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Disertační práce

P-selektin - nový protein v hemostáze

P-selectin - a novel protein in hemostasis

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PROHLÁŠENÍ

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OBSAH

1. ABSTRAKT

P-selektin je znám.jako adhezivní glykoprotein se vztahem k zánětlivé reakci organismu. První práce o možném vztahu P-selektinu ke krevnímu srážení byly publikovány v polovině devadesátých let. Popisovaly pokusy, kde inhibující protilátky P-selektinu způsobily blokování vzniku fibrinu a potlačily růst krevních sraženin. Bylo prokázáno, že P-selektin zvyšoval expresi tkáňového faktoru (TF) na monocytech. K potvrzení úlohy P-selektinu v hemostáze také přispělo naše zjištění, že nadprodukce solubilního P-selektinu (sP-sel) vede k hyperkoagulačnímu stavu krevní plazmy. Ve své disertační práci jsem se zabývala patofyziologií sP-sel a funkcí mikropartikulí s navázaným TF.

Podrobnou studií účinku sP-sel jsme zjistili, že za hyperkoagulační stav plazmy jsou zodpovědné prokoagulační mikropartikule (MPs), z nichž část nese na svém povrchu TF. Potvrdili jsme, že nedávno popsaný „blood-borne TF“, tj. cirkulující aktivní TF, může být indukován sP-sel. Produkce MPs byla potlačena „inhibující“ protilátkou proti PSGL-1, což je transmembránový receptor P-selektinu přítomný na monocytech a granulocytech. Tím jsem prokázala, že MPs se rekrutují z monocytárních buněk při vazbě P-selektinu na receptor PSGL-1. Zjištěných skutečností, že sP-sel produkuje MPs, jsem využila u pokusů, které měly zvrátit krvácivé projevy u transgenních zvířat s defektním genem pro FVIII. Po intravenózní aplikaci sP-sel došlo u myší s hemofilií A k signifikantnímu zkrácení krvácivosti ve srovnání s kontrolami. Výrazně se také zkrátil čas srážení plné krve. *In vitro* pokusy s krví pacientů s těžkou formou hemofilie A prokázaly signifikantní zvýšení množství MPs a aktivity TF. Sledovala jsem také, co se děje s MPs, které vznikly působením sP-sel. Mikropartikule, které jsem separovala z krve myší, jsem obarvila fluorescenčním barvivem a injikovala *i.v.* Označené MPs se okamžitě inkorporovaly do rostoucího trombu, který byl uměle indukován v mikrocirkulaci *m.cremaster* myši.

Přetrvávající zvýšená koncentrace sP-sel byla prokázána u pacientů s kardiovaskulárním onemocněním. Proto jsem se také zabývala kvantifikací MPs a TF u pacientů s koronárním syndromem. Ve studii bylo prokázáno signifikantně nižší množství MPs a aktivity TF u pacientů s akutním stavem koronárního syndromu ve srovnání s nemocnými ve stabilním stavu, nebo s jinou kardiovaskulární poruchou. Výsledek výrazně koreloval s dřívějším poznatkem, že MPs se rychle inkorporují do míst, kde vznikají destičkové agregáty, které jsou velmi časté u akutního koronárního syndromu

Výsledky mé disertační práce přispěly k vysvětlení funkce sP-sel v hemostáze a staly se podkladem dalších studií, které na ni navázaly a vysvětlily intracelulární mechanismus vzniku monocytárních MPs působením P-selektinu.

2. ÚVOD

2.1. P-SELEKTIN – KRÁTKÝ PŘEHLED

P-selektin je člen rodiny selektinů, adhezivních receptorů, které se vážou na specifické cukerné zbytky glykoproteinů, např. PSGL-1. GPIbα(Ley, 2003) Transmembránový glykoprotein P-selektin (P-sel) se nachází v alfa-granulech krevních destiček a ve Weibel-Paladeho tělíscích endotelových buněk (Johnston and McEver,1989). Při aktivaci buňky např. trombinem nebo histaminem je rychle translokován do plazmatické membrány, kde se účastní adheze leukocytů z krevního oběhu. P-sel sprostředkuje rolování leukocytů po povrchu aktivovaného endotelu a jejich extravazaci, což je část obranného mechanismu organismu při zánětlivé reakci (Madyas and Wagner, 1993). Solubilní forma P-sel (sP-sel) vzniká odštěpováním plazmatické části P-sel z membránového povrchu a byla detekována v lidské i myší plazmě (Dunlop and Berndt, 1992; Hartwell and Wagner, 1998). Enzym, který to způsobuje, dosud nebyl popsán. U lidí byla též popsána alternativní forma P-selektinu, která je exprimována bez transmembránové oblasti.

Zvýšená hladina sP-sel byla nalezena u mnoha konsumpčních destičkových onemocnění jako např. u DIC(diseminovaná intravaskulární koagulace), TTP(trombotická trombocytopenická purpura) a HIT (heparinem indukovaná trombocytopenie) (Chong and Chesterman, 1994). Zvýšená hladina byla popsána u dalších onemocnění, jako jsou kardiovaskulární onemocnění, diabetes a hypertense. Bylo prokázáno, že zvýšená hladina sP-sel je rizikovým faktorem pro infarkt myokardu a iktus (Ridker, 2001). Úloha P-sel v hemostáze začala být evidentní při výzkumu transgenních myší, které neměly žádný P-sel. Zvířata měla prodloužený čas krvácivosti, tvořila nedostatečné množství fibrinu v krevních sraženinách a destičky v krevních sraženinách byly jinak uspořádány než u kontrolních zvířat (Subramaniam and Wagner, 1996).

Ačkoliv je cirkulující sP-sel potenciálně aktivní, protože lektinová i EGF (epidermal growth factor-like) vazebné domény jsou zachovány (Mehta and McEver, 1996), nebylo do počátku 21.stolení téměř nic známo o jeho biologické aktivitě. Impuls k výzkumu sP-sel daly poznatky ze studie transgenní myši bez

intracelulární části proteinu, proto byla nazvána „delta-cytoplasmic tail“ myš (DCT). Endotel této myši konstitutivně exprimoval P-sel, který byl následně uvolňován do krevního řečiště. DCT myš měla trvale 3-4x větší koncetraci sP-sel než kontrolní myši.

2.2. TKÁŇOVÝ FAKTOR- INICIÁTOR A REGULÁTOR KOAGULACE

Tkáňový faktor (TF) je také znám jako trombokináza, tromboplastin, CD142 nebo FIII. Je transmembránový glykoprotein, který je vysoce exprimován v mozku, srdci, ledvinách, děloze, placentě a v matrix cévní stěny. TF je hlavní iniciátor koagulační kaskády. Tvoří s faktorem VII(FVII), který aktivuje FX a FIX. Následují složité komplexní mechanismy, jejichž konečným produktem je trombin, který přeměňuje koagulační faktor fibrinogen na fibrin, aktivuje krevní destičky a účastní se dalších procesů, které jsou pro- i protikoagulační. Povrch endotelu cévní stěny a monocyty za normálních podmínek TF neexprimují. Mnoho let se předpokládalo, že malé množství TF detekované v krevním oběhu nebylo biologicky aktivní. Průkopnická práce skupiny Yale Nemersona (Giesen and Nemerson, 1999) prokázala, že existuje „blood born TF“-aktivní TF, který je trvale přítomný v krevním oběhu. „Blood born-TF“ je tkáňový faktor, který je vázaný na mikropartikule (MPs). Tyto MPs s navázaným TF (TF-MPs) byly prokázány v krevních sraženinách a přítomnost TF-MPs je nutná pro správný a organizovaný růst krevní sraženiny. Předokládá se, že většina TF-MPs pochází z periferních monocytů. V poslední době byly také prokázány TF-MPs se znaky endotelových buněk i krevních destiček (Zillmann et.all, 2001). O funkci TF-MPs se stále diskutuje. Z výsledků studií je ale evidentní, že TF-MPs mají nezaměnitelnou roli při růstu trombů v situacích, kde je málo aktivovaných endotelových buněk nebo je endotel pokryt krevními destičkami a fibrinem (Myers and Wakefield, 2003). Velké množství TF-MPs bylo nalezeno v aterosklerotických placích (Mallat and Tedgui, 1999; Marmur and Taubman, 1996). TF-MPs jsou cirkulující rezervoár vždy přítomného aktivního TF, který výrazně dramaticky vzroste u onemocnění s protrombotickými symptomy.

TF je jedinný koagulační faktor, jehož kompletní deficit nebyl nikdy popsán. Kompletní deficit TF u transgenních myší je letální, „low TF“myši (1% TF) mají těžké, často letální krvácení.

2.3. MIKROPARTIKULE V HEMOSTÁZE

Mikropartikule (MPs), malé membránové vesikuly lipoproteinové povahy, které měří 0,1 až 1,0 μm v průměru, byly poprvé popsány Wolfem v roce 1967. Mluvilo se o nich jako o „prachu“, „buněčném odpadu", což vyjadřovalo tehdejší náhled na jejich užitečnost. MPs se oddělují z endoteliálních buněk, monocytů, granulocytů, lymfocytů, erytrocytů a krevních destiček po jejich aktivaci nebo při apoptose. Protože si na svém povrchu nesou znaky svého původu, je možné předpokládat, že jsou produkovány k zachování jejich informace, kvalitativně i kvantitativně. MPs, i když mají stejný původ, mohou mít jiné složení proteinů, lipidů a antigenů. MPs mohou na svém povrchu nést i patogenní částice. Mateřská buňka kontroluje počet a složení MPs (Hugel and Freyssinet, 2005). Navázáním do buněčné membrány mohou stimulovat buňku k produkci látek, které běžně neprodukuje, např. cytokiny, adhesivní molekuly, růstový faktor a TF.

MPs pocházející z krevních destiček obsahují receptor GP IIb-IIIa, který je schopen vázat fibrinogen, pokud jsou destičky aktivované trombinem nebo kolagenem. V případě, že jsou destičky aktivované komplexem C5b-9, MPs fibrinogen nevážou (Morel and Freyssinet, 2004).

