Úloha proteinázami-aktivovatelného receptoru-2 (PAR-2)

v patogenezi lidských onemocnění

Disertační práce

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OBSAH

Seznam užitých zkratek:

PAR - Proteinase activated receptor

DMEM - Dulbecco's Modified Eagle Medium

FCS - fetální telecí sérum

SD – standardní odchylka

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

DNA – deoxyribonukleová kyselina

RNA – ribonukleová kyselina

PCR – polymerázová řetězová reakce.

RT – reversní transkripce

IL – interleukin

CNS – centrální nervový systém

c-erbB2 (HER2/Neu) – onkogen c-erbB2

p53 – supresorový protein p53

Ki-67 – proliferační marker

GTP (GDP) - guanosine tri(di)phosphate

HIV – virus lidské imunodeficience

TBS – tris buffer saline pufr

ICAM – Intracellular Adhesion Molecule

TGF - Transforming Growth Factor

MAP(K) - mitogen-activated protein (kinase)

MDA MB-231, MCF-7 – buněčné linie karcinomu mléčné žlázy

MKN-1, STKM-1 - buněčné linie karcinomu žaludku

ERK - extracellular signal-regulated kinase

1. Úvod

1.1 Úvod do problematiky

Serinové proteázy tvoří rodinu proteolytických enzymů, které jsou charakterizovány jedinečnou katalytickou triádou Ser-His-Asp, jež je schopna účinně hydrolyzovat kovalentní peptidové vazby. Serinové proteázy jsou produkovány ve formě zymogenů nebo inaktivních prekurzorů, které se stanou fyziologicky účinnými procesem zvaným „limitovaná proteolýza" nebo zymogenní aktivace. U savců mají serinové proteázy mnoho fyziologických funkcí. Podílejí se například na regulaci krevního srážení a na fibrinolytické rovnováze, degradují neuropeptidy nebo slouží jako modulátory imunitní odpovědi při zánětlivých procesech.

Účinnými antagonisty serinových proteáz jsou tři typy proteázových inhibitorů lišící se mechanismem účinku. Nerovnováha mezi hladinami těchto inhibitorů a příslušných proteáz vede k poruchám imunitní/zánětlivé odpovědi a v konečném důsledku může vyústit v onemocnění. Studie z nedávné doby navíc jasně dokumentují, že do interakce proteáz a jejich inhibitorů vstupuje skupina specifických receptorových molekul, které mají po aktivaci sérovými proteázami schopnost se aktivně účastnit regulačních procesů souvisejících s buněčnými funkcemi. Jsou to s G-proteiny spřažené receptorové molekuly, jež je možné rozdělit nejméně do dvou velkých skupin: urokinázové receptory a proteinázami-aktivovatelné receptory (proteinase-activated receptors - PARs).

1.2 Rodina proteázami-aktivovatelných receptorů.

PAR jsou v podstatě ubikvitní. Jsou přítomny zejména na imunokompetentních buňkách, a to jak normálních, tak i nádorově transformovaných, na endotelových a svalových buňkách

velkých i drobných cév. Přítomnost PAR byla imunohistochemicky prokázána rovněž na střevních epiteliích, epiteliích žláz s vnitřní i vnější sekrecí, na keratinocytech, fibroblastech a dalších buňkách (1-7).

PAR patří do relativně nově definované podrodiny s G-proteinem spřažených receptorových molekul. Tyto mají sedm transmembránových domén a jsou aktivovatelné jedinečným procesem, který spočívá v rozpoznání specifické sekvence v oblasti NH_2 konce receptorové molekuly, následném rozštěpení a prezentací nového NH_2 konce. Nově vzniklý NH_2 konec pak má funkci tzv. „vázaného ligandu", který se váže s extracelulární doménou (nejspíše v oblasti 2. kličky) štěpené receptorové molekuly (**Obr. 1**). Uvedený intramolekulární aktivační proces je následován interakcí s G proteiny a spouští řadu různých signálních drah. PAR jsou tedy receptory, jejichž ligand je fyzickou součástí molekuly receptoru (8).

V současné době rozeznáváme čtyři různé PAR. PAR1, PAR3 a PAR4 jsou cílovým místem působení trombinu či kathepsinu G. PAR2 je k trombinu resistentní, avšak může být aktivován trypsinem, tryptázou žírných buněk, faktorem Xa, akrosinem nebo neuronální serinovou proteinázou.

Recentní studie prokázaly, že existuje větší počet různých mechanismů, které regulují stimulaci PAR a ukončují PAR-spuštěnou signalizaci. Dostupnost PAR molekul na buněčné membráně je významně regulována intracelulárním transportem receptorových molekul z intracelulárních depozit. Obdobně dostupnost G-proteinů a přítomnost G-proteinových kináz výrazně modifikuje aktivitu PAR.

V nedávné době byly syntetizovány specifické umělé oligopeptidy, které sekvencí aminokyselin odpovídají vázaným ligandům. Tito receptoroví agonisté mají schopnost aktivovat PAR, avšak v řádově vyšších koncentracích než serinové proteázy jako takové.

Také jejich zvýšená citlivost k aminopeptidázám omezuje praktické využití těchto působků v experimentálních podmínkách.

1.2.1 PAR-1

Extracelulární NH_2-konec lidského PAR-1 obsahuje štěpné místo pro α-trombin ($LDPR^{41}$! S^{41} FLLRN), po kterém následuje sekvence reziduí s negativním elektrickým nábojem (D^{51}.KYEPF^{56}) (9), která je velmi podobná COOH-části antikoagulancia pijavek hirudinu. Hirudin inhibuje trombin analogickou vazbou na stejném místě. Doména D^{51}.KYEPF^{56} reaguje (stejně jako hirudin) s oblastí vážící anionty na molekule α-trombinu a pravděpodobně indukuje konformační změny receptorové molekuly, která pak přizpůsobí své štěpné místo katalytickým doménám α-trombinu. Aktivace PAR-1 γ-trombinem, který neobsahuje oblast vážící anionty, je 100-krát slabší. Delece domény D^{51}.KYEPF^{56} vede ke ztrátě aktivovatelnosti trombinem (10). Trombin tedy štěpí PAR-1 na NH_2-konci. Syntetické, volné peptidové ligandy mohou aktivovat PAR-1 i bez štěpení receptoru (11); ve srovnání s proteinázami jsou však velice slabými agonisty, jejichž působení je možné v mikromolárních koncentracích, kdežto vlastní serinové proteinázy účinkují v koncentracích nanomolárních. Důvodem je zřejmě nedokonalá prezentace solubilního peptidu vazebné doméně ve srovnání s "vázaným ligandem". Navíc jsou solubilní peptidy v krevní plazmě bezprostředně proteolyticky degradovány aminopeptidázami (12).
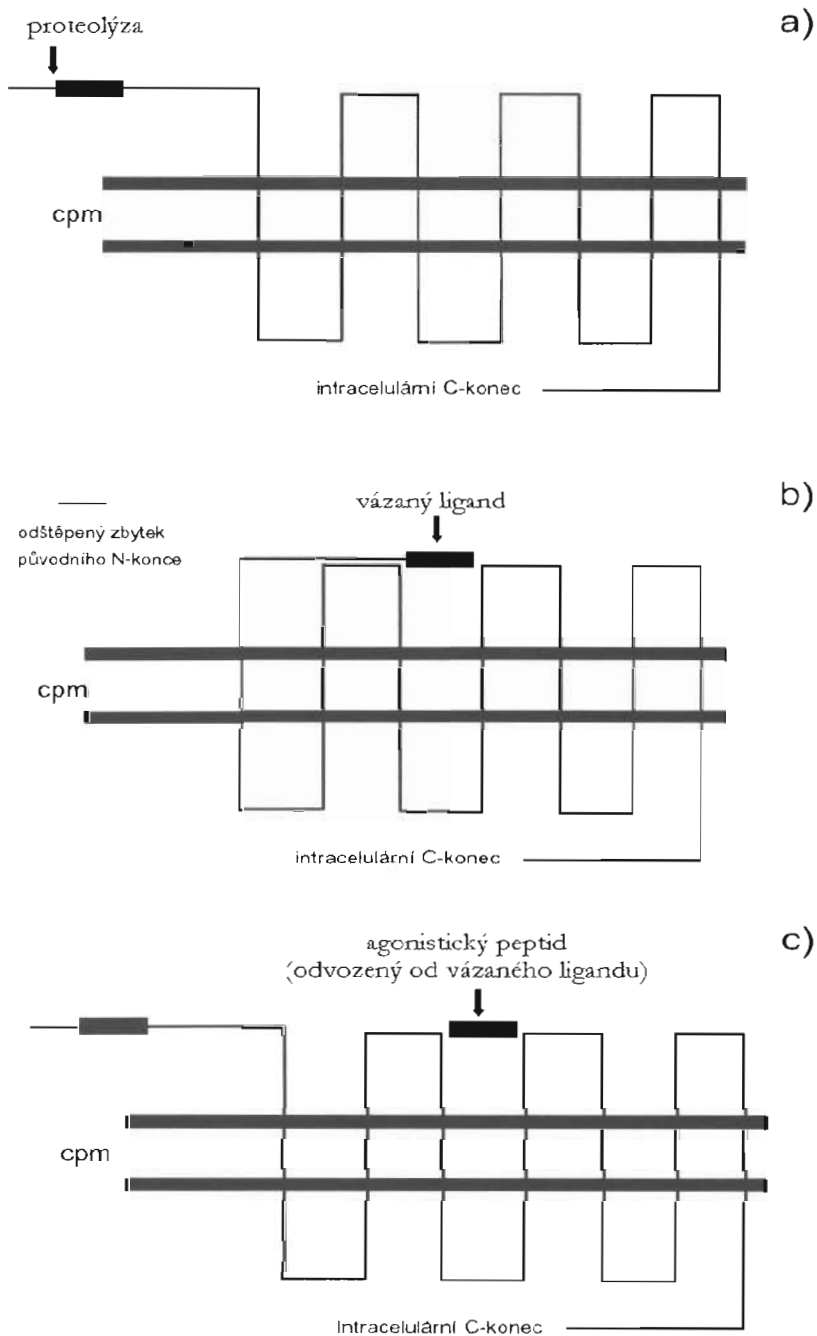
Obr. 1. Mechanismus aktivace PAR: V první fázi dochází ke specifickému odštěpení oligopeptidu z N-konce (a). Vázaný ligand (nový N-konec) po konformační změně molekuly aktivuje receptor reakcí s jeho 1. a 2. extracelulární doménou (b). Receptor je aktivovatelný i volným (syntetickým) oligopeptidovým ligandem bez proteolýzy N-koncové části molekuly (c). Upraveno podle Dery O, Corvera C, Bunnett NW. Proteinase-activated receptors: novel mechanismus of signaling by serine proteases. Am J Physiol 1998; 274:C1429–C1452.

štěpné místo   interakce s oblastí vážící anionty na molekule trombinu   2. extracelulární klička

PAR1	P*ESKATNATLDPR	SFLLRN	PN	DKYEPFWEDEEKNES	PAR1 QTIQVPGLNITTCHDVLNETLLEG
PAR2	Q*GTNRSSKGR	SLIGKV	DG	-----------------------	PAR2 QTIFIPALNITTCHDVLPEQLLLVG
PAR3	NDTNNLAKPTLPIK	TFRGAP	PNS	FEEFP FSALE	PAR3 QEYYLVQPDITTCHDVHNTCESSS

doména vázaného ligandu

interakce s oblastí vážící anionty na molekule trombinu
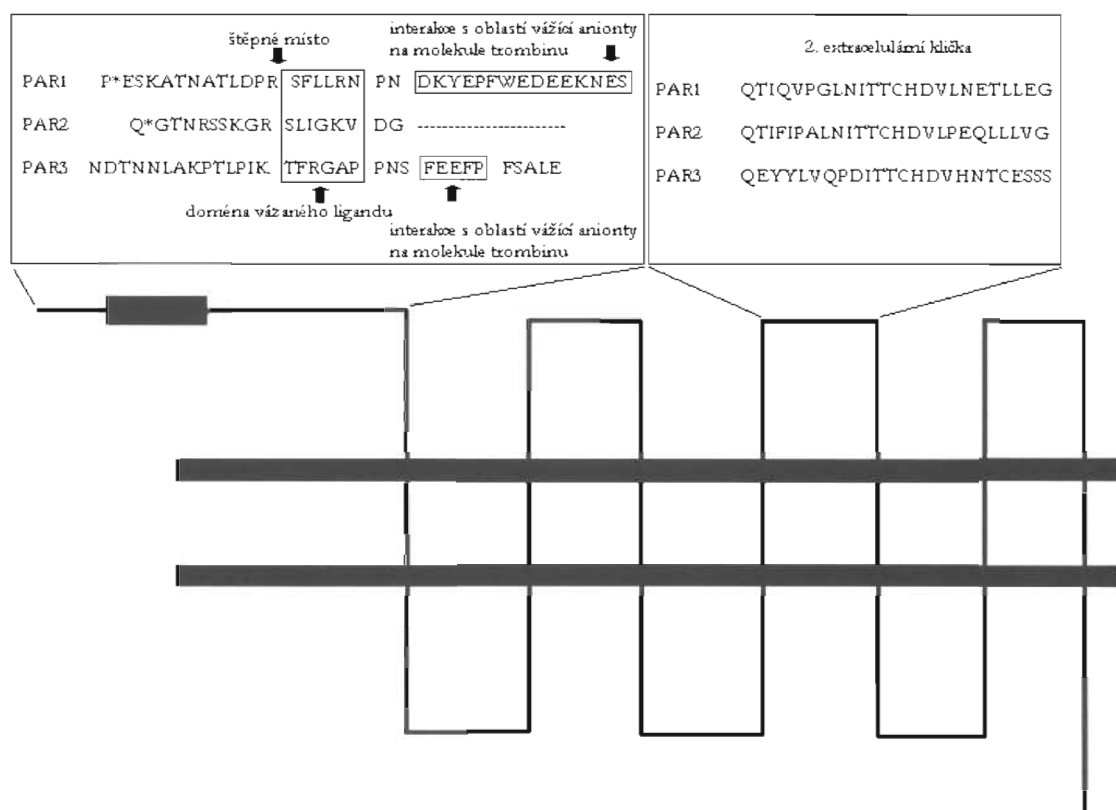
Obr. 2. Struktura PAR: Sekvence aminokyselin N-konce a 2. extracelulární domény jsou pro aktivaci PAR nejpodstatnější. Rámečky označují domény vázaných ligandů a oblasti se záporným nábojem reagující s trombinem (v případě PAR-1 a PAR-3). Upraveno podle Dery O, Corvera C, Bunnett NW. Proteinase-activated receptors: novel mechanismus of signaling by serine proteases. Am J Physiol 1998; 274:C1429-C1452.

1.2.2 PAR-2

Druhý člen rodiny proteinázami aktivovatelných receptorů, PAR-2, byl identifikován při PCR screeningu myšího genomu za použití primerů pro druhou a šestou transmembránovou doménu receptoru pro neurokinin 2 (13). Ukázalo se, že je zde kódován

protein s typickými charakteristikami receptoru spřaženého s G-proteinem a se stupněm identity s lidským PAR-1 dosahujícím 30%. Myší PAR-2 obsahuje štěpné místo pro trypsin (SKGR^{34} ! S^{35} LIGR). Později byl PAR-2 identifikován i u lidí a potkanů (14). Trypsin, podobně jako trombin v případě PAR-1, štěpí v určitém místě PAR-2 a umožňuje expozici vázaného ligandu jeho vlastní extracelulární doméně (15). Syntetické peptidy (SLIGRL u myší, SLIGKV u lidí) rovněž aktivují PAR-2 bez potřeby štěpení vlastního receptoru (16,17). PAR-1 a PAR-2 jsou tedy aktivovány stejným mechanismem - štěpením receptoru a expozicí vázaného ligandu s jedním podstatným rozdílem: neexistují doklady pro interakci trypsinu s jinou částí receptoru, než je oblast štěpená.

PAR-2 je exprimován v podstatě ubikvitně. Byla popsána jeho exprese v celé řadě buněčných typů jako jsou endotelie, buňky hladké svaloviny, osteoblasty, buňky imunitního systému zahrnující T-lymfocyty, neutrofilní i eosinofilní granulocyty nebo žírné buňky (6,13,17,23,26,30,31,38) a dále v mozku, oku, dýchacích cestách, gastrointestinálním traktu, pankreatu, ledvinách, játrech. Molekulu PAR-2 neexprimují například krevní destičky (39).

1.2.3 PAR-3 a PAR-4

Existence dalších receptorů pro trombin, PAR-3 a PAR-4, byla doložena pomocí primerů k různým doménám PAR-1 a PAR-2. Trombin štěpí sekvenci LPIK^{38} ! T^{39} FRG PAR-3. Stejně jako v případě PAR-1, obsahuje PAR-3 místo podobné hirudinu (FEEFP), které reaguje s α-trombinem. Na rozdíl od PAR-1 však peptidy analogické vázanému ligandu receptor neaktivují. Stereochemická změna molekuly v důsledku vazby trombinu je zde nezbytná (18).

1.3 Signální mechanismy zprostředkované PAR-2 a jejich biologické funkce

Jiné proteinázy (katepsin X, proteináza 3, elastáza lidských leukocytů) štěpí PAR v jiném než aktivačním místě a receptor tedy není aktivován (19,20).

Aktivace PAR vede ke konformační změně heterodimerického G proteinu, který katalyzuje výměnu GDP za GTP na α-podjednotce G proteinu. α-podjednotka a βγ-heterodimer aktivují četné efektorové enzymy nebo iontové kanály až do hydrolýzy GTP. G protein se pak navrací do inaktivního stavu. Nejvíce byl dosud sledován přenos signálu prostřednictvím aktivace PAR-1 (21).

V případě PAR-2 bylo publikováno jen několik prací, které sledovaly přesné signalizační kaskády působení PAR-2. PAR-2 interagují s podtypy Gq/G11, čímž zasahují do aktivace kalciové signalizační dráhy a zřejmě i s G0/Gi. Vztah vazby PAR-2 k jiným G proteinům je nejistý. Interakce PAR-2 s Gi a Gq nejspíše ovlivňuje aktivaci fosfolipázy C, proteinkinázy C a MAPkinázovou signalizační dráhu. Všechny tyto signální dráhy ovlivňují široké spektrum cílových buněčných funkcí včetně regulace buněčného cyklu, regulace transkripční aktivity nebo buněčné motility. V dalších pracích byly publikovány poznatky o jiných interakcích PAR-2 s některými dalšími významnými buněčnými signálními drahami ovlivňujícími tak zásadní fyziologické funkce jako je depolarizace neuronálních elementů, změny cytoskeletu v endoteliích nebo produkce cytokinů (18, 21-27). Schematické působení aktivace PAR-2 je shrnuto na obrázku č.3.

Trypsin

Gα12/13 Gαi Gαq

PLCβ

IkBα,β GEF Src
NFκB Rho Shc
Grb2 → Ras

SAPK DAG/IP₃
JNK

? ?

NFκB c-Fos c-Jun Raf PKC α,β,γ,ε,λ ? Ca²⁺

MEK NO

p38 ERK1/2

Kinázy
Iontové kanály
Uvolňování
neuropeptidů

Apoptóza, buněčná adheze, buněčný růst a diferenciace, kontrakce, sekrece,
imunomodulace, receptorová transaktivace, zánětlivá a imunitní odpověď
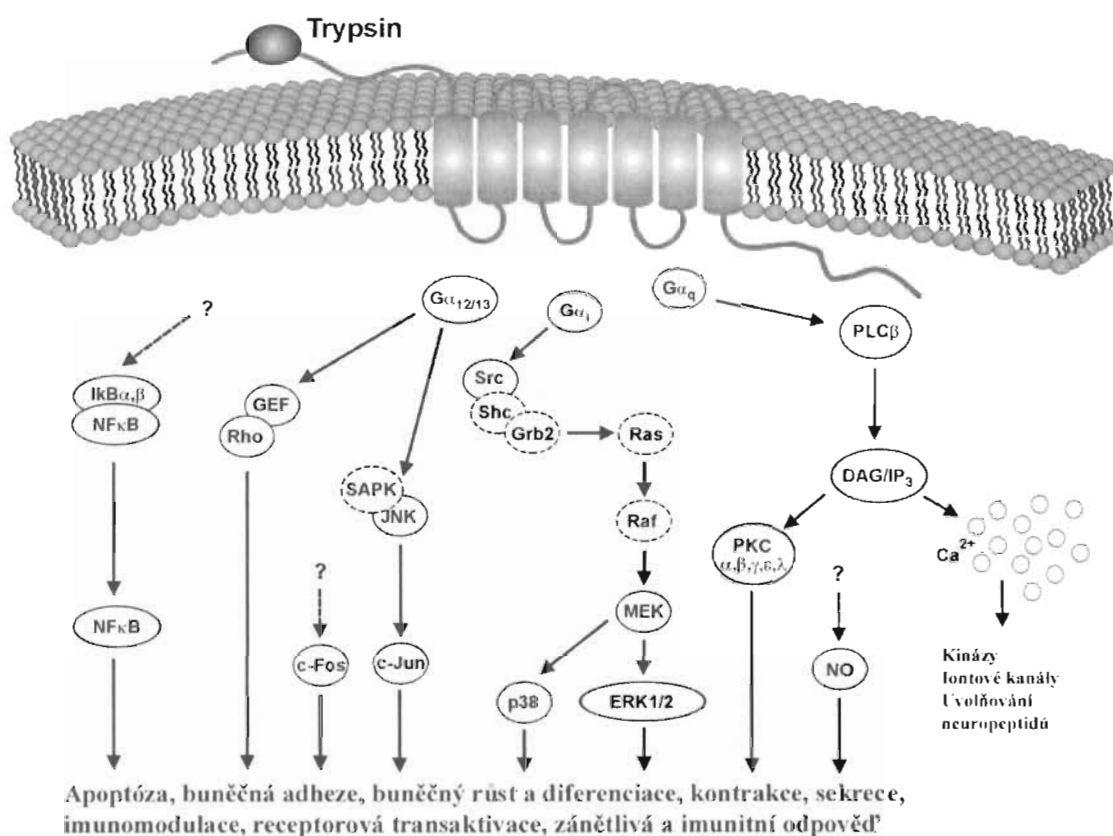
Obr. 3. Schéma nejvýznamnějších G-proteiny zprostředkovaných signálních drah spojených s aktivací PAR-2 v různých tkáních a buněčných typech. Upraveno podle Steinhoff et al.: Proteinase-activated receptors: Transducers of Proteinase-Mediated Signaling in Inflamation and Immune Response. Endocrine Reviews 2005; 26(1):1-43.

