UNIVERZITA KARLOVA V PRAZE

2. LÉKAŘSKÁ FAKULTA

Tkáňový faktor u akutních koronárních syndromů

Tissue factor in acute coronary syndromes

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1. Etiopatogeneze aterotrombózy, akutního koronárního syndromu, úloha tkáňového faktoru a cytoadhezivních molekul.

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1.1 Úvod

Ateroskleróza a její komplikace jsou na prvním místě v příčinách úmrtí v průmyslově rozvinutých zemích. V České republice je stále více než 50% úmrtí spojeno s kardiovaskulárními chorobami, hlavně s akutním koronárním syndromem. Přitom ještě před 100 lety byly kardiovaskulární choroby dle statistik příčinou přibližně 10% úmrtí. Tento strmý nárůst je dáván do spojitosti s výrazně vyšším výskytem rizikových faktorů akcelerované aterosklerózy – hypertenzí, hyperlipoproteinémií, kouřením, obezitou a diabetem. Tento trend se u nás zastavil na konci osmdesátých let a od té doby má klesající tendenci. Je to dáno úspěšnou primární i sekundární prevencí: důslednou léčbou hypertenze, změnou stravovacích návyků, která vedla k poklesu průměrné hladiny cholesterolu na 5.88 $mmol.l^{-1}$ a poklesem počtu kuřáků . Nedaří se však ovlivnit procento obezity (průměrný body mass index u mužů 28.1) a s ní spojené inzulinové rezistence (Bruthans 2000, Cífková *et al.* 2002).

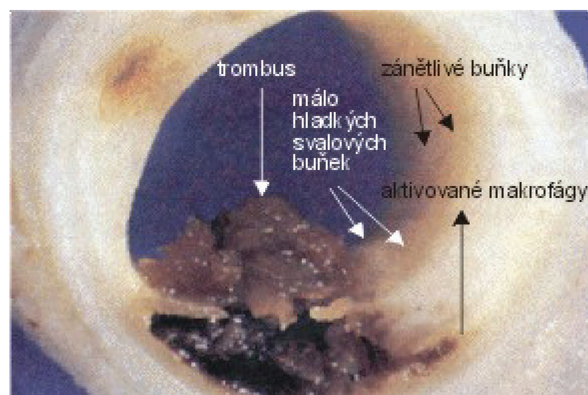
1.2 Patogeneze aterosklerózy

V 19. století byly postulovány dvě teorie patogeneze aterosklerózy: tzv. inkrustační z roku 1852, jejíž autorem byl Rokitanský a tzv. lipidová z roku 1856, jejímž autorem byl Wirchov. Základem těchto dvou teorií byla identifikace depozit fibrinu, inkorporace lipidů a tvorby extracelulární matrix v plátech. Navíc Wirchov poprvé použil termínu *endarteritis deformans*, vedoucí k teorii zánětu a tvořící spojnici k teorii Rosse, který považoval aterosklerózu jako odpověď cévy na její poškození o více než století později (Ross *et al.* 1976). Vzhledem k tomu, že zánětlivá reakce a akumulace lipidů jsou v přímé souvislosti

s rupturou aterosklerotického plátu a následnou tvorbou trombu (Falk *et al.* 1995, Libby 1995), hovoříme přesněji o aterotrombóze.

1.3. Aterotrombóza

Aterotrombóza je definována jako systémové onemocnění velkých a středně velkých tepen zahrnující karotické tepny, aortu, koronární a periferní tepny. Hlavními složkami aterotrombotických plátů jsou: 1) extracelulární pojivová matrix zahrnující kolagen, fibronektinové elastické fibrily a proteoglykany, 2) krystaly cholesterolu, estery cholesterolu a fosfolipidy, 3) buňky zahrnující z monocytů odvozené makrofágy, buňky hladké svaloviny a T lymfocyty, 4) trombotický materiál s depozity trombocytů a fibrinu (Stary *et al*. 1992, Schwartz *et al.* 1995, Daugherty et al. 2002, Libby *et al.* 2002). Aterotrombotické pláty jsou lokalizovány hlavně v intimně tepen, ale částečně zasahují i do medie a adventitie (Moreno *et al.* 2002). Uvedená morfologie je znázorněna na Obr. 1.



Obr. 1 Jednotlivé složky neobturujícího trombu nasedajícího na prasklý aterosklerotický plát.

1.4. Patofyziologie akutního koronárního syndromu

V patofyziologii akutního koronárního syndromu se uplatňují tři základní mechanizmy: 1) ruptura aterosklerotického plátu, 2) nasedající trombóza (okludující či neokludující) a 3) vazokonstrikce. Tvorba trombu na prasklém aterosklerotickém plátu je klíčová v dalším rozvoji akutního koronárního syndromu a progresi aterosklerózy (Fuster *et*

al. 1992, Badimon *et al.*1993). Trombogenicita aterosklerotické léze je určována charakterem a rozsahem obsahu plátu spolu s rheologickými a koagulačními charakteristikami krve. Je známo, že u 30-40% koronárních trombóz se nepodařilo identifikovat prasklý plát jako podklad trombózy (Farb *et al.* 1996). Trombotické komplikace tedy tvoří samostatný, i když úzce propojený mechanizmus vzniku akutního koronárního syndromu.

1.5. Trombotické komplikace

1.5.1. Akutní intrakoronární trombóza

Ruptura aterosklerotického vulnerabilního plátu vede ke změně jeho prostorového uspořádání a stává se spouštěcím mechanizmem pro vznik arteriálního trombu (Falk *et al.* 1995). Tato rychlá změna prostorového uspořádání vede buď k totální okluzi nebo subokluzi koronární arterie, která je patofyziologickým podkladem vzniku akutního koronárního syndromu. Mnohem častěji se jedná o vznik murálního trombu bez uzávěru tepny, který se klinicky nemanifestuje. Tento proces vede ke zhojení plátu spojené se zvětšením jeho obsahu, což vede k další progresi aterotrombózy (Fuster *et al.* 1992, Theroux *et al.* 1998). Tabulka 1 shrnuje základní faktory, které přispívají ke stabilitě či nestabilitě trombu a tím vedou k různé intenzitě klinické manifestace akutního koronárního syndromu. Základem zůstává původní, více než 100 let stará Wirchovem postulovaná trombotická trias: poškození cévní stěny, změna rheologických poměrů a aktivace systémových faktorů krve – Tabulka 1.

Složky cévní stěny
- Ateroskleróza
  - Stupeň ruptury či fisury plátu
- Přítomnost zánětu
  - Složení plátu (lipidového jádra)
  - Makrofágy a generace mikropartikulí (tkáňového faktoru)
- Poškození cévní stěny v důsledku koronární intervence
  - Ruptura plátu v důsledku balónkové angiopastiky či implantace stentu
  - Poškození hladkých svalových buněk (trombin)

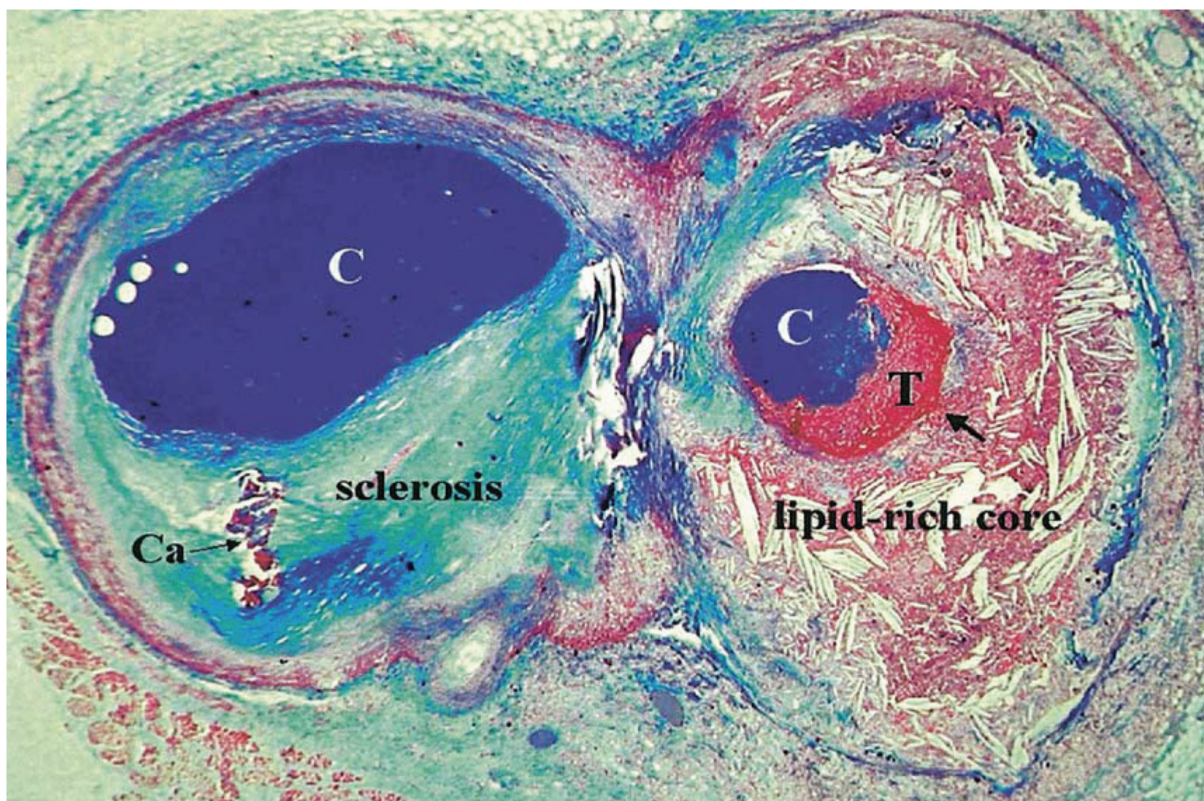
Rheologické poměry
- Vysoké střižné síly
  - Významná stenóza (změněná geometrie, reziduální tromby)
  - Vazokonstrikce (serotonin, tromboxan A2, trombin, dysfunkční endotel)
- Turbulentní proudění
  - Bifurkační stenózy, excentrické pláty
- Zpomalení toku po intervencích (reziduální disekce)

Systémové faktory cirkulující krve
- Metabolické či hormonální faktory
  - Dyslipidémie (hypertriglyceridémie, zvýšené hladiny LDL cholesterolu, oxidovaný LDL cholesterol, snížený HDL cholesterol, lipoprotein A)
  - Diabetes mellitus (glykosylace)
  - Katecholaminy (kouření, stres, kokain)
  - Systém renin-angiotensin
- Plazmatické složky hemostázy
  - Tkáňový faktor - TF, faktor VII - fVII, fibrinogen, trombin, inhibitor plazminogenového aktivátoru 1 – PAI 1, inhibitor tkáňového faktoru – TFPI
  - Infekce (Chlamydia pneumonie, Cytomegalovirus, Helicobacter pylori)
  - Buněčné složky krve (monocyty, leukocyty)

Tabulka 1: Wirchovova trias – faktory trombogenicity

1.5.1.1. Trombogenní substrát závislý na ruptuře plátu

Jak vyplývá z Tabulky 1, expozice aterogenních hmot z prasklého aterosklerotického plátu je pro vznik arteriálního trombu klíčová. Pláty jsou však velmi heterogenní a to i v rámci nálezů u jednotlivce, odpovídající individuálnímu stupni progrese aterosklerózy – Obr. 2.



Obr.2. Aterotrombóza: různorodá směs chronických a akutních trombotických změn. Příčný řez tepennou bifurkací zobrazuje na kolagen bohatý (modře) plát v ramus circumflexus (vlevo) a na lipidy bohatý prasklý aterosklerotický plát s neokluzivní trombózou nasedající v boční větvi (vpravo). C = kontrastní látka v lumen; Ca = kalcifikace; T = trombóza. Převzato z Falk E, Prediman S, Fuster V. Coronary plaque disruption. Circulation 1995;92:657-71.

Je známo, že složení plátu je odpovědné za jeho vulnerabilitu a tím je přímo spojeno s rozvojem akutního koronárního syndromu při jeho ruptuře. Nejčastěji se jedná o pláty s vysokým obsahem lipidů. Jejich trombogenicita je navíc modulována obsahem tkáňového faktoru, který je přítomen v oblastech s velkou infiltrací makrofágy (Toschi *et al* 1997, Marmur *et al.* 1996).

1.5.1.2. Rheologické poměry a trombóza

Stupeň stenózy a změna geometrie uvnitř tepny po ruptuře plátu ovlivňuje rychlost průtoku, kdy jeho zpomalení přispívá k akutnímu ukládání destičkových depozit a tím další progresi okluze. Tak dochází k dalšímu omezení průtoku tepnou. Většina destiček se ukládá

na vrcholu prasklého plátu, kde díky změně geometrie jsou největší střižné síly (Frojmovic *et al.* 2002). Navíc rychlá tvorba trombu vede k vazokonstrikci na základě uvolnění serotoninu a tromboxanu A2 z trombocytů (Willerson *et al.* 1989).

1.5.1.3. Systémová prokoagulační aktivita

Jak již bylo zmíněno, přibližně u třetiny intrakoronárních trombóz se jedná o vznik trombózy na povrchových erozích plátů. Vznik těchto trombů je tedy závislý na systémových prokoagulačních vlastnostech krve v rámci hyperkoagulačního stavu (Fuster *et al.* 1992). Na systémové prokoagulační aktivitě se podílejí dva základní mechanizmy: rizikové faktory a cirkulující tkáňový faktor.

Na zvýšené trombogenicitě krve se významně spolupodílejí změny v lipidovém metabolizmu, kouření cigaret, hyperglykémie, hemostáza a řada dalších rizikových faktorů (Rauch *et al.* 2001, Kullo *et al.* 2000, Edelberg *et al.* 2001). Zvýšené hladiny LDL cholesterolu zvyšují trombogenicitu krve a narůstání trombu (Rauch *et al.* 2000). Kouření zvyšuje hladinu cirkulujících katecholaminů, vede k aktivaci trombocytů a zvyšuje hladiny fibrinogenu (Miller 1992). Zvýšená trombogenicita krve je popisována u nemocných se špatně kompenzovaným diabetem (Meigs *et al.* 2000). Současné práce ukazují, že zvýšené hladiny LDL cholesterolu, kouření a diabetes mohou tvořit společnou patofyziologickou osu. Společným jmenovatelem by mohla být aktivace leukocyto-trombocytárních interakcí spojených s uvolněním tkáňového faktoru a výsledné trombinové aktivace (Sambola *et al.* 2003).

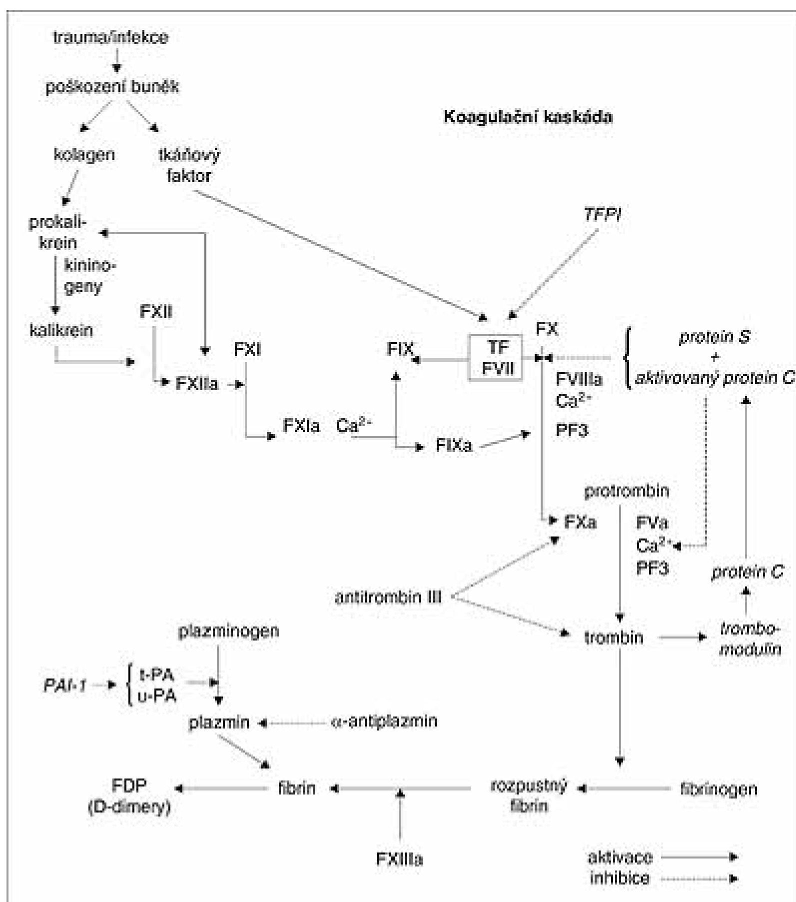
Otázka cirkulujícího tkáňového faktoru jako druhého mechanizmu, který se uplatňuje v systémové prokoagulační aktivitě, je tématem této dizertační práce.

1.6. Tkáňový faktor

1.6.1. Koagulační kaskáda

Cirkulující krev je kapalina, ale jakmile unikne z poraněné cévy, rychle se sráží a tím zabraňuje dalším krevním ztrátám. Na druhé straně může dojít k patologická aktivaci srážení krve uvnitř cév. Důsledky v koronárním řečišti byly diskutovány v předešlých odstavcích.

Schopnost krve rychle se srazit je její fundamentální vlastností a byla předmětem obsáhlého vědeckého zkoumání. Za desítky let vědci identifikovali množství plazmatických proteinů, které se účastní na tvorbě krevní sraženiny. Průlomem bylo postulování kaskádové hypotézy, jejíž autoři poprvé prokázali, že srážení je kaskádovitý děj, sestávající se z několika aktivačních kroků, kdy každý je umožněn proteolytickou konverzí zymogenu na odpovídající aktivní serinovou proteázu (Davie, Ratnoff 1964, Mac Farlane 1964). Celá složitá kaskáda je zobrazena na Obr. 3



Obr. 3 Schéma koagulační kaskády
TFPI = tissue factor pathway inhibitor, Ca^{2+} = kalciové ionty, F = koagulační faktory, Fa = aktivované koagulační faktory, PAI-1 = plazminogen activator inhibitor 1, t-PA = tissue plasminogen activator, u-PA = urokinase plasminogen activator, FDP = fibrin degradační produkty, TF = tkáňový faktor,
PF3 = platelet factor 3.