Při tvorbě MPs dochází k určité asymetrii v membráně buněk a negativně nabité fosfolipidy, hlavně fosfatidylserin (PS), z vnitřní strany membrány se objeví na vnější. Proto jsou MPs silně prokoagulační. Některé MPs pocházející z endotelu nebo monocytů jsou ale i fosfatidylserin negativní.

MPs jsou přítomny v normální plazmě, zvýšené množství mikropartikulí lze nalézt u řady onemocnění. Jsou to srpkovitá anémie, preeklampsie, sepse, diabetes typu 1 i 2, hypertenze, lupus anticoagulans, lupus erythematodes a další. Při velkém zvýšení množství MPs v plazmě mohou nastat trombotické problémy (Morel and Toti, 2008).

Kromě MPs uvolňují krevní destičky po aktivaci ještě 10x menší částice, které se nazývají exosomy. Zdrojem exosomů jsou nejčastěji multivezikulární tělíska uvnitř destičky. Jsou obtížně sledovatelné, protože jsou pod rozlišovací schopností průtokového cytometru. Bylo prokázáno, že obsahují větší koncentraci tetraspan proteinu CD63 a P-sel.

3. CÍLE PRÁCE

Cílem mé disertační práce bylo postupně objasnit několik otázek:

1. Jakou roli hraje sP-sel při vzniku hyperkoagulačního stavu;

2. Co způsobuje hyperkoagulační stav.

Odpovědi na tyto otázky jsou v první uvedené publikaci.

Na základě zjištěných výsledků byl můj další cíl zjistit

3. Jaký je receptor sP-sel, zodpovědný za indukci exprese TF-MPs a MPs,

4. Zda je možné ovlivnit krvácivý stav aplikací vysoké dávky sP-sel.

Výsledky byly publikovány ve druhé uvedené publikaci.

V poslední publikaci jsem se zabývala protrombotickým potenciálem sP-sel, MPs a TF-MPs u pacientů s kardiovaskulárním onemocněním.

Cílem bylo zjistit zda:

5. Mohou být TF nebo TF-MPs být markerem závažnosti kardiovaskulárního onemocnění.

6. Jaká je úloha TF- MPs a MPs v kardiovaskulárním onemocnění.

4. VÝSLEDKY

4.1. SOLUBILNÍ P-SELEKTIN A HYPERKOAGULACE

Andre,P., Hartwell,D., **Hrachovinova,I.,** Saffaripour,S., and Wagner,D.D.(2000) **Pro-coagulant state resulting from high levels of soluble P-selectin in blood.** PNAS. 97(25).13835-13840.

Koncentrace solubilních adhezivních receptorů vzrůstá za patologických podmínek, ale jejich význam je zatím neznámý. Solubilní P-selektin (sP-sel) se odštěpuje z povrchu aktivovaných destiček a endoteliálních buněk. Geneticky upravené myši bez cytoplazmické části P-selektinu (DCT) produkují 3-4x více sP-sel v plazmě než myši normální.

Metodika a výsledky: Pozorovali jsme, že DCT myši tvoří fibrinová vlákna rychleji a ve větším množství. V *ex vivo* perfuzní komůrce došlo k většímu depozitu fibrinu v místě vytvořeného trombu než u normální myši (WT) a žádný fibrin se neobjevil u myší, které byly geneticky upraveny tak, že neměly žádný P-selektin. Stejně *in vivo*, při Shwartzmanově reakci došlo k výrazně menšímu krvácení u DCT myší než u WT. Krvácení bylo naopak mnohem větší u myší deficitních na P-selektin. Infuze P-sel-Ig chiméry měla ten samý protektivní efekt na WT myš, jako byl pozorován u DCT myši. To ukazovalo na skutečnost, že tento efekt je způsoben sP-sel myši, kterým byla aplikována sP-sel-Ig chiméra nebo DCT myši měly mnohem vyšší koncentraci prokoagulačních mikropartikulí a jejich plazma koagulovala v průměru o 1 minutu rychleji než plazma WT myší. Tento prokoagulační fenotyp DCT myši byl potlačen čtyřdenní aplikací PSGL-1(inhibitor P-selektinu).

Závěr: Myslíme si, že sP-sel by neměl být považován jenom za marker zánětu a aktivace krevních destiček. Naše výsledky prokázaly, že může přímo indukovat prokoagulační aktivitu vázanou na cévní a trombotické choroby.

Pro-coagulant state resulting from high levels of soluble P-selectin in blood

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Abbreviations: sP-sel, soluble P-selectin; P-sel-Ig, P-selectin-Ig fusion protein; TF, tissue factor; WT, wild-type; P2/2, P-selectin-deficient; DCT, mice that express P-selectin lacking the cytoplasmic domain; PPP, platelet-poor plasma; PSGL, P-selectin glycoprotein ligand.

The plasma concentration of soluble adhesion receptors is increased under pathological circumstances, but their function remains enigmatic. Soluble P-selectin (sP-sel) is shed from activated platelets and endothelial cells. Mice genetically engineered to express P-selectin without the cytoplasmic tail (DCT) constitutively show a 3- to 4-fold increase of sP-sel in plasma. We observed that the DCT mice formed fibrin very readily. In an *ex vivo* perfusion chamber, there was more fibrin deposited at the site of platelet thrombus formation than in wild type (WT), whereas no fibrin deposits were detected using P-selectin-deficient blood during the same interval. Similarly, *in vivo*, the hemorrhage produced by local Shwartzman reaction was smaller in the DCT mice than in WT. In contrast,we previously showed hemorrhage to be more prominent in P-selectin knock-out mice. Infusion of mouse P-sel-Ig chiméra produced the same protective effect in WT mice as seen in the DCT mice, indicating that the effect was due to increased levels of sP-sel. Mice infused with P-sel-Ig showed significantly more fibrin deposited on the luminal face of the injured vessels than kontrol mice. Plasma from DCT mice or mice infused with P-sel-Ig contained higher concentration of pro-coagulant microparticles and clotted one minute faster than WT. This pro-coagulant phenotype of DCT mice could be reversed by a 4-day treatment with PSGL-Ig, a P-selectin inhibitor. We propose that sP-sel should no longer be considered only as a marker of inflammation or platelet activation, but also as a direct inducer of pro-coagulant activity associated

P-selectin is a member of the selectin family localized in the membranes of a-granules of platelets and the Weibel-Palade bodies of endothelial cells (1). A soluble form of P-selectin can be found in the plasma as a circulating protein (2). In vivo, two main physiological roles are attributed to the integral membrane form of P-selectin. First, in inflammation, P-selectin is redistributed onto the surface of activated endothelial cells where it mediates the rolling of leukocytes (3). Second, in thrombosis,P-selectin expressed on activated platelets present in a thrombus supports the recruitment of leukocytes (4). Soluble P-selectin (sP-sel) of healthy individuals has been suggested to originate from the alternatively spliced form found in endothelial cells and platelets (5). Alternatively, elevated levels of sP-sel may reflect platelet activation (6) because P-electin is proteolytically shed from the plasma membrane in vivo shortly after activation (7, 8). Therefore, plasma levels of sP-sel have been considered a useful tool to predict thrombotic consumptive platelet disorders (9–12), but they can also reflect endothelial cell activation (13, 14). Although the circulating form of P-selectin is potentially active because only the lectin and epidermal growth factor (EGF) domains are required to bind its receptor, P-selectin glycoprotein ligand-1 (PSGL-1) (15), the biological role of sP-sel and similarly that of other soluble adhesion receptors circulating in blood is not known (16). Previous work in our laboratory has shown that P-selectindeficient mice exhibit a slightly prolonged bleeding time, as well as an increased hemorrhagic response in a local Shwartzman reaction (17), suggesting that P-selectin could play a role in hemostasis. To further evaluate this possibility, we studied the hemostatic properties of mice genetically engineered to expressP-selectin without the cytoplasmic domain (DCT mice) (18). In these mice, P-selectin is constitutively expressed on the surface of the endothelial cell and shed from the plasma membrane, leading to a 3- to 4-fold increase of sP-sel in plasma. We now report that the increased levels of sP-sel accelerate hemostasis in these mice. Similarly, wild-type (WT) animals infused with a P-selectin-Ig fusion protein (P-sel-Ig) chimera entered a procoagulant state.

Materials and Methods

Reagents. Human IgG1 was from Sigma, and P-sel-Ig was from PharMingen. The P-sel-Ig is composed of N-terminal fragment of mouse P-selectin including the first two complement-binding domains fused to the Fc region (hinge, C1 and C2) of human IgG1 (19). PSGL-Ig (a generous gift from Genetics Institute, Cambridge, MA) is composed of the first 47 aa from the N-terminal end of mature human PSGL-1 fused to the Fc region of human IgG1 (20). The control protein (control-Ig, Genetics Institute) is a murine IgG2a produced in Chinese hamster ovary (CHO) cells. The protein has been mutated in the FcgRI and C91q binding sites to inhibit Fc binding and complement directed cytolysis. The same sites were mutated in the human PSGL-Ig molecule.

Mice. C57BLy6Jy129Sv mice, WT and DCT, (18) were compared. C57BLy6J mice were used as recipient for the injection of P-sel-Ig, human IgG1, PSGL-Ig, and control-Ig. Animals were housed at the Center for Blood Research, Harvard Medical School. Experimental procedures were approved by the Animal Care and Use Committee of the Center for Blood Research.

Ex Vivo Perfusion Chamber. Glass capillary tubes (0.56 mm inner diameter) were coated with 1 mgyml type III fibrillar collagen (Sigma) as described (21). Mice were nesthetized with 2.5% tribromoethanol (0.15 mly10 g). Non-anticoagulated blood was collected from the vena cava by using a 25G butterfly needle, and perfused through the collagen-coated perfusion chamber via silastic tubing. A flow rate of 220 mlymin was established for 2 min by a syringe pump mounted distal to the chamber, resulting in a 212 s21 shear rate. Immediately after the blood perfusion, the thrombotic deposits formed on the collagen surface were rinsed for 20 s with PBS and fixed in ice-cold 2.5% acodylatebuffered glutaraldehyde (pH 7.4) at the same shear rate. The perfusion chamber was then removed and fixed in a freshly prepared fixative for 24 h at 4°C. En face visualization of thethrombotic deposits was performed under light microscopy after epon embedding.

