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Molekuly „DASH systému“ v lokálních a systémových patogenetických procesech revmatoidní artritidy

"DASH molecules " in local and systemic pathogenetic processes of rheumatoid arthritis

Disertační práce

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Abstrakt

Biologická funkce řady lokálních mediátorů účastnících se na patogenezi revmatoidní artritidy (RA) je kontrolována proteolytickým odštěpením dvou aminoterminálních aminokyselin prostřednictvím dipeptidylpeptidázy-IV (DPP-IV) a molekul vykazujících DPP-IV-podobnou enzymovou aktivitu ("Dipeptidylpeptidáze-IV Aktivitou a/nebo Strukturou Homologní molekuly- DASH).

Cílem této disertační práce bylo identifikovat spektrum DASH molekul podílejících se na celkové DPP-IV-podobné enzymové aktivitě u nemocných s RA v periferní krvi a synoviální tekutině a srovnat ho s kontrolní skupinou pacientů trpících osteoartrózou (OA) a dále popsat vztah těchto molekul k aktivitě onemocnění u RA.

Prokázali jsme, že dominantním nositelem DPP-IV-podobné enzymové aktivity v plazmě a synoviální tekutině u pacientů s RA je kanonická DPP-IV. DPP-IV-podobná aktivita a kanonická DPP-IV byla dále detekována na povrchu mononukleárních buněk v krvi i synoviální tekutině

U pacientů s RA jsme pozorovali sníženou DPP-IV-podobnou enzymovou aktivitu i expresi DPP-IV na mononukleárních buňkách synoviální tekutiny. U těchto pacientů navíc zastoupení DPP-IV+ T lymfocytů v synoviální tekutině negativně korelovalo s koncentrací prozánětlivého mediátoru SDF (stromal cell-derived factor-1α), který je substrátem DPP-IV. V krevní plazmě jsme u pacientů s RA prokázali významně nižší aktivitu a koncentraci DPP-IV ve srovnání s OA a negativní korelaci mezi koncentrací C reaktivního proteinu (CRP) a enzymovou aktivitou DPP-IV. Nepozorovali jsme rozdíl v DPP-IV-podobné enzymové aktivitě ani expresi na krevních mononukleárních buňkách (BMNC) mezi pacienty s RA a OA. Při intraindividuálním porovnání došlo u pacientů s poklesem aktivity RA k poklesu DPP-IV na BMNC a současně nárůstu plazmatické DPP-IV.

Tyto výsledky svědčí pro možný vztah DPP-IV k aktivitě RA a možnou využitelnost tohoto enzymu při monitoringu léčby. Pozorovaná snížená dostupnost DPP-IV na mononukleárních buňkách v synoviální tekutině navíc naznačuje její možnou přímou patogenetickou roli na lokální úrovni prostřednictvím omezení degradace SDF a tím zvýšení jeho prozánětlivého působení.

Abstract

The biological half-life of several pro-inflammatory mediators involved in the pathogenesis of rheumatoid arthritis (RA) is controlled by molecules exhibiting dipeptidyl peptidase-IV (DPP-IV)-like enzymatic activity (Dipeptidyl peptidase-IV activity and/or structure homologues- DASH).

The aim of this thesis was to identify the molecular source of the DPP-IV-like enzymatic activity in the peripheral blood and synovial fluid in patients with rheumatoid arthritis as compared to control patients with osteoarthritis (OA), and to evaluate the association of DPP-IV with the disease activity.

We found that the main source of the DPP-IV-like enzyme activity in the plasma and in the synovial fluid in patients with RA is the canonical DPP-IV. DPP-IV-like enzymatic activity and canonical DPP-IV were also detected on the cell surface of blood and synovial fluid mononuclear cells.

Significantly lower DPP-IV-like enzymatic activity and DPP-IV expression in the synovial fluid mononuclear cells was found in RA as opposed to OA patients. In the synovial fluid of RA patients there was also a negative correlation between the concentration of the pro-inflammatory DPP-IV substrate SDF (stromal cell-derived factor-1α) and the proportion of the DPP-IV+ T cells. The blood plasma DPP-IV-like enzymatic activity and concentration were lower in patients with active RA as compared to OA, while there were no differences in DPP-IV expression on the blood mononuclear cells (BMNC). In a follow-up study, intraindividual comparison in patients with disease remission revealed that there was an increase of the blood plasma DPP-IV and a decrease of DPP-IV on BMNC in RA patients during the less active phase of their disease.

The association between RA activity and the changes in the blood plasma and the blood mononuclear cell DPP-IV in individual patients supports the possible role of DPP-IV as a disease activity marker. The lower local availability of DPP-IV in the synovial fluid in RA may in addition participate on the disease progression by the reduced degradation of the pro-inflammatory chemokine SDF.

Zkratky

BMNC	mononukleární buňky periferní krve
BSA	hovězí sérový albumin
CD	diferenciační skupina (clusters of diferenciation)
CRP	C-reaktivní protein
CXCR4	CXC chemokinový receptor 4
DAS28	disease activity score 28
DASH	dipeptidylpeptidáze-IV aktivitou a/nebo strukturou homologní molekuly
DPP	dipeptidylpeptidáza
ELISA	enzyme-linked immunosorbent assay
FAP	fibroblastový aktivační protein-α
FMNC	mononukleární buňky synoviální tekutiny
FW	rychlost sedimentace červených krvinek
FSC	forward scatter detektor umístěn v ose dopadajícího paprsku
IC50	koncentrace inhibitoru potřebná k inhibici 50% enzymové aktivity
MAb	monoklonální protilátka
MFI	medián intenzity fluorescence
mRNA	mediátorová ribonukleová kyselina
NK1	neurokininový receptor 1
OA	osteoartróza
PB	periferní krev
PBS	fosfátový pufr
RA	revmatoidní artritida
RANTES	regulated upon activation normal T-cell expressed and secreted
SDF	stromal cell-derived factor-1α/ CXCL 12
SF	synoviální tekutina
SP	substance P
SSC	side scatter detektor v úhlu 90° na dopadající paprsek
Tc	cytotoxické T lymfocyty
Th	pomocné T lymfocyty

VAS	vizuální analogová škála
VIP	vasoaktivní intestinální peptid

Klíčová slova

Dipeptidylpeptidáza

Dipeptidylpeptidáze-IV aktivitou a/nebo strukturou homologní molekuly

Revmatoidní artritida

Osteoartróza

Mononukleární buňky

Substance P

Stromal cell-derived factor 1-α

Synoviální tekutina

Periferní krev

Key words

Dipeptidyl peptidase

Dipeptidyl peptidase-IV activity and/or structure homologues

Rheumatoid arthritis

Osteoarthritis

Mononuclear cells

Substance P

Stromal cell-derived factor 1-α

Synovial fluid

Peripheral blood

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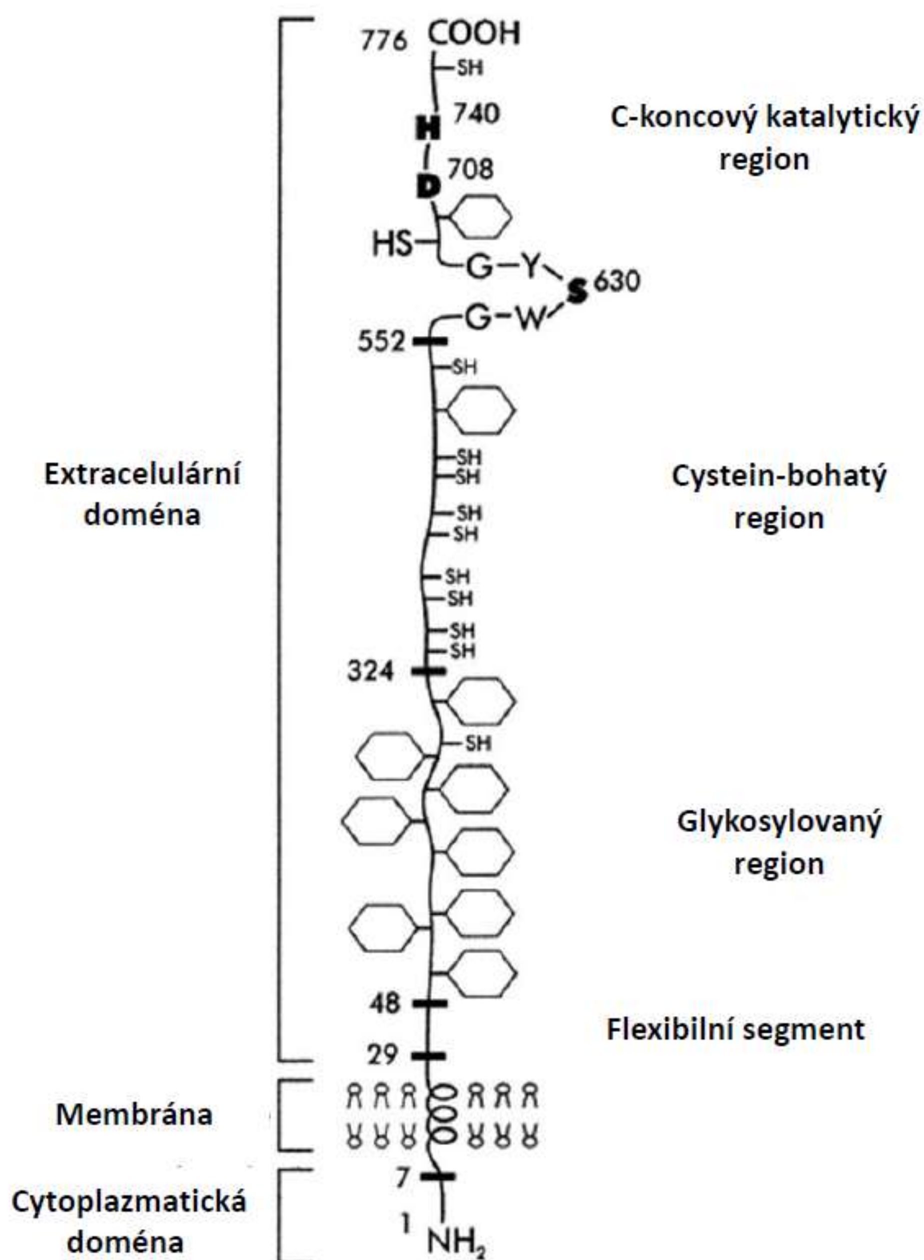
Úvod

Revmatoidní artiritida (RA) je zánětlivé autoimunitní onemocnění, jehož patogeneze není dosud zcela objasněna, nicméně za rizikové faktory lze považovat genetickou predispozici (zejména exprese HLA-DR4), pohlavní hormony a infekční nebo jiné antigeny ovlivňující imunitní systém. U pacientů s RA dochází vlivem autoimunitního procesu především k destrukci kloubů, a následně i postižení dalších orgánů (McInnes a Schett 2011). Rozvoj zánětlivého poškození u RA je spojený s infiltrací buněk do synoviálního prostředí kloubu, přičemž významnou roli v těchto procesech hrají T lymfocyty (Loetscher a Moser 2002). Buňky v synoviálním prostředí secernují prozánětlivé cytokiny, což stimuluje migraci dalších buněk do synoviální tekutiny i tkáně a vznik uskupení buněk podobných sekundárním lymfatickým orgánům. V důsledku zvýšené dostupnosti prozánětlivých mediátorů dochází k stimulaci synovialocytů a fibroblastů podporující formování zánětlivého pannu, který produkuje kolagenasy a stromelysin, jež jsou zodpovědné za postupnou destrukci chrupavek a kostí. (Loetscher a kol., 2002, McInnes a kol., 2011). Řada z těchto mediátorů jako např. SDF (stromal cell-derived factor-1α), SP (substance P), VIP (vasoaktivní intestinální peptid), RANTES (regulated upon activation normal T-cell expressed and secreted), má svůj biologický poločas kontrolovaný DPP-IV-podobnou enzymovou aktivitou (Ohnuma a kol., 2011). Jejich změněná dostupnost tak mimo jiné může vznikat i v důsledku změny proteolytického opracování a vést k ovlivnění progrese onemocnění (Sedo a kol., 2005).

SDF (Stromal cell-derived factor 1-α) je chemoatraktant produkovaný synoviálními buňkami a endotelem (Bradfield a kol., 2003). V patogenesi RA se SDF podílí především na stimulaci infiltrace lymfocytů a monocytů/makrofágů do zanícené synovie (Wolf a kol., 2008). Infiltrující buňky (zejména CD4+ T lymfocyty) exprimují membránový receptor pro SDF – CXCR4 (Bradfield a kol., 2003). Exprese CXCR4 je rovněž zvyšována parakrinním působením některých dalších lokálních mediátorů, které jsou přítomné v zánětlivém kloubu a předpokládá se, že osa CXCR4 - SDF je zásadně významná pro setrvání imunitních buněk v synoviálním prostředí. To ve svém důsledku vede k další perpetuaci zánětlivého procesu způsobujícím následnou destrukci kloubů (Pablos a kol., 2003, Proost a kol., 2006).

Neuropeptid **Substance P (SP)** je prozánětlivý mediátor interagující s tachykinovými receptory NK1, NK2 a NK3, nicméně nejvyšší afinitu má k receptoru NK (Maggi 1995). Vyšší koncentrace SP byla ve srovnání s kontrolními skuinami nalezena v synoviální tekutině pacientů s RA (Westermark a kol., 2001) . SP vykazuje prozánětlivé účinky na monocyty, lymfocyty, ale též synovialocyty v prostředí zanícené synoviální tkáně. To vede k další produkci řady prozánětlivých cytokinů, kolagenáz a adhezivních molekul, podílejících se na formování synoviálního pannu a tím dalším rozvoji vlastního onemocnění (Lambert a kol., 1998)

Dipeptidylpeptidáza-IV (DPP-IV, CD26, EC 3.4.14.5, Obrázek 1) odštěpuje díky své hydrolytické aktivitě N-terminální dipeptid z peptidů majících jako předposlední aminokyselinu prolin nebo alanin. Po dlouhá léta byla DPP-IV považována za jedinou molekulu vykazující takovouto enzymovou aktivitu. Další studie ovšem postupně prokázaly existenci dalších molekul nesoucích podobnou enzymovou aktivitu a rovněž několik molekul bez charakteristické hydrolytické aktivity, ovšem DPP-IV sekvenčně podobných. Studium vlastností a biologických funkcí těchto molekul vedlo k definici „Dipeptidylpeptidáze-IV podobných strukturou a/nebo aktivitou homologních“ (**DASH)** molekul (Sedo a Malík 2001). Většina těchto molekul tedy vykazuje DPP-IV-podobnou enzymovou aktivitu, přičemž ovšem některé své biologické funkce vykonávají tyto molekuly nezávisle na své vnitřní enzymové aktivitě. DASH molekuly jsou často multifunkční proteiny, nesoucí různé biologické funkce, v závislosti na exprimující tkáni či buněčném systému, ale i subcelulární lokalizaci a kontextu daného mikroprostředí. DASH a molekuly s nimiž funkčně interagují (substráty, receptory), představují „**DASH systém**“, jehož (de)regulace se pravděpodobně uplatňuje v řadě patogenetických procesů (Sedo a kol., 2001).



Obrázek 1. Dipeptidylpeptidáza-IV (DPP-IV, CD26, EC 3.4.14.5). DPP-IV je transmembránový protein II. typu obsahující 6 aminokyselin dlouhou cytoplazmatickou doménu, extracelulární doménu s krátkým flexibilním segmentem a C-koncovým regionem se serin-proteázovým typem katalytického centra. Serin^{630} tvoří katalytickou triádu s kyselinou asparogovou^{708} a histidinem^{740}. Převzato z Mentlein 1999 (Mentlein 1999).

DPP-IV poprvé identifikovali na základě její proteolytické aktivity Hopsu-Havu a Glenner v jaterních homogenátech (Hopsu-Havu a Glenner 1966). DPP-IV pak byla následně identifikována a její protein charakterizován v celé řadě tkání i buněk (např. epitel, endotel, glie, buňky imunitního systému) za fyziologických i patologických okolností (Lambeir a kol., 2003, Sedo a kol., 2008) a její solubilní forma se také vyskytuje v tělních tekutinách včetně krevní plazmy (Sedo a kol., 2005, Balaziova a kol., 2006). Tato multifunkční molekula svojí enzymovou aktivitou vykonává různé imunoregulační funkce a mnoho úloh též zastává v metabolismu živin i patogenezi diabetu mellitu.

V imunitním systému je kanonická DPP-IV identická s diferenciačním antigenem CD26. Je exprimována zejména CD4+CD45RO+ paměťovými T lymfocyty a je asociována s Th1 imunitní odpovědí. Obvykle nedetekovatelná, nebo jen velmi málo exprimována je DPP-IV na B-lymfocytech, NK buňkách a na monocytech zdravých jedinců (Ohnuma a kol., 2011). K její upregulaci dochází na aktivovaných T lymfocytech (Morimoto a kol., 1989). DPP-IV je rovněž ko-stimulační molekulou T lymfocytů podílející se na jejich aktivaci a proliferaci. Tyto účinky jsou pravděpodobně závislé na její intrinsické enzymové aktivitě (Ohnuma a kol., 2008, Ohnuma a kol., 2011).

V *in vitro* studiích byl použitím DPP-IV inhibitorů prokázán pokles produkce některých cytokinů (IL-2, IL-6, IL-10, IL-12 a IFN gamma) a zvýšení produkce supresivního cytokinu transformujícího růstového faktoru beta (Ansorge a kol., 1997, Reinhold a kol., 1997, Reinhold a kol., 1997, Reinhold a kol., 1998, Lendeckel a kol., 2003) Vzhledem k funkčnímu významu v imunoregulacích je v současnosti DPP-IV v imunitním systému intenzivně studována v souvislosti s možnými důsledky její systémově farmakologicky navozené inhibice při léčbě diabetu mellitu 2. typu gliptiny (Stulc a Sedo 2010).

Mezi neenzymové účinky DPP-IV lze zařadit její interakci s adenosin deaminázou, dále i s membránově vázanou protein. tyrosin fosfatázou CD45 či její vazbu ke kolagenu a fibronektinu (Tanaka a kol., 1994, Tanaka a kol., 1995, Aytac a

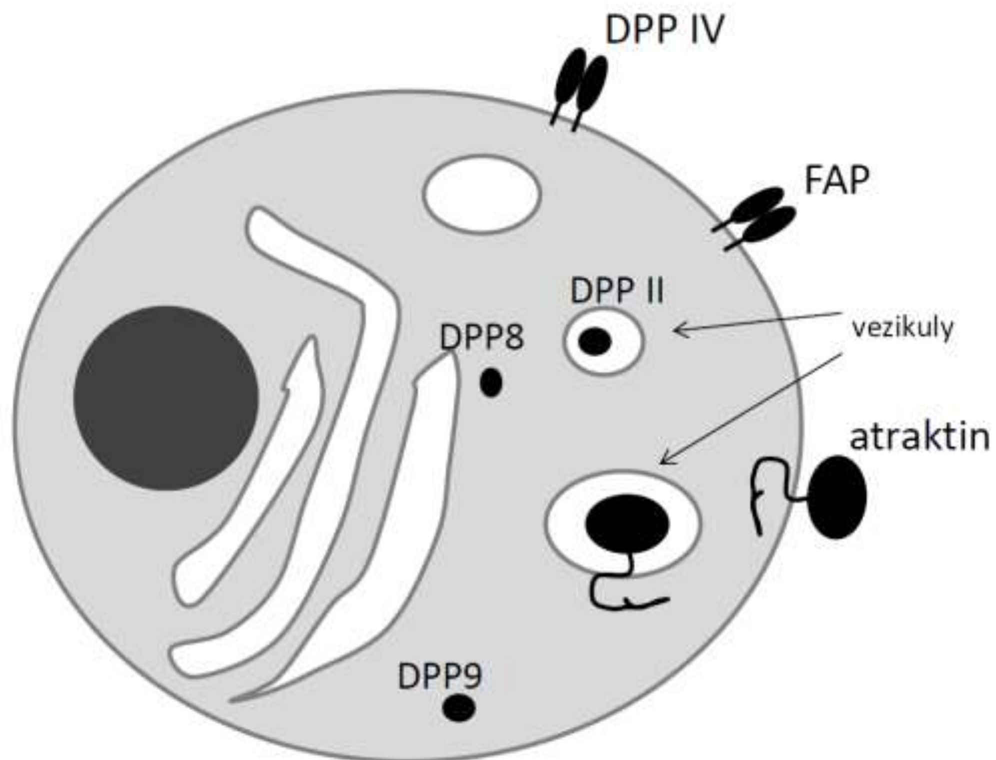
Dang 2004). DPP-IV takto zprostředkovává kontakt mezi buňkami navzájem i mezi buňkami a extracelulární matrix. Její interakce s proteiny extracelulární matrix jsou významné pro invazi a migraci nádorových buněk a tím i pro další metastazování nádorů, stejně tak jako pro migrace lymfocytů do zánětlivé tkáně synovie (Dang a Morimoto 2002, Kikkawa a kol., 2003, Kikkawa a kol., 2005).

Změny exprese DPP-IV byly popsány v biopsiích z různých typů lidských tumorů, kdy u některých z nich je považována za možný prognostický ukazatel (Tanaka a kol., 1995, Busek a kol., 2004) DPP-IV byla studována v transformovaných nervových buňkách, gliálních buňkách či melanomech. Potlačení tumorigeneze v důsledku re-exprese DPP-IV bylo pozorováno na buňkách melanomu či neuroblastomu (Wesley a kol., 1999, Arscott a kol., 2009). Nicméně v pokročilých stadiích melanomu spojeném již s tvorbou metastáz, exprese DPP-IV tento protinádorový efekt spojený se změnami invazivity nevykazuje (Pethiyagoda a kol., 2000)

Zvýšená exprese kanonické DPP-IV byla pozorováno v tkáních pocházejících z gliomů s vyšším stupněm malignity dle WHO (Stremenova a kol., 2007). V permanentních i primárních liniích odvozených z těchto nádorů byl pozorován negativní efekt DPP-IV-podobné enzymové aktivity na růst buněk in vitro (Busek a kol., 2012, Příloha 3).

.

Kromě kanonické DPP-IV/CD26 patří mezi DASH molekuly (Obrázek 2) například Fibroblastový aktivační protein alfa (FAP), DPP-II, DPP8 a DPP9, dříve byl mezi ně řazen i atraktin (Wrenger a kol., 2006, Wrenger a kol., 2006).



Obrázek 2. Buněčná lokalizace DASH molekul. DPP-IV a FAP jsou lokalizovány na buněčné membráně, jejich aktivní doména je orientována extracelulárně. Atraktin je exprimovaný na membráně i v intracelulárních vesikulech. DPP8 a DPP9 jsou typicky cytosolické enzymy. DPP-II je lokalizována v intracelulárních vezikulech. (DPP - Dipeptidylpeptidáza, FAP – fibroblastový aktivační protein alfa), převzato a upraveno dle Wrenger a kol. 2006, Maes a kol 2007.

Atraktin byl primárně identifikován jako solubilní DPP-IV-enzymově aktivní protein, který však nevykazuje strukturní homologii s kanonickou DPP-IV (Duke-Cohan a kol., 1995). Následné práce popisují i jeho expresi na buňkách periferní krve, zejména na monocytech (Wrenger a kol., 2006). Další studie však zpochybnily existenci jeho intrinsické proteolytické aktivity, ale prokázaly jeho schopnost zvyšovat specifickou aktivitu kanonické DPP-IV, pravděpodobně přímou intermolekulární interakcí obou molekul, zprostředkovanou CUB doménou Atraktinu (Jonathan-Duke Cohan, osobní sdělení). Friedrich a kol. (2007) izolovali atraktin z plazmy periferní krve a prokázali, že atraktin nemá podíl na DPP-IV-podobné enzymové aktivitě a jeho regulační účinky jsou spíše důsledky neenzymových interakcí (Friedrich a kol., 2007).

Dipeptidylpeptidáza-II (DPPII, „Quiescent cell“ prolinová dipeptidáza) je intracelulární proteáza s pH optimem v kyselé oblasti (Underwood a kol., 1999) . Při neutrálním pH má stejnou substrátovou specifitu jako DPP-IV, avšak v buňkách je tato molekula lokalizována ve vesikulech (Chiravuri a kol., 2000).

Dipeptidylpeptidáza 8 a 9 (DPP8 a DPP9) jsou DASH molekuly vykazující DPP-IV-podobnou exopeptidásovou aktivitu i strukturní homologii (21% resp. 19%) s kanonickou DPP-IV (Sedo a kol., 2001, Qi a kol., 2003). Oba enzymy jsou exprimovány v buňkách imunitního systému a epiteliálních strukturách různých tkání (Qi a kol., 2003, Bank a kol., 2011). Maes a kol (2007) díky frakcionaci leukocytů prokázali, že molekuly DPP8 a DPP9 jsou dominantně lokalizovány v cytosolu, zatímco kanonická DPP-IV je exprimována především v membránové frakci (Maes a kol., 2007). Ojedinělé je pozorování Bankové et al. (Bank a kol., 2011) popisující v nepermeabilizovaných myších splenocytech a permanentních buněčných liniích Jurkat (T lymfoblastoidní linie) a H9 (embryonální kmenové buňky) DPP-IV-podobnou aktivitu inhibovatelnou specifickými inhibitory DPP8 a 9. Autoři spekulují, zda se jedná o primárně extracelulární lokalizaci nebo o sekundární interakci původně extracelulárně secernované molekuly s plazmatickou membránou. Struktura molekul DPP8 a 9 navíc neobsahuje typickou transmembránovou doménu (Abbott a kol., 2000, Qi a kol., 2003)

Fibroblastový aktivační protein alfa (FAP), známý též pod názvem sepráza, je strukturně vysoce homologní s kanonickou DPP-IV a je považován za produkt její genové duplikace (Busek a kol., 2004). Kromě DPP-IV-podobné enzymové aktivity vykazuje i endopeptidázovou gelatinázovou aktivitu (Aertgeerts a kol., 2005). Stejně jako DPP-IV bývá jeho solubilní forma secernována buňkami a nacházíme jej též v plazmě periferní krve (Lee a kol., 2006). Exprese FAP bývá spojována s migračním fenotypem buněk a jeho lokalizace byla prokázána např. v invadopodiích maligních buněk a spolu s dalšími secernovanými proteázami hraje roli v degradaci složek extracelulární matrix (Sedo a kol., 2001, Ramirez-Montagut a kol., 2004). FAP je exprimován nejen v řadě tumorů, ale i aktivovanými fibroblasty synoviální tkáně a u pacientů s osteoartrózou byla prokázána jeho role v destrukci chrupavky (Bauer a kol., 2006, Ospelt a kol., 2010).

Ghersi a kol popisují formace membránově vázaných komplexů FAP s DPP-IV a jejich vliv na migraci a invazi buněk (Ghersi a kol., 2002, Ghersi a kol., 2006). V našich studiích (Balaziova a kol., 2011, Příloha 2), jsme prokázali ko-expresi DPP-IV a FAP na primárních kulturách i permanentních liniích odvozených z gliálních tumorů.

V posledních letech došlo k značnému rozšíření poznatků o možném využití diagnostického a terapeutického potenciálu DASH molekul, stejně tak jako ve studiu jejich funkčního významu v patogenezi řady onemocnění. U pacientů s RA byl pozorován pokles solubilní DPP-IV-podobné enzymové aktivity v periferní krvi ve srovnání s kontrolami a rovněž vyšší intenzita exprese na mononukleárních buňkách periferní krve (Balaziova a kol., 2006, Ohnuma a kol., 2011). Někteří autoři se rovněž snažili najít rozdíly v těchto parametrech mezi pacienty s vyšší/nižší aktivitou onemocnění (Gerli a kol., 1996, Ulusoy a kol., 2012) či jejich vztah ke klinickým parametrům souvisejícími s aktivitou RA.

Jen málo prací se však zaměřilo u pacientů s revmatoidní artritidou na konkrétní charakterizaci DASH molekul, které by byly dostupné buďto v solubilní formě cirkulující v tělních tekutinách nebo jako membránově vázané molekuly a svojí DPP-

IV-podobnou enzymovou aktivitou by se tak mohly přímo podílet na proteolytickém opracování lokálních mediátorů. Dosud publikované výsledky (Gerli a kol., 1996, Balaziova a kol., 2006) navíc naznačují, že lze očekávat jejich odlišné chování a úlohu na systémové úrovni tj. v periferní krvi a v lokálním synoviálním mikroprostředí. Dosud nebyla provedena longitudiální studie, která by se zaměřila na intraindividuální charakterizaci molekul DASH systému u jednotlivých pacientů v závislosti na vývoji aktivity jejich onemocnění.

Hypotéza

Ve srovnání s pacienty s osteoartrózou (OA) jsou u pacientů trpících revmatoidní artritidou (RA) pozorovány významné rozdíly DPP-IV na systémové (periferní krev) a lokální (kloubní výpotek) úrovni.

Důsledkem je pravděpodobně změna opracování biologicky aktivních peptidů přítomných jak v cirkulaci, tak v kloubním prostředí přítomnou solubilní, nebo na buněčných površích lokalizovanou DPP-IV-podobnou enzymovou aktivitou a tím i výsledný patogenetický efekt - perpetuace a rozvoj zánětlivého procesu.

Identifikace molekul "DASH systému", jejichž exprese je v souběhu nebo v důsledku zmíněné dysregulace změněna, může vést k nalezení nových diagnostických přístupů a nebo prognostických úvah a současně k návrhu nových terapeutických modalit, regulujících specificky aktivitu patogeneticky významných DASH molekul.

Cíle práce

I. **Identifikace DASH molekul nesoucích DPP-IV-podobnou enzymovou aktivitu v plazmě a na povrchu mononukleárních buněk periferní krve a synoviální tekutiny a jejich reakčních partnerů**

II. **Porovnání exprese a DPP-IV-podobné aktivity vybraných DASH molekul, jejich substrátů a receptorů u pacientů s revmatoidní artritidou (RA) ve srovnání s pacienty s osteoartrózou (OA) na lokální a systémové úrovni**

III. **Intraindividuální posouzení vztahu vybraných DASH molekul v periferní krvi ke klinickému průběhu RA**

Materiál a metody

Pacientský soubor

V Revmatologickém ústavu v Praze byla rekrutována skupina pacientů s aktivní RA (diagnostika dle standardních kritérií (Arnett a kol., 1988) a kontrolní skupina tvořená pacienty s osteoartrózou (OA), kteří měli v době zařazení do studie výpotek v kolenním kloubu (Tabulka 1).

Aktivita revmatoidní artritidy byla hodnocena na základě tzv. DAS28 skóre (Prevoo a kol., 1995) vypočítaného z hodnoty plazmatické koncentrace C-reaktivního proteinu (CRP), pacientem uvedené hodnoty VAS (vizuální analogové škály) a počtu bolavých a oteklých kloubů (http://www.das-score.nl/).

Tabulka 1. Klinické charakteristiky studovaných skupin pacientů.