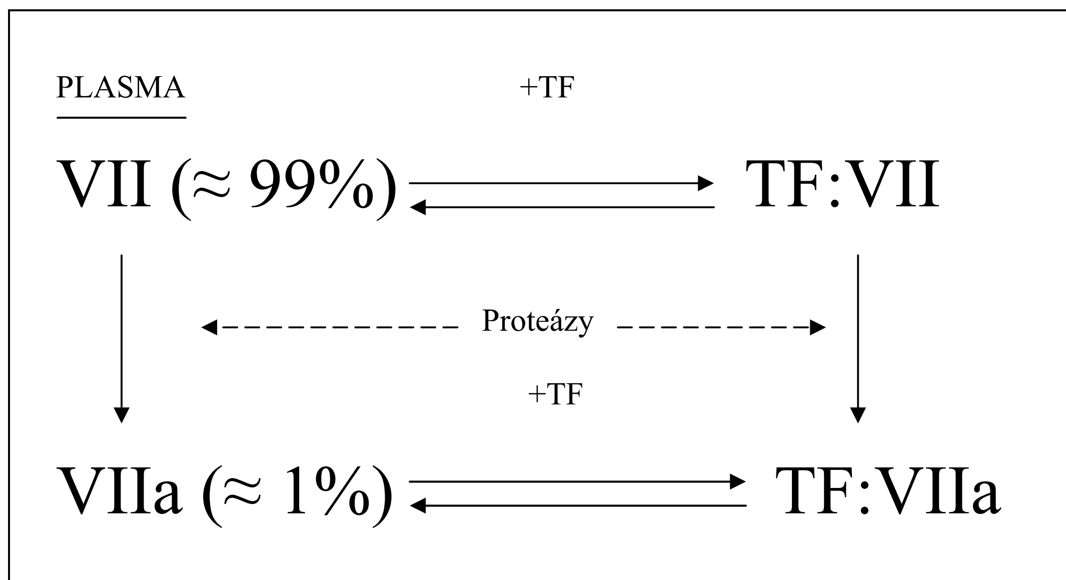
Existují dvě cesty aktivace koagulační kaskády: vnitřní (kontaktní) a vnější, kde ústřední roli hraje tkáňový faktor. Vnitřní (kontaktní) se tak nazývá, protože je vnitřní vlastností plazmy. Projeví se srážením, když krev či plazmu umístíme do skleněné trubičky. Kontaktní je přesnější název, protože je aktivována kontaktem krve či plazmy s umělým povrchem, kupříkladu sklem. Kontaktní cesta aktivace se jeví v normální hemostáze jako nedůležitá, neboť nemocní s kompletně nefunkční kontaktní cestou aktivace (díky těžkým vrozeným defektům některého z kontaktních faktorů, například faktoru XII) netrpí žádnými klinickými projevy krvácení. Vnější cesta (cesta tkáňového faktoru) se aktivuje, když se krev dostane do kontaktu s buňkami exprimujícími tkáňový faktor. Dojde k vazbě tkáňového faktoru s faktorem VII (respektive jeho aktivní formou VIIa) a tím ke spuštění koagulační kaskády.

1.6.2. Aktivace koagulační kaskády tkáňovým faktorem

První enzym v koagulační kaskádě se skládá ze dvou podjednotek: serinové proteázy = koagulační faktor VII a proteinového kofaktoru = tkáňový faktor. Tento enzym je dík tomu, že tkáňový faktor je integrální součástí lipidové membrány, pevně navázán k buněčnému povrchu. Samotný volný aktivovaný faktor VII (VIIa) je slabým enzymem, ale v okamžiku, kdy se naváže na tkáňový faktor, stává se nejsilnějším aktivátorem koagulační kaskády.

Všechny serinové proteázy koagulační kaskády (včetně faktoru VII) cirkulují v plazmě ve formě inertních zymogenů. Navíc většina kofaktorů se vyskytuje v plazmě ve formě inertních prekurzorů (výjimkou je právě tkáňový faktor; ke své aktivaci nepotřebuje proteolýzu). Mnoho let tedy nebylo zřejmé, jak se může aktivovat koagulační kaskáda

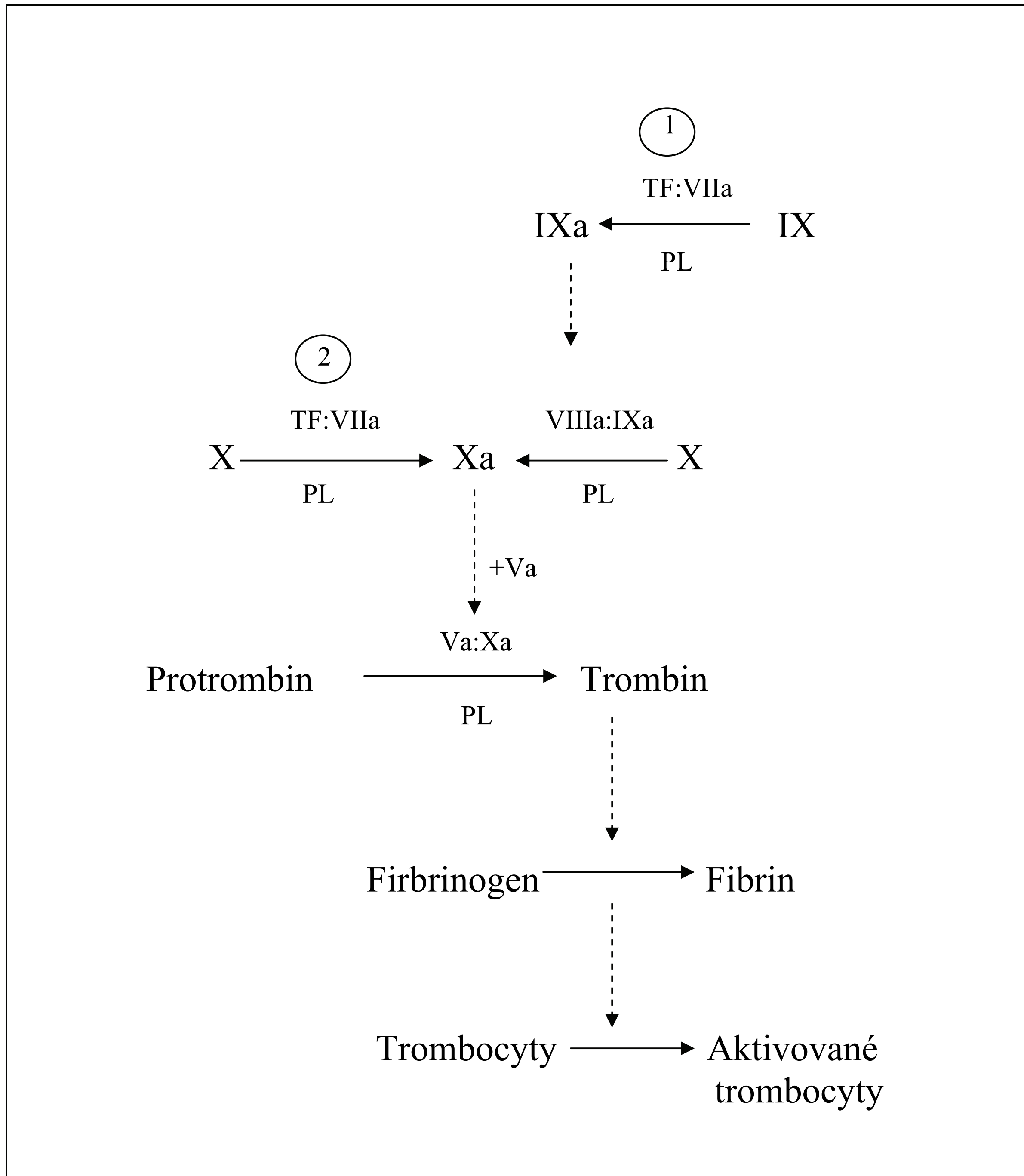
pouhým shromážděním inertních prekurzorů. Nakonec byla nalezena odpověď: v cirkulaci je vždy přítomno malé množství aktivovaného faktoru VII (VIIa). Samotný plazmatický poločas většiny serinových proteáz je velmi krátký – řádově měřený v sekundách. Výjimkou je právě volný cirkulující faktor VIIa díky tomu, že nereaguje s proteázovými inhibitory v plazmě (Kondo, Kisiel 1987). Tkáňový faktor může vázat jak faktor VII, tak faktor VIIa. Jakmile je faktor VII navázán na tkáňový faktor, dochází k jeho proteolýze na faktor VIIa (Nemerson, Repke 1985). Jsou tedy dvě cesty vzniku komplexu tkáňový faktor/faktor VIIa. Jedna je přímá vazba aktivovaného faktoru VIIa cirkulujícího v plazmě na tkáňový faktor, druhá je vazba neaktivního faktoru VII na tkáňový faktor a jeho sekundární aktivace – Obr 4.



Obr 4: Dvě cesty formace komplexu tkáňového faktoru a aktivovaného faktoru VII (TF:VIIa). Plazma obsahuje směs přibližně 99% zymogenní formy faktoru VII (VII) a přibližně 1% aktivní formy faktoru (VIIa). Obě formy se reverzibilně váží na tkáňový faktor se stejnou afinitou. Zymogenní forma je na aktivní formu štěpena různými proteázami v plazmě a po vazbě na tkáňový faktor je faktor VII omezenou proteolýzou ihned aktivován.

Komplex tkáňový faktor/faktor VIIa může spouštět koagulační kaskádu dvěma způsoby. Prvním mechanizmus spočívá v aktivaci faktoru IX omezenou proteolýzou. Nově vytvořený aktivovaný faktor IX (IXa) se na fosfolipidovém povrchu spojí se svým kofaktorem, aktivovaným faktorem VIII (VIIIa) a vytvořený komplex IXa/VIIIa následně katalyzuje konverzi faktoru X na aktivovaný faktor Xa. Druhým mechanizmem je přímá aktivace faktoru X. Tato cesta aktivace probíhá hlavně v podmínkách *in vitro* – Obr. 5.

Tyto poznatky nás vracejí zpět k otázce inicializace koagulační kaskády. To znamená: odkud pochází první proteázová aktivita, jestliže všechny enzymy a jejich kofaktory cirkulují ve formě neaktivních prekurzorů? Jak bylo zmíněno výše, v krvi nacházíme stopová množství aktivovaného faktoru VII (VIIa), tvořící přibližně 1% celkové koncentrace faktoru VII (Morrissey *et al.* 1993). Jakmile se tedy dostane tkáňový faktor do kontaktu s plazmou, tvoří komplex jak s faktorem VII, tak s faktorem VIIa. Tyto nízké hladiny komplexu tkáňový faktor/faktor VIIa jsou patrně právě spouštěcím mechanizmem cestou zpětné aktivace faktoru VII na aktivní faktor VIIa. V podmínkách *in vitro* byla identifikována celá řada koagulačních proteáz schopných aktivovat faktor VII: faktory IXa, Xa a XIIa, trombin a plazmin. Navíc je schopen komplex tkáňový faktor/faktor VIIa autoaktivační reakce faktoru VII ve vazbě komplexu tkáňový faktor/faktor VII (Neuenschwander *et al.* 1993). Dosud není přesně známo, která z výše uvedených proteáz hraje hlavní úlohu v aktivaci faktoru VII zpětným mechanizmem, ale většina autorů se kloní ke klíčové úloze faktoru Xa. Též není přesně známo, kde či jak se tvoří toto malé množství faktoru VIIa. Řešení však napovídá studium nemocných s hemofilií B (postrádajících faktor IX), kdy jejich hladiny faktoru VIIa tvoří pouze přibližně 10% normálu (Wildgoose *et al.* 1992). Tento poznatek naznačuje, že faktor IX (hlavně v aktivované formě IXa) se významně účastní na tvorbě tohoto stopového množství cirkulujícího faktoru VIIa.

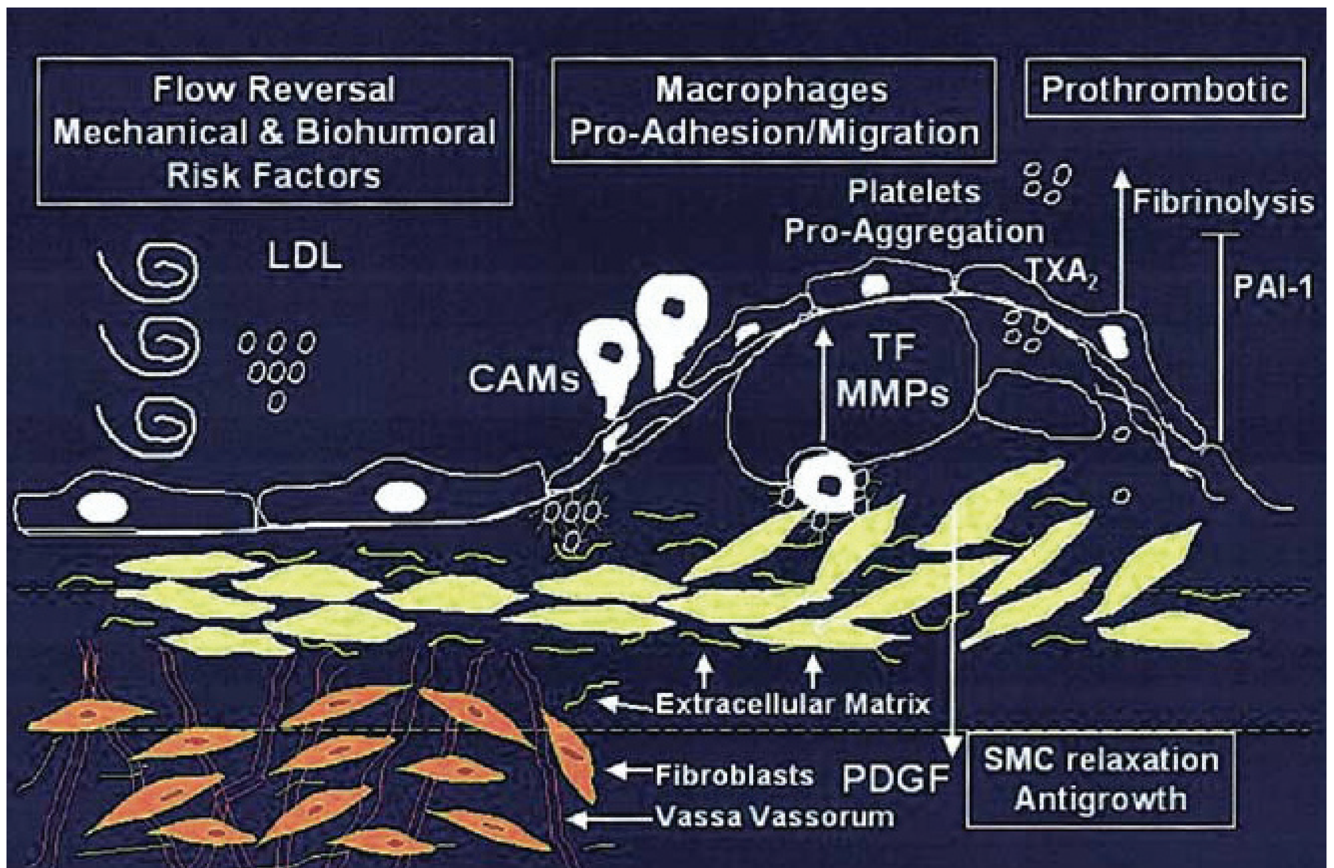


Obr. 5 Zjednodušené schéma koagulační kaskády zahájené tkáňovým faktorem (TF) a aktivovaným faktorem VII (TF:VIIa). Komplex TF:VIIa může aktivovat koagulační kaskádu buď aktivací faktoru IX (reakce 1), nebo faktoru X (reakce 2). Nezávisle na mechanizmu iniciace, všechny aktivační cesty vedou k tvorbě aktivovaného faktoru X (Xa), který reaguje na fosfolipidovém povrchu (PL) se svým proteinovým kofaktorem (Va) a katalyzuje konverzi protrombinu na trombin, který je zodpovědný za konverzi fibrinogenu na fibrin. Navíc trombin je silným aktivátorem krevních destiček.

1.7. Endoteliální dysfunkce a cytoadhezivní molekuly

Celý proces aterogeneze začíná jako porucha funkce endotelu (endoteliální dysfunkce). Endotel je dynamický parakrinní a endokrinní orgán regulující protizánětlivé, mitogenní a kontraktilní charakteristiky cévní stěny a též hemostatické procesy uvnitř cévy (Bonetti *et al.* 2003). Za tyto procesy je zodpovědná molekula oxidu dusnatého (NO) (Ignarro 2004).

Dysfunkce endotelu, charakterizovaná poklesem syntézy NO, vede ke zvýšené propustnosti cévní stěny, oxidací cirkulujících lipoproteinů, průnikem monocytů a zánětem, proliferací hladkých svalových buněk, depozity extracelulární matrix, vazokonstrikcí a protrombotickým stavem uvnitř cévního lumen (Voetsch *et al.* 2004). Endoteliální buňky odpovídají na oscilace střižných sil, které jsou způsobeny změnou laminárního proudění na turbulentní, změnami v expresi různých genů. Pravděpodobný mechanizmus této genové modulace je zprostředkován přes elementy, reagující přímo na tuto změnu, které následně ovlivňují genovou expresi (Malek *et al.* 1999). Aktivace endotelových buněk vede k expresi tzv. cytoadhezivních molekul (CAM) ze skupiny selektinů (P-selektin, E-selektin). Tyto proteiny umožňují vazbu monocytů na povrch aktivovaných endoteliálních buněk. Exprese selektinů je regulována transkripčním nukleárním faktorem (TNFκ-B) s následnou expresí dalších CAM (ICAM-1 = intercellular cytoadhesive molecule, VCAM-1 = vascular cytoadhesive molecule) (De Caterina *et al.* 2001). Tyto cytoadhezivní molekuly umožňují průnik adherovaných monocytů přes endotelovou membránu do cévní stěny a tím přispívají aterogenezi - Obr. 6.



Obr. 6 Dysfunkční endotel s turbulentním prouděním krve, depozity LDL cholesterolu, expresí cytoadhezivních molekul, migrací makrofágů, tkáňového faktoru a metaloproteináz matrix vedoucí k proliferaci hladkých svalových buněk a neovaskularizaci vasa vasorum. LDL = low density lipoproteins, CAMs = cytoadhesive molecules, TF = tissue factor, TXA_2 = tromboxan A_2, PAI-1 = plasminogen activator inhibitor 1, SMC = smooth muscle cell, PDGF = platelet derived growth factor. Převzato z Falk E, Prediman S, Fuster V. Coronary plaque disruption. Circulation 1995;92:657-71.