Determination of sP-sel and Fibrinogen Levels in Plasma. Determination of the level of sP-sel was performed by using a modified sandwich ELISA procedure previously described (18). Calculations of the amount of sP-sel in the serum samples were made by comparing the specific sP-sel values with a standard curve of titrated P-sel-Ig. The plasma level of fibrinogen was measured according to Sigma Diagnostics Procedure No. 886.

Local Shwartzman Reaction. Twelve- to fourteen-week-old agematched WT and DCT male mice were primed on day 0 by a s.c. injection of Escherichia coli LPS 055:B5 (Difco) at 100 mgy mouse in 0.1 ml of sterile PBS. Twenty-four hours later (day 1), recombinant murine TNF-a (Genzyme) at 0.3 mgymouse was injected at the same site (17). On day 2, the hemorrhagic lesions were examined and scored on a scale of 0 to 4 without knowledge of genotypes. Hematoxylin-eosin-stained paraffin sections were prepared from the lesion site, and the degree of inflammatory cell infiltration as well as hemorrhage were scored microscopically, on a scale of 0 to 4 (17).

Immunohistology. Paraffin sections from the Shwartzman lesion site were de-paraffinized, sequentially blocked with avidin D solution and biotin blocking solution (Vector Laboratories) and stained with a rabbit anti-human fibrinogen (1:1000 dilution; Dako), which crossreacts with mouse fibrin fibrinogen. Sections were then sequentially treated with a biotinylated goat anti-rabbit antibody (Zymed), and an ABC mix solution (Vector Laboratories). Development was done by treating the sections with an AEC substrate kit for horseradish peroxidase (Vector Laboratories). Sections were counterstained with hematoxylin.

Plasma Clotting Time Assay. One milliliter of blood was drawn from the retro-orbital enous plexus by using plain microhematocrit capillary tubes (VWR Scientific) and collected into polypropylene tubes (Eppendorf; Marsh Biochemical Products, Rochester,NY) containing 10% final volume of acid-citrate-dextrose (ACD; 38 mM citric acidy75 mM trisodium citratey100 mM dextrose). Platelet-poor plasma (PPP) was prepared by centrifugation at 1,500 3 g for 25 min. PPP was centrifuged once more for 2 min at 15,000 3 g to remove contaminating cells from the plasma. Plasma clotting was induced under stirring conditions (800 rpm) at 37°C in an aggregometer (Sienco, Inc., Wheat Ridge, CO) by adding a volume of prewarmed 20 mM CaCl2 solution to an equal volume of plasma in a siliconized tube. The time (in seconds) needed to clot was determined.

Flow Cytofluorometry of Plasma Micro-particles. PPP was prepared as above. Three hundred microliters was obtained per mouse. PPP from three mice was pooled, diluted 1:3 with a Hepes buffer [10 mmol/L Hepesy5 mmol/L KCLy1 mmol/L MgCl2y136 mmol/L NaCl (pH 7.4)], and centrifuged for 1.5 h at 100,000 3 g. The supernatant was removed, and the pelleted microparticles were resuspended in 120 ml of a 10

mM Hepes, 136 mM NaCl (pH 7.4) buffer. Flow cytometric analysis was performed on a Becton-Dickinson FACSCalibur with CELLQUEST software (Becton- Dickinson). The light scatters and fluorescent channels were set at logarithmic gain (forward scatter was E00 with a threshold of 12 and sideward scatter was 300). To count the total microparticle population, 30-ml aliquots were incubated for 15 min in the dark with 0.25 mgyml calcein AM (Molecular Probes). The total number of events was counted for a set interval of 10 s Samples were stained for 20 min at room temperature with a rat monoclonal anti-murine Mac-1 (CD11byCD18, 10 mgyml; Boehringer Mannheim) and with a sheep anti-rabbit tissue factor IgG (5 mgyml; American Diagnostica, Greenwich, CT), which recognizes mouse tissue factor. A phycoerythrin-conjugated goat anti-rat-IgG (1:200; Immunotech-Coulter, Marseille) and FITC-conjugated rabbit anti-sheep IgG (1:1000; Zymed) were second antibodies. Rat IgG (Sigma) and FITC-conjugated sheep IgG (Caltag Laboratories, South San Francisco, CA) were control antibodies. For analysis of microparticles in plasma of DCT mice treated with PSGL-Ig, 200 ml of blood was collected by retro-orbital puncture on day 0. PPP was obtained, and 40 ml was diluted in 260 ml PBS and immediately analyzed for microparticle number by FACS. Mice were then infused i.v. (days 0 and 2) with 10 mg/kg PSGL-Ig or control-Ig. On day 4, 200 ml of blood was collected from the other eye, and the number of microparticles was determined.

Tissue Factor Actvity in PPP. PPP was prepared from pooled plasma of WT and DCT mice. A first centrifugation step at 12,000 3 g was performed to remove contaminating cells. The supernatant was then diluted in 20mMHepes, 1mMEDTA (pH 7.2) solution and ultracentrifuged at 200,000 3 g for 1.5 h. The pelleted microparticles were resuspended (1y2 of the initial volume) as described above. Determination of tissue factor activity of the microparticles solution was evaluated by its ability to promote the activation of factor X (150 nM) by factor VIIa (5 nM) in the presence of 1 mMCaCl2. A chromogenic substrate of factor Xa, Spectrozyme fXa, was added (0.3 mM). The linear changes in absorbance at 405 nM were recorded by using a plate reader equipped with kinetics software (DYNEX Technologies, Chantilly, VA). The changes directly correlate with the concentration of factor Xa generated in the assay.

Results

Excessive Fibrin Deposition on Platelet Thrombi Formed in a Flow Chamber Perfused with DCT Blood. We compared the thrombotic process in WT, P-selectin-deficient (P2/2), and DCT mice by perfusing non-anticoagulated blood through glass capillaries coated with fibrillar collagen type III. Platelet deposits were observed in all capillaries with no significant differences in size, but, surprisingly, we observed striking differences in the amount of deposited fibrin (Table 1, Fig. 1). Perfusion of WT blood showed microscopically visible depositions of short fibrin tails in only 4y11 cases. We did not observe any fibrin tails when blood from P2/2 mice was perfused under identical conditions. On the other hand, long fibrin tails distal to the thrombi were found in eight of nine chambers perfused with blood from DCT mice. It should be noted that, when fibrin was detected, it always originated from the adhering platelets (Fig. 1), confirming that the collagen surface is not pro-coagulant by itself (22). Because the activated platelets from WT and DCT mice have similar P-selectin expression on the plasma membrane (18), we hypothesized that the difference in fibrin formation at the platelet thrombi most likely reflected a function of sP-sel in plasma. As described previously (18), plasma of DCT mice contains elevated levels of a 100-kDa P-selectin fragment. We determined by comparison to known concentrations of P-sel-Ig that the concentration of sP-sel is approximately 1 mgyml in DCT mice and 0.3 mgyml in WT mice. We also checked that there were no significant differences in fibrinogen levels betweenWT and DCT mice (367 ± 24 and 344 ± 14 mgydl, respectively, for each group,bn= 13).

sP-sel Reduces Hemorrhage and Affects Fibrin Deposition in a Local Shwartzman Reaction. The local Shwartzman reaction is a hemorrhagic and necrotic lesion that can be induced by endotoxin and cytokines and is attributed to a multitude of interactions between platelets, neutrophils, and endothelium (23). P2/2 mice display increased hemorrhage in this model (17). To evaluate a potential role for sP-sel in hemostasis, local Shwartzman reaction was studied in DCT mice, as well as WT mice of similar genetic background. Macroscopic evaluation of the injection sites revealed that, in contrast with P2/2 mice, the average size of the hemorrhagic lesion in DCT mice was reduced to about 50% of WT (Table 2). This reduction in hemorrhage was also observed by microscopic examination of paraffin sections (Table 2). To determine whether sP-sel was the cause of this protective effect, we studied the local Shwartzman reaction in C57yBL6J WT

mice injected with 1.2 mgyg body weight of either P-sel-Ig or control IgG1 1 h before the challenge dose of tumor necrosis factor-a (TNFa) was administered. Infusion of P-sel-Ig significantly reduced the size of hemorrhagic lesions measured both macroscopically and evaluated microscopically (Table 2). Immunohistological staining for fibrin at the lesion site revealed that the P-sel-Ig-treated mice had more vessels in the lesion withfibrin deposited on the luminal surface of the vessel wall than did IgG1-infused mice (Fig. 2 A and B). In animals treated with IgG1, the fibrin was preferentially deposited outside the injured vessel (Fig. 2 A and B). This suggested that the reduction in the hemorrhage was probably due to an increased or more rapid deposition of fibrin on the damaged vessel. The degree of inflammatory cell infiltration was the same in WT and DCT animals. A slight reduction in infiltration was observed in the P-sel-Ig-treated group compared with the IgG1-treated group (Table 2). This reduction might be explained by the occupancy of PSGL-1 receptors by the injected P-sel-Ig chimera, which could affect the leukocyte rolling. The infused P-sel-Ig concentration in plasma could be higher than the level of sP-sel found in the plasma of DCT mice.

Shorter Plasma Clotting Time in DCT Mice and in Mice Infused with P-sel-Ig. To evaluate whether the pro-coagulant activity seen in DCT mice or mice infused with P-sel-Ig is intrinsic to plasma of these mice, we have measured the plasma clotting time. Plasma clotting time of DCT mice (214 6 7 s) was significantly shorter than that of WT mice (276 6 8 s) (Fig. 3A). Adding P-sel-Ig to the plasma ofWT mice just before the clotting assay did not change the clotting time (not shown), showing that sP-sel does not directly affect coagulation. On the other hand, injection of P-sel-Ig into WT mice 22 h before collecting the blood samples reduced the clotting time by 1 min, i.e., to 218 6 14 s, a time similar to DCT plasma (Fig.3B). This time was also significantly shorter than that obtained with the plasma from the IgG1-treated mice (369 6 21 s). Our results indicate that the presence of high levels of sP-sel in mice increases the coagulability of their plasma. Interestingly, when plasmas of WT and DCT mice were ultra-centrifuged at 200,000 3 g for 1 h, clotting times of both supernatants were dramatically increased and were no longer different (DCT, 1064 ± 79 s, n = 4; WT, 1033 ± 141 s, n = 3; P= 0.84). This indicated that the pro-coagulant activity found in DCT mice was probably associated with the microparticles that were removed by ultracentrifugation.