	Revmatoidní artritida (n=27)	Osteoartróza (n=15)
Věk	59 ± 13	62±11
Muži/Ženy	7/20	6/9
Délka onemocnění (roky)	12.5±12.4	-
Počet bolavých kloubů (0-28)	9±6.3	-
Počet oteklých kloubů (0-28)	11± 6.7	-
DAS 28	5.8±1.1	-
FW (mm/h)	56±28.2	10±6.4
CRP (mg/l)	44±26	5± 4

DAS 28, disease activity score; CRP, C-reaktivní protein, FW, rychlost sedimentace červených krvinek. Hodnoty jsou uvedeny jako medián±SD.

Následné (follow-up) vyšetření bylo u pacientů s RA provedeno v době, kdy neměli kloubní výpotek a uplynulo alespoň 6 měsíců od vstupního vyšetření a zařazení do studie. Intraindividuální změny studovaných parametrů byly hodnoceny u pacientů vykazujících alespoň mírný pokles aktivity onemocnění, definovaný jako změna DAS28 skóre (pokles DAS28 větší než 0.6 oproti vstupnímu vyšetření, jestliže současná

hodnota je menší než 5.1 a nebo jako pokles DAS28 větší než 1.2 jestliže současná hodnota je větší než 5.1, Tabulka 2) (https://www.rheumatology.org/practice/clinical/indexes/members/Disease_Activity_Score_Sheet.pdf).

Tabulka 2. Klinická charakteristika skupiny pacientů s RA vykazující alespoň mírný pokles aktivity onemocnění ve follow-up vyšetření.

	Vstupní hodnoty	Follow-up
Věk	60±16	61±16
Muži/Ženy	5/13	5/13
Délka onemocnění (roky)	4.5±13.3	5.5±13.5
Počet bolavých kloubů (0-28)	9±6	2±3
Počet oteklých kloubů (0-28)	11.5±6.5	2.5±6.6
DAS 28	5.66±0.97	3.49±1.22
FW (mm/h)	56±27.7	30±24.1
CRP (mg/l)	51.08±24.49	7.31±19.13

DAS 28, disease activity score; CRP, C-reaktivní protein, FW, rychlost sedimentace červených krvinek. Hodnoty jsou uvedeny jako medián±SD.

V letech 2011-2012 (5 let po vstupním odběru) pak u všech pacientů zařazených do studie bylo provedeno kontrolní rentgenologické vyšetření k posouzení přítomnosti eroze kloubů. Pouze u pěti pacientů byla pozorována progrese erozí kloubů (Tabulka 3).

Tabulka 3. Radiologicky hodnocená progrese erozí kloubů u pacientů zahrnutých do studie

Kód pacienta	Pohlaví	Stupeň eroze		
		Vstupní vyšetření	Follow-up vyšetření	Vyšetření po 4 letech
1	žena	I	II	III
22	žena	I	I	II
34	žena	III	IV	IV
35	muž	II	IV	IV
47	muž	I	I	III

Pacientům byla při vstupním vyšetření odebrána periferní krev a synoviální tekutina. V rámci follow-up vyšetření pak byl proveden pouze odběr periferní krve. Studie byla schválena etickou komisí Revmatologického ústavu v Praze a byla v souladu s Helsinskou deklarací. Všichni pacienti podepsali informovaný souhlas.

Biologický materiál

Izolace mononukleárních buněk

Periferní krev byla odebrána do „BD Cell preparation tube“ (BD Biosciences, USA) obsahující jako protisrážlivou látku heparin a ficoll pro efektivní gradientovou separaci. Vacutainer byl centrifugován 20 minut při teplotě 20°C a 2000g kdy došlo k oddělení erytrocytů a vytvoření vrstvy BMNC

Synoviální tekutina byla po punkci výpotku z kolene odebrána do sterilních „BD Vacutainer“ (BD Biosciences, USA) obsahujících heparin. Mononukleární buňky byly získány gradientovou centrifugaci (40 minut při teplotě 20°C a 1000g) na gradientu vytvořeného pomocí Ficoll-Paque Plus (GE Healthcare, Sweden).

Vyizolované buňky byly odebrány, dvakrát promyty roztokem PBS a následně byla stanovena jejich koncentrace na přístroji Z2 Coulter (Beckman Coulter,USA). Viabilita izolovaných buněk byla potvrzena testem s Trypanovou modří.

Příprava plazmy a synoviální tekutiny pro analýzy

Periferní krev i synoviální tekutina byly po odebrání do BD vacutaineru s heparinem uloženy na led. Pro stanovení koncentrace Substance P byl vždy 1ml heparinizované krve ihned po odběru přenesen do zkumavky obsahující aprotinin (0,014 TIU/ml vzorku, Sigma Aldrich, USA). Všechny typy vzorků byly následně centrifugovány (15 minut, 4°C, 850g) a supernatanty odebrány. Vzorky pro analýzu DPP-IV-podobné enzymové aktivity byly ihned analyzovány, alikvoty pro stanovení koncentrace DPP-IV, FAP, SDF a SP byly zamrazeny na -80°C.

Analýza buněk průtokovou cytometrií

Pro imunofenotypizační studie byly použity vzorky heparinizované periferní krve (PB) nebo synoviální tekutiny (SF) získané z kloubního výpotku. Vzorky SF byly centrifugovány (5 min, 4 °C, 233 g), pelet promyt a resuspendován v RPMI 1640 mediem (Lonza, Švýcarsko) a zfiltrován (50 µm cup filcons, BD Biosciences, USA). 50µl vzorku bylo inkubováno 30 minut ve tmě při laboratorní teplotě se směsí protilátek (Tabulka 4).

Tabulka 4. Protilátky pro průtokovou cytometrii

Protilátka	Zdroj	Ředění	Klon	Fluorochrom	Výrobce
anti-CD3	myš	1 : 20	SK7	PerCP	BD Bioscience
anti-CD4	myš	1 : 20	SK3	APC	BD Bioscience
anti-CD8	myš	1 : 20	SK1	PE-Cy7	BD Bioscience
anti-CD14	myš	1 : 20	M5E2	APC-Cy7	BD Bioscience
anti-CD26/DPP-IV	krysa	1 : 20	222113	FITC	RD systems
anti-CXCR4	myš	1 : 20	12G5	PE	RD systems
anti-FAPα stalk region	králík	1 : 50	-	-	Abcam
anti-FAPα spacer region	králík	1 : 50	-	-	Abcam
anti-NK1 receptor	králík	1 : 50	-	-	Abcam
anti-rabbit IgG H&L	koza	1 : 50	-	FITC	Abcam

PerCP - Peridinin chlorofyl protein; APC, Alofykocyanin; PE-Cy7, Fykoerytrin - Cyanin-7; APC-Cy7, Alofykocyanin -Cyanin-7; FITC, Fluorescein isothiokyanát; PE, Fykoerytrin

Erytrocyty ve vzorcích byly lyzovány pomocí BD FACS Lysing solution (BD Biosciences, USA) a vzorky byly následně promyty roztokem BD Cell wash (BD Biosciences, USA). V případě nepřímé imunofluorescence byla po tomto promytí

provedena 30 minut dlouhá inkubace ve tmě a při laboratorní teplotě se sekundární protilátkou (Tabulka 4). Analýza byla prováděna na přístroji BD FACS Canto (BD Biosciences, USA) se softwarem Diva 5 pro akvizici. K vlastnímu kvantitativnímu vyhodnocení pak byl používán software FlowJo (TreeStar, Inc. USA). Výsledky jsou vyjádřeny jako % pozitivních buněk ze sledované populace, nebo jako podíl mediánu intenzity fluorescence (MFI) pozitivních a negativních populací buněk.

Stanovení DPP-IV-podobné enzymové aktivity

DPP-IV-podobná enzymová aktivita byla stanovována spektrofluorimetricky za přítomnosti 50 μmol/l 7-(Glycyl-L-Prolylamido)-4-methylkumarinu (Bachem AG, Švýcarsko) jako substrátu. Detekce byla prováděna při excitační vlnové délce 380 nm a emisní 460 nm na spektrofluorimetru Perkin Elmer LS50B (Perkin Elmer, USA). Aktivita byla měřena v krevní plazmě, supernatantu synoviální tekutiny a ve viabilních mononukleárních buňkách periferní krve a synoviální tekutiny při pH 7,4 a 37°C. Pro zjištění celkové aktivity byly pak buňky permeabilizovány pomocí 0.1% Triton X-100 (Balaziova a kol., 2006). Inhibiční studie byly prováděny s použitím specifických inhibitorů DPP-IV a DPP8/9, získaných v rámci spolupráce s Ferring Research (USA) (Stremenova a kol., 2007) .

Izolace a kvantifikace celkové RNA, real-time RT PCR

Mononukleární buňky vyizolované z periferní krve i synoviální tekutiny byly resuspendovány v 2,5 ml izoloačního činidla TriZol (Life Technologies, USA), celková RNA byla izolována činidlem TriZol dle instrukcí výrobce. Koncentrace celkové RNA byla stanovena kitem RiboGreen RNA Quantitation Kit (Life Technologies, USA). Ke studiu exprese DPP-IV, FAP, NK1R, CXCR4 a β-aktin (interní referenční transkript) mRNA byly využity gen kódující region-specifické priméry a fluorogenní TaqMan próby, připravené v programu Primer Express (Applied Biosystems - Life Technologies, USA). Jejich přehled je uveden v tabulce 5. Exprese byla kvantifikována spřaženou real time RT-PCR s využitím ThermoScript One-Step System (Life

Technologies, USA). Analýzy byly prováděny v dubletech v MicroAmp Optical 96-jamkové desce na přístroji ABI PRISM 7700 Sequence Detection System (Applied Biosystems - Life Technologies, USA). Exprese cílových transkriptů byla normalizována na expresi mRNA β-aktinu použitím ΔCt metody (Livak a Schmittgen 2001)

Enzyme-linked immunoassay (ELISA)

DPP-IV, FAP a SDF

Pro stanovení koncentrace DPP-IV, FAP a SDF byly použity komerčně dodávané kity „DuoSet“ firmy RD Systems (USA) DPP-IV/CD26 DY1180, FAP DY3715 a CXCL12/ SDF DY350. Příprava všech reagencií byla provedena dle instrukcí výrobce. Vzorky heparinizované plazmy i synoviální tekutiny byly pro stanovení DPP-IV a FAP naředěny 1000x, pro analýzu SDF 2x roztokem 1% BSA v PBS. 96-jamková destička byla přes noc inkubována na třepačce při pokojové teplotě se 100 μl roztoku protilátky, 4x opláchnuta roztokem PBS s 0,05% Tween-20 na přístroji Hydroflex (Tecan, Švýcarsko) a hodinu blokována roztokem 1% BSA v PBS. Po promytí byly do jamek naneseny vzorky i standardy v duplikátech a dvě hodiny inkubovány na třepačce při pokojové teplotě. Po promytí byla do jamek nanesena detekční protilátka a inkubována (2 hodiny, pokojová teplota). Poté byly jamky promyty a 20 minut (SDF 40 minut) inkubovány ve tmě s roztokem Streptavidin-HRP (součást kitu) a následně se 100 μl substrátu (DY999, RD systems USA), připraveného smícháním roztoků „Color reagent A“ (stabilizovaný peroxid vodíku) a „Color reagent B“ (stabilizovaný tetrametylbenzidin) v poměru 1:1 (20 mintu DPP-IV a FAP, 40 minut SDF). Reakce byla zastavena přidáním 50 μl 2N kyseliny sírové. Optická densita byla měřena na přístroji Sunrise (Tecan, Švýcarsko) při vlnové délce 450nm s korekcí na 540nm.

Substance P

Pro stanovení koncentrace byl použit Substance P Parameter Assay Kit firmy RD systems (USA) KGE007, který funguje na principu kompetitivního enzymového imunostanovení. Do každé jamky bylo dle instrukcí napipetováno 50 µl naředěné plazmy (1:1 s roztokem „Calibrator diluent“, součást kitu), 50 µl primární protilátky a 50 µl konjugované Substance P. Deska s jamkami byla 3 hodiny inkubována na třepačce při pokojové teplotě a 4x opláchnuta roztokem PBS s 0,05% Tween-20 na přístroji Hydroflex (Tecan, Švýcarsko). Do jamek bylo přidáno 200 µl substrátu (peroxid vodíku a tetrametylbenzidin v poměru 1:1, součást kitu). Reakce byla zastavena přidáním 50 µl 2N kyseliny sírové. Optická densita byla měřena na přístroji Sunrise (Tecan, Švýcarsko) při vlnové délce 450nm s korekcí na 540nm.

Statistická analýza

Statistická analýza byla prováděna v programu Statistica 12.0 (StatSoft, Inc., USA). Rozdíly mezi jednotlivými skupinami jsou hodnoceny pomocí Mann-Whittneyho U-testu a v případě intraindividuálního posuzování změn u pacientů pak použitím Wilcoxnova párového testu, korelace byly analyzovány pomocí Spearmanova korelačního koeficientu.

Tabulka 5. Přehled primérů a prób pro real time RT-PCR

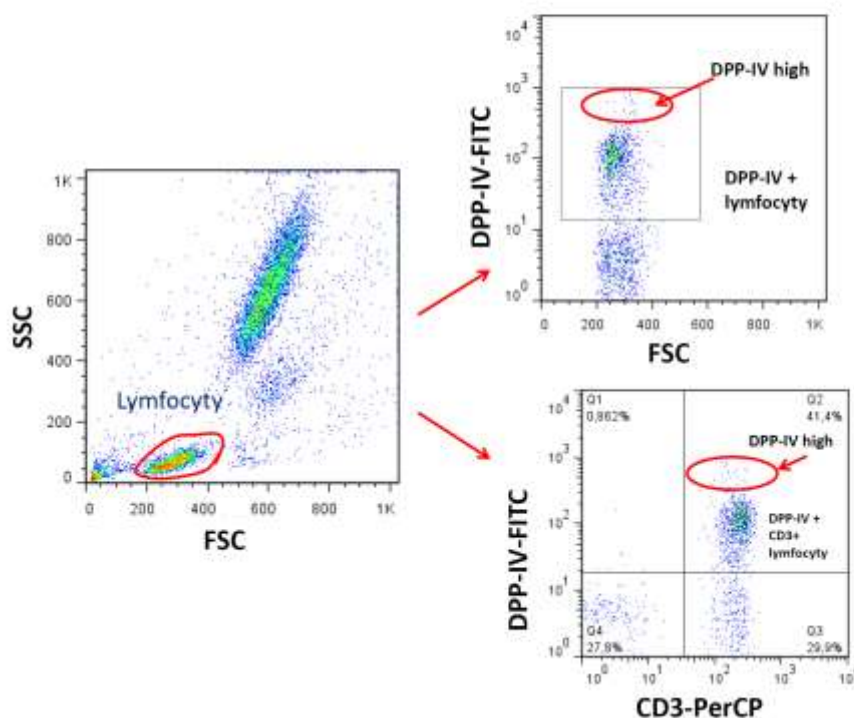
Transkript	GeneBank Vstupní číslo.	Sekvence a finální koncentrace primérů a TaqMan prób
DPP-IV	NM_001935	Forward primér: 5'-TGGAAGGTTCTTCTGGGACTG-3', 200 nmol/l Reverse primér : 5'-GATAGAATGTCCAAACTCATCAAATGT-3', 200 nmol/l TaqMan próba : 5'-(6-FAM)CACCGTGCCCGTGGTTCTGCT(TAMRA)-3', 200 nmol/l
FAP	NM_004460	Forward primér : 5'-TGCCACCTCTGCTGTGC-3', 200 nmol/l Reverse primér : 5'-GAAGCATTCACACTTTTCATGGT-3', 200 nmol/l TaqMan próba : 5'-(6-FAM)TGCATTGTCTTACGCCCTTCAAGAGTTC(TAMRA)-3', 200 nmol/l
NK1	NM_001058	Forward primér : 5'-CAGTGGTGAACTTCACCTATGCT-3', 400 nmol/l Reverse primér : 5'-GATGTATGATGGCCATGTACCTATC-3',400 nmol/l TaqMan próba : 5'-(6-FAM)TCCACAACTTCTTTCCCATCGCCG(TAMRA)-3', 200 nmol/l
CXCR4	NM_001008540	Forward primér : 5'-CATGGGTTACCAGAAGAAACTGA-3', 400 nmol/l Reverse primér: 5'-GACTGCCTTGCATAGGAAGTTC-3', 400 nmol/l TaqMan próba : 5'-(6-FAM)CACCTGTCAGTGGCCGACCTCCT(TAMRA)-3', 200 nmol/l
β-Aktin	NM_001101	Forward primér : 5'-CTGGCACCCAGCACAATG-3', 200 nmol/l Reverse primér : 5'-GGGCCGGACTCGTCATAC-3', 200 nmol/l TaqMan próba : 5'-(VIC)AGCCGCCGATCCACACGGAGT(TAMRA)-3', 200 nmol/l

Výsledky

I. Identifikace DASH molekul nesoucích DPP-IV-podobnou enzymovou aktivitu v plazmě a na povrchu mononukleárních buněk periferní krve a synoviální tekutiny a jejich reakčních partnerů

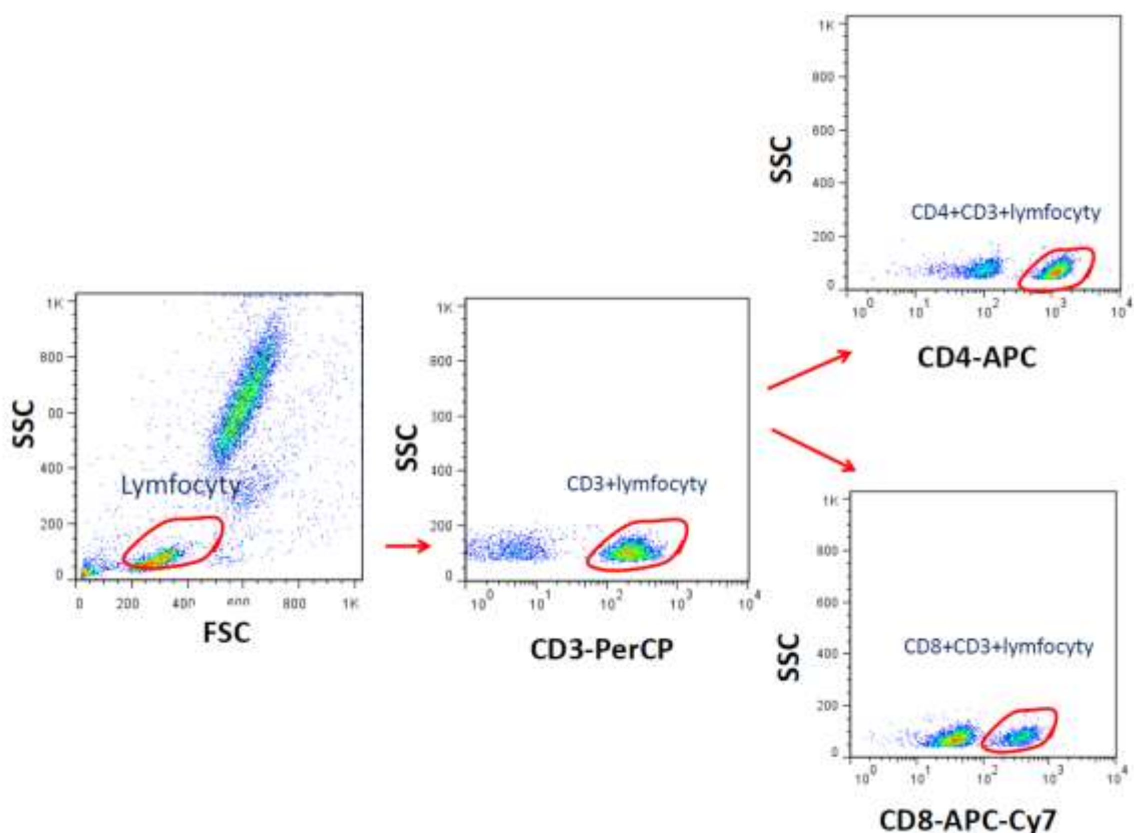
Detekce DASH molekul na mononukleárních buňkách

Metodou průtokové cytometrie byla analyzována přítomnost DPP-IV, FAP, CXCR4 a NK1 receptoru na populacích lymfocytů a v monocytech v periferní krvi a synoviální tekutině pacientů s RA a OA. Lymfocyty byly definovány dle optických parametrů a následně s využitím monoklonální protilátky anti-CD3 jako T lymfocyty (Obrázek 3).



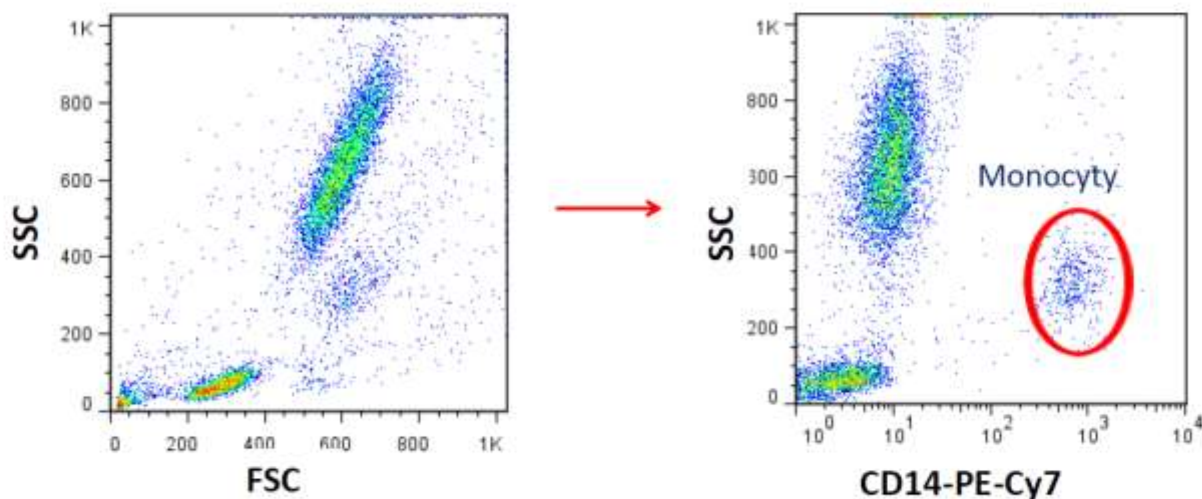
Obrázek 3. Gatovací strategie pro identifikaci lymfocytární populace a exprese DPP-IV. DPP-IV je dominantně lokalizována na CD3+ lymfocytech, zatímco na CD3- lymfocytech je prakticky nedetekována. V některých vzorcích byla patrná přítomnost DPP-IV vysoce pozitivní (high) populace CD3+lymfocytů. Typický experiment.

Subpopulace lymfocytů (Obrázek 4) byly definovány jako Th (CD4+CD3+lymfocyty) a Tc (CD8+CD3+lymfocyty). Monocyty byly identifikovány jako CD14+ buňky (Obrázek 5 a 6)

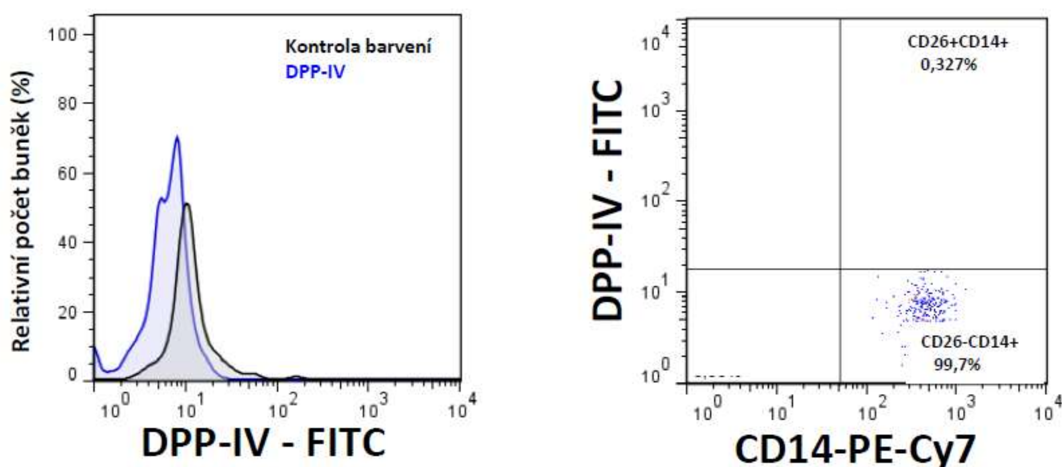


Obrázek 4. Gatovací strategie pro identifikaci T lymfocytárních subpopulací CD4+ a CD8+ buněk . Typický experiment.

Na všech analyzovaných vzorcích periferní krve i synoviální tekutiny pacientů s RA i OA byla detekována přítomnost DPP-IV a receptoru CXCR4. Zatímco CXCR4 byl exprimován na lymfocytech i monocytech, DPP-IV byla dominantně (95-100%) přítomná na CD3+ lymfocytech. Pozitivita detekovaná na CD3- lymfocytech a monocytech (definovaných jako CD14+ buňky) byla spíše výjimečná a na hranici detekce. Ve většině vzorků nebyla hodnotitelná tzv. DPP-IV high populace pozitivních buněk (Obrázek 3).



Obrázek 5. Gatovací strategie pro identifikaci monocytární populace. Monocyty byly charakterizovány jako CD14+buňky. Typický experiment.

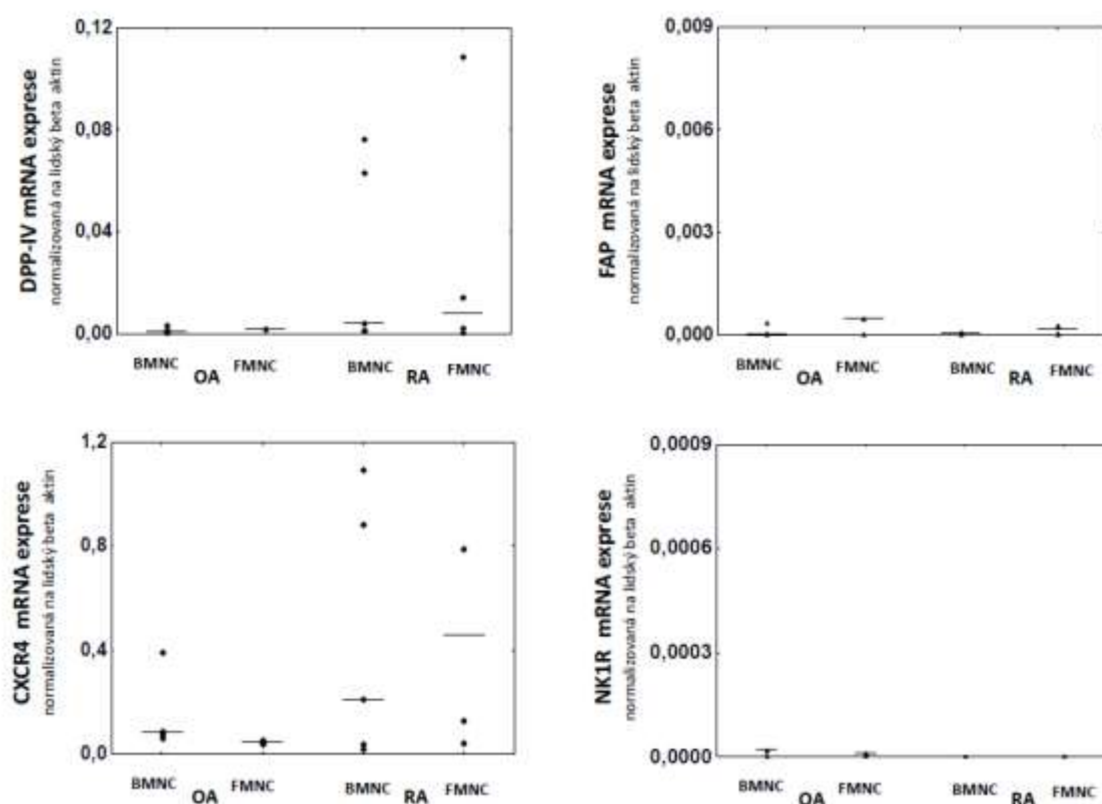


Obrázek 6. Detekce exprese DPP-IV na monocytech (CD14+ buňkách).

Není detekovatelná změna intenzity fluorescence po obarvení monocytů protilátkou proti DPP-IV s fluorochromem FITC oproti kontrole barvení (vlevo) ani subpopulace DPP-IV pozitivních buněk (dot-plot, vpravo). Typický experiment.

Na vzorcích BMNC ani FMNC nebyla metodou průtokové cytometrie prokázána přítomnost FAP ani receptoru NK1 žádnou z použitých protilátek. Proto jsme se rozhodli ověřit na několika vzorcích separovaných BMNC a FMNC případnou expresi FAP a NK1-R transkriptu pomocí real-time RT-PCR. V grafu jsou uvedeny relativní hodnoty exprese jednotlivých transkriptů normalizovaných na hodnotu exprese beta-aktinu (Obrázek 7). Tyto výsledky potvrdily, že FAP a NK1R (NK1 receptor) nebyly v mononukleárních buňkách přítomny ani na úrovni transkriptu.

Na všech vzorcích BMNC i FMNC byla nalezena povrchově vázaná DPP-IV-podobná enzymová aktivita, jejímž zdrojem je tedy pravděpodobně kanonická DPP-IV.



Obrázek 7. Exprese mRNA DPP-IV, FAP, NK1R a CXCR4 v mononukleárních buňkách normalizovaná na lidský beta aktin. Na mononukleárních buňkách periferní krve (BMNC) i synoviální tekutiny (FMNC) byla u pacientů s revmatoidní artritidou (RA) i osteoartrózou (OA) potvrzena přítomnost transkriptu DPP-IV a CXCR4, zatímco exprese FAP a NK1R zde prokázána nebyla.

Detekce molekul DASH systému v plazmě periferní krve a solubilní komponentě synoviální tekutiny

Ve všech vzorcích plazmy periferní krve i solubilní (bezbuněčné) komponentě synoviální tekutiny byla detekována DPP-IV-podobná enzymová aktivita, která byla velmi dobře inhibovatelná DPP-IV specifickým inhibitorem (IC50 pro rekombinantní protein 2 nmol/l vs. 4,8 nmol/l ve vzorcích plazmy a 5,8 nmol/l pro solubilní frakci SF). Pro specifický inhibitor DPP8/9 (IC50 pro rekombinantní protein DPP8 je 4 nmol/l a pro DPP9 20 nmol/l) byly naměřeny hodnoty IC50 546 nmol/l ve vzorcích plazmy a 498 nmol/l pro solubilní frakci SF svědčící proti významné přítomnosti těchto enzymů v námi analyzovaném materiálu.

Koncentrace DPP-IV stanovená metodou ELISA signifikantně korelovala s DPP-IV-podobnou enzymovou aktivitou v plazmě ($r=0.83$, $p<0.001$) i v synoviální tekutině ($r=0.61$, $p<0.001$). FAP byl ve vzorcích s využitím ELISA rovněž detekovatelný, avšak jeho koncentrace byla několikanásobně nižší než koncentrace DPP-IV a s DPP-IV-podobnou enzymovou aktivitou nekoreloval (Tabulky 6 a 7). Z těchto výsledků vyplývá, že dominantním nositelem DPP-IV-podobné enzymové aktivity v plazmě periferní krve a solubilní komponentě synoviální tekutiny je kanonická DPP-IV.

