the NH_2-terminal amino acid sequence TGLPSVRP, i.e. they were both generated by trypsin cleavage between Arg^{79} and Thr^{80}. The smaller of these two fragments (band 2, ~ 48 kDa) extended approximately up to amino acid 540. The most COOH-terminally located fragment that we identified in trypsin digests started with Ala^{629} (AVAEP, Fig. 1, gel 4, band 3). This fragment, representing the second half of the COOH-terminal globular domain, was not recognized by mAb 12-C4. Thus, it could be deduced that the epitope for mAb 12-C4 was localized to the region between Asp^{290} (an approximate end of the 22-kDa fragment recognized exclusively by mAb 17-C10) and Ala^{629}. All four of the large fragments recognized by mAb 12-C4 spanned the whole calcium-binding domain of type 3 repeats and extended into the first third of the COOH-terminal globular domain. However, 12-C4 was the only mAb that recognized a recombinant COOH-terminal domain of human COMP (Glu^{525}–Ala^{757}) (M. Briggs and P. Holden, personal communication). Considering all these results together, the epitope for the mAb 12-C4 can be localized to the beginning of the COOH-terminal globular domain.

A schematic diagram summarizing the results of epitope mapping is shown in Fig. 2.

3.2. Steric competition experiments

The steric competition experiments were set up in the format of an antibody capture assay, using one unlabeled mAb and a second biotinylated mAb, with streptavidin-horseradish peroxidase conjugate for detection. The combination of an unlabeled mAb with the same mAb labeled with biotin was used as control; for all three mAbs, the level of this "self-competition" was 100%. There was no competition evident among the various pairwise combinations of different mAbs, indicating that their epitopes did not overlap (Fig. 3).

3.3. Sandwich ELISA for COMP

We elected to use the mAb 16-F12 as the first (capture) mAb in the sandwich ELISA because its epitope was mapped to the NH_2-terminal helical domain that is presumably resistant to proteolytic cleavage. To test mAbs 12-C4 or 17-C10 as the second mAb in the assay, a collection of clinical samples was measured using both combinations of mAbs, i.e. either unlabeled 16-F12 × biotinylated 12-C4 or unlabeled

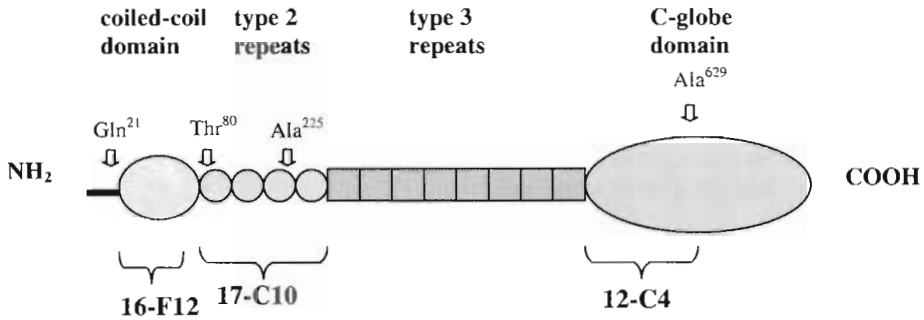

Fig. 2. Domain structure of the subunit of COMP. The deduced locations of the epitopes for mAbs 16-F12, 17-C10, and 12-C4 are indicated together with positions of amino acids that represent the first amino acids of the NH_2-ends of identified tryptic fragments (Gln^{21}, Thr^{80}, Ala^{225}, and Ala^{629}). The first 20 amino acids represent the signal sequence. The domain of type 2 repeats is also designated as EGF-like domain and the domain of type 3 repeats is also designated as calmodulin-like or calcium-binding domain.

16-F12 × biotinylated 17-C10, and the results were compared using linear regression (Fig. 4). The biotinylated second mAbs were interchangeable ($r^2 = 0.811$; $P < 0.0001$). Thus, in all the subsequent analyses described below, COMP was measured with mAb 17-C10 as the second mAb in the assay.