Elevated Levels of sP-sel Induce an Increase in Pro-Coagulant Microparticles.To nvestigate further the possibility that the observed pro-coagulant activity is associated with microparticles, we measured the number of microparticles present in the plasma ofWT and DCT mice by FACS analysis (Table 3). Plasma from DCTmice presented a 2-fold increase in the total number of microparticles compared with WT. As observed in the plasma clotting time assay, infusion of P-sel-Ig inWT mice induced a phenotype similar to that of the DCT mice, increasing the number of microparticles by 4-fold compared with the IgG1-treated mice (Table 3).

We identified a population of Mac-1 (aMb2 integrin) positive microparticles in WT mice, which showed their leukocyte origin (Fig. 4). This population was increased in DCT mice, and, interestingly, 13 ± 3.5% of all microparticles (n= 3) stained for both Mac-1 and tissue factor (TF) (Fig. 4).

PSGL-Ig Reduces the Pro-Coagulant Activity of DCT Mice. We further evaluated whether infusion of a soluble form of the P-selectin receptor PSGL-1 (PSGL-Ig) would prevent the generation of the pro-coagulant state in DCT mice. We found that a 4-day treatment with PSGL-Ig significantly reduced the amount of microparticles and increased the plasma clotting time of DCT mice. Control-Ig had no such effect (Fig. 5 A and B). The clotting time of the PSGL-Ig-treated DCT was slightly longer than that of WT mice of similar age, indicating that the excess of microparticles observed in DCT mice was indeed due to sP-sel action.

Discussion

The presence of soluble adhesion receptors in plasma has been widely reported, and it is often increased in pathological or stress conditions (24, 25). The physiological importance of these soluble receptors is not known. Until now, only speculations existed about the function of sP-sel. It was proposed that it may have an anti-inflammatory function by preventing leukocyte-vessel wall interactions because sP-sel can bind PSGL-1 on leukocytes (26, 27). This hypothesis is also supportedby the fact that sP-sel binding to PSGL-1 activates leukocytes (28), and activated leukocytes may have a reduced ability to adhere to endothelium in vivo (29). It was also proposed that sP-sel could limit the extent of thrombosis by preventing additional leukocyte recruitment. On the other hand, sP-sel was shown to induce tissue factor expression on monocytes in vitro (30), indicating that it could have such an activity in

vivo. Interestingly, infusion of anti-P-selectin antibodies in vivo, reduced fibrin deposition on dacron graft implanted within an arteriovenous shunt in baboons (4).

In this study, we found that sP-sel constitutes an endogenous activator of the coagulation process through the generation of circulating micro-particles in plasma. Low levels of microparticles derived from platelets, leukocytes, and endothelial cells have been found in plasma of healthy individuals (31–34), and elevated numbers of microparticles circulate in the blood of patients with acute coronary syndromes and meningitis (35, 36). There are several explanations for a role of microparticles in modulating the coagulation process in vivo. First, microparticles carry pro-coagulant activity notably through the expression of prothrombinase activity on their membrane (37). Second, monocyte- derived microparticles were shown to activate endothelial cells in vitro, leading to expression of TF (32). In our study, we have found that elevated amount of sP-sel in DCT mice enhanced the generation of leukocyte-derived microparticles (Mac-11), some of which also expressed TF (Fig. 4). Functional TF activity in microparticles in whole blood from normal individuals has been recently reported (38, 33). In certain clinical conditions, such as meningococcal sepsis, the amount of pro-coagulant micro-particles originating from either platelets or granulocytes is increased 10- to 100-fold (36). In DCT mice, only 13% of microparticles expressed tissue factor, which may explain why these mice do not show symptoms of disseminated intravascular coagulation [the differential cell count (18) and the fibrinogen level were normal].

In the perfusion chamber model, it appeared that the procoagulant microparticles were preferentially recruited to thrombi, probably via a Mac-1yfibrinogen interaction (39), or via a CD15yP-selectin interaction (40). An increased concentration of leukocyte-derived micro-particles, especially when bound to platelets, would further accelerate the coagulation process on activated platelets (41, 42), via the TFyFVIIa pathway, as reported by Giesen and colleagues (33). A second line of evidence for an increased pro-coagulant activity at the site of injury comes from the local Shwartzman reaction model. An intense staining for fibrinyfibrinogen was found frequently on the luminal surface of the vessels in the lesions of WT mice infused with P-sel-Ig but not in control animals. This may be explained again by the recruitment of micro-particles to activated platelets as described above.

Thus, we have found a role for sP-sel in plasma in promoting coagulation that is different from its usual role as surface receptor involved in leukocyte recruitment and transmigration (43). High levels of sP-sel observed in thrombotic consumptive platelet disorders such as disseminated intravascular coagulation and thrombotic thrombocytopenic purpura could be in part responsible for the generalized hypercoagulable state associated with these diseases (9). In addition, sP-sel could contribute to the formation of fibrin tails, which are often associated with thrombi formed under high shear rates (44). Moreover, elevated levels of sP-sel might be involved in the recurrent thrombotic process of angina (45) and in restenosis (46). Our findings support and provide an explanation for the promising results of antithrombotic therapies directed against P-selectin in various thrombotic models such as stroke (47), or deep vein thrombosis (48). A reduction in numbers of circulating pro-coagulant microparticles by PSGL-Ig could explain its efficacy in accelerating thrombolysis (20) and reducing venous thrombosis in vivo (49). On the other hand, sP-sel could be also considered as an adjunctive treatment for patients with congenital bleeding disorders such as hemophilia, for example, to enhance hemostasis before a surgical procedure. We would like to propose that sP-sel in plasma should no longer be viewed as a simple marker of platelet or endothelial activation, but rather as a direct inducer of a pro-coagulant state. It is possible that other adhesion receptors may have new yet unsuspected functions when present in their soluble form.

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4.2. MODULACE KRVÁCIVÉHO STAVU POMOCÍ SP-SELEKTINU

Hrachovinova, I., Cambien, B., Hafezi-Moghadam, A., Kappelmayer, J., Camphausen, RT., Widom, A., Xia, L., Kazazian, HH Jr, Schaub, RG., McEver, RP., Wagner, DD.(2003) **Interaction of P-selectin and PSGL-1 generates microparticles that correct hemostasis in a mouse model of hemophilia A.** Nat Med., 9(8),1020-1025.

Vysoká hladina sP-sel je vázaná na trombotické onemocnění a může být rizikovým markerem pro kardiovaskulární onemocnění. Myši se zvýšenou hladinou sP-sel mají zvýšené množství prokoagulačních mikropartikulí v plazmě.

Metodika a výsledky: Prokázali jsme, že sP-sel-Ig chiméra přes svůj receptor PSGL-1 indukovala tvorbu prokoagulačních mikropartikulí v lidské krvi. Následně, že transgenní myši bez PSGL-1 receptoru (Psgl-1–/–) produkují menší množství MPs a po aplikaci sP-sel nedošlo k nárůstu MPs, jak tomu bylo u kontrolních zvířat. Při aplikaci mikropartikulí do místa vznikajícího trombu došlo k jejich okamžitému navázání a navýšení generace trombinu v místě. Při infuzi SP-sel-Ig do hemofilických myší došlo k 20násobnému nárůstu počtu MPs spolu s nárůstem aktivity TF. To signifikantně urychlilo kinetiku tvorby fibrinu v hemofilické myši a normalizovalo krvácivost.

Závěr: Léčba P-sel–Ig může představovat nový přístup ke kontrole krvácivých komplikací u hemofilie A.

P-selectin/PSGL-1 interaction generates microparticles that correct hemostasis in a mouse model of hemophilia A

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Abstract

High plasma levels of soluble P-selectin are associated with thrombotic disorders and may predict future cardiovascular events. Mice with high levels of soluble P-selectin have increased numbers of microparticles in plasma. We now show in human blood that P-selectin-immunoglobulin chimeras (P-sel–Ig) induced formation of procoagulant microparticles through P-selectin glycoprotein ligand-1 (PSGL-1). In addition, *Psgl-1*$^{-/-}$ mice produced fewer microparticles after P-sel–Ig infusion and did not spontaneously increase their microparticles count in old age as do wild-type mice. Injected microparticles specifically bound to thrombi and thus could be involved in thrombin generation at sites of injury. Infusion of P-sel–Ig into hemophilia A mice produced a twenty-fold increase over control immunoglobulin in microparticles containing tissue factor. This significantly improved the kinetics of fibrin formation in the hemophilia A mice and normalized their tail-bleeding time. P-sel–Ig treatment could become a new approach to sustained control of bleeding in hemophilia.

Introduction

P-selectin is a member of the selectin family localized in the membranes of α-granules of platelets and the Weibel-Palade bodies of endothelial cells[1]. P-selectin supports adhesion of leukocytes to activated platelets present in thrombus[2] or to activated endothelial cells, where it mediates leukocyte rolling[3]. A soluble form of P-selectin found in plasma[4] is potentially active because it possesses the lectin and epidermal growth factor (EGF) domains required to bind its receptor, P-selectin glycoprotein ligand -1 (PSGL-1)[5]. A high level of shedding of soluble P-selectin in mice expressing P-selectin without the cytoplasmic domain (ΔCT mice)[6] is associated with a procoagulant state in these animals[7]. Higher levels of soluble P-selectin in blood are observed also in thrombotic consumptive disorders such as disseminated intravascular coagulation , thrombotic thrombocytopenic purpura , and heparin-induced thrombocytopenia, all known for generalized hyper-coagulable states[8]. On the other hand, prophylactic infusion of monoclonal antibody to P-selectin reduces the extent of fibrin deposition on dacron grafts in baboons[2] and diminishes experimentally induced venous thrombosis in baboons[9], rats or cats[10].

The cause of the hyper-coagulable state in ΔCT mice and mice infused with recombinant P-selectin-Ig chimera (P-sel–Ig) is a higher level of circulating microparticles, some of which contain tissue factor[7]. Tissue factor is a trans-membrane protein that initiates hemostasis[11]. The tissue factor positive microparticles also express the integrin αMβ2 (Mac-1) indicating that they originate from leukocytes[7].