Koncentrace biologicky aktivních substrátů DPP-IV SDF a SP byly ve všech našich vzorcích nad detekčním limitem použité metody, avšak dostupné protilátky proti SDF a SP neumožňují rozlišení peptidů biologicky aktivních (intaktních) a inaktivovaných (proteolyticky opracovaných) DPP-IV-podobnou enzymovou aktivitou. Stanovená koncentrace je tak spíše nepřímou informací o celkovém množství a obratu prozánětlivého mediátoru než o jeho biologicky aktivní frakci.

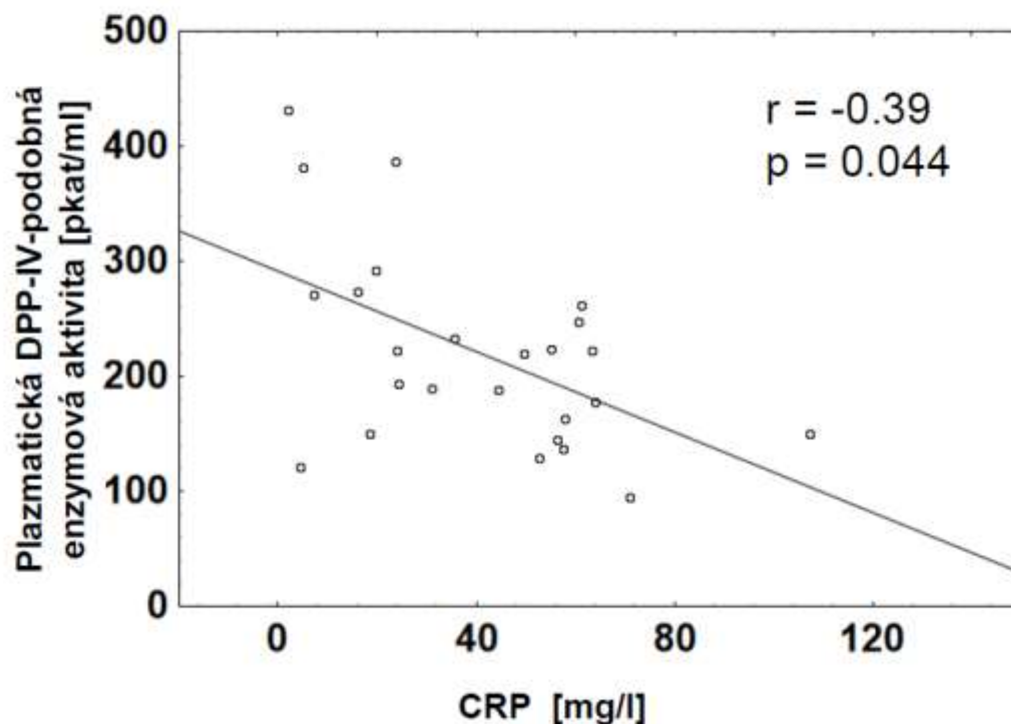
II. Porovnání exprese a DPP-IV-podobné aktivity vybraných DASH molekul, jejich substrátů a receptorů u pacientů s revmatoidní artritidou (RA) ve srovnání s pacienty s osteoartrózou (OA) na lokální a systémové úrovni

Periferní krev

Ve vzorcích BMNC pacientů s aktivní RA ve srovnání s nemocnými s OA nebyl pozorován statisticky významný rozdíl v DPP-IV-podobné enzymové aktivitě ani procentuálním zastoupení DPP-IV či CXCR4 pozitivních buněk (Tabulka 6).

Pacienti s aktivní RA ve srovnání s kontrolní skupinou ovšem vykazovali signifikantně nižší plazmatickou DPP-IV-podobnou enzymovou aktivitu i koncentraci DPP-IV v krevní plazmě (Tabulka 6). Tato aktivita u pacientů s RA významně negativně korelovala s koncentrací proteinu akutní fáze CRP ($r = -0.39$ $p<0.05$, Obrázek 8).

U pacientů s RA byla oproti kontrolní skupině pozorována vyšší plazmatická koncentrace SDF i SP, ale nebyla zde nalezena žádná statisticky významná korelace ani s DPP-IV-podobnou enzymovou aktivitou ani s ELISA stanovenými koncentracemi DPP-IV a FAP. Rovněž nebyl pozorován statisticky významný vztah obou prozánětlivých mediátorů k přítomnosti DPP-IV a receptoru CXCR4 na povrchu krevních mononukleárních buněk.



Obrázek 8. Negativní korelace koncentrace CRP a DPP-IV-podobné enzymové aktivity v plazmě periferní krve pacientů s RA. Spearmanův korelační koeficient

Synoviální tekutina

Signifikantně nižší DPP-IV-podobná enzymová aktivita i množství DPP-IV pozitivních buněk bylo pozorováno ve vzorcích FMNC u pacientů s RA ve srovnání s OA (Tabulka 7). Ve FMNC nemocných s RA byla navíc pozorována i nižší intenzita exprese u DPP-IV pozitivních lymfocytů (vyjádřená jako medián intenzity fluorescence - MFI pozitivní/negativní populace; medián±SD 34.2±14.0 v RA vs. 65.3±30.6 v OA, p= p<0.05) než u pacientů s OA. Stejně jako v periferní krvi, byla u pacientů s OA i v buňkách ze synoviální tekutiny DPP-IV dominantně exprimována na CD4+ lymfocytech, zatímco u pacientů s RA nebyla podobná převažující exprese DPP-IV na CD4+ v porovnání s CD8+ lymfocyty synoviální tekutiny pozorována (Příloha 5, Obrázek 9),

Tabulka 6. Porovnání exprese a DPP-IV-podobné enzymové aktivity vybraných DASH molekul a přítomnost jejich substrátů a receptorů u pacientů s revmatoidní artritidou ve srovnání s pacienty s osteoartrózou v periferní krvi

	Revmatoidní artritida	Osteoartróza	Statistická analýza
BMNC			
DPP-IV-podobná enzymová aktivita pkat/10^5 buněk	**0.15±0.075**	**0.18±0.079**	**n.s.**
DPP-IV+ lymfocyty (%)	**46.7±14.9**	**50.9±18.4**	**n.s.**
DPP-IV+ monocyty (%)	**nedetekovatelné**	**nedetekovatelné**	
FAP+ lymfocyty (%)	**nedetekovatelné**	**nedetekovatelné**	
FAP+ monocyty (%)	**nedetekovatelné**	**nedetekovatelné**	
CXCR4+ lymfocyty (%)	**28.5±25.5**	**20.0±18.2**	**n.s.**
CXCR4+ monocyty (%)	**44.5±26.5**	**43.0±24.6**	**n.s.**
NK1R+ lymfocyty (%)	**nedetekovatelné**	**nedetekovatelné**	
NK1R+ lymfocyty (%)	**nedetekovatelné**	**nedetekovatelné**	
Plazma			
DPP-IV-podobná enzymová aktivita pkat/ml	**220.15±83.6**	**376.9±144.9**	**p<0.001**
DPP-IV koncentrace ng/ml	**465.1±215.6**	**953.3±368.4**	**p<0.001**
FAP koncentrace ng/ml	**56.7±47.3**	**73.3±30.7**	**n.s.**
SDF koncentrace pg/ml	**170.0±214.0**	**50.0 ± 51.0**	**p<0.05**
SP koncentrace pg/ml	**495.0±83.6**	**262.0±220.7**	**p<0.05**

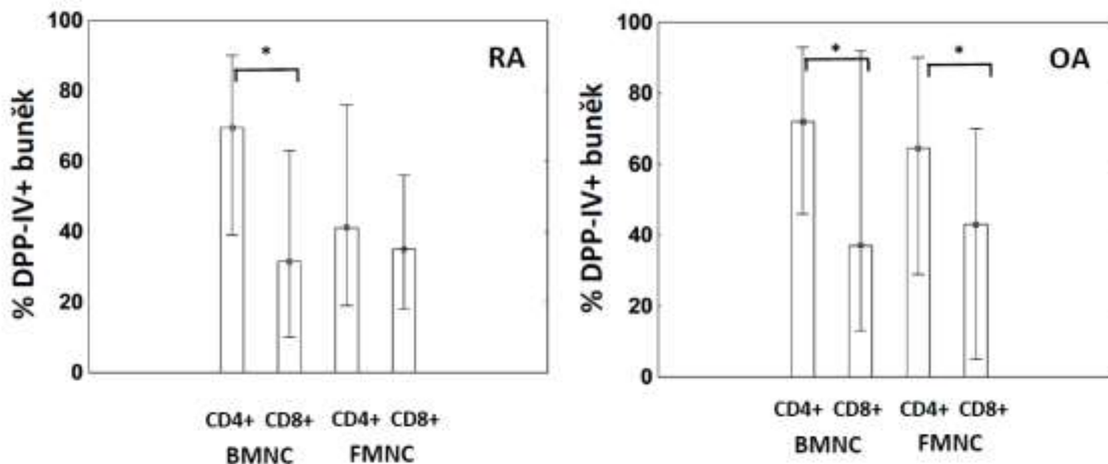
BMNC – mononukleární buňky periferní krve, DPP-dipeptidylpeptidáza, FAP – fibroblastový aktivační protein alfa, SDF - Stromal cell-derived factor 1-α, SP – substance P, n.s. – statisticky nevýznamný rozdíl mezi skupinami, hodnoty jsou vyjádřeny jako medián±SD

DPP-IV-podobná enzymová aktivita, stejně jako ELISA stanovená koncentrace DPP-IV a FAP v solubilní komponentě synoviální tekutiny mezi oběma skupinami nemocných nevykazovaly signifikantní rozdíl (Tabulka 7)

Tabulka 7. Porovnání exprese a DPP-IV-podobné enzymové vybraných DASH molekul a přítomnost jejich substrátů a receptorů u pacientů s revmatoidní artritidou ve srovnání s pacienty s osteoartrózou v synoviální tekutině

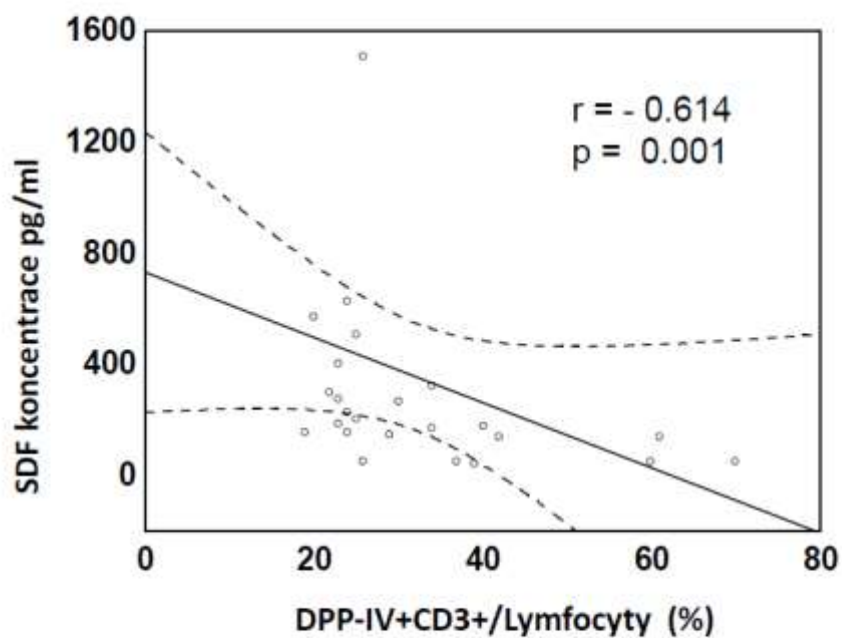
	Revmatoidní artritida	**Osteoartróza**	**Statistická analýza**
FMNC			
DPP-IV-podobná enzymová aktivita pkat/10^5 buněk	**0.16±0.16**	**0.37±0.84**	**p<0.05**
DPP-IV+ lymfocyty (%)	**33.7±15.9**	**52.6±16.4**	**p<0.05**
DPP-IV+ monocyty (%)	**nedetekovatelné**	**nedetekovatelné**	
FAP+ lymfocyty (%)	**nedetekovatelné**	**nedetekovatelné**	
FAP+ monocyty (%)	**nedetekovatelné**	**nedetekovatelné**	
CXCR4+ lymfocyty (%)	**38.5±22.6**	**23.0±24.5**	**n.s.**
CXCR4+ monocyty (%)	**37.0±23.8**	**37.0±20.9**	**n.s.**
NK1R+ lymfocyty (%)	**nedetekovatelné**	**nedetekovatelné**	
NK1R+ lymfocyty (%)	**nedetekovatelné**	**nedetekovatelné**	
Solubilní komponenta			
DPP-IV-podobná enzymová aktivita pkat/ml	**90.9±33.9**	**96.5±30.8**	**n.s.**
DPP-IV koncentrace ng/ml	**315.0 ± 152.0**	**293.0 ± 101.0**	**n.s.**
FAP koncentrace ng/ml	**58.0 ± 38.0**	**49.0 ± 41.0**	**n.s.**
SDF koncentrace pg/ml	**174.0±513.0**	**55.0 ± 219.0**	**p<0.05**
SP koncentrace pg/ml	**459.0±126.5**	**256.0±192.7**	**p<0.05**

FMNC – mononukleární buňky synoviální tekutiny, DPP-dipeptidylpeptidáza, FAP – fibroblastový aktivační protein alfa, SDF - Stromal cell-derived factor 1-α, SP – substance P, n.s. – statisticky nevýznamný rozdíl mezi skupinami, hodnoty jsou vyjádřeny jako medián±SD



Obrázek 9. Procentuelní zastoupení DPP-IV pozitivních buněk v rámci CD4+ a CD8+ populací lymfocytů. BMNC – mononukleární buňky periferní krve, FMNC – mononukleární buňky synoviální tekutiny, DPP-IV –dipeptidylpeptidáza-IV, RA – revmatoidní artritida, OA – osteoartróza, * p< 0.05, Manm-Whitneyův U test

U pacientů s RA byla pozorována vyšší koncentrace SDF i SP ve srovnání s kontrolní skupinou. Na rozdíl od systémové úrovně (periferní krve), na lokální úrovni (v kloubním výpotku) koncentrace SDF v solubilní komponentě u pacientů s RA významně negativně korelovala s množstvím DPP-IV+ CD3+lymfocytů synoviálního výpotku (R = - 0.614; p = 0.001, Obrázek 10, Příloha 1)



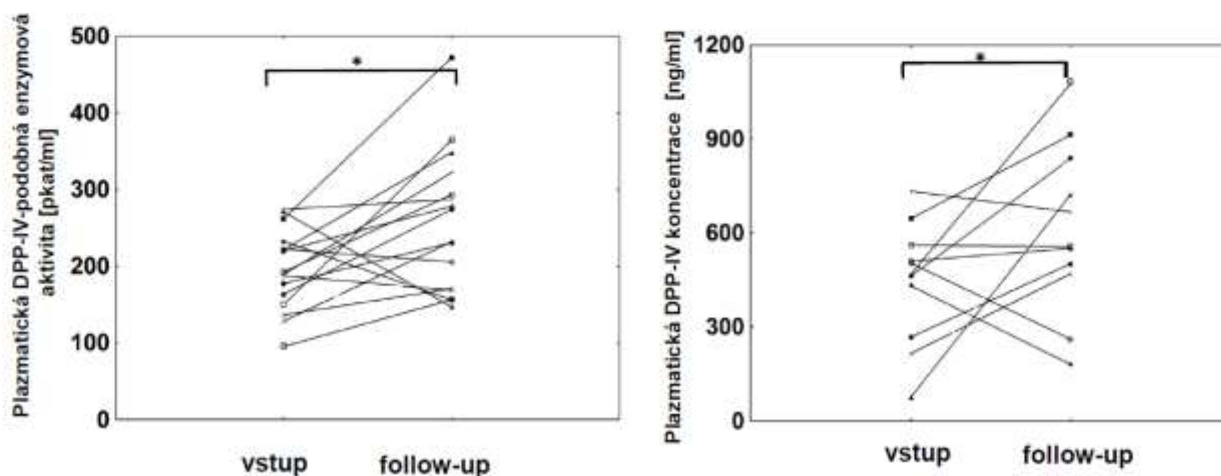
Obrázek 10. Negativní korelace procentuálního zastoupení DPP-IV+CD3+lymfocytů (DPP-IV+T lymfocytů) a koncentrace SDF (stromal cell-derived factor 1-α) v synoviální tekutině pacientů s RA. Spearmanův korelační koeficient.

III. Intraindividuální posouzení vztahu DASH molekul ke klinickému průběhu RA

Jako dominantní nositel DPP-IV-enzymové aktivity v krevní plazmě i na mononukleárních buňkách periferní krve u pacientů s RA byla v první části studie identifikována kanonická DPP-IV. Proto byla intraindividuální sledování vztahu DASH molekul ke klinickému průběhu RA zaměřena na studium exprese a enzymové aktivity kanonické DPP-IV.

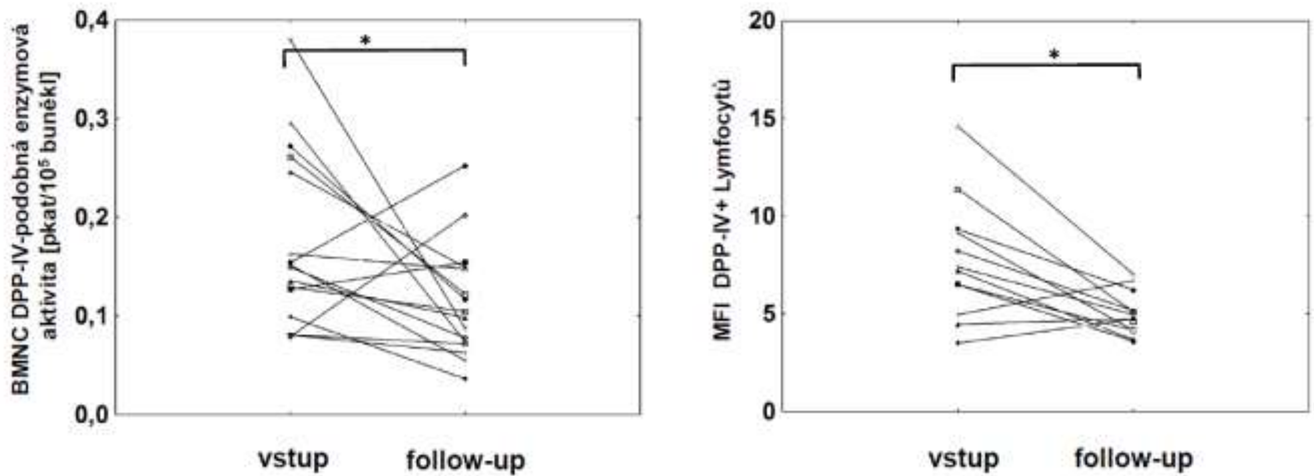
Pro analýzu byli ze skupiny pacientů s aktivní RA vybráni ti, u nich došlo k definovanému zlepšení (tj. poklesu aktivity onemocnění – viz Materiál a Metody, https://www.rheumatology.org/practice/clinical/indexes/members/Disease_Activity_Score_Sheet.pdf). U 13 pacientů našeho experimentálního souboru (72%), bylo toto zlepšení stavu onemocnění asociováno se vzestupem plazmatické DPP-IV-podobné enzymové aktivity alespoň o 20% ve srovnání se vstupní hodnotou u daného pacienta. U 3 pacientů (17%) zůstala aktivita nezměněna a u 2 (11%) poklesla.

Celkově lze říci, že plazmatická DPP-IV-podobná enzymová aktivita vzrostla na 141±46% (medián±SD, p=0.011) v porovnání se vstupními hodnotami jednotlivých pacientů. Podobný vzestup na 168±25% (medián±SD, p=0.033) vstupních hodnot individuálních pacientů byl pozorovaný i v případě koncentrace proteinu plazmatické DPP-IV stanovené metodou ELISA (Obrázek 11). Navzdory tomuto pozorování, vzestup plazmatické DPP-IV u pacientů s poklesem aktivity RA medián této hodnoty zůstává asi o 30% nižší než u pacientů bez zánětlivého kloubního onemocnění.



Obrázek 11. Intraindividuální porovnání plazmatické DPP-IV u pacientů s poklesem aktivity RA. Plazmatická DPP-IV-podobná enzymová aktivita a DPP-IV koncentrace analyzované u pacientů vykazujících pokles aktivity RA. Znázornění individuálních vstupních a follow-up hodnot. * p< 0.05, Wilkoxonův párový test

Opačný trend byl u nemocných s poklesem aktivity RA pozorován v BMNC (Obrázek 12). Povrchová DPP-IV-podobná enzymová aktivita BMNC poklesla na 66±56% (medián±SD, p=0.018) individuálních vstupních hodnot pacientů. Procentuální zastoupení DPP-IV pozitivních lymfocytů u jednotlivých pacientů sice signifikantně pokleslo o 11±80% (medián±SD, p=0.029), ale větší a významnější změny byly pozorovány v expresi DPP-IV kvantifikované jako medián intenzity fluorescence, který poklesl na 63±31% vstupních hodnot (medián±SD, p=0.005).



Obrázek 12. Intraindividuální porovnání přítomnosti DPP-IV na povrchu mononukleárních buněk periferní krve (BMNC) u pacientů s poklesem aktivity RA. DPP-IV-podobná enzymová aktivita povrchu BMNC a medián intensity fluorescence (MFI) DPP-IV+ lymfocytů u pacientů vykazujících pokles aktivity onemocnění RA. Znázornění individuálních vstupních a follow-up hodnot. * $p < 0.05$, Wilkoxonův párový test

Závěrem lze říci, že pokles aktivity RA individuálních pacientů byl spojen s nárůstem plazmatické DPP-IV a poklesem DPP-IV mononukleárních buněk periferní krve.

Diskuze

Několik dřívějších studií, včetně prací naší laboratoře, poukazuje na možnou roli dipeptidylpeptidáze-IV aktivitou a/nebo strukturou homologních molekul (DASH) v autoimunitních zánětlivých onemocněních jako např. systémový lupus erythematodes a revmatoidní artritida (Hagihara a kol., 1987, Gotoh a kol., 1989, Kobayashi a kol., 2002).

Cílem této disertační práce bylo identifikovat spektrum DASH molekul podílejících se na celkové DPP-IV-podobné enzymové aktivitě u nemocných s RA v periferní krvi a synoviální tekutině a srovnat ho s kontrolní skupinou pacientů trpících osteoartrózou (OA) a dále posoudit možný vztah těchto molekul k aktivitě RA. Námi provedené inhibiční studie za využití specifických inhibitorů spolu s ELISA analýzami potvrdily dominantní podíl kanonické DPP-IV na celkové DPP-IV-podobné enzymové aktivitě jak v periferní krvi tak i synoviální tekutině a to i včetně významné korelace mezi DPP-IV-podobnou enzymovou aktivitou a koncentrací DPP-IV proteinu. Tato pozorování jsou v souladu s prací Durinx a kol. (Durinx a kol., 2000) v níž autoři popisují 95% podíl kanonické DPP-IV na celkové DPP-IV-podobné enzymové aktivity v krevní plazmě. Naše imunodetekční studie, stejně tak jako expresní studie na úrovni mRNA, navíc neprokázaly přítomnost FAP na mononukleárních buňkách jak v periferní krvi, tak v synoviální tekutině, proto i zde je vysoce pravděpodobné, že membránově lokalizovaná DPP-IV-podobná enzymová aktivita BMNC a FMNC je pravděpodobně dominantním atributem kanonické DPP-IV. Pro další porovnání jsme se tedy zaměřili na studium kanonické DPP-IV v periferní krvi a synoviální tekutině u pacientů s RA ve srovnání s OA a následně na vlastní dynamiku DPP-IV u jednotlivých pacientů ve vztahu k aktivitě jejich onemocnění.

U pacientů trpících RA byla prokázána nižší plazmatická DPP-IV-podobná enzymová aktivity ve srovnání s kontrolní skupinou s nezánětlivým kloubním onemocněním (Busso a kol., 2005, Balaziova a kol., 2006). Toto pozorování jsme dále rozvinuli v této práci, kdy pozorovaná plazmatická DPP-IV-podobná enzymová aktivita i koncentrace proteinu DPP-IV byly o 50% nižší ve srovnání s pacienty s osteoartrózou (Příloha 4). Ačkoliv Cuchacovich a kol. (Cuchacovich a kol., 2001) prokázali, že u pacientů s RA (na rozdíl od zdravých kontrol) dochází k hypersialylaci DPP-IV a tím ke

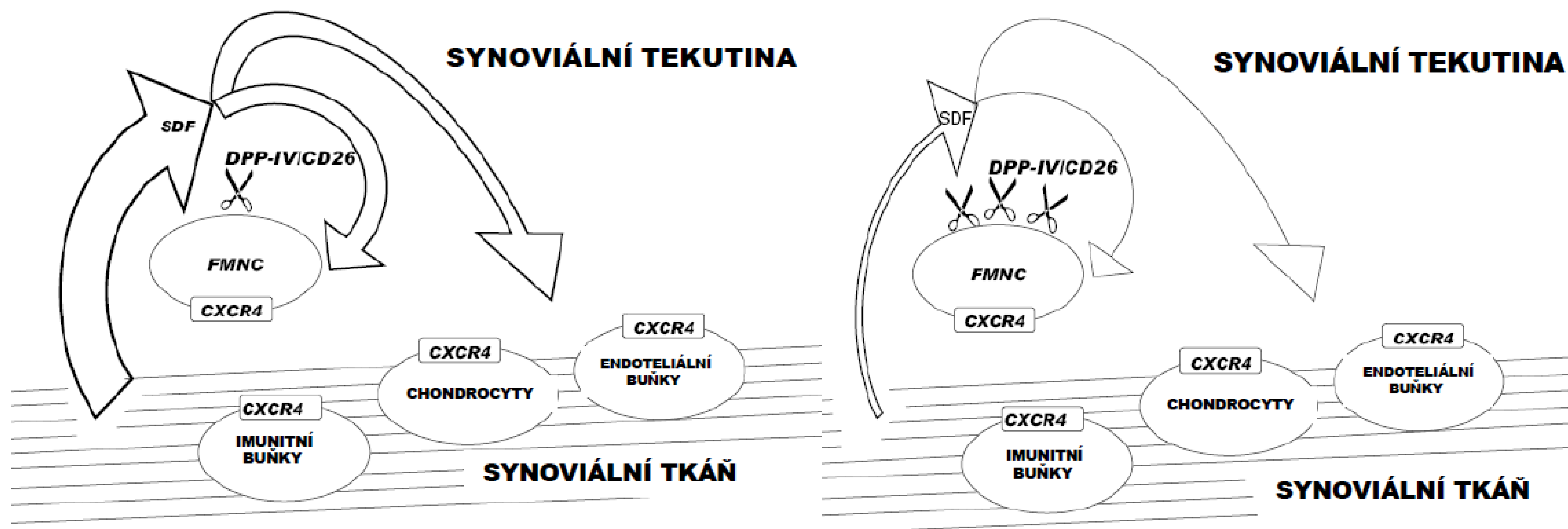
snížení její specifické enzymové aktivity, naše výsledky v souladu s dalšími (Cordero a kol., , Busso a kol., 2005, Ulusoy a kol., 2012) ukazují, že nižší enzymová aktivita pozorovaná u RA pacientů je z velké části způsobena spíše poklesem koncentrace proteinu DPP-IV v plazmě. Funkční důsledek tohoto pozorování však stále zůstává neobjasněn. Nižší DPP-IV-podobná enzymová aktivita byla některými autory popisována i v synoviální tekutině pacientů s RA ve srovnání s OA (Gotoh a kol., 1989, Sedo a kol., 2005), nicméně tento závěr nebyl u všech autorů jednoznačný (Busso a kol., 2005) a zřejmě může záviset i na způsobu vyjádření specifické enzymové aktivity (přepočet na objem nebo na koncentraci celkového proteinu ve vzorku; vzhledem k tomu, že celková koncentrace proteinu je ve výpotku u pacientů s RA významně vyšší než u OA, enzymová aktivita vyjádřená ve vztahu k celkové koncentraci proteinu mohou zdánlivě maskovat skutečný proteolytický potenciál DPP-IV přítomné v synoviální tekutině). V naší studii nebyl pozorován významný rozdíl mezi DPP-IV-podobnou enzymovou aktivitou synoviální tekutiny RA a OA pacientů vyjadřovanou na jednotku objemu výpotku (Sromova a kol., 2010, Příloha 1).

Signifikantní vzestup exprese antigenu DPP-IV na CD4+ lymfocytech byl popsán u pacientů s chronickou (medián trvání onemocnění 11.5 let) revmatoidní artritidou (Ellingsen T 2007), zatím co u krátkodobě diagnostikovaných pacientů (< 6 měsíců), signifikantní rozdíl ve srovnání se zdravými kontrolami pozorován nebyl (Ellingsen a kol., 2012). V naší studii byli jako kontrolní skupina použiti pacienti s osteoartrózou. Nepozorovali jsme signifikantní rozdíl v proporci DPP-IV pozitivních lymfocytů ani v intenzitě exprese DPP-IV mezi oběma pacientskými skupinami. Variabilita v délce trvání onemocnění, rozdíly v použitých protilátkách anti-DPP-IV a rozdílné kontrolní skupiny (pacienti s osteoartrózou vs. zdravé kontroly) použité v naší studii mohli zapříčinit pozorovaný rozdíl mezi našimi výsledky a daty v citované literatuře. V souladu s dříve publikovanými výsledky naší laboratoře (Balaziova a kol., 2006), jsme nepozorovali signifikantní rozdíly v DPP-IV-podobné enzymové aktivitě na mononukleárních buňkách periferní krve mezi RA a OA pacienty, zatímco v FMNC byla pozorována významně nižší DPP-IV-podobná enzymová aktivita u pacientů s RA. Tato nižší enzymová aktivita korespondovala i s nižším procentuálním zastoupením DPP-IV+ lymfocytů i intenzitě exprese DPP-IV (MFI) na těchto buňkách (Sromova a kol., 2010, Příloha 1). Naše pozorování podporují i dřívější práce Muscata a kolektivu, kteří pozorovali procentuálně menší populaci DPP-IV+CD3+FMNC vykazující též nižší intenzitu fluorescence u

pacientů s aktivní RA ve srovnání s OA. (Muscat a kol., 1994), avšak v další práci částečně poukazují na fakt, že přesné počty pozitivních buněk jsou do jisté míry závislé i na typu použité protilátky (Gerli a kol., 1996). DPP-IV pozitivita na buňkách je spojována s migračním fenotypem buněk infiltrujících revmatoidní synovii (Ohnuma a kol., 2006). Tento jev může být příčinou relativního úbytku DPP-IV+ FMNC v kloubním výpotku u RA pacientů pozorovaných v této studii, avšak nelze vyloučit ani primární pokles exprese DPP-IV FMNC u pacientů s RA (Sromova a kol., 2010, Příloha 1).

Na buňkách periferní krve je DPP-IV exprese dominantně přítomna na CD4+ T lymfocytech (Morimoto a kol., 1989). Podobnou distribuci jsme v naší studii pozorovali u OA BMNC i FMNC, nikoliv však u RA, kde DPP-IV pozitivita byla rovnoměrně rozložena mezi CD4+ a CD8+ buňky i když relativní proporce obou těchto subpopulací ve výpotku se mezi oběma skupinami nemocných významně nelišila (Příloha 5, Obrázek 9).