1.8. Mikropartikule

Buňky v klidovém stavu zachovávají membránovou asymetrii – cholinfosfolipidy (sfyngomyelin a fosfatidylcholin) jsou na vnějším povrchu membrán, zatímco většina aminofosfolipidů (fosfatidylserin a fosfatidyletanolamin) jsou skryty na vnitřní straně. Tato asymetrie je ATP dependentní aktivní proces. Po aktivaci buněk či jejich apoptóze a následném vzestupu koncentrace intracelulárního vápníku dojde ke ztrátě této asymetrie. To vede k přesunu fosfatidylserinu na povrch buňky, což následně vede ke zvýšené náchylnosti těchto buněk k fagocytóze makrofágy (Piomelli, Seaman 1993). Ztráta membránové lipidové

asymetrie během buněčné aktivace či apoptózy je doprovázena oddělováním mikropartikulí z buněčného povrchu. Mikropartikule jsou uvolňovány z různých typů buněk (trombocytů, T a B lymfocytů, monocytů, erytrocytů) a z buněk cévní stěny (endoteliálních buněk a buněk hladkého svalstva) (Zwaal, Schroit 1997, Miyazaki *et al.* 1996). Na svém povrchu pak mikropartikule exprimují charakteristické antigeny buněk svého původu, což umožňuje jejich specifickou detekci.

Morfologicky jsou mikropartikule malé vezikuly (< 1µm) Apoptóza, na rozdíl od buněčné nekrózy, je fyziologicky naprogramovaná buněčná smrt, která je aktivována či suprimována intracelulárními buněčnými pochody jako odpověď na extracelulární podněty okolních buněk či samotné intracelulární podněty. Základní rozdíl mezi mikropartikulemi uvolňovanými během apoptózy a během aktivace buněk spočívá v přítomnosti, respektive absenci DNA. Na rozdíl od mikropartikulí uvolňovaných během apoptózy, mikropartikule uvolňované během aktivace buněk (typicky při procesu aterotrombózy) DNA neobsahují (Hristov *et al.* 2004). Dalším rozdílem mezi apoptózou a buněčnou aktivací z hlediska mikropartikulí spočívá ve faktu, že při aktivaci buněk se mikropartikule syntetizují.

Bylo potvrzeno, že tvorba trombocytárních mikropartikulí je výsledkem zvýšení intracelulární koncentrace vápníku (Pasquet *et al.* 1996) a současné expozice prokoagulačních fosfolipidů jak na povrchu mikropartikulí, tak na povrchu aktivovaných trombocytů (Comfurius *et al.* 1990, Thiagarajan, Tait 1991) po stimulaci trombocytů například kombinací trombinu a kolagenu.

Dalšími buňkami schopnými exprimovat mikropartikule jsou endoteliální buňky. Stimulačními faktory jsou tumor necrosis factor a další cytokiny, bakteriální lypopolysacharidy či proteiny komplementu (Bomeli *et al.* 1997, Hamilton *et al.* 1990).

Monocyty po stimulaci lypopolysacharidy uvolňují mikropartikule a též na svém povrchu exprimují tkáňový faktor, CD 14 a alfa $_2$ integriny. Všechny tyto faktory mohou

přispívat k iniciaci koagulační kaskády a integrin-dependentních adhezních reakcí (Robinson, Stephens 1992, Satta *et al.* 1994).

Produkce mikropartikulí je rovněž indukována změnami v průtoku krve například při turbulentním proudění za stenózou, což způsobuje aktivaci krevních destiček a následnou akumulaci mikropartikulí derivovaných z trombocytů (Holme *et al.* 1997).

1.8.1. Mikropartikule a aterotrombóza

Mikropartikule generované z aktivovaných trombocytů na svém fosfolipidovém povrchu tvoří vazebné místo pro aktivované faktory Va, VIIIa, IXa, Xa. Tím získávají mikropartikule prokoagulační vlastnosti (Gilbert *et al.* 1991, Hoffman *et al.* 1992). Úloha ve fyziologii koagulace je dokumentována faktem, že deficience trombocytárních mikropartikulí vede ke krvácivým projevům (Castaman *et al.* 1997). Trombocytární mikropartikule se váží na subendoteliální matrix a adherují k trombinem aktivovaným endoteliálním buňkám. Zde, pravděpodobně expozicí glykoproteinů IIb/IIIa na svém povrchu, umožňují adhezi a agregaci trombocytů (Siljander *et al.* 1996, Holme *et al.* 1998).

Mikropartikule z jiných buněčných elementů jsou schopny vázat tkáňový faktor, což vede přes aktivaci koagulační kaskády k výsledné arteriální trombóze (Morrissey 2001). Aktivní forma tkáňového faktoru vyžaduje přítomnost fosfatidylserinu, který je exprimován jak na apoptotickém buněčném povrchu, tak na povrchu mikropartikulí. Tento tkáňový faktor patrně hraje určující roli v tvorbě arteriálního trombu po ruptuře aterosklerotického plátu (Mallat *et al.* 1999).

Interakce mezi trombocyty a leukocyty (v klinické praxi možné spojení mezi trombózou a zánětem) byla *in vivo* prokázána na základě interakce P-selektinu trombocytů a P-selektin-glykoproteinové ligandy (PSGL-1) na straně mikropartikulí generovaných z

leukocytů (Falati *et al.* 2003). Kromě této přímé vazby ještě existuje mezi leukocyty a trombocyty transfer tkáňového faktoru (Rauch *et al.* 2000).

Výše uvedené fakta svědčí pro klíčovou úlohu mikropartikulí a tkáňového faktoru v procesu aterotrombózy a orchestraci simultánně probíhajících trombotických a zánětlivých procesů.

2. Cíl práce

Intrakoronární trombóza s následnou okluzí koronární tepny je patofyziologickým substrátem akutního koronárního syndromu. Tkáňový faktor, mikropartikule a cytoadhezivní molekuly (spolu s trombocyty) hrají klíčovou úlohu v iniciální fázi tvorby trombu.

Cílem naší práce bylo zjistit, zda lze tyto patofyziologické poznatky dokumentovat v lokální a systémové cirkulaci u nemocných s angiograficky prokázanou koronární nemocí a nalézt jejich korelaci s klinickou manifestací akutního koronárního syndromu.

Konkrétně jsme testovali následující hypotézy:

1. Hladina tkáňového faktoru stanovená na základě ELISA metody nereflektuje (vzhledem k jeho mnohočetným biologickým úlohám) specifickou trombogenní aktivitu vyjádřenou při akutním koronárním syndromu.
2. Vzhledem k nespecifičnosti ELISA metody nelze nalézt patofyziologicky zdůvodnitelné korelace mezi koncentracemi jednotlivých selektinů a koncentrací cytoadhezivní molekuly ICAM-1 stanovené touto metodou u nemocných se stabilní anginou pectoris a u nemocných s akutním koronárním syndromem.
3. Hladiny tkáňového faktoru stanovené na základě jeho aktivity, která koreluje specificky s jeho trombotickým potenciálem, koresponduje s vyjádřeným klinickými parametry.
4. Změna počtu mikropartikulí, které jsou zdrojem aktivního tkáňového faktoru v procesu aterotrombózy, koresponduje se změnami v jeho aktivitě.

3. Metodologické aspekty a výsledky

Vzhledem k tomu, že v naší práci spolu část metodologická a část zahrnující výsledky úzce souvisejí (a částečně se i překrývají), jsou zde obě diskutovány zároveň. Detaily použitých metod, včetně statistického zpracování jsou rozvedeny v příslušných přílohách. Zde jsou podrobněji zmíněny hlavně dva aspekty významné z hlediska stanovení tkáňového faktoru – heterogenita výsledků při použití ELISA metody a otázka metody měření tkáňového faktoru na základě jeho aktivity ve vztahu k trombogennímu potenciálu přítomnému v plazmě.

Práce je rozdělena do dvou částí (příloha 1 a příloha 2). Druhá část práce byla koncipována na základě výsledků zjištěných v první části.

Výzkum probíhal na 2. lékařské fakultě Univerzity Karlovy v Praze a byl schválen lokální etickou komisí. Všichni nemocní podepsali informovaný souhlas se vstupem do studie a prováděnými výkony.

3.1. Stanovení hladiny tkáňového faktoru na základě měření antigenu

V posledních letech bylo publikováno několik prací zaměřených na otázku změny koncentrace tkáňového faktoru u akutního koronárního syndromu. Hladiny byly stanoveny na základě ELISA metody. Tyto práce jsou nadále citovány v písemnictví, byť jejich výsledky nejsou konzistentní.

V roce 1998 byla publikována práce (Misumi *et al.* 1998), zahrnující 21 nemocných s nestabilní anginou pectoris, 27 nemocných se stabilní anginou pectoris a 27 kontrolních vzorků. Hladiny antigenu tkáňového faktoru byly ve skupině nestabilní anginy pectoris 240±75 pg/ml, ve skupině stabilní anginy pectoris 184± 46 pg/ml a v kontrolní skupině

177±37 pg/ml ($p<0.01$). Nebyl nalezen rozdíl mezi kontrolní skupinou a skupinou se stabilní anginou pectoris.

Často citovanou prací je publikace z roku 1999 (Soejima *et al.* 1999), která zahrnovala 51 nemocných s anginou pectoris a jako kontrolní skupina sloužily vzorky u 55 nemocných s atypickými bolestmi na hrudi. Ve skupině nestabilní anginy pectoris byly hodnoty antigenu tkáňového faktoru 238±54 pg/ml ($p<0.001$), ve skupině se stabilní anginou pectoris 189±30 pg/ml ($p<0.001$). V kontrolní skupině byla hodnota 180±30 pg/ml. V samotné diskuzi dané práce je zmíněno, že tkáňový faktor v plazmě je směs fragmentů tkáňového faktoru pocházející jak z apoptózy různých buněk, tak z vysoce trombogenních mikropartikulí.

V roce 2000 vyšla práce (Falciani *et al.* 1998), která zahrnovala 55 nemocných s ischemickou chorobou srdeční (18 s nestabilní anginou pectoris, 24 s klidovými stenokardiemi a 13 nemocných po infarktu myokardu). Kontrolní skupina byla tvořena 48 zdravými dobrovolníky. Hladiny antigenu tkáňového faktoru v plazmě byly u nemocných s ischemickou chorobou srdeční signifikantně zvýšeny (median 215.4 pg/ml; rozmezí 72.6 to 834.3 pg/ml; kontrolní skupina: median 142.5 pg/ml; rozmezí 28.0-255.3 pg/ml) ($p<0.001$). Práce uzavírá, že nemocní s ICHS mají v plazmě zvýšenou hladinu tkáňového faktoru (přes extrémní rozptyl hodnot).

V roce 2005 byla publikována práce holandských autorů (van der Putten *et al.* 2005), která zkoumala klinickou konsekvenci stanovení antigenu tkáňového faktoru jako parametru pro diagnostiku akutního koronárního syndromu. Zahrnovala celkem 90 nemocných, používala vysoce senzitivní imunoassay a následně zkoumala hladinu tkáňového faktoru. Výsledek byl negativní – nebyla nalezena korelace mezi akutním koronárním syndromem a vstupní hladinou tkáňového faktoru.

Další práce z roku 2005 (Ikonomidis *et al.* 2005) byla zaměřena na hladinu tkáňového faktoru během zátěžového dobutaminového testu. Autoři uzavírají, že nemocní s poruchou

kinetiky při zátěži měli zvýšené hladiny tkáňového faktoru. Nicméně již v editorialu se uvádí, že tkáňový faktor byl u některých nemocných s poruchou kinetiky sice lehce zvýšen po zátěži, ale někteří nemocní z této skupiny nevykazovali žádnou změnu v hladině tkáňového faktoru. Možným vysvětlením je odlišný fenotyp. A opačně, někteří nemocní bez poruchy kinetiky vykazovali po zátěži dramatický pokles hladin tkáňového faktoru, zatímco u některých nemocných se hladina neměnila. Výsledky tedy vykazují značnou heterogenitu. Dokud nebudou tyto otázky zodpovězeny, nelze zatím tkáňový faktor použít jako biomarker ischémie (Christenson *et al.* 2005).

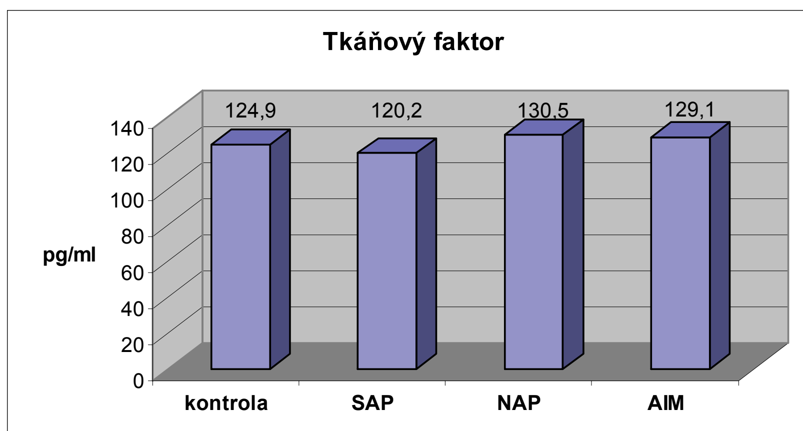
V letošním roce byla publikována AteroGene Study (Morange *et al.* 2007), zkoumající prognostický význam hladin tkáňového faktoru a jeho inhibitoru (TFPI) na osud nemocných s ischemickou chorobou srdeční. Dle publikovaných výsledků se vstupní hladiny tkáňového faktoru ve skupině se stabilní anginou pectoris a akutním koronárním syndromem nelišily. Hodnoty TFPI signifikantně stoupaly ve skupině se stabilní anginou pectoris, nestabilní anginou pectoris a infarktem myokardu. Ti nemocní s akutním koronárním syndromem, kteří měli vstupně vyšší hodnotu tkáňového faktoru, však měli horší prognózu se zvýšeným rizikem kardiovaskulárního úmrtí po dobu dvouletého sledování.

Tyto výsledky z literatury ukazují na limitace stanovení hladiny tkáňového faktoru vyplývající ze samé podstaty použité ELISA metody, kdy jakékoliv proteinové struktury mající strukturou podobnou tkáňovému faktoru (tedy i jeho fragmenty) jsou identifikovány protilátkou a tedy nevypovídají nic o jeho samotném trombogenním potenciálu.

3.1.1. Vlastní výsledky

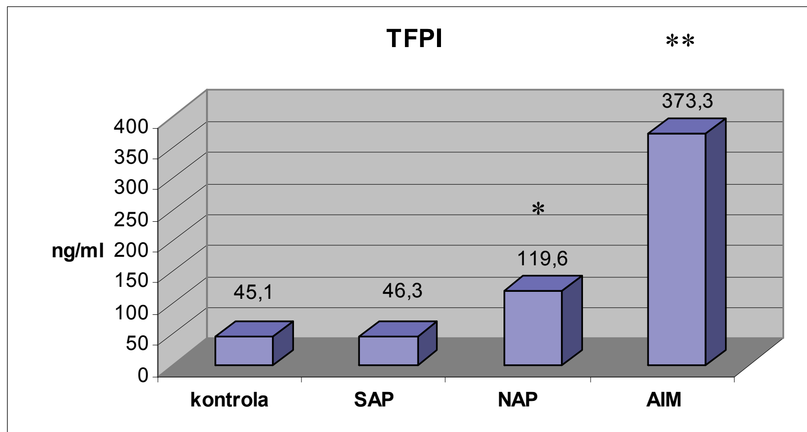
V první části naší práce (viz příloha 1) jsme se zabývali otázkou, zda lze u nemocných s akutním koronárním syndromem nalézt korelaci mezi klinickou manifestací ischemické

choroby srdeční a hodnotami tkáňového faktoru, inhibitoru tkáňového faktoru (TFPI), hladinami P a E selektinu a cytoadhezivní molekulou ICAM-1. Celkem jsme v této fázi vyšetřili 50 nemocných a 10 nemocných bez angiograficky dokumentované koronární nemoci sloužilo jako kontrolní skupina. Naše výsledky potvrzují již výše zmíněnou heterogenitu výsledků, kdy se hodnoty koncentrace tkáňového faktoru mezi jednotlivými podskupinami nelišily (Graf 1). V souhlase s literárními daty jediným signifikantně zvýšeným parametrem ve vztahu ke klinické manifestaci ischemické choroby srdeční byl TFPI, jehož hodnota byla nejvyšší v podskupině s akutním infarktem myokardu a nejnižší u kontrolní podskupiny (Graf 2). Zřejmě je to dáno tím, že TFPI se (na rozdíl od tkáňového faktoru) neúčastní dalších biologických procesů a je tedy pro proces trombózy specifický. Jeho koncentrace dobře koreluje s jeho biologickou funkcí.



Graf 1: Koncentrace tkáňového faktoru stanovené na základě ELISA metody v jednotlivých podskupinách. Výsledky se od kontrolní skupiny statisticky neliší (p=ns).

SAP stabilní angina pectoris; NAP nestabilní angina pectoris; AIM akutní infarkt myokardu.



Graf 2: Koncentrace TFPI stanovené na základě ELISA metody v jednotlivých podskupinách. Výsledky se od kontrolní skupiny statisticky liší na hladině významnosti p< <0.05 (*), respektive 0.01 (**).

TFPI tissue factor pathway inhibitor; SAP stabilní angina pectoris; NAP nestabilní angina pectoris; AIM akutní infarkt myokardu.

V první části naší práce se tedy nepodařilo prokázat rozdíly v hladině tkáňového faktoru v plazmě. Vysvětlení je možné trojí:

a) žádné rozdíly v hladině tkáňového faktoru v plazmě reálně neexistují
b) tkáňový faktor v plazmě vykazuje takovou heterogenitu a interindividuální variabilitu, že nelze na základě jeho měření predikovat rozdíly v rámci jednotlivých klinických manifestací ischemické choroby srdeční
c) ELISA metoda není vhodná pro stanovení specifického trombogenního potenciálu cirkulujícího tkáňového faktoru

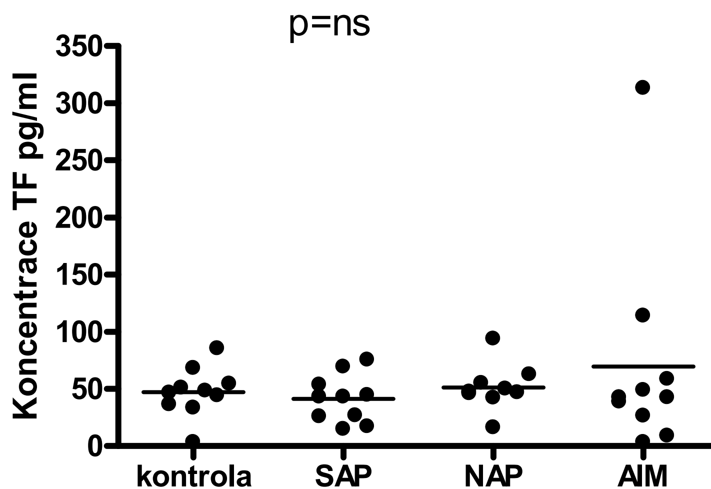
Na dané otázky jsme se pokusili odpovědět ve druhé části naší práce.