The mechanisms through which P-selectin induces the formation of tissue factor-containing micro-particles are not known. PSGL-1 is a potential candidate receptor for this process as it is a known physiological ligand for P-selectin. PSGL-1 is a homodimeric mucin expressed on the majority of leukocytes[1]. Binding of P-selectin to PSGL-1 initiates signaling in leukocytes such as protein-tyrosine phosphorylation, MAP kinase activation and increased αMβ2 binding activity[12,13]. In the present study we demonstrate that the P-selectin-induced generation of pro-coagulant microparticles is also mediated by its interaction with PSGL-1. In addition we show that the generation of tissue factor-containing micro-particles through the P-selectin/PSGL-1 interaction could potentially be used to treat bleeding disorders resulting from defective coagulation, such as hemophilia.

Results

P-sel–Ig induces microparticles in human blood

We previously observed that infusion of P-sel–Ig in mice induces the formation of procoagulant microparticles[7]. In order to investigate the molecular mechanisms involved in the procoagulant function of P-sel–Ig, we studied the

process *in vitro* using human cells. Human blood was treated with human P-sel–Ig, which was made by fusing the extracellular portion of P-selectin and the Fc portion of human IgG_1[14], or control IgG (control-Ig) (**Fig.1a**). Whole blood clotting time in the P-sel–Ig treated group was progressively shortened in comparison to blood treated with control-Ig. The response was also dose-dependent. While Fig. 1 shows results with 15 μg/ml of the proteins, significant differences were also observed with 1 or 5 μg/ml, but the time required for the effects to be apparent was longer (not shown). Blood treated with 15 μg/ml P-sel–Ig for 8 h clotted 3 times faster than control blood (**Fig.1a**). Thus the procoagulant activities of P-sel–Ig directly involve the blood cells and do not require the vessel wall or other organs.

In order to see the effect of P-sel–Ig on microparticle generation (released into plasma), we measured clotting time of plasma obtained from P-sel–Ig-treated and control-Ig-treated whole blood. Fibrin formation in plasma of P-sel–Ig-treated samples became significantly faster after 6 h incubation, consistent with P-sel–Ig generating procoagulant microparticles (**Fig.1b**). Microparticles bear tissue factor *in vivo*[7,15]; therefore we asked whether an increase in tissue factor could be responsible for the P-sel–Ig-mediated reduction of clotting time. Microparticles pelleted by high spin centrifugation were tested for tissue factor activity by measuring their capacity to activate factor X and determining the activity of the resulting active enzyme (Xa). The Xa activity of microparticles obtained from P-sel–Ig-treated samples was higher than from control-Ig-treated samples indicating that they contained more active tissue factor (P-sel–IgG-treated samples 17.3 ± 1.8 mOD/min, control-Ig-treated samples 9.4 ± 2.4 mOD/min, $n = 5$, $P < 0.04$). This indicates that P-sel–Ig promoted the formation of tissue factor-positive microparticles from human cells *in vitro*.

PSGL-1 mediates the microparticle formation

PSGL-1 is a well-recognized receptor for P-selectin[1]. We investigated its role in P-sel–Ig-mediated microparticle generation. Inhibitory monoclonal antibody PSG3, binding to the sulfotyrosine domain of PSGL-1, completely inhibited the shortening of whole blood clotting time (**Fig. 1c**) and of the plasma clotting time (**Fig. 1d**) induced by P-sel–Ig. This indicates that PSGL-1) was the main receptor for P-sel–Ig-mediated generation of procoagulant activity. Control antibody had no effect on coagulation.

In order to show that the engagement of PSGL-1 also mediates the procoagulant activity of P-sel–Ig *in vivo*, we injected mouse P-sel–Ig, containing the lectin domain, the EGF-like repeat, and the first two complement-binding domains of mouse P-selectin fused to the Fc region of human IgG_1[16], into 2–3 month old mice lacking PSGL-1 and their wild-type siblings. The number of microparticles before injection in these mice was similar between the two genotypes (**Table 1a**). Twenty-two h after infusion of P-sel–Ig, the total number and the percentage of tissue factor-positive microparticles in plasma were determined by flow cytometry. Both the total number of microparticles and the percentage of microparticles bearing tissue factor were significantly higher in the wild-type than in the *Psgl-1*$^{-/-}$ mice after P-sel–Ig treatment ($P < 0.03$ and $P < 0.02$, respectively, **Table 1b**) indicating that PSGL-1 is necessary for the procoagulant microparticles production.

Older mice tend to have more circulating microparticles and higher soluble P-selectin levels than younger mice (Hrachovinova and Wagner, unpublished observation). This was the case also for wild-type *Psgl-1*$^{+/+}$ mice (**Table 1a**). Doubling the age of the wild-type mice more than doubled the concentration of circulating microparticles, while no significant increase was observed in the *Psgl-1*$^{-/-}$ mice. This suggests that PSGL-1 is involved in the age-dependent spontaneous increase in procoagulant activity in animals. Age is a known risk factor for thrombotic events[17].

Microparticles incorporate in a growing thrombus

Our results point to a P-selectin/PSGL-1-dependent pathway for the generation of procoagulant microparticles both *in vitro* and *in vivo*. However, little is known about the physiologic function of these microparticles and whether they contribute to thrombus formation in live animals. Using intravital microscopy we examined the interaction of microparticles with a growing thrombus in ferric chloride injured vessels[18]. We isolated microparticles from the plasma of □CT mice[6]. These mice have higher concentrations of soluble P-selectin and higher numbers of circulating procoagulant microparticles[7]. The microparticles were labeled with calcein AM, washed and introduced into the microcirculation of the mouse-cremaster muscle[19], concomitant to induction of injury and thrombus formation. The specific accumulation of the fluorescently-labeled microparticles in the thrombus (**Fig. 2**) demonstrated the preferential binding of the free-flowing microparticles onto

activated platelets/fibrin in comparison to areas of the vessel free of thrombus. These results provide support to the notion that procoagulant microparticles contribute their activity to the growing thrombus.

P-sel–Ig improves phenotype of hemophilia A mice

Infusion of sP-sel–Ig reduces plasma clotting time in wild-type mice[7]. We asked whether the generation of procoagulant microparticles through the P-selectin/PSGL-1 interaction could improve the phenotype of an animal with a major bleeding disorder such as hemophilia A. Factor VIII-deficient mice (FVIII $^{-/-}$) were injected with P-sel–Ig or control-Ig and mice were bled six h later. Platelet poor plasma was prepared to determine partial thromboplastin time (APTT) which is prolonged in Factor VIII-deficiency[20] and plasma clotting time. APTT in FVIII-deficient mice was not modified by control-Ig-treatment (37.9 ± 1 s vs. 38 ± 1.5 s) but it was significantly reduced by P-sel–Ig-treatment to 31.5 ± 0.7 s, n = 7 and 11, P < 0.02. Wild type value was 23.2 ± 0.5 s. Plasma clotting time of control-Ig-treated mice was 316 ± 16.5 sec and that of P-sel–Ig-treated mice was shorter, 249.5 ± 9.5 s, P < 0.006. Wild-type value was 263.5 ± 12 s. Thus infusion of P-sel–Ig partially corrected APTT in the hemophilia A mice and shortened plasma clotting time to wild-type values.

To study the parameters of whole blood clotting in the hemophilia A mouse model, the mice were treated as above and blood collected 6 or 72 h later. The onset of clotting (Clotting time) and the rate of clotting were determined (**Table 2a** and **Fig. 3a**). While infusion of control-Ig did not affect these parameters, P-sel–Ig rapidly improved both parameters and their values became similar to wild type 72 h after infusion. The numbers of microparticles in platelet poor plasma of hemophilia A mice treated for 6 h was determined by flow cytometry (**Fig. 3b**). There were three times as many microparticles in the plasma of P-sel–Ig-treated mice and the percentage of microparticles positive for tissue factor was increased seven fold over control mice.

To evaluate whether excessive bleeding in the hemophilia A mouse [21] could be prevented by infusion of P-sel–Ig, tail bleeding time was measured 6 h after treatment with P-sel–Ig or control-Ig (**Fig. 4**). Since the majority of Factor VIII-deficient mice could not arrest their bleeding, their tails were cauterized at 15 min. Treatment with P-sel–Ig normalized the bleeding time of hemophilia A mice and none of the treated mice needed cauterization.

P-sel–Ig affects human hemophilia A blood To verify that patients with hemophilia A can also respond to soluble P-selectin by producing procoagulant microparticles from their blood cells, we obtained blood samples from three patients with FVIII levels < 1% of normal. The blood was incubated with 15 μg/ml P-sel–Ig or control-Ig for 6 h. Platelet poor plasma (PPP) was then prepared and numbers of microparticles and their tissue factor activity determined (**Table 2b**). Both microparticle number and FXa generation were significantly higher in the samples treated with P-sel–Ig (P < 0.04 and P < 0.05, respectively), indicating that patients with hemophilia A might also respond to P-sel–Ig treatment.

Discussion

Previous studies in our laboratory demonstrated that infusion of P-sel–Ig into a wild-type mouse generates procoagulant microparticles[7]. In this study we reproduced these results *in vitro* using human blood. This indicates that the stimulus to generate microparticles is directly from P-selectin onto the blood cells and not through some intermediate cell type such as the endothelium. The appearance of procoagulant activity in the plasma (microparticles) was slightly delayed compared to that in the whole blood (**Fig. 1a** and **b**). This could be consistent with induction of tissue factor synthesis and its subsequent release into plasma as a component of cell membrane-derived microparticles. The tissue factor-containing microparticles are likely of leukocyte origin because they also contain the leukocyte-specific integrin Mac-1[7] and they are generated through P-selectin binding to PSGL-1 (**Fig. 1d**) which is primarily a leukocyte receptor. The monocyte synthesizes tissue factor after stimulation with various inflammatory agents[22], which may include P-selectin[23].