We were also interested in knowing if our previous results obtained with a competitive inhibition ELISA using a single mAb 17-C10 [17,21] were comparable to results obtained with the newly developed sandwich ELISA. Fig. 5 depicts COMP levels in 93 samples from our collection of OA and RA sera measured with both methods. Standards ranged from 400 to 12.5 ng/ml and serum samples in both ELISA formats were diluted similarly (10 ×, 20 ×, and 40 ×). Although the absolute values of the serum levels were shifted with higher values yielded by the sandwich ELISA, the results of the two assay methods strongly correlated when compared by means of linear regression ($r^2 = 0.836$; $P < 0.0001$). Thus, conclusions resulting from our studies comparing serum COMP levels in patient cohorts should be similar with both ELISA formats.

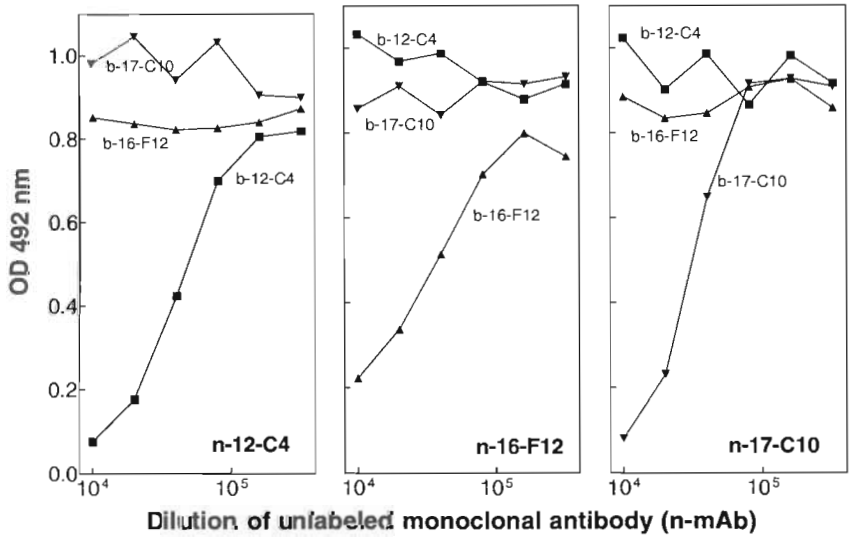

Fig. 3. Competition experiments. Each panel shows the results of competition for binding to COMP-coated plates between an unlabeled mAb (n-mAb, as indicated) and each of the three biotinylated mAbs (b-mAb, as indicated). Unlabeled mAbs were tested at six different dilutions (10,000 ×, 20,000 ×, 40,000 ×, 80,000 ×, 160,000 ×, and 320,000 ×), biotinylated mAbs were always added at an identical dilution (10,000 ×).

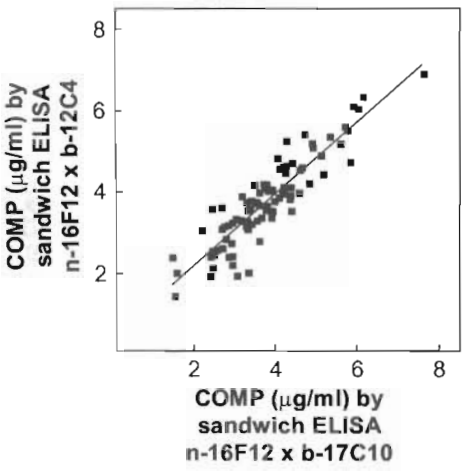

Fig. 4. Linear regression comparing COMP levels (µg/ml) measured in 93 samples of sera of OA and RA patients by sandwich ELISA with the mAb 16-F12 as the unlabeled capture mAb (n-mAb) and either mAb 17-C10 or mAb 12-C4 as the biotinylated second mAb (b-mAb). Regression statistics: slope = 0.8845 ± 0.04475; *Y*-intercept = 0.4359 ± 0.1754; *X*-intercept = − 0.4928; $r^2 = 0.811$; Sy.x = 0.4608; $F = 390.7$; $P < 0.0001$.