SDF, biologický substrát DPP-IV, dosahuje vyšších koncentrací v synoviální tekutině nemocných s RA než s OA (Wei a kol., 2006, Kim a kol., 2007). Tato zvýšená dostupnost SDF v synoviálním prostředí je považována za faktor vedoucí ke zvýšené neovaskularizaci, infiltraci imunitních buněk do synoviální membrány a k destruktivnímu účinku na chondrocyty (Pablos a kol., 2003, Bauer a kol., 2006). Tento efekt zvýšeného uvolňování SDF, substrátu DPP-IV, ze zánětlivé tkáně může být dále potencován námi pozorovanou nižší přítomností DPP-IV na FMNC přítomných v synoviální tekutině (Sromova a kol., 2010, Příloha 1 Obrázek 13). V průběhu pěti let po ukončení studie došlo k progresi eroze kloubů u sledované skupiny pacientů pouze u pěti. Pravděpodobně i z tohoto důvodu jsme při porovnání ostatních sledovaných parametrů na této malé skupině nemohli pozorovat žádný významný vztah k námi sledovaným parametrům. Soubor nemocných by z tohoto hlediska vyžadoval další sledování.



Obrázek 13. Hypotetizovaná vzájemná interakce DPP-IV a SDF-CXCR4 signalizace v synoviálním mikroprostředí. V synoviální tkáni pacientů s RA je buňkami produkováno a secernováno více SDF než u OA. Ačkoliv DPP-IV-podobná enzymová aktivita v synoviální tekutině se u pacientů s RA a OA neliší, nižší hydrolytický potenciál FMNC může u pacientů s RA zvyšovat dostupnost aktivního SDF a tím i vlastní parakrinní účinky tohoto mediátoru a potenciovat tak další prozánětlivé procesy. FMNC: mononukleární buňky synoviální tekutiny; SDF: stromal cell-derived factor-1; DPP-IV: dipeptidylpeptidáza-IV (Sromova a kol., 2010, Příloha 1)

Některé publikované studie spekulují možný vztah DPP-IV a aktivity RA. Cordero a kol. (Cordero a kol., 2001) popsali negativní korelaci mezi koncentrací proteinu DPP-IV v krevním séru a počtem oteklých kloubů, avšak nepozorovali rozdíly v sérové koncentraci DPP-IV mezi skupinami s aktivní a neaktivní RA. Podobně ani Ulusoy a kol (Ulusoy a kol., 2012) nepozorovali významný vztah DPP-IV v krevním séru s aktivitou RA. Vyšší exprese DPP-IV v T lymfocytech byla pozorována u pacientů s aktivní RA ve srovnání s pacienty s méně aktivní RA (Muscat a kol., 1994, Gerli a kol., 1996). Dříve publikované výsledky naší pracovní skupiny prokázaly negativní korelaci mezi enzymovou aktivitou plazmatické DPP-IV a koncentrací CRP (Balaziova a kol., 2006). Dosud prováděné studie vycházely většinou z interindividuálních skupinových porovnání, která vzhledem k poměrně značné populační variabilitě přítomnosti DPP-IV v biologickém materiálu mohou ve statistických porovnáních vést k falešně negativním výsledkům. Pokusili jsme se tedy charakterizovat vztah DPP-IV a aktivity RA na intraindividuální bázi. V průběhu naší studie měli jednotliví pacienti rozdílnou léčbu, v závislosti na klinickém posouzení ošetřujícího lékaře (glukokortikoidy, metotrexát, anti-TNF alfa a anti-CD20 protilátky, leflunomid, sulfasalazin případně jejich kombinace). Navzdory heterogenitě použité léčby jsme ve follow-up vyšetření pozorovali signifikantní vzestup plazmatické DPP-IV-podobné enzymové aktivity i koncentrace u pacientů s definovaným poklesem aktivity RA. Podobný vzestup DPP-IV-podobné enzymové aktivity, spojený s posunem z kyselých do více neutrálních glykoforem cirkulující DPP-IV, byl již dříve popsán u nemocných s klinickým zlepšením RA navozeným anti-TNF cílenou terapií (Mavropoulos a kol., 2005).

Kromě těchto změn v hladině solubilní DPP-IV jsme v naší studii pozorovali, že i DPP-IV-podobná enzymová aktivita na BMNC a exprese na lymfocytech klesá souběžně s poklesem DAS28, tedy se snížením aktivity RA u jednotlivých pacientů. Intraindividuální pokles exprese DPP-IV antigenu byl nejvíce patrný na CD4+ T lymfocytech (p=0.055), což je v souladu s popsanou dominantní přítomností DPP-IV v této lymfocytární subpopulaci u RA pacientů (Muscat a kol., 1994).

Závěry

- Kanonická DPP-IV je hlavním nositelem DPP-IV-podobné enzymové aktivity v krevní plazmě, synoviální tekutině a velmi pravděpodobně i na povrchu BMNC a FMNC u nemocných s RA (i OA).

- U nemocných s RA ve srovnání s OA je významně nižší enzymová aktivita a koncentrace DPP-IV v krevní plazmě a exprese a aktivita DPP-IV na FMNC. U pacientů s RA nebyl pozorován statisticky významný rozdíl v expresi a aktivitě DPP-IV na BMNC oproti kontrolní OA skupině.

- Intraindividuálně pozorované snížení exprese a aktivity DPP-IV na BMNC, a vzestup koncentrace i aktivity plazmatické DPP-IV spojené s poklesem aktivity onemocnění a negativní korelace plazmatické aktivity DPP-IV s CRP naznačují vztah tohoto enzymu k aktivitě RA a jeho možnou využitelnost při sledování léčby.

- Nižší dostupnost DPP-IV na FMNC může vést k omezení degradace SDF a tím ke zvýšení jeho prozánětlivého působení v synoviálním prostředí.

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Přílohy

Dipeptidyl peptidase-IV in synovial fluid and in synovial fluid mononuclear cells of patients with rheumatoid arthritis.

Sromova L, Mareckova H, Sedova L, Balaziova E, Sedo A.

Clin Chim Acta. 2010 Aug 5;411(15-16):1046-50

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Coupled expression of dipeptidyl peptidase-IV and fibroblast activation protein-α in transformed astrocytic cells.

Balaziova E, Busek P, Stremenova J, Sromova L, Krepela E, Lizcova L, Sedo A.

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Dipeptidyl peptidase-IV inhibits glioma cell growth independent of its enzymatic activity.

Busek P, Stremenova J, Sromova L, Hilser M, Balaziova E, Kosek D, Trylcova J, Strnad H, Krepela E, Sedo A.

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Intraindividual changes of dipeptidyl peptidase-IV in peripheral blood of patients with rheumatoid arthritis are associated with the disease activity

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Dipeptidyl peptidase-IV in synovial fluid and in synovial fluid mononuclear cells of patients with rheumatoid arthritis

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ABSTRACT

Background: Dipeptidyl peptidase-IV (DPP-IV) enzymatic activity controls biological halftime of multiple local mediators. Its deregulation is associated with pathogenesis of several autoimmune diseases, including rheumatoid arthritis (RA). Although DPP-IV is the canonical representative of the group, a number of other proteins have been shown to have similar enzymatic activity. This study was aimed to identify the molecular source of DPP-IV activity in synovial fluid (SF) and fluid mononuclear cells (FMNC) in patients with RA and osteoarthritis (OA). In addition, the association of DPP-IV and the concentration of stromal cell-derived factor-1α (SDF), DPP-IV substrate, were evaluated.

Methods: DPP-IV activity was measured by the kinetic fluorimetric method. The expression of studied molecules in FMNC and their concentrations in SF were assayed using flow cytometry and ELISA respectively.

Results: DPP-IV activity in SF, dominantly derived from the canonical DPP-IV, does not significantly differ between RA and OA. However, a significantly lower DPP-IV activity and expression in FMNC was found in RA as opposed to OA patients. Negative correlation between SDF concentration in SF and the relative amount of CD3+CD26+ cells was observed.

Conclusions: We report decreased presence of DPP-IV/CD26 in CD3+ FMNC in RA, which also may participate on impaired balance of SDF concentration in SF.

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1. Introduction

Dipeptidyl peptidase-IV (DPP-IV, EC 3.4.14.5, identical to CD26 differentiation antigen) is an almost ubiquitous multifunctional plasma membrane peptidase. Within the immune cells, it is typically localized in lymphocytic elements. Furthermore, a soluble form of DPP-IV, known to enhance proliferation of T-cells in response to specific antigens, is present in blood plasma [1]. DPP-IV was formerly believed to be the only enzyme capable to cleave N-terminal dipeptides from substrates with proline aminoacyl residue on the penultimate position. Yet, further research discovered a growing panel of molecules possessing DPP-IV-like enzymatic activity and a varying structure similarity, with specific cell type and tissue distribution. This fact led to the definition of the novel molecular group of multifunctional "Dipeptidyl peptidase-IV activity and/or structure homologous" (DASH) molecules [2], also including fibroblast activation protein-α (FAP) and DPP-II, DPP8, DPP9 apart from the canonical DPP-IV. As opposed to the intracellularly localized DASH, both DPP-IV and FAP are expressed as plasma membrane ectoproteases with soluble counterparts present in the body fluids and thus available to process local humoral mediators [1]. In the last decade, great strides have been made to exploit diagnostic potential of DPP-IV/CD26 and FAP [3] as well as to understand their roles in pathogenesis of a number of diseases, including rheumatoid arthritis [1,4].

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by infiltration of T-cells and monocytes into the synovium and proliferation of synoviocytes. Although its etiology has not yet been fully elucidated, there is vast evidence for the participation of multiple humoral circuits within the systemic, e.g. blood circulation as well as local, e.g. synovial, environment. A dysregulation of biologically active mediators may, among others, be the consequence of their improper proteolytic processing. A number of proinflammatory peptides supposed to be involved in the pathogenesis of rheumatoid arthritis, have their biological half-life controlled by limited proteolysis executed by DPP-IV-like hydrolytic activity [5]. In this respect, the chemokine stromal cell-derived factor

Abbreviations: DASH, dipeptidyl peptidase-IV activity and/or structure homologous; DPP-IV, dipeptidyl peptidase-IV; ELISA, enzyme-linked immunosorbent assay; FAP, fibroblast activation protein-α; FMNC, synovial fluid mononuclear cells; MFI, median fluorescence intensity; OA, osteoarthritis; RA, rheumatoid arthritis; SDF, stromal cell-derived factor-1α/CXCL 12; SF, synovial fluid.

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1-alpha (SDF), a crucial mediator controlling the influx of lymphocytes and monocyte/macrophages into the inflamed synovium and thus triggering joint destruction [6], belongs among the best natural substrates of DPP-IV.

Decreased, severity and treatment dependent DPP-IV enzymatic activity in the blood plasma [7,8] and increased expression of DPP-IV in the blood mononuclear cells (BMNC) [9,10] in active RA patients were already reported by several authors. However, there is just sparse evidence about the DPP-IV within the "local environment" of synovial fluid (SF) and fluid mononuclear cells (FMNC). The main goal of this study was therefore to assess the representation of both plasma membrane and soluble forms of DPP-IV and FAP in FMNC and SF, respectively, in patients with active RA. Additionally, the association of DPP-IV and concentration of SDF in synovial fluid was evaluated due to the fact that increased concentration of DPP-IV-controlled chemokine SDF was previously observed in SF of RA patients.

SF and FMNC of patients with osteoarthritis (OA), which possess substantially lower inflammatory component in terms of aggressiveness, progressivity, erosive potential and systemic inflammation, were used as controls.

2. Materials and methods

The study was approved by the Institutional ethic committee and was conducted in accordance with the Declaration of Helsinki. All patients signed informed consent. Patients with active RA were diagnosed according to standard criteria of the American College of Rheumatology [11]. All had active disease despite their treatment. The control group was comprised of patients with OA with knee effusion (Table 1). SF was collected into a vacutainer with sodium heparin as an anticoagulant under sterile conditions. FMNC were isolated by discontinuous Ficoll-Paque density centrifugation (Ficoll-Paque Plus, GE Healthcare, Sweden). Isolated cells were counted on a cell counter Z2 (Beckman Coulter, USA) and their viability was determined using the trypan blue exclusion.

DPP-IV enzymatic activity in SF and plasma membrane of viable FMNC was measured by a continuous rate fluorimetric assay (Spectrofluorimeter Perkin Elmer LS50B) with 7-(glycyl-prolylamido)-4-methylcoumarin (Bachem, Switzerland), final concentration of 50 μmol/L, as a substrate. Inhibition studies were performed as described previously [12]. The release of NH_2Mec was detected at excitation and emission wavelengths of 380 and 460 nm, respectively [13]. The total protein concentration in all samples was measured with Lowry's reagent [14].

Immunophenotypization was performed by a flow cytometer FACS Canto (BD Biosciences, USA) with the Diva software for acquisition and FlowJo (TreeStar Inc.) for data evaluation. For this purpose, synovial fluid was centrifuged (5 min, 4 °C, 233 g), the cell pellet was resuspended in RPMI 1640 (Lonza, Switzerland) and the resulting suspension was filtered (50 μm cup falcons, BD Biosciences, USA). 50 μL of the filtrate was incubated for 30 min with a mixture of

Table 1
Clinical characteristics of patients.

Patients	Age (years)	Female/male	Treatment (n)	CRP (mg/L)	DAS 28
	Mean (SD)			Mean (SD)	Mean (SD)
RA (n = 25)	59 (14)	20/5	Methotrexate (12) Glucocorticoids (19) Anti-TNF (5) Anti-CD 20 (1) Antimalarics (1) Leflunomide (3) Sulfasalazine (4)	37 (22)	5.8 (1.1)
OA (n = 16)	64 (11)	11/5		4 (3)	–

DAS28, disease activity score; OA, osteoarthritis; RA, rheumatoid arthritis; CRP, C-reactive protein; TNF, tumor necrosis factor.

antibodies. Mouse anti-CD3-PerCP, anti-CD4-APC, anti-CD8-APC-Cy7 and anti-CD14-PE-Cy7 antibodies from BD Bioscience, rat anti-DPP-IV/CD26-FITC and mouse anti-CXCR4-PE from RD systems were used in titres 1:20. Primary rabbit anti-FAP stalk region and anti-FAP spacer region and secondary goat anti-rabbit IgG H&L-FITC from Abcam were used in titres 1:50. Lymphocyte subsets were identified by gating analysis and fluorescence profiles obtained for 10 000 cells of each sample.

SDF, CD26 and FAP concentrations were determined by the sandwich enzyme-linked immunosorbent assay ("Duo set" ELISA kits – DY350, DY1180, DY3715 from RD Systems, UK), according to the manufacturer's instructions. A microplate reader Sunrise (Tecan, Switzerland) was used to measure absorbance at 450 nm. A wavelength correction was performed at 570 nm.

The Statistica 8.0 software (StatSoft, Inc., USA) was used for statistical analyses. Differences between the groups were evaluated with the

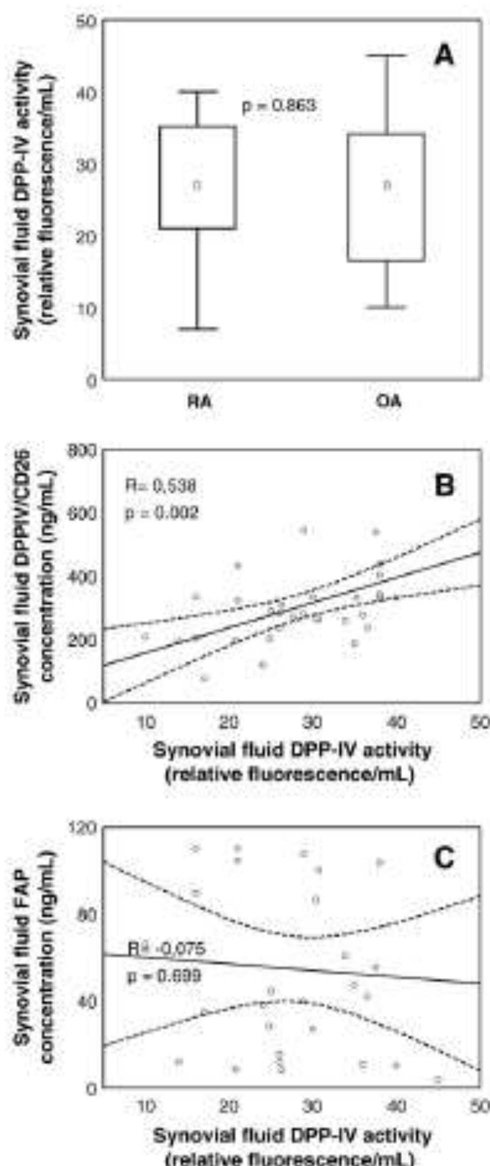

Fig. 1. DPP-IV enzymatic activity in synovial fluid (A) and its correlation with DPP-IV/CD26 (B) and FAP (C). Squares: median values; boxes: middle 25–75% of measured values; bars, minimal resp. maximal values; *: significant difference between experimental groups, p: the probability of difference.

Mann–Whitney test; correlations were assessed by Spearman's correlation coefficient.

3. Results

DPP-IV enzymatic activity in SF was detected in all patients included in the study, and was very similar in both RA and OA groups (Fig. 1A). In line with that, the immunodetection of neither CD26 (RA: 315 ± 152 ng/mL; OA: 293 ± 101 ng/mL) nor FAP (RA: 58 ± 38 ng/mL; OA: 49 ± 41 ng/mL) in SF did not demonstrate a significant difference between the diagnoses. DPP-IV enzymatic activity in SF tightly correlated with the concentration of CD26 (Fig. 1B), but not with FAP (Fig. 1C). Inhibition studies using specific inhibitor of DPP8/9 argued against significant participation of both enzymes on the total DPP-IV-like enzymatic activity in SF (not shown).

A significantly lower FMNC surface DPP-IV enzymatic activity (Fig. 2A) as well as the presence of CD26 positive cells (Fig. 2B) was observed in FMNC derived from RA patients than OA ones. DPP-IV/CD26 immunostaining in FMNC provided qualitatively identical pattern as in BMNC (not shown). No detectable immunopositivity of FAP was found using two commercially available primary antibodies, generated against different molecular domains of the molecule and reliably recognizing FAP in other cell systems. As expected, immunophenotypization studies demonstrated a significant majority of CD26 expression among CD3+ elements, ranging between 30 and 70% in individual patients, while the CD3− lymphocytes were CD26 negative with the antibody used. Cell surface DPP-IV enzymatic activity of FMNC correlated with the relative number of the CD3+ population ($R = 0.452$, $p = 0.02$). The double positive CD3+CD26+ population possessed significantly lower median fluorescence intensity (MFI; $p = 0.012$) in RA patients compared to the OA group (Fig. 2D). In contrast with OA patients, where the CD26 immunopositivity of T-cells was prevalently associated with CD4+ elements, in RA synovial fluid T-cells CD26 was distributed almost evenly between both CD4+ and CD8+ subpopulations (not shown). CD26 expression was below the detection limit in B and NK cells and reached up to 15% positive elements in monocytic CD14+ population, without significant differences between RA and OA (not shown).

As shown in Fig. 3A, a significantly higher ($p = 0.003$) SDF concentration was observed in synovial fluid of RA (median = 174 pg/mL, mean = 421 pg/mL) patients than in OA (median = 55 pg/mL, mean = 105 pg/mL) patients. Significant negative correlation ($R = -0.614$; $p = 0.001$) between the SDF concentration in synovial fluid and the amount of CD3+CD26+ cells in RA was demonstrated (Fig. 3B).

4. Discussion

In this study, we identified molecular source of DPP-IV enzymatic activity in SF and in plasma membrane of FMNC in RA and OA. Furthermore, we demonstrated lower representation of DPP-IV in FMNC derived from RA patients, as compared with OA patients. In addition, we observed for the first time inverse correlation of CD26+CD3+ FMNC and SDF concentration in SF in RA patients.

While the circulating T-cells and vascular endothelia seem to be the main sources of DPP-IV and FAP occurring in blood plasma [15,16], the origin of both molecules in synovial fluid remains enigmatic. Lower DPP-IV enzymatic activity in synovial fluid of RA patients compared to the OA, was noted in some reports [1,17], yet it was not confirmed by others [18]. In this respect, data available so far may depend whether the fluid volume or its protein concentration are considered as the factors to express specific DPP-IV activity. Since the total protein concentration is significantly higher in RA than in OA effusions, specific activity expressed relative to the protein may seemingly camouflage the real proteolytic potential of DPP-IV present in SF. In our study a significant difference between the DPP-IV enzymatic activity in synovial fluids of RA and OA patients has not been observed. Moreover, quantitative abundance of CD26 over FAP,

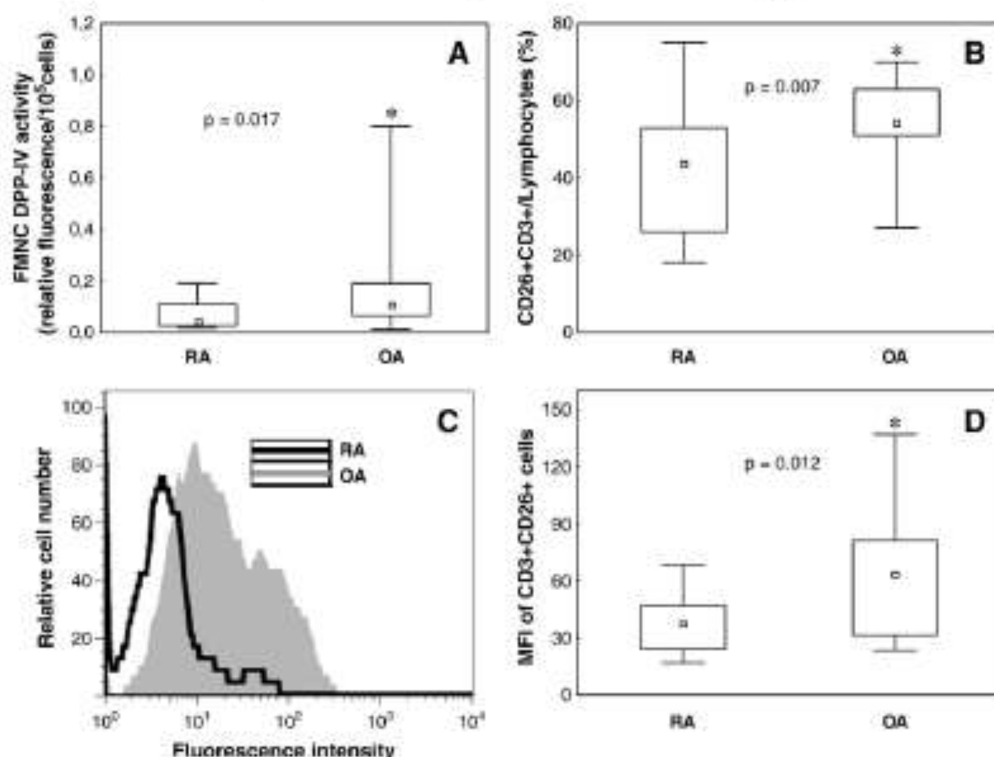

Fig. 2. DPP-IV in synovial fluid mononuclear cells of RA and OA patients. (A) DPP-IV enzymatic activity of FMNC surface. (B) Relative representation of CD26+ lymphocytes. (C) Typical pattern of DPP-IV/CD26 expression on RA and OA FMNC. (D) Median fluorescence intensity of CD26+CD3+ cells. Squares: median values; boxes: middle 25–75% of measured values; bars: minimal resp. maximal values; p: the probability of difference between experimental groups.

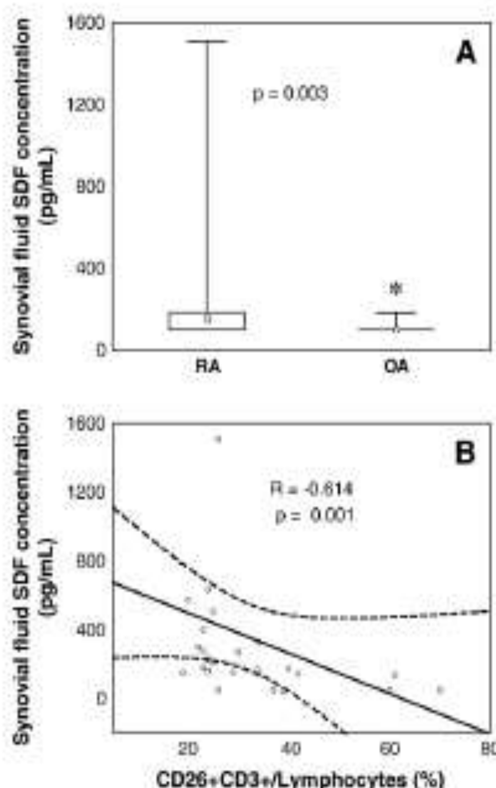

Fig. 3. Stromal cell-derived factor-1α (SDF) concentration in synovial fluid of rheumatoid arthritis (RA) and osteoarthritis (OA) patients (A) and its correlation with the relative representation of CD26+CD3+ cells (B). Squares: median values; boxes: middle 25–75% of measured values; bars: minimal resp. maximal values; p: the probability of difference between experimental groups.

the former correlating with DPP-IV hydrolytic activity, about two orders of magnitude higher DPP-IV than FAP affinity toward the Gly-Pro N-terminal sequence of the substrates [19] and results of inhibition studies using DPP8/9 inhibitor argue for canonical DPP-IV as the dominant part of DASH hydrolytic potential in SF.

Similarly, the correlation of DPP-IV enzymatic activity with CD26 immunopositivity, associated with negative results of FAP immunodetection and previously observed absence of FAP transcripts [20], suggests that canonical DPP-IV is the major, if not exclusive, molecule processing DASH substrates by the FMNC surface. Lower DPP-IV enzymatic activity of RA FMNC corresponds with the lower expression of DPP-IV/CD26 in CD3+ cells. This result supports the previous report of Muscat et al. [9], which claims smaller CD26+CD3+ FMNC population, exhibiting significantly lower median fluorescence intensity in patients with active RA compared to the OA, although his own later paper [10] partially challenged that, pointing to the dependence of results on the particular type of an anti-DPP-IV/CD26 antibody used. Immunohistochemical studies of other authors have revealed an abundant presence of CD26+ cells in the synovial tissue. In such cases, CD26+ phenotype is associated with the "migratory" phenotype of activated elements tending to infiltrate the rheumatoid synovium [21]. This phenomenon may be the cause of relative depletion of CD26+ FMNC in RA observed in our study. However, primary downregulation of CD26 expression by FMNC in RA cannot be excluded on the basis of clinical data.

Typically, in human peripheral blood, CD26 expression is dominantly restricted to the CD4+ helper/memory population of T-cells [22]. A similar distribution was observed in our study in OA FMNC, but not in RA where the CD26 positivity almost evenly spreads between CD4+ and CD8+ cells, while the relative proportion of both populations within the FMNC remains similar in both diseases. A clinical approach does not allow a clear-cut decision on whether the observed differences in the relative CD26 antigen representation within the CD4+ and CD8+ FMNC subpopulations are a consequence of specific regulation in the given cells or differing redistribution of a particular population between the synovial fluid/synovial tissue compartments.

Together, DPP-IV in FMNC and its soluble counterpart in SF seem to represent a dominant DASH processing local humoral mediators in the SF environment, while FAP, known to be abundantly expressed in rheumatoid synovial tissue and possessing also the endopeptidase collagenolytic activity, in addition to the DPP-IV-like one is probably mostly involved in the cleavage of structural proteins [4,23].

SDF, mostly synthesized and secreted into the synovial environment by synoviocytes [24,25], reaches higher concentrations in synovial fluid in RA than in OA patients [6,24]. Although commercially available anti-SDF antibodies do not discriminate intact and DPP-IV-truncated SDF, its increased availability in the synovial fluid and consequent amplification of its signalling has been noted by several studies [26]. Apart from that, an augmented expression of the CXCR4 receptor in synovial CD4+ cells of RA patients was observed [26]. The substantial role of boosted SDF-CXCR4 signalling axis in the processes of RA pathogenesis such as e.g. neovascularization, homing of fluid immune cells to the rheumatic synovium, and the antiapoptotic effect on T-cells as well as its harmful effect on chondrocytes was previously affirmed by several studies [24,26]. The role of SDF signalling via CXCR7 in FMNC still remains as a hypothetical option. Besides the increased spill out from the inflamed synovial tissue, the impaired degradation of SDF caused by the lower DPP-IV activity of FMNC might be considered a cause of their own higher sensitivity to SDF signalling. Moreover, although the FMNC surface DPP-IV only represents about 20% of the activity of synovial fluid of the respective volume (not shown), it might still be significant also for quantitative scavenging of SDF from SF.

Interestingly, ligand induced internalization of the CXCR4, SDF cognate receptor, associated with down regulation of DPP-IV/CD26 has been observed in human lymphocytes [27]. Although we have not observed significant difference in CXCR4 expression in RA and OA FMNC (unpublished results), this mechanism may also account for the decreased CD26 immunopositivity and DPP-IV enzymatic activity in FMNC exposed to increased SDF concentration in RA SF.

DPP-IV inhibition recently represents the novel therapeutic regimen in diabetes mellitus type 2 and its clinical relevance is also speculated in the treatment of several autoimmune and rheumatic diseases [1]. The ambiguous role of DPP-IV in such processes however deserves caution. DPP-IV is known to be positively involved in the stimulation of cellular immunity while due to its proteolytic activity, it mitigates the proinflammatory effect of a number of cytokines [18]. Consequently, while the systemic application of DPP-IV inhibitors demonstrated a potent anti-arthritic effect in several models hence there is growing evidence that the local – synovial – DPP-IV inhibition may be counterproductive, due to the protection of proinflammatory mediators–DPP-IV substrates [1]. Moreover, the inhibitors thought to be DPP-IV specific, may have broader off-target inhibition range within the DASH group. For example, inhibition of FAP expressed in synovial tissue may abrogate its destructive effect on the cartilage and bone of arthrotic joints [23]. Such cross/inhibition of other DASH molecules can also explain the anti-arthritic effects of DPP-IV inhibitors demonstrated in DPP-IV knockout animals [1].

In conclusion, we report a markedly lower DPP-IV enzymatic activity in the cell surface of FMNC in RA in comparison to the OA patients and identified its molecular source as a canonical DPP-IV. The lower DPP-IV hydrolytic potential of FMNC might relatively decrease effective SDF disposal, at least in their immediate environment, and thus favour their "paracrine" activation (Fig. 4). Further studies will however be required to assess the functional relevance of this hypothesis.