Crosslinking of PSGL-1 molecules on the leukocyte by dimeric or oligomeric forms of P-selectin may be needed for signal transduction[12,24] and therefore for microparticles generation. In our experiments, the infused P-sel–Ig is dimeric as it contains the Ig backbone. The transmembrane form of P-selectin on platelets and endothelial cells is dimeric [25,26]. The valency of circulating P-selectin is less clear. Recombinant forms of soluble P-selectin that lack the transmembrane domain are monomeric in aqueous buffers[25]. P-selectin may be monomeric in plasma although calcium dependent lectin-like domains, such as found in the selectins, commonly associate with

each other to form dimers and trimers[27]. At least part of the P-selectin in human plasma is an alternatively spliced secreted molecule that lacks the transmembrane domain[28], and at least part of the P-selectin in mouse plasma results from proteolytic shedding of the extracellular domain without the transmembrane domain[6]. Activated platelets shed microparticles which contain P-selectin. In plasma, such microparticles might present multivalent P-selectin that could effectively signal through PSGL-1 on leukocytes. Similarly, P-selectin on stimulated endothelium might signal to the leukocytes. To determine whether plasma P-selectin lacking the transmembrane domain is sufficient for signaling, we are preparing a transgenic mouse producing the extracellular fragment of the protein from liver cells.

Two different approaches were used to show that PSGL-1 is the crucial receptor for generation of procoagulant activity by P-selectin. First, the formation of procoagulant activity by P-selectin *in vitro* was inhibited fully by an inhibitory antibody to PSGL-1 (**Fig. 1c** and **d**). This antibody binds to the sulfated region of PSGL-1 that is recognized by the P-selectin lectin domain[1]. The antibody blocks binding of platelets to leukocytes but does not induce leukocyte aggregation or stimulate the cells to release IL-8. The second line of evidence came from studies with PSGL-1-deficent mice[29]. Infusion of P-sel–Ig into wild-type littermates generated five times more tissue factor-positive microparticles than infusion into mice of the same age lacking PSGL-1 (**Table 1b**). More interestingly the spontaneous increase in microparticle number seen in mice with old age was prevented by the lack of PSGL-1 (**Table 1a**).

Thus P-selectin/PSGL-1 interaction likely modulates coagulation at two levels: 1) P-selectin may regulate the overall procoagulant state of an animal. The level of the constitutively produced microparticles may reflect the health and age of the animal. Soluble P-selectin is increased in many diseases including atherosclerosis[8,30,31]. In atherosclerosis, soluble P-selectin is mostly derived from endothelial cells[32]. High levels of soluble P-selectin are predictive of future cardiovascular events [33,34]. Tissue factor-containing microparticles could be at least in part responsible for these clinical observations. 2) P-selectin may further increase procoagulant activity of an animal after injury, perhaps to further stabilize the fibrin clot and the platelet plug. This delayed effect of P-selectin/PSGL-1, requiring formation of new tissue factor-containing microparticles, would be proportional to the extent of injury, i. e. the numbers of activated platelets and/or endothelial cells which shed their P-selectin[32,35]. This may explain why anti-P-selectin therapy promotes dissolution of thrombi[36-38].

For the microparticles to be active in thrombus stabilization, they have to be recruited into the forming platelet plug. This was first demonstrated by Nemerson and colleagues in a flow chamber *in vitro*[15] and we now show the same phenomenon *in vivo* (**Fig. 2**). Tissue factor was observed in thrombi formed *in vivo* but its origin from plasma or vessel wall was uncertain[39]. We show that purified fluorescently-labeled microparticles specifically accumulated in the growing thrombus. *In vitro*, this recruitment was shown to be mediated by P-selectin[40]. Thus the adhesion mechanisms of recruitment of the procoagulant microparticles derived from leukocytes are likely very similar to those of leukocytes themselves. Therefore, P-selectin/PSGL-1 interaction may have a dual function, first in generation of the procoagulant microparticles and second in their recruitment to the growing thrombus.

The data discussed in the previous sections suggest that in old and/or diseased animals or humans elevated levels of soluble P-selectin point to a poor prognosis as they indicate a procoagulant state. However, there may be instances where such an elevated thrombotic potential may have therapeutic benefit. One such instance may be in patients with defective intrinsic coagulation such as the deficiencies of Factor VIII or Factor IX in hemophilia A and B. In these individuals, constitutively higher levels of soluble P-selectin may improve the capacity to generate thrombin through the extrinsic pathway and alleviate the bleeding tendency. We tested this hypothesis using an animal model of hemophilia A[20]. Infusion of P-sel–Ig, in contrast to control-Ig, improved all clotting/bleeding parameters tested. Within a few hours of infusion, blood of the P-sel–Ig-treated animals began to clot sooner and the rate of clotting was higher than in the control-Ig-treated hemophilia A mice (**Fig. 3a** and **Table 2a**). This was because tissue factor-containing microparticles were being generated (**Fig. 3b**). As a result, the infinite bleeding time found in the majority of the hemophilia A mice was shortened to wild-type length within 6 h after infusion of P-sel–Ig (**Fig. 4**). The template-induced skin bleeding time used in humans mostly measures platelet adhesion. Since platelet adhesion is not substantially modified by the absence of FVIII, the bleeding time in humans with hemophilia A is

normal. Bleeding time in mice, measured after snipping part of the tail while transecting both veins and arteries, is a much more severe injury, sensitive to levels of coagulation factors[41].

Currently, human hemophilia A patients are treated with recombinant Factor VIII. Unfortunately a significant fraction (20–50%) of hemophilia A patients develops antibodies to the factor making further treatment difficult[42,43], while a smaller fraction (2–5%) of hemophilia B patients develop inhibitors[44]. In these hemophilia patients treatments that bypass Factor VIII or IX, such as recombinant activated Factor VII are being used. At the recommended dose and the necessary frequency of treatment, due to the short half life of the factor, the cost of a single episode of bleeding can exceed $ 50,000[42]. The activity of P-selectin would also bypass Factor VIII or IX by producing tissue factor. Perhaps the much longer half life of P-sel–Ig (1–3 weeks) would reduce the cost of treatment significantly so that it could become more generally available. Also, since hemophilia patients would not perceive P-selectin as foreign, they would be less likely to develop antibodies to the protein. The *in vitro* studies we performed on blood samples from 3 severe hemophilia A patients (**Table 2b**) show both an increase in microparticle production and microparticle –tissue factor activity after P-sel–Ig treatment. These preliminary results suggest that administration of P-sel–Ig to hemophilia patients might also generate procoagulant microparticles.Our results indicate that interactions between P-selectin and PSGL-1 play an important role in regulating coagulation by inducing the release of procoagulant microparticles from leukocytes. This procoagulant activity could be either therapeutically reduced[7] with inhibitors of P-selectin/PSGL-1 binding, or enhanced by infusion of P-sel–Ig. This unexpected function of P-selectin/PSGL-1 provides new possibilities to treat disorders of coagulation.

Methods

Mice. Mice deficient for P-selectin glycoprotein ligand-1 (*Psgl-1*$^{-/-}$) and their littermate controls (*Psgl-1*$^{+/+}$)[29], mice expressing P-selectin without the cytoplasmic domain (ΔCT)[6] and Factor VIII-deficient mice (less than 1% Factor VIII activity) with targeted disruption of exon 16 were described[20]. Experimental protocols were approved by the Animal Care and Use Committees of the Center for Blood Research and the Oklahoma Medical Research Foundation.

Blood sampling and *in vitro* incubation. We obtained informed consent from healthy donors and hemophilia A patients and approval from the Institutional Review Board of the Center for Blood Research and Institute of Hematology and Blood Transfusion. Patients (aged 38, 45 and 58 years) were at least three days without treatment with any FVIII concentrate, had normal platelet counts and FVIII activity was < 1%. Blood was collected into 10 ml Vacutainer glass tubes containing 1.5 ml ACD (Becton Dickinson) and 0.8 ml was aliquoted into 2.0 ml sterile plastic tubes. Incubation was performed with either 15 μg/ml of human P-selectin-Ig chimera (P-sel–Ig)[14] or human IgG_1 (Sigma). Samples were inverted two times every 30 min. Blood was aliquoted for whole blood clotting time assay (see below) or recentrifuged platelet poor plasma (RPPP) was prepared by centrifugation at 1,500g for 25 min and then at 10,000g for 5 min..

Determination of clotting times. Whole blood clotting time was measured in a Sonoclot (Sienco). 280 μl of the whole blood was added to 20 μl of 150 mM $CaCl_2$ in 37 °C cuvettes. Clotting time (T) was determined as the time until fibrin formation, clotting rate (R) as a percentage of the peak amplitude per min. Plasma clotting time was determined in an aggregometer(Sienco)[7].

Microparticles preparation and flow cytometry. 40 μl of RPPP was added to 260 μl PBS and analyzed by flow cytometry. The total number of events was counted for 10 s (ref. 7). Washed microparticles were prepared by ultracentrifugation of RPPP and resuspended in PBS in 1/3 of initial volume as described[7]. Microparticles from mice were stained with sheep anti-rabbit tissue factor IgG (5 μg/ml, American Diagnostica), which recognizes mouse tissue factor, or with sheep IgG (5μg/ml, Sigma) and fluorescein-conjugated donkey anti-sheep-IgG (Cortex Biochem).

Tissue factor activity of microparticles. Activity was evaluated by ability to activate Factor X (150nM) by Factor VIIa (5 nM) in the presence of 1 mM $CaCl_2$. Chromogenic substrate of Factor Xa, Spectrozyme FXa, was added (0.3 mM). The linear changes in absorbance at 405 nm were recorded with a plate reader with kinetic software (DYNEX Technologies). The changes in mOD/min directly correlated with Factor Xa generated.

Anti-PSGL-1 treatment. PSG3 is a human monoclonal antibody to human PSGL-1 generated by Wyeth Research (Cambridge, USA) and Cambridge Antibody Technology (Melbourn, United Kingdom). This inhibitory IgG1 antibody was mutated to reduce Fc receptor binding and

complement activation. PSG3 was expressed in CHO cells. The purified antibody is highly specific for human and non-human primate PSGL-1. PSG3 does not induce production of IL8 from leukocytes *in vitro* nor *in vivo* after infusion into primates. PSG3 was added to human blood (15 μg/ml) and incubated 30 min at 37 °C before treatment with human P-sel–Ig.