3.4. Intra- and inter-assay variability

We prepared a large volume of "control" sera which were stored in aliquots at − 70 °C. Each day, a fresh aliquot was thawed and six repeats were measured on one plate for 20 consecutive working days (20 plates). Mean COMP concentration determined by these measurements was 1.19 µg/ml; the values were further used to calculate intra- and inter-assay variability and these were found to be 6.3% and 7.5%, respectively.

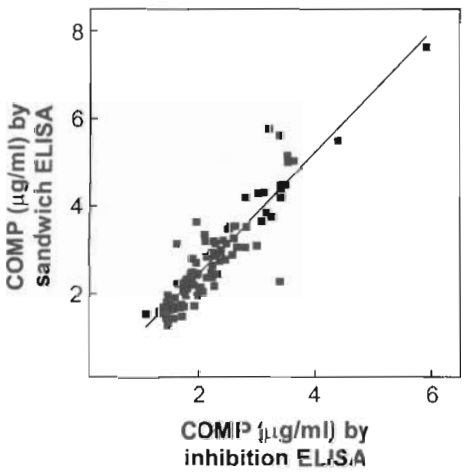

Fig. 5. Linear regression comparing COMP levels (µg/ml) measured in 93 samples of sera of OA and RA patients by sandwich ELISA (mAbs 16-F12 and b-17-C10) with levels measured by inhibition ELISA (mAb 17-C10). Regression statistics: slope = 1.374 ± 0.06380; *Y*-intercept = − 0.2504 ± 0.1544; *X*-intercept = 0.1823; $r^2 = 0.836$; Sy.x = 0.4755; $F = 463.5$; $P < 0.0001$.

3.5. COMP levels in SFs and sera

COMP levels in SFs of control subjects and patients with OA or RA are shown in Fig. 6A. Mean COMP levels in these groups differed significantly (ANOVA, $P < 0.0001$). Control subjects had higher mean COMP levels than OA patients ($P < 0.05$) or

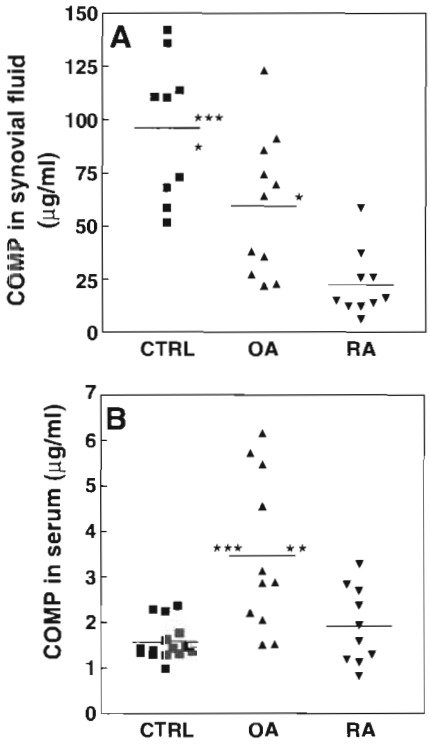

Fig. 6. (A) Synovial fluid concentration of COMP in control subjects (CTRL, $n = 8$) and patients with osteoarthritis (OA, $n = 11$) and rheumatoid arthritis (RA, $n = 10$). The horizontal bars indicate the mean of each group. ***(CTRL) $P < 0.001$ compared with RA; *(CTRL) $P < 0.05$ compared with OA; *(OA) $P < 0.05$ compared with RA. (B) Serum concentrations of COMP in control subjects (CTRL, $n = 15$) and patients with osteoarthritis (OA, $n = 11$) and rheumatoid arthritis (RA, $n = 10$). The horizontal bars indicate the mean of each group. ***(OA) $P < 0.001$ compared with CTRL; **(OA) $P < 0.01$ compared with RA.