1.3.1 PAR-2 v zánětlivé a imunitní odpovědi.

V cévních strukturách působí aktivace PAR-2 celkově prozánětlivě zejména svým vasodilatačním účinkem následovaným extravazací plasmatických proteinů a proliferací endotelií (1). V buňkách imunitního systému je PAR-2 exprimován v rozsáhlém spektru buněčných typů. Ve zvířecím modelu byl prokázán účinek aktivace PAR-2 na migraci a funkci leukocytů. Vergnolle v roce 1999 prokázala významné zvýšení migrace, rollingu a adheze leukocytů po intraperitoneální aplikaci agonistického peptidu (SLIGRL-NH₂) u novorozených krys (18). Recentně publikované práce prokazují významný vliv aktivace

PAR-2 v regulaci aktivity eosinofilních granulocytů a tím účast v patogenezi alergických onemocnění. Miike et al. prokázali degranulaci eosinofilních granulocytů a produkci superoxidu po aktivaci PAR-2 (22). V T-lymfocytárních buněčných liniích indukuje aktivace PAR-2 trypsinem nebo agonistickým peptidem mobilizaci kalciových iontů (26). Ferrell et al. ozřejmili zásadní úlohu PAR-2 v patogenezi a progresi akutního chronického zánětlivého postižení kloubů na modelu indukované arthritidy (33). Podrobněji byla zkoumána role PAR-2 v patofyziologii kožních zánětlivých procesů. Stimulace PAR-2 hraje významnou úlohu v rozvoji experimentálně navozené alergické kontaktní dermatitidy na modelu PAR-2 deficientních myší, kde Seeliger et. al prokázali, že PAR-2 se účastní vzniku edému, extravazace plazmy a zvýšené produkci hladin některých cytokinů (například IL-6), adhezních molekul (ICAM-1) a selektinů (E-selektin) (34). Ve stejné práci byly obdobné mechanismy účinku stimulace PAR-2 pozorovány i v lidské kůži jednak při studiu změn cévní stěny v modelu in vivo, jednak v kožních biopsiích zdravých dobrovolníků. O působení PAR-2 v patofyziologii zánětlivých onemocnění dýchacího traktu bylo publikováno několik prací, jejichž výsledky nejsou zcela jednoznačné a mnohdy jsou i protichůdné. PAR-2 je široce exprimován v celé řadě buněčných typů, které se nacházejí v dýchacích cestách. Aktivace PAR-2 může mít u zánětů dýchacích cest jak prozánětlivý, tak i protizánětlivý efekt. Tento ambivalentní vztah bývá vysvětlován širokým spektrem zkoumaných tkání, buněk a modelových stavů (zejména akutní vs. chronický zánět) při popisu účinků PAR-2 v zánětlivých onemocněních dýchacích cest. Současný pohled na působení PAR-2 u těchto onemocnění vychází ze skutečnosti, že PAR-2 působí jako prozánětlivý faktor v časných fázích zánětu a naopak v pozdních fázích převažuje jeho efekt potizánětlivý. Uvedená představa ovšem platí pro „obvyklou" zánětlivou odpověď. U zánětlivých procesů s poruchou základních regulačních

mechanismů zřejmě převažuje prozánětlivý efekt aktivace PAR-2, což bylo demonstrováno zejména v modelu astmatu jako představitele autoimunitního onemocnění (31,35).

V trávícím traktu je PAR-2 široce exprimován na různých strukturách. Nedávná pozorování nasvědčují významné úloze aktivace PAR-2 trypsinem v produkci některých metabolitů kyseliny arachidonové, zejména PGE_2 a PGF_{1a} s celkovým cytoprotektivním a protizánětlivým účinkem (36). V pankreatické tkáni je PAR-2 exprimován mnoha buněčnými typy a to jak ve strukturách acinárních, tak i ve strukturách duktů. V endokrinní části pankreatické tkáně jsme prokázali expresi PAR-2 v buňkách Langerhansových ostrůvků. Trypsin jako přirozený ligand PAR-2 může být v pankreatické tkáni aktivován za fyziologických nebo patologických podmínek, zejména pak při zánětlivých onemocněních pankreatu, kdy je trypsin aktivován předčasně. Je zřejmé, že aktivní trypsin a jeho schopnost aktivace PAR-2 v pankreatické tkáni hrají významnou úlohu v zánětlivých onemocněních pankreatu, zejména pak v průběhu akutní pankreatitidy (66).

Přítomnost PAR-2 v CNS byla prokázána v řadě buněčných typů, například neuronálních elementech, gliálních buňkách, meningoteliálních elementech, endoteliích atd. v mnoha oblastech a strukturách mozku a míchy, zejména v hipokampální formaci, amygdale, thalamu, hypothalamu, stratu a isokortexu. Obdobně i v periferním nervovém systému je exprese PAR-2 velmi široká (37,38,50). Účinky aktivace PAR-2 mohou mít v různých buněčných typech různých struktur CNS velmi rozdílné účinky. PAR-2 aktivace v hipokampálních neuronech má cytotoxický efekt v přímé závislosti na koncentraci. PAR-2 je vysoce exprimován v různých oblastech mozku po hypoxickém a hypoglykemickém inzultu (47). V současné době jsou široce diskutovány vlivy působení serinových proteáz a aktivace jejich receptorů v etiopatogenezi a progresi různých neurodegenerativních onemocnění. Nejlépe byl dosud popsán efekt aktivace PAR v myším modelu Parkinsonovy nemoci. V tomto modelu dochází po aplikaci serinových proteáz do oblasti substantia nigra

k selektivní buněčné smrti populace dopaminergních neuronů. Důležitou roli zde zřejmě hraje aktivace mikrogliálních elementů. Účast serinových proteáz a aktivace jejich receptorů v patogenezi Alzheimerovy nemoci byla také opakovaně studována. Bylo prokázáno, že proteolytická aktivita některých serinových proteáz prostřednictvím aktivace PAR přímo zasahuje do tvorby patologických konformerů tau proteinu a amyloid prekurzorového proteinu a tím se podílí na vzniku senilních plak a neurofibrilárních klubek. Relativně nedávno byla popsána úloha PAR-1 v neurodegenerativních změnách při encefalitidě související s nákazou virem HIV-1 (49). V experimentálních modelech ischemických cévních mozkových příhod byly rovněž pozorovány změny v expresi PAR (50). PAR-2 se uplatňuje i v patofyziologii bolesti a nocicepce. Aplikace agonistického peptidu v hladinách, které nevyvolávaly zánětlivou odpověď, vedla v myším modelu k dlouhotrvající hyperalgézii, která byla mnohem účinnější než obecně používané stimulátory hyperalgézie jako např. PGE2. Navíc byla stejnými autory v myší míše prokázána up-regulace c-fos jako markeru aktivace nociceptivních neuronů po aplikaci PAR-2 agonistického peptidu (51-53).

1.3.2 PAR při radiačním poškození tkání

Ve vztahu k radiačnímu poškození jsou zajímavé zejména poznatky o vlivu PAR na cévy a krevní oběh. *In vivo* dochází působením trypsinu nebo SLIGRL cestou aktivace PAR-2 na endoteliích k dilataci bazilární arterie potkanů (12). Naopak vliv trombinu a trypsinu zprostředkovaný PAR se prostřednictvím hladkých svalových buněk medie projevuje vazokonstrikcí (23). V případě radiačního poškození se exprese PAR-1 zvyšuje v endoteliálních a svalových buňkách a provází neointimální formaci a změny v hladkých svalech medie (54). Trombin působí zároveň i jako mitogenní faktor pro buňky hladké

svaloviny cév, které jsou významným činitelem postiradiační cévní sklerotizace a fibrotizace (24). PAR navíc regulují permeabilitu kapilár a jejich aktivací dochází k tvorbě edému (25).

PAR jsou exprimovány i na zánětlivých elementech, kde jejich aktivace vede ke stimulaci těchto buněk (26,27). Jiné studie dokládají, že buněčná odpověď imunokompetentních buněk na stimulaci PAR-1 α-trombinem i SLIGRL závisí na stupni její aktivace (28). Trombin tak zprostředkovává přímou funkční vazbu mezi systémem hemostázy a imunitním systémem. Stimulace tvorby cytokinů na některých buňkách v průběhu radiačního poškození dokládá podíl PAR i na regeneračních a reparačních procesech. Aktivace PAR vede na fibroblastech ke zvýšené tvorbě kolagenu a následné fibróze (29,30,54).

Asi nejkomplexnější studii mezi vztahem PAR a radiačního poškození publikovali Wang et al. na modelu ozářeného krysího střeva. V tomto modelu autoři prokázali výrazné zvýšení exprese PAR-1 po ozáření, které bylo závislé na dávce. Takto významné zvýšení produkce PAR-1 pak dávají do souvislosti s aktivací celého komplexu následných dějů, jejichž přímým důsledkem je postiradiační endotelová dysfunkce s poruchou mikrocirkulace následovaná cévní sklerotizací. Výsledkem celého procesu je postiradiační střevní fibrotizace (54).

V souhlase s těmito pozorováními jsou i výsledky naší práce, kdy jsme prokázali výrazné zvýšení exprese PAR-2 na strukturách krysího mozku po ozáření.

1.4   PAR-2 v procesu vzniku a progrese nádorové transformace.

Mikroprostředí nádoru významnou měrou ovlivňuje vlastnosti nádorových buněk a tím i biologické chování nádorů. Bylo zjištěno, že v tomto mikroprostředí se vyskytují různé druhy serinových proteáz, které mohou aktivovat PAR. Nádorové buňky samy mohou

produkovat a ve skutečnosti často i produkují významná množství serinových proteáz, ktcré zpětně ovlivňují buněčný růst nádorových elementů, hrají důležitou roli v procesu degradace extracelulární matrix, zvyšují invazivitu nádorových elementů a jejich metastatický potenciál, podílejí se na angiogenezi, remodelaci tkání a na dalších důležitých dějích souvisejících s progresí nádorových onemocnění (55).

PAR-1 i PAR-2 jsou exprimovány v širokém spektru nádorových buněk. Opakovaně byly popsány v buněčných kulturách karcinomu mammy, tlustého střeva, žaludku, prostaty, plic a dalších (56-58). Kromě vlastních nádorových elemetů se PAR vyskytují i v makrofázích, endoteliích, buňkách hladké svaloviny a fibroblastech nádorového stromatu (56). Několikanásobné zvýšení exprese PAR-1 a PAR-2 v porovnání s jejich expresí nemaligními alveolárními buňkami bylo prokázáno v modelech karcinomu plic. V plicních nádorech byla zaznamenána i zvýšená produkce trypsinu a jiných serinových proteáz (55).

Metastazování nádorů vyžaduje celou řadu složitých pochodů včetně uvolňování proliferujících buněk z nádorové masy, degradaci nádorového stromatu a bazálních membrán a zvýšenou buněčnou motilitu. Bylo přesvědčivě prokázáno, že serinové proteázy a aktivace jejich receptorů se na těchto dějích významnou měrou podílejí. Darmoul et al. v nedávné minulosti zjistili, že trypsin, který účinkuje jako přirozený ligand PAR-2, je významným růstovým faktorem pro buňky karcinomu tlustého střeva (59). Ve své další práci prokázali, že agonisté PAR-2 transaktivují cestou aktivace receptoru pro epitelový růstový faktor (EGF-R) kaskádovitým dějem, který zahrnuje štěpení a uvolňování TGF alfa prostřednictvím matrix-metaloproteinázového komplexu. EGF-R se pak stane aktivním a spuštěním MAPK kaskády odstartuje buněčnou proliferaci (60). Kromě toho prokázali i vliv aktivace PAR-2 na další významnou buněčnou signalizační dráhu. Použitím specifických farmakologických a protilátkových blokátorů ozřejmili, že aktivace PAR-2 vede přímo k fosforylaci ERK1/2. Význam tohoto silně potentního aktivovaného

mitogenního faktoru v mechanismech regulujících buněčný cyklus a buněčnou proliferaci je dobře známý (60).

V modelech karcinomu mléčné žlázy Ge et al. prokázali, že více invazivní nádorové buněčné kultury (MDA MB-231) produkují větší množství serinových proteáz než buněčné linie karcinomu mammy s nižším metastatickým potenciálem (MDA MB-484). Zároveň prokázali, že aktivace PAR-2 trypsinem v tomto modelu vede k vyšší metastatické aktivitě buněčné linie tvorbou pseudopodií po PAR-2 zprostředkované fosforylaci ERK1/2 a aktivaci beta-arrestinových molekul, jež jsou klíčovými molekulami transcelulárního transportního systému (61). Hjortoe et al. na modelu stejné nádorové buněčné linie mammárního karcinomu prokázali interakci mezi aktivací PAR-2 a tkáňového faktoru (TF) zprostředkovanou proteázovou molekulou faktoru VIIa vedoucí ke zvýšené produkci některých cytokinových molekul. V tomto případě byla nejvýznamnější zvýšená exprese potentního chemokinu - interleukinu 8 (IL8), který zprostředkovává vyšší invazivitu nádorových elementů, angiogenezi a buněčnou migraci (62).

Další pozorování účinku aktivace PAR na růst a metastatický potenciál nádoru byla prováděna na karcinomech žaludku. Miyata et al. prokázali, že aktivace PAR-2 prostřednictvím integrinu alfa5beta1 zvyšuje adhezivitu nádorových buněk k fibronektinu a vitronektinu a zvyšuje také proliferaci buněčné linie karcinomu žaludku MKN-1 (63). Jiná studie na buněčné linii karcinomu žaludku STKM-1 prokázala souvislost mezi autokrinní produkcí trypsinu nádorovými elementy a zvýšenou schopností invazivního růstu (64).

1.5   Karcinomy mléčné žlázy

Zhoubné nádory mléčné žlázy patří mezi nejčastější maligní novotvary a jsou jednou z nejčastějších příčin úmrtí na nádorové onemocnění v ženské populaci (65). Největší skupinu maligních novotvarů mléčné žlázy představují zhoubné epitelové nádory - karcinomy. Karcinomy se na základě mikroskopického hodnocení dělí na neinvazivní (in situ) karcinomy a na karcinomy invazivní. Mikroskopická invaze je definována jako přesvědčivá invaze nádorových buněk do stromatu, bez ohledu na přítomnost in situ komponenty. Podle cytoarchitektonických hledisek se invazivní kacinomy mléčné žlázy rozdělují do několika typů, avšak více než ¾ z nich představuje klasický, jinak dále nespecifikovaný, typ duktálního karcinomu. Pro určení prognózy nádorového onemocnění je rozhodující hodnocení tzv. prognostických faktorů, z nichž k nejvýznamnějším patří určení klinického a klinicko-patologického stádia onemocnění, které zahrnuje velikost nádoru a přítomnost metastatických nádorových ložisek v regionálních lymfatických uzlinách či ve vzdálených orgánech. K dalším důležitým prognostickým hlediskům patří i stupeň diferenciace (grade) nádoru a exprese některých klíčových proteinů. Mikroskopický grade v současnosti nejužívanějšího skórovacího systému posuzuje 3 základní kriteria (Nottinghamská modifikace původní Bloom-Richardsonovy klasifikace). Je to tendence k tvorbě tubulárních formací, míra jaderné nepravidelnosti a mitotická aktivita. Výsledný počet dosažených bodů rozdělí nádory na nejlépe diferencované Grade I až po nejhůře diferencované Grade III. Užitečnost a reproducibilita tohoto skórovacího systému byly opakovaně ověřeny. V současné době by informace o gradingu měla být rutinní součástí zprávy patologa o nálezu karcinomu mléčné žlázy.

Z hlediska exprimovaných proteinů patří v současné době k rutinně stanovovaným exprese estrogenového a progesteronového receptoru, exprese proteinu p53 a exprese onkogenu c-

erb-B-2. Zatímco zjištění akumulace proteinu p53 v cytoplasmě či jádrech nádorových buněk, která může souviset s mutací genu Tp53, má spíše význam obecného epidemiologického rizikového faktoru, přítomnost onkogenu c-erb-B2 a zejména steroidních receptorů má přímé využití i pro volbu příslušné terapeutické strategie. „Zlatým standardem" určování exprese estrogenního a progesteronového receptoru je v současné rutinní praxi patologa imunohistochemická detekce, většinou hodnocená semikvantitativně. U onkogenu c-erb-B2 je prováděna imunohistochemická detekce proteinu specifickou monoklonální protilátkou s relativně jednotným přístupem k hodnocení. V prokazatelně pozitivních případech by před nasazením specifického chemoterapeutika měla být ověřena exprese tohoto onkogenu ještě metodou fluorescenční in situ hybridizace (FISH).

Z hlediska určení prognózy platí, že v případě stoupajícího gradu se prognóza zhoršuje, obecně lepší prognózu mají tumory s detekovanou expresí estrogenního a progesteronového receptoru a proteinu c-erbB2 (HER2/Neu). Exprese onkogenu c-erbB2 také relativně dobře koreluje s mikroskopicky hodnocenou diferenciací nádoru (gradingem), vztah exprese steroidních receptorů a mírou diferenciace nádorové populace není zcela jednoznačný.

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2. Cíle práce

2.1 Vyhodnocení změny exprese PAR-2 v zánětlivé lézi – Zvířecí model experimentálně vyvolané akutní pankreatitidy.

2.2 Vyhodnocení změny exprese PAR-2 v radiační lézi - Imunohistochemická lokalizace PAR-2 na strukturách tkání CNS v časném postiradiačním období u myší kmene C57Bl/6.

2.3 Vyhodnocení změny exprese PAR-2 v nádorové lézi - Exprese PAR-2 v infiltrativním duktálním karcinomu mammy a vliv trypsinu na růst a metabolismus buněčné linie mammárního karcinomu MDA MB-231.

3.   Výsledky.

3.1.1   T. Olejár, R. Matěj, M. Zadinová, P. Poučková: Taurocholate-induced acute pancreatic lesion in Wistar rats: Immunohistochemical observation of proteinase-activated receptor 2.

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Research Article

Expression of Proteinase-Activated Receptor 2 During Taurocholate-Induced Acute Pancreatic Lesion Development in Wistar Rats

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Abstract

Background: Proteinase-activated receptor 2 (PAR-2) is a G-protein coupled transmembrane receptor activated by trypsin by site-specific cleavage. Its presence on pancreatic structures was demonstrated in the past. PAR-2 physiologically involves in duct/ acinary cells secretion, arterial tonus regulation or capillary liquid turnover. During development of acute pancreatitis/ acute pancreatic lesion (APL) these mentioned structures are influenced by very high concentration of trypsin due to its increased basolateral secretion into the interstitium. The aim of our study as presented was to investigate whether PAR-2 is also involved in APL following changes of PAR-2 expression.

Methods: APL was investigated in Wistar rats after injection of 0.1 mL taurocholate into the ductus choledochus. Anatomy, histology, reverse transcriptase polymerase chain reaction (RT PCR) as well as immunohistochemistry and Western-blot analysis of pancreatic tissue were performed using antibody mapping of the new NH_2 terminal of PAR-2 after trypsin cleavage. Results from control rats and d 1 or d 4 rats after taurocholate injection were compared.