PSGL-1 $^{-/-}$ **mice evaluation.** Number of procoagulant microparticles was measured by FACS in diluted plasma from *Psgl-1*$^{+/+}$ and *Psgl-1*$^{-/-}$ mice 9–12 and 17–20 weeks old. The younger mice were then injected i.v. with 1.2 mg/kg of mouse P-sel–Ig [7]. Twenty-two h later blood was taken, plasma isolated, and washed microparticles prepared. The number of microparticles was determined by FACS and microparticles were stained for tissue factor.

Intravital microscopy of microparticle recruitment. Washed microparticles isolated from blood from ΔCT mice were stained with calcein AM (Molecular Probes), washed two times by ultracentrifugation and resuspended in PBS. C57Bl6/J mice were anesthetized with Avertin (2.5%). The cremaster muscle was prepared[19,45]. A heparinized catheter was placed into the proximal part of the right femoral artery[19] and the microparticles were infused over 5 min using a syringe pump (Harvard Apparatus). Ferric chloride (30 μl of a 250 mM solution) was applied topically[18] and venules (diameters 25–45 μm) were recorded[45].

Hemophilia A mice phenotype correction analysis. Mice were injected i.v. with 1.2 mg/kg of mouse P-sel–Ig or human IgG_1 (Sigma). Whole blood clotting time and tissue factor-bearing microparticles in plasma were measured as above. Tail bleeding time was as described[18]. For APTT test, mice were bled to ACD (1/10). PPP was prepared and APTT was determined with APTT reagent (IL).

Endotoxin screening. Endotoxin in reagents and buffers was found < 10 pg/ml with CoaTest (Chromogenix, Haemochrom Diagnostica).

Statistical analysis. Results are expressed as mean ± SEM and analyzed using the unpaired Student's *t*-test. In Figure 1, ANOVA test was applied with Tukey-Kramer post test.

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4.3.TF A MIKROPARTIKULE U PACIENTŮ S KORONÁRNÍM SYNDROMEM

Maly, M**., Hrachovinova, I.,** Tomasov, P., Salaj, P., Hajek, P., Veselka, J.(2009) **Patients with acute coronary syndromes have low tissue factor activity and microparticle count, but normal concentration of tissue factor antigen in platelet free plasma : a pilot study.** Eur.J.Haematol., 82(2), 148-153.

Tkáňový factor (TF) je hlavní iniciátor koagulační kaskády. Stanovení TF v případě akutního koronárního syndromu je logisticky komplikované. Z tohoto důvodu jsme měřili paralelně antigen a aktivitu TF společně s prokoagulačními mikropartikulemi v plazmě pacientů s akutním koronárním syndromem.
Metody: Krev 40 pacientů byla odebrána jak z koronárního sinu tak z periferní žíly(femorální žíla). Aktivita TF byla měřena expresí FXa za přítomnosti FVIIa. Množství MPs bylo detekováno průtokovou cytometrií. Koncenrace TF byla stanovena ELISA testem.

Výsledky: AktivitaTF u skupiny pacientů se stabilní anginou pectoris se nelišila od kontrolní skupiny (18.12 ± 3.35 mOD⁄min vs. 17.72 ± 4.05 mOD⁄min, respectively), ale byla signifikantně vyšší než u skupiny s nestabilní anginou pectoris (7.62 ± 4.19 mOD⁄min) a infarktem myokardu(MI) (3.56 ± 3.85 mOD⁄min), ($P < 0.05$). Výsledky náběrů z koronárního sinu a femorální žíly se nelišily. Množství MPs klesalo shodně s tíží akutního koronárního syndromu: kontrolní skupina, 520 ± 172; stabilní angina pectoris, 532 ± 167; nestabilní angina pectoris, 392 ± 142; a MI skupina, 165 ± 30 ($P < 0.05$). Nenašli jsme žádné rozdíly v množství TF měřeného ELISA metodou.

Závěr: Naše výsledky ukazují na fakt, že prokoagulační MPs s navázaným TF jsou pravděpodobně v případě akutního koronárního syndromu vychytávány z plazmy interakcí s krevními buňkami..

Patients with Acute Coronary Syndromes Have Low Tissue Factor Activity and Microparticle Count, but Normal Concentration of Tissue Factor Antigen in Platelet Free Plasma—a Pilot Study.

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Key words: Tissue factor; Microparticles; Platelets; Acute coronary syndrome

Abstract:
Objectives: Tissue factor is a main initiator of coagulation cascade. It´s determination in conditions of acute coronary syndrome is logistically difficult. Hence, in our study, the activity and the concentration of tissue factor and the count of microparticles in the platelet free plasma were determined.
Methods: Blood was drawn from both coronary sinus and femoral vein circulation in a cohort of 40 patients. Tissue factor activity was measured by activation of factor X in the presence of factor VIIa, whereas microparticles were detected using flow cytometry. Tissue factor antigen coken-trations were determined using the ELISA test.
Results: Tissue factor activity in the stable angina subgroup was not significantly different from the control group (18.12 ± 3.35 mOD/min vs. 17.72 ± 4.05 mOD/min, respectively), but it was significantly lower in the unstable angina (7.62 ± 4.19 mOD/min) and myocardial infarction (3.56 ± 3.85 mOD/min) subgroups ($p < 0.05$). Results from the coronary sinus and femoral vein circulations were not significantly different. The count of microparticles decreased according to the severity of the acute coronary syndrome: control group, 520 ± 172; stable angina subgroup, 532 ± 167; unstable angina subgroup, 392 ± 142; and myocardial infarction subgroup, 165 ± 30 ($p < 0.05$). There were no significant differences in concentrations of tissue factor antigen in four subgroups.
Conclusions: These results suggest that the procoagulant tissue factor-bearing microparticles could be recruited from platelet free plasma by interaction with platelets and blood cells in the conditions of acute coronary syndrome.

Introduction

Tissue factor (TF) is considered to be the major regulator of normal hemostasis and thrombosis (1, 2). Recent evidence highlights the role of the blood-borne pool of TF (3). This TF fraction is bound to the procoagulant microparticles (MPs). The main source of circulating MPs is the membrane of activated platelets (4). Increased levels of MPs with procoagulant potential were identified in the peripheral circulating blood of patients with acute coronary syndromes (ACS) (5). In addition, high levels of shed apoptotic MPs and TF activity were found in the extracts from atherosclerotic plaques (6, 7). Microparticles are able to transfer their procoagulation potential to the target cells by binding on its cell membrane and cause the initiation and propagation of the thrombus formation (8).

Determination of TF in whole blood is methodologically difficult. There is necessity of immediate processing of the samples to avoid the artificial activation of the platelets with nonphysiological sequestration of the microparticles. In the conditions of ACS is impossible to plan out the timing of sampling. Hence, in our study, the activity of TF, the count of microparticles, and the concentration of TF antigen in the platelet free plasma (PFP) - which allows the storage of frozen samples - were determined. According to our knowledge, study determining TF activity in PFP in patients with ACS is not yet published.

Materials and methods:

Forty prospectively selected patients (22 men, 18 women, 64.5 ± 10.71 years) who were undergoing coronary angiography in the Department of Cardiology, University Hospital Motol, Prague, were enrolled in this study. Cardiovascular risk factors (diabetes, hypertension, smoking, and hypercholesterolemia) were determined. The blood from both the coronary sinus and femoral vein in four matched subgroups of patients with the following symptoms were sampled: 1) no significant coronary artery disease and no symptoms of angina pectoris, defined as normal coronary angiogram (control), 2) stable angina pectoris (SAP), 3) unstable angina pectoris–Braunwald class III (UAP), and 4) myocardial infarction (MI) with ST elevations, undergoing primary percutaneous coronary intervention (PCI). Blood was sampled as follows: from the coronary sinus using Amplatz coronary catheter (AL1, F4, Cordis Corporation, FL, USA) and from the femoral vein just before coronary angiography. All patients with coronary heart disease received standard antithrombotic therapy within one hour before blood sampling, i.e. low-molecular-weight heparin to the subgroup with UAP and a bolus of unfractioned heparin to the subgroup with MI

undergoing primary PCI. Patients received 100 mg aspirin daily and were not pretreated with clopidogrel. GP IIb/IIIa blockers were not used. PCI was performed in six patients (60%) in the SAP subgroup. Seven patients (70%) in the UAP subgroup were treated with PCI, whereas 3 patients (30%) of this subgroup underwent surgical revascularization. There were ten patients (100%) treated with primary PCI in the MI subgroup of patients. In the control group, eight patients (80%) underwent coronary angiography due to chest pain that was confirmed not to be of ischemic origin, one patient (10%) had severe aortic stenosis, and one patient (10%) had pulmonary hypertension. All the patients provided their informed consent before participation in the study. The study was approved by the institutional Ethics Committee on human research.

Preparation of the samples:

All the samples were prepared and assayed without the knowledge of their respective subgroups. Within 30 min of sampling, a volume of 10 ml of citrated blood (3.2%) from the coronary sinus and femoral vein samples was centrifuged twice for 15 minutes at 3,000×g to remove the platelets present in plasma. PFP was prepared and was immediately aliquoted in eppendorf tubes. Samples in aliquots were stored at –75°C until they were used for ELISA tests, microparticles counting, or preparation of washed microparticles. Samples for ELISA tests and microparticles counting were then warmed for 10 min in 37°C and were immediately diluted to working concentration and used. For each assay, a previously unthawed aliquot was used. Washed microparticles were prepared by centrifugation of PFP (diluted 1:10 in a HEPES buffer) for 1 hour at 100,000×g. Supernatant was discarded. The pelleted microparticles were resuspended in 2 ml of the HEPES buffer and were centrifuged again. Washed microparticles were resuspended in HEPES in 1/3 initial volume of plasma. This suspension of microparticles was used for the measurement functional activity of TF.

TF activity:

The TF activity was evaluated by its ability to activate factor X (150 nM) through the limited amount of factor VIIa (5 nM) in the presence of 1 mM $CaCl_2$ using 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 using an ELISA plate reader. The changes in mOD/min directly correlated with the amount of FXa generated as we have published previously (9).