Acknowledgment

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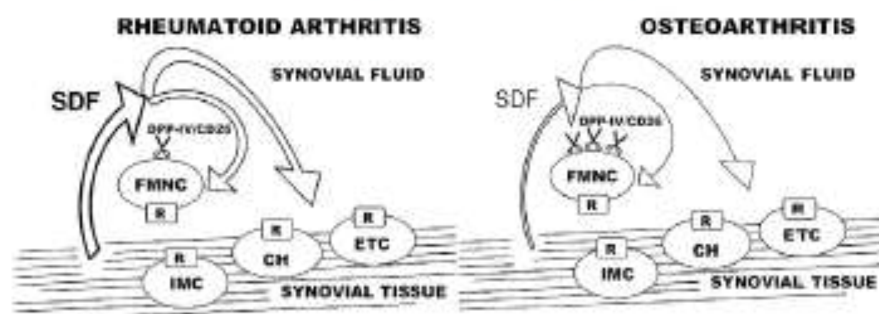

Fig. 4. Hypothesized crosstalk of DPP-IV and the SDF-CXCR4 axis in synovial fluid environment. SDF synthesis and release from cell populations resident in rheumatoid synovial tissue are augmented. Although the DPP-IV activity of SF in RA is similar to that in OA, the lower DPP-IV hydrolytic potential of FMNC might relatively decrease effective SDF disposal and thus favour their own functional "paracrine" activation and possibly also reinforcement of synovial tissue proinflammatory reactions. FMNC: fluid mononuclear cells; IMC: immune cells; CH: chondrocytes; ETC: endothelial cells; SDF: stromal cell-derived factor-1α/CXCL12; R: SDF receptor; DPP-IV/CD26: dipeptidyl peptidase-IV.

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Příloha 2

Coupled expression of dipeptidyl peptidase-IV and fibroblast activation protein-α in transformed astrocytic cells.

Balaziova E, Busek P, Stremenova J, Sromova L, Krepela E, Lizcova L, Sedo A.

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Coupled expression of dipeptidyl peptidase-IV and fibroblast activation protein-α in transformed astrocytic cells

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Abstract Dipeptidyl peptidase-IV (DPP-IV) and fibroblast activation protein-α (FAP) are speculated to participate in the regulation of multiple biological processes, because of their unique enzymatic activity, as well as by non-hydrolytic molecular interactions. At present, the role of DPP-IV and FAP in the development and progression of various types of tumors, including glioblastoma, is intensively studied, and their functional crosstalk is hypothesized. In this article, we describe the correlative expression of DPP-IV and FAP mRNA in primary cell cultures derived from human glioblastoma and associated expression dynamics of both molecules in astrocytoma cell lines depending on culture conditions. Although the molecular mechanisms of DPP-IV and FAP co-regulations remain unclear, uncoupled expression of transgenic DPP-IV and the endogenous FAP suggests that it occurs rather at the transcriptional than at the posttranscriptional level. Understanding of the expressional and functional coordinations of DPP-IV and FAP may help clarify the mechanisms of biological roles of both molecules in transformed astrocytic cells.

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Keywords DASH · Dipeptidyl peptidase-IV · Fibroblast activation protein-α · Glioblastoma

Introduction

The plasma membrane-bound serine protease dipeptidyl peptidase (DPP)-IV (EC 3.4.14.5) was discovered on the basis of its unique substrate specificity as a protease cleaving X-Pro dipeptides from the amino-terminus of peptides and proteins. However, a number of molecules constituting a group of "dipeptidyl peptidase-IV activity and/or structure homologues" (DASH) were discovered subsequently. In addition to the canonical DPP-IV, this group comprises another plasma membrane-bound protease, fibroblast activation protein-α (EC 3.4.21.-; FAP), and the intracellularly localized DPP8, DPP9, quiescent cell proline dipeptidase (QPP), and thymus-specific serine protease [1]. Moreover, in the late 1990s, few reports suggested the existence of 82-kDa plasma membrane DPP-IV-like active protein DPP IV-β in the immune cell lines. Until now, it has been neither sequenced nor cloned [2].

Owing to its capacity to process a number of biologically active peptides, involved in the processes of cell growth, migration, invasion, and neovascularization, DPP-IV is believed to be a significant player in cancer pathogenesis [3]. FAP, in addition to the DPP-IV-like exopeptidase activity, also possesses proline-specific endopeptidase activity, and hence was suggested to participate in the degradation of the extracellular matrix during tissue remodeling and cancer cell invasion [3]. Moreover, some biological roles of DPP-IV as well as FAP seem to be independent of their enzymatic activity [4].

Since the DPP-IV protein has 54% amino acid sequence identity with FAP and both genes are localized close to

each other on chromosome 2 [5], they are thought to be a product of gene duplication [6]. Co-expression of DPP-IV and FAP was described in multiple transformed cell types [7–10]. We described a correlation between the expression of DPP-IV and FAP in the human glioblastoma [11]. In past, FAP was shown to participate on the processes of glioma cell invasion [12]. In addition, heteromeric DPP-IV/FAP complexes, possessing both DPP-IV-like exopeptidase and proline-specific endopeptidase enzymatic activity, are suspected to influence the migratory and invasive potential of fibroblasts and endothelial cells [8, 13, 14].

In this study, we demonstrate the coupled expression of DPP-IV and FAP in transformed glial cells.

Materials and methods

Cell lines, primary cell cultures, and sample preparation

U138MG and U87MG cell lines derived from the human malignant glioma classified as WHO grade IV (WHO classification 2007) were obtained from ATCC (UK). U87MG possess epithelial-like morphology, with a tendency to produce long slender processes. U138MG are typically polygonal cells with cytoplasmatic processes. Both cell lines in culture form netlike formations, not reaching the full "cobblestone" confluence.

Cell lines U138MG, U87MG, and U87MG cells transfected using a vector containing full length DPP-IV cDNA were cultured in the DMEM (Sigma, Czech Republic) either supplemented with 10% FCS (Sigma) or in serum-free conditions, the latter as a model of adaptive cell differentiation [15].

Primary cell cultures were derived from 15 human glioblastomas. Fresh tissue samples were cut into small pieces and cultured in DMEM, supplemented with 10% FCS, with the addition of Streptomycin and Penicillin G (Sigma).

Cell lysates were prepared on ice in a lysis buffer (10 mM Tris–HCl pH 7.5, containing 1 mM EGTA, 1 mM Na_2EDTA, 1% Triton X-100, 0.1% SDS, and 10% glycerol) supplemented with 25 μM pepstatin A, 200 μM AEBSF and 50 μM E-64 and cleared by centrifugation at 27,000 g, 4°C for 30 min.

In order to separate the soluble and membrane subcellular fractions, the cells were resuspended in distilled water supplemented with 25 μM pepstatin A, 200 μM AEBSF, and 50 μM E-64, disrupted by sonication and centrifuged at 250 g, 4°C, 10 min, to spin down the nuclei. The following ultracentrifugation of the resulting supernatant at 136,000 g, 4°C, 30 min, produced the soluble fraction. The pellet after further resuspension in the lysis buffer with proteases' inhibitors and corresponding ultracentrifugation was then used to produce solubilized membrane fraction.

Construction of a DPP-IV cDNA vector and cell transfection

U87MG cells were transfected with human DPP-IV using the Mifepristone-inducible Gene Switch System (Invitrogen, Czech Republic) and the Lipofectamine TM2000 according to the manufacturer's instructions [16].

FISH

To detect chromosome 2, centromeric DNA probes CEP 2 (D2Z1), Spectrum Orange and CEP 18 (D18Z1) Spectrum Green (control) were employed according to the manufacturer's recommendations (Abbott Molecular, USA). BAC probes (RP11-576I16 for FAP and RP11-178A14 for DPP-IV, Pentagen, Czech Republic) were used for localizing the DPP-IV and the FAP genomic DNA sequences.

RT-PCR

The isolation of total RNA, RT-PCR quantification of the DPP-IV, FAP, and internal reference β-actin transcripts were performed as described previously [16].

Gel filtration chromatography and enzymatic activity assays

To separate and identify the DPP-IV-like exopeptidase and the proline-specific endopeptidase enzymatic activities, gel filtration chromatography on a Sephacryl S-300 (Pharmacia, Sweden) was utilized as described previously [17].

Enzymatic activity assays

The exopeptidase and the endopeptidase enzymatic activities in gel filtration fractions and cell suspensions were determined with the fluorogenic substrates glycyl-L-prolyl-7-aminomethylcoumarine (H-Gly-Pro-AMC) and *N*-benzyloxycarbonyl-glycyl-L-prolyl-7-aminomethylcoumarine (Z-Gly-Pro-AMC) (Bachem, Switzerland), respectively, using the continuous rate fluorimetric assay as described previously [17, 18]. Cell surface activity was recorded in the whole cell suspension, total activity after the addition of Triton X-100 in the same sample, with continual data acquisition [15].

ELISA

The levels of DPP-IV and FAP proteins were determined using the DuoSet ELISA development system human

DPPIV/CD26 and human FAP kits (R&D Systems, UK), respectively, according to the manufacturer's instructions. The microplate reader Sunrise (Tecan, Switzerland) was used to measure the absorbance of samples at 450 nm.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde. The immunodetection of DPP-IV and FAP was performed by an overnight incubation at 4°C with the respective monoclonal primary antibodies: mouse anti-human CD26 (clone M-A261, 1:100; Abcam, UK) and rat anti-human FAP-α (clone D28, 1:200; Vitatex, USA). This step was followed by incubation with the Alexa Fluor 488-labeled goat anti-mouse IgG and the Alexa Fluor 546-labeled goat anti-rat IgG (1:1000 and 1:600, respectively, Molecular Probes/Invitrogen). In controls, the primary antibodies were omitted. Mounted cover slips were observed and photographed using laser-scanning microscope Olympus IX81 (Olympus, Czech Republic).

Results

A significant correlation between DPP-IV and FAP was observed both at the transcription and translation levels in the primary cell cultures derived from the human glioblastoma multiforme. While the expressions of both DPP-IV and FAP mRNA templates correlated with cell surface as well as the total cellular DPP-IV-like hydrolytic activity, on the protein level only DPP-IV expression correlated with the enzymatic activity (Table 1).

The dynamic association of DPP-IV with FAP expression was demonstrated in a model of adaptive differentiation known to lead to the increase of cellular DPP-IV-like enzymatic activity [15]. For this purpose, U87MG and U138MG cell lines, both co-expressing DPP-IV and FAP and exhibiting a presence of the respective gene loci in the proper localization on the chromosome 2 (Fig. 1), were used. Upregulation of the cell surface DPP-IV-like activity was associated with the increase of DPP-IV and FAP mRNAs and proteins in both cell lines (Figs. 2, 3; Table 2).

Table 1 Correlations of DPP-IV and FAP transcripts, proteins, and DPP-IV-like enzymatic activity in primary cell cultures derived from human glioblastomas

	Transcript		Protein		Cell surface DPP-IV-like enzymatic activity	Total DPP-IV-like enzymatic activity
	DPP-IV	FAP	DPP-IV	FAP		
Transcript						
DPP-IV	x	$R = 0.58$ $P < 0.0001$ $N = 47$	$R = 0.67$ $P < 0.004$ $N = 16$	n.s.	$R = 0.56$ $P < 0.0001$ $N = 47$	$R = 0.613$ $P < 0.0001$ $N = 47$
FAP		x	n.s.	n.s.	$R = 0.47$ $P < 0.0001$ $N = 47$	$R = 0.50$ $P < 0.003$ $N = 47$
Protein						
DPP-IV			x	$R = 0.497$ $P < 0.016$ $N = 23$	$R = 0.753$ $P < 0.0001$ $N = 16$	$R = 0.79$ $P < 0.0001$ $N = 16$
FAP				x	n.s.	n.s.
Cell surface DPP-IV-like enzymatic activity					x	$R = 0.97$ $P < 0.0001$ $N = 48$

N Number of cases, *R* Spearman's correlation coefficient, *P* significance of correlation; *n.s.* not significant

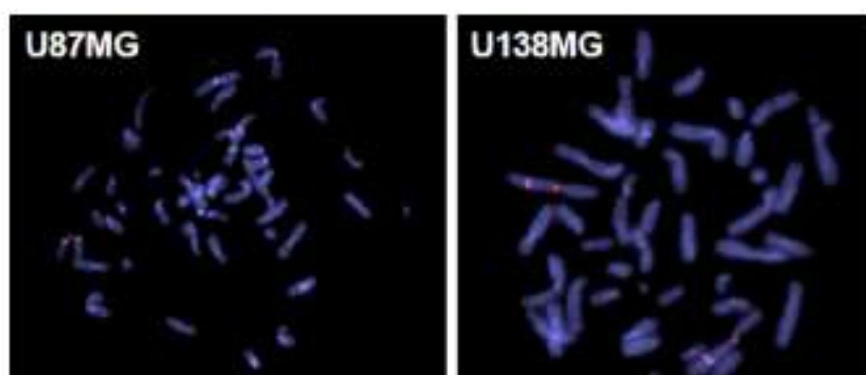

Fig. 1 Genomic localization of DPP-IV (*green*) and FAP (*pink*) loci on the chromosome 2 detected by FISH in U87MG and U138MG cell lines. Centromeric probe in *red*

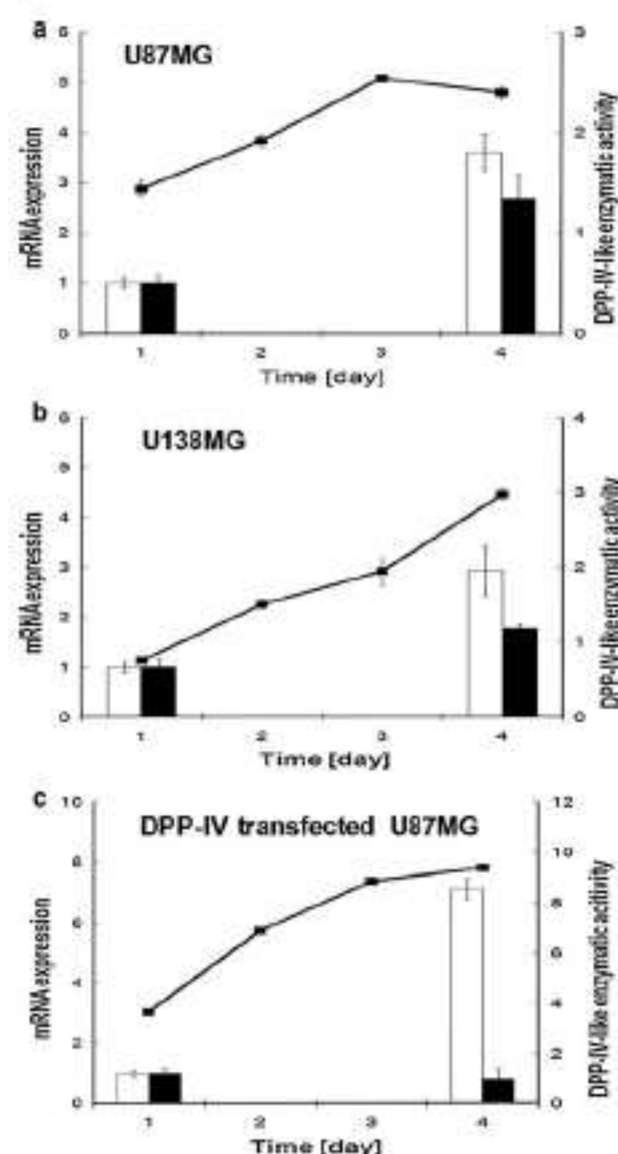

Fig. 2 Relative increment (fold change) of DPP-IV (*white bar*) and FAP (*black bar*) mRNA expressions, and the cell surface DPP-IV-like activity (*filled square*). **a** U87MG and **b** U138MG cells cultured in serum-free DMEM compared to those grown in 10% FCS-supplemented DMEM. **c** DPP-IV transfected U87MG cells treated by 0.25 nM Mifepristone to upregulate transgenic DPP-IV compared to the untreated counterparts of the control

To distinguish DPP-IV and FAP within the whole DPP-IV-like activity, the gel filtration separation was introduced before the enzymatic assay. The elution pattern of exopeptidase DPP-IV-like activity using H-Gly-Pro-AMC demonstrated two molecular weight (MW) forms of about 410–610 kDa and 130–230 kDa MW, the former about four times higher and the latter about 30% lower in cells cultured in serum-free media compared to the standard culture conditions (Fig. 4). In the same setting, using, however, Z-Gly-Pro-AMC as a substrate targeting the proline-specific endopeptidase activity, MW peaks corresponding to 410–610 kDa and MW 60–90 kDa were detected. In U87MG cells, cultured in serum-free media, both the DPP-IV-like exopeptidase and the proline-specific endopeptidase activities increased in fractions corresponding to MW of about 410–610 kDa (Fig. 4). To identify the subcellular localization of the given enzyme activity, gel filtration was also performed using the isolated plasma membrane and soluble fractions. DPP-IV-like exopeptidase and the proline-specific endopeptidase activities eluting in 410–610 kDa MW-region were dominantly represented in the plasma membrane subcellular fraction, while the DPP-IV-like exopeptidase and the proline-specific endopeptidase activities of 130–230 kDa MW and 60–90 kDa, respectively, occured mostly in the soluble fraction (Fig. 5).

As opposed to U87MG cells, the proline-specific endopeptidase activity in the MW-region of about 410–610 kDa was not detectable in U138MG cells, under neither the growth factors proficiency nor the deficiency culture conditions, while the DPP-IV-like exopeptidase activity in U138MG cells rose similarly as in U87MG cells (not shown).

The changes in DPP-IV and FAP expression, induced by cultivation under growth factors deficiency conditions were reverted by an addition of 10% FCS into the culture media.

In DPP-IV transfected U87MG cells, neither the upregulation of FAP mRNA and protein expression nor the increase of relevant proline-specific endopeptidase activity was observed upon the induction of transgenic DPP-IV overexpression (Figs. 2, 4; Table 2).

Discussion

The role of DPP-IV and FAP in multiple physiological as well as pathological processes, including regulation of growth, migration, and invasion of transformed cells, has been revealed and their functional crosstalk is hypothesized. In this article we describe for the first time the coupled expression of DPP-IV and FAP in human transformed astrocytic cells.

The previous study by the authors demonstrated a correlation between DPP-IV and FAP transcripts in the human glioblastoma multiforme [11]. To better characterize the nature of this observation and to assess the coincidence of both molecules in transformed astrocytes, further studies using glioblastoma-derived primary cell cultures were performed. A correlation of DPP-IV and FAP mRNA between themselves, as well as with the appropriate protein was confirmed and indirectly supports co-regulation of both molecules. Significant correlation of the DPP-IV protein expression with the corresponding enzymatic

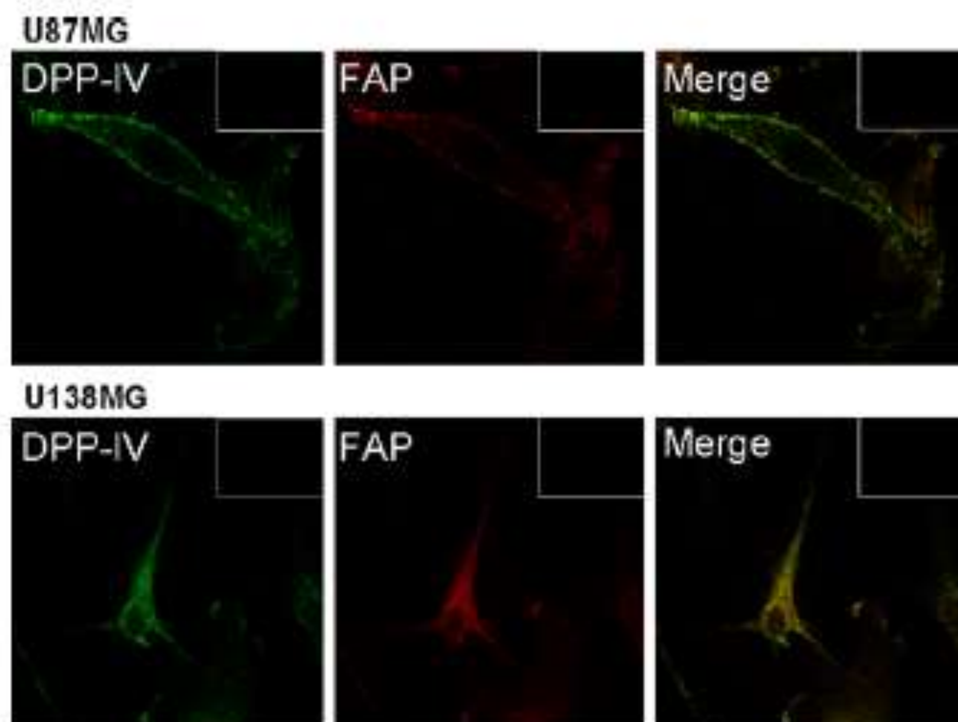

Fig. 3 Immunodetection of DPP-IV (*green*) and FAP (*red*) in U87MG and U138MG cell lines. Coverslip with adhered cells was incubated in serum-free DMEM for 72 h. *Insets*: staining controls

Table 2 DPP-IV and FAP immunopositivity (ELISA) in U87MG, U138MG, and DPP-IV transfected U87MG glioma cell lines

	Culture conditions	DPP-IV Mean ± SD	FAP (ng/mg protein)
U87MG	SFM[a]	56.1 ± 0.5	90.5 ± 12.3
	FCS[b]	13.5 ± 6.7	23.3 ± 1.2
U138MG	SFM[a]	66.4 ± 1.2	108.7 ± 0.7
	FCS[b]	36.6 ± 3.3	88.4 ± 1.8
Transfected U87MG	Mifepristone[c]	163.4 ± 1.7	5.4 ± 1.7
	Controls[d]	3.8 ± 0.7	8.4 ± 1.8

[a] Serum-free media; [b] DMEM supplemented with 10% foetal calf serum; [c] DPP-IV transfected U87MG cells stimulated to express transgene DPP-IV by 0.25 nM Mifepristone; [d] Untreated DPP-IV transfected U87MG cells

activity argues in support for canonical DPP-IV which represents a dominant source of the DPP-IV-like enzymatic activity in the primary cell cultures derived from the glioblastoma multiforme.

To exemplify the dynamic aspects of DPP-IV and FAP coupled expression, permanent glioblastoma cell lines possessing the characteristic genomic localization of DPP-IV and FAP were used. The cells cultured under the conditions of the adaptive cell differentiation were known to increase their DPP-IV-like activity [15]. In such settings, an increase of the cell surface DPP-IV-like enzymatic activity is consistently associated with the coupled upregulation of DPP-IV and FAP transcripts and proteins in both cell lines. In order to discriminate participation of proteolytically active FAP from the whole DPP-IV-like activity and to eliminate its superposition with the canonical DPP-IV, the proline-specific endopeptidase enzymatic activity characteristic for FAP was measured using Z-Gly-Pro-AMC as a substrate [18]. However, a possible interference with the prolyl-endopeptidase (PEP; EC 3.4.21.26), responsible for substantial part of the cellular proline-specific endopeptidase activity in biochemical assay had to be considered. Thus, to distinguish both endopeptidase activities known to differ in their MW, gel filtration was used. In U87MG cells cultured in serum-free media, the increments of both the DPP-IV-like exopeptidase and the proline-specific endopeptidase activities were observed in the MW-region of about 410–610 kDa, namely, in samples from the plasma membrane fractions, which corresponds well with the expected DPP-IV and FAP subcellular localization. Although both DPP-IV and FAP are typically described as homodimers with MW of 180–220 kDa [1] their higher oligomeric complexes, possessing MW of about 480–820 kDa occur [8]. Another proline-specific endopeptidase activity peak, present in the elution profiles of soluble but not plasma membrane subcellular fractions and showing MW of about 80 kDa, corresponds with the cytosolic PEP [19]. Significant increase of the DPP-IV-like activity in 410–610 kDa MW-region, which is attributable mostly to the plasma membrane DPP-IV-like species, corresponds with the upregulation of DPP-IV and FAP transcripts and protein occurring in serum-free conditions. In addition, a minor decrease in the 130–230 kDa peak was observed, representing mostly intracellular forms of the DPP-IV-like activity. The roles of modified expression of

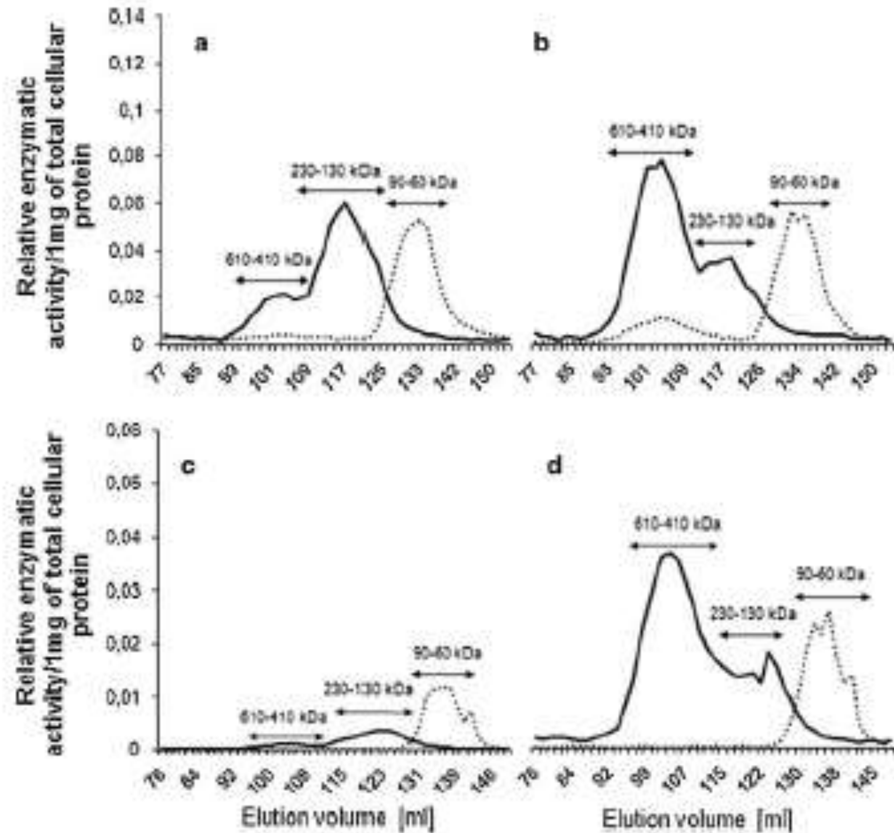

Fig. 4 Elution profile of the total DPP-IV-like exopeptidase (*full line*) and proline-specific endopeptidase (*dotted line*) activity in U87MG cell line cultured **a** in 10% FCS supplemented DMEM, **b** serum-free DMEM, **c** DPP-IV transfected U87MG untreated cells of the control and **d** DPP-IV transfected U87MG cell treated by 0.25 nM Mifepristone to upregulate transgenic DPP-IV expression

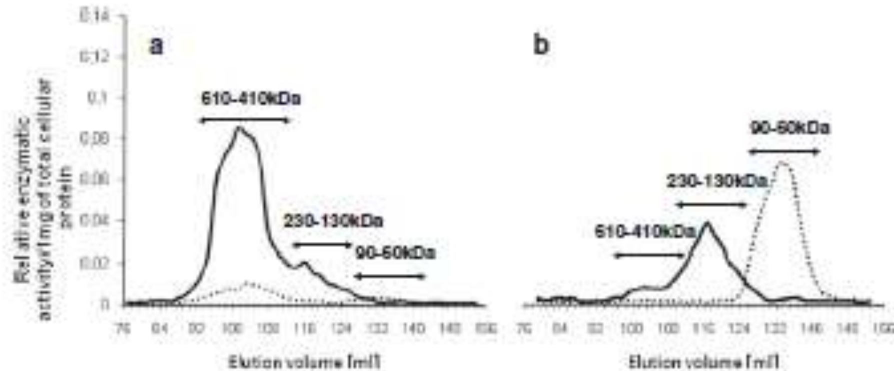

Fig. 5 Elution profile of the plasma membrane **a** and soluble **b** DPP-IV-like exopeptidase (*full line*) and proline-specific endopeptidase (*dotted line*) activity in U87MG cell line cultured in serum-free DMEM

DPP8/9 and/or changed proportion of the intracellular/plasma membrane localized DPP-IV on this phenomena remain under dispute.

Although we did not observe the increase of proline-specific endopeptidase activity which is attributable to FAP in U138MG cell line, an abundant expression of FAP transcript accompanied by a high FAP protein concentration was found. There are at least eleven known alternative splice variants of the FAP primary transcript, some of them encoding isoforms devoid of enzymatic activity [The AceView Database, http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/av.cgi?db=human&c=Gene&l=FAP, 20, 21]. Thus, the lack of the characteristic enzymatic activity might be caused by the expression of enzymatically inactive FAP variant(s) in U138MG cells; however, the data to confirm that explanation are missing.

An uncoupled expression of the endogenous FAP and transgenic DPP-IV in transfected cells, where the DPP-IV gene is inserted away from its physiological genomic context, suggests that the co-regulation of both molecules is more likely a result of a joint control of both genes' expressions than a consequence of an indirect reciprocal regulation, involving changes of their mRNA and/or protein.

Taken together, the associated dynamics of DPP-IV and FAP expressions at the transcriptional and translational levels in the glioblastoma cell lines further argues in favor of their putative joint involvement in biological processes.

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Příloha 3

Dipeptidyl peptidase-IV inhibits glioma cell growth independent of its enzymatic activity.

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Dipeptidyl peptidase-IV inhibits glioma cell growth independent of its enzymatic activity

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ABSTRACT

Malignant gliomas exhibit abnormal expression of proteolytic enzymes that may participate in the uncontrolled cell proliferation and aberrant interactions with the brain extracellular matrix. The multifunctional membrane bound serine aminopeptidase dipeptidyl peptidase (DPP)-IV has been linked to the development and progression of several malignancies, possibly both through the enzymatic and nonenzymatic mechanisms.

In this report we demonstrate the expression of DPP-IV and homologous proteases fibroblast activation protein, DPP8 and DPP9 in primary cell cultures derived from high-grade gliomas, and show that the DPP-IV-like enzymatic activity is negatively associated with their *in vitro* growth. More importantly, the DPP-IV positive subpopulation isolated from the primary cell cultures using immunomagnetic separation exhibited slower proliferation. Forced expression of the wild as well as the enzymatically inactive mutant DPP-IV in glioma cell lines resulted in their reduced growth, migration and adhesion *in vitro*, as well as suppressed glioma growth in an orthotopic xenotransplantation mouse model.

Microarray analysis of glioma cells with forced DPP-IV expression revealed differential expression of several candidate genes not linked to the tumor suppressive effects of DPP-IV in previous studies. Gene set enrichment analysis of the differentially expressed genes showed overrepresentation of gene ontology terms associated with cell proliferation, cell adhesion and migration.

In conclusion, our data show that DPP-IV may interfere with several aspects of the malignant phenotype of glioma cells in great part independent of its enzymatic activity.

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1. Introduction

Gliomas rank among the deadliest human malignancies. The median survival for the most common grade IV tumors (glioblastoma multiforme) is one year despite multimodality treatment with surgery, chemotherapy and radiotherapy (Wen and Kesari, 2008). Disease recurrence is almost a rule due to the uncontrolled proliferation of glioma cells that extensively infiltrate the surrounding brain parenchyma. In addition to genetic alterations, deregulated expression of para- as well as autocrine mediators and their receptors (Hoelzinger et al., 2007), components of the extracellular matrix and proteolytic enzymes jointly contribute to the malignant phenotype of glioma cells (Rao, 2003; Louis et al., 2002; Levicar et al., 2003).