Results: Much higher positivity on acinary/ duct cells was observed in APL induced animals than in controls. Similar findings were noticed on arterial smooth muscle cells. Surprisingly, parallel to the exocrine pancreas and vessel findings, enhanced Langerhans' islet cell positivity was observed in experimental animals.

Conclusions: Based on these results, we have demonstrated that during APL development PAR-2 expression increases. This effect is caused by conformational changes after PAR-2 activation, and the new NH_2 terminal of activated receptor presentation. We suggest that PAR-2 physiological functions are enhanced during APL development.

Key Words: Proteinase-activated receptor 2; trypsin; acute pancreatitis; acute pancreatic lesion.

Introduction

Necrosis of parenchyma and/or fatty tissue caused by digestive enzymes (trypsin, lipase and others) and

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following local disturbance of blood supply are the major signs in acute pancreatitis. Macroscopically, oedema focal necroses and haemorrhage are observed in the developed disease. Microscopically, necroses of parenchyma and fatty tissue of pancreas are seen. Ghost lines of necrotic fatty cells or only a pale eosinophylic material is found in fatty tissue. In these foci, fatty acid crystals and hematoidin pig-

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ment may appear. Necrosis of glandular parenchyma is in the beginning characterized as coagulation necrosis circumscribed with the edge of polynuclear leucocytes (1).

From experimental studies in animal models, it is known that axial trans-Golgi transport of proenzymes into the acinary lumen fails and basolateral secretion into the interstitium of gland increases (2). This failure can be induced by obstruction (cholelithiasis, spasm of papilla Vateri), toxically (alcohol), or by ischaemia (vasospasm, shock). Morphologically, this axial transport failure appears as dilatation of the Golgi apparatus (3). The state of organ damage depends on the amount of activated enzymes in the interstitium of gland. On the other hand, the drainage quality of a given region performed by lymphatic vessels is also very important in the development of the disease. In the organ as a whole, lymphatic drainage depends on the quality of local blood circulation, state of vascular barrier, capillary turnover, and even on the quality of lymphatic vessels.

Following systemic inflammatory responses are those caused by inflammatory cytokines (IL-1, IL-4, IL-6, IL-8, IL-10, TNF-α and others) produced in damaged pancreas (4,5). Increased density of adhesion molecules (CD54, CD62E, CD62L) on endothelial cells closes the *circulus vitiosus* of inflammatory response. Production of all these substances is related to oxidative stress (6), but also to certain pancreatic enzymes activated in the interstitium.

It was presented that one of the pancreatic enzymes, trypsin, modulates many biological processes via specific proteinase-activated receptor 2 (PAR-2). It belongs to a family of G-protein coupled receptors activated by tethered ligand sequences within the amino terminus made accessible by site-specific proteolysis (7).

Trypsin activates PAR-2 by the mediation of a unique process inherent in the recognition of the receptor by enzyme, subsequent cleavage at a specific site of the NH_2 terminus, and presentation of a new NH_2 terminus that behaves as a tethered ligand. This ligand interacts with the extracellular domain of the receptor molecule. Thus, PAR-2 is a receptor whose ligand is a physical part of the receptor molecule (8). This receptor was previously described on physiologic as well as malignant immunocompetent cells, on endothelial and muscle cells of major as well as minor vessels. Its presence was also immunohistochemically demonstrated on intestinal epithe-

lial cells, epithelial cells of exocrine organs (including pancreatic duct and acinary cells or pancreatic tumour cells [7,9]), keratinocytes, fibroblasts, and other cells (10). From this point of view, our study was to describe the immunohistochemical presence of activated PAR-2 in an animal model of acute pancreatitis/ acute pancreatic lesion (APL) induced by intraductal injection of taurocholate. We have also utilized RT-PCR to confirm presence of PAR-2 in pancreatic tissue, mainly in isolated Langerhans islets at the mRNA level.

Materials and Methods

Animals

We abided by the guidelines on animal experimentation of the 1st Medical School, Charles University, Prague, and all procedures were licensed by the institutional animal experimentation review committee.

Inbred Wistar rats (AnLab Ltd.—Charles River) were used for the experiment. Rats were bred in an SPF animal breed with a radiation-sterilized bedding (SAWI— Research Bedding), and fed with chow also sterilized by radiation. Autoclaved water was provided *ad libitum*. In tested groups, rats were injected with 0.1 mL of taurocholate into the *ductus choledochus* in thiopental induced anesthesia. After the taurocholate injection, time dependent and control observation was performed.

1. Control group: control rats were sacrificed, acute pancreatitis was not induced.
2. d 1 group: rats were sacrificed 1 d after taurocholate injection into the ductus choledochus.
3. d 4 group: rats were sacrificed 4 d after taurocholate injection into the *ductus choledochus*.

All animals were subjected to evisceration of the pancreas, and these were consequently fixed in buffered formaldehyde (10%) for histological and immunohistochemical staining.

Langerhans' Islets Isolation

Islets were isolated as previously described (11,12). Male Wistar rats (AnLab Ltd.—Charles River) 10–12 wk old, weighing 250–300 g, were used as a source of islets. In brief, 10 mL of collagenase solution 1 mg/mL (Sevac) was used for pancreas dissension and Ficoll gradient centrifugation

(Ficoll 400 L, Sigma) was used for islets purification. After isolation, islets were deeply frozen and stored for RT-PCR investigation.

Histology

Fixed samples of tissue were embedded in paraffin blocks. Tissue slides, 7 μm thick, were cut and stained with hematoxilin-eosin (HE).

Immunohistochemistry

Tissue slides (7 μm) were deparaffinized and transferred into a water solution. Consequently, they were boiled in citrate buffer (pH 7.6) 3 × 5 min in a microwave oven. Endogenous peroxidase was blocked with 0.05 mg of natrium azid and 5 mL of hydrogen peroxide in 50 mL of demineralized water. Non-specific positivity was blocked with 150 μL of rabbit serum in 10 mL of tris-buffered saline (TBS) for 30 min.

Goat polyclonal primary antibody mapping the new NH_2 terminus (SLIGRLETQPPITGKGVPVC) of murine and rat PAR-2 (PAR-2 (S-19): sc-8207 Santa Cruz Biotechnology, Inc.) was diluted 1 : 500 in 5% fetal bovine serum in TBS and put on slides for overnight at 4°C. Consequently, slides were incubated with biotinylated secondary antibody (rabbit anti-goat) and then with streptavidin-biotin conjugated horseradish peroxidase (VECTASTAIN ABC Kit, VECTOR Laboratories Ltd.).

Slides incubated with secondary antibody only were used as control of specificity.

Diaminobenzidine was used as a chromogen and Harris hematoxilin as a counterstain.

Western Blotting Analysis

Deeply frozen pancreatic tissue was homogenized, dissolved in 2X Tris-glycine SDS sample buffer (Novex), and boiled for 5 min. Equally 20 μg of protein was fractioned on SDS-PAGE and blotted to nitrocellulose membrane (S&S NC) using Mini TransBlott® Cells (BioRad). After visualization by Poceau S staining (Sigma) membranes were blocked with 5% nonfat dried milk in PBS with 0.1% Tween 20 at 4°C overnight. Goat polyclonal primary antibody (PAR-2 (S-19): sc-8207 Santa Cruz Biotechnology, Inc.) was diluted 1 : 1000 in 5% nonfat milk in T-PBS. The filters were incubated for 1 h and washed 6 × 10 min in T-PBS. The secondary antibody, peroxidase-conjugated (AffiniPure Rabbit Anti-Goat IgG (H+L), Jackson ImmunoResearch

Laboratories, Inc.) was diluted 1 : 10 000 in T-PBS. The filters were incubated for 45 min and washed in T-PBS 6 × 10 min. Peroxidase activity was detected by Amersham's ECL+, following the manufacturers protocol.

RT-PCR

Deeply frozen pancreatic tissue was homogenized (approx 50 mg). Isolated, deeply frozen Langerhans islets were used whole. Total RNAs were isolated using TRIzol® Reagent (Life Technologies, GIBCO BRL) according to the producers protocol. Concentration of RNA was done on spectrophotometer 280/260 nm. Primers (13) for PAR-2 were 5'-ACCCC GCCGTGATTTACATGGC-3', 5'-GCCGGGAA CAGGAAGACTC-3' (Life Technologies, GIBCO BRL). Primers for β-actin served as internal control 5'-GGACGACATGGAGAAGATCTG, 3'-CCGCT CGTTGCCAATAGTGAT (Life Technologies, GIBCO BRL). For reverse transcription, the Superscript II kit (Life Technologies, GIBCO BRL) was used. For PCR, the PCR Core Kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals) was used. Amplification cycles consisted of 45 sec at 93°, 45 sec at 55°C, and 1 min at 72°C for 30 cycles. PCR reaction was performed on a PCR thermocycler (MJ Research). Products were analyzed electrophoretically on 1% agarose gel with ethidium bromide.

Results

Anatomy

In both taurocholate induced APL groups (d 1 and d 4), blood congestion of pancreatic parenchyma and fatty necroses of peripancreatic adipose tissue was observed.

Histology

In the d 1 group, only oedema of interstitium accompanied by a small number of inflammatory cells (polynuclear leukocytes and macrophages) and mainly centrolobular vacuolisation of acinary cells were mostly observed. In the d 4 group, much more pronounced inflammatory cellular response with interstitial cells (fibroblasts) activation, necrotic loci, and repair/ proliferation changes in exocrine glandular parenchyma were observed, as shown previously (data not shown).

Immunohistochemistry

In the control group, only a weak positivity of PAR-2 was noticed on acinary cells. In high-power observation, only disperse intracellular granular positivity was observed in cells (Fig. 1A). The acinary cells in experimental groups showed a significant increase of immunohistochemical positivity with basolateral accentation in early stages (one day after induction of APL), and diffuse on d 4 after induction of acute pancreatic lesion. In high-power observation, this was represented by enhanced basolateral or diffuse granular positivity, respectively (Fig. 1B,1C). Only weak and disperse positivity of PAR-2 was noticed on duct cells in control rats (Fig. 1D). In experimental groups, a diffuse enhanced presence on duct cells was demonstrated (Fig. 1E). In the control group, mainly high endothelial positivity was demonstrated on venules, very low on arteries and veins (data not shown). Presence of PAR-2 on smooth muscle cells of vessels was not significant in control rats. In experimental groups, endothelial presence of PAR-2 was also observed. However, PAR-2 positivity on arterial endothelial cells was also slightly enhanced in comparison to controls. Furthermore, in experimental groups, a significant membrane presence of PAR-2 was demonstrated on arterial smooth muscle cells (Fig. 1F). A significant difference of PAR-2 presence was noticed on Langerhans islet cells of control and experimental groups, respectively. In the control group, only discrete positivity of PAR-2 was observed in Langerhans islet cells (Fig. 1G). Compared to controls, diffusely immunohistochemically stained Langerhans islet cells appeared in both experimental groups (Fig. 1H,1I). This PAR-2 positivity was enhanced on capillary poles of islet cells. In experimental groups, new elements such as inflammatory cells (membrane positivity mainly macrophages, data not shown), fibroblasts (Fig. 1E), non-specific granulation tissue (data not shown), and proliferating/regenerating exocrine epithelial cells (Fig. 1I) appears PAR-2 positive as well.

Western-Blot Analysis

Western-blot analysis confirms immunohistochemical positivity of PAR-2 in the pancreatic tissue. Fig. 1 describes the presence of PAR-2 proteins in control (Fig. 2A), d 1 after taurocholate induction of APL (Fig. 2B) and d 4 after taurocholate induction of APL (Fig. 2C).

RT PCR

PAR-2 presence was observed in control (Fig. 3A) and taurocholate-induced APL tissue of rats (Fig. 3B,3C). No significant changes in relative production of PAR-2 were observed in d 1 or d 4 after induction of APL with taurocholate. Presence of PAR-2 was also confirmed from the specimen of isolated Langerhans' islets (Fig. 4).

Discussion

From the presented results, studied PAR-2 immunohistochemical positivity increases in development of APL simultaneously on pancreatic structures. No marked change in PAR-2 mRNA production is not surprising. The self-stimulation of this G-protein coupled receptor on the mRNA level is not known from the literature, although its activation leads to a large number of highly interconnected cytoplasmic signaling routes. The amount of mRNA synthesis, in general, says nothing about final membrane localization of this receptor, its cleavage and consequent physiological or pathophysiological functions.

Changes in protein immunohistochemical positivity are related to intracellular receptor redistribution and new epitope presentation after conformational changes following specific receptor activation *(14)*. As the antibody is mapping the epitope of the new NH_2 terminus of PAR-2 of rat and mouse origin *(15)*, immunohistochemistry demonstrates activated receptors. Our results demonstrate that PAR-2 is strongly activated during acute pancreatitis/APL development.

In general, PAR-2 mediation causes different intracellular responses, including the phosphatidylinositol system activation on epithelial-intestinal epithelial cells, resulting in the intracellular utilization of arachidonic acid and secretion of prostaglandins *(16)*. It is suggested that PAR-2 activation by trypsin participates in the process of absorption in a nutritional way. From this point of view, the major function of PARs in the digestive system seems to be, in general, the stimulation of nutritional transport. Some authors suggested, investigating pancreatic duct cells in vitro, that activation of intracellular biochemical processes in these cells by trypsin could also participate in tissue debris elimination in APL *(17,18)*. It is suggested that duct
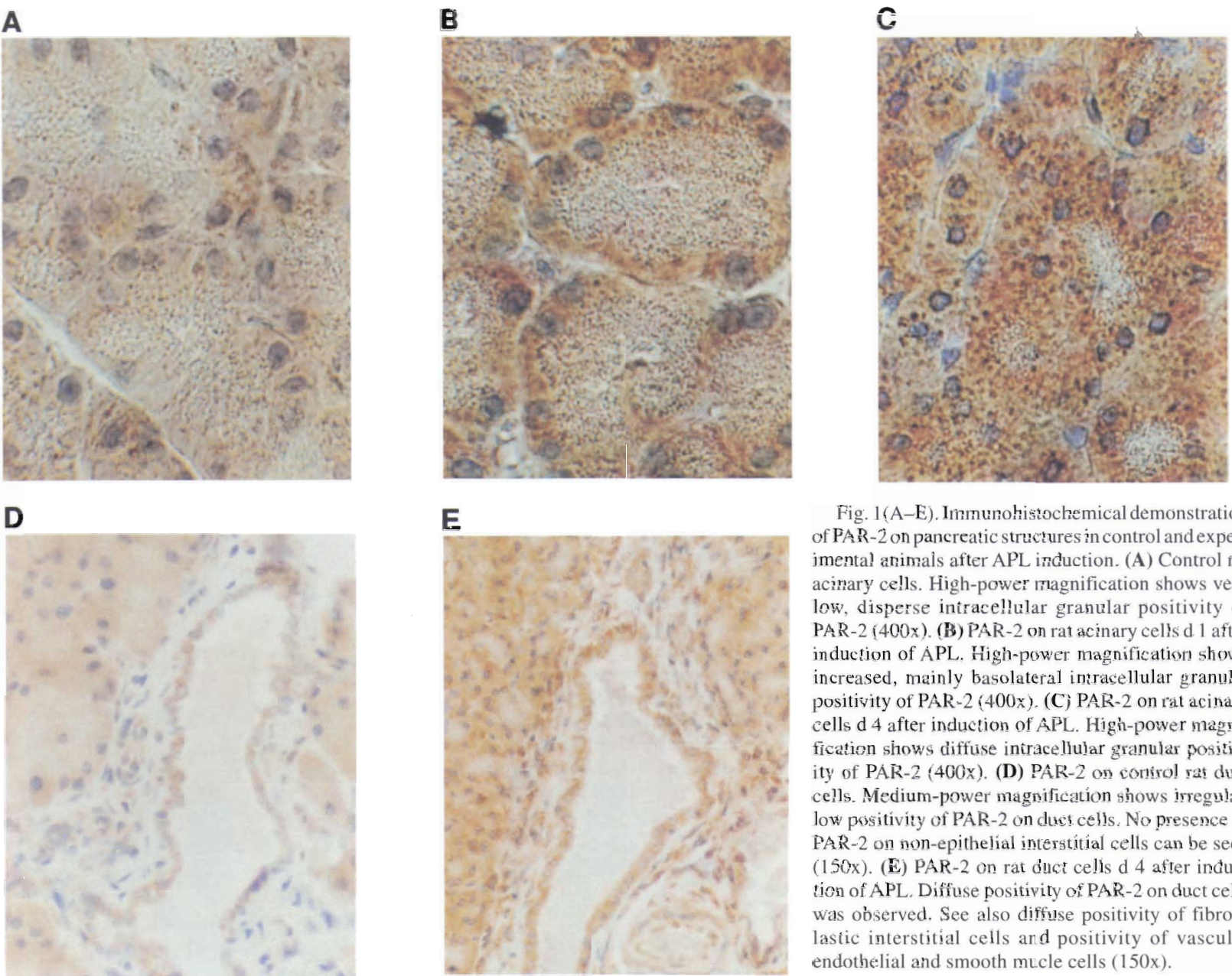
Fig. 1(A–E). Immunohistochemical demonstration of PAR-2 on pancreatic structures in control and experimental animals after APL induction. (A) Control rat acinary cells. High-power magnification shows very low, disperse intracellular granular positivity of PAR-2 (400x). (B) PAR-2 on rat acinary cells d 1 after induction of APL. High-power magnification shows increased, mainly basolateral intracellular granular positivity of PAR-2 (400x). (C) PAR-2 on rat acinary cells d 4 after induction of APL. High-power magnification shows diffuse intracellular granular positivity of PAR-2 (400x). (D) PAR-2 on control rat duct cells. Medium-power magnification shows irregular, low positivity of PAR-2 on duct cells. No presence of PAR-2 on non-epithelial interstitial cells can be seen (150x). (E) PAR-2 on rat duct cells d 4 after induction of APL. Diffuse positivity of PAR-2 on duct cells was observed. See also diffuse positivity of fibroblastic interstitial cells and positivity of vascular endothelial and smooth mucle cells (150x).