Microparticles:

The number of microparticles was analyzed using flow cytometry. To count the total microparticle population, 40 µl aliquots were incubated for 15 min in the dark with 0.25 µg/ml calcein AM (Molecular Probes). Forty microliters of plasma sample was diluted with 260 µl of a 0.2-µm filtered phosphate buffered saline (PBS), and it was immediately analyzed for number of microparticles by Fluorescent Activated Cell Sorting (FACS) (10). Flow cytometric analysis was performed on a Becton-Dickinson FACSCalibur with CELLQUEST software (Becton-Dickinson). The light scatters and fluorescent channels were set at logarithmic gain (forward scatter was E00 with a threshold of 12, and sideward scatter was 300). The total number of events was counted for a set interval of 10 s.

TF antigen:

TF antigen was measured. A commercially available enzyme-linked immunosorbent assay (ELISA) kit (ImubindTM; American Diagnostica, Greenwich, CT, USA) was used according to the manufacturer's instructions to measure TF antigen in plasma. In brief, samples were diluted in the ratio of 1:4 in PBS (pH 7.4) containing 0.1% Triton X-100 and 1% (w/v) bovine serum albumin and incubated overnight in the wells of a microtiter plate precoated with murine antihuman TF monoclonal antibody for antigen capture. After detection of bound antigen by biotinylated polyclonal anti-TF F(ab')2, streptavidin conjugated horseradish peroxidase was added to complete formation of the antibody fragment-enzyme detection complex. Following the stepwise addition of tetramethylbenzidine chromogenic substrate and sulfuric acid stopping solution, absorbance of yellowish colored solutions were read at 450 nm. Mean optical densities of duplicate incubations were referred to a standard curve obtained by serial concentrations (0–1,000 pg/ml) of nonlipidated, full-length recombinant human TF. Normal range of TF antigen in human plasma was established on 45 normal donors of plasma.

Statistical analysis:

The repeated measures ANOVA model with group (control, SAP, UAP, and MI), subject, and type of matrix (coronary sinus and femoral vein circulations) as factors and group vs. matrix interaction followed by Bonferroni multiple comparisons were used for evaluation of differences between the groups, subjects, and types of matrix. 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 from Manugistics (Rockville, MD, USA) and NCSS (Kaysville, UT, USA) was used for the analysis.

Results:

There were no significant differences between groups in their age and the status of hypercholesterolemia, diabetes mellitus, hypertension, and smoking (Table 1). Significantly lower levels of TF activity and the microparticles in subgroups of the patients with ACS were found, whereas no statistically significant differences in the same between their coronary sinus and femoral vein circulations were observed. No significant differences in the concentration of TF antigen across the subgroups were observed.

TF activity:

In the PFP from coronary sinus samples of patients with SAP, the TF activity was not significantly different from the patients in the control group (18.12 ± 3.35 mOD/min vs. 17.72 ± 4.05 mOD/min, respectively). However, the TF activity was significantly lower in the UAP (7.62 ± 4.19 mOD/min) and MI (3.56 ± 3.85 mOD/min) subgroups compared with the control ($p < 0.05$) and SAP groups ($p < 0.05$). There were no significant differences in the TF activity in patients with UAP vs. MI (Fig. 1).

In the PFP from femoral vein samples, the TF activity (17.22 ± 2.91 mOD/min) in the control group was not statistically different from that (17.52 ± 2.41 mOD/min) in the SAP subgroup. However, again, the TF activity was significantly lower in the UAP (8.28 ± 4.75 mOD/min) and MI (2.55 ± 3.56 mOD/min) subgroups compared with the control ($p < 0.05$) and SAP groups ($p < 0.05$). There were significant differences in the TF activity in patients with UAP vs. MI ($p < 0.05$).

Microparticles:

Plasma microparticles in the PFP were analyzed by the FSC and SSC scale, gate analysis was used to quantify the large microparticles that were, as we previously reported (10), found to be linked to procoagulant activity. In the control group, there were 520 ± 172 MPs; in the SAP subgroup, 532 ± 167; in the UAP subgroup, 392 ± 142; and in the MI subgroup, 165±30. Among these, the last value was statistically significant compared with the control group (Fig. 2).

TF antigen:

In the PFP from coronary sinus samples, the concentrations of TF antigen were 47.07 ± 21.57 pg/ml in control group, 79.24 ± 65.29 pg/ml in SAP group, 50.72 ± 19.29 pg/ml in UAP group, and 69.68 ± 90.76 pg/ml in MI group. In the PFP from femoral vein samples, the concentrations of TF antigen were 48.24 ± 32.76 pg/ml in control group, 41.33 ± 20.7 pg/ml in SAP group, 47.44 ± 25.88 pg/ml in UAP group, and 59.5 ± 75.43 pg/ml in MI group. The differences were not significantly different among all four subgroups of patients in both the coronary sinus and femoral vein circulation.

Discussion

The present study shows five times less TF activity in the PFP prepared from the whole blood samples of the patients with ACS than in the patients with SAP, which correlates with a three-fold decrease of microparticles in patients presenting with MI. The results should be interpreted very cautiously, since PFP was not yet used for measurements of TF activity in patients with ACS, and the numbers of each group participants are limited. However, despite the mentioned limitations, it appears to be in accordance with the current concept of cell-based coagulation (3). The model assumes that the active TF that is bound to MPs is recruited from the circulating blood by binding onto the surface of activated cells (platelets, monocytes, and endothelial cells), where it forms a highly procoagulant thrombogenic core (11).

The main source of circulating MPs is the membrane of activated platelets (4). The additional sources of MPs are leukocytes, erythrocytes, and endothelial cells (8). The interaction between MPs bearing TF and platelets is provided by the interaction of P-selectin glykoprotein ligand-1 and P-selectin on the surface of the activated platelets. A platelet adhesion molecule P-selectin is neceséry for TF accumulation and its incorporation into the thrombus after endothelial injury (11). Also, the accumulation of hematopoietic cell-derived TF in the developing thrombus correlates with the kinetics of MPs accumulation before the leukocyte–thrombus interaction (8). Because of the absence of the main pool of the TF Bering cells in the PFP, the decrease of MPs and TF aktivity in PFP of the patients with ACS appears to support this model.

In addition, unlike TF activity, TF antigen does not, show significant differences when its concentration in the PFP of patients presenting with SAP and ACS are compared. The potential explanation is based on the presence of various types of TF molecules in the circulation (active and non-active) (12, 13), all of which can be detected using antibodies in a commercial immunoassay and are the potential source of the

absence of signifiant differences in the TF antigen in ACS settings, as we have reported previously (14). In the study of Morange (15), where was investigated the prognostic value of TF antigen, on admission similarly no significant differences in TF levels were observed between SAP and ACS patients.

Further investigation aims to focus on the identification of specific subpopulations of microparticles. In a recent study (16), it was shown that there is a difference in the origin of microparticles in an 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 prove the decrease of these specific subpopulations of microparticles in patients with ACS.

There is a limitation in subgroup of ACS patients – the influence of heparin therapy and the heparin-induced release of TF pathway inhibitor (TFPI). This TFPI release can mitigate the TF consumption. It is established that TFPI, free or associated with microparticles, could naturally inhibit TF activity. Nevertheless, it was reported that this inhibition reached almost about 50% (17). Because five times less TF activity in the ACS group compared with the SAP group has been observed in this study, it seems that TFPI inhibition of TF is only partial.

Despite above mentioned limitations, the results of this study are a piece of knowledge in patho-physiology of blood coagulation and may support the concept of close interactions of TF procoagulant MPs with platelets and other blood cells in the settings of ongoing thrombus formativ as a substrate of ACS.

The results of the investigation in the field of TF-mediated coagulation led to the development of novel therapeutic strategies. The clinical trial was completed with Sunol-cH36, which is the chimeric monoclonal antipody to TF. In this PROXIMATE-TIMI 27 trial – focused on the safety profile – was found, that there is a direct dosedependent antiocoagulant effect of this agent and was postulated, that mucosal bleeding may reflect the antiplatelet effect resulting from networking between the coagulation cascade and platelets pathways (18). This concept implies very promising further direction of the strategies in the antithrombotic treatment in ACSs.

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5. DISKUSE A ZÁVĚR

Výsledky jednotlivých studií, které jsou součástí mé disertační práce, prokázaly, že P-sel hraje důležitou roli v hemostáze. Solubilní P-sel generuje prokoagulační a TF-pozitivní mikropartikule. Receptorem P-selektinu je při tomto procesu P-selektin glykoprotein ligand (PSGL-1).

Recentní práce (Del Conde, 2005; Bernimoulin, 2009), které se podrobně zabývaly působením P-sel na monocyty, prokázaly, že vazba P-sel-PSGL-1 indukuje exposici fospatidylserinu na povrchu monocytů a že vzniklé MPs se liší podle typu induktoru. MPs, které vznikly působením LPS (liposacharid) se kvalitativně lišily od těch, které byly indukované P-sel. Tyto výsledky potvrdily specifickou funkci MPs vzniklých působením P-sel. Není ale možné odlišit, zda se jedná o sP-sel nebo P-sel, který je stále součástí povrchu krevních destiček.

Hyperkoagulační potenciál získaný zvýšenou koncentrací sP-sel byl využit pro potlačení krvácivých projevů u transgeních myší s hemofilií A. K této studii nás vedlo také naše zjištění, že pacienti s těžkou formou hemofilie A mají na rozdíl od kontrol bez krvácivého onemocnění signifikantně nižší množství TF-MPs. I když naše výsledky na myších byly přesvědčivé a došlo k normalizaci některých koagulačních parametrů, z hlediska použití v lidské medicíně existuje několik závažných překážek. Např. mechanismus vzniku prokoagulačních mikropartikulí je zdlouhavý - trvá několik hodin. Solubilní P-sel může sloužit pouze jako podpůrný mechanismus pro snížení krvácivé schopnosti.

Větší význam pro medicínskou praxi má vysvětlení mechanismu inkorporace MPs do vznikajícího trombu, který hraje významnou roli při dodávání TF do destičkové sraženiny. Popsání tohoto mechnismu mělo logický výstup v testování látek, které by této inkorporaci zabránily. Tím by se potlačil růst trombů, případně vznik reokluzí. Několik příznivých výsledků použití rekombinantního PSGL-1, přímých inhibitorů P-sel nebo petidů blokujících vazbu P-sel - PSGL-1. již bylo publikováno (Myers and Wakefield, 2002).

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7. SEZNAM PUBLIKACÍ

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