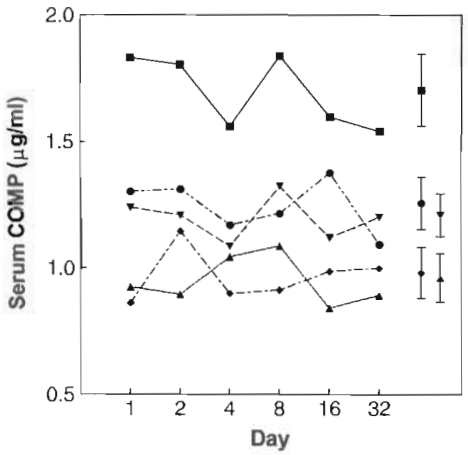

Fig. 7. Serum COMP levels in five volunteers sampled on days 1, 2, 4, 8, 16, and 32. The means ± S.D. of levels in these six consecutive collections are shown on the right.

RA patients ($P<0.001$), and OA patients had higher mean COMP levels than RA patients ($P<0.05$) (Bonferroni multiple comparisons post hoc test).

COMP levels in sera of control subjects and patients with OA or RA are shown in Fig. 6B. Mean COMP levels in these groups differed significantly (ANOVA, $P<0.001$). OA patients had higher mean COMP levels than control subjects ($P<0.001$) or RA patients ($P<0.01$); mean COMP levels in sera of control subjects and RA patients did not differ (Bonferroni multiple comparisons post hoc test).

3.6. Day-to-day and diurnal variability of COMP levels in sera

Blood of five volunteers with no obvious joint pathology was collected on 6 consecutive days within 1 month (days 1, 2, 4, 8, 16, and 32). Sera were stored frozen at −70 °C until the last sample was collected then the whole set was assayed simultaneously (Fig. 7). The subjects displayed little variation in COMP level during the month of sampling. This is the first experimental confirmation that serum COMP levels can be compared with radiographs or clinical indices obtained within a month of blood collection. The blood from four of these volunteers was collected into two tubes on the last day of sampling: one was used to prepare serum, the other, a heparin-coated tube, was used to prepare plasma. When compared, COMP levels measured in serum and plasma did not significantly differ (not shown).

On another occasion, blood of 20 volunteer hospital patients (5 OA, 8 RA, and 7 patients with various

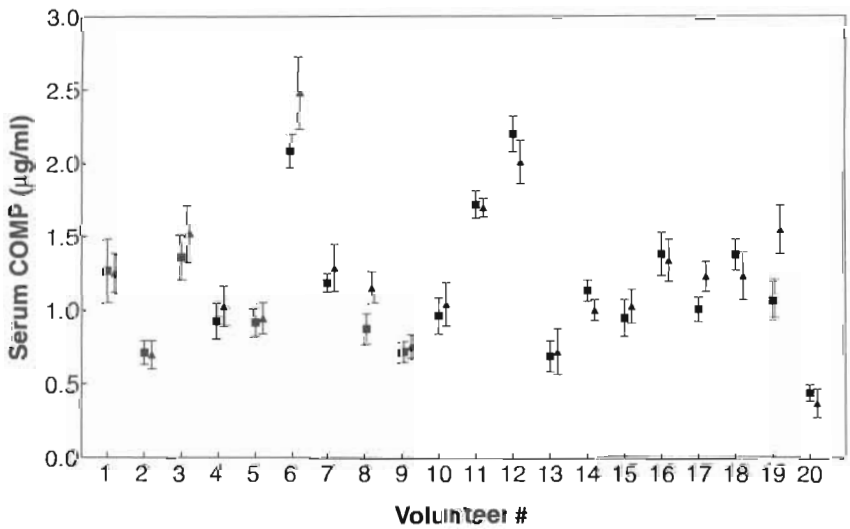

Fig. 8. Serum COMP levels in 20 volunteers sampled twice in a single day. Fasting serum (■) was collected in the morning and the second serum sample was collected approximately 2 h after lunch (▲). Each sample was assayed six times; the points and error bars represent means ± S.D. of these six measurements. Volunteers 1–5 represent patients with OA, volunteers 6–13 represent patients with RA, and volunteers 14–20 represent patients with various diagnoses.

diagnoses) was collected twice within 1 day. The first sampling was done in the morning (AM fasting blood), the second sampling was done in the afternoon (PM), approximately 2 h after lunch. COMP levels in these morning and afternoon sera were compared (Fig. 8). The AM and PM levels did not differ in 16 patients, but the PM levels were significantly higher in 4 patients (20%). Whether this increase is related to food intake, physical activity, or to some other factor is currently open to speculation. However, in light of this diurnal variability, we recommend using fasting sera collected in the morning for COMP measurements in clinical studies.