3.2. Stanovení hladiny tkáňového faktoru na základě měření aktivity

Vzhledem k výše uvedené heterogenitě výsledků jsme se rozhodli v druhé části naší práce zaměřit na zjištění potenciálních rozdílů hladiny tkáňového faktoru (pokud existují) u dalšího souboru nemocných při použití metody, která je schopna měřit schopnost tkáňového faktoru indukovat tvorbu faktoru Xa a tím tedy specificky podchytit jeho trombogenní potenciál. Metoda spočívá na chromatografickém principu, který se liší od metody stanovení antigenu na základě protilátek. Princip metody spočívá v simulaci koagulačního procesu, známého z experimentů. Po dvojí centrifugaci (nejprve celé krve a následně plazmy) rychlostí tři tisíce otáček po dobu 15 minut získáme plazmu, která již neobsahuje trombocyty. Ty jsou hlavním zdrojem potenciálních falešných výsledků (vzhledem ke své schopnosti se aktivovat kontaktem a následnou degranulací s uvolněním mikropartikulí). Takto připravené vzorky se následně zpracují přidáním do roztoku, který obsahuje 5 nM faktoru VIIa, 150 nM faktoru X a 1 mM $CaCL_2$. Tato směs je přenesena na titrovací destičku. Do této směsi je přidáno 0.3 mM chromogenního substrátu - Spectrozyme fXa. Přítomností aktivovaného faktoru Xa (která je dána proběhlou reakcí tkáňový faktor/faktor VIIa + faktor X na titrovací destičce) se změní jeho chromogenní vlastnosti. Zaznamenávají se lineární změny adsorbance, vyjádřené v mOD/min. Tyto změny přímo korelují s množstvím vytvořeného faktoru Xa a tím odpovídají aktivitě tkáňového faktoru, který tuto tvorbu podmiňuje. Nejedná se tedy o přímé stanovení hladiny tkáňového faktoru, ale o relativní srovnání jeho aktivity vyjádřené schopností štěpit faktor X na aktivovaný faktor Xa (Giesen *et al.* 1999, André *et al.* 2000).

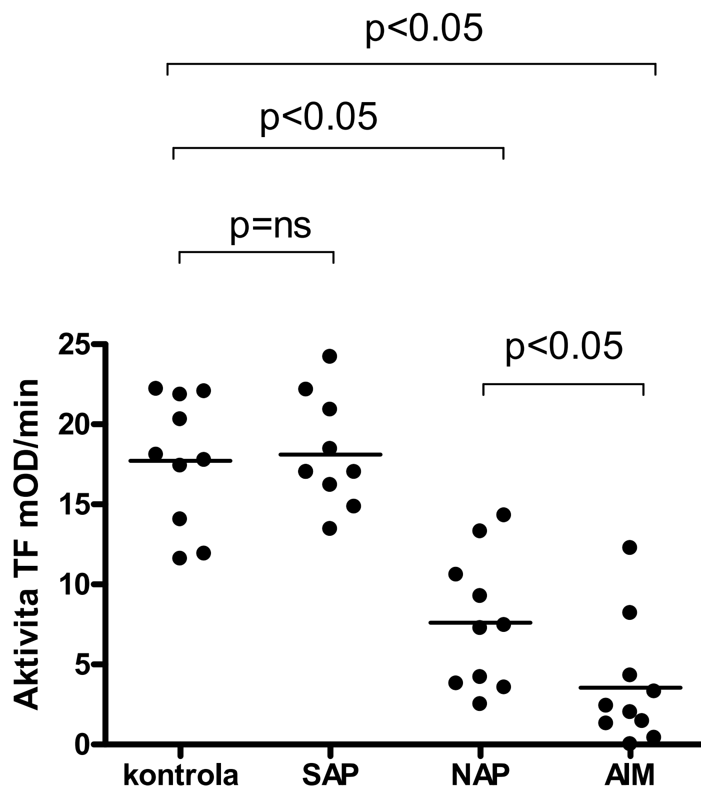
3.2.1. Vlastní výsledky

Na základě výše uvedených fakt jsme koncipovali druhou část naší práce. Ve čtyřech skupinách – vyšetřovaní s normální koronární angiografií jako kontrolní skupina (n=10), skupině se stabilní anginou pectoris (n=10), skupině s nestabilní anginou pectoris (n=10) a skupině s akutním infarktem myokardu (n=10) – jsme stanovili aktivitu tkáňového faktoru a počet mikropartikulí. Aktivita byla stanovena na základě dříve popsané metody a počet mikropartikulí na základě průtokové cytometrie. Hladinu P-selektinu jsme v této části nestanovovali, neboť z první části bylo zřejmé, že ELISA metodou nejsme schopni detekovat významné rozdíly (viz příloha 1). Odběry a další charakteristiky souboru jsou uvedeny v příloze 2. Zde jsou krátce diskutovány výsledky (Graf 3, 4).



Graf 3: Koncentrace tkáňového faktoru stanovené na základě ELISA metody v jednotlivých podskupinách.

SAP stabilní angina pectoris; NAP nestabilní angina pectoris; AIM akutní infarkt myokardu.



Graf 4: Hodnoty aktivity tkáňového faktoru stanovené na základě měření chromogenních substrátů v jednotlivých podskupinách.
SAP stabilní angina pectoris; NAP nestabilní angina pectoris; AIM akutní infarkt myokardu.

Z grafu 3 vyplývá, že jsme v našem druhém souboru opět nenalezli rozdíly v koncentraci antigenu tkáňového faktoru, což potvrzuje výsledky z první části práce. Nicméně graf 4 ukazuje statisticky významné rozdíly v aktivitě tkáňového faktoru a to opačné, než jsme předpokládali. V literárním úvodu jsme již uvedli, že dosud publikované práce buď žádné rozdíly v koncentraci tkáňového faktoru nenalezly, nebo nalezly u akutních koronárních syndromů koncentrace zvýšené. V této části naší práce jsme zjistili, že aktivita tkáňového faktoru se s tíží klinického obrazu akutního koronárního syndromu v plazmě snižovala. Toto překvapivé zjištění má možné patofyziologické vysvětlení

v jeho konzumpci z plazmy a inkorporaci do vznikajícího trombu u akutního koronárního syndromu (rozsah trombózy u akutního infarktu myokardu s ST elevacemi je větší než u nestabilní anginy pectoris a aktivita proto nižší). Aktivita tkáňového faktoru se mezi podskupinou se stabilní anginou pectoris a kontrolní podskupinou nelišila. Tento nález je opět konzistentní, neboť u nemocných se stabilní anginou pectoris není trombóza (jako substrát akutního koronárního syndromu) přítomna.

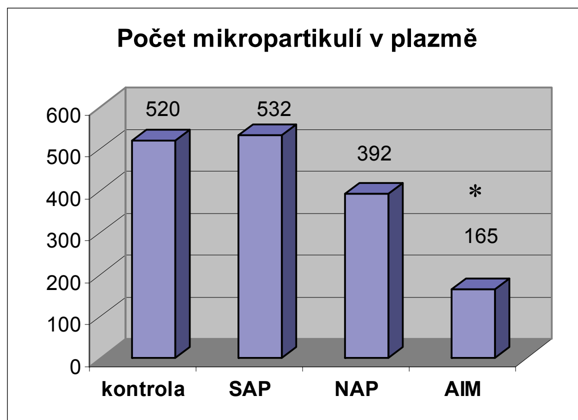
3.3. Trombogenní mikropartikule, P-selektin, PSGL-1

Tkáňový faktor, který má specifický trombogenní potenciál, je v plazmě vázán na cirkulující mikropartikule. Jak bylo zmíněno výše, zdrojem mikropartikulí v plazmě jsou buď apoptotické buňky, nebo stimulované trombocyty, leukocyty či monocyty. Otázkou je, co vede k této stimulaci. V tomto procesu aktivace hraje ústřední roli P-selektin a jeho receptor, PSGL-1 (P-selektin glycoprotein ligand-1). P-selektin je člen skupiny selektinů a je lokalizován na membráně α-granul trombocytů a na Weibel-Paladiho tělískách endoteliálních buněk (Mc Ever 2001). P-selektin usnadňuje adhezi leukocytů s aktivovanými trombocyty ve vznikajícím trombu (Palabrica *et al.* 1992), či s aktivovanými endoteliálními buňkami, kde umožňuje tzv. rolling leukocytů po jejich povrchu (Mayadas *et al.* 1993). Solubilní forma P-selektinu přítomná v plazmě (Dunlop *et al.* 1992) je částečně aktivní, neboť obsahuje domény nutné k vazbě na jeho receptor PSGL-1. Mechanizmus, kterým P-selektin vede k tvorbě mikropartikulí obsahujících tkáňový faktor není přesně znám. Patrně se jedná o jeho vazbu na PSGL-1 přítomný na straně leukocytů. Vazba P-selektinu na tento receptor iniciuje intracelulární aktivaci, vedoucí k produkci výše zmíněných mikropartikulí (Hrachovinova *et al.* 2003). Monocyty

jsou k produkci tkáňového faktoru stimulovány kromě P-selektinu (Celi *et al.* 1994) též různými zánětlivými mediátory (Gregory *et al.* 1989).

3.3.1. Vlastní výsledky

K potvrzení nálezu snížené aktivity tkáňového faktoru na základě jeho předpokládané konzumpce trombem jsme stanovili počet mikropartikulí ve vzorcích z jednotlivých skupin pomocí průtokové cytometrie. Nalezli jsme snížený počet mikropartikulí u akutního koronárního syndromu, což je konzistentní s výše uvedeným nálezem aktivity tkáňového faktoru (Graf 5).



Graf 5: Počet mikropartikulí stanovený na základě průtokové cytometrie

SAP stabilní angina pectoris; NAP nestabilní angina pectoris; AIM akutní infarkt myokardu; (* $p<0.05$ v porovnání s kontrolní skupinou).

4. Souhrn a závěr

Na počátku práce jsme postulovali celkem čtyři hypotézy:

1. *Hladina tkáňového faktoru stanovená na základě ELISA metody nereflektuje (vzhledem k jeho mnohočetným biologickým úlohám) specifickou trombogenní aktivitu vyjádřenou při akutním koronárním syndromu.*

Dle našich výsledků se koncentrace neliší. Celkem bylo vyšetřeno 100 vzorků (60 v první části a 40 v druhé části) a neprokázali jsme statisticky významné rozdíly. Výsledky známé z literatury jsou heterogenní, v některých byly rozdíly nalezeny, v jiných nikoliv. Domníváme se, že ELISA metoda není vhodná ke stanovení koncentrace tkáňového faktoru z hlediska jeho trombogenního potenciálu.

2. *Vzhledem k nespecifičnosti ELISA metody nelze nalézt patofyziologicky zdůvodnitelné korelace mezi koncentracemi jednotlivých selektinů a koncentrací cytoadhezivní molekuly ICAM-1 stanovené touto metodou u nemocných se stabilní anginou pectoris a u nemocných s akutním koronárním syndromem.*

Naše výsledky nepotvrdily, že by existovaly vztahy mezi koncentrací selektinů, koncentrací cytoadhezivní molekuly ICAM-1 a klinickou manifestací akutního koronárního syndromu. Byť z diskuze a literárních údajů jsme očekávali, že hladiny P-selektinu při akutním koronárním syndromu budou stoupat, v naší práci jsme tento nález nepotvrdili.

3. *Hladiny tkáňového faktoru stanovené na základě jeho aktivity, která koreluje specificky s jeho trombotickým potenciálem, koresponduje s vyjádřeným klinickými parametry.*

Potvrzení této hypotézy je základním poselstvím celé práce. Aktivita tkáňového faktoru založená na jeho schopnosti aktivovat faktor X na faktor Xa v plazmě u akutních koronárních syndromů klesá, což je nový poznatek, dosud v literatuře nepublikovaný. Tento nález je konzistentní se současnými patofyziologickými poznatky z oblasti výzkumu trombózy a jejího průběhu.

4. *Změna počtu mikropartikulí, které jsou zdrojem aktivního tkáňového faktoru v procesu aterotrombózy, koresponduje se změnami v jeho aktivitě.*

Počet mikropartikulí jako zdroje tkáňového faktoru, stanovený průtokovou cytometrií, se konzistentně snižoval se snižující se aktivitou tkáňového faktoru v plazmě v závislosti na tíži klinické manifestace akutního koronárního syndromu.

Závěrem lze shrnout, že byly nalezeny statisticky významné rozdíly v aktivitě tkáňového faktoru u nemocných s akutním koronárním syndromem konzistentně doprovázené změnami v počtu mikropartikulí, jako jeho zdroje. ELISA metoda u stejného vzorku nemocných neprokázala změny v koncentraci antigenu tkáňového faktoru. Aktivita tkáňového faktoru s tíží klinického obrazu v plazmě klesala, stejně tak počet mikropartikulí, patrně v souvislosti s inkorporací do vznikajícího trombu.

5. Poděkování

Děkuji profesoru MUDr. Janu Vojáčkovi, DrSc. a profesoru RNDr. Václavu Hamplovi, DrSc., kteří byli mými školiteli za odborné a metodické vedení. Děkuji profesoru MUDr. Janu Hergetovi, DrSc. za podporu a pomoc při konečném sestavování celé práce.

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7. Seznam příloh

Tato práce byla prezentována jako dvě původní práce (příloha 1,2) a jedna rešeršní práce (příloha 3). Kromě toho byly výsledky prezentovány jako posterová sdělení na dvou mezinárodních kardiologických kongresech (Great Wall International Congress of Cardiology 2005, Congress of European Society of Cardiology 2007).

Příloha 1:

Tissue factor, Tissue Factor Pathway Inhibitor and Cytoadhesive Molecules in Patients with an Acute Coronary Syndrome.

Příloha 2:

Patients with Acute Coronary Syndromes Have Low Activity of Tissue Factor on Microparticles and Low Count of Microparticles in Circulating Blood.

Příloha 3:

The role of Tissue Factor in thrombosis and haemostasis.

Příloha 4:

Životopis autora.

Příloha 5:

Seznam publikací autora.

Příloha 1:

Maly M, Vojacek J, Hrabos V, Kvasnicka J, Salaj P, Durdil V. Tissue factor, tissue factor pathway inhibitor and cytoadhesive molecules in patients with an acute coronary syndrome. Physiol Res 2003;52(6):719-28.

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Tissue Factor, Tissue Factor Pathway Inhibitor and Cytoadhesive Molecules in Patients with an Acute Coronary Syndrome

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Summary

The tissue factor plays a crucial role in initiating blood coagulation after plaque rupture in patients with acute coronary syndrome. It is abundant in atherosclerotic plaques. Moreover, P-selectin, some cytokines, endotoxin and immune complexes can stimulate monocytes and induce the tissue factor expression on their surface. The aim of the study was to compare plasma levels of the tissue factor, tissue factor pathway inhibitor, P-selectin, E-selectin and ICAM-1 in patients with acute myocardial infarction, unstable angina pectoris, stable coronary artery disease and normal control subjects. In addition, plasma levels of the tissue factor, tissue factor pathway inhibitor, P-selectin, E-selectin and ICAM-1 were measured in the blood withdrawn from the coronary sinus in a subgroup of patients with unstable angina pectoris and stable coronary artery disease in which the difference between concentrations in the coronary sinus and systemic blood was calculated. A significant increase in tissue factor pathway inhibitor plasma levels was detected in patients with acute myocardial infarction (373.3±135.1 ng/ml, $p<0.01$) and unstable angina pectoris (119.6±86.9 ng/ml, $p<0.05$) in contrast to the patients with stable coronary artery disease (46.3±37.5 ng/ml) and normal subjects (45.1±14.3 ng/ml). The plasma levels of tissue factor pathway inhibitor were significantly increased both in the coronary sinus and systemic blood in the patients with unstable angina pectoris. There was only a non-significant trend to higher plasma levels of the tissue factor in patients with acute myocardial infarction and unstable angina pectoris as compared to the patients with stable coronary artery disease and normal subjects, the values being 129.1±30.2 pg/ml, 130.5±57.8 pg/ml, 120.2±45.1 pg/ml and 124.9±31.8 pg/ml, respectively. Plasma levels of soluble P-selectin was only slightly, but non-significantly higher in patients with unstable angina pectoris and stable coronary artery disease (184.2±85.4 ng/ml and 201.6±67.9 ng/ml, respectively) than in patients with the acute myocardial infarction (157.4±88.4 ng/ml) or normal subjects (151.4±47.1 ng/ml). The difference in plasma levels of soluble ICAM-1 between the blood withdrawn from the coronary sinus and systemic circulation correlated significantly with the corresponding difference in plasma levels of soluble P-selectin and E-selectin. In conclusion, the tissue factor and the tissue factor pathway inhibitor play a crucial role in the initiation of arterial thrombosis. The tissue factor pathway inhibitor levels are increased both in the systemic blood and in the coronary sinus of patients with the acute coronary syndrome.

Key words
Tissue factor • Tissue factor pathway inhibitor • Cytoadhesive molecules • Acute coronary syndrome

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Introduction

The tissue factor plays a crucial role in the initiation of blood coagulation after the plaque rupture in patients with acute coronary syndromes (Rapaport and Rao 1995). It was well documented that tissue factor is abundant in atherosclerotic plaques and its contact with circulating blood after the plaque rupture leads to activation of the coagulation cascade (Toschi *et al.* 1997). Moreover, P-selectin, some cytokines, endotoxin and immune complexes can stimulate monocytes and induce the tissue factor expression on their surface (Celi *et al.* 1994).

The aim of our study was to measure the plasma levels of the tissue factor, tissue factor pathway inhibitor, soluble P-selectin, soluble E-selectin and soluble ICAM-1 in patients with acute myocardial infarction and unstable angina pectoris and compare them with those found in a control groups of patients with stable coronary artery disease and normal healthy subjects.

Patients and Methods

Patients.