F

G

H

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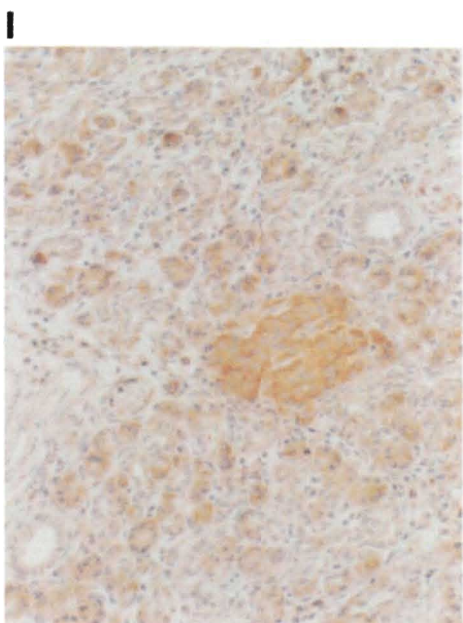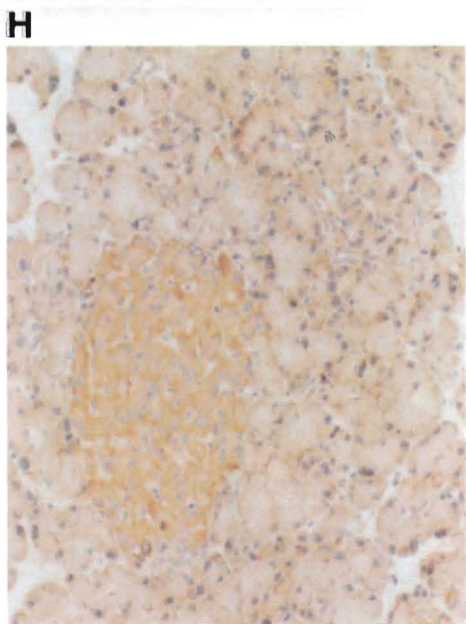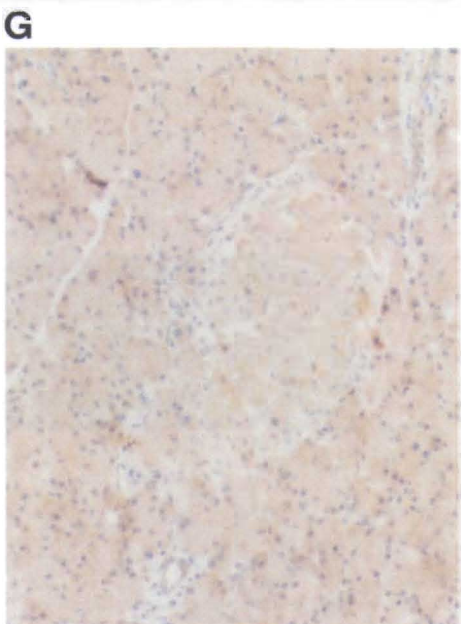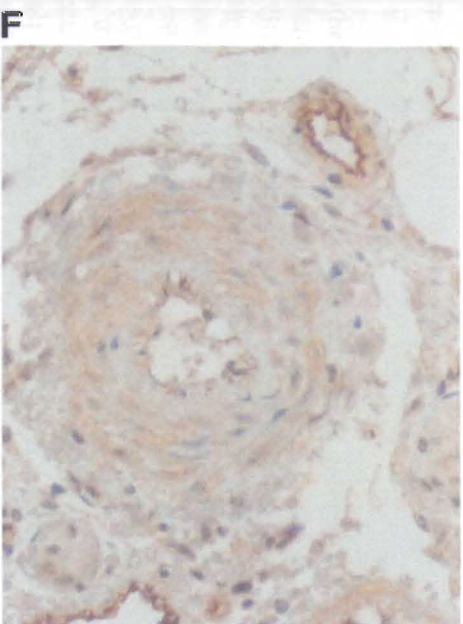
Fig. 1(F–I). **(F)** Detailed observation of vessels in rat peripancreatic fatty tissue d 4 after induction of APL. Significant membrane positivity of PAR-2 was observed on arterial smooth muscle cells, however, this positivity on endothelial cells was only moderate. Moderate positivity was observed on veins in contrary to very high endothelial positivity on venules (150x). **(G)** PAR-2 on Langerhans islet cells of a control rat. Very low positivity is present in low-power magnification (150x). **(H)** PAR-2 on rat Langerhans islet cells d 1 after induction of APL. Low-power magnification shows diffusely increased positivity of PAR-2 Langerhans islet cells with prevalence on vascular poles of cells. Increased basolateral positivity of acinary cells can also be seen (150x). **(I)** PAR-2 positivity of Langerhans islets remaining in damaged exocrine pancreatic tissue of rats d 4 after induction of APL. See also irregular positivity of PAR-2 on repair and/or proliferating cells of a damaged part of exocrine pancreatic tissue (100x).
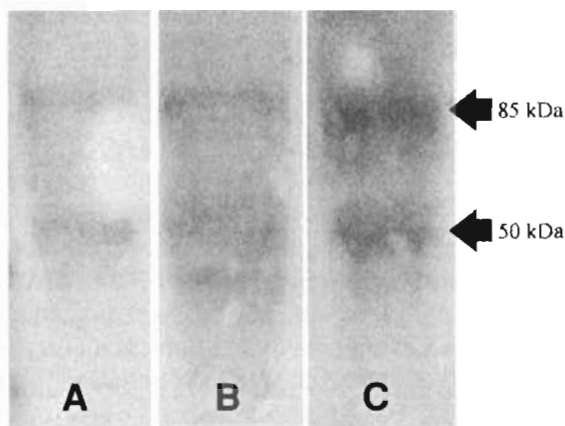
Fig. 2. Western blot analysis demonstrates presence of PAR-2 in pancreatic tissue of control (**A**). d 1 (**B**) and d 4 (**C**) rats after taurocholate-induced APL. Arrows indicate analysis of differentially glycosylated forms of PAR-2 expressed in pancreatic tissue (85 kDa and 50 kDa).
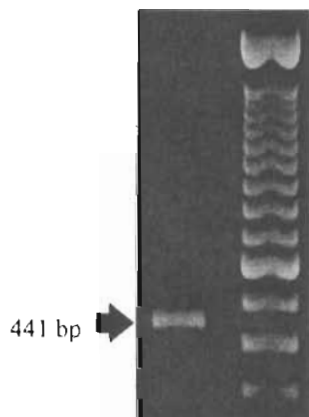

Fig. 4. RT PCR products of 441 bp demonstrate presence of mRNA PAR-2 from isolated Langerhans islets.

epithelium participates in reduction of interstitial oedema via activation of PAR-2.

Granular cytoplasmatic (basolateral or furthermore diffuse during APL development) presence of activated PAR-2 in acinary cells can be explained by enhanced transcellular transport of endocytic vesicles containing active pancreatic enzymes, including trypsin. Trypsin containing granules probably represent PAR-2 positivity similar to tryptase-positive granules in mast cells that function to sustain further mast cells degranulation upon exocytosis[19]. From this point of view, previous results
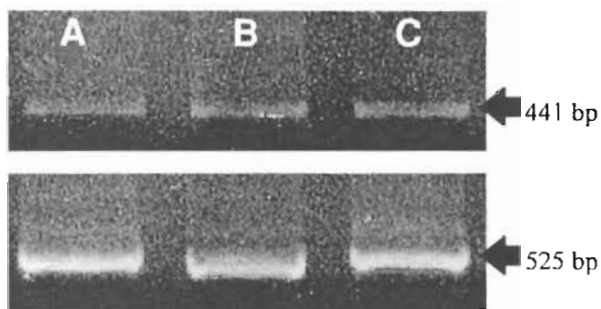
Fig. 3. RT PCR products of 441 bp demonstrate presence of mRNA PAR-2 in pancreatic tissue of control (**A**), d 1 (**B**) and d 4 (**C**) rats after taurocholate-induced APL. β-actin served as an internal control (RT PCR products of 525 bp).

regarding positive modulation of melanin ingestion into keratinocytes by PAR-2 *(20,21)*, and PAR-2 presence on intestinal epithelial cells, regulating their function *(16)* suggest its general role in the processes of endocytosis, transcellular transport, and also exocytosis. Previously described proliferating action of trypsin (*[22]*, but also thrombin activating PAR-1, *[23]*) on different cells could be caused only by enhanced PAR-2 mediated vesicle internalization. In pancreatic cells, intake of trypsin from interstitium and transcellular transport is physiologically involved in the enteropancreatic circuit of trypsin *(24)*. Although this circuit is not physiologically important in general, because only a small amount of serum trypsin is secreted by the exocrine part of the pancreas *(25)*, in pathologic conditions (during APL), intake of trypsin-rich fluid and its transcellular transport enhancement after stimulation of PAR-2 is obvious *(18)*. Similar cytoplasmatic posititivity (mainly basolateral) of PAR-1 (similar receptor activated by another proteolytic enzyme: thrombin) can also be seen in other cells during inflammation (in kidney proximal tubular cells, *[26]*).

PAR-2 presence on Langerhans' islets and its function was not described previously. Whether the enhanced PAR-2 positivity in inflammatory conditions can be related clinically to the diabetes onset during severe acute pancreatitis/APL or onset of diabetes in course of chronic pancreatitis *(27)* should be resolved in the future.

PAR-2 positivity on endothelial cells and mainly on smooth muscle vascular cells higher in APL than

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3.1.2   Radoslav Matěj, Daniel Housa, Tomáš Olejár: Acute pancreatitis: Proteinase activated receptor - 2 as Dr. Jekyll and Mr. Hyde - Mini-review.

Acute pancreatitis: Proteinase-activated receptor – 2 as Dr. Jekyll and Mr. Hyde

Mini-review

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Running title: PAR-2 in Acute Pancreatitis

Key words: PAR-2, acute pancreatitis, trypsin

Summary

„Proteinase-activated" receptor 2 (PAR-2) is a G-protein coupled transmembrane receptor with seven transmembrane domains activated by trypsin. It has been shown in the pancreatic tissue that PAR-2 is under physiological conditions involved in duct/acinary cells secretion, arterial tonus regulation and capillary liquid content turnover. These above mentioned structures play an important role during the development of acute pancreatitis (AP) and are profoundly influenced by the high concentration of trypsin enzyme after its secretion into the interstitial tissue from the basolateral aspect of acinar cells. It is, among the other factors, the increase in interstitial trypsin concentration followed rapidly by PAR-2 action on pancreatic vascular smooth muscle cells that initiates ischaemic changes in pancreatic parenchyma and that leads finally to the necrosis of the pancreas. Consequent reperfusion perpetuates changes leading to the AP development. On the contrary, PAR-2 action on both exocrine and duct structures seems to play locally a protective role during the AP development. Moreover, PAR-2 action is not confined to pancreas but it contributes to systemic vascular endothelium and immune cells activation that triggers systemic inflammatory response syndrome (SIRS) contributing to high early mortality rate in severe disease.

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I

Acute pancreatitis

Depending on the severity, cytologic changes range from apoptosis (Bhatia 2004) to necrosis of parenchyma and/or fatty tissue caused by digestive enzymes and following local disturbance of blood supply are the major signs of severe "acute pancreatitis". Grossly, oedema, focal necrosis and hemorrhage are observed in the fully developed disease in the pancreatic region during the surgery or autopsy. Microscopically, there are necrosis of parenchyma and fatty tissue of pancreas visible together with the remnants of necrotic fatty cells and a pale eosinophilic (light pink) material in fatty tissue. In these foci, fatty acid crystals and hematoidin pigment as a sign of hemorrhage may appear. Necrosis of glandular parenchyma is in the beginning characterised as coagulation necrosis circumscribed with the edge of polynuclear leukocytes (Thomas 1989). Nevertheless, the disease is not confined to the pancreas alone but is always accompanied with pulmonary, renal, cardiovascular, central nervous and coagulation system injury potentially resulting in multiorgan dysfunction syndrome (MODS) with a high mortality rate.

It is known from experimental studies in animal models of acute pancreatitis that axial trans-Golgi transport of proenzymes into the acinary lumen fails and basolateral secretion into the interstitium of pancreatic gland increases (Rattner 1996). This failure can be induced by obstruction (bile stones, spasm of papilla Vateri, etc.), toxic substances (alcohol) or by ischaemia (vasospasm, shock, severe atherosclerosis). Morphologically, this axial transport failure appears as dilatation of Golgi complex (Cook *et al.* 1996). The state of organ damage depends on the account of activated enzymes in the interstitium of gland. On the other hand, drainage quality of region performed by lymphatic vessels is important in development of disease, too. In the organ as a whole, lymphatic drainage depends on quality of local blood circulation, status of vascular barrier and capillary turnover even on the quality of lymphatic vessels.

In the past, intracellular premature activation of trypsin protease leading to acinary cell necrosis was discussed as a causative agent of acute pancreatitis, however this theory failed in the scope of coagulation necrosis of cells related much more to the organ ischemic injury. $\alpha 1$ anti-trypsin deficiency

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was suspected mainly as a cause of the chronic pancreatitis and a link to acute disease can be found in literature (Novis *et al.* 1975), however other studies have not proved any correlation between pancreatitis and α1 anti-trypsin deficiency (Braxel *et al.* 1982, Witt *et al.* 2002) . Also the mast cell activation could be involved in the initiation of AP and the early phase of AP-induced MODS, however, mechanisms seem to be complex and are still to be elucidated (Dib *et al.* 2002). Also apoptotic cell death together with caspase-cascade activation and cytochrome C release from mitochondria, related to the mild and moderate pancreatitis, is currently discussed in literature (Bhatia 2004, Gukovskaya and Pandol 2004). Nevertheless ischemic changes by themselves do not result in AP but consequent reperfusion of an organ accompanied with free radicals release is necessary for inducing AP.

Following systemic inflammatory responses are caused by inflammatory mediators (IL-1, IL-4, IL-6, IL-8, IL-10, TNF-α and others) produced in damaged pancreas (McKay *et al.* 1996, Scholmerich 1996). Increase in density of adhaesion molecules (CD54, CD62E, CD62L) on the surface of endothelial cells closes the self-inducing circle of inflammatory response. Production of inflammatory substances is related to oxidative stress (Sweiry and Mann 1996) but also to certain pancreatic enzymes activation in the interstitium. The role of trypsin was investigated in relation to mediation of the local and systemic mediated inflammatory response (Hartwig *et al.* 2004).

Proteinase-activate receptor – 2

It was presented that one of the pancreatic enzymes, trypsin, modulates many biological processes by acting on specific proteinase-activated receptor 2 (PAR-2). PAR-2 belongs to a family of G protein-coupled receptors activated by tethered ligand sequences within the N-terminal that is made accessible after the site-specific cleavage of the protein (Bohm *et al.* 1996).

Trypsin activates PAR-2 by the mediation of a unique process inhering in the recognition of the receptor by enzyme, subsequent cleavage at the specific site of NH_2-terminal and presentation of a new NH_2 terminal, which behaves as a tethered ligand (see Fig. 1). This ligand interacts with the extracellular

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domain of receptor molecule. Thus, PAR-2 is a receptor, whose ligand is a physical part of the receptor molecule (Dery *et al.* 1998). This receptor was previously described on normal as well as malignant immunocompetent cells, on endothelial and muscle cells of major as well as minor vessels. Its presence was also immunohistochemically demonstrated on intestinal epithelial cells, epithelial cells of exocrine organs (including the pancreatic duct and acinary cells or pancreatic tumor cells) (Kaufmann *et al.* 1998), keratinocytes, fibroblasts and further cells of stomach, small intestine, colon, liver and kidney (Nystedt *et al.* 1995).

General Function of Proteinase-activate receptor – 2

As mentioned above, PAR-2 is expressed on variety cells with wide spectrum of cellular rersponses after activation. The function and biology of PAR-2 is reviewed in detail elsewhere (Cottrell *et al.* 2003, Dery *et al.* 1998, Steinhoff *et al.* 2005).

How trypsin acts on pancreatic structures and the role of PAR-2 in physiological conditions and during acute pancreatitis development

Vascular effect of Proteinase-activate receptor – 2 activation

PAR-2 is strongly activated during AP development (Olejar *et al.* 2001). PAR-2 presence on endothelial cells and mainly on smooth muscle vascular cells suggests that in AP development, one of the most discussed causal mechanism in acute pancreatitis – ischaemic-reperfusion injury (Toyama *et al.* 1996) – might be contributed by the activation of PAR-2 receptors after increased basolateral secretion of trypsin. Thus, PAR-2 activation on smooth muscle cells causes vasoconstriction (Moffatt and Cocks 1998). However, vasoconstriction and ischaemy doesn't cause the AP by itself, as already mentioned above. On the contrary, PAR-2 activation on endothelial cells causes pronounced vasodilatation *via* nitric oxide release (Cheung *et al.* 1998). We hypothesize that vascular effect of PAR-2 on vessels of different caliber leading to constriction or dilatation could be the major point

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starting the cascade of changes resulting finally, depending on severity, to the pancreatic cell apoptosis/ necrosis via ischemia-reperfusion mechanism mentioned above. Furthermore, PAR-2 activation primarily increases vascular permeability in general which could be probably linked also to edematous changes at the beginning of AP development (Kawabata *et al.* 1998). High concentrations and PAR-2 activation on vessels might be the leading mechanism causing pancreatic ischemy during AP initiation. Attacks of pain during chronic pancreatitis exacerbation could be also related to simple organ ischemy, probably without reperfusion mechanism leading to major necrosis as during AP. PAR-2 activation also increases IL-6 production (Chi *et al.* 2001), induce von Willebrand factor release, and serve as a mitogen for human umbilical vein endothelial cells (HUVEC) (Mirza *et al.* 1996, Nystedt *et al.* 1996, Storck *et al.* 1996).

Epithelial effect of Proteinase-activate receptor – 2 activation

PAR-2 seems to confer surprisingly protective effect on acinar cells during bile-induced cell damage and on the pancreatic ducts, acting therefore as a double-edged sword both in inducing and attenuating cellular damage (Kong *et al.* 1997). However, in this experiment, trypsin in the circulation of rats with taurocholate-induced severe acute pancreatitis reached levels sufficient to activate endothelial, and immune cells to stimulate nitric oxide and interleukin-8 production respectively. The activation of systemic PAR - 2 by circulating protease-activated receptor 2 agonists induced a hemodynamic response similar to that observed in rats with severe acute pancreatitis. Furthermore, certain authors hypothesize that trypsin, acting *via* PAR-2, might regulate the severity of that disease. They found that experimental acute pancreatitis is more severe in PAR-2(-/-) than in '*wild*' mice and that in vivo activation of PAR-2, achieved by parenteral administration of the PAR-2 activating peptide SLIGRL-NH2, reduces the severity of pancreatitis. This indicates that PAR-2 exerts also a protective effect in pancreatitis development and that activation of PAR-2 ameliorates pancreatitis in regards of the potential therapeutic use (Sharma *et al.* 2005).

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Some authors suggested, investigating pancreatic duct cells *in vitro* that activation of intracellular biochemical processes in these cells by trypsin could participate also in tissue debris elimination in AP (Kawabata *et al.* 2000, Nguyen *et al.* 1999). It is suggested that duct epithelium participates in the disappearance of interstitial edema by activation of PAR-2. In pancreatic acinal cells, intake of trypsin from interstitium and transcellular transport is physiologically involved in enteropancreatic circuit of trypsin (Beynon and Kay 1976). Although this circuit is not physiologically important under physiological circumstances because only a small amount of serum trypsin is secreted by the exocrine part of the pancreas. In pathologic conditions (during AP), intake of trypsin-rich fluid and its transcellular transport enhancement after PAR-2 stimulation is obvious. A similar pattern of cytoplasmatic posititivity (mainly basolateral) of PAR-1 (similar receptor activated by another proteolytic enzyme thrombin) can be seen also in other cells during inflammation (in kidney proximal tubular cells) (Grandaliano *et al.* 2000). The role of PAR-1 in the development of AP and its relation to thrombin content in interstitial edematous fluid during AP development is the aim of further studies (unpublished data). However, from present data we may conclude comprehensive action of PARs (Macfarlane *et al.* 2001). In general, PAR-2 mediation participates on different intracellular responses including the phosphatidyl-inositol system activation on epithelial-intestinal epithelial cells, which results in the intracellular utilization of arachidonic acid and secretion of prostaglandins (Kong *et al.* 1997). It is suggested that PAR-2 activation by trypsin contributes to the process of absorption in a nutritional way. From this point of view, the major function of PARs in the digestive system seems to be the stimulation of nutritional transport.

Lectures from knock-out models: Proteinase-activate receptor – 2 in general immunology

Variety of PAR-2 deficient murine models were investigated under different conditions to unravel putative physiological roles of PAR-2. The effect of PAR-2 comprises an amazing amount of effects on different functions of organism. Kawagoe et al. (Kawagoe *et al.* 2002) found that PAR-2 might play a significant role in type IV allergic dermatitis as PAR-2 defficient mouse ear treated with

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hypersensitivity inducing topical agents showed attenuated signs of inflammation as compared with wild type. Partially overlapping work of Seeliger et al. (Seeliger *et al.* 2003) confirmed the previous results. Allergic airway inflammation was used by Schmidlin et al. (Schmidlin *et al.* 2002) to show, among others, a diminished eosinophil infiltration in mice lacking PAR-2. Ferrel et al. (Ferrell *et al.* 2003) demonstrated a role for PAR-2 in mediating chronic inflammation in monoarthritis model with a significant reduction of joint swelling and no signs of articular destruction even at the microscopical level. Noorbaksh et al. (Noorbakhsh *et al.* 2005) described more severe neuroinflammation and neuronal loss in PAR-2 null animals in their work on HIV associated dementia. Work of Ley group (Lindner *et al.* 2000) showed a delayed onset of inflammation as examined by leukocyte rolling. Another work on ischemic brain injury (Jin *et al.* 2005) revealed the greater size of infarction focus and TUNNEL counted cells in knockout mice.

Proteinase-activate receptor – 2: Direct link between enzymatic digestion and immune system

Presence of PAR-2 on inflammatory cells was also demonstrated in the past. It causes intracellular Ca^{++} mobilisation, prostaglandin and cytokine synthesis on Jurkat T-cells (Mari *et al.* 1996) or intracellular Ca^{++} mobilisation on human granulocytes isolated *ex vivo* (Howells *et al.* 1997). As discussed above, in the rat model of acute pancreatitis, trypsin in the circulation of rats with taurocholate-induced severe acute pancreatitis reached levels sufficient to activate endothelial and immune cells to stimulate nitric oxide and interleukin-8 production (Namkung *et al.* 2004). It is suggested that high amounts of cytokines produced in AP (McKay *et al.* 1996) are induced by PAR-2 activation, too. PAR-2 plays also an important role in the genesis of hypotension associated with endotoxic shock (Cicala *et al.* 1999).

PAR-2 is present also on fibroblasts. It is known that PAR-2 action on fibroblasts causes mitotic response, collagen synthesis and scar formation (Akers *et al.* 2000). As the fibrosis and reparation changes are obvious in chronic pancreatitis histologic samples, PAR-2 action may contribute to this effect as well.

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Link between inflammation and pain via PAR - 2 activation is also suggested, however, the PAR - 2-mediated role for trypsin in the pathogenesis of pancreatic pain is independent on its inflammatory effect (Hoogerwerf *et al.* 2004, Hoogerwerf *et al.* 2001).

Conclusion

Presence of PAR-2 expression on both vascular structures, and acinary and duct epithelium in acute pancreatitis was described in the past. This suggests an important role of trypsin-activated receptors in induction/development and/or regeneration/repair/cellular protection in acute pancreatitis. Recently published data show, that PAR-2 plays both beneficial and harmful effect in AP development. Moreover, PARs contribute to systemic changes in AP development and leading to multiple organ dysfunction syndrome and eventually death. Local and systemic structures influenced by the PAR-2 activation during acute pancreatitis are summarized in Table 1. Despite all the above described effect of PAR-2 activation, the vascular action leading to the pancreatic ischemic disturbance could be the leading mechanism starting consequent local changes resulting in acute pancreatitis development.