In our previous work, we detected dipeptidyl peptidase (DPP)-IV-like enzymatic activity in permanent glioma cell lines (Sedo et al., 2004) as well as in astrocytic tumors *in situ* (Stremenova et al., 2007). This enzymatic activity is an attribute of a limited number of proteases such as the canonical DPP-IV (CD26, EC 3.4.14.5) and homologous proteases fibroblast activation protein (FAP), DPP8 and DPP9 belonging to the MEROPS (http://merops.sanger.ac.uk) S9B subfamily (Sedo and Malik, 2001). The most thoroughly characterized of these molecules is DPP-IV, a multifunctional plasma membrane-bound serine dipeptidyl aminopeptidase. It is also found as a cleaved ectodomain in body fluids (Durinx et al., 2000) and is thought to proteolytically modify and thus fine tune the bioavailability and receptor binding of a large number of biologically active peptides in the pericellular space (Mentlein, 1999).

Abbreviations: DPP, dipeptidyl peptidase; FAP, fibroblast activation protein; SDF-1, stromal cell derived factor; SP, substance P; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; BSA, bovine serum albumin; MACS, magnetic cell sorting; bFGF, basic fibroblast growth factor.

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In addition to the well-studied incretins (McIntosh, 2008), the DPP-IV substrates also include several poly- and oligopeptides strongly associated with the malignant behavior of glioma cells, such as the chemokine stromal cell-derived factor (CXCL12, SDF-1) and the neuropeptide substance P (SP) (Mentlein, 1999; Bajetto et al., 2006; Palma and Maggi, 2000). Furthermore, DPP-IV executes several of its functions by protein–protein interactions that are independent of its intrinsic enzymatic activity. The binding partners of DPP-IV include proteins of the extracellular matrix, CD45, caveolin-1, thrombospondin, adenosine deaminase and plasminogen (Ohnuma et al., 2008; Liu et al., 2009; Gonzalez-Gronow et al., 2008). We speculated that plasma membrane localized DPP-IV-like enzymatic activity as well as nonproteolytic protein–protein interactions of DPP-IV may represent an important mechanism regulating the growth properties of human glioma cells (Busek et al., 2004, 2008).

In the current study we explore the role of DPP-IV in gliomagenesis by using primary cell cultures derived from high-grade gliomas and glioma cell lines inducibly expressing DPP-IV. We show that the DPP-IV-like enzymatic activity in primary cell cultures is negatively associated with their *in vitro* growth and DPP-IV overexpressing glioma cells exhibit decreased proliferation *in vitro*. In addition, forced expression of both enzymatically active and enzymatically inactive DPP-IV suppressed glioma growth in an orthotopic xenotransplantation mouse model.

2. Materials and methods

2.1. Glioma primary cell cultures and cell lines

Primary cell cultures were derived from tumor tissue samples collected from patients undergoing astrocytic tumor resection at the Department of Neurosurgery, Hospital Na Homolce in Prague, Czech Republic. The study was approved by the Institutional ethics committee and was conducted in accordance with the Declaration of Helsinki; all biopsy donors gave full informed consent. Each fresh tissue sample was sectioned into small pieces and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, Czech Republic) supplemented with 20% fetal bovine serum (FBS, Sigma), 100 µg/mL Streptomycin (Sigma) and 100 U/mL Penicillin G (Sigma). After 5–7 days, the explants were removed and the medium was replaced with DMEM supplemented with 10% FBS and antibiotics. GFAP expression was detected with a variable intensity in the majority of primary cell cultures supporting their glial origin.

Human glioma cell lines U373, T98G and U87 were from ATCC (LGC Standards, Middlesex, UK) and were cultured under standard conditions in DMEM supplemented with 10% FBS.

The CD26 positive cell subpopulation was isolated using MACS (magnetic activated cell sorting) by incubating the cells with a mouse anti-CD26 antibody (clone M A261, Acris, Germany) and then Dynabeads pre-coated with anti-mouse IgG antibodies (Invitrogen, CA, USA). After isolation, the cells were expanded and subcultured before ascertaining their growth properties.

2.2. Construction of vectors with enzymatically inactive mutant DPP-IV

The catalytic Ser^{630} of the full-length human DPP-IV inserted in the pGENE or pTRE-Tight vector was mutated to Ala by site-directed mutagenesis (Quik Change II, Agilent Technologies, Inc., CA, USA) using the following primers: forward 5′-GAATTGCAATTTGGGGCTGGGCATATGGAGGGTACGTAACCTC-3′ and reverse 5′-GAGGTTACGTACCCTCCATATGCCCAGCCCCAAATTCGAATTC-3′ (GeneriBioTech, Czech Republic).

The presence of the anticipated mutation was verified by DNA sequencing using automated DNA sequencer (ABI Prism 3100, Life Technologies Corporation, CA, USA).

2.3. Transfected cells

U373, T98G and U87 cells were transfected with DPP-IV using the mifepristone inducible Gene Switch system (Invitrogen), as described previously (Busek et al., 2006). Mifepristone (Invitrogen) in concentrations of 0.025–5 nmol/L was used to induce DPP-IV expression. In addition, a tetracycline inducible expression system (Clontech, CA, USA) was utilized. U373 cells were transfected with 4 µg of the regulatory pTet-On-Advanced plasmid and selected clones were co-transfected with the pTRE-Tight vector containing either the wild-type full-length human DPP-IV or the enzymatically inactive DPP-IV carrying an active site S630A substitution, and a linear Hygromycin marker (Clontech) using Lipofectamine 2000 (Invitrogen). Stable transfected clones inducibly expressing DPP-IV were subsequently selected with 400 µg/mL G418 (Sigma) and 200 µg/mL Hygromycin B (Invitrogen).

2.4. DPP-IV-like enzymatic activity assay

The cell surface DPP-IV-like enzymatic activity was measured in suspensions of viable cells by a continuous-rate fluorimetric kinetic assay using a plasma membrane impermeable (Bank et al., 2011) H-Gly-Pro-7-amino-4-methylcoumarin (Bachem, Bubendorf, Switzerland) as a substrate at pH7.5 and 37 °C; the total DPP-IV-like enzymatic activity was measured under the same conditions after permeabilization of the cells with 0.1% Triton X-100 (Sedo et al., 1989).

2.5. Characterization of the growth properties of glioma primary cell cultures

Cells were grown in DMEM supplemented with 10% FBS and counted every 2–3 days using a Coulter Counter Z2 (Beckman Coulter, CA, USA). The population doubling time was determined from the least square regression fit of the exponential part of the growth curve. The clonogenic assay was performed by seeding cells at a density of 50 and 150 cells/cm^2 in triplicates and counting the colonies after 2–5 weeks.

2.6. Growth of DPP-IV transfected cells, co-culture experiments

Glioma cells were grown in the presence of various concentrations of the inducing agent mifepristone and in some experiments together with a DPP-IV inhibitor Diprotin A (Bachem; 5 mmol/L).

In co-culture experiments, 4000 cells/well of wild type cells, DPP-IV transfected cells or a mixture of 1:1 wild type:DPP-IV transfected U373 cells were seeded in 96-well plates. After 24 h, the medium was exchanged with or without the addition of mifepristone, and the cells were allowed to grow for additional 72 h.

For quantification, cells were fixed and stained with methylene blue (5 g/l in 50%, v/v, ethanol) at the indicated time points, lysed with 1% sodium dodecyl sulfate and the relative cell number was determined by reading absorbance at 630 nm using a 96-well plate reader (Sunrise; Tecan, Männedorf, Switzerland). In some experiments, the cells were counted using a Coulter Counter Z2 (Beckman Coulter) to verify the results of colorimetric quantification.

2.7. Cell cycle analysis

Nuclear DNA in ethanol fixed cells was stained for 1 h with 50 µg/mL propidium iodide (Sigma) in PBS with 0.1% NaN_3 and 1 mg/mL bovine serum albumin (BSA; Sigma) in the presence of

1 mg/mL RNAse A (Sigma). Samples were analyzed using the flow cytometer FACS Canto II with Diva software (Becton Dickinson) for data acquisition. Histograms were analyzed using the Dean-Jett-Fox model in Flow-Jo (TreeStar Inc., OR, USA).

2.8. Migration assay

6×10^4 cells in DMEM were applied to the cell culture inserts with 8 μm pores (Becton Dickinson) and allowed to migrate for 24 h. Nonmigrated cells were removed using a cotton swab; cells on the lower surface of the inserts were fixed with 5% glutaraldehyde in PBS and stained with methylene blue. Five microscopic fields per insert were counted manually.

2.9. Adhesion assay

Cell adhesion was assessed in BioCoat™ Fibronectin 96-well plates (Becton Dickinson). 5×10^4 cells were added into the wells blocked with 0.1% BSA in DMEM for 15 min, and allowed to attach at 37 °C for the indicated times, after which the nonadherent cells were gently removed with three PBS washes. The adhered cells were fixed with 5% glutaraldehyde and stained with methylene blue. Colorimetric quantification was performed as described above.

2.10. Orthotopic xenograft glioma model

The experimental use of animals was approved by The Commission for Animal Welfare of the First Faculty of Medicine of the Charles University in Prague and The Ministry of Education, Youth and Sports of the Czech Republic according to the animal protection laws.

Male adult NOD.129S7(B6)-Rag1tm1Mom/J mice (The Jackson Laboratory, ME, USA) weighting approximately 25 g were used. All animals were anesthetized prior to surgery. 10^6 DPP-IV transfected U373 cells in 5 μL of DMEM were injected with a Hamilton syringe 1.2 mm anterior from the bregma and 2.5 mm sagital from the midline to a depth of 3 mm using a stereotactic device (Stoelting Co., IL, USA).

The expression of DPP-IV in the experimental group was initiated with the appropriate induction agent 1–3 days after cell implantation and maintained till the sacrification of animals. Mifepristone in sesame oil (Sigma) was administered intraperitoneally at a dose of 270 μg/kg (100 μL total volume) three times a week; the control group received 100 μL of the sesame oil alone at the same intervals. Doxycyclin hyclate (Sigma) was administered in drinking water at a concentration of 2 mg/mL; the doxycyclin solution was changed three times a week.

2.11. Tumor volume assessment

Serial 25 μm thick coronal sections were cut on a cryostat at −20 °C. Every fifth section was stained with hematoxylin and eosin and digitized at 20× magnification. Photographs were used for unbiased tumor volume estimation according to the Cavalieri principle (Mayhew and Olsen, 1991).

2.12. Immunodetection of DPP-IV and GFAP

For flow cytometric detection of DPP-IV, cells were fixed in 2% paraformaldehyde, stained for 30 min at room temperature with a phycoerythrin conjugated monoclonal anti-CD26 antibody (clone 222113, 1:40, RD Systems, MN, USA); samples were analyzed using FACS Canto II as described above. For immunocytochemistry, cells were grown on glass coverslips, air-dried at 4 °C, blocked with 3% heat inactivated FBS and incubated overnight at 4 °C with the primary antibody (anti-CD26 [clone M A261, 1:100, Acris, Herford, Germany]; anti-GFAP [GF-01, 1:200, Exbio, Czech Republic]) and then for 1 h at room temperature with the corresponding Alexa Fluor 488 conjugated secondary antibody (Invitrogen). The primary antibodies were omitted in the staining control. Slides were mounted in Aqua Polymount (Polysciences, PA, USA) and viewed on

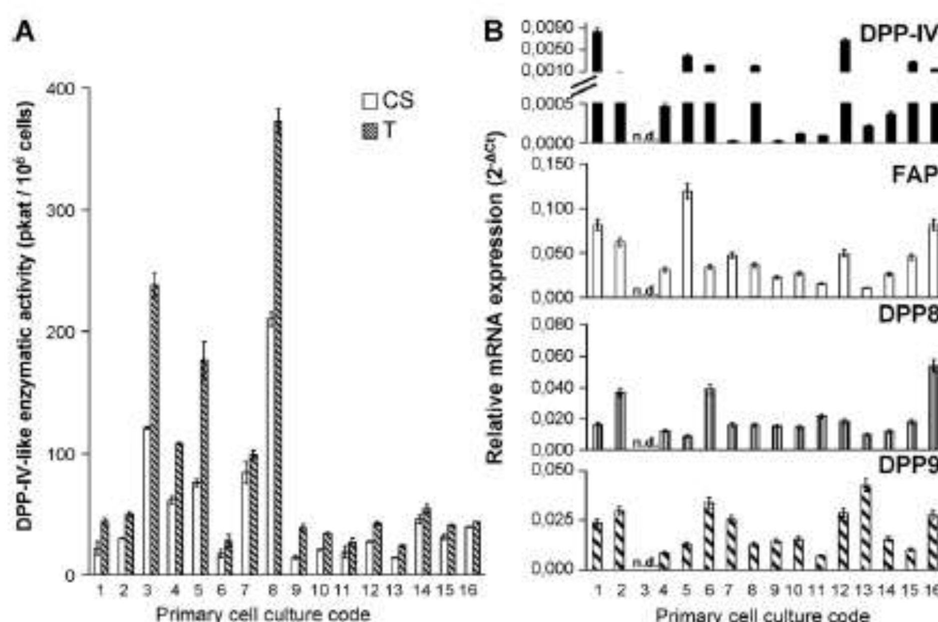

Fig. 1. Dipeptidyl peptidase (DPP)-IV and homologous proteases in glioma primary cultures. (A) DPP-IV-like enzymatic activity and (B) expression of mRNAs encoding proteases known to exhibit the DPP-IV-like activity in primary cell cultures derived from high-grade gliomas. CS: cell surface; T: total DPP-IV-like enzymatic activity. The expression of the investigated mRNAs was normalized to the expression of human β-actin using the ΔCt method. n.d.: not determined; bar graphs depict mean ± SD.

the Olympus IX70 microscope equipped with the DP30BW camera or the Olympus IX81 confocal microscope (FluoView 300, Olympus, Czech Republic).

2.13. DPP-IV-like catalytic histochemistry

25 μm brain sections were fixed in a 1:1 mixture of acetone and chloroform for 2 min at 4 °C and incubated with 7-(glycyl-L-prolylamido)-4-methoxy-β-naphthylamide hydrochloride (0.83 mmol/L, Sigma) as a substrate and Fast Blue B (Sigma) in PBS (pH 7.4) at room temperature for several minutes (Lojda, 1981).

2.14. Real time RT-PCR and microarray analysis (see supplementary methods)

The expression of DPP-IV, FAP, DPP8 and DPP9 was quantified as described previously (Stremenova et al., 2007; Busek et al., 2008).

For the microarray analysis, 0.75 μg of the amplified RNA from control and induced (72 h, 1 nmol/L mifepristone) DPP-IV transfected U373 cells was hybridized on Illumina HumanRef-8 v3 Expression BeadChip (Illumina, CA, USA) according to the manufacturer's instructions. Wild type, untransfected U373 cells were processed identically to correct for the effects of the inducing agent itself. The data were deposited to the ArrayExpress database under the accession number E-MTAB-583.

Functional annotation and gene set enrichment analysis (GSEA) was performed on transcripts with Storey's q-value ≤0.1 using the DAVID database (Huang et al., 2009, http://david.abcc.ncifcrf.gov/summary.jsp).

2.15. Statistical analysis

All statistical analyses were performed using the Statistica software (StatSoft CR s.r.o., Czech Republic) and a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. DPP-IV-like enzymatic activity is associated with slower growth in glioma primary cell cultures

In order to study the function of DPP-IV in a model that closely resembles the heterogeneous cell populations present in gliomas *in vivo*, we examined the DPP-IV-like activity and growth properties of 16 primary cell cultures derived from high-grade gliomas. Primary cell cultures in early passage expressed DPP-IV mRNA as well as mRNAs encoding several proteases known to exhibit the DPP-IV-like enzymatic activity (Fig. 1B). Expression of the canonical DPP-IV was further confirmed using flow immunocytometry and immunocytochemistry (Supplementary Fig. 1). The cell surface DPP-IV-like enzymatic activity (Fig. 1A), probably representing the sum of enzymatic activities of the membrane bound DPP-IV and FAP, was variable and correlated rather poorly with the expression of the corresponding transcripts (DPP-IV mRNA $r = 0.17$, not significant ($p = 0.55$), FAP mRNA $r = 0.51$, $p < 0.05$). mRNA expression of the intracellularly localized DPP8 and DPP9 did not correlate with the DPP-IV-like enzymatic activity.

There was a statistically significant negative correlation of the cell surface DPP-IV-like enzymatic activity and the ability to form colonies ($r = -0.52$, $p < 0.05$). In addition, there was a trend for an increase in the doubling time (median 102.5 vs. 143 h, $p = 0.08$, Mann–Whitney test) in primary cell cultures with high DPP-IV-like enzymatic activity (Fig. 2). We further isolated the DPP-IV expressing cells using immunomagnetic separation (MACS) from some of the primary cell cultures and assessed their growth properties. The resulting DPP-IV positive subpopulation exhibited minimal *in vitro* growth or progressively lost the DPP-IV-like enzymatic activity (Supplementary Fig. 2). These results corroborate the association between DPP-IV and decreased growth of the primary cell cultures.

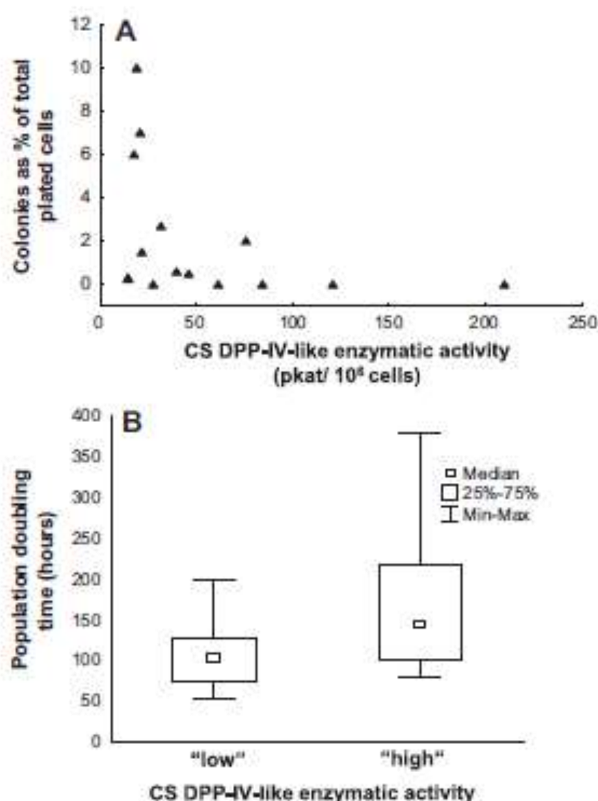

Fig. 2. Growth properties of primary cell cultures derived from high-grade gliomas in relation to their cell surface (CS) dipeptidyl peptidase (DPP)-IV-like enzymatic activity. (A) Negative correlation of the CS DPP-IV-like enzymatic activity and the ability to form colonies ($r = -0.52$, $p < 0.05$, Spearman correlation coefficient). (B) A trend for shorter population doubling time of primary cell cultures with "low" CS DPP-IV-like enzymatic activity. An arbitrary threshold for categorizing the cells as having "low" (median = 19.8 pkat/10^6 cells, $n = 8$) or "high" (median = 68.3 pkat/10^6 cells, $n = 8$) CS DPP-IV-like enzymatic activity is based on the median CS DPP-IV-like enzymatic activity of all investigated primary cell cultures (30.3 pkat/10^6 cells).

3.2. DPP-IV overexpression in glioma cells leads to decreased cell growth and a cell cycle block

The inherent heterogeneity of primary cell cultures together with the possible contribution of DPP-IV activity by multiple molecular species precluded a precise analysis in these cells of the effects of DPP-IV on glioma growth. We therefore utilized model glioma cell lines, which frequently exhibited low to undetectable endogenous DPP-IV, and transfected them with DPP-IV using a mifepristone inducible expression system. Transfected cells exhibited a concentration dependent increase of the DPP-IV enzymatic activity (Fig. 3A) that appeared within hours after the addition of the inducing agent mifepristone; DPP-IV expression was further confirmed by flow immunocytometry and immunocytochemistry (Supplementary Fig. 4A).

DPP-IV overexpression in glioma cells led to a decreased *in vitro* cell growth in different clones of T98G, U373 (Fig. 3B) and U87 cells (not shown).

In order to analyze the mechanisms of the growth inhibitory effect of DPP-IV, we performed the cell cycle analysis in DPP-IV

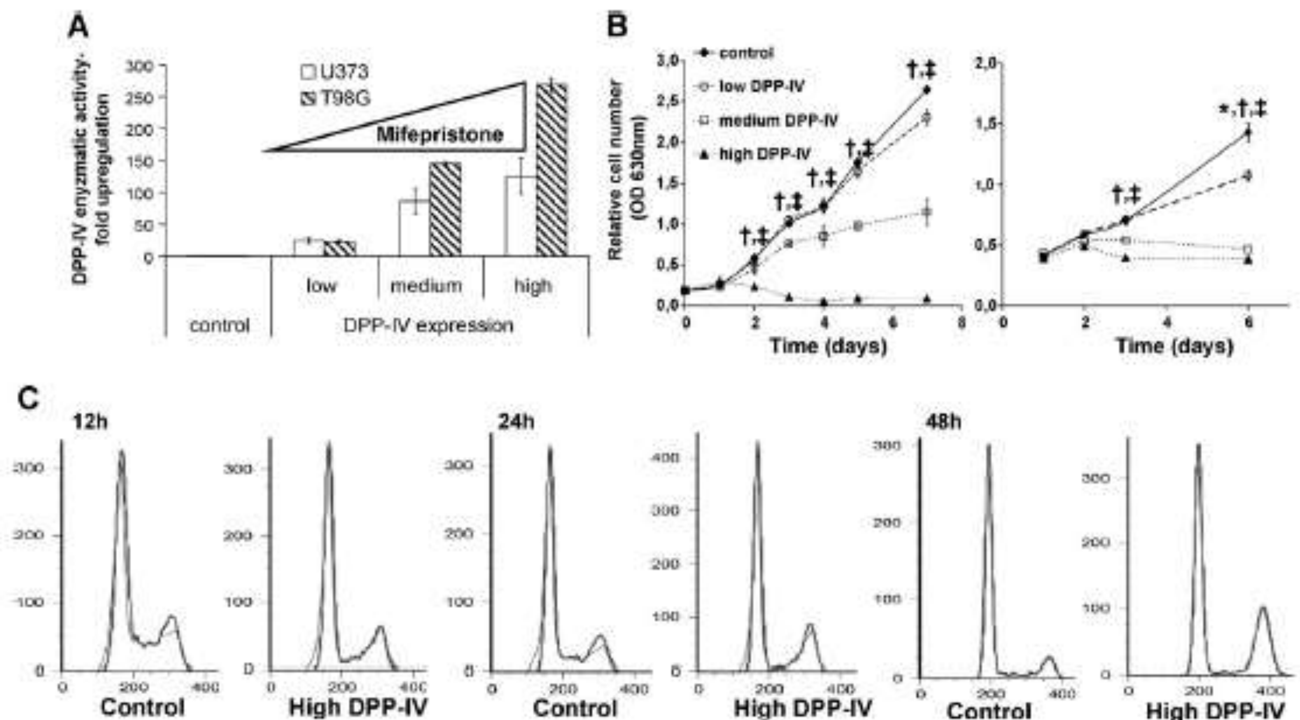

Fig. 3. Effect of dipeptidyl peptidase (DPP)-IV on glioma cell growth and cell cycle. (A) Concentration dependent increase in DPP-IV enzymatic activity in transfected glioma cell lines. DPP-IV enzymatic activity was measured 48 h after the addition of increasing concentrations of the inducing agent mifepristone. Bar graphs depict mean ± SD. (B) Growth curves of DPP-IV transfected U373 (left panel) and T98G (right panel) cells. DPP-IV expression was induced with various concentrations of mifepristone, cells were fixed and stained with methylene blue followed by colorimetric quantification. *,†,‡ $p < 0.05$ for control vs. low, medium and high DPP-IV expression respectively (repeated measurement ANOVA, Tukey *post hoc* test). (C) Flow cytometric analysis of the cell cycle in DPP-IV transfected U373 cells at various time points after the induction with mifepristone. Representative histograms (vertical axis – number of cells, horizontal axis – relative fluorescence intensity) of control cells and cells with induced DPP-IV expression are shown. Control: transfected cells not induced with mifepristone.

overexpressing cells. The proportion of the cells in the S phase declined early after DPP-IV induction and a G2/M block developed within 24–48 h (Fig. 3C). Apoptotic cell death did not significantly contribute to the growth inhibition as we did not observe a significant G0/G1 subpeak or an increase in annexin V staining (not shown). The changes were not due to the expression-inducing agent itself since mifepristone in concentrations up to 10 nmol/L had no effect on the growth or cell cycle in untransfected glioma cells (Fig. 4B and data not shown) and the concentrations used were much lower than those described to have pharmacological effects on glioma cells (Pinski et al., 1993).

We further aimed at elucidating, whether the effects of DPP-IV on glioma cells might be linked to an increased breakdown of putative soluble growth factor(s) and thus depend on its intrinsic enzymatic activity. Culturing the DPP-IV expressing cells in the presence of Diprotin A, an inhibitor that completely abrogated their DPP-IV enzymatic activity at the concentrations used, only mildly affected the growth decrease (Fig. 4A). We also observed no changes in the growth or the morphology of the wild type, untransfected cells when these were co-cultured with DPP-IV overexpressing cells using cell culture inserts (Supplementary Fig. 3). In addition, when wild type and transfected cells were seeded as a mixture at various ratios, which allowed direct interaction of the cells, the observed decline in cell numbers upon the addition of mifepristone corresponded to the decreased growth of transfected cells (Fig. 4B). Finally, expression of an enzymatically inactive mutant DPP-IV with catalytic site S630A substitution hampered the growth and induced a G2/M cell cycle arrest in U373 cells similarly to the wild type DPP-IV (data not shown). Collectively, these data suggest that the effect of DPP-IV on the growth of glioma cells *in vitro* is in large part independent of its enzymatic activity.

DPP-IV was described to interact with proteins of extracellular matrix such as collagen and fibronectin (Loster et al., 1995; Cheng et al., 2003) with possible effects on cell adhesion and migration. However, using cell adhesion and migration assays we observed decreased adhesion and spreading on fibronectin (Fig. 5A) as well as decreased migration (Fig. 5B) and collagen I induced haptotaxis (not shown) in glioma cells with high DPP-IV expression.

To identify potential functional partners and molecular mechanisms underlying the observed effects of DPP-IV in our model, we compared the whole genome expression profile in induced, DPP-IV overexpressing U373 cells and uninduced control cells. Gene set enrichment analysis performed on differentially expressed genes identified overrepresentation of genes linked to cell proliferation, cell adhesion, migration and regulation of cell development and neuron differentiation (Supplementary Tables 1–4). Transcripts for several growth factor receptors (e.g. PDGFRA, CALCRL, GRPR), proteins promoting cell cycle progression (e.g. CCND1, CDK6, PTP4A3) and involved in cell adhesion (CD97, COL8A1, COL13A1, NLGN1, NLGN4X, PCDH20, SCARF2, NrCAM) were downregulated (Supplementary Table 1). DPP-IV overexpression also led to the elevation of several putative or proven glioma associated tumor suppressors such as BEX2 (Foltz et al., 2006), RAP1GAP (Zheng et al., 2009), DUSP26 (Patterson et al., 2010), SYT13 (Jahn et al., 2010), TSPYL2 (Tu et al., 2007). On the contrary, several genes typically overexpressed in gliomas (e.g. CALCRL, COL8A1, HAS2, NES, RRM2; Cancer Genome Atlas Research Network, http://cancergenome.nih.gov/) were downregulated. Expression of DPP-IV in glioma cells *in vitro* thus reverses several changes in the expression profile typical of glioblastoma multiforme.

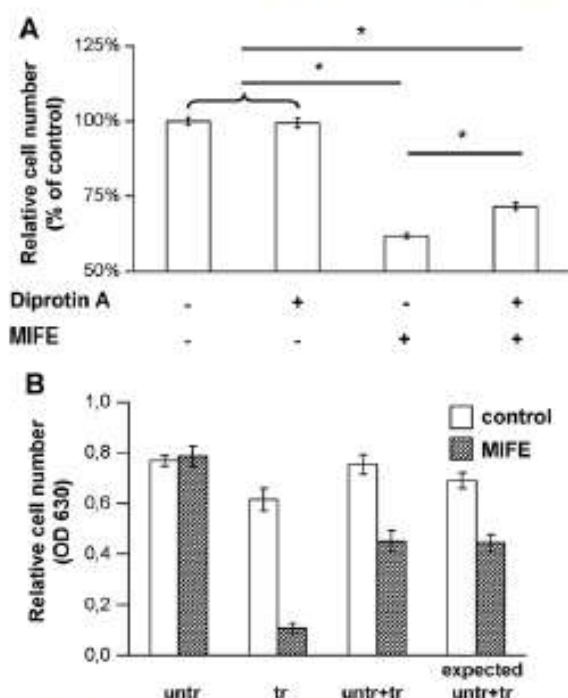

Fig. 4. Effect of dipeptidyl peptidase (DPP)-IV-like enzymatic activity inhibition on the growth of DPP-IV overexpressing glioma cells. (A) Control uninduced U373 cells and cells induced with 1 nmol/L mifepristone (MIFE) to achieve medium overexpression of DPP-IV were cultured for 72 h in the presence or absence of 5 mmol/L DPP-IV inhibitor Diprotin A. Mean ± SEM of 3 experiments performed in hexaplicates, $^*p < 0.05$, ANOVA, Tukey *post hoc* test. (B) Co-culture of untransfected (untr) and DPP-IV transfected (tr) U373 cells. "untr + tr" – cell suspensions of "untr" and "tr" mixed 1:1. "expected untr + tr" – calculated based on the values in "untr" and "tr" without co-culture. 2.5 nmol/L mifepristone was added as indicated to induce DPP-IV expression in transfected cells; cells were grown for 72 h. Bar graphs depict mean ± SD.

3.3. Forced DPP-IV expression reduces glioma growth in an orthotopic xenograft model

To test the effect of DPP-IV on tumor growth *in vivo*, transfected U373 cells inducibly expressing DPP-IV were orthotopically implanted into immunodeficient mice and DPP-IV expression was induced with mifepristone. DPP-IV induction *in situ* was confirmed by enzyme catalytic histochemistry (Fig. 6B) and immunohistochemistry (Supplementary Fig. 4C). Tumors developed in control mice as well as in animals with DPP-IV expressing cells and both exhibited features typical of high-grade gliomas, *i.e.* high cellularity and infiltrative growth with occasional necrotic areas (Supplementary Fig. 4D). The volume of tumors overexpressing DPP-IV was decreased by 42 ± 18% (mean ± SD; $p < 0.05$; $N = 16$ per experimental group) (Fig. 6A) compared to controls 5 weeks after implantation, accompanied by a statistically significant decrease of the Ki67 labeling index (median 23.1% *vs.* 18.5%, $p < 0.05$; data not shown).

To verify these results we used the tetracycline inducible expression system for transgenic DPP-IV expression. Although this expression system achieved much lower DPP-IV expression compared to the mifepristone system (Supplementary Fig. 4A and B), thus reducing the putative risk of non-specific effects of protein overexpression, we observed similar decrease in the size of the implanted tumors. Importantly, identical results were also obtained with U373 cells transfected with an enzymatically inactive mutant DPP-IV (Fig. 6A).