Patients who underwent coronary angiography as candidates for percutaneous transluminal coronary intervention or coronary bypass surgery were included in the study. There were patients with a ST segment elevation due to acute myocardial infarction lasting less than 12 h in the subgroup A. Subgroup B comprised patients with class IIIb unstable angina pectoris according to Braunwald classification (Braunwald 1989), whereas patients with chronic stable coronary artery disease were in subgroup C.

Moreover, a group of healthy young subjects without history or clinical findings suggesting a presence of heart disease served as controls (subgroup D). Blood sampling was performed in all patients from the peripheral or central vein in the fasting state after at least 30 min at rest.

In addition, samples in subgroup B and C were withdrawn from the coronary sinus by means of a catheter (right coronary Amplatz I, Goodal-Lubin or multipurpose catheter) introduced from the jugular or femoral vein. A blood sample from systemic circulation was obtained at the same time. No patient was treated by unfractionated heparin at the time of sampling.

All patients gave their informed consent and the study was approved by the Hospital Ethical Committee.

Methods

The sampling of coronary sinus and systemic circulation blood preceded the coronary angiography. Blood was collected into a 3.8 % trisodium citrate anticoagulant solution in the proportion of 9:1. The sample was then centrifuged at 3000 rpm for 10 min and the plasma stored frozen.

The plasma level of the tissue factor was determined by means of the IMUBIND® Tissue Factor ELISA Kit (American Diagnostica). The kit employs a murine anti-human tissue factor monoclonal antibody for antigen capture. Plasma samples were incubated in microtest wells precoated with capture antibody. Once captured, the tissue factor was detected using a biotinylated antibody fragment that specifically recognizes the bound tissue factor. The subsequent binding of horseradish peroxidase conjugated streptavidin completes the formation of the antibody-enzyme detection complex. The reaction with added substrate (perborate/3, 3´, 5, 5´-tetramethyl-benzidine) turns blue. The sensitivity is increased by addition of 0.5 M sulfuric acid stop solution turning the color to yellow. Quantitative data were obtained by measuring the solution absorbance at 450 nm and relating it to the standard curve. All assays were performed in duplicate. The plasma levels of the tissue factor pathway inhibitor was measured using IMUBIND® Total Tissue Factor Pathway Inhibitor (TFPI) ELISA Kit (American Diagnostica). This sandwich immunoassay makes it possible to quantitate tissue factor pathway inhibitor in the plasma. It detects both intact and truncated forms of TFPI as well as complexes with the tissue factor (TF) and factor VII (TF/VIIa/TFPI). Binary complexes with factor Xa (TFPI/Xa) and quaternary complexes with tissue factor, factor VIIa and factor Xa (TF/VIIa/TFPI/Xa) are also recognized by this ELISA kit, but with a slightly lower sensitivity. The lower limit of detection for this assay is 0.360 ng TFPI/ml sample. This kit employs a rabbit anti-human tissue factor pathway inhibitor polyclonal antibody as the capture antibody. Its specificity for native complexes and truncated antibody was confirmed by Western blot analysis. Diluted plasma samples were incubated in micro-test wells precoated with the capture antibody. Tissue factor pathway inhibitor is detected using a biotinylated monoclonal antibody specific for the Kunitz domain 1. The subsequent steps were identical with those described above.

Human soluble P-selectin was assessed using Parameter® Human sP-selectin Immunoassay (R&D Systems). This 1.25 hour solid state ELISA measures

soluble P-selectin in cell culture supernatants, serum and plasma. Plasma was collected using citrate as an anticoagulant and within 30 min centrifuged at 1000 *x g*. The kit contains recombinant human soluble P-selectin and antibodies raised against the recombinant factor. This assay employs the quantitative sandwich immunoassay technique.

Similarly, the levels of soluble E-selectin were determined by means of the Parameter® Human sE-selectin Immunoassay (R&D Systems) and the levels of soluble ICAM-1 by means of Parameter® human sICAM-1 Immunoassay (R&D Systems). All assays were performed in duplicate.

Differences between the plasma concentrations in coronary sinus and systemic circulation were calculated for soluble ICAM-1, soluble P-selectin, soluble E-selectin, tissue factor and tissue factor pathway inhibitor. These differences were expressed as values relative to their original concentrations in the systemic blood *(CS – SYST)/SYST* where CS = plasma concentrations in the blood drawn from the coronary sinus and SYST = plasma concentrations in the peripheral blood.

Data were expressed as means ± SEM. Statistical evaluation included the analysis of variance and Student's t test for the assessment of differences of continuous variables. Correlations were analyzed using linear regression. A $p<0.05$ value was considered significant.

Results

Altogether 50 patients and 10 normal subjects were studied. Ten patients (6 males, 4 females, age 62.3±3.4 years) with acute myocardial infarction comprised group A. Group B included 23 patients with unstable angina pectoris (17 males, 6 females, mean age 63.6±8.1 years). Group C consisted of 17 subjects with chronic stable ischemic heart disease (13 males, 4 females, mean age 65.5±9.3 years). The demographic characteristics are shown in Table 1. There were 10 healthy subjects (2 males, 8 females, age 33.8±8.2 years) in the group D.

The plasma levels of tissue factor and tissue factor pathway inhibitor in the studied groups are shown in Figures 1 and 2. The subgroups A and B displayed a significant rise in plasma levels of the tissue factor pathway inhibitor (Fig. 2). The tissue factor pathway inhibitor plasma levels was 373.3±135.1 ng/ml, ($p<0.01$) in patients with acute myocardial infarction, 119.6±86.9 ng/ml, ($p<0.05$) in unstable angina pectoris, 46.3±37.5 ng/ml in patients with stable coronary artery disease and 45.1±14.3 ng/ml in normal subjects. The plasma levels of tissue factor pathway inhibitor were significantly increased both in the coronary sinus and systemic blood in the patients with unstable angina pectoris.

Table 1. Demographic data about the present cohort

n =	**Group A** **10**	**Group B** **23**	**Group C** **17**
Males	6	17	13
Females	4	6	4
Previous myocardial infarction	0	10	9
Angina pectoris			
CCS 1,2	-	-	9
CCS 3	-	-	1
CCS 4	-	-	0
Smokers	6	2	1
Diabetes	1	6	7
Hypertension	3	14	8
Hyperlipoproteinemia	4	6	8
Beta-blockers	3	15	6
Ca^{++} channel blockers	1	3	4
ACE inhibitors	3	6	8
Statins	2	4	2
Antiplatelet drugs	2	18	14
Low molecular weight heparins	0	7	1

There was only a non-significant trend to higher plasma levels of tissue factor in patients with acute myocardial infarction and unstable angina pectoris as compared to the patients with stable coronary artery disease and normal subjects, the values being 129.1±30.2 pg/ml, 130.5±57.8 pg/ml, 120.2±45.1 pg/ml and 124.9±31.8 pg/ml, respectively. Plasma levels of soluble P-selectin was only slightly, but non-significantly higher in patients with unstable angina pectoris and stable coronary artery disease (184.2±85.4 and 201.6±67.9 ng/ml) than in patients with the acute myocardial infarction (157.4±88.4 ng/ml) or normal subjects (151.4±47.1 ng/ml). The plasma levels of soluble E-selectin were 50.2±17.6, 33.2±12.9, 42.0±15.6 and 43.1±11.8 ng/ml, whereas the plasma concentrations of the soluble ICAM-1 were 308.6±68.7, 290.0±72.5, 247.8±54.1 and 303.7±38.6 ng/ml in the subgroup A, B, C and D, respectively.

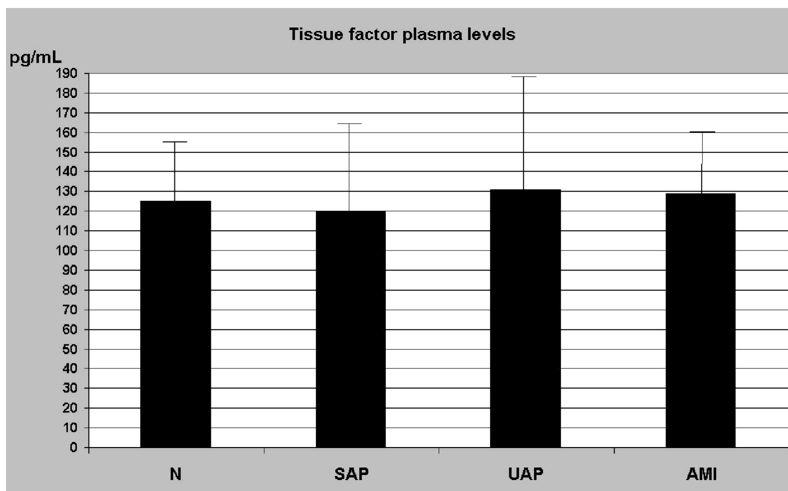

Fig. 1. *Plasma levels of the tissue factor in the studied subgroups (N = normal subjects, SAP = stable coronary artery disease, UAP = unstable angina pectoris, AMI = acute myocardial infarction). Mean values ± SEM..*

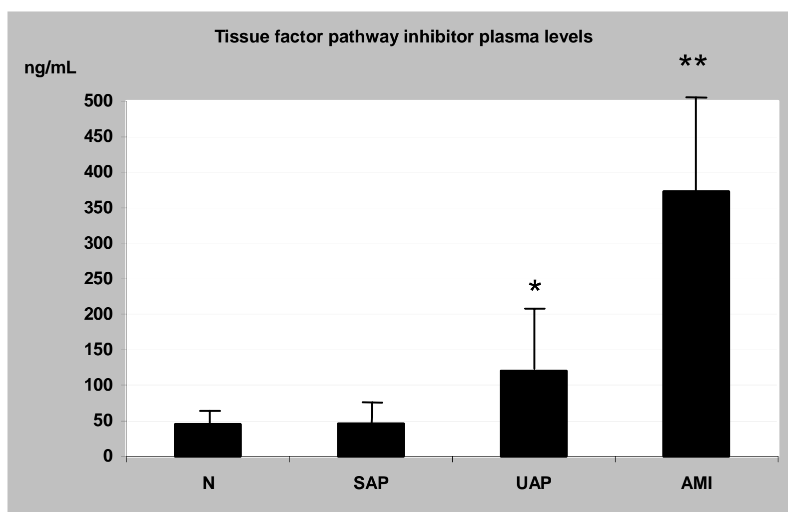

Fig. 2. *Plasma levels of the tissue factor pathway inhibitor in the studied subgroups (N = normal subjects, SAP = stable coronary artery disease, UAP = unstable angina pectoris, AMI = acute myocardial infarction). Mean values ± SEM. * p<0.05; ** p<0.01*

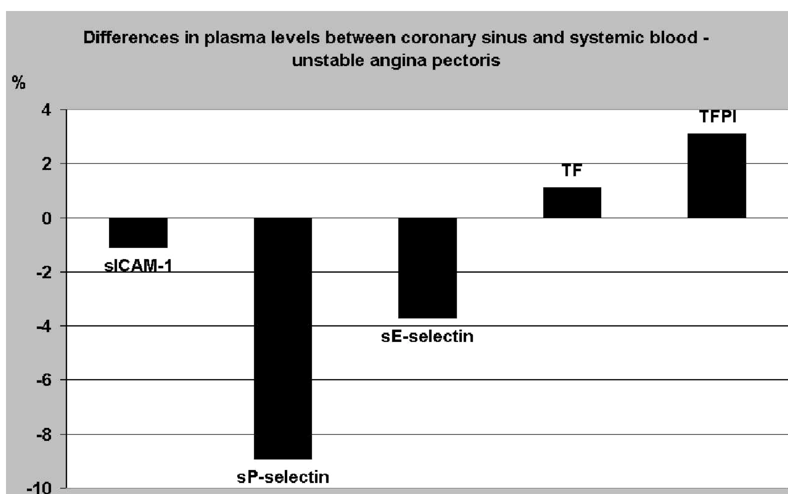

Fig. 3. *Relative differences in the soluble ICAM-1, soluble P-selectin, soluble E-selectin, tissue factor and tissue factor pathway inhibitor between coronary sinus and systemic circulation plasma concentrations in patients with unstable angina pectoris (group B). [TF = tissue factor; TFPI = tissue factor pathway inhibitor; ICAM-1 = intercellular adhesion molecule-1, s = soluble].*

The relative differences in the plasma levels of tissue factor, tissue factor pathway inhibitor, soluble P-selectin, soluble E-selectin and soluble ICAM-1 between coronary sinus and systemic blood plasma concentrations in patients with unstable angina pectoris and stable coronary artery disease are shown in Figures 3 and 4. Non-significant tendency to increase plasma levels of tissue factor, tissue factor pathway inhibitor and

soluble ICAM-1 were noticed in subgroup B, whereas the trend to non-significant decrease in coronary sinus plasma concentration of soluble P-selectin was present in subgroup C. The coronary sinus – systemic blood differences in the plasma levels of the soluble ICAM-1 were positively related to those of soluble E- and P-selectin (Figs. 5 and 6). The tissue factor levels in coronary sinus displayed a trend to an inverse relation to the levels of soluble P-selectin in coronary sinus (Fig. 7). The correlation was, however, non-significant.

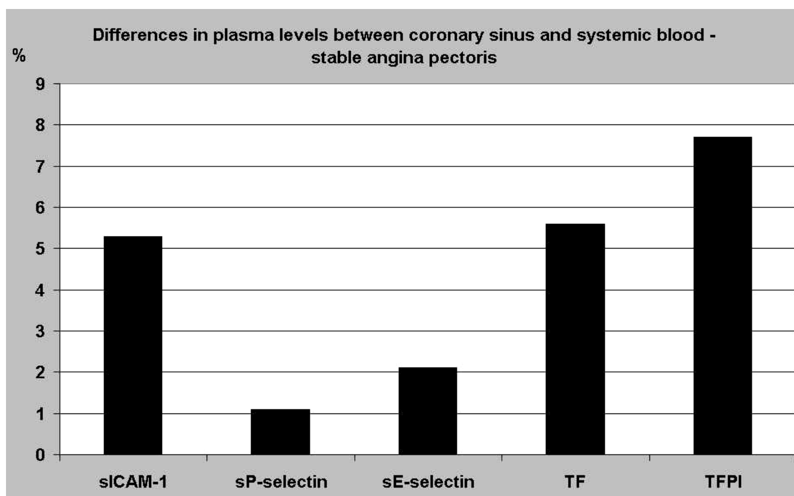

Fig. 4. *Relative differences in the soluble ICAM-1, soluble P-selectin, soluble E-selectin, tissue factor and tissue factor pathway inhibitor between coronary sinus and systemic circulation plasma concentrations in patients with stable coronary artery disease (group C).*

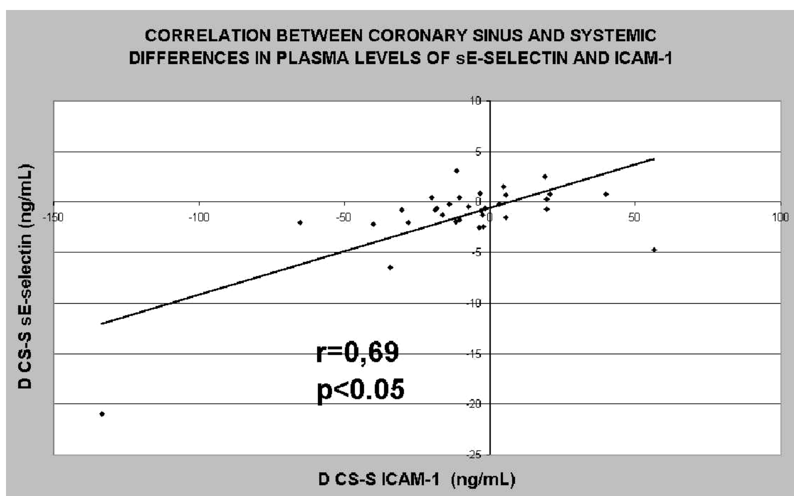

Fig. 5. *Correlation between soluble ICAM-1 and soluble E-selectin differences between coronary sinus and systemic blood concentrations. [D CS – S = differences in plasma levels between coronary sinus and systemic blood in ng/ml].*

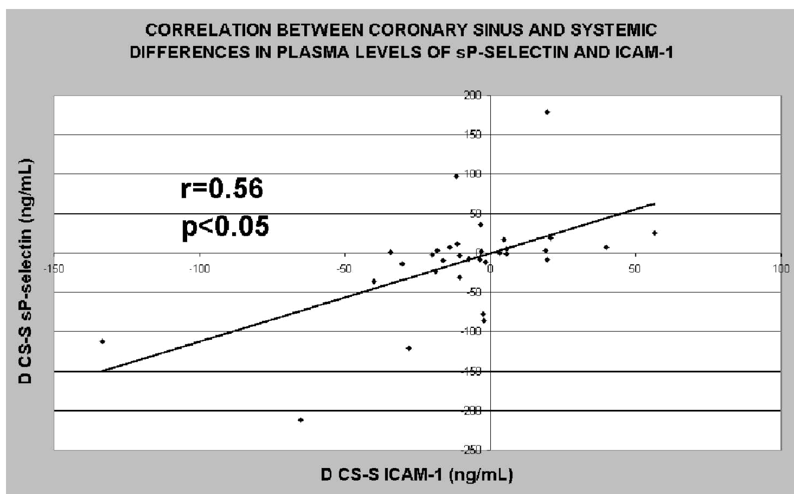

Fig. 6. *Correlation between soluble ICAM-1 and soluble P-selectin differences between coronary sinus and systemic blood concentrations.*

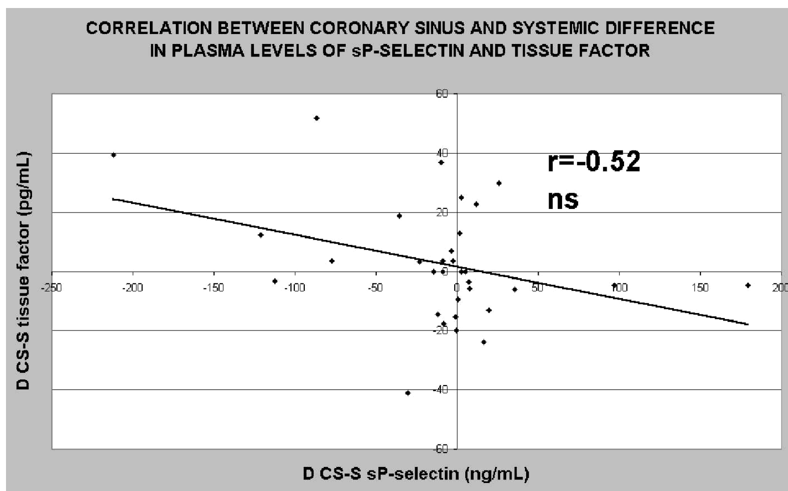

Fig. 7. *Correlation between tissue factor and soluble P-selectin differences between coronary sinus and systemic blood concentrations. [D CS – S tissue factor = difference in plasma levels of the tissue factor between coronary sinus and systemic blood in pg/ml; D CS – S sP-selectin = difference in plasma levels of the soluble P-selectin between coronary sinus and systemic blood in ng/ml; ns = non-significant].*

Discussion

The tissue factor is a membrane protein spontaneously expressed in many cells. Its release into circulating blood is responsible for the activation of coagulation cascade (Rapaport and Rao 1995). The tissue factor is present in the subendothelium, and any damage to the endothelial layer in experimental animal models triggers thrombus formation (Weiss *et al.* 1994). Although monocytes and endothelial cells do not produce tissue factor under normal conditions, expression of the tissue factor on the surface of these cells can be upregulated under certain circumstances. P-selectin, some cytokines, endotoxin and immune complexes can stimulate monocytes and induce tissue factor expression on their surface (Celi *et al.* 1994). Thus, the systemic activation of blood coagulation in patients suffering from disseminated intravascular coagulation is almost entirely attributable to the elevated production of tissue factor (Levi and Ten Cate 1999). Peripheral blood monocytes express tissue factor when stimulated, and together with the tissue factor of monocyte-derived macrophages in arterial plaques (Wilcox *et al.* 1989) may provide the primary source of tissue factor in patients with coronary artery disease. Fibroblasts and smooth muscle cells within the vessel wall constitutively express tissue factor and may serve as an additional source (Iakhiaev *et al.* 1999). Atherosclerotic plaques contain a substantial amount of tissue factor. When exposed to circulating blood after the plaque rupture it binds to factor VIIa, a vitamin K-dependent enzyme, which circulates in low concentration in the blood (Toschi *et al.* 1997). The catalytic subunit – factor VIIa is a soluble plasma protease that forms the first step in the blood clotting cascade together with the regulatory subunit cell – surface integral membrane protein – tissue factor (Morrissey *et al.* 1997). This complex of factor VIIa and tissue factor subsequently activates factor IX and factor X and thus initiates the fibrin formation pathway.