Acknowledgement

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Table 1. Involvement of pancreatic and systemic structures in pathological processes during acute pancreatitis development

Vascular effect of PAR-2

Effect	Structure	Reference
vasoconstriction	muscularis propria	Moffatt et al.1998
vasodilatation/ hypotension	endothelium (systemic)	Cheung et al. 1998
oedema formation	lymphatics	Kawabata et al. 1998
hypotension	endothelium ? (systemic)	Cicala et al. 1999

Nociceptive effect of PAR-2

Effect	Structure	Reference
pain induction	duct cells (intraductal activation of PAR-2)	Hoogerwerf et al. 2001, 2004

Secretion (protective) effect of PAR-2

Effect	Structure	Reference
increase of pancreatic exocrine secretion	acinary cells	Kawabata et al. 2000
clearance of toxins and debris	duct cells (basolateral activation)	Nguyen et al. 1999

Pro-inflammatory role of PAR-2

Effect	Structure	Reference
NO and IL-8 production	systemic effect	Namkung et al. 2004

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Fig. 1. Schematic description of PAR-2 activation
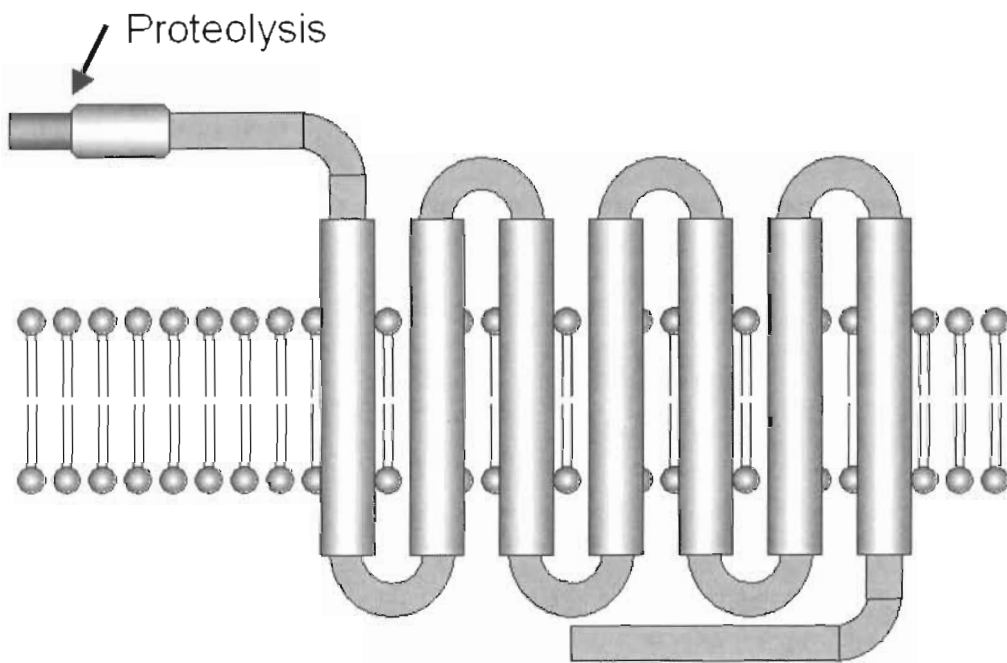
a) in the first phase, the specific oligopeptide is cleaved from the N-terminal part of the receptor and tethered ligand is expressed.
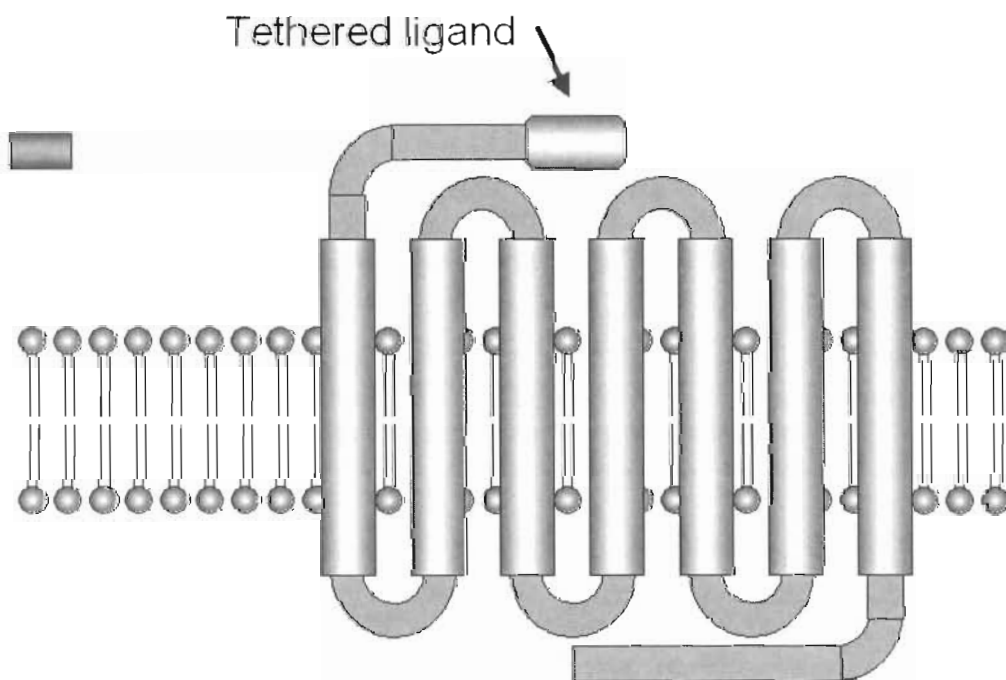
b) in the second phase, the tethered ligand activates the receptor in the reaction with its 1st and 2nd extracellular domain.

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Fig. 2. Schematic description of acute pancreatitis development.
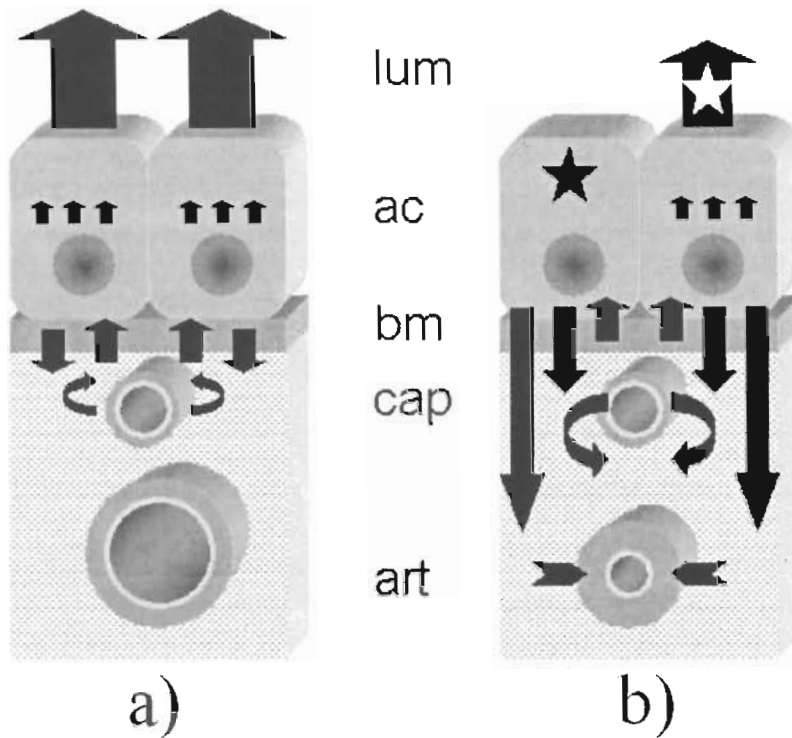
a) Physiologically, the majority of acinary cells proenzyme/ enzyme production after stimaltion is secreted intraluminary. These enzymes go from acini to duodenum *via ductus choledochus*.Only a small part of proenzymes/enzymes enters interstitium. Enzymes secreted basolaterally are together with nutrients reinternalised into the acinary cells. Internalisation is also regulated by PAR-2 stimulation by interstitially activated trypsin.

b) During pathological conditions (cholelithiasis, ethanol abusus, etc.), the physiological transport of proenzymes is blocked on different levels. Basolateral secretion of enzymes is markedly enhanced over their internalisation. Pronounced stimulation of PAR-2 on capillary cells by activated trypsin causes edema formation. Activation of PAR-2 on smooth muscle cells causes marked vasoconstriction, whereas activation of PAR-2 on endothelial cell leading to vasodilation.

Abbreviations: lum – acinary lumen, ac – acinary cells, bm – basal membrane, cap – capillary, art – artery, asterisk presents block of transcellular enzymatic transport

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PROTEINASE-ACTIVATED RECEPTOR-2 EXPRESSION ON CEREBRAL NEURONES AFTER RADIATION DAMAGE: IMMUNOHISTOCHEMICAL OBSERVATION IN WISTAR RATS

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Summary: *Radiation damage results in blood-brain barrier damage followed by blood plasma transfer into the neuropil. The transferred liquid contains high amounts of biologically active substances/proteinases including factor Xa and a free pool of serum trypsin, which is not bound to antiproteases (α_1 AT, α_2-macroglobulin). The aim of this study was to follow up expression of proteinase-activated receptor-2 (PAR-2) in the brains of Wistar rats after single exposure to radiation at 26 Gy (^Co, 23 min, 15 sec). After irradiation, the animals were sacrificed on days 10, 20, 30 and 40. Control rat brains served as negative control. Coronal sections of caudal diencephalons were investigated using histology and immunohistochemistry. Polyclonal goat specified the antibody against the NH-end of murine and rat PAR-2. Significant PAR-2 membrane positivity of scattered swollen neurons in deeper cortical layers was found in irradiated animals compared with controls. Although this membrane positivity was noticed in all irradiated animals, the most prominent occurred on day 30. Diffuse cytoplasmic positivity was also demonstrated on shrunken neurons in the cortex and hippocampus. Increased cytoplasmic and polarized membrane positivity was also noticed on the neurons of hypothalamic nuclei. The causal relationship between blood-brain barrier damage, PAR-2 activation and neurodegeneration has not yet been verified. However, the present findings indicate that PAR-2 mediates a certain cellular response. It remains to be demonstrated whether this is a response to higher concentrations of factor Xa, a free pool of trypsin or other unknown possible proteinases in brain tissue; whether changes in PAR-2 expression are consequences of direct radiation damage to neuronal cells; whether this reaction is protective; and whether primary PAR-2 activation results in neuronal damage.*

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Introduction

Proteinase-activated receptors (PAR) are essentially ubiquitous. They are particularly present on normal and malignant immunocompetent cells, as well as on the endothelial and muscle cells of major and minor vessels. Their presence was also demonstrated immunohistochemically on intestinal epithelial cells, epithelial cells of endocrine as well as exocrine organs, keratinocytes, fibroblasts and further cells including neurons and glia (1, 2). PAR-2 belongs to a family of PARs activated by specific serum or tissue proteinases (PAR-2 is activated by trypsin, factor Xa and mast cell tryptase, PAR-1 and PAR-3 are activated by serum thrombin) (3, 4). These proteinases activate PAR-2 through the mediation of a unique process characterized by the recognition of the receptor by an enzyme, subsequent cleavage at a specific site on the NH-2 terminal of the receptor and presentation of a new NH-2 terminal, which behaves as "a tethered ligand" and which binds to the extracellular domain of the split molecules. Thus, PAR-2 is a receptor, whose ligand is a physical part of the receptor molecule (5).

Radiation has previously been reported to cause damage to the blood-brain barrier and consequent leakage of serum proteins into the neuropil (6). This was demonstrated mainly on serum albumin (7, 8). However, many other biologically active substances pass through the vascular barrier, such as serum proteinase thrombin, factor Xa and even pancreatic trypsin. Part of the digestive enzyme, trypsin, is bound in serum to the a_2-macroglobulin and other enzyme inhibitors, while part is free in the blood stream (9). In the case of vascular radiation damage, serum-free trypsin together with other serum proteinases (factor Xa or others) passing through the blood-brain barrier could act on vessels, neurones or other brain structures.

Material and methods

Animals: This study was performed in accordance with the guidelines on animal experimentation of the First Medical School, Charles' University, Prague, Czech Republic and all procedures were approved by the animal experimentation review committee.

Inbred Wistar rats were used for the experiments. Rats were bred in an SPF animal breed with radiation-sterilized bedding (SAWI Research Bedding, manufacturer) and were fed with chow that was sterilized by radiation. Autoclaved water was provided *ad libitum*.

The rats were anesthetized with intraperitoneal thiopental (50 mg/kg) and were exposed to a brain dose of 26 Gy (^{60}Co was used, radiation distance 121.5 cm, radiation input 0.3248 Gy/min). Fifty animals were included in the experiments. All the animals were divided into groups of 10 animals each and were sacrificed on days 10, 20, 30 and 40 after irradiation. Ten nonirradiated rats were used as controls and were sacrificed on day 0 of the experiment.

Histology. Rat brains were fixed in 10% formaldehyde buffered transcardially and after evisceration in 10% formaldehyde for 48 h. Fixed samples of coronal brain sections of caudal diencephalons were embedded in paraffin blocks. Tissue slides, 6 m thick, were cut and stained with hematoxylin-eosin.

Immunohistochemistry. Tissue slides taken from experimental and control animals (6 m) were deparafinized and transferred into the water solution. Then they were boiled in citrate buffer (pH 7.6) for 3 x 5 min in a microwave oven. Endogenous peroxidase was blocked with 0.05 mg of natrium azide and 5 ml of hydrogen peroxide in 50 ml of demineralized water. Nonspecific positivity was blocked with 150 l of rabbit serum in 10 ml of TBS for 30 min.

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Goat polyclonal primary antibody for mapping the new NH$_2$ terminal (SLIGRLETQPPITGKGVPVC) of murine and rat PAR-2 (PAR-2 [S-19]: sc-8207 Santa Cruz Biotechnology, Inc.) was diluted 1:500 in 5% fetal bovine serum in TBS and put on slides overnight in 4 °C. Subsequently, slides were incubated with biotinylated secondary antibody (rabbit antigoat) and then with streptavidin-biotin conjugated with horseradish peroxidase (Vectastain ABC Kit, Vector Laboratories Ltd.).

Slides incubated with secondary antibody were used only as a control for specificity. Diaminobenzidine was used as a chromogen and Harris' hematoxylin as a counterstain.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Deeply frozen pancreatic, kidney, heart and brain tissues of control rats were separately homogenized. Total RNAs were isolated using TRIzol®Reagent (Life Technologies, GIBCO BRL) according to the manufacturer's protocol. Concentration of RNA was performed on a spectrophotometer 280/260 nm. Primers for PAR-2 (10) were 5'-ACCC-CGCCGTGATTTACATGGC-3', 5'-GCCGGGAACAG-GAAGACTC-3', (Life Technologies, GIBCO BRL). For reverse transcription, Superscript II kit (Life Technologies, GIBCO BRL) was used. For the PCR, PCR Core Kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals) was used. Amplification cycles consisted of 45 sec at 93 °C, 45 sec at 55 °C and 1 min at 72 °C for 30 cycles. PCR reaction was performed on a PCR thermocycler (MJ Research). Products were analyzed electrophoretically on 1% agarose gels with ethidium bromide.

Results

Histology. Pronounced edema was noticed in all irradiated mice. This was observed mainly perivascularly, surrounding spastic intrathecal arteries (mainly the hippocampal region) and also along penetrating arterioles of white matter of centrum semiovale. This edema was mostly mild but in certain cases, irradiated brains presented an even, perivascular spongiform appearance. The tissue edema was noticed also periventricularly and was most pronounced along the third ventricle.

Dystrophic and disperse or locally diffuse necrotic neurons were observed in all hippocampal structures without predilection. In the brain cortex of irradiated rats, swollen neurons with tigrolysis and empty nucleus were seen. Shrunken eosinophilic neurons were also seen in the brain cortex.

Microvacuolized cells of the plexus chorioideus were observed with an increase on day 20 and resolution on day 40. Disperse apoptotic ependymal cells were also observed with accentuation on days 30 and 40. None of the above-mentioned changes were observed in control brains.

Immunohistochemistry. PAR-2 positivity was found on dystrophic and/or necrotic cortical neurons in irradiated animals. This positivity was related to cell membranes in the case of swollen/dystrophic neurons and was diffuse and intracellular in the case of shriveled/necrotic neurons (Fig. 1). Although membrane positivity was noticed in all irradiated animals, the most frequent was on day 30. Unaffected neurons exhibited only a weak intracellular positivity similar to that in nonirradiated neurons (see below). Membrane/intracellular positivity was not observed in nonirradiated brains, where only weak intracytoplasmic positivity of PAR-2 was noticed (not shown). Diffuse intracellular positivity of PAR-2 was also seen in shrunken neurons of the hippocampus, including their axons (Fig. 2). As with cortical neurons, unaffected hippocampal cells exhibited only weak intracellular positivity corresponding to nonirradiated brains.
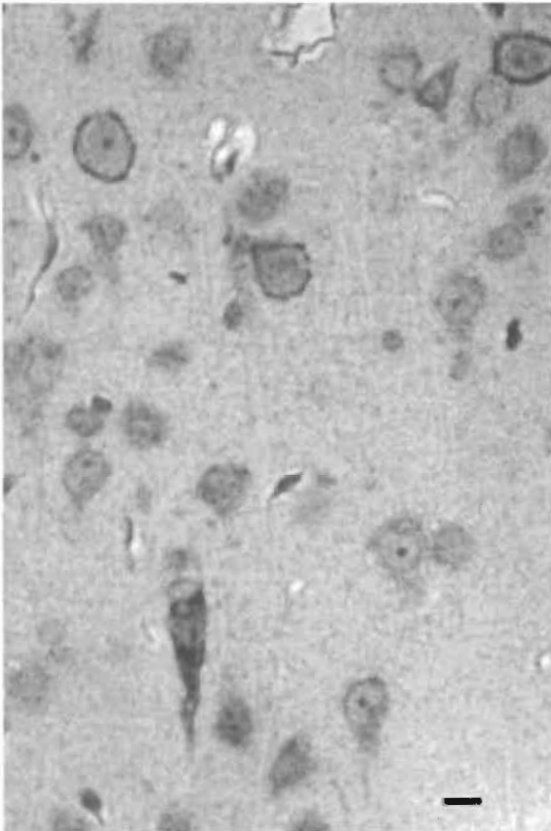
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Fig. 1 Immunohistochemical observation of proteinase-activated receptor (PAR)-2 in rat brain 30 days after irradiation (26 Gy). Goat polyclonal antibody against rat PAR-2 was used; high-power magnification (x400); scale bar equal to 10 μm.

Fig. 2 Immunohistochemical observation of proteinase-activated receptor (PAR)-2 in rat brain 30 days after irradiation (26 Gy). Goat polyclonal antibody against rat PAR-2 was used; high-power magnification (x400); scale bar equal to 10 μm.

In contrast with control brains, experimental brains showed intracellular and membrane-accented as well as site-polarized positivity of PAR-2 was observed on the neurosecretory cells of hypothalamic nuclei (Fig. 3).