Neither mifepristone nor doxycycline alone affected the size of tumors originating from untransfected U373 at the doses used for the induction of DPP-IV expression (not shown).

These data demonstrate that DPP-IV functions as an inhibitor of glioma cell growth *in vivo* and that these growth inhibitory effects are independent of its intrinsic enzymatic activity.

4. Discussion

Several proteases were shown to participate on the promotion of malignancies due to their involvement in the regulation of cell proliferation, invasion into surrounding tissue or support of neovascularization (Kessenbrock et al., 2010). This predominant view of tumor associated proteases as molecules that support tumor progression may however be oversimplified. Protease inhibitors have so far failed in halting tumor progression in clinical trials and several reports demonstrate that proteases may act as tumor suppressors (reviewed in Lopez-Otin and Matrisian, 2007). Abnormal expression of DPP-IV and its association with malignant transformation was demonstrated in a number of malignancies including brain tumors (Stremenova et al., 2007). DPP-IV-like enzymatic activity and DPP-IV expression are often increased in tumor tissue (Sedo et al., 2008), but the clinical implications and the biological effects of DPP-IV on transformed cells are diverse. On the one hand, DPP-IV expression is associated with a more malignant behavior in some T cell malignancies (Sato et al., 2005), thyroid cancer (Hirai et al., 1999), gastrointestinal stromal tumors (Yamaguchi et al., 2008), and has recently been described as a marker of a subpopulation of colorectal cancer stem cells responsible for the metastatic spread of the disease (Pang et al., 2010). On the other hand, DPP-IV was reported to act as a tumor suppressor in ovarian (Kajiyama et al., 2002), prostate (Wesley et al., 2005) as well as non-small cell lung cancer cells (Wesley et al., 2004) and also in the tumor cells derived from neuroectoderm such as melanoma (Wesley et al., 1999) and neuroblastoma (Arscott et al., 2009). The underlying mechanisms of these disparate effects on cancer cells are only scarcely understood and are likely dependent on the cell type and the molecular context within the tumor microenvironment. In neuroblastoma and prostate cancer cells, DPP-IV may proteolytically inactivate the growth promoting and prometastatic chemokine CXCL12 (SDF-1) (Arscott et al., 2009; Sun et al., 2008). However, in several experimental models (Wesley et al., 1999, 2004; Pethiyagoda et al., 2000) overexpression of a mutant, enzymatically inactive form of DPP-IV was shown to produce similar results to the enzymatically active DPP-IV suggesting that in addition to the inactivation of biologically active peptides, nonproteolytic mechanisms must contribute to its tumor suppressive effects. DPP-IV overexpression in cell lines frequently triggers profound changes in cell morphology, growth, migration or invasion (Yu et al., 2010), which may reflect the changed expression of adhesion molecules (*e.g.* E cadherin, Kajiyama et al., 2003), CD44 (Wesley et al., 2004), protease inhibitors (Kajiyama et al., 2003), altered expression and subcelullar localization of bFGF (Wesley et al., 2005) or upregulation of the related protease FAP (Wesley et al., 1999, 2004).

We previously reported a grade dependent increase of the DPP-IV-like enzymatic activity in the human glioma tissue (Stremenova et al., 2007). In the present study we therefore aimed at determining the role of DPP-IV and its intrinsic enzymatic activity in the malignant behavior of glioma cells.

We observed varying cell surface DPP-IV-like enzymatic activity and DPP-IV expression on the mRNA as well as protein level in primary cell cultures derived from high-grade gliomas. Somewhat surprisingly, higher DPP-IV-like enzymatic activity was associated with slower proliferation of primary cell cultures at early passage. Although DPP-IV mRNA expression did not correlate with the DPP-IV-like enzymatic activity in the set of primary cell cultures at early passage that were used in this study, we later observed a modest

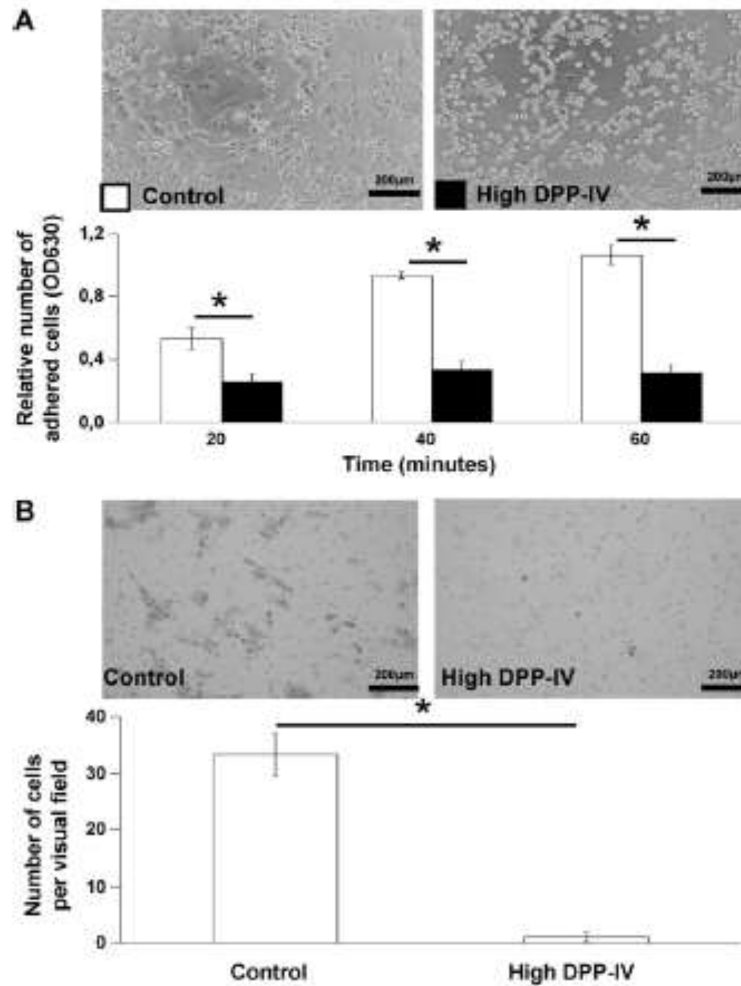

Fig. 5. Effect of dipeptidyl peptidase (DPP)-IV on glioma cell adhesion and migration. (A) Cell adhesion was evaluated 20, 40 and 60 min after seeding the DPP-IV transfected U373 cells into fibronectin-coated wells. Insets – phase contrast microphotographs of cells that were allowed to attach for 180 min without washing. $^*p < 0.05$, repeated measurement ANOVA. (B) Cell migration was evaluated by a modified Boyden chamber assay using tissue culture inserts with 8 µm pores. The assay was performed in quadruplicates. Insets – representative microphotographs of the transmigrated cells. $^*p < 0.05$, Mann–Whitney test; bar graphs depict mean ± SD.

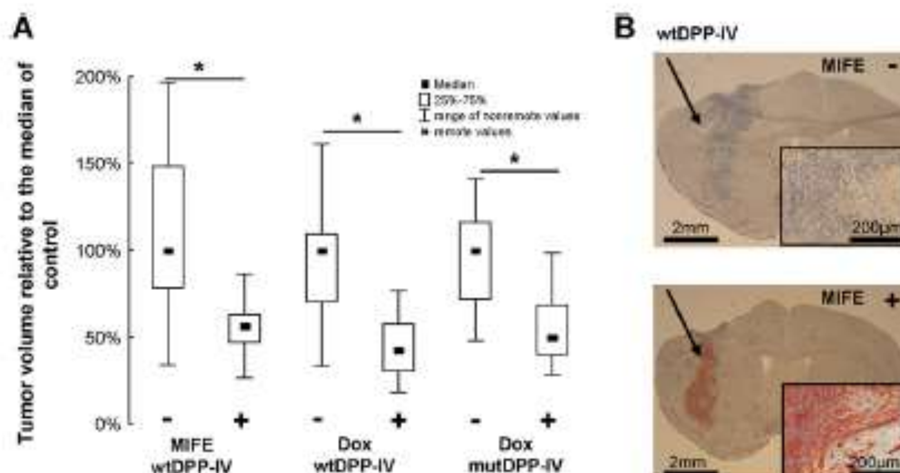

Fig. 6. Effect of dipeptidyl peptidase (DPP)-IV on glioma growth *in vivo*. (A) Tumor volume in mice orthotopically implanted with U373 cells inducibly expressing enzymatically active (wtDPP-IV) or enzymatically inactive (mutDPP-IV) DPP-IV. DPP-IV expression was induced by mifepristone (MIFE) or doxycycline (Dox) as indicated. A total of 14–19 animals per experimental group were analyzed. (B) Representative microphotographs of control and DPP-IV expressing tumors (arrows) with the detection of the DPP-IV-like enzymatic activity by enzyme histochemistry (red); nuclei were counterstained with hematoxylin. $^*p < 0.05$, Mann–Whitney test.

correlation ($r = 0.56$, $p \leq 0.05$, $n = 47$) in a substantially extended panel of primary cultures (Balaziova et al., 2011). In addition to the canonical DPP-IV (CD26), the cell surface DPP-IV-like enzymatic activity may in part reflect the presence of FAP. FAP was detected on the surface of glioma cells by Mentlein et al. (2011) and is increased in 40–60% of patients with glioblastoma (Stremenova et al., 2007; Mentlein et al., 2011; Dolznig et al., 2005; The Cancer Genome Atlas Research Network, 2008). So far, there are no data on the possible effects of FAP on glioma cell proliferation and a recent report by Mentlein et al. (2011) suggests that FAP may rather be involved in glioma cell invasion. There is one report suggesting that a minor fraction of the typically intracellularly localized DPP8 and DPP9 might also be loosely bound on the cell surface of immune cells under certain circumstances (Bank et al., 2011). However, the washing steps preceding the enzymatic assay in our studies and no correlation of the DPP8 and DPP9 mRNA expression with the DPP-IV-like enzymatic activity make their putative contribution less probable.

Importantly, the DPP-IV positive subpopulation isolated from our primary cell cultures using immunomagnetic separation showed decreased growth. The antiproliferative effect of CD26 binding, which was previously demonstrated for certain anti-CD26 antibodies in renal cell carcinoma and mesothelioma cells (Inamoto et al., 2006, 2007), cannot be completely ruled out. However, the higher proliferation of the CD26 negative subpopulation compared to the parental population before MACS indirectly argues that it is rather CD26 itself than the antibody binding that is responsible for the slower cell growth. In addition, we routinely use the respective anti CD26 antibody for the enrichment of CD26 transfected cells without significant effects on cell proliferation.

The observed negative association of DPP-IV and glioma cell growth is also consistent with our reports of DPP-IV upregulation in serum withdrawal-induced differentiation and growth arrest of glioma cells (Sedo et al., 2004; Balaziova et al., 2011).

To directly address the role of DPP-IV in glioma cells we transfected glioma cell lines with DPP-IV using an inducible expression system. Consistently with the primary cell culture data, we observed a diminished cell growth in cells expressing high levels of transgenic DPP-IV, which was associated with delayed progression of the cell cycle as suggested by the decreased proportion of cells in the S phase and a subsequently developing G2/M block. Similarly to the previous studies in lung cancer cell lines and melanoma (Wesley et al., 1999, 2004; Pethiyagoda et al., 2000), the effects of DPP-IV were largely independent of its intrinsic enzymatic activity as demonstrated using enzymatically inactive DPP-IV with an active site S630A substitution and coculture experiments. These results suggested that similarly to other cancer cell types (Arscott et al., 2009; Wesley et al., 1999, 2004, 2005), DPP-IV may function as a negative growth regulator also in glioma cells. We therefore tested the importance of DPP-IV for *in vivo* growth of glioma induced by orthotopic implantation of glioma cells into immunodeficient mice. Compared to the controls, tumor size was significantly reduced in animals with the overexpression of the transgenic DPP-IV in implanted cells. Similar results were obtained with two different expression systems with differing levels of the transgene expression and irrespective of the DPP-IV enzymatic activity. This further supports specific growth inhibitory effect of DPP-IV on glioma cells and its independence on the enzymatic activity.

The whole genome expression profiling of glioma cells overexpressing DPP-IV revealed alterations of several pathways critical for cell proliferation, as well as cell–cell and cell–extracellular matrix interactions. In addition, DPP-IV overexpression reverted the expression of a number genes typically overexpressed in gliomas. Somewhat surprisingly, DPP-IV overexpression was also accompanied by the upregulation of a number of growth factors (e.g. BMP4, BDNF, FGF18, GPI, IL11, PDGFB, TGFB3), invasion promoting genes (MMP15) and genes that may support glioma cell survival (ACSL5; Mashima et al., 2009). These gene expression changes may indicate activation of compensatory mechanisms counteracting the observed growth inhibitory effects of DPP-IV. Functional validation and determining the biological relevance of these changes require further studies. To the best of our knowledge, this is the first study on the changes in the whole genome expression profile induced by DPP-IV in cancer cells.

The growth inhibitory effects of DPP-IV in glioma cells might seemingly be in contradiction to the observation of higher DPP-IV expression and activity in glioma tissue homogenates (Stremenova et al., 2007). The "net" pro- or anti-oncogenic effects of proteases seems to represent an outcome of several factors including their differing functions in individual cell populations of both the tumor parenchyma and stroma, and varying (in)dependence of these functions on the intrinsic enzymatic activity. For example, forced expression of MT1-MMP was described to cause glioma cell death, although its presence in the tumor microenvironment promoted tumor expansion (Markovic et al., 2009). Thus, DPP-IV may – independent of its enzymatic activity – negatively influence the proliferation of glioma cells, slowing their growth possibly as a part of an adaptive response to the limited nutrition supply or hypoxia (Dang et al., 2008), yet support angiogenesis or promote intratumoral deregulation of immune response through the proteolytic processing of neuropeptides and chemokines (Ghersi et al., 2001; Mentlein, 1999). By degrading the chemokines such as CXCL12 (SDF-1), DPP-IV might also impair the recruitment of tumor suppressive neural precursor cells (Chirasani et al., 2010; Charles et al., 2011) and as a result promote glioma progression. Understanding of the complex role and the molecular mechanisms by which DPP-IV participates on gliomagenesis would allow its rational therapeutic targeting with the advantage of the availability of several specific, clinically tested DPP-IV inhibitors that are used in patients with type 2 diabetes (Neumiller et al., 2010).

Taken together, in the present work we demonstrate a growth inhibitory effect of DPP-IV on glioma cells. We show for the first time that primary cell cultures derived from high-grade gliomas express several molecules with DPP-IV-like enzymatic activity, which is negatively associated with their proliferation. DPP-IV overexpression in glioma cell lines does not promote their malignant behavior but rather slows their growth and may decrease their migration and adhesion. Our data also indicate that nonproteolytic mechanisms are important for these effects of DPP-IV in glioma cells. The whole genome expression profiling of DPP-IV overexpressing glioma cells revealed several candidate genes that shed light on the molecular pathways potentially affected by DPP-IV and will serve as a lead for further functional studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2012.01.011.

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Příloha 4

Intraindividual changes of dipeptidyl peptidase-IV in peripheral blood of patients with rheumatoid arthritis are associated with the disease activity

Sromova L., Busek P., Sedova L., Sedo A

Under re-review in BMC Musculoskeletal Disorders

Intraindividual changes of dipeptidyl peptidase-IV in peripheral blood of patients with rheumatoid arthritis are associated with the disease activity

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patients as evidenced by a decrease in the cell surface DPP-IV-like enzymatic activity as well as the median fluorescence intensity of DPP-IV staining in lymphocytes (median±SD 66±56%, p=0.018 and 63±31% of the patient's entry values, p=0.005, respectively).

Conclusions: The association between RA activity and the changes in blood plasma and blood mononuclear cell DPP-IV in individual patients supports the possible role of DPP-IV in RA pathogenesis.

Key words

Rheumatoid arthritis activity; CD26; Dipeptidyl peptidase; Osteoarthritis; Blood mononuclear cells

Introduction

Dipeptidyl peptidase-IV (DPP-IV, CD26, EC 3.4.14.5) is a plasma membrane peptidase that selectively cleaves an N-terminal dipeptide from peptides with a proline or alanine residue in the penultimate position. DPP-IV is widely expressed on epithelial and endothelial cells and a soluble form can be found in body fluids including blood plasma, where it represents at least 95% of the DPP-IV-like enzymatic activity [1]. In the immune system, DPP-IV is expressed preferentially by the CD4+CD45RO+ memory T cells, is associated with the Th1 response and its expression is upregulated following T cell activation [2]. In contrast, DPP-IV is low or undetectable on B-cells, NK cells and monocytes in healthy adults [3]. DPP-IV functions as a T cell co-stimulatory molecule, is involved in the T cell activation and proliferation, and these effects seem to be dependent on its intrinsic enzymatic activity [4, 3]. In *in vitro* studies, some of the DPP-IV inhibitors were demonstrated to suppress the production of various cytokines and enhance the production of the suppressive cytokine transforming growth factor beta [5].

Changes in the expression and/or the blood plasma concentration of DPP-IV are associated with several diseases including rheumatoid arthritis (RA) [6], an autoimmune inflammatory disorder characterized by synovial inflammation leading to cartilage destruction as well as systemic manifestations. The pathogenesis of RA has not been clearly elucidated so far, but involves an interplay of predisposing genetic factors, sex hormones and possibly an infectious or another immunity triggering agent that ultimately lead to an inappropriate activation of the immune system and perpetuating inflammation [7]. Given the role of DPP-IV in T cell activation [8] and its ability to cleave numerous pro-inflammatory peptides involved in the pathogenesis of RA [6], several studies examined its possible significance in the disease progression. The results of these studies, including ours, have so far revealed

(although variably) a decreased DPP-IV-like enzymatic activity in the mononuclear cells isolated from the synovial fluid of RA patients [9, 10] as well as decreased blood plasma DPP-IV enzymatic activity/concentration [10-12]. An increase of DPP-IV was reported in blood mononuclear cells and especially CD4+ T lymphocytes [13-16]. The literature data on the association of these changes with the disease activity are inconsistent [12, 10, 17, 16]. Furthermore, it is currently unclear whether the changes of DPP-IV in blood plasma and peripheral mononuclear cells relate to the disease activity on the intraindividual basis.

Materials and methods

27 patients with RA diagnosed according to the standard criteria of the American College of Rheumatology were examined during the active phase of their disease with joint effusion (active RA), 15 patients with osteoarthritis served as controls (Table 1.). RA disease activity was evaluated using the DAS28 score based on the blood plasma concentration of the C-reactive protein (CRP), patient-assessed visual analogue scale (VAS) and swollen and tender joint counts (http://www.das-score.nl/). The follow-up examination in RA patients was performed after joint effusion regression and at least 6 months after the first investigation. Intraindividual changes of the studied parameters were evaluated in patients with at least a moderate improvement of the disease activity as defined by the change of the DAS28 score (a decrease in DAS28 >0.6 if the current value was <5.1 or a decrease in DAS28 >1.2 if the current value was >5.1). The study was approved by the Institutional ethics committee and was conducted in accordance with the Declaration of Helsinki. All patients signed informed consent.

Peripheral blood was collected under sterile conditions into BD Vacutainer (BD Biosciences, USA) with sodium heparin as an anticoagulant. Blood mononuclear cells (BMNC) were isolated by discontinuous Ficoll-Paque density centrifugation in the "cell

preparation tube" with sodium heparin (CPT, BD Biosciences, USA). Isolated BMNC were counted on a cell counter Z2 (Beckman Coulter, USA); there were more than 95% of viable cells as determined by trypan blue exclusion.

The DPP-IV-like enzymatic activity in the heparinized blood plasma and in BMNC was measured by a continuous rate fluorimetric assay with 7-(glycyl-prolylamido)-4-methylcoumarin (Bachem, Switzerland, final concentration 50 μmol/L), as a substrate at pH 7.5 and 37 °C. The release of 7-amino-4-methylcoumarin was monitored at excitation and emission wavelengths of 380 and 460 nm, respectively (Spectrofluorimeter Perkin Elmer LS50B). The cell surface DPP-IV-like enzymatic activity in BMNC was determined as the activity of viable cells, the total DPP-IV-like enzymatic activity was measured under the same conditions after permeabilization of the cells with 0.1% Triton X-100 [10].

Immunophenotypization of the BMNC was performed by a flow cytometer FACS Canto (BD Biosciences, USA) with the Diva software for acquisition and FlowJo (TreeStar Inc.) for data evaluation. 50 μL of the peripheral blood was incubated for 30 min at room temperature with mouse anti-CD3-PerCP, anti-CD4-APC, anti-CD8-APC-Cy7 and anti-CD14-PE-Cy7 (all from BD Biosciences), rat anti-DPP-IV/CD26-FITC (RD systems). All antibodies were used in the titre of 1:20. Lymphocyte subsets were identified by gating analysis and fluorescence profiles were obtained for 10 000 cells in each sample. Median fluorescence intensity (MFI) of DPP-IV/CD26 expression in lymphocytes was calculated as a ratio of the median fluorescence intensity of the DPP-IV/CD26 positive and negative lymphocyte populations.

Blood plasma DPP-IV concentration was determined by the sandwich enzyme-linked immunosorbent assay ("Duo set" ELISA kit – DY1180, RD Systems, UK), according to the manufacturer's instructions. A microplate reader Sunrise (Tecan, Switzerland) was used to measure the absorbance at 450 nm, a wavelength correction was performed at 570 nm.

The Statistica 12 software (StatSoft, Inc., USA) was used for statistical analyses. The Wilcoxon pair test and the Mann-Whitney U test were used as appropriate. Correlations were analyzed by the Spearman's correlation coefficient.

Results

DPP-IV in patients with active rheumatoid arthritis

Compared to patients with osteoarthritis (OA), rheumatoid arthritis (RA) patients recruited in the active phase of their disease exhibited significantly lower blood plasma DPP-IV-like enzymatic activity (median±SD 220.15±83.6 pkat/mL in RA vs. 376.9±144.9 pkat/mL in OA, $p<0.001$) and blood plasma DPP-IV concentration (median±SD 465.1±215.6 ng/mL in RA vs. 953.3±368.4 ng/mL in OA, $p<0.001$) (Figure 1). The blood plasma DPP-IV-like enzymatic activity correlated with DPP-IV concentration determined by ELISA ($r=0.83$, $p<0.001$). In RA patients, there was a negative correlation between the DPP-IV-like enzymatic activity and the CRP concentration (Figure 2). These results were consistent with the previously published data, including ours [10, 6, 18].

Neither the DPP-IV-like enzymatic activity in blood mononuclear cells nor the DPP-IV expression in lymphocytes determined by flow cytometry were significantly different in patients with active RA compared to OA (data not shown).

Intraindividual changes of DPP-IV in blood plasma and in blood mononuclear cells parallel disease activity in RA

To evaluate whether the changes in the blood plasma DPP-IV reflect disease activity in individual patients, a follow-up evaluation was performed in 18 patients with RA during a less active phase of their disease as defined by the regression of joint effusion and improved

DAS28 score compared to the entry values. The disease activity decreased (Table 2) as evidenced by the reduction of the markers of inflammation CRP and ESR. In 13 patients (72%), this improvement was associated with a rise in the blood plasma DPP-IV-like enzymatic activity of at least 20% compared to the patient's entry values. The enzymatic activity remained unchanged in 3 patients (17%) and decreased in 2 (11%). Overall, the blood plasma DPP-IV-like enzymatic activity rose to 141±46% (median±SD, p=0.011) compared to the entry values of individual patients. A similar increase to 168±25% (median±SD, p=0.033) of the patient's entry values was observed for the blood plasma DPP-IV concentration as determined by ELISA. The changes of these parameters in individual patients are shown in Figure 3. Despite this observed increase of the blood plasma DPP-IV, the median of the values in the RA patients in the less active state of the disease remained approximately 30% below the levels in the OA patients (data not shown), most likely due to the background of the RA disease activity.

In contrast to the blood plasma DPP-IV, an inverse trend was seen in the BMNC (Figure 4). The plasma membrane as well as the total DPP-IV-like enzymatic activity in BMNC decreased to 66±56% (median±SD, p=0.018) and 52±64% (p=0.005), respectively, of the entry values of individual patients. Using flow cytometry, DPP-IV/CD26 was detected in lymphocytes and only exceptionally a very low positivity was observed in monocytes (data not shown). The percentage of CD26 positive lymphocytes was statistically significantly decreased compared to the individual patient entry values (p=0.029, data not shown). Larger changes were however observed in the quantity of DPP-IV/CD26 expression as determined by median fluorescence intensity, which was decreased to 63±31% of the patient's entry values (median±SD, p=0.005). Taken together, the decrease of RA disease activity in individual patients was accompanied by a rise of the blood plasma DPP-IV and a reduction of DPP-IV expression in BMNC.

Discussion

Dipeptidyl peptidase-IV (CD26) is a costimulatory molecule and a known marker of activated T lymphocytes. It is also involved in the specific processing of several proinflammatory mediators by its unique proteolytic activity [6]. Several studies have therefore explored the role of DPP-IV in autoimmune and inflammatory diseases, including systemic lupus erythematodes and RA [12, 19, 20]. RA patients exhibit lower blood plasma DPP-IV-like enzymatic activity compared to the controls [12, 21, 22, 18]. This was also confirmed in our present study where both the DPP-IV-like enzymatic activity and DPP-IV concentration were almost 50% lower compared to patients with osteoarthritis. Although Cuchcovich et al demonstrated that DPP-IV is hypersialylated in RA patients compared to healthy controls and has lower specific enzymatic activity [23], our data in agreement with other reports [18, 17, 11] show that the lower enzymatic activity observed in RA patients is in large part caused by the decreased blood plasma concentration of the DPP-IV protein. The pathogenetic relevance of this consistently observed phenomenon nevertheless remains elusive.

Significant increase of the DPP-IV/CD26 antigen expression in CD4+ T cells was reported in patients with chronic (median disease duration 11.5 years) rheumatoid arthritis [14], while early after diagnosis (< 6 months), there were no differences in comparison to healthy controls [24]. In our study, patients with osteoarthritis were used as a control and we observed neither a significant difference in the proportion of DPP-IV positive lymphocytes nor in the intensity of DPP-IV expression between both patient groups (data not shown). The variability of disease duration, different antibodies utilized for DPP-IV/CD26 quantification and different control group (osteoarthritis patients vs. healthy individuals) used in our study may cause the difference between our results and the above cited literature data. In line with our previously published data [10], we did not observe any substantial difference in the DPP-

IV-like enzymatic activity in blood mononuclear cells between RA and OA patients (data not shown).

Several previous studies speculated the possible relation of DPP-IV and the activity of RA. Cordero et al [17] described the inverse correlation between the blood serum DPP-IV concentration and the number of swollen joints, but did not observe differences in the blood serum DPP-IV concentration in the groups of patients with active as compared to inactive RA. Similarly, Ulusoy et al [11] did not find a significant association between DPP-IV blood serum concentration and RA disease activity. Higher expression of DPP-IV in T cells was detected in patients with active RA as compared to the less active RA [13, 15]. Our previously published data showed a negative correlation between the DPP-IV-like blood plasma enzymatic activity and CRP [10]. The main focus of our current study was to extend and strengthen this observation by describing intraindividual changes of DPP-IV in relation to the disease activity. During the study, the patients received various types of therapy based on the clinical judgment of the attending physician (glucocorticoids, methotrexate, anti-TNF alpha and anti-CD20 antibodies, leflunomide, sulfasalazine and their combinations). Despite this heterogeneity of the administered treatment, we observed a significant intraindividual increase in the blood plasma DPP-IV enzymatic activity and concentration in follow-up examinations in patients with decreased RA activity. A similar increase of the DPP-IV enzymatic activity, accompanied by a shift from acidic to more neutral glycoforms of the circulating DPP-IV, was previously observed in patients with clinical improvement after anti TNF alpha therapy [25].

In addition to the changes in the levels of the circulating DPP-IV, our study suggests that both the DPP-IV-like enzymatic activity in BMNC and its expression in lymphocytes are decreased along with an improvement in the DAS28 score compared to the entry values in individual patients. The intraindividual decrease of the DPP-IV/CD26 antigen expression was

most notable in the CD4+ T cells (p=0.055, data not shown) which is in agreement with the reported dominant presence of DPP-IV/CD26 in this lymphocyte subpopulation in RA patients [13].

Conclusions

Blood plasma DPP-IV enzymatic activity and concentration are lower in RA patients during the active phase of the disease compared to non-inflammatory arthritis patients. In addition, our intraindividual comparison demonstrates elevation of the blood plasma DPP-IV associated with the clinical improvement in the RA patients. Concurrently, the DPP-IV/CD26 in peripheral blood mononuclear cells decreases. These results further support the possible role of DPP-IV in the pathogenesis of RA probably due to its participation in inflammatory processes.

List of abbreviations

BMNC	peripheral blood mononuclear cells
CRP	C-reactive protein
DAS28	disease activity score 28
DPP-IV	dipeptidyl peptidase-IV
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
MAb	monoclonal antibody
MFI	median fluorescence intensity
OA	osteoarthritis
RA	rheumatoid arthritis

VAS visual analogue scale

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LSr and PB performed biochemical assays, flow cytometry studies and statistical analysis. LSe carried out the diagnostics and clinical examinations. AS designed and coordinated the study. LSr, PB and AS wrote the manuscript, all authors approved the final manuscript.

Acknowledgement

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Figure legends

Figure 1. Blood plasma DPP-IV-like enzymatic activity and concentration in RA and OA patients. In the box plots, the medians are depicted as small squares, interquartile range (25-75th centile) as boxes and bars extend from the minimum to maximum values. * $p < 0.001$, Mann-Whitney U test.

Figure 2. Negative correlation between the blood plasma DPP-IV-like enzymatic activity and CRP concentration in RA patients. Spearman's correlation coefficient is shown.

Figure 3. Blood plasma DPP-IV in RA patients with clinical improvement of the disease. Blood plasma DPP-IV-like enzymatic activity and DPP-IV concentration were determined in patients exhibiting at least a moderate improvement of the disease, individual entry and follow-up values are depicted. * $p < 0.05$, Wilcoxon pair test.

Figure 4. DPP-IV in blood mononuclear cell (BMNC) in RA patients with clinical improvement of the disease. Cell surface DPP-IV-like enzymatic activity and the median fluorescence intensity (MFI) of DPP-IV in lymphocytes were determined in patients exhibiting at least a moderate improvement of the disease, individual entry and follow-up values are depicted. * $p < 0.05$, Wilcoxon pair test.

Table 1. Clinical characteristics of the patients included in the study.