The tissue factor/factor VIIa complex catalyzed activation of factor IX can be inhibited by a complex formed by tissue factor pathway inhibitor and factor Xa (Lindhout *et al.* 1995, Camici and Sagripanti 1999). Successive steps involved in this process are the following: 1) formation of the factor VIIa/tissue factor complex; 2) activation of factor Xa; 3) formation of the TFPI/factor Xa complex; 4) inactivation of tissue factor/factor VIIa complex by the TFPI-factor Xa complex; 5) formation of the reversible quaternary complex consisting of tissue factor, factor VIIa, TFPI and factor Xa (Camici and Sagripanti 1999).

Tissue factor production by activated circulating blood particles (Giesen *et al.* 1999) promotes thrombi formation in microcirculation of patients with acute coronary syndrome. Tissue factor is the key initiator of monocyte-mediated coagulation and monocyte-platelet interaction. Monocytes stimulated with lipopolysaccharide express tissue factor and elicit a pronounced fibrin deposition and platelet-thrombus formation (Barstad *et al.* 1995). Plasma markers of coagulation (fibrinopeptide A) and platelet activation (β-thromboglobulin) showed a significant increase after lipopolysaccharide stimulation. This can be almost completely blocked by anti-tissue factor monoclonal antibody (Barstad *et al.* 1995). Elevated plasma levels of tissue factor and large amounts of monocyte procoagulant

activity have been documented in patients with unstable angina pectoris (Gori *et al.* 1999). Heparin is able to blunt the production of tissue factor by monocyte *in vitro*. Heparin treatment is associated with a decrease in tissue factor plasma levels as well as monocyte procoagulant activity in patients with unstable angina pectoris (Gori *et al.* 1999).

Factor X activation that occurs in response to tissue factor/factor VIIa complex is abolished in the presence of heparin and this effect requires both antithrombin and tissue factor pathway inhibitor. Antithrombin in the presence of heparin blocks the activation of the tissue factor/factor VII complex, whereas tissue factor pathway inhibitor inhibits the tissue factor/factor VIIa complex that is generated (Jesty *et al.* 1996). Several tissue factor/factor VIIa inhibitors are being developed, including the protein-based inhibitors such as NAPc2, Corsevin M, FFR-FVIIa, and Tifacogin (Girard and Nicholson 2001).

The tissue factor pathway inhibitor is now recognized as a major physiological anticoagulant that acts as a natural tissue factor/factor VIIa inhibitor (Lindahl 1997). Immunodepletion of tissue factor pathway inhibitor lowers the threshold by which tissue factor induces disseminated intravascular coagulation (Sandset 1996). Infusion of a recombinant tissue factor pathway inhibitor was found to protect against thrombosis and disseminated intravascular coagulation in different experimental models (Sandset 1996).

Human tissue factor pathway inhibitor is a plasma protease inhibitor that consists of three tandem Kunitz-type inhibitor domains. The first and second Kunitz-type domains are involved in the inhibition of factor VIIa/tissue factor complex and factor Xa on the cell surface, respectively (Hamamoto *et al.* 1993). Tissue factor pathway inhibitor has a dual inhibitory function it inhibits the complex factor VIIa/ tissue factor and directly inhibits factor Xa by binding at or near its active serine site. Tissue factor pathway inhibitor promotes the binding of Xa to monocytes (Li *et al.* 2001). The recovery of Xa activity from Xa/TFPI complex may be related to the cleavage of tissue factor pathway inhibitor by monocyte proteases. This process may add to monocytes procoagulant activity, apart from tissue factor expression on their surface (Salemink *et al.* 1998).

Tissue factor pathway inhibitor circulates in blood in several forms, mostly as a complex with LDL-HDL-VLDL. Approximately 10 % of tissue factor pathway inhibitor are carried by platelets, and are released once the platelets are activated by thrombin. Consequently, an elevated level of tissue factor pathway inhibitor is present at the site of platelet aggregation. Heterozygous deficiency of tissue factor pathway inhibitor in mice was associated with a greater atherosclerotic burden involving the carotid and common iliac arteries and shortened the time before an occlusive thrombosis occurred after photochemical atherosclerotic plaque injury (Westrick *et al.* 2001). Continuous intravenous infusion of unfractionated heparin increased the levels of tissue factor pathway inhibitor more than twofold (Brown and Kuter 2001). Its level remained high during the infusion, but returned to baseline soon after the infusion had been stopped. On the other hand, therapeutic doses of low molecular weight heparin resulted in significantly weaker tissue factor pathway inhibitor induction. Tissue factor pathway inhibitor is released from the vascular endothelium after an injection of heparin and concentrates at sites of tissue damage and ongoing thrombosis. The cessation of a treatment with unfractionated heparin, but not low molecular weight heparin, given in therapeutic doses was associated with a progressive depletion of tissue factor pathway inhibitor (Sandset *et al.* 2000). This phenomenon might be responsible for the observed rebound activation of coagulation after withdrawal of unfractionated heparin. Partial depletion of intravascular pools of tissue factor pathway inhibitor during repeated or continuous intravenous infusion of heparin in man has been reported (Hansen *et al.* 1996). This might explain the attenuation of the tissue factor pathway inhibitor contribution to the antithrombotic effect of heparin. Subnormal levels of tissue factor pathway inhibitor increase the risk of disseminated intravascular coagulation in septic patients, and the risk of occlusive thrombi over damaged vascular intima or fissured atherosclerotic plaques (Abilgaard 1995). Elevated plasma levels of tissue factor pathway inhibitor have been documented in patients with unstable angina pectoris (Gori *et al.* 1999).

In the present study, the tissue factor plasma level increased only marginally in patients with acute coronary syndrome and there was no further rise even in the blood withdrawn from coronary sinus. Nevertheless, the increased plasma levels of another marker of ongoing thrombosis, the tissue factor pathway inhibitor, were found both in patients with the acute myocardial infarction and unstable angina pectoris. The increase was detected in both the peripheral blood and the blood withdrawn from the coronary sinus. Plaque thrombosis is the cause of the acute coronary syndrome, and presumably blood-borne rather than plaque-derived tissue

factor is responsible for the detected elevation in our patients. On the other hand, while no changes in tissue factor coronary sinus plasma level were noticed in patients with stable ischemic heart disease, slightly elevated values of soluble P-selectin were detected in this subgroup. This suggests that some platelet activation takes place in both stable and unstable coronary artery disease, but tissue factor pathway inhibitor upregulation in coronary vascular bed only dominates in patients with acute coronary syndrome. The changes in the level of soluble ICAM-1 were related to the changes of soluble P- and E-selectin, but the changes of soluble P-selectin were only marginally and inversely related to the changes in tissue factor. Detectable circulating soluble form represents only a portion of native P-selectin. Because it lacks the transmembrane anchoring domain, it is conceivable that a consumption of the soluble form of P-selection takes place while the tissue factor on monocytes is being upregulated in patients with an acute coronary syndrome.

The role of adhesion molecules is to mediate interactions of cells with extracellular matrix or with other cells. The immunoglobulin superfamily of proteins contains a large class of adhesion molecules with multiple immunoglobulin-like domains. Adhesion of monocytes and neutrophils to endothelial cells is probably one of the first steps in the pathway leading to plaque rupture and acute coronary thrombosis with clinical manifestation of an acute coronary syndrome. Apart from plaque rupture, an interaction between activated platelets, monocytes and neutrophil leukocytes is responsible for the growth of the thrombus and for possible propagation of the thrombus into the microvasculature. Soluble isoforms of these adhesion molecules believed to be shed from the surface of activated cells can now be quantified in peripheral blood (Gearing and Newman 1993).

P-selectin is a cell surface glycoprotein that plays a critical role in the migration of lymphocytes into tissues. It is found constitutively in a preformed state in the Weibel-Palade bodies of endothelial cells and in α-granules of platelets. This stored P-selectin is mobilized to the cells surface within minutes in response to a variety of inflammatory and trombogenic agents. The mobilized P-selectin is apparently present on the cell surface for only a few minutes after which it is recycled to intracellular space. P-selectin also binds monocytes and neutrophils to activated platelets and is responsible for incorporation of leukocytes into the growing thrombus. Circulating soluble P-selectin has a smaller molecule than native P-selectin, because it lacks the transmembrane anchoring domain. ICAM-1 also appears either in the form of a transmembrane protein (mICAM-1) or in circulating soluble form (sICAM-1). Up-regulation of ICAM-1 expression is initiated by inflammatory cytokines (TNF-α, IFN-γ, IL-1), whilst down-regulation is mediated by anti-inflammatory agents. The soluble form arises either from proteolytic cleavage of mICAM-1 or may be synthesized *de novo* from alternatively spliced mRNA. E-selectin (Endothelial Leukocyte Adhesion Molecule-1) is a transmembrane glycoprotein expressed on endothelial cells after activation by some inflammatory cytokines (IL-1β, TNF-α) and by endotoxin. The expression is reaching a transitory maximum within 6 h after stimulation. The decline coincides with the shedding of its soluble form. The transmembranous form of E-selectin is a mediator of rolling movements of leukocytes and their attachment to the endothelium leading to a migration of leukocytes to the site of inflammation.

Platelet activation with elevated levels of P-selectin has been documented in patients with unstable angina pectoris (Singh *et al.* 1995). Plasma levels of soluble ICAM-1 and soluble P-selectin were also significantly higher in blood withdrawn from coronary sinus immediately after coronary angioplasty (Inoue *et al.* 1999). The increase persisted for 48 h with maxima at 48 h for ICAM-1 and at 24 h for P-selectin. None of these changes was observed in peripheral blood samples. The plasma levels of the soluble E-selectin did not change during 48 h after coronary angioplasty in blood samples taken from coronary sinus. Increased levels of some inflammatory markers including sICAM have been identified as risk factors for the development of the acute coronary syndrome (O'Malley *et al.* 2001). Soluble ICAM-1 monoclonal antibody has been shown to limit infarct size and reduce reperfusion injury (Simpson *et al.* 1988, Yamazaki *et al.* 1993).

In our study, no significant changes in soluble P-selectin, E-selectin or soluble ICAM-1 levels were found in patients with acute coronary syndrome. There were no changes of plasma levels of soluble E-selectin and ICAM-1 in the coronary sinus and only slight decrease of P-selectin in coronary sinus was detected. The decrease in soluble P-selectin in the coronary sinus showed a non-significant correlation with the increase of coronary sinus tissue factor plasma levels.

Tissue factor and its inhibitor play an important role in the pathogenesis of the acute coronary syndrome. Our results suggest that they act not only

locally but also as circulating elements. There were no significant differences in the local concentrations of tissue factor, soluble P-selectin, E-selectin or ICAM-1 in the coronary sinus and systemic blood in our patients. However, the plasma levels of tissue factor pathway inhibitor were significantly increased both in the coronary sinus and systemic blood in the patients with the acute coronary syndrome. As tissue factor/factor VIIa blockers are being developed and the role of the tissue factor pathway inhibitor in the therapeutic effects of heparin was recently recognized, the crucial role of the tissue factor in the development of arterial thrombosis might be not only theoretical in the near future, but we can expect also some therapeutic consequences in this respect.

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Reprint requests

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Příloha 2:

(odesláno do tisku)

Maly MA, Tomasov P, Hajek P, Blasko P, Hrachovinova I, Salaj P, Veselka J. Patients with Acute Coronary Syndromes Have Low Activity of Tissue Factor on Microparticles and Low Count of Microparticles in Circulating Blood.

Patients with Acute Coronary Syndromes Have Low Activity of Tissue Factor on Microparticles and Low Count of Microparticles in Circulating Blood

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Short title: Tissue factor and microparticles in acute coronary syndromes

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Summary:

Tissue factor (TF) is the main initiator of coagulation cascade. The TF activity, microparticles and the TF antigen in acute coronary syndromes patients were tested. Blood was drawn from local (coronary sinus) and systemic (femoral vein) circulation in the cohort of 40 patients. TF activity in stable angina pectoris patients was not significantly different from the control group of patients (18.12±3.35 mOD/min versus 17.72±4.05 mOD/min, respectively) and was significantly lower in the unstable angina pectoris subgroup (7.62± 4.19 mOD/min) and myocardial infraction subgroup (3.56± 3.85 mOD/min) ($p<0.05$). Results in systemic and local circulation were not significantly different. There were not significant differences in concentrations of TF antigen (48.24± 32.76 pg/ml in control subgroup, 41.33± 20.7 pg/ml in stable angina subgroup, 47.44± 25.88 pg/ml in unstable angina subgroup and 59.5± 75.43 pg/ml in myocardial infarction subgroup), neither in local nor systemic circulation. The count of microparticles was decreasing according to the severity of acute coronary syndrome: control 520±172, stable angina pectoris 532±167, unstable angina pectoris 392±142 and myocardial infarction 165±30, which last one was significant compared to control subgroup. These results suggest that the pro-coagulant TF microparticles could be consumed by the ongoing thrombus formation in acute coronary syndromes.

Key words: tissue factor activity, TF antigen, microparticles, acute coronary syndromes.

Introduction

Tissue factor (TF) is considered to be the major regulator of normal hemostasis and thrombosis (Nemerson 1988, Banner *et al.* 1996). The recent evidence highlights the role of the blood-borne pool of TF (Giesen *et al.* 1999). This TF fraction is bound to the procoagulant microparticles. The questionable issue still remains the orchestration of TF, microparticles and its incorporation to the growing arterial thrombus. It is of great interest, because TF there serves as a starter of coagulation cascade. In association with the coronary artery disease the high levels of shed apoptotic microparticles and TF activity were found in extracts from atherosclerotic plaques (Mallat *et al.* 1999, Marmur *et al.* 1996) and the increased levels of microparticles with procoagulant potential were identified in the peripheral circulating blood of patients with acute coronary syndrome (ACS) (Mallat *et al.* 2000). However, the only study of Van der Putten (Van der Putten *et al.* 2005), was focused on the TF activity on microparticles in the patients with ACS. These mentioned facts reflect the difficulties in the measuring this activity directly in the plasma, mainly in the presence of its natural plasma inhibitor – tissue factor pathway inhibitor (TFPI).

Interestingly, inconsistent results of studies dealing with coronary artery disease patients are usually based on the measurement of TF antigen, which could be a source of this inconsistency (Soejima *et al.* 1999, Maly *et al.* 2003, Van der Putten *et al.* 2005, Ikonomidis *et al.* 2005). This study was designed to find any relations between plasma TF activity on microparticles and TF antigen in local and systemic circulation, both in patients presented with acute coronary syndromes and stable angina pectoris. The count of microparticles was determined all at once.

Materials and Methods

Study population and blood sampling

Forty prospectively selected patients (22 males, 18 females, age 64.5 ±10.7 years) undergoing coronary angiography in our institution were enrolled. Cardiovascular risk factors (diabetes, hypertension, smoking, hypercholesterolemia) were determined. We sampled the blood from local (coronary sinus) and systemic (femoral vein) circulation in four matched subgroups of patients – with 1) no significant coronary artery disease defined as normal coronary angiogram and no symptoms of angina pectoris (control), with 2) stable angina pectoris (SAP), 3) unstable angina pectoris – Braunwald class III (UAP) and with 4) with myocardial infarction with ST elevations (MI) undergoing primary percutaneus coronary intervention (PCI). Blood was sampled from local circulation (coronary sinus) using Amplatz coronary catheter (AL1, F4, Cordis Corporation, FL, USA) and from systemic circulation with sheath inserted in femoral vein just before coronary angiography. All coronary patients were receiving standard antithrombotic therapy within 1 hour before blood sampling, i.e. low-molecular weight heparin in subgroup presented with UAP and bolus of unfractioned heparin in the subgroup presented with MI undergoing primary PCI. Patients were receiving aspirin 100 mg daily and were not pre-treated with clopidogrel. There was no use of GP IIb/IIIa blockers. PCI was performed in 6 patients (60%) in the subgroup of SAP. Seven patients (70%) of the subgroup of UAP were treated with PCI and 3 patients (30%) of this subgroup underwent surgical revascularization. There were 10 patients (100%) treated with primary PCI in the subgroup of MI patients. In the control group 8 patients (80%) underwent coronary angiography due to chest pain which was not confirmed to be of ischemic origin, 1 patient (10%) had severe aortic stenosis and 1 patient (10%) had pulmonary hypertension. The

hospital institutional review board approved this study. All patients provided their informed consent prior to study participation.