The supporting glial cells were immunohistochemically negative with the exception of oligodendroglia of white matter. Weak diffuse intracytoplasmic positivity of oligodendroglial cells had no relation to radiation damage in either irradiated or control brains. Other brain structures (arterioles, capillaries, meninges) showed no significant immunohistochemical positivity of PAR-2. However, enhanced positivity was observed on vacuolized cells of the plexus chorioideus in experimental brains when the plexus chorioideus of control brains were completely negative (Fig. 4).
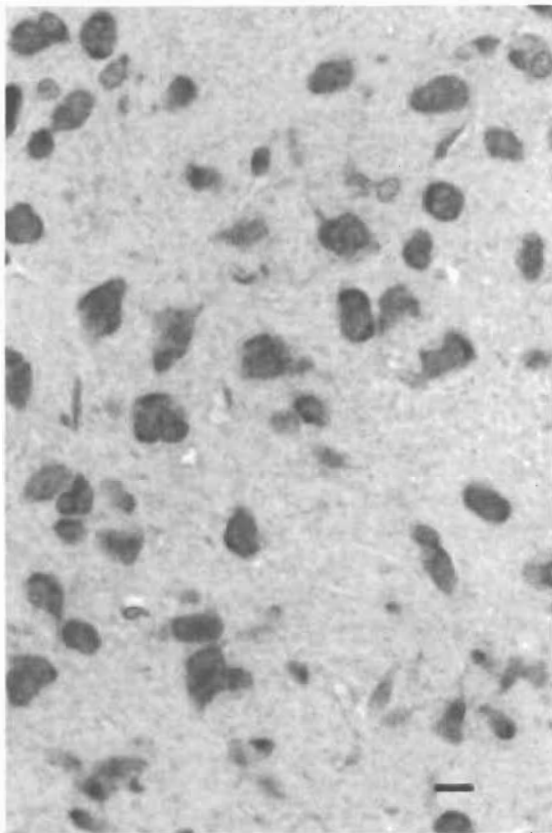
Fig. 3 Immunohistochemical observation of proteinase-activated receptor (PAR)-2 in rat brain 30 days after irradiation (26 Gy). Goat polyclonal antibody against rat PAR-2 was used; high-power magnification (x400); scale bar equal to 10 μm.
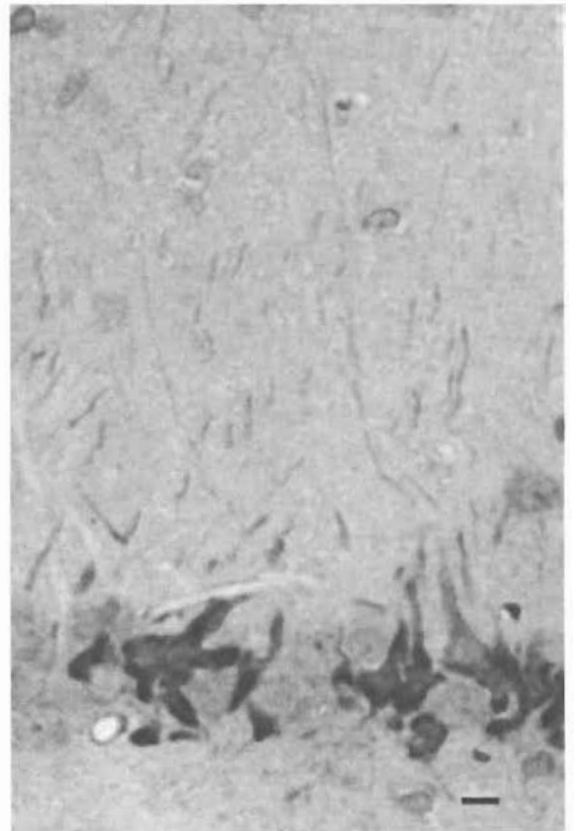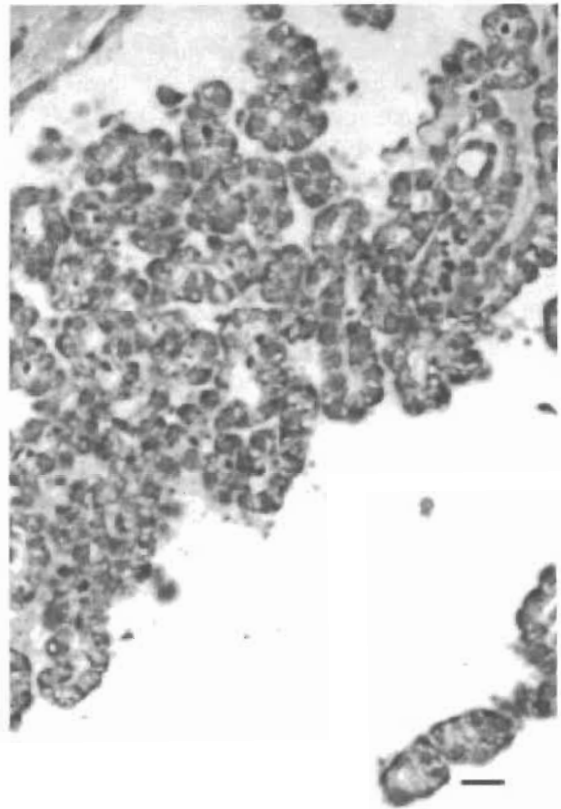
Fig. 4 Immunohistochemical observation of proteinase-activated receptor (PAR)-2 in rat brain 30 days after irradiation (26 Gy). Goat polyclonal antibody against rat PAR-2 was used; medium-power magnification (x60); scale bar equal to 25 μm.

No significant immunohistochemical presence of PAR-2 was found on other brain structures (arterioles, capillaries, astroglia, ependyma).

RT-PCR. RT-PCR products of 441 bp from pancreas, kidney, heart and brain confirmed PAR-2 presence/production in mRNA in these tissues from control rats (Fig. 5).
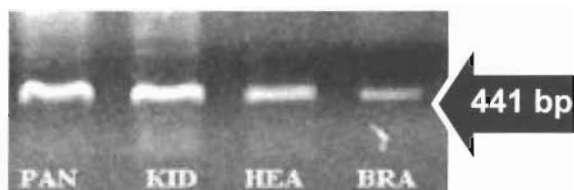
Fig. 5 Reverse transcriptase-polymerase chain reaction products of 441 bp from pancreas (PAN), kidney (KID), heart (HEA) and brain (BRA) confirm proteinase-activated receptor-2.

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Discussion

The histological appearance of radiation-induced edema has previously been described (11). Our observation confirms these findings: edema in deep or penetrating arteries and their spasm suggested ischemic involvement in radiation injury to the central nervous system (12). The acute demyelinization and necroses of centrum semiovale previously described after radiation damage could be caused by this ischemic mechanism (13). Necroses of hippocampal neurons together with the finding of some "red neurons" and other ischemia-related dystrophic changes in the cortex support the possible role of spastic vascular reaction in brain damage. Leakage of serum proteins into the brain tissue and its relationship with radiation-induced ischemia has also been previously demonstrated (14). Obviously, any vascular barrier damage causes a varying degree of vasoconstriction followed by perfusion failure and hypoxia. This was observed in experimental studies in an animal model of radiation damage in rats (15).

The most interesting of our results was the finding of membrane positivity of specifically cleaved/activated PAR-2 on damaged cortical neurons and on necrotic/dystrophic cells of the hippocampus. Changes in protein immunohistochemical positivity are related to intracellular receptor redistribution and new epitope presentation after conformational changes following specific receptor activation (16). As the antibody maps the epitope of the new NH_2 terminal of PAR-2 of rat and mouse origin (17), immunohistochemistry demonstrates activated receptors.

Our results demonstrate that PAR-2 is strongly activated during and after brain irradiation. The presence of PAR-2 was also confirmed in the mRNA of control rat brain (but also pancreatic, kidney and heart tissue) using RT-PCR. Damage to hippocampal neuronal cell lines after PAR-2 activation was previously described *in vitro* (11). However, for the first time we have demonstrated a similar finding *in vivo*. Further specific sharp membrane positivity of PAR-2 on cortical cells compared with cytoplasmic positivity on necrotic hippocampal neurons supports, through knowledge of the hippocampal neurons' susceptibility to damage (mainly to the ischemia [18]), the hypothesis of involvement of PAR-2 in neuronal cell death, as previously described (10). Taken together with previously published data on PAR-1 (thrombin receptor) on these cells, this finding is very interesting as thrombin is also suggested to be involved in neuronal death (19-21). However, PAR-2 activation on neuron surfaces also causes inhibition of their growth and degeneration (22). Enhanced glial fibrillary acid protein expression in astrocytes of transgenic mice expressing the human brain-specific trypsinogen IV was also observed (23). Although PARs were unknown at that time, the authors related their findings to neurodegenerative processes because prominent astrocytosis is seen in the brains of patients with AIDS dementia (24) and other viral infections (25), acute traumatic brain injury (26) and Alzheimer's disease (27).

PAR-2 positivity on the neurosecretory cells of hypothalamic nuclei in irradiated brains has not been previously described. This finding corresponds to our previous immunohistochemical observation of polarized intracellular PAR-2 positivity on neuroendocrine cells in pancreatic Langerhans' islets during acute pancreatitis development (28). We suggest that intracellular/polarized PAR-2 positivity could correlate with their significant role in intracellular/transcellular processes in general (29,30) and that the role of PARs increases during different pathological conditions. Finally, this suggestion could be confirmed by our finding of the huge immunohistochemical positivity of PAR-2 in cells of the plexus chorioideus in irradiated brains in contrast to their immunohistochemical negativity in control brains.

In conclusion, the results of the present study demonstrate PAR-2 mediation of certain cellular responses. It is unclear whether this is a response to higher concentration of factor Xa, a free pool of trypsin or other unknown possible proteinases in brain tissue or whether changes in PAR-2 expression are consequences of direct radiation damage of neuronal cells. The real role of activation of PAR-2 in pathological conditions could be elucidated through further study of the role of PAR-1 in brain ischemia and of the protective effect of low-dose stimulation of PAR-1 by thrombin in ischemia and the destructive effect of high-dose stimulation by the same enzyme in the same conditions (18).

Acknowledgment

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3.3 Radoslav Matěj, Petra Manďáková, Irena Netíková, Pavla Poučková and Tomáš Olejár: Proteinase-activated receptor – 2 expression in breast cancer and the role of trypsin on growth and metabolism of breast cancer cell line MDA MB-231.

Proteinase-activated receptor – 2 expression in breast cancer and the role of trypsin on growth and metabolism of breast cancer cell line MDA MB-231

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Abstract

Proteinase-activated receptor – 2 (PAR-2) is a ubiquitous surface molecule participating in many biological processes. It belongs to the family of G protein-coupled receptors activated by the site-specific proteolysis of trypsin and similar proteases. Altered function of PAR-2 has been described in different malignant tumours. In the present study, we investigated the expression of PAR-2 in breast cancer surgical specimens and the role of trypsin in breast cancer cell line MDA MB-231 proliferation and metabolism. A total of 40 surgical samples of infiltrative ductal breast cancer and breast cancer cell line were included in this study. We analysed PAR-2 expression by immunohistochemistry, RT-PCR and western blot. Activation of PAR-2 on cell line MDA MB – 231 was measured using calcium mobilisation assay determined by flow cytometry. MTT cell metabolism assay and cell count analysis were used to assess the trypsin influence on breast cancer cell line MDA MB – 231 proliferation. Immunohistochemical examination showed the expression of PAR-2 in all samples of breast cancer surgical specimens and cell lines in high level, this was confirmed by RT-PCR and western blot. Calcium mobilisation assay corroborated the activation of PAR-2 on cell line MDA MB – 231 either by trypsin or by agonistic peptide. Cell metabolism assay and cell count analysis showed significant differences of proliferative activity of breast cancer cells dependent on presence or absence of trypsin and serum in culture medium. PAR-2 is expressed in a high level in infiltrative ductal breast cancer tissue specimens. PAR-2 is expressed in studied breast cancer cell lines in a high level. PAR-2 is activated by trypsin and by agonistic peptide as well in the model of breast cancer cell line MDA MB-231. Activation of PAR-2 in vitro influences proliferative and metabolic activity of breast cancer cell line MDA MB-231. The action of trypsin is modified by the presence of serum which is a potential source of protease inhibitors.

Keywords: breast cancer, PAR-2, trypsin, expression

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Introduction

The role of different proteinases and their inhibitors in cancer development and malignant behaviour and the relationship of these enzymes to the prognosis of cancer is constantly disscussed in the literature. The role of tissue matrix metalloproteinases, cathepsins and other enzymes produced by cancer cells has been extensively investigated (Hojilla et al. 2003, Leeman et al. 2003, Roshy et al. 2003). However, many other enzymes are produced by malignant cells and/or tumour associated cells such as fibroblasts or immunocompetent cells. One of these enzymes, trypsin, is proteinase produced not only by exocrine pancreatic and intestinal Paneth's cells, but also by other epithelial tissues (bronchial epithelium) or connective brain cells, glias (Cederqvist et al. 2003 , Minn et al. 1998). Enhanced production of trypsin was reported in malignant cells (Uchima et al. 2003). Recently, a new function of this enzyme in cancer progression and development has been investigated. Trypsin and similar enzymes, such as mast cell tryptase or coagulation factor Xa, act on the cell surfaces via specific receptors. The action of these enzymes is physiologically regulated by peptides called antiproteases, normaly present in blood serum.

Proteinase-activated receptor – 2 (PAR-2) is ubiquitous surface molecule participating in many biological processes. It belongs to a family of G protein-coupled receptors (PAR's) activated by tethered ligand sequences within the amino terminal part of the molecule that is made accessible by the site-specific proteolysis. PAR-2 activation after the site specific proteolysis of N-terminal end by trypsin and presentation of tethered ligand sequence (SLIGKV) to extracellular domains of the receptor participates in the tissue growth and differentiation, regeneration and repair, inflammatory response regulation and also in malignant transformation (Macfarlane et al. 2001).

Infiltrative breast carcinoma is one of the most common human malignancy. Following standardized criteria it is possible to score the prognostic factors which depend on the histopathological grade and expression of several regulatory proteins (e.g. progesterone receptor, estrogene receptor and c-erb B2) (Rosai 2004).

The aim of our study was to characterize the expression of PAR-2 receptor in breast cancer tissue in the surgical samples of infiltrative ductal breast carcinoma with correlation to histopathological grade of the tumors and to the expression of other prognostic factors (estrogen and progesteron receptors). In the model of breast cancer cell line MDA MB-231, we studied the possibility of activation of PAR-2 receptor by trypsin and agonistic peptide

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(SLIGKV). The role of trypsin on the cell line metabolism and proliferative activity we assessed by MTT cell metabolism assay and cell count analysis.

Material and Methods

Breast cancer samples: 40 peroperative surgical samples of infiltrative ductal carcinomas were frozen in liquid nitrogene and routinelly processed for histopathological examination. During this diagnostical procedure, additional two sets of 20 ten-μm-thick sections were collected in sterille Eppendorf tubes, frozen in liquid nitrogen and stored at -80 °C for extraction of nucleic acids and proteins. Definitive diagnosis of infiltrative breast cancer was confirmed by microscopic evaluation of histological slides from formaline-fixed, paraffin embedded tissue samples. Standardized criteria were used to evaluate the prognostic factors (8) (Table 1.).

Cell cultures: MDA MB-231 and MCF-7 breast cancer cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) with High Glucose, supplemented with 10% fetal calf serum (FCS) and gentamicine in concentration 40μg/1ml. Cells were cultured according to routine practice.

Immunohistochemistry: Microscopic tissue slides 5 μm thick were deparaffinized, rehydrated and boiled in a citrate buffer (pH 7,6) 3x5 minutes in a microwave oven. Endogenous peroxidase was blocked with a water solution containing 0,01 % natrium azid and 1% hydrogen peroxide. Non-specific positivity was blocked by rabbit serum in TBS (150μl/10ml) for 30 minutes.

The slides were incubated overnight in 4°C with the goat polyclonal primary antibody mapping the new NH$_2$ terminal of human PAR-2 (clone N-19, sc-8206 Santa Cruz Biotechnology, Inc.) diluted 1:500 in 5% fetal bovine serum in TBS. The detection of immunostaining was performed using the Envision® kit and diaminobenzidine was used as a chromogen. Slides incubated with secondary antibody only and with nonspecific isotype-matched primary antibodies were used as control of specificity. Mayer's hematoxylin was used as a nuclear counterstain. As a negative control, slides of breast cancer sample were incubated with nonspecific isotype-matched primary antibodies and with secondary antibody, only.

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Western blotting analysis: Deeply frozen breast cancer tissue or cell suspension of breast cancer cell lines were homogenised, dissolved in 2X Tris-glycine SDS sample buffer (Novex) and boiled for 5 minutes. The concentration of total protein amount was measured spectrophotometrically using BSA kit (Pierce) following manufacturer's instructions. Equally, 20 µg of protein was fractioned on the SDS-PAGE and blotted to nitrocellulose membrane (S&S NC) using Mini TransBlott®Cells (BioRad). After visualisation by Poceau S staining (Sigma) membranes were blocked with 5% nonfat dried milk in PBS with 0.1% Tween 20 at 4°C overnight. Goat polyclonal primary antibody (PAR-2 (N-19): sc-8206 Santa Cruz Biotechnology, Inc.) was diluted 1:1000 in 5% nonfat milk in T-PBS. The filters were incubated for 1h and washed 6x 10 min in T-PBS. The secondary antibody, peroxidase-conjugated (AffiniPure Rabbit Anti-Goat IgG (H+L), Jackson ImmunoResearch Laboratories, Inc.) was diluted 1:10 000 in T-PBS. The filters were incubated for 45 min and washed in T-PBS 6x 10 min. Peroxidase activity was detected by Amersham's ECL+, following the manufacturer's protocol. Breast cancer sample incubated without primary antibody served as a negative control.

RT PCR: Deeply frozen breast cancer tissue and MDA MB-231 and MCF-7 breast cancer cell lines were homogenised (approx. 50 mg). Total RNAs were isolated using TRIzol®Reagent (Life Technologies, GIBCO BRL) according to the producer's protocol. Concentration of RNA was determined by spectrophotometer at 280/260 nm. Primers for PAR-2 were 5'-TTGCCTTCTTCCTGGAGTGC–3', 5'-TCCTGCAGTGGCACCATCCA–3' (Life Technologies, GIBCO BRL). For reverse transcription the Superscript II kit (Life Technologies, GIBCO BRL) was used. For the PCR, the PCR Core Kit (Roche Diagnostics GmbH, Roche Molecular Biochemicals) was used. Amplification cycles consisted of 45 sec. at 93°, 45 sec. at 55°C and 1 min. at 72°C for 30 cycles. PCR reaction was performed on a PCR thermo cycler (MJ Research). Products were analyzed electrophoretically on 1% agarose gel with ethidium bromide.

Calcium signalling assay: Concentration of calcium ions were determined by flow cytometry on FACS Calibur (Becton Dickinson, USA), using the Fluo-4 and FuraRed probes (Molecular Probes, Inc., Eugene, Oregon, USA). Combined cellular loading permits a sensitive ratiometric assay using visual illumination.
Cells MDA MB-231 (10^6 cells per sample) were two times washed and loaded with 4□l 100mM Probenecid, 1□l FuraRed and 0,4 □l Fluo-4 in the dark for 15min at the room

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temperature. The cells were washed, resuspended and put in the dark for 15 min at room temperature and than for 15 min at 37^0C. Finally the cell suspensions were measured and data were compared before and after activation by trypsin in final concentration 10^{-7} M and by agonistic peptide (SLIGKV – Neosystem SA, France) in final concentration 10^{-5} M. As a positive control of calcium signalling specificity were used ionomycin in concentration $1\mu g/100\mu l$ (Calbiochem, San Diego, California, USA).

Cell count assay: The cell growth analysis of the MDA MB-231 breast cancer cell line has been performed in the same manner as the cell cultures themselves in the cell culture wells. Into each well, 400 µl of the culture medium with cell suspension of 10^6 cells/ml was randomly injected into the each well. After one-day pre-incubation in the DMEM high glucose medium containing 10% FCS and gentamicine solution, cells were incubated for three days: a) DMEM high glucose medium containing 10% FCS and gentamicine solution in the presence of trypsin in PBS in final concentration 10^{-7} M, b) DMEM high glucose medium containing gentamicine solution without FCS in the presence of trypsin in PBS in final concentration 10^{-7} M, c) DMEM high glucose medium containing 10% FCS and gentamicine solution in the presence of PBS without trypsin, d) DMEM high glucose medium containing gentamicine solution without FCS in the presence of PBS without trypsin.
Finally, the cells were washed with PBS and trypsinized with 0.25% trypsin solution. After deliberation the cell suspension was measured by flow cytometry using FACS Calibur (Becton Dickinson, USA) under standard flow for 120 seconds. The average count of cells incubated in the common DMEM high glucose medium containing FCS without trypsin was used as a 100% standard and all individual measurements were compared to this stantadard in %. Each group of measurements was performed in cell suspension from 6 wells. The results were obtained from three independent experiments.

Cell metabolism assay: Metabolic activity of MDA MB-231 breast cancer cells was determined by a colorimetric MTT cell proliferation assay. MDA MB-231 cells were cultured in 96-well plates in M1H medium supplemented with 10% FCS. Briefly, after one day pre-incubation the cells were maintained for 1, 2 or 3 days in M1H or HBSS media in the presence or absence of trypsin (see bellow) at 37 °C, 10 µl of MTT (3-/4,5-dimethylthiazol-2-yl/-2,5,diphenyltetrazolium bromide, Sigma) solution (0,5mg/ml) in PBS (phosphate-buffered saline) were added and 6 hr later 100 µl of 10% SDS solution (lauryl sulfate) pH 5.3

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were added for overnight incubation. The intensity of blue colour dependent on the mitochondrial enzyme succinyldehydrogenase activity of cells in individual wells was measured by an ELISA reader (spectophotometry) at 570 nm.

Statistics:

Student's T-test and standard deviation (SD) were used for statistical analysis. For all tests a p-value of < 0.05 was considered as statistically significant.

All experimental research procedures reported in the manuscript has been performed with the approval of an appropriate ethics committee and in compliance with the Helsinki Declaration.