	Rheumatoid arthritis (n=27)	Ostheoarthritis (n=15)
Age	59 ± 13	62±11
Male/Female	7/20	6/9
Disease duration (years)	12.5±12.4	-
Swollen joint counts (0-28)	9±6.3	-
Tender joint counts (0-28)	11± 6.7	-
DAS 28	5.8±1.1	-
ESR (mm/h)	56±28.2	10±6.4
CRP (mg/L)	44±26	5± 4

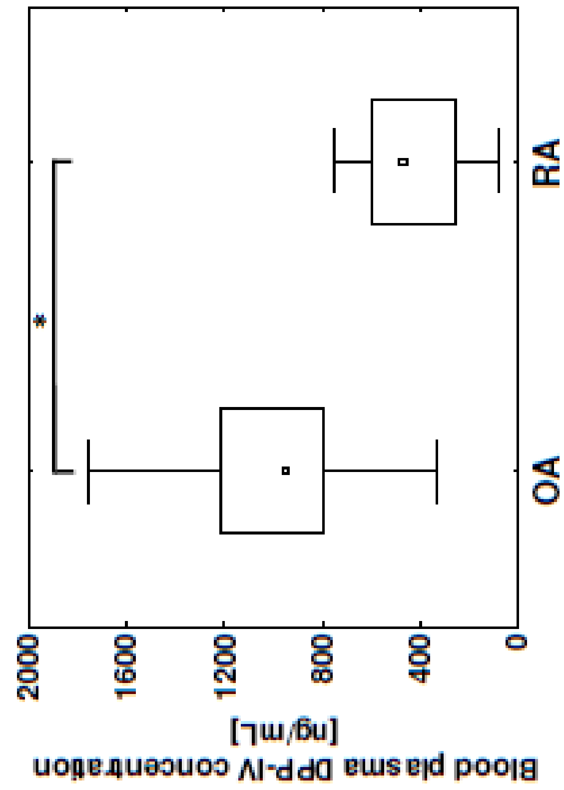
DAS 28, disease activity score; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate. The values are medians±SD.

Table 2. Clinical characteristics of the RA patients exhibiting at least moderate improvement at the follow-up examination.

	Entry Values	Follow-up
Age	60±16	61±16
Male/Female	5/13	5/13
Disease duration (year)	4.5±13.3	5.5±13.5
Swollen joint count (0-28)	9±6	2±3
Tender joint count (0-28)	11.5±6.5	2.5±6.6
DAS 28	5.66±0.97	3.49±1.22
ESR (mm/h)	56±27.7	30±24.1
CRP (mg/L)	51.08±24.49	7.31±19.13

DAS 28, disease activity score; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate. The values are medians±SD.

Figure 1



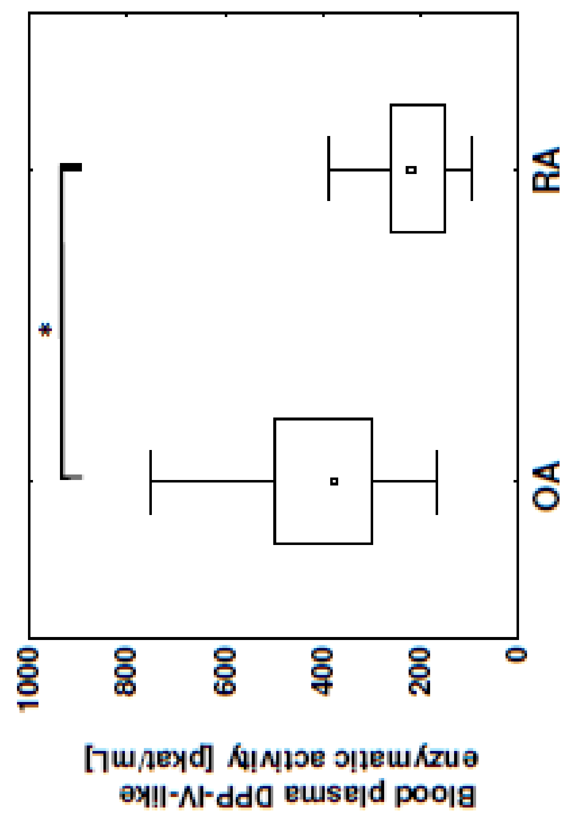


Figure 2

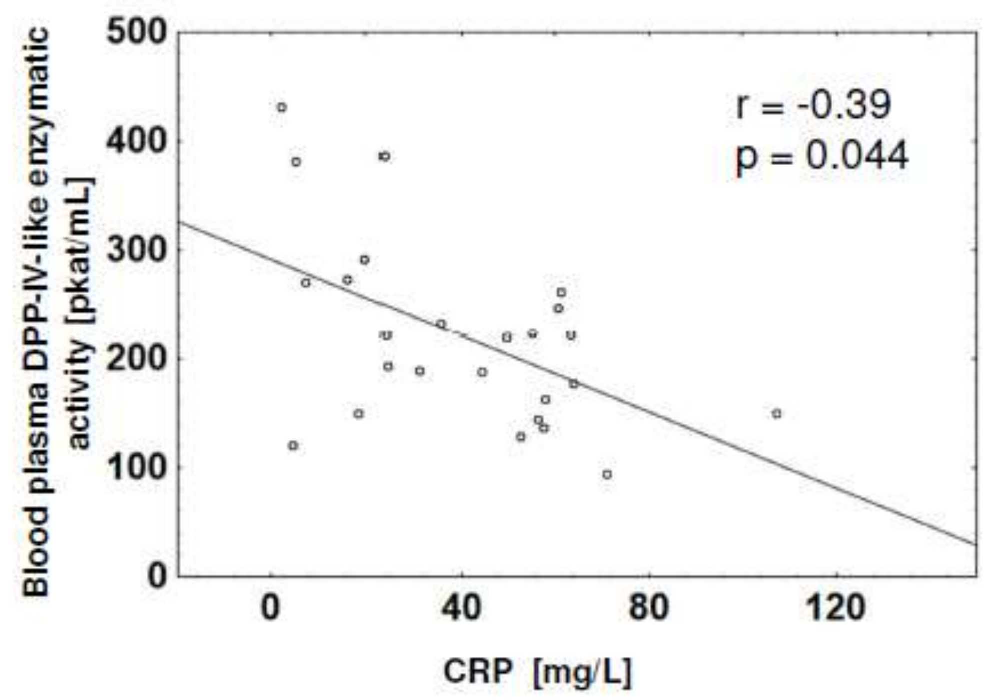


Figure 3

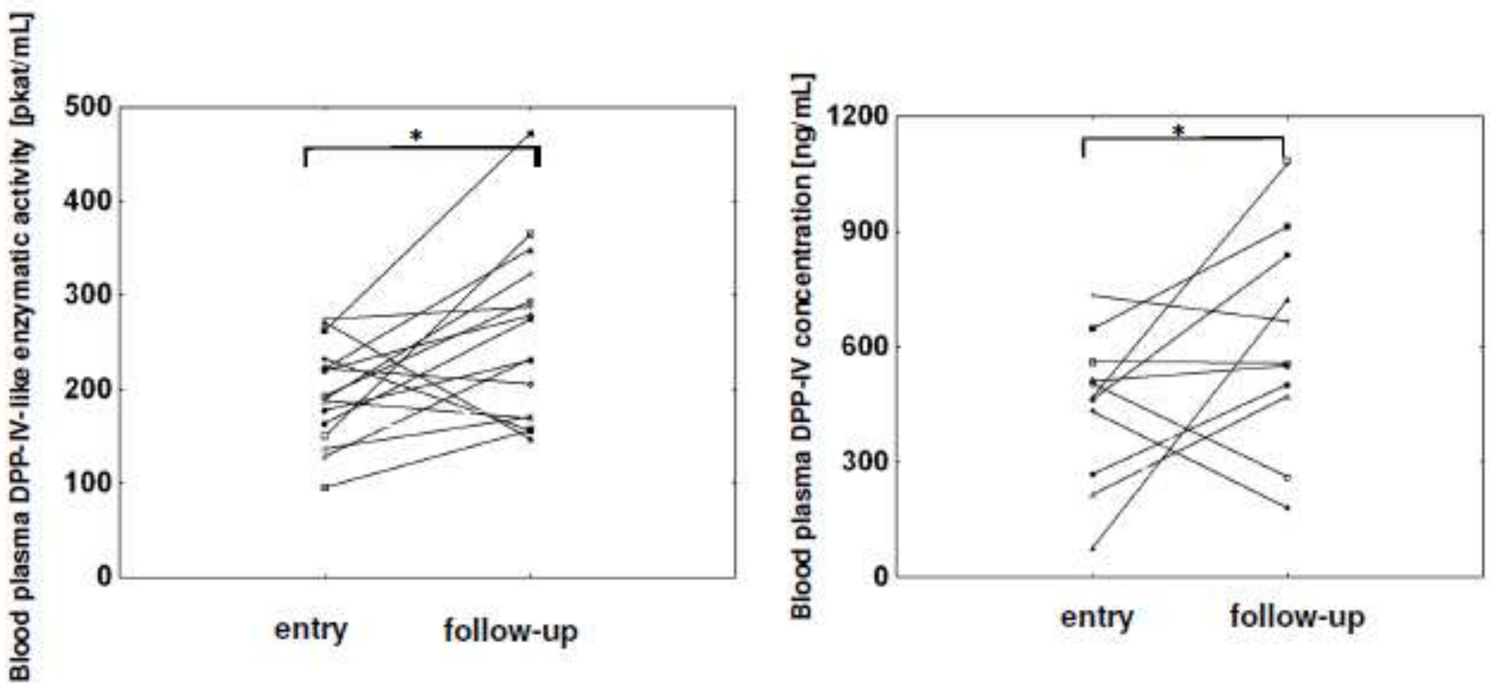
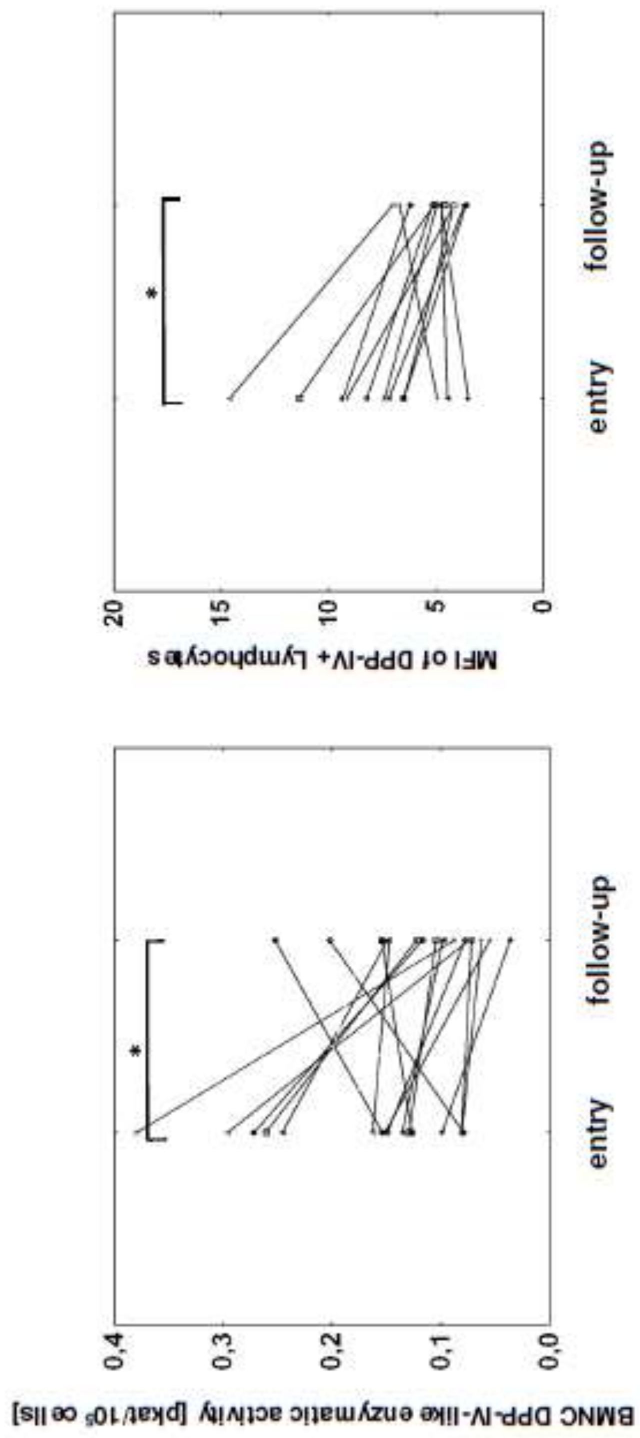


Figure 4



Příloha 5

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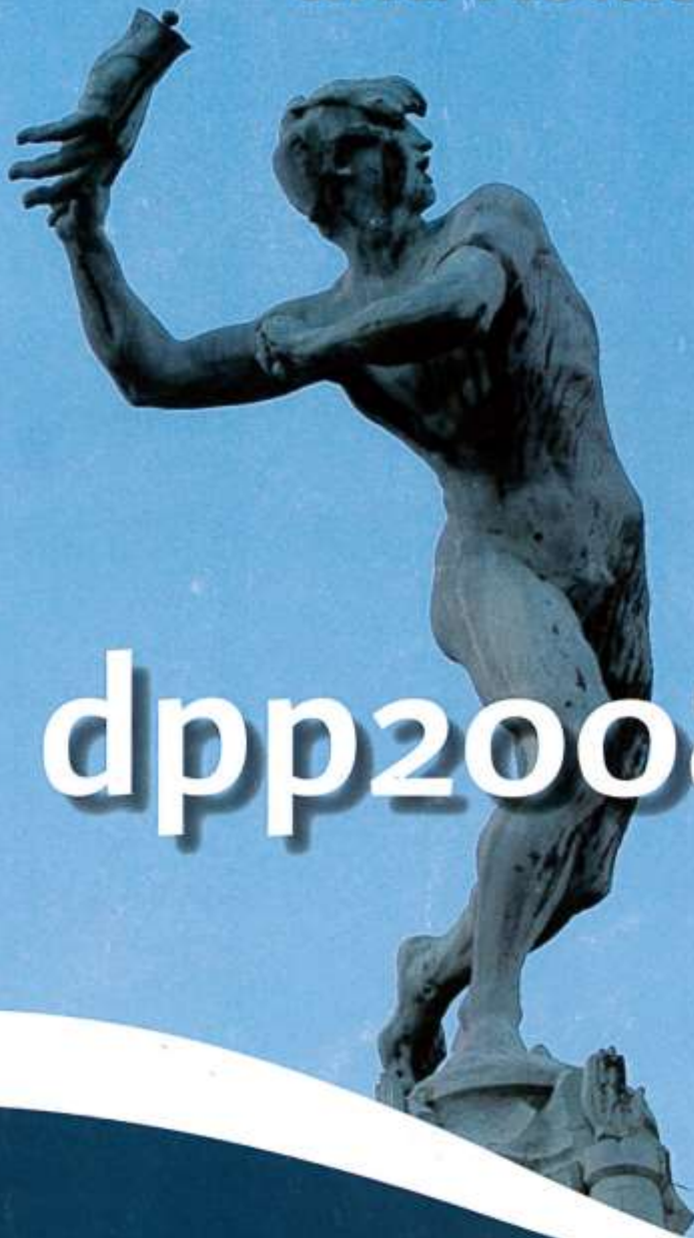

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Dipeptidyl peptidase-IV and Fibroblast activation protein in blood and synovial fluid mononuclear cells of patients with rheumatoid arthritis

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Evolutionary conserved proline residue at the penultimate position of the protein amino-terminus of many biologically active mediators (e.g. several cytokines, neuropeptides etc) represents a checkpoint avoiding proteolytic attack by majority of common proteases. Dipeptidyl peptidase-IV (DPP-IV/CD26) was for many years believed to be the unique proline specific protease, capable to cleave out X-Pro N-terminal dipeptides. However, other enzymes possessing DPP-IV-like enzymatic activity, sharing varying degree of structural homology, were found and classed as a "DPP-IV activity and/or structure homologues" (DASH) [1]. Several proinflammatory peptides as e.g. Substance P (SP) and Stromal cell-derived factor alpha (SDF), supposed to be involved in rheumatoid arthritis (RA) pathogenesis, have their biological half-lives controlled by dipeptidyl peptidase IV-like activity [2]. Indeed, changes of DPP-IV enzymatic activity in blood plasma, synovial fluid and namely in immune cells in RA patients were observed by several authors.

The aim of our study was to assess the expression of DPP-IV and Fibroblast activation protein alpha/Seprase (FAP), both being the plasma membrane bound DASH molecules, in blood and synovial fluid mononuclear cells (BMNC and FMNC respectively) in patients with active RA.

Significant DPP-IV-like enzymatic activity was observed in both BMNC and FMNC. Inhibition as well as immunodetection studies together with previously presented RT-PCR studies argued for dominant, if not absolute, prevalence of the canonical DDP-IV over the FAP. DPP-IV-like activity of BMNC correlated positively with CRP, suggesting increased activation of immune cells as a reflection of the disease severity. Such interpretation is also supported by inverse correlation of DPP-IV-like enzymatic activity in BMNC and blood plasma. As expected, results of colocalization studies demonstrated predominant (about five times) expression of CD26 in CD4 compared to CD8 positive BMNC. Surprisingly, CD26 was distributed almost evenly between both CD4 and CD8 positive FMNC subpopulations, despite the ratio of CD3+CD26+ and CD3+CD26- mononuclear cells was almost identical in peripheral blood and synovial fluid.

Our data, demonstrating disease course associated variation of DPP-IV enzymatic activity and expression supports its previously proposed pathogenetic role in RA. Furthermore, differing distribution of DPP-IV in immune cell subpopulations in peripheral blood and synovial fluid might argue for distinct functional mechanisms executed by the enzyme in the central and peripheral regulation of inflammatory processes.

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Dipeptidyl peptidase-IV and Fibroblast activation protein in blood and synovial fluid mononuclear cells of patients with rheumatoid arthritis

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Introduction:

Several proinflammatory peptides such as e.g. Substance P (SP) and Stromal cell-derived factor 1 alpha (SDF), suposed to be involved in the pathogenesis of rheumatoid arthritis (RA), have their biological half-lives controlled by dipeptidyl peptidase IV-like activity [1]. Changes of DPP-IV enzymatic activity in blood plasma, synovial fluid and namely in immune cells in RA patients were observed by several authors. The aim of our study was to assess the expression of DPP-IV and Fibroblast activation protein alpha/Seprase (FAP-α) in blood and synovial fluid mononuclear cells (BMNC and FMNC respectively) in patients with active RA.

Patients and methods:

Synovial fluid and peripheral blood were collected from patients with active rheumatoid arthritis. Patients (13 women, 4 men) were diagnosed according to the standard criteria of the American College of Rheumatology [2].

Mononuclear cells were isolated using Ficol Paque Plus (GE Healthcare, Sweeden). DPP-IV-like enzymatic activity and inhibition studies in cells and fluids were assessed by continuous rate fluorimetric assay (Spectrofluorimeter Perkin Elmer LS50B).

Immunophenotypisation studies were performed by flow cytometer (FACS Canto, BD Biosciences, USA) with software Diva for aquisition and FlowJo (TreeStar Inc.) for data evaluation.

Quantitative analysis of DPP-IV and FAP-α expression normalized to human β-actin mRNA ($2^{-\Delta\Delta CT}$ method) was performed by real time RT-PCR using Sequence Detection System ABI PRISM 7700 (Applied Biosystems).

Results:

1. DPP-IV-like enzymatic activity in blood plasma was significantly higher than the DPP-IV-like activity in synovial fluid (Fig. 1). According to the inhibition studies the majority of DPP-IV- like activity can be attributed to cannonical DPP-IV (Tab.1).

Figure 1.

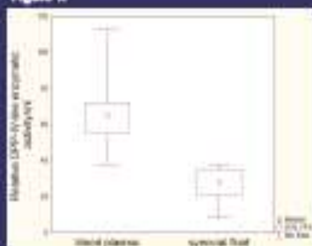
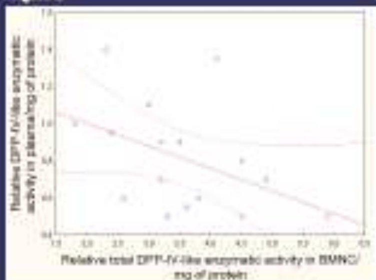


Table 1. IC50 values for specific DPP-IV and DPP8/9 inhibitions. IC50 values reported by the manufacturer for the respective recombinant proteins are 2nM for the DPP-IV inhibitor, 4nM for DPP8 and 20nM for DPP9 for the DPP8/9 inhibitor.

	Inhibitor IC50 (nM)	
Biological material	Anti DPP-IV	Anti DPP 8/9
Plasma	4.8	301.8
Synovial fluid	6.7	314.8
BMNC	55.0	185.0
FMNC	11.3	188.3

2. Negative correlation (R=-0,504; p=0,039) between the DPP-IV-like enzymatic activity of blood plasma and BMNC was observed (Figure 2). The previously described [1] inverse correlation between the biochemical marker of inflamation CRP and blood plasma DPP-IV-like enzymatic activity was confirmed (R=-0,694; p=0,001). Furthermore, we found a positive correlation between CRP and DPP-IV-like enzymatic activity of BMNC (R=0,555; p=0,025).

Figure 2.



3. The presence of canonical DPP-IV/CD26 in BMNC and FMNC was confirmed by flow cytometry (Fig. 3; one typical dot plot of 17 measurements) and by quantitative RT-PCR (Tab.2). On the contrary, the expression of FAP-α was low (Tab.2) and there were no FAP-α positive cells detected by flow cytometry (Fig. 4 ; one typical dot plot of 10 measurements).

Figure 3.

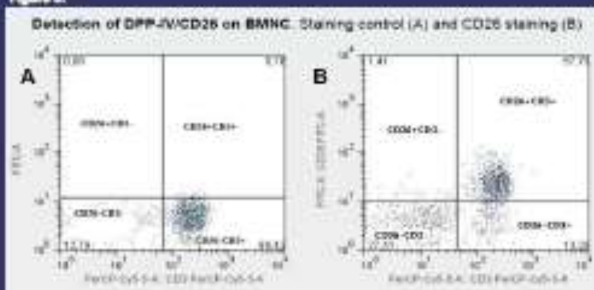
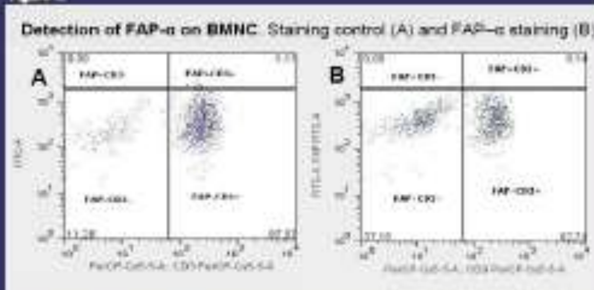


Figure 4.

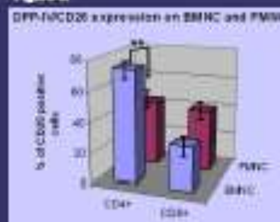


Gene	BMNC	FMNC
DPP-IV	[illegible]	[illegible]
FAP-α	[illegible]	[illegible]

Table 2. Quantitative analysis of DPP-IV and FAP-α expression normalized to human β-actin mRNA ($2^{-\Delta\Delta CT}$ method).

4. Flow cytometry studies demonstrated predominant expression of DPP-IV/ CD26 in CD4 compared to CD8 positive subpopulations of BMNC (Fig.5). Quantity of double positive CD4+CD26+ cells in FMNC is significantly lower than that in BMNC (p=0,001).

Figure 5.



Conclusions:

DPP-IV-like enzymatic activity of BMNC and FMNC is probably an attribute of canonical DPP-IV in patients with active RA.

Different distribution of DPP-IV in populations of BMNC and FMNC T-cells might argue for distinct functional roles executed by the enzyme in regulation of central and peripheral inflammatory processes.

Correlation between DPP-IV-like enzymatic activity in blood plasma and CRP suggests association of DPP-IV with RA severity.

References:

1. Sedo A, et al.: Arthr Res Ther 7: 253-269 2005
2. Arnett FC, et al.: Arthritis Rheum, 31 : 315-324, 1988

Contact: [illegible]

Acknowledgement: This work was supported by grant No [illegible], Grant Agency of Ministry of Health of Czech Republic. Inhibitors of DPP-IV and DPP8/9 were kindly provided by Ferring Pharmaceuticals, UK.

Příloha 6

11. Studentská vědecké konference 1.LF UK v Praze
2010 Praha, Česká republika

Abstrakt vítězné přednášky

11. STUDENTSKÁ VĚDECKÁ KONFERENCE

KONANÁ DNE 19. KVĚTNA 2010
POD ZÁŠTITOU DĚKANA 1.LF UK
PROF. MUDR. TOMÁŠE ZIMY, DRSC., MBA

SEKCE
POSTGRADUÁLNÍ KLINICKÁ II.
VÍTĚZNÉ PRÁCE

MUDr. Hana Skalická, 5. ročník - II. interní klinika – klinika kardiologie a angiologie 1. LF UK a VFN
školitel: MUDr. Jan Horák, CSc.
název práce: Hodnocení efektivity a bezpečnosti intrakoronární aplikace mononukleárních buněk kostní dřeně u pacientů s akutním infarktem myokardu s dysfunkcí levé komory: výsledky dvouletého sledování

Mgr. Lucie Šromová, 4. ročník - Laboratoř biologie nádorové buňky, Ústav biochemie a experimentální onkologie 1. LF UK a VFN
školitel: prof. MUDr. Aleksi Šedo, DrSc.
název práce: Dynamika dipeptidylpeptidázy – IV v krevní plazmě a na mononukleárních buňkách periferní krve u pacientů s revmatoidní artritidou v závislosti na aktivitě onemocnění

MUDr. Jana Vávrová, 2. ročník - Neurologická klinika 1. LF UK a VFN
školitel: prof. MUDr. Karel Šonka, DrSc., MUDr. David Kemlink, Ph.D.
název práce: Syndrom neklidných nohou u pacientů s roztroušenou sklerózou: epidemiologie a genetika

DYNAMIKA DIPEPTIDYLPEPTIDÁZY – IV V KREVNÍ PLAZMĚ A NA MONONUKLEÁRNÍCH BUŇKÁCH PERIFERNÍ KRVE U PACIENTŮ S REVMATOIDNÍ ARTRITIDOU V ZÁVISLOSTI NA AKTIVITĚ ONEMOCNĚNÍ

Autor: **Mgr. Lucie Šromová**

Školitel: **prof. MUDr. Aleksi Šedo, DrSc., Laboratoř biologie nádorové buňky, Ústav biochemie a experimentální onkologie 1. LF UK**

Úvod: Dipeptidylpeptidáza–IV (DPP–IV, identická s CD26) svojí proteolytickou aktivitou opracovává řadu pro-zánětlivých mediátorů (např. Substance P, Stromal cell derived factor 1–α (SDF)) účastnících se patogeneze revmatoidní artritidy (RA). Ve srovnání s kontrolní skupinou pacientů s osteoartrózou (OA), vykazují pacienti s RA nižší DPP–IV enzymovou aktivitu v plazmě a za použití některých typů protilátek anti-DPP–IV/CD26 naopak vyšší expresi na mononukleárních buňkách (MNC) v periferní krvi (Šedo A et al., Arthritis Res Ther 2005, 7: 253–69). Cílem této práce bylo posouzení změn exprese DPP–IV a koncentrace jejích biologicky aktivních substrátů v periferní krvi u pacientů s RA ve vztahu k aktivitě jejich onemocnění.

Metody: Klinická charakteristika pacientů: Do studie bylo na základě informovaného souhlasu zařazeno 32 pacientů s aktivní RA, kteří byli diagnostikováni podle standardních klinických kritérií (Arnett FC et al.: Arthritis Rheum, 1988, 31: 315–324). Kontrolní skupinu tvořilo 15 pacientů s osteoartrózou (OA). Při následnému vyšetření (follow-up) byli pacienti s RA rozděleni podle aktuální aktivity onemocnění do dvou skupin: s poklesem CRP větším než 50 % („významné zlepšení") resp. menším než 50 % („bez významného zlepšení") proti vstupní hodnotě (tabulka).

Pacienti	Věk (roky) Průměr (SD)	Ženy/Muži	CRP (mg/l) Průměr (SD)
Osteoartróza	66 (10)	9/6	5 (4)
Revmatoidní artritida: aktivní	59 (13)	24/8	43 (30)
vstupní hodnoty			
Revmatoidní artritida:			
hodnoty follow up			
Významné zlepšení	63 (16)	11/2	10 (10)
Bez významného zlepšení	61 (4)	6/2	51 (22)

DPP–IV enzymová aktivita v plazmě a na MNC byla stanovena kinetickou fluorimetrickou metodou za použití 7-(glycyl-prolylamido)-4-methylkumarinu jako substrátu. Exprese proteinu DPP–IV/CD26 na subpopulacích MNC byla kvantifikována průtokovou cytometrií a koncentrace proteinů DPP–IV/CD26, Substance P a SDF metodou ELISA. Statistická analýza byla prováděna v programu Statistica 8.0. Rozdíly mezi jednotlivými skupinami byly hodnoceny pomocí Mann-Whittneyho U-testu a Wilcoxonova párového testu, korelace pomocí Spearmanova korelačního koeficientu. Klinické parametry (CRP, DAS 28, FW, revmatoidní faktory) byly získány z klinického a laboratorního vyšetření ve spolupráci s Revmatologickým Ústavem v Praze.

Výsledky: V souladu s dříve publikovanými daty (Šedo A et al., Arthritis Res Ther 2005, 7: 253–69) byla pozorovaná významně nižší DPP–IV enzymová aktivita v plazmě pacientů s RA ve srovnání s OA (o 50 %, p = 0,0001) a statisticky významná negativní korelace mezi CRP a enzymovou aktivitou DPP–IV/CD26 (R = –0,471, p = 0,013) v plazmě pacientů s aktivní RA. Statisticky významná negativní korelace byla navíc zjištěna i mezi CRP a enzymovou aktivitou (R = –0,367, p = 0,014) a koncentrací DPP–IV/CD26 (R = –0,495, p = 0,002) v plazmě v celém souboru pacientů s RA (tj. pacienti s aktivní RA a „follow-up").

U pacientů s poklesem CRP během léčby (skupina „významné zlepšení") bylo pozorováno statisticky významné zvýšení DPP–IV enzymové aktivity (o 40 %, p = 0,019) i plazmatické koncentrace DPP–IV/CD26 (o 80 %, p = 0,001). Mezi pacienty s RA a OA nebyl pozorován statisticky významný rozdíl v DPP–IV enzymové aktivitě na MNC, ani v expresi membránově vázané DPP–IV/CD26, ovšem u pacientů s významným poklesem CRP (skupina „významné zlepšení") byl pozorován významný pokles DPP–IV enzymové aktivity na MNC (o 30 %, p = 0,023) a imunofenotypizačními studiemi prokázána i nižší exprese DPP–IV/CD26 (o 17 %, p = 0,014).

Naše výsledky potvrzují pozorování již dříve publikovaná různými autory (Šedo A et al., Arthritis Res Ther 2005, 7: 253–69), že koncentrace biologických substrátů DPP–IV – Substance P a SDF v plazmě jsou významně vyšší u pacientů s RA ve srovnání s pacienty s OA (Substance P o 60 % p = 0,022 a SDF o 200 % p = 0,006). U pacientů s RA s poklesem aktivity onemocnění jsme v našem souboru pozorovali statisticky významný pokles koncentrace Substance P (o 42 %, p = 0,001) i SDF (o 60 %, p = 0,003) v plazmě.

Závěr: Snížení exprese a aktivity DPP–IV/CD26 na MNC periferní krve s poklesem aktivity onemocnění a negativní korelace plazmatické aktivity a koncentrace DPP–IV s CRP naznačují vztah tohoto enzymu k aktivitě RA a jeho možnou využitelnost jako markeru úspěšnosti léčby.