Plasma Preparation and Microparticles Isolation

All samples were prepared and assayed without knowledge of belonging under subgroups. Volume of 10 ml citrated blood (3.2%) sampled from local and systemic circulation was within 30 min centrifuged 15 minutes at 2,000xg and collected plasma was centrifuged again 15 minutes at 2,000xg to avoid the presence of platelets in plasma. Samples were stored at –75°C in aliquots until microparticles were prepared. Washed microparticles were prepared by centrifugation of plasma diluted 1:10 in a HEPES-buffer (20mmol/l HEPES, 1mmol/l EDTA, pH 7.4) for 1 hour at 200,000xg , resuspended in a HEPES-buffer and centrifuged again for 1 hour at 200,000xg. Pelleted microparticles were resuspended in a buffer (10mmol/l HEPES, 5mmol/l KCl, 1mmol/l MgCl2, 136mmol/l NaCl, pH 7.4) in 1/3 of the initial volume of plasma.

TF activity, TF antigen and the count of microparticles

The activity of TF was measured on washed microparticles. The TF activity was evaluated by its ability to activate factor X (150 nM) by factor VIIa (5 nM) in the presence of 1 mM $CaCl_2$ by spectrophotometric method. A chromogenic substrate of factor Xa, Spectrozyme FXa, was added (0.3 mM; American Diagnostica, CT, USA). The linear changes in absorbance at 405 nm were recorded with an ELISA plate reader. The changes in mOD/min directly correlated with the amount of FXa generated (Hrachovinova *et al.* 2003). The TF concentration was evaluated in aliquoted stored plasma samples with ELISA kit (IMUBIND Tissue Factor ELISA, American Diagnostica, CT, USA). The number of

microparticles was analyzed by flow cytometry. Forty µl of plasma sample was diluted with 260µl PBS and analyzed for 10 s (Andre *et al.* 2000).

Statistical analysis

The repeated measures ANOVA model with group, subject and type of matrix as factors and group vs. matrix interaction followed by Bonferroni multiple comparisons was used for evaluation of differences between groups (control, SAP, UAP, MI) subjects and types of matrix (local circulation, systemic circulation). The results are expressed as mean of values. Statistical significance was assumed at the level $p< 0.05$. Statistical software Statgraphics Plus version 5.1 form Manugistics (Rockville, MD, USA) and NCSS (Kaysville, UT, USA) was used for the analysis.

Results

Demographic data show, that there were no significant differences in age, hypercholesterolemia, diabetes mellitus, hypertension and smoking. The data are shown in Table 1.

TF acitivity in local and systemic circulation

We found significantly lower levels of TF activity on microparticles in patients presented with ACS and no statistically significant differences in the TF activity in local and systemic circulation. In the local circulation the TF activity on microparticles in patients presented with a diagnosis SAP was not significantly different from control group patients (18.12±3.35 mOD/min versus 17.72±4.05 mOD/min, respectively). However, the TF activity

was significantly lower in UAP subgroup (7.62± 4.19 mOD/min) and MI subgroup (3.56± 3.85 mOD/min) compared with control subgroup ($p<0.05$) and SAP subgroup ($p<0.05$). In the patients with an ACS, a significant difference ($p<0.05$) was found in the subgroups UAP and MI (Figure 1). In systemic circulation the TF activity 17.22± 2.91 mOD/min in control group was not statistically different from 17.52± 2.41 mOD/min in SAP subgroup. The TF activity was significantly lower in UAP subgroup (8.28± 4.75 mOD/min) and MI subgroup (2.55± 3.56 mOD/min) compared with control subgroup ($p<0.05$) and SAP subgroup ($p<0.05$). There were significant differences in TF activity in the patients in UAP versus MI subgroups ($p<0.05$).

TF antigen in local and systemic circulation

The level of TF antigen was not significantly different among all four subgroups of patients. In systemic circulation the concentrations of TF were 48.24± 32.76 pg/ml in control subgroup, 41.33± 20.7 pg/ml in SAP subgroup, 47.44± 25.88 pg/ml in UAP subgroup and 59.5± 75.43 pg/ml in MI subgroup. There were no statistically significant differences in local and systemic circulation in all four subgroups.

The count of microparticles in plasma

Plasma microparticles were analyzed in FSC and SSC scale, the gate analysis was used to quantify the large microparticles previously found to be linked up to pro-coagulant activity. Plasma from MI patients presented 3-fold decrease of microparticles compared to control and SAP plasma samples. The results are shown in Table 2.

Discussion

The present study demonstrates five times less TF activity in circulating blood in patients with ACS than SAP, which correlates with 3-fold decrease of microparticles in patients presented with MI. These results seem to be in accordance with the current concept of cell-based coagulation which provides a new insight into the process of arterial thrombosis (Giesen *et al.* 1999, Falati *et al.* 2003). The model assumes that the active TF bound to microparticles, is consumed from circulating blood by binding on the surface of activated platelets, where it forms highly procoagulant thrombogenic place.

We further report that unlike TF activity, TF antigen does not show significant differences when compared its concentration in the plasma of the patients presented with SAP and the patients presented with ACS. The potential explanation is based on the presence of various types of TF molecule in circulation (active and non-active) (Soejima *et al.* 1999) which all can be detected with antibodies used in commercial immunoassay.

The next challenging issue is the heparin induced release of TFPI since all the ACS patients are generally treated with heparin. This TFPI release can mitigate the TF consumption. It is established that TFPI free or associated with microparticles could naturally inhibit the TF activity. Nevertheless, it was reported that this inhibition reached most about 50% (Steppich *et al.* 2005). Since we report five times less TF activity in ACS compared with SAP, it seems that TFPI inhibition of TF is only of partial influence.

Further investigation is to be focused on the identification of the specific subpopulations of microparticles. In the recent study (Leroyer *et al.* 2007). was demonstrated, that there is a difference in the origin of the microparticles in the atherosclerotic plaque (originates mainly from the smooth muscle cells) and in the blood (originates mainly from the thrombocytes). It would be of great interest to proof the decrease of these specific subpopulations of microparticles in acute coronary syndrome patients. The second point of

further investigation is, as the lowering of TF activity correlated with a clinical severity of ACS, to investigate the clinical consequences. Especially a blocking of TF positive specific microparticles in growing thrombus could bring a clinical significance.

The limitations of our study are absence of the identification of various subtypes of microparticles. Also the levels of TFPI in matched subgroups will be of interest to proof the mentioned marginal influence. Despite presented results are conclusive, the limited number of patients do not allow to generalize the concept of incorporation of the microparticles bearing TF to growing thrombus, which is the pathophysiologic substrate of acute coronary syndrome.

Our results suggest, despite mentioned limitations, that the pro-coagulant TF microparticles could be consumed from circulating by ongoing thrombus formation in acute coronary syndromes.

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Table 1: **Characteristics of study subgroups**

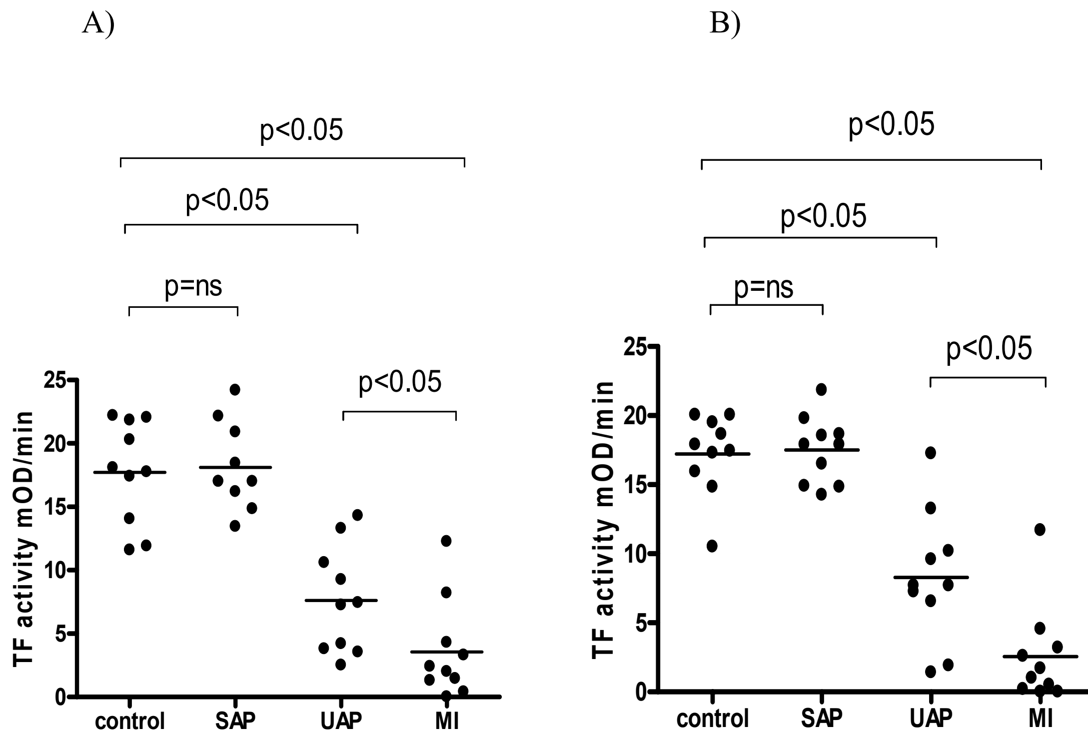
Variable	Control (n=10)	SAP (n=10)	UAP (n=10)	MI (n=10)
Age	61.7±10.4	65.2±2.84	66.1±4.18	65.5±3.9
Men/Women	4/6	7/3	5/5	6/4
Diabetes	1	4	4	1
Smoking	1	1	2	6
Hypercholesterolemia	4	8	4	3
Hypertension	7	8	9	6

Table 2: **The count of the microparticles in plasma**

Subgroup	N° Microparticles
Control	520±172
SAP	532±167
UAP	392±142
MI	165±30*

* $p < 0.05$ vs. corresponding control value; SAP, stable angina pectoris; UAP, unstable angina pectoris; MI, myocardial infarction. The results are expressed as a mean of values.

Figure 1: **TF activity in local and systemic circulation**



TF activity in patients with non-coronary heart disease (control) compared with stable angina pectoris (SAP), unstable angina pectoris (UAP) and myocardial infarction with ST elevations (MI) in A) local circulation sampled from coronary sinus; B) systemic circulation sampled from femoral vein.

Příloha 3:

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Physiological Research Pre-Press Article

The role of Tissue Factor in thrombosis and haemostasis.

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Short title:

The role of Tissue Factor in thrombosis and haemostasis.

Summary:

The tissue factor (TF) is one of the most important regulators of the arterial thrombosis. Because arterial thrombosis is the pathophysiologic background of acute coronary syndrome, the possible impact of blocking the arterial thrombosis on its beginning is a challenging question. The investigations TF brought a new concept of “cell based coagulation model” with highlighted question of the blood-borne TF as a source of TF in circulating blood. In this review are summarized essentials of pathophysiology, molecular structure, expression and distribution of TF, novel concept of blood borne TF and finally the possibilities of the inhibition of coagulation cascade with newly synthetized drugs.

Introduction.

Tissue factor (TF) is a major regulator of normal hemostasis and thrombosis (Nemerson 1988). TF, a membrane-bound molecule, acts as an essential cofactor with activated factor VIIa to form a complex that cleaves factors IX and X and activating the whole coagulation cascade (Banner *et al.* 1996). Recent investigations suggest the existence of a blood-borne pool of TF that may play a critical role in the propagation of thrombosis (Giesen *et al.*1999). These investigations revealed, that TF bound to procoagulant microparticles, which are present in the circulating blood under pathophysiological conditions, might have specific trombogenic potential (Nieuwland *et al.* 1997). In the concordance high levels of shed apoptotic microparticles and TF activity were found in extracts from atherosclerotic plaques (Malat *et al.* 1999, Marmur *et al.* 1996) and increased levels of microparticles with procoagulant potential were found in the peripheral circulating blood of patients with acute coronary syndrome (ACS) (Mallat *et al.* 2000).

Pathophysiology.

TF (also known as coagulation factor III or tissue thromboplastin) is a transmembrane glycoprotein responsible for the start of blood coagulation. The classical view of blood coagulation as a cascade of the activation of coagulation factors consists of intrinsic and extrinsic pathways. After vascular injury, TF, like the serine protease receptor for the coagulation factor VIIa (FVIIa), binds circulating FVII which then turns by allosteric activation into activated form FVIIa. Besides this activation, TF enhances proteolytic and amidolytic activity of FVIIa and TF/FVIIa complex, which subsequently turns factor X (FX) into FXa. FXa as a prothrombinase complex (FXa/FVa) cleaves prothrombin into thrombin and thrombin then cleaves fibrinogen into fibrin, completing the extrinsic pathway of blood coagulation. TF/FVIIa complex is also capable of turning zymogens FVII and at slower rate also factor IX (FIX) into their active forms FVIIa and FIXa (Ruf 1998). FIXa together with its cofactor VIIIa (FVIIIa) activates FX into FXa as well, thus explaining the role of TF in the intrinsic pathway of blood coagulation. Factor V (FV) and factor VIII (FVIII) are turned into their active forms by thrombin, providing thus a back-activation loop.

Nowadays the look upon blood coagulation is evolving into more complex models unifying the whole process and emphasizing the role of cells and activated platelets. A cell based model involves the series of reactions between the coagulation factors divided into three steps: the 1) initiation, 2) amplification and 3) propagation occurring on two principal cell surfaces: the TF bearing cells and the platelets (Walsh 2004). After the exposure of TF bearing cells to the bloodstream, TF/FVIIa complex is formed and FX is turned into FXa. However, the process is rapidly stopped by an endogenous inhibitor – tissue factor pathway inhibitor (TFPI) which forms an inactive quaternary TF/FVIIa/FXa/TFPI complex. By this way only small amounts of FXa are produced and only traces of thrombin are generated,

incapable to cleave enough fibrinogen into fibrin. Nevertheless the generated thrombin is sufficient to activate FV and FVIII, which then serve as cofactors and strongly enhance the production of FXa by FIXa/FVIIIa complex and subsequently the production of thrombin by FXa/FVa complex. Furthermore thrombin activates factor XI (FXI) into FXIa which provides another way of activation of FIX apart from the one by TF/FVIIa complex. This is in concordance with findings that people, deficient in contact factors (FXII, prekallikrein, high molecular weigh kininogen) which were claimed responsible for FXI activation, do not suffer from bleeding, while people deficient in FXI do, proving that there has to be another way of FXI activation. TF seems to serve as a sort of starter to produce minute amounts of thrombin before its pathway is switched off and the process then continues thanks to back-activation loops leading from thrombin to FIX, FVIII and FV (Walsh 2004, Frederick *et al.* 2005).

Molecular structure of TF.

TF is encoded by a 12.4 kb gene organized into six exons separated by five intones located on chromosome 1, at locus 1p22-23. The promoter region exhibits two binding sites for the transcription factor activator protein-1, one κB-binding site, three Egr-a binding sites and five Ap1 sites (Mackman *et al.* 1989). A binding site for nuclear factor of activated T cells was identified as well, overlapping the κB site (Armesilla *et al.* 1999).

TF is constitutively expressed in several cell types such as adventitial fibroblasts, smooth muscle cells and epithelial cells but extensive cell culture studies showed that TF promoter is capable to induce TF expression after stimulation (by LPS, IL-1β, TNF-α) on other cell types as well (e.g. monocytes) (Eilertsen *et al.* 2004).

TF is composed of an extracellular domain consisting of two fibronectin type III repeats (N-terminal domain TF1 and C-terminal domain TF2), a transmembrane domain and a short cytoplasmatic tail. The crystal structure of soluble tissue factor (sTF) showed the

interdomain angle to be about 120° (Harlos *et al.* 1994, Muller *et al.* 1994) and a structural relationship between TF and the cytokine receptors (Bazan *et al.* 1990). The close structural similarity of TF with cytokine receptors explains a receptor function of TF with intracellular signaling capacity. Several studies addressing this aspect of TF function prove the view that TF plays a role in biological actions unrelated to coagulation, such as tumor metastasis and angiogenesis (Rickles *et al.* 2003, Versteeg *et al.* 2003, Bromberg *et al.* 1995, Zhang *et al.* 1994).

Expression of TF.

TF is expressed on cells in the vessel wall (Wilcox *et al.* 1989) and is normally not found on cells that are in direct contact with the bloodstream (Kirchhofer and Banner 1997). Various endogenous (for example, TNF-α) and exogenous agonists (for example, lipopolysacharides) can induce TF expression on endothelial cells and monocytes (Camerer *et al.* 1996). This TF upregulation may play a role in thrombotic disorders. Furthermore, induction of TF expression on smooth muscle cells was observed in experimental restenosis, implicating tissue factor activity in thrombotic complications after angioplasty (Marmur *et al.* 1993, Speidel *et al.* 1995), and also in neointima proliferation (Jang *et al.* 1995). TF expression in atherosclerotic plaques was first described in 1989 (Wilcox *et al.* 1989), and subsequent studies identified extensive TF expression in human atherectomy specimens (Marmur *et al.* 1996, Annex *et al.* 1995), suggesting a function of the TF pathway in the development of coronary artery diseases.

Blood-borne TF.

Several studies show that an additional source of TF, known as blood-borne TF or plasma TF, may also contribute to thrombosis. According to them, in healthy subjects, TF

antigen is present in plasma at a mean level of 149 to172 pg/ml (Koyama *et al.* 1994, Albrecht *et al.* 1996). Some studies have shown that levels of blood-borne TF are increased in various disease states, such as atherosclerosis, sepsis, diabetes, and sickle cell disease (Misumi *et al.* 1998, Soejima *et al.* 1999, Mallat *et al.* 1999, Nieuwland *et al.* 2000, Diamant *et al.* 2002, Semenza *et al.* 1990). Also was shown the correlation between the levels of blood-borne TF and acute myocardial infarction (Nieuwland *et al.* 19974, Misumi *et al.* 1998, Seljeflot *et al.* 2003). In addition, inhibition of TF in a rabbit model of venous thrombosis reduced thrombus propagation (Himber *et al.* 1997).