Results

Immunohistochemistry: Different intensity of specific diffuse intracellular cytoplasmatic positivitity of PAR-2 was observed in the tumour cells of all breast cancer samples (Fig. 1a). Positive immunostaining of cancer cells in bioptic specimens was independent on the histopathological grade or the expression of other prognostic factors (progesteron and estrogen receptors). Positive immunostaining of stromal fibroblasts was also observed in evaluated tissue samples. Non-neoplastic connective tissue of the breast was constantly negative.

Western-blot analysis: Western-blot analysis confirms immunohistochemical positivity of expression of PAR-2 in patient's breast cancer samples and tissue culture cells MDA MB–231. MCF–7 culture cells as an additional model of breast cancer cell line showed strong positivity too. (Fig. 2).

RT PCR: PAR-2 mRNA presence was observed in breast cancer patient's samples and in tissue culture cells MDA MB–231. MCF–7 breast cancer cell line confirmed the expression of detectable amount of mRNA in the models of breast cancer cell lines (Fig. 3).

Ca++ mobilisation assay: Activation of PAR-2 was observed in MDA MB–231 cells using the flow cytometric method. Fig. 4a. demonstrates Ca++ mobilisation after addition of trypsin in final concentration $10^{-7}M$. Fig. 4b. demonstrates Ca++ mobilisation after addition trypsin agonistic peptide (SLIGKV) in final concentration $10^{-5}M$, ionomicin in concentration $1\mu g/100\mu l$ was used as a positive specific control.

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Cell metabolism assay: Fig. 5 presents one exemplary result of three independent experiments with the same trend. Arbitrary units were used for the spectrophotometric optic density evaluation.

As anticipated, a significant difference was found between the cells maintained in the medium containing 10% FCS (M1H) or missing 10% FCS (HBSS). In the FCS-free HBSS medium, continuous (linear) decrease of metabolic activity with statistically significant difference between the groups treated or untreated with 10^{-7}M trypsin (Fig. 5a) was observed. Higher metabolic activity was recorded in the trypsin group. Similarly, in the medium containing FCS (M1H), statistically significant difference between the groups treated or untreated with 10^{-7}M trypsin since the day 2 has been recorded with higher metabolic activity in trypsin group (Fig. 5b). In contrary to the HBSS groups, cells growing in M1H medium with trypsin tend to retain higher metabolic activity even after 3 days of exposition in comparison to trypsin-free group, where linear decrease of metabolic activity was recorded similarly to the HBSS groups. Higher metabolic activity of cells in trypsin treated groups was observed despite the decrease of cell growth observed in the cell count assay in serum-free medium.

Cell morphology analysis: As the activity of trypsin was blocked by the serum trypsin inhibitors presented in FCS, no difference was observed between cells cultured with addition of trypsin in PBS in final concentration 10^{-7}M or cultured in the DMEM high glucose medium containing of 10% FCS and gentamicine solution with the PBS only served as a control (Fig 6a,b,d). Their shape was regularly elongated, typical for normally cultured cells of MDA MB-231 cells. In the DMEM high glucose medium containing only gentamicine solution, without FCS, in the presence of trypsin, the cells changed their shape to round, small, poorly adhering comparing to control (Fig 6c). The total amount of cells was reduced in both serum-free groups.

Cell count assay: The cell growth of the MDA MB-231 breast cancer cell line in the full DMEM high glucose medium containing (a, c) or missing (b, d) 10 % FCS with (a, b) or without (c, d) presence of trypsin is summarized in Fig. 7. There was no significant difference (p>0,05) in the cell growth in groups growing in DMEM high glucose medium containing FCS with (a, 96,1%, SD 19,3) or without (c, 100%, SD 17,8) presence of 10^{-7}M trypsin. Cells growing in DMEM high glucose medium without FCS with (b, 12,3 %, SD 4,5) or without (d, 41,9 %, SD 22,5) presence of 10^{-7}M trypsin showed significant difference in the

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cell number after 3-day incubation (Fig. 7.). The absence of FCS changed the cell number during the short term 3-days cultivation.

Discussion

Our results demonstrate that PAR-2 is expressed in the breast cancer. PAR-2 was detected on the protein and also mRNA levels in breast cancer cell lines. In bioptic tissue samples, the expression of PAR-2 was detected independently on histopahological grade of the tumor, estrogen and progesteron receptor expression or on other prognostic factors. We also observed the influence of trypsin on metabolic activity and growth of the cell line isolated from breast cancer. This observation suggests possible increased expression of PAR-2 in breast cancer tissues and its interaction with trypsin to the initiation and/or progression of breast cancer. Our observation confirms previous study of D'Andrea et al. presenting immuniohistochemical positivity of breast cancer cells and stromal fibroblasts in the breast cancer patient samples (D'Andrea et al. 2001).

The discovery of PAR-2 receptor started the process of partial elucidation of the potential role of trypsin in the cellular signalling, tissue growth and/or malignant transformation (Nystedt et al. 1995, Nystedt et al. 1994). In the past, it was shown that tumor associated trypsin (TAT) is involved in a protease cascade stimulating tumor cell invasion and degradation of extracellular matrix in different cell lines (Koivunen et al. 1991). Previous studies demonstrated increased expression of PAR-2 in different malignant tissues and cell lines, including digestive tract cancer, sarcomas, leukemias, lymphomas and also malignant brain tumours of astrocytic origin (Steinhoff et al. 2005).

In recent studies, PAR-2 activation in IL-3 dependent murine lymphoma cell line BaF-3 resulted in cell proliferation (Mirza et al. 1997). However, the data obtained so far are still controversial.

In the gastric carcinoma cell line MKN-1, Miyata et al. demonstrated, after the trypsinogen-1 cDNA introduction, the stimulation of cellular growth and adhesion to fibronectin and vitronectin when trypsinogen activator enterokinase was added into the culture. In vivo (intraperitonealy) transplanted, these transfected MKN-1 cells produced solid tumors in adjacent organs (Miyata et al. 1998). In above mentioned MKN-1 cells, Miyata et al, 2000, demonstraded that trypsin stimulates integrin alfa5beta1-dependent adhesion to fibronectin and proliferation through the activation of PAR-2 (Miyata et al. 2000).

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Another study with STKM-1 gastric cancer cell line demonstrated that production of trypsin correlates with their malignant phenotype and invasive growth (Kato et al. 1998).

In the human colon cancer cells, proliferation was initiated by trypsin acting on PAR-2 (Darmoul et al. 2001). Authors suggested that trypsin could be considered as a growth factor. The same authors showed that colon cancer cell lines secrete trypsin in concentrations compatible with activation of PAR-2. They suggested possible autocrine/ paracrine regulation of PAR-2 acting by trypsin in colon cancer cells (Ducroc et al. 2002). The PAR-2 agonist peptide SLIGKV and trypsin also significantly increased cell proliferation in three pancreatic cancer cell lines SW1990, Capan-2, and Panc-1. In vivo, subcutaneous xenografted tumors showed significantly enhanced growth after treatment with agonist peptide (Shimamoto et al. 2004). Thus PAR-2 activated by trypsin plays an important role in promoting proliferation of pancreatic cancer.

Recently Ge et al. (Ge et al. 2004) presented that secretion of trypsin-like protease and its autocrine activation of PAR-2 in breast cancer cell line MDA MB-231 influences cell migration.

Our results showed that PAR-2 expressed in breast cancer cell line MDA MB-231 is activated by trypsin and agonistic peptide SLIGKV. Our results also demonstrate that trypsin inhibitors present in blood serum influence the action of trypsin on cultured cells. These results suggest that trypsin in investigated concentration doesn't have significant influence on the growth of MDA MB-231 breast cancer cell line under physiological conditions, where extracellular fluid containing serum with protease inhibitors is present. This suggests that different activity of proteinases and different consequent response of PAR-2 can be observed in particular human tissue compartments (blood, tumor enviroment, interstitium) *in vivo*. From this point of view, the activity of agonistic peptide doesn't seem to play any important role in physiological processes of the cell regulation and "real" trypsin or trypsin-like activity. The general role of PAR-2 in cancer initiation and development has to be evaluated in the presence/ absence of natural inhibitors. Although the PAR-2 activation by trypsin is predominantly considered stimulating tumor cells growth and invasiveness, inhibitory effect on tumor cells growth has been also reported. For instance, activation of PAR-2 on CAPA-2 pancreatic cancer cell line leads to the decrease of ^{3H}Thymidine incorporation into the cell related to inhibition of tumor cell growth (Kaufmann et al. 1998). Similarly, PAR-1, receptor of the same family activated by thrombin, is a potential tumour cell proliferating and invading agent (Henrikson et al. 1999, Wojtukiewicz et al. 1993). Another study demonstrated that PAR-1 signalling inhibits migration and invasion of breast cancer cells (Kamath et al.. 2001).

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The results of Yamashita and al. also support the notion that trypsin plays a tumor-suppressive role in human carcinomas as they found reduced production of trypsinogen accompanied by reduced PAR-2 expression in esophageal squamous cell carcinomas and 72 gastric adenocarcinomas (Yamashita et al.).

These controversal results sustain for further investigation of the role of PARs in the process of tumour cells migration, invasiveness and metastasis formation.

Conclusion

PAR-2 is expressed in breast cancer tissues independently of the routinely investigated prognostic factors. PAR-2 is expressed in the breast cancer cell lines. In in vitro conditions, PAR-2 is activated by trypsin and by agonistic peptide as well in the model of breast cancer cell line MDA MB-231. Activation of PAR-2 in vitro increases proliferative activity of breast cancer cell lines MDA MB-231 and this activation is modified by presence of serum as a potential source of protease inhibitors.

List of abbreviations

PAR-2 - Proteinase-activated receptor – 2, DMEM - Dulbecco's Modified Eagle Medium, FCS - fetal calf serum, SD - standard deviation, SDS-PAGE - Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Acknowledgement

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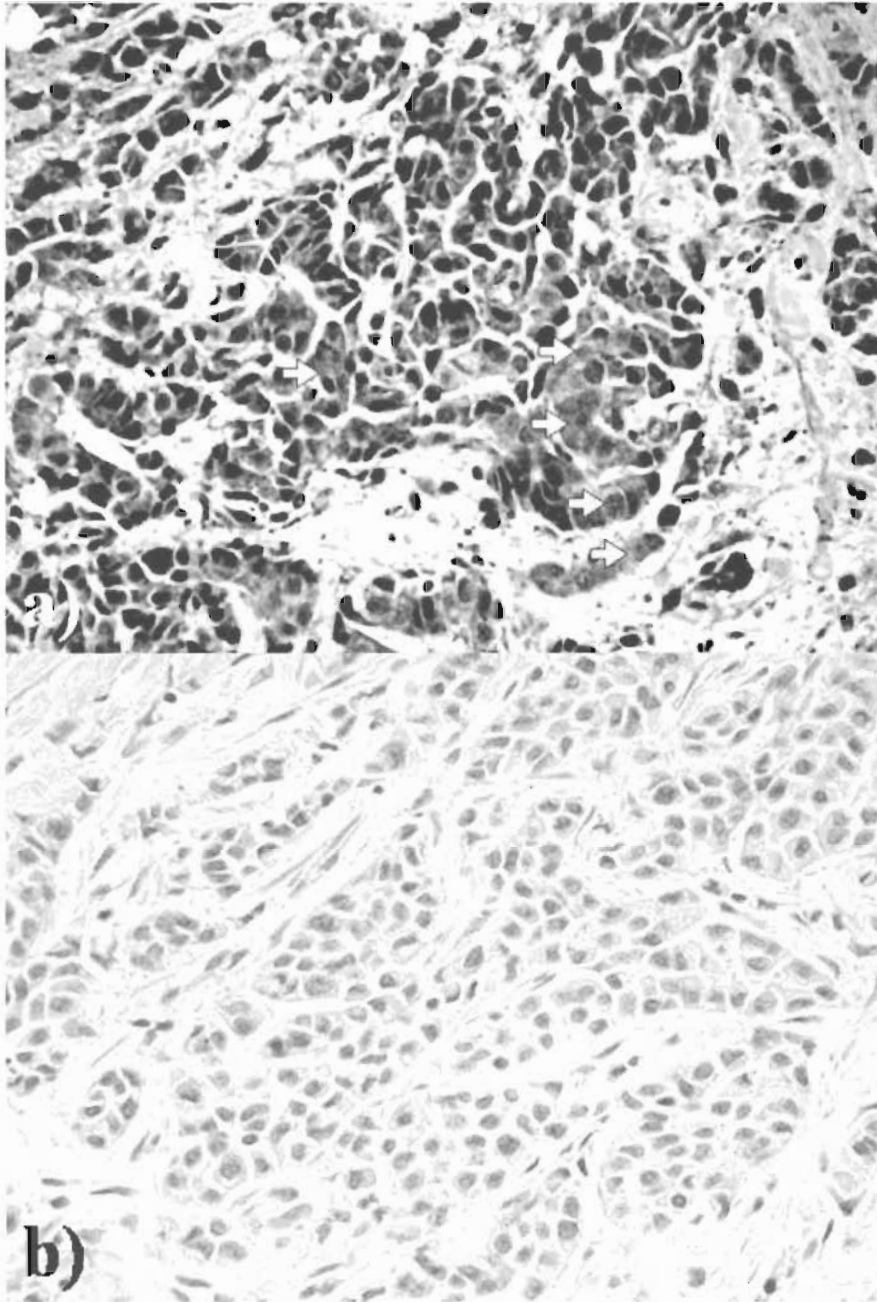
13

Fig. 1- a) Immunohistochemical positivity of PAR-2 on the samples of infiltrative ductal breast cancer. Diffuse intracellular positivity has been observed (arrows). **b)** Negative control slide of breast cancer sample incubated with nonspecific isotype-matched primary antibodies and with secondary antibody, only. Original magnification 400x.
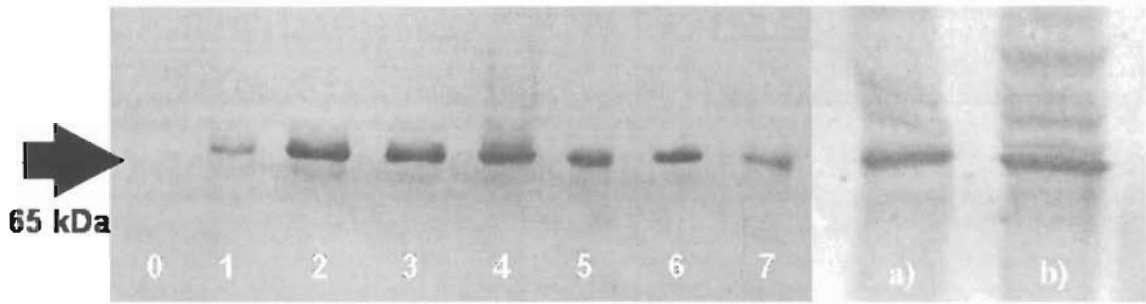
14

Fig. 2-Western blot analysis demonstrates presence of PAR-2 in samples of breast cancer patients (1 – 7) and in samples of tissue culture cells MDA MB – 231 (a) and MCF – 7 (b). Zero line indicates breast cancer sample incubated without primary antibody. Arrow indicates analysis of PAR-2 expressed in samples (65 kDa).

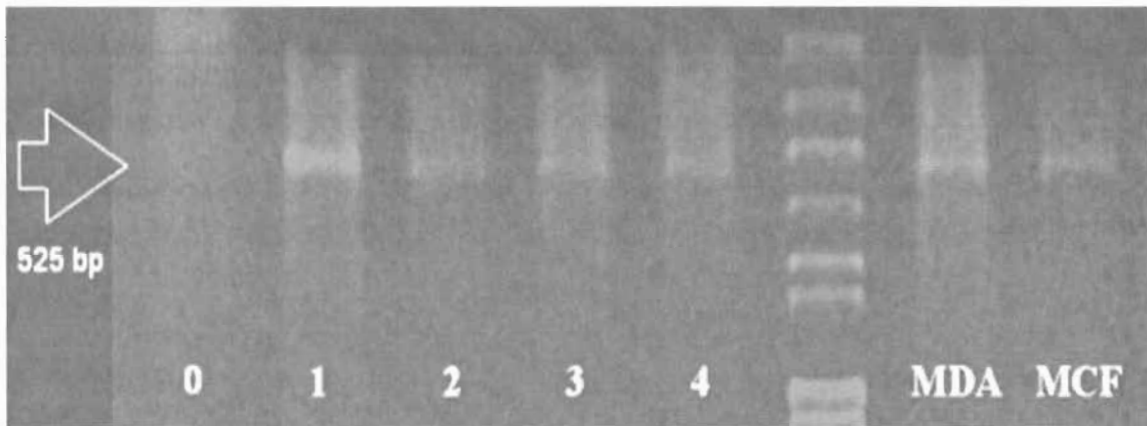

Fig. 3- RT PCR products of 525bp demonstrate presence of mRNA of PAR-2 in samples of breast cancer patients (1 – 4) and in samples of tissue culture cells MDA-MB – 231 and MCF – 7. Zero line demonstrates negative control of PCR reaction.

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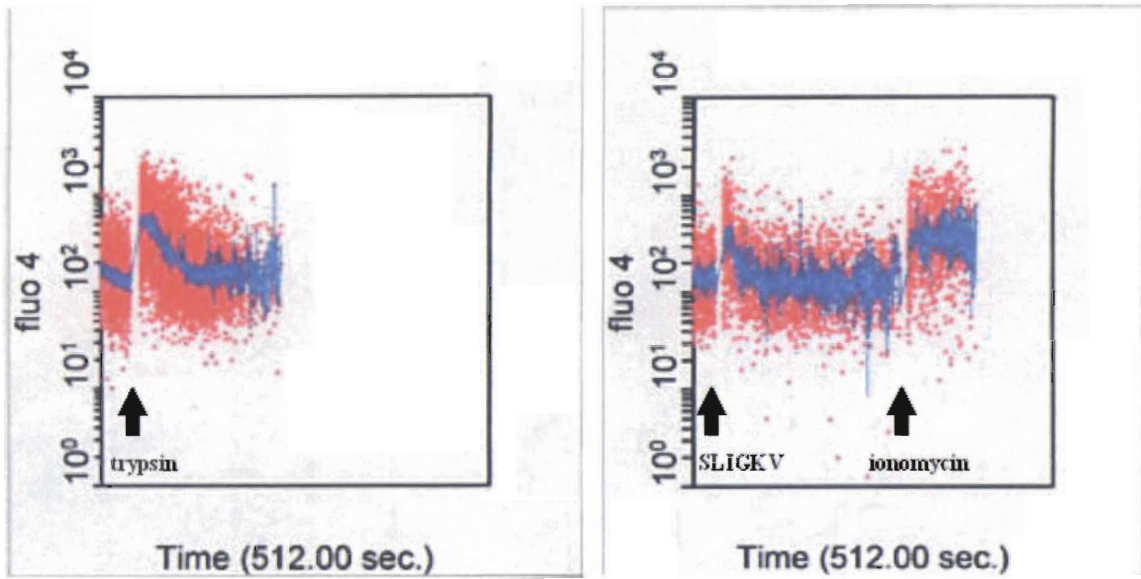**Fig. 4-** Ca++ mobilisation analysis. Activation of PAR-2 has been followed on MDA- MB – 231 cells using the flow cytometric method. Fig. 4a. demonstrates Ca++ mobilisation after trypsin activation in concentration of 10^{-7} M. Fig. 4b. demonstrates Ca++ mobilisation after trypsin agonistic peptide (SLIGKV) activation in concentration of 10^{-5} M. Ionomicin in concentration 1μg/100μl served as positive control.
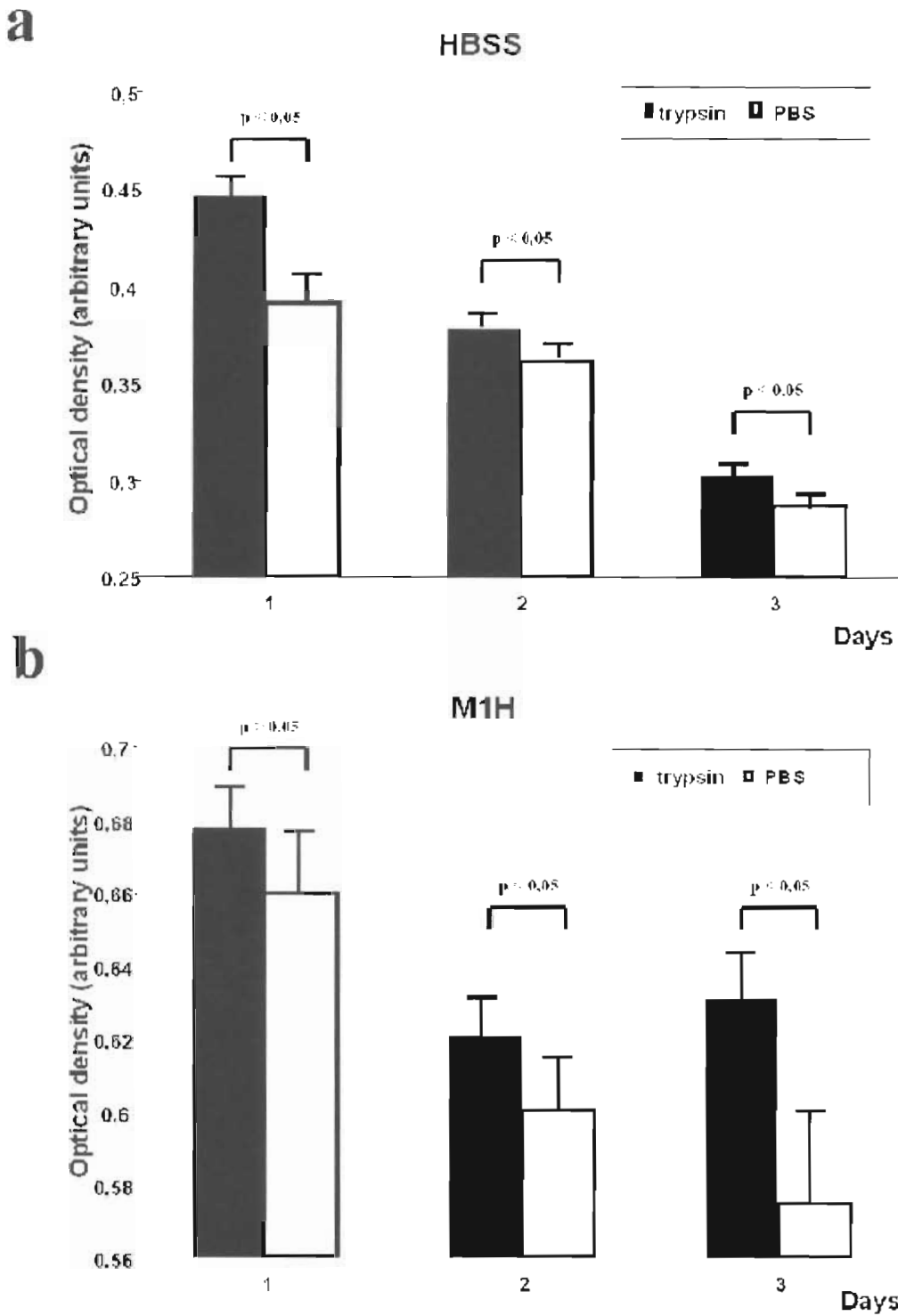
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Fig. 5- Cell metabolism assay shows statistically significant difference of metabolic activity of the cancer cell line MDA-MB-231 in serum-free medium (HBSS) with and without trypsin (Fig. 5a) and in medium containing serum (M1H) with and without presence of trypsin (Fig. 5b).