4. Discussion

We mapped the epitopes of three monoclonal antibodies to three different domains of the COMP subunit. In the course of pilot experiments, we observed an increased sensitivity of calcium-depleted COMP to digestion with trypsin. This is in agreement with recently published results of Chen et al. [2] who have suggested that in the presence of calcium, COMP folds into a more compact conformation with potential cleavage sites protected from a proteolytic attack.

We can only speculate upon the observed shift of absolute values of COMP concentrations connected with the transition from the inhibition to the sandwich ELISA, with consistently higher levels measured with the sandwich ELISA. A shift in the opposite direction would not be surprising as it would indicate that a portion of measured antigen represents fragments possessing the epitope for only one mAb and thus escaping detection by the sandwich assay. It is possible that non-specific low-level interaction of COMP with some serum component would prevent it from binding the mAb 17-C10, thus increasing the level of free antibody in an inhibition mixture and lowering apparent COMP levels measured by the inhibition ELISA relative to the sandwich ELISA. Others have also suggested the existence of such interactions [24]. The effect may be prevented in the sandwich ELISA because of higher avidity of COMP to mAb in this assay format, as complexes of antigen and antibody formed on a solid support are generally more stable than complexes formed, as in the inhibition ELISA, in a free solution. This assumption is further supported by the fact that in the inhibition ELISA, the titration curve observed for serum samples often does not exactly parallel the titration curve of the COMP standard, whereas, in the sandwich ELISA, subsequent dilutions of a serum sample always yield a curve that aligns exactly with the standard curve (not shown). We used a high molecular weight oligomeric COMP, isolated from normal articular cartilage, as a calibrator; the nature (e.g. extent of fragmentation) of the antigenic COMP in human sera is currently unknown.

The remarkable interchangeability of the sandwich ELISA using either mAb 12-C4 or mAb 17-C10 as the secondary antibodies indicates that antigenic COMP in human sera, as detected by this assay, is almost exclusively intact molecules containing both the NH_2-terminus (binding site for the capture mAb 16-F12) and the COOH-terminus (binding site for the detection mAb 12-C4). Hypothetical fragments containing intact NH_2-terminus and lacking the COOH-terminus (which would escape detection by the sandwich that uses mAb 12-C4 as the secondary antibody) are present, if at all, at a very low level within a range of the variability of the assay. The presence of hypothetical fragments lacking the oligomerizing NH_2-terminal domain, however, cannot be excluded.

The difference that we found in SF as well as serum COMP levels between OA and RA patients reached statistical significance even when the groups of patients that we compared were very small. This finding is quite encouraging, as it suggests that the assay might be able to distinguish between these two diagnoses even at the level of individual patients.

The highest COMP level in control SFs shown in Fig. 6 may appear somewhat surprising but one should bear in mind that the total volume of SF in normal subjects may be an order of magnitude lower than the SF volume present in the painful and swollen knees of RA and OA patients requiring therapeutic arthrocentesis. As we do not know the total volume of SF from which our OA and RA samples originated, we cannot calculate the total absolute quantity of COMP in normal and pathological SFs. We suppose, however, that for such measure, the total content of COMP might be lowest for SFs of control subjects. COMP levels in sera did not

correlate with SF levels in OA or in RA patients (not shown); the correlation might, hypothetically, have improved if correction of SF levels for total SF volume were possible.

In summary, we have developed and characterized a sandwich ELISA for COMP that permits sensitive and reproducible analysis of human sera and SFs. We anticipate that this assay will be a useful tool for clinical studies and will further help the understanding of joint tissue metabolism.

Acknowledgements

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