The role of blood-borne TF was strongly supported by a study measuring rate at which FX in the well-mixed clot supernatant permeates the clot and is converted to FXa on various in vitro prepared platelet-fibrin clots on TF/VIIa-coated surfaces. Paradoxically, the growing thrombus acted as a barrier, restricting the convective and diffusive exchange of substrates and coagulation products between the blood and reactive vessel wall, thus limiting the role TF from the vessel wall plays in thrombus growth. The apparent diffusion coefficients of FX(a) in fibrin and platelet-fibrin clots at 37°C was 2.3×10^{-7} and 5.3×10^{-10} cm2/second, respectively, indicating that the mean time required for FX(a), and likely FIX(a), to diffuse 1 mm in a fibrin clot is 4 hours, and in the presence of platelets, 3.6 months. As complete human thrombotic occlusion has been observed within 10 minutes, an alternative source of procoagulant activity that can localize to the outer surface of growing thrombi (such as platelet factor XI or blood-borne TF) appears to be essential for rapid thrombus growth (Hathcock and Nemerson 2004). One controversial issue is the form of blood-borne TF. Many studies have shown that TF circulates in blood in the form of cell-derived microparticles (Nieuwland *et al.* 1997, Combes *et al.* 1999, Berckmans *et al.* 2001, Sturk-Maquelin *et al.* 2003). Other groups suggest that TF is present in platelets (Muller *et al.* 2003, Camera *et al.* 2003, Siddiqui *et al.* 2002). Finally, it was recently proposed that an alternatively spliced form of soluble TF is the major

form of blood-borne TF (Bogdanov *et al.* 2003). Monocytes were shown to be capable of shedding microparticles exposing TF and phosphatidylserine together with adhesion molecules CD14, CD11a and CD18 after LPS stimulation (Eilertsen *et al.* 2005). It was found that this TF on microparticles became incorporated into spontaneous human thrombi, suggesting that TF may be transferred to platelets by interaction of CD15 with P-selectin exposed on activated platelets (Giesen *et al.* 1999). TF itself played a role of adhesion molecule necessary for this transfer. These findings were supported by the fact that anti-CD15 antibodies abolish 80% of the LPS-induced TF activity in monocytes of cell suspensions recombined with platelet-rich plasma (Halvorsen *et al.* 1993). Furthermore, mice lacking P-selectin or PSGL-1 developed platelet-rich thrombi with minimal TF and fibrin (Falati *et al.* 2003). Leukocyte-platelet interactions associated with the release of TF and subsequently thrombin are suspected to play an important role in various hyperthrombotic states in patients with high LDL cholesterol concentrations, cigarette smoking and diabetes mellitus, emphasizing thus the importance of not only the vulnerable or high-risk atherothrombotic lesions but also the relevance of hyperreactive or vulnerable blood and explaining a possible mechanism of thrombus formation in those cases of acute coronary syndromes that are based on only a superficial erosion of fibrotic plaque and not on a disruption of a lipid-rich plaque exposing the lipid core with high levels of TF .

Some amounts of microparticles with TF probably originate from apoptotic cells. The role of apoptosis in atherothrombosis is important both at the site of the plaque and in the circulation (Viles-Gonzalez *et al.* 2005). Apoptosis within atheroma involves all cell types and it has been demonstrated that it coexists with high levels of TF within the plaque. TF activity is dependent on the presence of phosphatidylserine, exposure of which is associated with apoptosis. The prothrombotic potential of apoptotic cells was shown to be present in the circulation as well when one group found high levels of endothelial membrane microparticles

in the peripheral circulating blood of patients with ACS, inferring that they might participate in the generation and perpetuation of intracoronary thrombi (Mallat *et al.* 2000).

Another significant issue with blood-borne TF is how to measure functional levels. It is clear that levels of blood-borne TF are very low compared with levels in the vessel wall, particularly in healthy subjects (Mackman 2004). In addition, microparticles in the blood have TF-dependent and TF-independent procoagulant activity (Berckmans *et al.* 2001, Aras *et al.* 2004). A recent study described a novel assay that overcomes this problem by measuring TF activity of selectively captured, TF-positive microparticles. This study also showed that elevation of procoagulant microparticles, a subset of which contained TF, restored hemostasis in hemophiliac mice (Hrachovinova *et al.* 2003). Moreover, FVIIa has proven to be a very effective hemostatic agent in hemophiliac patients and in trauma patients (Hedner 2000). The mechanism of action of FVIIa has been proposed to be independent of TF (Hoffman *et al.* 1994). However, the presence of blood-borne TF suggests that this conclusion should be re-evaluated, and it seems possible that the hemostatic protection provided by FVIIa may be, in part, caused by its binding to blood-borne TF (Mackman 2004).

Tissue-specific TF distribution.

TF exhibits a nonuniform tissue distribution with high levels in the brain, lung, and placenta, intermediate levels in the heart, kidney, intestine, uterus, and testes, and low levels in the spleen, thymus, skeletal muscle, and liver (Bach 1988, Mackman *et al.* 1993, Hartzell *et al.* 1989, Faulk *et al.* 1990, Drake *et al.* 1989). Immunohistochemical and in situ hybridization studies demonstrated that high levels of TF were expressed by astrocytes in the brain, alveolar

cells in the lung, trophoblasts in the placenta, epithelial cells surrounding organs and at body surfaces, adventitial fibroblasts surrounding blood vessels, and cardiac myocytes in the heart (Mackman *et al.* 1993, Drake *et al.* 1989, Fleck *et al.* 1990, Eddleston *et al.* 1993, Flossel *et al.* 1994, Luther *et al.* 1996). This cell type-specific distribution suggested that TF provides a "hemostatic envelope" to limit bleeding after vessel injury (Drake *et al.* 1989). The higher levels of TF in the brain, lung, placenta, heart, and uterus would provide additional hemostatic protection to these vital organs. In contrast, tissues that express low levels of TF, such as skeletal muscle and joints, rely on the FVIIIa/FIXa complex of the intrinsic pathway to prevent bleeding. Indeed, this model explains why hemophilia patients deficient in either FVIII or FIX frequently bleed into joints and soft tissues (Bolton-Maggs and Pasi 2003).

TF is constitutively expressed in cardiac myocytes but not in skeletal myocytes (Drake *et al.* 1989). The likely function of TF in the heart is to provide additional hemostatic protection. Low-TF mice had very low levels of TF in their hearts compared with the level of TF in the hearts of wild-type mice, suggesting reduced TF expression in cardiac myocytes. Importantly, low-TF mice and low-FVII mice exhibited hemosiderin deposition and fibrosis in their hearts. It is believed that the hemosiderin is derived from erythrocytes hemorrhaging into the myocardium and phagocyte digestion of the hematin. Indeed, occasional hemorrhages in the hearts of low-TF mice were observed (Pawlinski *et al.* 2002). Taken together, these results suggest that low-TF and low-FVII mice have impaired heart hemostasis. It has been found that over expression of murine TF in the cardiac myocytes abolishes fibrosis in the hearts of low-TF mice. These results indicate that TF expression by cardiac myocytes plays a key role in the heart, most likely by providing additional hemostatic protection to this vital organ that may be prone to mechanical injury of the vessels. In contrast, FIX deficient mice have normal hearts (Mumford and McVey 2004). These results suggest that the TF/FVIIa complex, but not the FVIIIa/FIXa complex, plays a critical role in heart hemostasis.

FIX and other factors of the amplification stage of blood coagulation normally provide an important back-activation loop, enhancing thus the thrombin generation, however some computer generated models showed that high concentrations of TF (for example in the conditions of the sepsis or endotoxemia) provide enough FXa and enough thrombin to generate a fibrin clot without this back-activation loop (Pawlinski and Mackman 2004).

Role of TF in thrombosis.

Aberrant TF expression triggers intravascular thrombosis associated with various diseases, such as atherosclerosis, cancer, and sepsis (Rickles *et al.* 2003, Tremoli *et al.* 1999, Rao 1992, Creasey *et al.* 1993). Importantly, inhibition of TF/FVIIa complex activity reduced coagulation and decreased mortality in animal models of sepsis (Taylor *et al.* 1991, Taylor *et al.* 1998). In atherosclerosis, TF is expressed (apart from other cells) by macrophage-derived foam cells within atherosclerotic plaques (Wilcox *et al.* 1989). These results strongly suggest that high levels of TF exposed upon plaque rupture trigger thrombosis and myocardial infarction. Thus, the classical view of TF is that it is expressed locally within an atherosclerotic lesion. Inhibition of TF would be expected to reduce thrombosis associated with a variety of diseases.

The role of TF and FVII in coronary no-reflow was also studied. The data suggest that active TF is released from dissected coronary atherosclerotic plaque and is one of the factors causing the no-reflow phenomenon (Bonderman *et al.* 2002).

Functional inhibition of TF with an anti-rabbit TF monoclonal antibody in a rabbit coronary ligation model has been shown to improve coronary blood flow after myocardial ischemia/reperfusion (I/R) injury. At-risk areas of myocardium showed increased TF expression in the sarcolemma of cardiomyocytes, which was associated with a low level of

extravascular fibrin deposition. Infarct size was reduced by 61% and 44%, respectively (Erlich *et al.* 2000).

Local inhibition of TF by both recombinant tissue factor pathway inhibitor and a polyclonal antibody against human TF reduces the thrombogenicity of disrupted human atherosclerotic plaques in an in vitro model of human atherosclerotic and normal arterial segments exposed to heparinized blood at flow conditions modeling medium-grade coronary stenosis (Badimon *et al.* 1999). The antithrombotic effects of the specific inhibition of plaque TF was assessed by reduction in the deposition of radiolabeled platelets and fibrinogen and immunohistochemical analysis of perfused arteries.

TF role in embryonic development.

As there are no known TF deficient humans, some groups studied the effect of knocked-out murine TF gene and they all reported high rate of lethality of the embryos (approximately 90%) (Carmeliet *et al.* 1996, Bugge *et al.* 1996). Defect of hemostasis was considered to be the cause of death, but flawed development of the yolk sac vasculature was also noted (Toomey *et al.* 1996). Controversial were findings that FVII deficient embryos survive the embryonic development (Rosen *et al.* 1997). Using a transgenic approach the role of several coagulation factors and PARs (protease-activated receptors) in the embryonic development was studied with the conclusion that the high rate of lethality of TF deficient embryos may be caused by loss of both hemostatic and non hemostatic pathways and an absence of maternal rescue of this transmembrane receptor (Carmeliet *et al.* 1996).

Inhibition of the early steps of the coagulation cascade.

Studying the role of TF in blood coagulation leads to new ideas in anticoagulation therapy. New drugs are expected to be found, with less side effects and stronger anticoagulation potential when acting at the beginning of the coagulation cascade.
The possible influence of TF transcription was reported by a group demonstrating that activation of liver X receptors (LXRs) LXRα and LXRβ suppresses TF expression. Treatment of mouse peritoneal macrophages with synthetic LXR agonist T0901317 or GW3965 reduced TF expression induced by pro-inflammatory stimuli. LXR agonists also suppressed TF expression and its activity in human monocytes. Human and mouse TF promoters contain binding sites for the transcription factors AP-1, NFκB, Egr-1 and Sp1, but no LXR-binding sites could be found. Cotransfection assays with LXR and TF promoter constructs revealed that LXR agonists suppressed LPS-induced TF promoter activity. Analysis of TF promoter also showed that inhibition of TF promoter activity by LXR was at least in part through inhibition of the NFκB signaling pathway. In addition, in vivo, LXR agonists reduced TF expression within aortic lesions in an atherosclerosis mouse model as well as in kidney and lung in mice stimulated with LPS. These findings indicate that activation of LXR results in reduction of TF expression, which may influence atherothrombosis in patients with vascular disease (Terasaka *et al.* 2005).

A lot of effort has been made to inhibit the TF gene expression at the translational level using various approaches involving ribozymes, antisense technologies and RNA interference (Armesilla *et al.* 1999, Zhang *et al.* 1996, Cavusoglu *et al.* 2002). However these

techniques seem to have limited efficiency and need to develop an effective and safe delivery mechanism (Armesilla *et al.* 1999).

Direct blocking of the TF, FVIIa or TF/FVIIa complex activity can be achieved by various inhibitors. One way of blocking the TF mediated start of coagulation is using a specific antibody as in a recently published study in which one group used Sunol-cH36, a chimeric monoclonal antibody to TF, which blocks binding of factor X to the TF/VIIa complex and completed a trial of Sunol-cH36 in humans. The safety and pharmacokinetics of Sunol-cH36 were assessed in an open-label, dose-escalating trial among subjects with stable coronary artery disease. No major bleeding (≥2 g/dl hemoglobin decline) was reported. Spontaneous minor bleeding was observed with a dose-related pattern. The majority of spontaneous bleeding episodes were clinically consistent with platelet-mediated bleeding (e.g. gum, tongue) without thrombocytopenia. Sunol-cH36 exhibited dose-dependent anticoagulant effects and the mucosal bleeding observed with this potent inhibitor of thrombin generation may reflect antiplatelet effects resulting from networking between the coagulation cascade and platelet pathways that could prove clinically relevant with this novel class of anticoagulants (Morrow *et al.* 2005).

A recombinant version of the truncated, extracellular form of TF (hTFAA) has been reported to function as an antagonist of membrane-bound TF as well (Kelley *et al.* 1997). Promising investigation was presented of a selective small-molecule TF inhibitor PHA-927F capable of efficiently inhibiting or preventing acute thrombosis without any increase in risk of bleeding thanks to its great selectivity against TF when compared to other coagulation factor as a result of extensive crystal structure studies of similar molecules (Frederick *et al.* 2005, Suleymanov *et al.* 2003).

A study was published showing the effect of active site-inhibited FVIIa (FVIIai) binding to TF. FVIIai blocks binding of the corresponding enzyme (FVIIa) or zymogen

(FVII) forms of FVII and inhibits coagulation. Although several studies have suggested that FVIIai may have superior anticoagulation effects in vivo a challenge for use of FVIIai is the cost of production. The study reported the properties of dimeric forms of FVIIai that are cross-linked through their active sites. Dimeric wild-type FVIIai was at least 75-fold more effective than monomeric FVIIai in blocking FVIIa association with TF. The dimer of a mutant FVIIai with higher membrane affinity was 1600-fold more effective. Anticoagulation by any form of FVIIai differed substantially from agents such as heparin and showed a delayed mode of action. Coagulation proceeded normally for the first minutes, and inhibition increased as equilibrium binding was established. It is suggested that association of FVIIa(i) with TF in a collision-dependent reaction gives equal access of inhibitor and enzyme to TF. Assembly was not influenced by the higher affinity and slower dissociation of the dimer. As a result, anticoagulation was delayed until the reaction reached equilibrium. Properties of different dissociation experiments suggested that dissociation of FVIIai from TF occurred by a two-step mechanism. The first step was separation of TF/FVIIa(i) while both proteins remained bound to the membrane, and the second step was dissociation of the FVIIa(i) from the membrane. These results suggest novel actions of FVIIa(i) that distinguish it from most of the anticoagulants that block later steps of the coagulation cascade (Stone *et al.* 2005).

Some studies of a novel class of peptide exosite inhibitors of FVIIa (like E-76, A-183 or extended A-183X) have been performed. Among these, A-183X is capable of almost complete in vitro inhibition of TF/FVIIa activity. The NAPc2 (Nematod Anticoagulant Protein c2) isolated from the canine hookworm Ancylostoma caninum, binds to FXa and then to TF/FVIIa complex and forms an inactive quaternary complex (Walsh 2004, Frederick *et al.* 2005).

One publication investigated the effect of inhibition of TF by recombinant tissue factor pathway inhibitor (rTFPI) on thrombus formation and intimal hyperplasia in a porcine model

of coronary balloon angioplasty (Roque *et al.* 2000). Tissue factor pathway inhibitor (TFPI) is the principle physiologic inhibitor of the TF-factor VII/VIIa complex and is found mainly in endothelial cells. TFPI therapy has been proven beneficial in deep vein thrombosis (Holst *et al.* 1994), in preventing arterial reocclusion after fibrinolysis (Abendschein *et al.* 1995) and in reducing intimal hyperplasia in experimental models of arterial injury (Oltrona *et al.* 1997, Jang *et al.* 1995). Using an ex vivo perfusion system it has been shown previously that specific inhibition of TF with TFPI reduces thrombogenicity of disrupted human aortic atherosclerotic plaques (Badimon *et al.* 1999).

The potential use of TFPI after angioplasty was also studied in a rabbit model, when the TFPI gene in a liposome and in an adenoviral vector was locally administered after iliac and carotid artery angioplasty and markedly reduced restenosis (Yin *et al.* 2002, Zoldhelyi *et al.* 2001).

Conclusion

In this review are summarized essentials of the physiology and the pathophysiology of TF in terms of the literature sources. But still, until today, there are many questions to be answered: the source and the level of TF in healthy subjects, the method how to measure the "trombogenic" potential of TF because its role in many biological processes, the "normal" levels, the possible impact of risk factors and possibility of risk stratification of "healthy" subjects according to the levels of TF like marker of the blood with procoagulant potential. Answering these questions and impact of the blocking of TF as a therapeutical approach in acute coronary syndromes is a challenging field of investigation in blood coagulation.

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Příloha 5: Kompletní bibliografie autora

Krátká sdělení, editorial:

1. **Malý M**: Workshop intervenční kardiologie, Praha 2000. Cor Vasa 2000;42: K 105.
2. **Malý M**: Workshop intervenční kardiologie 2001. Cor Vasa 2002;44:K4.
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Abstrakta v impactovaných časopisech:

1. Vojáček J, **Malý M**, Hraboš V, Mates M, Hájek P, Durdil V, Kvasnička J, Salaj P: Tissue factor, tissue factor pathway inhibitor and cytoadhesive molecules in patients with acute coronary syndrome. Eur Heart J 2002;Suppl 8/9:21.
2. Mates M, Hrabos V, **Maly M,** Hajek P, Durdil V, Fiedler J, Vojáček J. Mid-term follow-up after deffering coronary intervention on the basis os myocardial fractional flow reserve. Am J Cardiol 2003;92:202.
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4. **Maly M,** Veselka J, Blasko P, Tomasov P, Hrachovinova I. Elisa Metod does not detect differences in the level of tissue factor between patiens with acute coronary syndrome and patiens with normal coronary angiogram. Clin Car 2005;28(II):65.
5. Veselka J, **Maly M**, Duchonova R, Palenickova J, Zemanek D, Tesar D, Blasko P, Hajek P. Age influences the early course of hemodynamic and morphologic changes in patiens undergoing alcohol septal ablation for obstructive hypertrophic cardiomyopathy. Clin Car 2005;28(II):99.

Publikace v periodiku s impact faktorem:

1. **Maly M,** Vojacek J, Hrabos V, Kvasnicka J, Salaj P, Durdil V. Tissue factor, tissue factor pathway inhibitor and cytoadhesive molecules in patients with an acute coronary syndrome. Physiol Res 2003;52(6):719-28.
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