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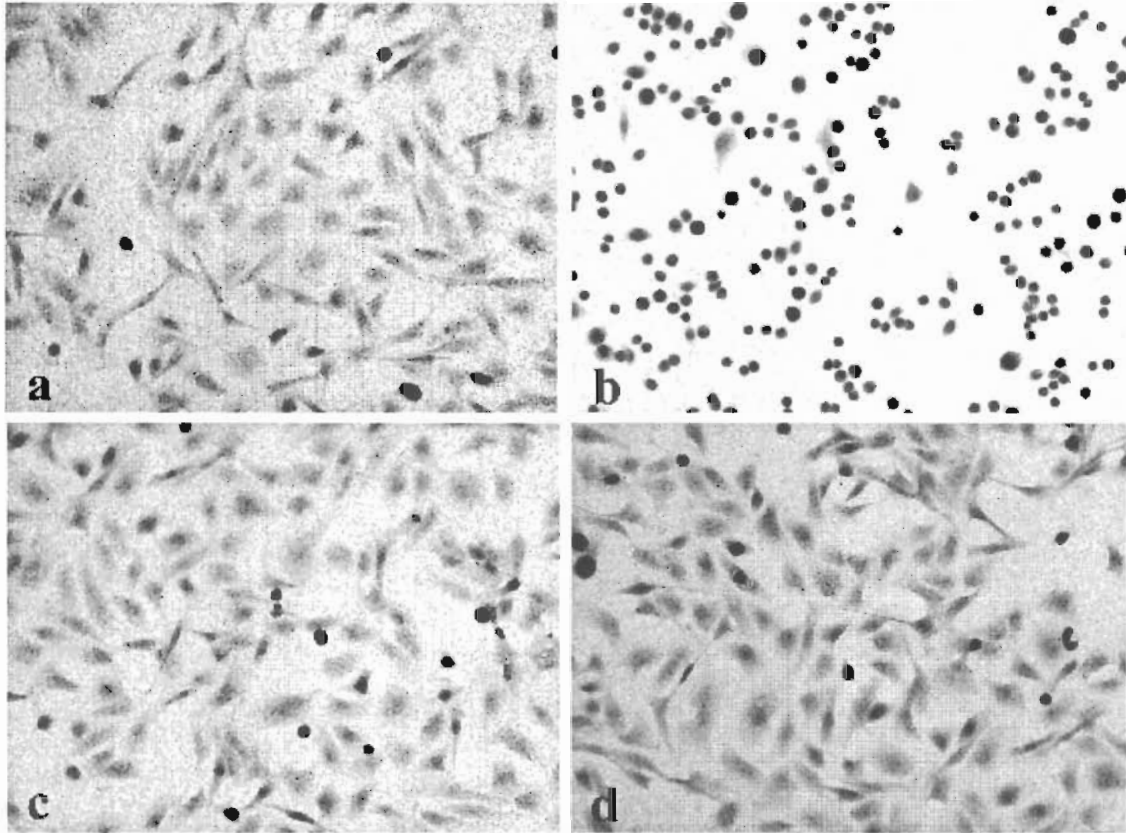
Fig. 6- Three days incubation of MDA MB – 231 breast cancer cells in the DMEM high glucose medium containing 10% FCS and 1%gentamicin solution in the presence of 10^{-7} M trypsin in PBS (a) or PBS only (c) as a control. No difference between the two groups has been observed. Three days incubation of MDA MB – 231 breast cancer cells in the DMEM high glucose medium containing only 1% gentamicin solution without FCS in the presence of 10^{-7} M trypsin in PBS (b) or PBS only (d) as a control. In the presence of trypsin, the cells change their shape to round, small poorly adhering. This change has not been observed in the absence of trypsin. The total amount of cells was reduced in both serum free groups.

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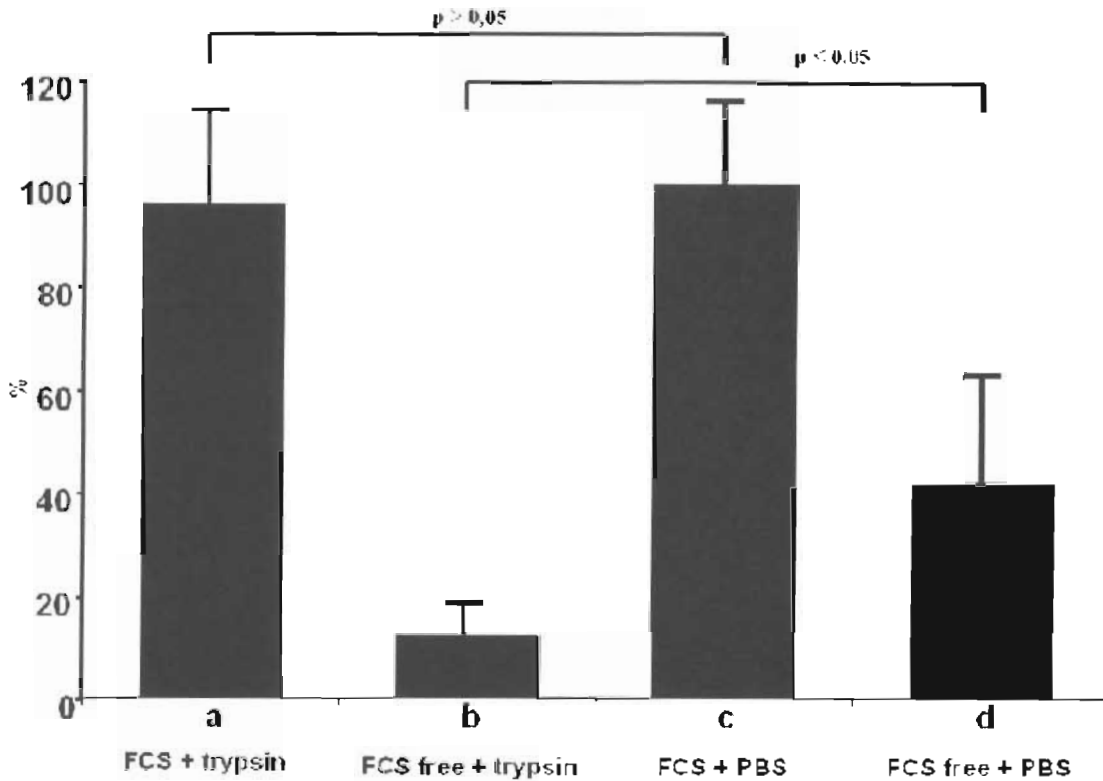
Fig.7- The cell growth of the MDA MB-231 breast cancer cell line in the full DMEM high glucose medium containing (a, c) or missing (c, d) 10% FCS with (a, b) or without (c, d) presence of trypsin. There was no significant difference (p>0,05) found in the cell growth in groups growing in DMEM high glucose medium containing FCS with (a, 96,1%, SD 19,3) or without (c, 100%, SD 17,8) presence of 10^{-7}M trypsin.
Cells growing in DMEM high glucose medium without FCS with (b, 12,3%, SD 4,5) or without (d, 41,9%, SD 22,5) presence of 10^{-7} M trypsin showed significant difference in the cell amount after 3-day incubation.

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Table 1 – Breast cancer surgical samples specification.

Histopathological grade	
Grade I	15
Grade II	12
Grade III	13
Total	40
Expression of prognostic factors	
Estrogen receptor	32
Progesterone recptor	30
P-53 protein	25
c-erb B2 protein	30
Total	40

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4. Shrnutí a zhodnocení cílů práce.

4.1 Vyhodnocení změny exprese PAR-2 v zánětlivé lézi – Zvířecí model experimentálně vyvolané akutní pankreatitidy.

V naší práci jsme demonstrovali signifikantní zvýšení exprese aktivovaného receptoru PAR-2 nejen na cévních strukturách ale také na epiteliálních elementech výstelky pankreatických acinárních a duktálních struktur v průběhu akutní léze pankreatu. Také jsme jako první prokázali přítomnost tohoto receptoru v endokrinní části pankreatické tkáně – Langerhansových ostrůvcích. Výsledky naší práce dokládají význam role receptoru aktivovaného prostřednictvím trypsinu v indukci (a vývoji) a v regeneraci (a hojení) procesu akutní pankreatitidy. Navíc je pravděpodobné, že změny v expresi PAR-2 v epitelové výstelce acinů exokrinní části pankreatu mají vztah i k některým dalším morfologicky pozorovaným změnám jako jsou například vakuolizace cytoplazmy a nekróza epitelií a v pozdějších fázích i reparace pankreatické tkáně. Zároveň je zřejmé, že aktivace PAR-2 se uplatňuje již v počátečných fázích vývoje akutní pankreatitidy. Naše výsledky jsou tedy plně v souladu s novým trendem komplexního pojetí akutní pankreatitidy jako složité souhry mnoha účinných signálních faktorů, které nejen modifikují rozsah a charakter postižení pankreatické tkáně, ale uplatňují se i na úrovni celého organismu. Naše práce rovněž dokládá, že aktivace PAR-2 má v procesu vzniku a vývoje akutní pankreatitidy duální charakter a že se aktivace receptoru se spuštěním celé řady efektorových drah neúčastní pouze progrese onemocnění, ale je důležitým faktorem úpravy a hojení. Je zřejmé, že látky schopné aktivovat/inhibovat proteázové receptory, jsou perspektivní pro budoucí široké využití k modifikaci rozvoje akutní pankreatitidy i následných reparativních procesů. Dosud ne zcela jasně definovaný charakter účinku

aktivace PAR-2 však zatím brání širšímu využití agonistů/antagonistů trypsinu v protekci a léčbě akutní pankreatitidy.

4.2 Vyhodnocení změny exprese PAR-2 v radiační lézi - Imunohistochemická lokalizace PAR-2 na strukturách tkání CNS v časném postiradiačním období u myší kmene C57Bl/6.

Výsledky naší práce dokládají význam aktivace PAR-2 v procesu reparace struktur CNS po poškození radiační zátěží. Zřetelné zvýšení exprese aktivovaného receptoru na membránových strukturách morfologicky postižených neuroektodermových elementů je v souladu s předchozími in vitro studiemi na liniích hipokampálních neuronů. Zvýšená imunohistochemická pozitivita aktivovaného PAR-2 pozorovaná zejména na dystrofických hipokampálních neuronech potvrdila význam aktivace proteázami aktivovaných receptorů in vivo. Navíc uvedené pozorování koreluje s obecně známou vnímavostí hipokampálních neuronálních struktur na poškození různými etiopatogenetickými agens. Zvýšená exprese aktivovaného PAR-2 byla pozorována i na dalších strukturách v odlišných oblastech CNS. Velmi zajímavá a překvapující byla například masivní pozitivita buněk plexus chorioideus v porovnání s kontrolou. Aktivace PAR-2 nejspíše souvisí s již dříve popsanými intracelulárními/transcelulárními mechanismy, jež se uplatňují v rámci regenerace mozkové tkáně po poškození různými noxami. Účast PAR-1 receptoru v patogenezi a progresi mozkové ischemie byla studována podrobně. Uvedené poznatky i výsledky naší práce podporují předpoklad významné role PAR v rozvoji poškození mozkové tkáně prostřednictvím ovlivnění dalších etiopatogenetických mechanismů Tím se rozšiřuje možnost budoucího potenciálního použití agonistů/antagonistů proteázami aktivovaných

receptorů v prevenci a léčbě natolik epidemiologicky významných skupin onemocnění jako jsou cerebrovaskulární nemoci.

4.3 Vyhodnocení změny exprese PAR-2 v nádorové lézi - Exprese PAR-2 v infiltrativním duktálním karcinomu mammy a vliv trypsinu na růst a metabolismus buněčné linie mammárního karcinomu MDA MB-231.

Přítomnost aktivovaného receptoru PAR-2 ve všech vyšetřených bioptických vzorcích invazivního duktálního karcinomu mammy, bez ohledu na expresi prognosticky významných proteinů či stupeň diferenciace i ve dvou modelových liniích mammárních karcinomů dokládá význam tohoto receptoru v iniciaci a progresi nádorového onemocnění mléčné žlázy. Navíc jsme v in vitro modelu specificky ověřili v nádorových elementech přítomnost funkčního receptoru, který má schopnost po specifické aktivaci měnit jejich proliferační aktivitu. Význam aktivace PAR-2 pro ovlivnění migrační aktivity buněk karcinomu mammy byl prokázán v recentní studii stejně jako význam PAR-2 v procesu adheze nádorových buněk k molekulám mezibuněčné hmoty. Ve vztahu k těmto předchozím výsledkům, jež dokládají významnou úlohu aktivace trypsinového receptoru v procesu invazivity růstu nádoru a zakládání vzdálených metastáz, naše pozorování přispívá k objasnění vlivu aktivace PAR na proliferaci buněk karcinomů mléčné žlázy. Účast PAR-2 v klíčových dějích kancerogeneze a progrese karcinomů mléčné žlázy předurčuje tento receptor pro budoucí potenciální farmakologické ovlivnění aktivátory/inhibitory proteázami aktivovaných receptorů. Naše výsledky však zároveň ukazují, že toto využití musí respektovat zejména fyziologickou přítomnost velkého počtu různých sérových antiproteáz v organismu, které účinky těchto typů farmak mohou významně ovlivňovat.

5. Summary

It was presented that one of the pancreatic enzymes, trypsin, modulates many biological processes by acting on specific proteinase-activated receptor 2 (PAR-2). PAR-2 belongs to a family of G protein coupled receptors activated by tethered ligand sequences within the N-terminal, which is made accessible after the site-specific cleavage of the protein. Trypsin activates PAR-2 by the mediation of a unique process inhering in the recognition of the receptor by enzyme, subsequent cleavage at the specific site of NH2-terminal and presentation of a new NH2 terminal, which behaves as a tethered ligand. This ligand interacts with the extracellular domain of receptor molecule. Thus, PAR-2 is a receptor, whose ligand is a physical part of the receptor molecule. This receptor was previously described on normal as well as malignant immunocompetent cells, on endothelial and muscle cells of major as well as minor vessels. Its presence was also immunohistochemically demonstrated on intestinal epithelial cells, epithelial cells of exocrine organs, keratinocytes, fibroblasts and other cell types in stomach, small intestine, colon, liver and kidney. PAR-2 is expressed on various cells with a wide spectrum of cellular responses after activation.

In the first part of this work we focused on the role of PAR-2 during the process of acute pancreatitis. An animal model of acute pancreatitis induced by taurocholate injection to ductus choledochus of Wistar rats was used. Much higher positivity for PAR-2 on acinary/ duct cells was observed in APL induced animals than in controls. Similar findings were noticed on arterial smooth muscle cells. Surprisingly, parallel to the exocrine pancreas and vessel findings, enhanced Langerhans' islets cell positivity was observed in experimental animals. Presence of PAR-2 expression on both vascular structures, and acinary and duct epithelium in acute pancreatitis suggests an important role of trypsin-activated receptors in induction/development and/or regeneration/repair/cellular protection in acute pancreatitis.

Recently published data show that PAR-2 plays both beneficial and harmful effect in AP development. Moreover, PARs contribute to systemic changes in AP development and leading to multiple organ dysfunction syndrome and eventually death. Despite all the above described effect of PAR-2 activation, the vascular action leading to the pancreatic ischemic disturbance could be the leading mechanism starting consequent local changes resulting in acute pancreatitis development.

The aim of the second part of the study was to characterize the expression of proteinase-activated receptor-2 (PAR-2) in the brains of Wistar rats after single exposure to radiation at 26 Gy (^Co, 23 min, 15 sec). After irradiation, coronal sections of caudal diencephalons were investigated using histology and immunohistochemistry. Significant PAR-2 membranous positivity of scattered swollen neurons in deeper cortical layers was found in irradiated animals compared with controls. Although this membrane positivity was noticed in all irradiated animals, the most prominent occurred on day 30. Diffuse cytoplasmic positivity was also demonstrated on shrunken neurons in the cortex and hippocampus. Increased cytoplasmic and polarized membrane positivity was also noticed on the neurons of hypothalamic nuclei. The causal relationship between blood-brain barrier damage, PAR-2 activation and neurodegeneration has not yet been verified. However, the present findings indicate that PAR-2 mediates a certain type of cellular response. It remains to be demonstrated whether this is a response to higher concentrations of factor Xa, a free pool of trypsin or other unknown possible proteinases in brain tissue; whether changes in PAR-2 expression are consequences of direct radiation damage to neuronal cells; whether this reaction is protective; and whether primary PAR-2 activation results in neuronal damage.

Altered function of PAR-2 has been described in different malignant tumours. In the last part of the study, we investigated the expression of PAR-2 in breast cancer surgical specimens and the role of trypsin in breast cancer cell line MDA MB-231 proliferation and metabolism. A

total of 40 surgical samples of infiltrative ductal breast cancer and breast cancer cell line were included in this study. We analyzed PAR-2 expression by immunohistochemistry, RT-PCR and western blot. Activation of PAR-2 on cell line MDA MB – 231 was measured using calcium mobilisation assay determined by flow cytometry. MTT cell metabolism assay and cell count analysis were used to assess the trypsin influence on breast cancer cell line MDA MB – 231 proliferation.

Immunohistochemical examination showed the expression of PAR-2 in all samples of breast cancer surgical specimens and cell lines in high level, this was confirmed by RT-PCR and western blot. Calcium mobilisation assay corroborated the activation of PAR-2 on cell line MDA MB – 231 either by trypsin or by agonistic peptide. Cell metabolism assay and cell count analysis showed significant differences of proliferative activity of breast cancer cells dependent on presence or absence of trypsin and serum in culture medium. The results of this study showed that:

PAR-2 is expressed in a high level in infiltrative ductal breast cancer tissue specimens.

PAR-2 is expressed in studied breast cancer cell lines in a high level.

PAR-2 is activated by trypsin and by agonistic peptide as well in the model of breast cancer cell line MDA MB-231.

Activation of PAR-2 in vitro influences proliferative and metabolic activity of breast cancer cell line MDA MB-231.

The action of trypsin is modified by the presence of serum which is a potential source of protease inhibitors.

In conclusion, we would like to sum up that PAR-2 is an important receptor involved in many types of essential pathophysiological processes, as inflammation, reactions to radiation damage or in cancerogenesis. Further studies should be performed in the near future to complete our understanding of all possible PAR-2 actions. This will enable to utilize the

knowledge on the effects of activators/inhibitors of PAR that could be used as potential powerful drugs influencing the course of different pathological processes.

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